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BIOTECHNOLOGICAL STRATEGIES TO IMPROVE SAFETY AND QUALITY IN FOOD PRODUCTS

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SUMMARY

The purpose of this PhD research is to facilitate the development of green and successful strategies for the control of undesirable microorganisms in food products. It is well known that many stress resistant bacteria are able to contaminate food products and produce their spoilage or, worse still, be a potential source of human illness. In the last decade, illnesses resulting from food borne pathogens have been higher than in the past and have become one of the most widespread public health problems in the world. Contextually, contaminations with spoilage microorganisms remain a major threat for the industry and food-based market, so much so, that consumers are not only paying more attention to the risk of foodborne pathogens but also the safety of chemical preservatives that are used to control undesirable microorganisms. It is, therefore, essential to find a satisfactory solution and useful strategy to prevent or reduce the incidences of pathogens or spoilage microorganisms. In the last two decades, much attention has been focused on food bio preservation, a "*green strategy*" that can assure shelf-life extension and food safety using microorganisms or their antimicrobial compounds. Lactic acid bacteria could be considered an ideal choice for application as protective cultures in food products and, more specifically, *Lactobacillus plantarum* is the most versatile and widespread species. Several screening processes are developed in order to select the most appropriate effective strains to be used as protective cultures, including the production of bacteriocins, BLIS, organic acids, hydrogen peroxide as well as short chain fat acids. However, these screening programmes are labour intensive and a large number of strains isolated from different food matrices are assessed, thereby requiring more expensive investments in order to avoid unsatisfactory results. These findings call for a more simplified and useful approach when searching for new protective strains, taking into account that food stress conditions strongly influence the development of specific microbial strains. It would be extremely interesting to ascertain the effect of different environments on the selection of strains able to exert antimicrobial activities and, therefore, this research looks at the correlation between the *Lb. plantarum* strain isolated from hard environments and the ability to produce antimicrobial compounds. In addition, the main antimicrobial compound produced by producers *Lb. plantarum* strains and its mode of action was also investigated. This

dissertation assesses the observations and the main significant results were reported in six chapters.

Chapter 1 is an overview of the biocontrol strategies developed. More specifically, advancements in control strategies based on natural compounds and living organisms and/or their antimicrobial products (biocontrol, or bio preservation) were highlighted. These natural preservation methods are regarded as health-friendly by consumers, and are expected to have a lower impact on food nutritional and sensory properties. In addition, they may reduce the processing costs and, at the same time, extend the product shelf life period. However, until now, several issues, such as the high minimal inhibitory concentration levels, the stability of antimicrobial compounds, the knowledge of action mode, as well as the relation between microbial growth and compound formation kinetics, still remain unclear, making the individuation of a simplified screening procedure necessary. In **Chapter 2** are reported the objectives of PhD research.

Chapter 3 considers the relation between antimicrobial properties of *Lb. plantarum* strains and their source of isolation. For this purpose, a total of 110 *Lb. plantarum* strains were used as antagonistic strains (producers) against 33 undesirable microorganisms (indicators), including both moulds and bacteria. The antimicrobial activity exerted by cells, cell free supernatants (CFS), neutralized CFS (nCFS) or CFS added with α-chymotrypsin, proteinase K, and trypsin (pCFS) of the producer strains was evaluated by the spot-on-the-lawn and by the agar well diffusion assay. Moreover, the inhibition effects expressed by cell free supernatant (CFS) and by neutralized cell free supernatant of selected strains was evaluated in culture-broth expressed against strains belonging to *Ps. fluorescens*, *B. thermosphacta* and *L. innocua*.

The preliminary results achieved by the evaluation of the antimicrobial effects expressed by CFS and the correspondent neutralized CFS support the hypothesis that the inhibition was due to the production of extracellular compounds having neither acid (such as lactic acid, that represent the principal extracellular metabolites produced by *Lb. plantarum*) nor proteinaceous nature. In addition, the results evidenced that the inhibitory effect produced by certain *Lb. plantarum* strains also remains at higher pH values. Therefore, the comparison between the inhibitory effects produced by CFS and lactic acid could provide more information on the antimicrobial compound. Moreover, in order to better appreciate differences between lactic acid and CFS, the most lactic acid resistant strains among the indicators should be chosen. *L. innocua* strains are well

known for their acid stress resistance. The relative results evidenced that the inhibitory effect of CFS from *Lb. plantarum* H_BB1 against *L. innocua* was due to the synergic presence of more than one inhibitory substance. A more in depth, further investigation evidenced that in addition to lactic acid, the CFS might also possess another compound of acid nature. On the basis of this evidence, PLA in the cells free supernatant from strains (able to produce antimicrobial effects), was evaluated. The results highlighted significant differences among the assayed strains showing that PLA production is strain-dependent. In addition, for the first time, a relation between PLA-producing strains and isolation environment of the strains was highlighted. In fact, those environments characterised by harsh conditions (high ethanol levels, low pH and high sugar levels), such as wines and honey, harboured a higher number of antagonistic strains than other fermented matrices (e.g. cheese, sourdoughs or fermented sausages). This could be due to selective pressures which are more accentuated in wines and honey than in the other food matrices researched.

The most important scientific enrichment produced by the activities in Chapter 1 is attributable to results highlighting that the choice of the source of isolation could be an important preliminary tool for the individuation of antagonistic strains. However, the correlation between *Lb. plantarum* PLA formation ability and their isolation sources would lead to opening new frontiers in understanding the PLA formation process.

PLA formation seems to be linked to stress response mechanisms performed by *Lb. plantarum*. However, no information with regards the LAB stress response and PLA production is available in literature and little information is reported on the relation between the microbial growth phase and PLA formation. Even if the prevailing opinion in the scientific community believes that PLA formation is related to LAB growth arrest, the linkage to metabolic pathways involved in its stationary phase has not been clarified. Little information, if any, can be found on the optimal pH condition of PLA metabolic pathway in the *Lactobacillus* species.

With this in mind, the research reported in **Chapter 4** focuses on the effect of growth phase and on the PLA formation by *Lb. plantarum* H_BB1. Moreover, cultivation conditions that were able to assure the highest PLA levels were investigated. The production of PLA by *Lb. plantarum* H_BB1 was preliminarily investigated in MRS broth. The comparison between PLA behaviour and growth curves evidenced that the PLA accumulation begins immediately after the end of the lag phase and reached the highest levels between the exponential and the stationary phase. As far as I know, these

results show, for the first time, that the PLA production is strictly related to the growth and to the exponential phase of *Lb. plantarum*. More specifically, the results obtained in the present study suggest that the PLA could assume new metabolic meanings. In fact, the accumulation from the beginning of the exponential phase highlight typical behaviour of a primary metabolite. On the other hand, the highest production rate between the exponential and stationary phase suggests that PLA production could assume a key role in the acid stress response. In fact, in the transition between exponential and stationary phase, pH showed values similar to the *pKa* of lactic acid. On the basis of the above reported statements, the evaluation of ecological factors on the PLA formation process appear essential. More precisely, sub-lethal pH could positively affect some metabolic pathways in *Lb. plantarum*. For this purpose, PLA production by *Lb. plantarum* was assayed in different cultural conditions (MRS acidified to pH 4.0 and to pH 3.5) and the results suggest that the metabolic pathway involved in PLA formation is tied to the energetic metabolism of growing cells. Key reactions of PLA formation, such as the regeneration of NAD^+ levels, the transamination reaction (where the α-ammino group is transferred to a keto acid acceptor) and the deamination reactions with NH_3 and amino acceptor regeneration, found different linkages with typical metabolic activities of growing cells. The results evidenced that, in no way, could the PLA formation be related to cell growth arrest. Whereas, its formation could represent an adaptation response of growing cells to acid stress. In fact, evaluating the behaviour of ratio between PLA (g/L) and biomass (g/L) levels, the highest performances were detected when the strain was cultivated in MRS pre-acidified to pH 4.0. On the basis of the above reported results, the PLA could be considered a "primary-like metabolite" of *Lb. plantarum* in sub-optimal pH condition and may open new horizons to the development of an advanced optimal design for maximum PLA production.

Moreover, the evidences reported in state-of-the-art Chapter 1, together with data specified in Chapters 2 and 3 suggest the opportunity to use PLA as anti-*Listeria* compounds. To date, antimicrobial activity of PLA, including anti-*Listeria* ability, was well recognized. Whereas, little information is available on the PLA anti-*Listeria* mechanism. Some results reported in literature suggest that the PLA mode of action could be similar to lactic acid. Nevertheless, considering the chemical structure of PLA, an action mode which is different from the lactic acid and more similar to phenolic acids could also be hypothesized. In **Chapter 5**, the anti-*Listeria* mechanism of 3-

phenyllactic acid was investigated and, hence, the antimicrobial effect of PLA was evaluated to different pH. Moreover, the PLA anti-*Listeria* has been compared with those expressed by the lactic acid and the better studied hydroxybenzoic and hydroxycinnamic acids. *Listeria innocua* was chosen as the indicator to investigate the antimicrobial mechanism of PLA. *Listeria innocua* is regarded as a non-pathogenic indicator for the presence of *Listeria monocytogenes* in foods.

This study has evidenced that PLA, which, for many years, has been considered an antifungal metabolite, is also able to inhibit bacteria cells. Moreover, very low concentrations were required to produce anti-*Listeria* activity. MIC values of about 0.47 or 0.94 mg/mL appear compatible with the maximum PLA production revealed in *Lb. plantarum* cultures. In fact, this PhD research (chapters 2 and 3), recognizes that *Lb. plantarum* strains produced up to 0.12 or 0.23 mg/mL and this production level could be increased two or even tenfold when specific cultural strategies were applied. Therefore, a resolution to the gap between PLA required to assure antimicrobial activity and the PLA levels detected in fermentation batches seems possible. This gap has long proven to be a serious obstacle when applying PLA producing bacteria as protective or as anti-*Listeria* cultures in food characterized by neutral or sub-acid pH. Moreover, the relation between pH values and anti-*Listeria* activity of PLA was clarified. A relation between MICs and pH values was found and a significant reduction in PLA anti-*Listeria* activity was detected at the highest pH values tested.

In order to understand the PLA mode of action, the anti-*Listeria* effect produced by PLA at pH 5.5 was compared to the effect illustrated by lactic acid to the same pH value. Lactic acid was the best choice as its antimicrobial mechanism is well known. The results evidenced that the antimicrobial action of PLA was substantially different from LA. The different and more successful effect produced by PLA must be due to its amphiphilic properties resulting from the hydrophobic group-benzene ring and hydrophilic group-carboxy in its chemical structure. These properties would allow an interaction with the lipid and protein in cytoplasmic membrane as well as an interaction with genomic materials. Therefore, a comparison between PLA and the more studied phenolic acids (hydroxybenzoic and hydroxycinnamic) should be researched. Furthermore, to better understand the antimicrobial effect of PLA on *L. innocua*, further experiments were conducted using three phenolic compounds (gallic, ferulic and caffeic acid). The results generated from both an MIC and MBC survival test, highlighted that the gallic acid and PLA showed the most performing anti-*Listeria* activity. However,

the differences between PLA and GA in death kinetic parameters suggest that PLA produces anti-*Listeria* activity through a specific mechanism which is somewhat different from those usually adopted by other phenolic compounds. Antimicrobial activity of phenolic acids involves several mechanisms of action such as permeability destabilization or the rupture of the cytoplasmic membrane as well as enzymes inhibition through nonspecific interaction. It is possible to hypothesize that PLA utilizes more than one of these pathways but differently from the other phenolic compounds. More exhaustive results were obtained by the evaluation of the effect of phenolic acids on surface charge and loss of cellular content. In fact, zeta potential measurements demonstrated that after phenolic acids exposure, the cells become more ($P<0.05$) negatively charged when exposed to PLA. While in the presence of other phenolic compounds (GA, CA, FE) no variation in charge was detected. This fact may open new horizons to the understanding of the PLA anti-*Listeria* mechanism. It is possible to surmise that the PLA anti-*Listeria* action is also associated with the affinity with cell surface and the interaction PLA-cell surface could contribute to the damage of cellular structures. The rupture of cellular structures was also supported by the results of the cellular content loss. The results could help to explain the differences in the anti-*Listeria* mechanism of phenolic compounds. Hydroxybenzoic and hydroxycinnamic acid seem to induce an alteration in membrane permeability without causing its rupture. Whereas, PLA having the main targets in cellular surface and in cytoplasmic membrane, leads to a severe rupture of the cellular structures. All these evidences contribute to the enrichment of scientific knowledge in the anti-*Listeria* mechanism of PLA and highlighted that PLA effectiveness is superior to that expressed by other preservative acids. Finally, in **Chapter 6** are reported the general conclusions of PhD research.

CHAPTER 1

STATE OF THE ART

1.1 Quality and safety in food

The terms “food quality” and “food safety” mean different things to different people. Quality has a vast number of meanings and can encompass parameters as diverse as organoleptic characteristics, physical and functional properties, nutrient content and consumer protection from fraud. Furthermore, it can cover political and social issues such as wages paid to farm workers, geographical issues such as controlled appellations, and religious issues such as halal and kosher. Safety is more straightforward, relating to the content of various chemical and microbiological elements in food (Saraoui et al., 2017). Food quality and safety take on greater scope as the global food supply evolves. Even the concept of a nutrient has changed in recent years (Fageria, 2016). In the not-so-distant past, a nutrient was simply defined as a substance that an organism had to obtain from its surroundings for growth and sustenance for life. Now, many components of foods such as polyphenols, which are not necessary for livelihood, are characterized as nutrients (Ozdal et al., 2016). The same is true for isoflavones, coumestrol, non-provitamin A carotenoids and other phytochemicals. Those who do not characterize these compounds as nutrients are inclined to call them beneficial bioactive components. Yet there can be too much of a good thing, and benefit can turn to risk, even for conventional nutrients. The ever-growing interest in nutrients and related substances, and the increasing amounts used in foods (such as fortificants, antioxidants, etc.) and supplements, led to develop a model for establishing safe upper levels of intake of nutrients and related substances (Yates et al., 2017).

1.1.1 Food chain approach

During the last couple of decades, the credibility of the food industry was heavily challenged after a number of food crises, such as Bovine Spongiform Encepha- lopathy (BSE) or mad cow disease, Dioxin in chicken feed, Food-and-Mouth Disease (FMD) and issues such as the use of Genetically Modified (GM) crops in foods. The outbreak of foodborne illnesses such as salmonella, campylobacter and *Escherichia coli* O157:H7 also further increase consumer concerns over the safety and quality of food. As a consequence of food scandals and incidents, customers call for high quality food with integrity, safety guarantees and transparency (Trienekens and Zuurbier, 2008). Therefore, keeping safety and quality along the food supply chain has become a significant challenge. In response to growing food safety issues, the laws, policies and standards regarding food safety and quality management have been developed for the food industry. Quality assurance has become a cornerstone of food safety policy in the food industry that started to implement integrated quality and food safety management systems. To supply top quality, safe and nutritious foods, as well as rebuild public confidence in the food chain, the design and implementation of whole chain traceability from farm to end-user has become an important part of the overall food quality assurance system (Opara, 2003). FAO (2003) stated managing food safety and quality as a shared responsibility of all actors in the food chain including governments, industry and consumers. Compositional changes, for better or for worse, can be introduced at each and every link in the food chain. Adopting a food chain framework goes beyond ensuring the safety of food (Aung and Chang, 2014). It facilitates a more general approach to quality in agriculture and food safety and quality systems that will comprise government, industry and consumer participation. This implies potential future shifts in the agricultural sectors of many countries. For example, plant breeders are using genetic resources to increase the nutrient contents of foods at source. Farmers are also exploring new farming and technological choices to meet demands for a safe and healthy diet in response to new regulations and standards, changing global consumption patterns, improved market access and value added opportunities, as well as increasing concerns over the sustainability of existing agricultural systems. The FAO's "Strategy for a Food Chain Approach to Food Safety and Quality is a framework document designed to encourage the development of future strategic direction" (FAO, 2003) which broadly outlines the most important issues in the development of a food chain approach to food

safety, while the broader implications of a food chain approach on production and post-production systems, biosecurity and nutrition are addressed in other Committee on Agriculture (COAG) documents. The FAO recognizes the need to more fully incorporate a food chain approach in its food quality, safety and nutrition strategies and acknowledges that this revised strategic direction will require an integrated and preventative approach to the management of food safety, meeting sustainability concerns and building on aspects of the implementation of international commitments. While the developments may be largely beneficial, the composition of the foods need to be monitored to ensure that no harm comes to the consumers.

1.1.2 Food safety versus food quality and new requirement

Food safety and food quality are two important terms which describe aspects of food products and the reputations of the processors who produce food. The Codex Alimentarius Commission (CAC, 2003) defines food safety as an assurance that food will not harm the consumer when it is prepared and/or eaten according to its intended use. Food safety refers to all hazards, whether chronic or acute, that may cause food to be harmful to the consumer. It is not negotiable and a global issue affecting billions of people who suffer from diseases caused by contaminated food. Both developed and developing countries share concerns over food safety as international food trade and cross-border movements of people and live animals increase (Asian Productivity Organisation, 2009). In industries, such as telecommunications, software development and airlines, security is the principal driver for traceability, in contrast to the food industry where the safety is a genuinely important issue (Opara, 2003).

Food safety hazards may occur at a variety of stages in the food chain. Therefore, food safety is a responsibility to be shared by producers, processors, distributors, retailers, and consumers alike. An important preventative approach that may be applied at all stages of the food chain regards the traceability of food products and the ability of food facilities to provide information about their sources, recipients, and transporters are essential to ensure the safety of food supply (Levinson, 2009).

Quality icdefined by the International Organization for Standardization as “the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs” (Van Reeuwijk, 1998). Quality can also be defined as “conformance to

requirements", "fitness for use" or, more appropriately, in foodstuffs, "fitness for consumption" (Kafetzopoulos et al., 2014). Thus, quality can be described as the requirements necessary to satisfy the needs and expectations of the consumer (Ho, 1994; Peri, 2006). However, food quality is very general, implying many expectations which can differ from consumer to consumer. Quality includes attributes that influence a product's value to the consumer. Quality does not refer solely to the properties of the food itself, but also to the ways in which those properties have been achieved (Morris and Young, 2000).

Many experts have argued that safety is the most important component of quality, since a lack of safety can result in serious injury and even death for the consumer. Safety differs from many other quality attributes since it is difficult to observe. A product can appear to be of high quality (i.e. well coloured, appetizing and flavourful, etc.), however, it can be unsafe as it may be contaminated with undetected pathogenic organisms, toxic chemicals, or physical hazards (UN, 2007). Rohr et al. (2005), Grunert (2005) and Pinto et al. (2006) agreed that food safety has become an important food quality attribute.

Defects and improper food quality may result in consumer rejection and lower sales, while food safety hazards may be hidden and go undetected until the product has been consumed. If detected, serious food safety hazards may result in market access exclusion and major economic losses and costs. Since food safety hazards directly affect public health and economies, achieving correct food safety must always take precedence over achieving high levels of other quality attributes (UN, 2007). These two have obvious links, but food quality is primarily an economical issue decided by the consumer, while food safety is a governmental commitment to ensure that the food supply is safe for consumers and meets regulatory requirements (Sarig, 2003). Quality is seen to lead to taste, health, safety and pleasure. Similarly, safety is seen to be the consequence of control, origin, best before date and quality, while resulting in health and perceiving a feeling of calm. Both quality and safety are interrelated and linked to trust and confidence (Rijswijk and Frewer, 2006).

On the basis of the above reported statement, in the food safety field, quantitative microbial risk assessment (QMRA) has been applied for many years. This approach is primarily aimed at assessing the influence of different control measures on level of safety, rather than an absolute assessment of risk and, in addition, can thus provide an objective and scientific basis for risk management decisions (Guillier et al., 2016).

However, an integrated public health assessment also depends on other elements such as sustainability of food chains and green label food. Food sustainability is becoming an increasingly important issue because food systems are not sustainable in terms of their consumption of resources, their impact on ecosystems or their effect on health and social equality (Esnouf, 2013). This interest is confirmed by the growing number of call for research projects dedicated to the sustainable food production suggesting several topic such as carbon labeling and reducing food waste. Is widly reported that the use of energy, resources and the emission of Green House Gases (GHG) in the entire food cycle, including production, consumption, and transportation is unavoidable. The initiatives to use carbon labelling (i.e. carbon footprints of the products) and conception of food miles (the distance that food is transported as it travels from producer to consumer) indicate that the food chain needs more environmentally friendly solutions to reduce the environmental impacts such as pollution and global warming. The wastage of food and resources used for growing unused products are also a big issue for the environment. In many countries, one of the problems concerning food safety and quality is food spoilage. Food spoilage is wasteful, costly and can adversely affect trade and consumer confidence (Flores and Tanner, 2008).

On the basis of the above reported statements, the knowledge of the main spoilage and pathogens bacteria as well as the study of advances in food technologies represents a crucial step.

1.1.3 Spoilage and pathogen microrganisms

Many food products are perishable by nature and require protection from spoilage during their preparation, storage, and distribution, in order to give them a desidered shelf life. The demand for minimally processed, easily prepared, and ready-to-eat fresh food products, the globalization of food trade, and distribution from centralized processing, pose major challenges for food safety and quality. Food products can be subjected to contamination by bacteria and fungi. Many of these microorganisms can cause undesirable reactions that deteriorate the flavor, odor, color, sensorial and textural properties of foods. Microbial growth is a major concern because some microorganisms can potentially cause food-borne illness. In packaged foods, the growth and survival of common spoilage and patoghenic microorganisms such as *Listeria monocytognes*, *Escherichia Coli* O157, *Salmonella*, *Staphylococcus aureus*, *Bacillus cereus*,

Campylobacter, *Clostridium perfringens*, *Aspergillus niger*, and *Saccharomoyces cerevisae* are affected by a variety of intrinsic factors, such as pH values and the presence of oxygen or by extrinsic factors associated with storage conditions, including temperature, time and relative humidity (Singh et al., 2003; Lòpez-Malo et al., 2005)

1.1.3.1 Spoilage microorganisms

Many studies have reported the main bacterial species associated with various types of spoilage from a large range of foodstuffs. The ability of some species to grow under or resist the harsh conditions encountered during processing and storage has been described. For various foods of animal origin, Gram-positive bacteria, and especially Firmicutes, have often been reported as spoilers (Bron and Kleerebezem, 2011). In particular, lactic acid bacteria (LAB), encompassing *Lactococci*, *Lactobacilli*, *Leuconostoc*, *Weissella*, and *Carnobacteria* species, as well as *Enterococci* are frequently associated with spoilage. In fact, although LAB are generally beneficial for food and are used for the fermentation of a variety of food and raw materials, where they contribute to flavor, texture and shelf-life (Bron and Kleerebezem, 2011), some species can play a significant role in food spoilage and decay. This is the case, for instance, of *Lactobacillus alimentarius*, known as a specific spoilage organism in marinated herring (Lyhs et al., 2001), and of *Lactobacillus sakei* and *Lactobacillus curvatus*, also found in the spoilage microbiota of herring (Lyhs and Bjorkroth, 2008). *L. sakei* was reported as predominant in the spoilage microbiota of sliced, vacuum-packed, smoked, oven-cooked turkey breast fillets, which developed sour spoilage flavors (Samelis et al., 2000). *L. curvatus* was also found to be one of the specific spoilers in cold-smoked salmon (Jørgensen et al., 2000), as was *L. sakei* (Stohr et al., 2001; Joffraud et al., 2006). The latter was also isolated in the spoilage microbiota of brined and drained shrimp stored under modified atmosphere packaging (Mejlholm et al., 2012, 2008). *Carnobacterium* is another LAB genus commonly involved in the spoilage process of food, with notably *Carnobacterium divergens* and *Carnobacterium maltaromaticum* known to dominate the spoilage microbiota of different meat and seafood products, particularly those packed under vacuum or modified atmosphere (Laursen et al., 2005; Leisner et al., 2007; Vasilopoulos et al., 2008). Other LAB genera and species have also been incriminated in the spoilage process of various food

products. *Enterococcus faecalis* and *Enterococcus faecium* can be involved in the greening of meat products (Foulquié-Moreno et al., 2006).

In addition to LAB species, other Gram-positive bacteria can play a significant role in food spoilage. One of the most prominent is the psychrotrophic species *Brochothrix thermosphacta*, known as an important spoiler bacterium of various food matrixes (Rattanasomboon et al., 1999; Russo et al., 2006). *B. thermosphacta* is a ubiquitous microorganism throughout the meat production chain, from animal to food. It was isolated from beef carcasses during boning, dressing and chilling. Moreover, lairage slurry, cattle hair, rumen content, walls of slaughterhouses, hands of workers, air in the chill room, neck and skin of the animals as well as the cut muscle surfaces have all been shown to be contaminated by this organism (Nychas et al., 2008). In vacuum, packed meat products or modified atmosphere, *B. thermosphacta* can dominate the spoilage microbiota at the expense of other genera, such as *Carnobacterium*, *Lactobacillus* or *Leuconostoc* (Borch et al., 1996; Ercolini et al., 2006). *B. thermosphacta* was also found in the spoilage microbiota of raw turkey breasts (Samelis et al., 2000), pork meat (Bohaychuk and Greer, 2003) and cooked ham (Samelis et al., 1998). It was identified as the dominant spoiler in freshly prepared chicken products (Liang et al., 2012) and was responsible for a cheesy/dairy off-odor and discoloration of chilled vacuum-packaged lamb meat (Gribble and Brightwell, 2013). Seafood products also provide a favorable niche for *B. thermosphacta* development, i.e. neutral pH, high water activity (Aw) and high content of low molecular weight compounds such as free amino acids and nucleotides (Jeyasekaran et al., 2006).

More recently, *B. thermosphacta* was found to be predominant in meat and coral of king scallops (*Pecten maximus*), during storage under air at low temperature (Coton et al., 2013). The growth of *B. thermosphacta* in vacuum-packed meat products depends largely on the amount of available oxygen remaining in the package. *B. thermosphacta* may become the dominant spoilage species when oxygen is present, but is displaced by *Lactobacillus* species under anaerobic conditions. The spoilage potential of *B. thermosphacta* in vacuum-packed meat products is thus influenced by factors that control the level of oxygen in the pack like, for example, the film permeability, the residual gas composition and the pack integrity (Gribble and Brightwell, 2013). In vacuum packs, there is often enough residual oxygen for *B. thermosphacta* to metabolize offensive off-odors. The anaerobic growth of *B. thermosphacta* at chill temperatures is also reduced by meat pH values of less than 5.8; consequently *B.*

thermosphacta is most often associated with the early spoilage of high pH meat cuts of lamb and beef (Bell, 2001; McClure et al., 1993).

Several species have been reported as responsible for the so-called blown-pack defect of vacuum-packed food due to gas production. This defect is mostly associated with chilled fresh meat particularly beef, lamb and venison, but was also reported to have been found in cooked meat products (Broda et al., 1996). The causative agents include psychrotrophic *Enterobacteriaceae* or *Clostridium s*pecies such as *Clostridium estherteticum* (Brightwell et al., 2007; Hernandez-Macedo et al., 2012; Yang and Badoni, 2013). Other *Clostridium* species (*Clostridium algidixylanolyticum*, *Clostridium frigidicarnis*, *Clostridium frigoris*, *Closdridium gasigenes*, and *Clostridium algidicarnis*) may also cause blown pack spoilage of vacuum-packed beef (Silva et al., 2011; Yang and Badoni, 2013). In addition, the acid tolerant species *Clostridium perfringens*, *Clostridium barati*, and *Clostridium butyricum* have been reported as the causative agents of the spoilage of canned pasteurized mung bean sprouts, stored under acidic conditions (de Jong, 1989).

Gram-negative bacteria with species belonging to the genera *Serratia, Hafnia*, and *Pseudomonas* have also often been incriminated. Some species like *Shewanella baltica* and *Photobacterium phosphoreum*, typical of sea water or sediment, have been specifically reported in seafood spoilage. *Shewanella* sp., notably *Shewanella putrefaciens* or *S. baltica*, are typical spoilers of fresh marine fish (Gram and Huss, 1996; Broekaert et al., 2011), such as halibut (Hovda et al., 2007b), sea bass (Papadopoulos et al., 2003), mullet (Pournis et al., 2005), swordfish (Pantazi et al., 2008), salmon filets (Macé et al., 2013), and sea bream (Tryfinopoulou et al., 2007). *Shewanella* have also been found on smoked fish such as blue cod or salmon (Penney et al., 1994; Joffraud et al., 2001).

Enterobacteriaceae (Enterobacteria) can also play a key role in food spoilage due to their ability to metabolize amino acids to malodorous volatile compounds, such as foul-smelling diamines and sulphuric compounds (Baylis, 2006; Borch et al., 1996; García-López et al., 1998; Samelis, 2006). A range of enterobacteria may grow on chilled meats: *Hafnia alvei*, *Pantoea agglomerans*, *Rahnella* spp., *Serratia* spp., and *Yersinia enterocolitica* are frequently reported at the end of chilled storage (Sade et al., 2013).

Pseudomonas spp., particularly *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas fragi*, also contribute to a large extent to the spoilage process of food. These are the predominant spoilers of proteinaceous raw foods stored under aerobic

refrigerated conditions (Nychas et al., 2008), especially aerobically chill-stored beef (Doulgeraki and Nychas, 2013; Liu et al., 2006), sea-food (Gram and Huss, 1996; Macé et al., 2013; Reynisson et al., 2008), poultry (Dominguez and Schaffner, 2007; Mellor et al., 2011), and milk (Quigley et al., 2013; Stevenson et al., 2003). In fact, as already well described by Arslan et al. (2011), *Pseudomonas* spp. may cause spoilage of dairy products through the production of extracellular enzymes that are heat-stable and can therefore remain active during pasteurization or ultra-high-temperature treatments. *P. fluorescens* also plays an important role in the spoilage of aerobically stored chicken meat by producing bio-surfactants which provide a competitive advantage to producing strains in a number of ways e.g. enhancement of water-immiscible substrate bioavailability and of adhesion to surfaces, antibiotic activity or motility facilitation (Mellor et al., 2011). *P. fragi* is involved in the spoilage of fresh food products, such as milk, stored under refrigerated aerobic conditions (Ercolini et al., 2007) where it can produce thermo-resistant extracellular enzymes, causing spoilage and structural defects in pasteurized and ultra-high-temperature-treated milk (De Jonghe et al., 2011; Dogan and Boor, 2003; Marchand et al., 2009). Other *Pseudomonas* species were reported to express a significant spoilage potential of milk, such as *Pseudomonas gessardii*, *Pseudomonas lundensis*, and *P. fluorescens*-like (De Jonghe et al., 2011; Marchand et al., 2009). In vegetables, several species of *Pseudomonas* have been identified as spoilage bacteria, including *Pseudomonas chlororaphis*, particularly effective on many vegetables such as lettuce, red mustard seeds, broccoli, onion, potato, or carrot, by producing plant cell wall-degrading enzymes (PCWDEs) like pectate lyase (Lee et al., 2013). Some *Pseudomonas* species can also produce biofilm on food and food-processing equipment (Kerekes et al., 2013). The biofilm-forming bacterial cells can communicate through a density-dependent cell-to-cell communication mechanism, quorum sensing, notably by releasing signaling molecules, N-acyl homoserine lactones (AHLs), into their environment. Gram et al. (2002) reported the widespread occurrence of AHLs in fish products, poultry and vacuum-packed meat. AHL production is a widespread phenomenon in food-spoiling bacteria.

1.1.3.2 Pathogens bacteria

Many stress resistant bacteria are able to contaminate food products and be a potential source of human illness. In the last decade, illnesses resulting from food borne pathogens have been higher than in the past and have become one of the most widespread public health problems in the world.

Escherichia coli

Escherichia coli are a group of motile, Gram-negative facultative anaerobes that are part of the natural microflora in the intestinal tracts of humans and other warm-blooded animals (Montville et al., 2012). While most strains are harmless, some are pathogenic and cause a diarrheal infection ranging from mild to severe, with complications arising in some individuals. These pathogenic strains are largely transmitted via the fecal-oral route and have been categorized into groups based on their virulence, pathogenic mechanisms, clinical syndromes and serotypes. The diarrheagenic *E. coli* includes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC). Combined, strains of diarrheagenic *E. coli* have been estimated to cause over 200,000 cases of foodborne disease each year in the US, resulting in close to 2,500 hospitalizations (Scallan et al., 2011). EHEC strains are able to produce cytotoxic factors named verotoxins or Shiga toxins (Stx), and infection with these strains can lead to serious complications, such as hemorrhagic colitis, kidney failure, HUS, thrombotic thrombocytopenia purpura (TTP) and death (FDA, 2012). *Escherichia coli* O157:H7 is the predominant serotype of EHEC implicated in human illness, accounting for about 75% of infections globally, and it has a very low estimated infectious dose of 10–100 cells (FDA, 2012). *E. coli* O157:H7 generally grows best around 37°C (Doyle and Schoeni, 1984); however, the optimal growth temperature varies depending on the strain (Gonthier et al., 2001). Although *E. coli* O157:H7 is not able to maintain growth at reduced temperatures (e.g. 10 °C), it is able to survive freezing for long periods of time (Doyle and Schoeni, 1984). *Escherichia coli* O157:H7 is also exceptionally tolerant of acidic conditions compared to most other foodborne pathogens, with growth occurring as low as pH 4.0–4.5 (Montville et al., 2012). Interestingly, sublethal exposure to one type of environmental stress has been shown to

increase the overall resistance of this organism to other types of stressors, thereby enhancing its survival in these circumstances (Franz and van Bruggen, 2008).

Early outbreaks of foodborne illness from *E. coli* O157:H7 were predominantly associated with the consumption of undercooked beef products. While beef products continue to be implicated in many *E. coli* O157:H7 infections, there has been an increasing trend for contamination of fruit and vegetables as well, such as unpasteurized juices, fresh lettuce, sprouts and bagged spinach (FDA, 2012). Fresh produce may become contaminated with this pathogen in the pre-harvest environment through contact with fecal material from sources such as domestic livestock and wildlife (Mandrell, 2009). For example, during a 2006 outbreak investigation of *E. coli* O157:H7 in spinach, matches to the outbreak strain were detected in feral swine feces, cattle feces and soil samples collected near the spinach field (Jay et al., 2007). Some potential means of dispersal for *E. coli* O157:H7 into production fields include watersheds, aerosols, animal effluents or animal intrusion (Fremaux et al., 2008b; Mandrell, 2009). Once in the soil, *E. coli* can survive for extended periods of time, potentially contaminating fruit and vegetables (Fremaux et al., 2008b) and migrating to other areas, such as groundwater (van Elsas et al., 2011).

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, non-spore-forming bacterium that is ubiquitous in nature and can withstand adverse environmental conditions (Montville et al., 2012). This organism is motile, facultatively anaerobic and psychrotrophic, with a growth temperature range of between 0–45 °C. *Listeria monocytogenes* demonstrates tolerance to salt, dehydration and acidic conditions, and can survive for extended periods in or on food, soil, plants and hard surfaces. *Listeria monocytogenes* has several known animal reservoirs, such as cattle, deer and goats, and can also proliferate in water environments, including surface waters of canals and lakes, ditches and sewers. Thirteen serotypes of *L. monocytogenes* have been recorded, but the majority of human cases (95%) have been associated with serotypes 1/2a, 1/2b or 4b (Montville et al., 2012). While human infections with *L. monocytogenes* are less numerous than those with other foodborne pathogens, the effects can be severe and it is one of the leading causes of death from foodborne illness in the US (FDA, 2012). Cases of listeriosis range from mild, self-limiting gastroenteritis to serious, invasive infections with

complications such as meningitis, septicemia and abortion or stillbirth in pregnant women. Listeriosis is primarily associated with ready-to-eat-foods, milk products, cheeses, meat and poultry products and seafood. As it does not survive most heat treatments, *L. monocytogenes* usually contaminates these foods in the post-processing environment, such as through food-contact surfaces or cross-contamination with raw materials (Montville et al., 2012). It can also be found in foods that are not heat-treated prior to consumption, such as unpasteurized milk and cheeses made from unpasteurized milk (Silk et al., 2013). There is an emerging link between *L. monocytogenes* and fresh produce outbreaks. For example, a 2011 outbreak of *L. monocytogenes* in fresh whole cantaloupe represented the deadliest outbreak of foodborne listeriosis in the USA (Laksanalamai et al., 2012) and the first outbreak of this pathogen in a whole fruit raw agricultural commodity (FDA, 2011). Contamination was found to be most likely due to factors associated with the growing environment, the packing facility and the process for cold storage. Additional outbreaks linking *L. monocytogenes* to fresh produce include an outbreak of raw sprouts in 2008–2009 and pre-cut celery in 2010 (Gould et al., 2011; Silk et al., 2013).

Salmonella spp.

Non-typhoidal *Salmonella* spp. are the leading cause of foodborne illnesses, hospitalization and death among bacterial pathogens in the US, with over 1 million cases annually (Scallan et al., 2011). Like *E. coli*, *Salmonella* are motile, Gram-negative facultative anaerobic bacteria that belong to the family Enterobacteriaceae and exhibit optimal growth around 37°C (Montville et al., 2012). These organisms are classified into over 2,500 serovars of two species: *S. enterica* and *S. bongori*. While both species are known pathogens, *S. enterica* is responsible for the majority of *Salmonella* associated foodborne illnesses, with 99% of reported human isolates in the USA belonging to *S. enterica* subsp. *enterica* (CDC, 2011). Non-typhoidal *Salmonella* strains are widespread in nature, including pond-water sediment, and can colonize the intestinal tracts of vertebrate animals, including livestock, wildlife, domestic pets and humans (FDA, 2012). These strains exhibit a great deal of diversity, with variations in factors such as metabolic properties, survivability and virulence factors (Montville et al., 2012). For example, some strains of *Salmonella* are capable of growth at psychrotrophic temperatures (2°C) while others have been found to grow at elevated temperatures (up

to 54°C). In general, *Salmonella* are resilient to environmental conditions and are able to persist in the environment outside an animal host, with extended survival times as compared to *E. coli* in terrestrial habitats (Franz and van Bruggen, 2008; van Elsas et al., 2011). Similar to *E. coli* O157:H7, *Salmonella* has demonstrated an ability to grow in acidic environments and increased survival under adverse conditions following exposure to acid stress (Montville et al., 2012). Increased temperatures can further enhance the ability of *Salmonella* to demonstrate resistance to environmental stresses (Montville et al., 2012). These bacteria are spread to humans through the fecal-oral route and cause a gastrointestinal illness called salmonellosis which is usually self-limiting in healthy individuals. Salmonellosis has traditionally been associated with animal products, such as undercooked poultry and eggs; however, more recently other food items have also been implicated, including fresh produce, nuts and spices. The presence of *Salmonella* in the outdoor environment and its transmission among animals make it a problem in the food supply. Incidences of salmonellosis have been correlated to increased ambient temperatures in studies carried out in regions of Australia, Canada and Europe (Tirado et al., 2010). For example, a time-series analysis carried out among 10 European countries found that, for most of the countries analyzed, each 1°C increase in temperature above a 6°C threshold corresponded to a 5–10% increase in cases of salmonellosis (Kovats et al., 2004). However, these associations may be related to seasonal effects on human behavior rather than direct effects of climate on the dispersal and persistence of the pathogen in the environment.

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive, non-spore-forming facultative anaerobe that is ubiquitous in the outdoor environment, including soil, dust, water and air (FDA, 2012). This organism is also part of the natural microbiota associated with humans and animals and is predominately found on the skin, hair or in the nasal passages (Le Loir et al., 2003; Madigan et al., 2012). *Staphylococcus aureus* typically enters foods through contamination from humans, animals, or the environment (FDA, 2012). *Staphylococcus aureus* causes a number of human illnesses, including foodborne intoxication due to the ingestion of highly heat-stable enterotoxins produced in foods. Staphylococcal foodborne illness is associated with acutely intense gastroenteritis, including vomiting and diarrhea, which is normally of short duration and self-limiting in healthy

individuals. Foods commonly associated with S. aureus intoxication are those that require substantial handling for preparation and those that are held at ambient temperatures for extended periods, such as dairy products, meat and poultry products, ready-to-eat salads, such as egg, tuna and potato and bakery produce. *Staphylococcus aureus* is non-motile and mesophilic, with optimal growth occurring at around 35°C. It is among the most highly resistant non-spore-forming human pathogens and is exceptionally osmotolerant. For example, it can survive in a dry state for extended periods of time and is able to grow in foods with water activity as low as 0.83–0.86 (FDA, 2012; Montville et al., 2012).

1.1.4 Advances in Food Preservation Technologies

Advances in food technologies reflect the changes in market requirement. Food quality requirements change constantly and, compared to foods from recent years, they now encompass desires for foods that are convenient to store and use and yet are better quality, "fresher", "more natural" and "healthier" than hitherto. At the same time, increased awareness of the risks of food poisoning has ensured that a high degree of assurance and, indeed improvement, of safety are perceived as key requirements too.
The main thechnologies that are employed to preserve the quality and microbiological safety of foods include:

1. procedures that prevent the access of microorganisms to foods in the first place;
2. procedures that inactivate them should they nevertheless have gained access;
3. procedures that prevent or slow down their growth should they have gained access and not been inactivated.

Whilst the currently used traditional preservation procedures continue to act in one of these three ways, there has recently been a reawakening of interest in the modification of these technologies, mainly in the direction of reducing the severity of the more extreme techniques. These modifications are being sought primarily to improve the quality of food products and, principally, in order to meet the requirements of consumers through the avoidance of the extreme use of any single technique. In addition to the modified techniques, but with the same objective of improving food quality, radically new techniques are also being researched and applied. For both the modified and the new techniques it is imperative that they deliver, not only the

promised improvements in quality, but also an equivalent or preferably an enhanced level of safety compared with the procedures that they replace.

Only some of the existing technologies for food preservation act primarily by restricting the access of microorganisms to foods except at the terminal phase of production of thermally processed foods, so that packaging restricts access.

There are more precedures that act via inactivation but still, considering the tonnes of foods treated, only heat is used substantially.

There are currently many more procedures available to slow down or prevent the growth of microrganisms in foods, including those that rely on control of the environment (e.g. temperature control), those that result from particular methods of processing (e.g. microstructure control) and those that depend on the intrinsic properties built in to particular formulated foods (e.g. control by the adjustment of water activity or pH value).

1.1.4.1 New and improved techniques

With regards procedures that restrict the access of microorganisms to foods, the use of aseptic packaging techniques for thermally processed foods has expanded greatly in recent years, both in the numbers of applications and the numbers of alternative techniques that are commercially available.

With regards to the improvement of techniques for the inactivation of microrganisms in foods, most effort and new application has concerned thermal processing. A particular aim has been to minimise damage to product quality. This is being pursued in two, often complementary, ways. Firstly, by the wider application of more high temperature-short time processing, with associated aseptic packaging where relevant. Secondly, by delivering heat in new ways, e.g. by microwaves or by electrical resistance ("ohmic") heating of foods, which allow better control of heat delivery and minimise over-cooking that commonly occurs in more conventional thermal processes. An important safety consideration that must be borne in mind is the overall reduction in total heat delivery to foods that will result from the wider application of these techniques, as target F0 values are more and more stringently achieved.

The use of ionising radiation to preserve foods or to eradicate pathogens from them, is already well established. In addition to its value as a preservation technique, it offers a very effective route for the reduction in food poisoning, e.g. via the irradiation of the

often *Salmonella* and *Campylobacter* contaminated foods such as poultry and other foods of animal origin. Whilst the use of radiation continues to grow worldwide, negative consumer reaction in many countries holds back its wider use.

Radically new procedures for the inactivation of microrganisms in foods include two other physical procedures that offer alternatives to heat: the use of high hydrostatic pressure and the use of high voltage electric pulses. Both techniques are highly effective in inactivating vegetative cells of bacteria, yeasts and filamentous fungi, at pressures and at voltage gradients that are compatible with the retention of high quality in some foodstuffs. However, bacterial spores remain more difficult to control with these procedures, and their use for the preservation of foods other than relatively short shelf-life or products in which spores are not a problem because they are inhibited by the intrinsic properties of the food (e.g. low pH or low water activity) must await further research.

Finally, concerning novel inactivation procedures, the effectiveness of ultrasonic radiation in inactivating the vegetative forms of microorganisms has been well known for many years. However, recent research showing that its efficacy can be enhanced by the simultaneous application of, relatively low, hydrostatic pressure, is leading to a re-evaluation of its potential as a food preservation aid.

A particularity is that an important new inactivation technique has been developed from surface decontamination procedures that can be applied to meat and poultry carcasses, and to other animal-derived foods which are known to be potentially contaminated with enteric pathogens. In many countries, unacceptably high levels of enteric infection in the human population still occur, and the situation is getting worse rather than better. Many food microbiologists have come to realise that although improved hygiene education and the application of Hazard Analysis and Critical Control Point techniques etc. may all help to improve food poisoning statistics, a major reduction will only be achieved if such new elimination techniques are employed. If the organisms concerned did not enter the home or the catering establishment etc. in the first place, then the momentary lapses of hygiene that always occur, at some frequency or other, would be of little consequence.

With regards procedures that slow down or prevent the growth of microorganisms in foods, major successes have been accomplished and new applications are steadily being introduced, through the use of “combination preservation” techniques or “hurdle technology”. This has been supported by a greatly improved understanding of the

principles underlying the stability and safety of an enormous number of combination-preserved foods that are traditional and indigenous to different parts of the world. It has also been supported by the beginning of an understanding of how many of these combination procedures act at the cellular level, which often seems to involve "multitarget" interference with the various homeostatic mechanisms that are fundamental to the reaction of microrganisms to the stresses to which the food technologist exposes them in foods.

Though still a relatively new technology, modified atmosphere packaging has grown rapidly in use in some countries, particularly for the extension of the high-quality shelf-life of certain chill-stored foods. It remains, however, seldom used in other countries. Again, considering its wide use, it is surprising that a full understanding of how modified atmospheres (particularly the carbon dioxide component that most of them contain) that exert their inhibitory effects at the level of cell biochemistry, have not yet been worked out. Elucidation of the mechanisms of action could lead to improved means for effective application.

To some extent, interest in naturally occuring antimicrobial systems has expanded in recent years, in response to consumer requirements for fresher, more natural additive-free foods. With a few notable exceptions, very few of these systems have yet been taken through to application. However, the substantial research efforts underway on animal derived, plant derived and microorganism derived antimicrobial systems, are demonstrating the efficacy of a wide range of natural mechanisms, many of which have potential for use in food preservation. So far, few such natural systems have been included as components in combination studies, i.e. such as additional "hurdles". This is a pity, because the food technologist would have many opportunities to use these systems in a wide range of combinations with other potential inhibitors. Furthermore, it is arguable that although *in vitro* studies are necessary to investigate mechanisms of action and for intense genetical and biochemical studies, too few studies of natural systems have yet to be undertaken using actual foodstuffs. Sound and extensive food studies are essential prerequisites for food manufacturers before they commit to expending efforts or making the necessary investment to bring new preservation systems into successful application.

1.2 Biocontrol

Foodborne diseases are among the most serious and costly public health concerns worldwide, being a major cause of morbidity. In spite of modern technologies, good manufacturing practices, quality control and hygiene and safety concepts, the reported numbers of foodborne illnesses and intoxications have nevertheless increased over the past decade. The most common foodborne infections in the European Union (EU) are caused by bacteria, namely *Campylobacter*, *Salmonella* and *Listeria*, and viruses. They are reported to affect over 380,000 EU citizens each year (EFSA, 2009).

Food market globalization, the introduction of novel foods, new manufacturing processes and the growing demand for minimally processed, fresh-cut and ready-to-eat products may require a longer and more complex food chain, increasing the risk of microbiological contamination. Thus, novel and complementary food preservation technologies that comply with these demands from "farm to fork" are continuously in demand. Among alternative food preservation technologies, particular attention has been paid to biopreservation to extend the shelf-life and enhance the hygienic quality, minimizing the impact on the nutritional and organoleptic properties of perishable food products. Biopreservation rationally exploits the antimicrobial potential of naturally occurring (micro-) organisms in food and/or their metabolites with a long history of safe use.

Microbes elicit a variety of mechanisms that facilitate colonization and prevalence in ecological niches. These include adherence, competition for available nutrients, production of toxic metabolites, and secretion of dedicated antimicrobial substances such as antibiotics and bacteriocins. The wise exploitation of these mechanisms of microbial interference can be beneficial to both human and animal health as well as the economy. The transmission of foodborne pathogens through the food chain is still an unresolved issue. The globalization of the food market, and the new trends in food production and distribution, together with changes in consumer habits and population susceptibility (such as the elderly or immuno-compromised people) are always highlighted as being the main contributing factors. In addition, the substantial economic losses because of spoilage of raw materials or processed products and the costly recalls because of microbial contamination are matters of concern in a world that periodically faces economic crisis and increasingly suffers from population overgrowth, malnutrition and overexploitation of natural resources. In developing countries, the incidence of

illnesses caused by foodborne pathogens in younger people also has a clear influence on malnutrition, which in turn has a negative impact on health status and cognitive potential.

Among the wide array of strategies currently being used or proposed for food preservation, control strategies based on natural compounds and living organisms and/or their antimicrobial products (biocontrol, or biopreservation) have been used since ancient times (such as in food fermentation) and are becoming increasingly popular for several reasons: firstly, natural preservation methods are regarded as health-friendly by consumers, and are expected to have a lower impact on the food nutritional and sensory properties (as opposed to chemical or physico-chemical treatments); secondly, they may decrease the processing costs while at the same time extending the product shelf life period, they do not require advanced technological equipment or skills and therefore can be exploited by smaller economies; thirdly, they may offer new opportunities to solve emerging issues such as the increase of antibiotic resistance in the food chain, the need to improve animal productivity by natural means, or the control of emerging pathogens.

1.2.1 Natural substances

In recent years, aromatic plants and their extracts have been examined for their effectiveness for food safety and preservation applications (Fisher and Phillips, 2008; Gyawali and Ibrahim, 2014; Prakash et al., 2015) and have received attention as growth and health promoters (Brenes and Roura, 2010). Most of their properties are due to their essential oils (EOs) and other secondary plant metabolite components (Brenes and Roura, 2010). Phytochemicals, such as EOs, are naturally occurring antimicrobials found in many plants that have been shown to be effective in a variety of applications by decreasing growth and survival of microorganisms (Callaway et al., 2011). In addition, EOs exhibit antimicrobial properties that may make them suitable alternatives to antibiotics (Chaves et al., 2008). These potential attributes and an increasing demand for natural food additive options have led to an interest in the use of EOs as potential alternative antimicrobials (Fisher and Phillips, 2008; Solórzano-Santos and Miranda-Novales, 2012). There has been an extensive search for potential natural food additive candidates that retain a broad spectrum of antioxidant and antimicrobial activities while possessing the ability to improve the quality and shelf life of perishable foods (Fratianni

et al., 2010). Moreover, the emergence of bacterial antibiotic resistance and negative consumer attitudes toward food preservatives has led to an increased interest in the use of plant components that contain EOs and essences as alternative agents for the control of food spoilage and harmful pathogens (Burt, 2004; Fisher and Phillips, 2008; Nostro et al., 2004; Shelef, 1983; Smith-Palmer et al., 1998; 2001; Tremonte et al. 2016).

Natural compounds with animal, plant or microbiological origins have been used in order to kill or at least prevent the growth of pathogenic microorganisms (Juneja et al., 2012; Li et al., 2011; Muthaiyan et al., 2011; Roller and Lusengo, 1997; Tiwari et al., 2009). A number of naturally occurring antimicrobial agents are present in animal and plant tissues, where they probably evolved as part of their hosts defense mechanisms against microbiological invasion and exist as natural ingredients in foods (Sofos et al., 1998; Dias et al., 2012).

1.2.1.1 Plant extracts

Plant extracts have shown considerable promise in a range of applications in the food industry and several plant extracts enjoy GRAS status. The antimicrobial activities of plant extracts may reside in a variety of different components, and several extracts, owing to their phytochemical constituents, have been shown to have antimicrobial activity. The antibacterial activity is most likely due to the combined effects of adsorption of polyphenols to bacterial membranes with membrane disruption and subsequent leakage of cellular contents (Ikigai et al., 1993; Otake et al., 1991), and the generation of hydroperoxides from polyphenols (Akagawa et al., 2003).

Plant extracts also showed antimicrobial activity against a wide range of fungi (Davidson and Parish, 1989; Grange and Ahmed, 1988; Jayaprakasha et al., 2001; Negi et al., 2002; Tremonte et al.; 2016); antioxidant and antimutagenic activities (Boubaker et al., 2011; Cherdshewasart et al., 2009; Horn and Vargas, 2003; Jayaprakasha et al., 2002, 2006, 2007; Negi et al., 2003b, 2010) and inhibited lipid oxidation in foods (Shan et al., 2009).

Dietary herbs and spices have been traditionally used as food additives throughout the world not only to improve the sensory characteristics of foods but also to extend their shelf life by reducing or eliminating the survival of pathogenic bacteria. Many herbs and spices extracts possess antimicrobial activity against a range of bacteria, yeast and molds (Beuchat 2001; Friedman et al., 2002, 2004; Raybaudi-Massilia et al., 2009;

Tajkarimi et al., 2010). Herbs and spices are rich in phenolic compounds and besides exerting an antimicrobial effect, they can preserve foods by reducing lipid oxidation as they are reported to have significant antioxidant activity (Scwarz et al., 2001; Shahidi et al., 1997; Shan et al., 2009; Tanabe et al., 2002; Yanishlieva et al., 2006). A wide variety of phenolic substances derived from herbs and spices possess potent biological activities, which contribute to their preservative potential (Surh, 1999; Careaga et al. (2003; Ahn et al., 2007; Lee et al., 2009; Tornuk et al., 2011;) reported that a range of plant extracts were useful in reducing pathogens associated with cooked beef.

Although numerous studies have been done in-vitro to evaluate the antimicrobial activity of plant extracts, very few studies are available for food products, probably because plant extracts did not produce as marked inhibitions as many of the pure compounds in foods did. The reduced effectiveness may be attributed to the use of crude extracts in most studies. The crude extracts generally contain flavonoids in glycosidic form, where the sugar present in them decreases effectiveness against some bacteria (Kapoor et al., 2007; Parvathy et al., 2009; Rhee et al., 1994).

1.2.1.2 Essential oils

These naturally occurring antimicrobials have extensive histories of their use in foods and can be identified from various components of the plants leaves, barks, stems, roots, flowers and fruit (Erasto et al., 2004; Rahman and Gray, 2002; Zhu et al., 2004). Essential oils are not strictly oils, but are often poorly soluble in water like oils. Essential oils often have a pleasant odor and sometimes a distinctive taste and are therefore used in significant amounts in the flavoring and perfume industries (Burt, 2004). Essential oils are usually prepared by fragrance extraction techniques such as distillation (including steam distillation), cold pressing, or extraction (maceration) (Burt, 2004; Edris, 2007; Faleiro, 2011; Kelkar et al., 2006; Shannon et al., 2011a; Solórzano-Santos and Miranda-Novales, 2012). Typically, EOs are highly complex mixtures of often hundreds of individual aroma compounds. Herbs and spices commonly used in foods have provided most of the EOs that have been studied for their antimicrobial activity (Cueva et al., 2010; Negi, 2012; Tajkarimi et al., 2010).

Essential oils have been documented to be effective antimicrobials against several foodborne pathogens including *E. coli* O157:H7, *Salmonella Typhimurium*, *S. aureus*, *L. monocytogenes*, *Campylobacter* and others (Callaway et al., 2011). Friedman et al.,

(2002) tested 96 EOs and 23 oil compounds against *Campylobacter jejuni*, *E. coli* O157:H7, *L. monocytogenes* and *Salmonella enterica* and found that 27 EOs and 12 compounds had some activity against all 4 bacterial genera. The oils with the most activity included ginger root, jasmine, carrot seed, celery seed, and orange bitter oils (*C. jejuni*); oregano, thyme, cinnamon, bay leaf, clove bud, lemon grass, and allspice (*E. coli* O157:H7); bay leaf, clove bud, oregano, cinnamon, allspice and thyme (*L. monocytogenes*); thyme, oregano, cinnamon, clove bud, allspice, bay leaf, and marjoram (Soković et al., 2010; Cherrat et al., 2014; Sangwan et al., 2008; Callaway et al., 2011; Chalova et al., 2010; Fisher and Phillips, 2008; Di Pasqua et al., 2006; Chikhoune et al., 2013; Settanni et al., 2012; Li and Chiang, 2012; Sun, 2007).

1.2.1.3 Phenolic compounds

Phenolic compounds secondary plant metabolites, are important determinants in the sensory and nutritional quality of fruit, vegetables and other plants. (Tomas-Barberan et al., 2000; Lapornik et al., 2005). Phenolic compounds are widely distributed in plants, such as fruit, vegetables, tea, olive oil, tobacco and others. The plant kingdom offers a wide range of natural antioxidants. Consequently, antioxidants have become an essential part of the preservation technology and contemporary health care. The potential toxicity of some synthetic antioxidants, however, has intensified research efforts to discover and utilise antioxidants from natural sources, such as fruit and vegetables (Popa et al., 2007; Zhang et al., 2009).

These compounds possess an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of a complex high-molecular mass polymer (Balasundram et al., 2006).

As a large group of bioactive chemicals, they have diverse biological functions and may act as phytoalexins (Popa et al., 2008), antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, amongst other things (Naczk and Shahidi, 2006). These bioactive properties allow these compounds to play an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators (Popa et al., 2002; Bravo, 1998), besides contributing to the colour and sensory characteristics of fruit and vegetables (Alasalvar et al., 2001).

In particular, natural phenols were reported to have excellent properties as food preservatives (Valenzuela et al., 1992) as well as playing an important role in the protection against a number of pathological disturbances, such as atherosclerosis, brain dysfunction and cancer (Gordon, 1996). Moreover, polyphenols have many industrial applications, for example, they may be used as natural colourants and preservatives for foods, or in the production of paints, paper, and cosmetics.

Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings they contain and to the structural elements that bind these rings to one another. The main groups of polyphenols are: flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes and lignans.

1.2.1.3.1. Flavonoids

More than 8,000 polyphenolics, including over 4,000 flavonoids have been identified, and the number is still growing (Harborne et al., 1999). Flavonoids can be further classified into anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols (Tsao and Yang, 2003).

Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C. The aromatic ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway (Merken and Beecher, 2000).

Variations in the substitution patterns of ring C result in the major flavonoid classes, i.e., flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, and anthocyanidins (Hollman and Katan, 1999), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne et al., 1999). Substitutions to rings A and B give rise to different compounds within each class of flavonoids (Pietta, 2000). These substitutions may include oxygenation, alkylation, glycosylation, acylation, and sulphonation (Balasundram et al., 2006).

Flavonoids are especially important antioxidants due to their high redox potential, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelating potential (Tsao and Yang, 2003).
Flavonoids are the most commonly found phytochemicals and typically these chemicals help to protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury (Cook and Samman, 1996). When consumed regularly by humans, flavonoids have helped cause a reduction in the incidences of diseases such as cancer and heart disease (Beecher, 2003; Cook and Samman, 1996; Liu et al., 2008). There is currently great interest in flavonoid research due to the possibility of improved public health through diet, where preventative health care can be promoted through the consumption of fruit and vegetables (Caridi et al., 2007).

1.2.1.3.2 Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols, which may be present in plants in both free and bound forms (Robbins, 2003). Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds (Zadernowski et al., 2009). The different forms of phenolic acids result in varying suitability to different extraction conditions and different susceptibilities to degradation (Ross et al., 2009). Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have the C6–C1 structure in common. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6–C3), caffeic, ferulic, *p*-coumaric and sinapic acids being the most common representatives (Bravo, 1998).

Ferulic acid. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a phenolic acid ubiquitously existing in the plant kingdom, which can be absorbed by the small intestines and excreted through urine. It is one of the most abundant phenolic acids in plants, varying from 5 g kg^{-1} in wheat bran to 9 g kg^{-1} in sugar-beet pulp and 50 g kg^{-1} in corn kernel (Kroon et al., 1997; Rosazza et al., 1995). In plants, ferulic acid is rarely found in the free form. It is usually found as ester cross-links with polysaccharides in the cell wall, such as arabinoxylans in grasses, pectin in spinach and sugar beet and xyloglucans in bamboo (Iiyama et al., 1994). It can also cross-link with proteins

(Figueroa-Espinoza et al., 1999). The cross-linking property of ferulic acid with both polysaccharides and proteins suggests that it can be used in the preparation of complex gels in food applications.

In recent years, there have been an increasing number of reports on the physiological functions of ferulic acid and its derivatives in humans. Many applications of ferulic acid in the food industry have also been discovered.

Ferulic acid can be used to preserve food because of its antioxidant and antimicrobial activities and it can also inhibit the growth of bacteria, fungi and yeasts. Ferulic acid is an active ingredient of extracts of some plants showing antimicrobial activity.

Gallic acid. Gallic acid (3,4,5-trihydroxybenzoic acid) and its structurally related compounds are found widely distributed in fruits and plants. Gallic acid, and its catechin derivatives are also present as one of the main phenolic components of both black and green tea. Esters of gallic acid have a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharmaceutical industry. In addition, gallic acid is employed as a source material for inks, paints and colour developers. Studies utilising these compounds have found them to possess many potential therapeutic properties including anti-cancer and antimicrobial properties.

Tannins. Tannins are relatively high molecular compounds which constitute the third most important group of phenolics and may be subdivided into hydrolysable and condensed tannins (Porter, 1989). Proanthocyanidins (condensed tannins) are polymeric flavonoids. Although the biosynthetic pathways for flavonoid synthesis are well understood, the steps leading to condensation and polymerisation have not been elucidated. The most widely studied condensed tannins are based on flavan-3-ols (-)-epicatechin and (+)-catechin. Hydrolysable tannins are derivatives of gallic acid (3,4,5 trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins (Hagerman, 2002). Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants. Because of the varied biological roles that tannins can play and because of the enormous structural variation, it has been difficult to develop models that would allow an accurate prediction of their effects in any system. An important goal of

future work on the biological activities of tannins is the development of structure/activity relationships so that biological activities can be predicted (Hagerman, 2002).

Stilbenes and lignans. Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol, that exists in both cis and trans isomeric forms, mostly in glycosylated forms (Delmas et al., 2006). It is produced by plants in response to infection by pathogens or to a variety of stress conditions (Bavaresco, 2003). It has been detected in more than 70 plant species, including grapes, berries and peanuts.
Lignans are produced by the oxidative dimerisation of two phenylpropane units; they are mostly present in nature in the free form, while their glycoside derivatives are only a minor form. Interest in lignans and their synthetic derivatives is growing because of potential applications in cancer chemotherapy and various other pharmacological effects (Saleem et al., 2005).

1.3 Microbial cell factories for biocontrol

Microbes may produce a wide spectrum of antimicrobial substances. Most studies have focused on antimicrobials produced by lactic acid bacteria (LAB) and associated bacteria such as the propionic acid bacteria and the bifidobacteria. The decreased pH value and antibacterial activities of organic acids produced by LAB are the main mechanisms for biopreservation of fermented foods. Specific strains of LAB may also produce other inhibitory substances (such as diacetyl, reuterin, reutericyclin), antifungal compounds (such as propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides, and 3-hydroxy fatty acids), bacteriocins and bacteriocin-like inhibitory substances (BLIS), which can be exploited against foodborne pathogens and spoilage bacteria (Figure 1.1).

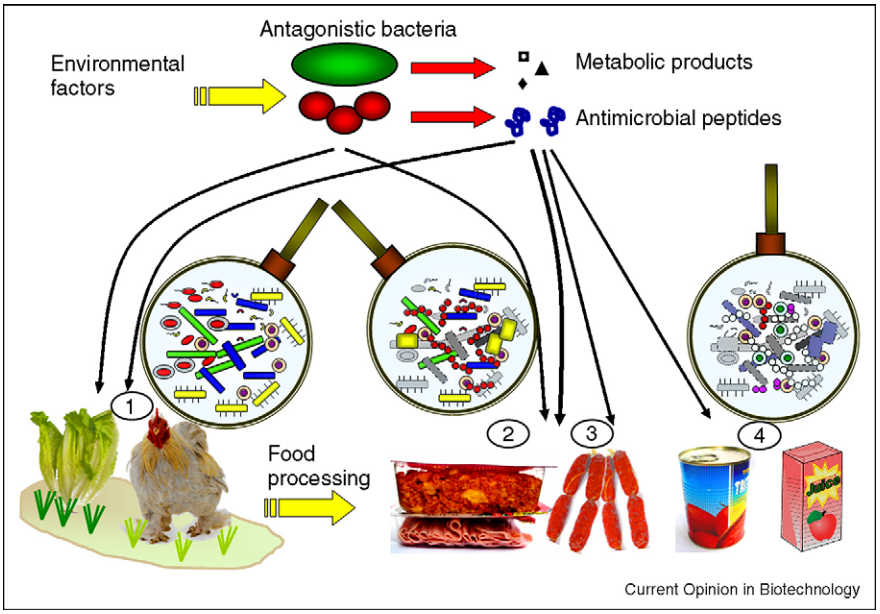

Figure 1.1. Biocontrol of pathogenic bacteria through the food chain using microbial antagonistic bacteria and/or their antimicrobial products. Antagonistic strains can be applied: (1) as living cultures on livestock and fresh produce; (2) as protective cultures on ready-to-eat food products; (3) as starter or protective cultures in fermented foods. They are expected to grow and produce antimicrobial substances in situ, displacing unwanted bacteria. Alternatively, food-grade preparations containing antimicrobials produced at industrial scale by antagonistic strains can be applied as biopreservatives or as food additives to inhibit transmission of food-borne and/or spoilage bacteria through the food chain (1–4). Since the food microbiota may change considerably from farm to fork, biocontrol strategies must be designed specifically for each type or category of food product.

LAB are the most widely used bacteria as starter cultures for the industrial processing of fermented dairy, meat, vegetable and cereal products. Despite the starter culture addition, non-starter lactic acid bacteria (NSLAB), originating from the raw material and environment, grow out during fermentation and may reach higher numbers than the starters. Reduction of pH and conversion of sugars to organic acids are the primary

preserving actions that these bacteria provide to fermented food. However, many kinds of food are still fermented naturally, without the use of starter cultures, by autochthonous lactic acid bacteria, which form the characteristic properties of the products. These natural isolates of lactic acid bacteria from spontaneous fermentations could be used as specific starter cultures or as adjunct strains, after phenotypic and genotypic characterisation, and they represent a possible source of potentially new antimicrobial metabolites (Maric, 1984; Wouters et al., 2002; Topisirovic et al., 2006). In addition, the application of lactic acid bacteria and their antimicrobial metabolites in the prevention of food spoilage and the extension of the shelf life of food that is ready to eat, fresh-tasting, nutrient and vitamin rich, minimally processed and biopreserved are the major challenges for the current food industry (Gálvez et al., 2007). The use of bacteriocin-producing lactic acid bacteria as protective strains or bacteriocins in form of purified or concentrated compounds as biopreservatives to control undesirable bacteria remains a primary focus of researches related to food safety and quality (Havelaar et al., 2009).

In the concept of functional food, especially in the dairy industry, there is an increasing interest for probiotic products that contain lactic acid bacteria of intestinal origin. Probiotic lactic acid bacterial strains must be chosen according to accurate selection criteria in order to survive the transition through gastrointestinal tract and preferably colonize the intestinal tract for a sufficiently long period to achieve the desired healthy effect (Suskovis et al., 2001). One of the most important properties of probiotics is its protection against pathogens in the intestinal tract of the host. The role of antimicrobial compounds produced by probiotic strains such as prophylactic agents against enteric infections is crucial and well documented (Kos et., 2008; Frece et al., 2009; Saulinier et al., 2009).

The antimicrobial activity of starter cultures and probiotic bacteria has been attributed to the production of metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, other low molecular mass compounds with antimicrobial activity and bacteriocins (Vanderbergh, 1993; Brkić et al., 1995). Industrial potential of antimicrobials from lactic acid bacteria is illustrated in Figure 1.2.

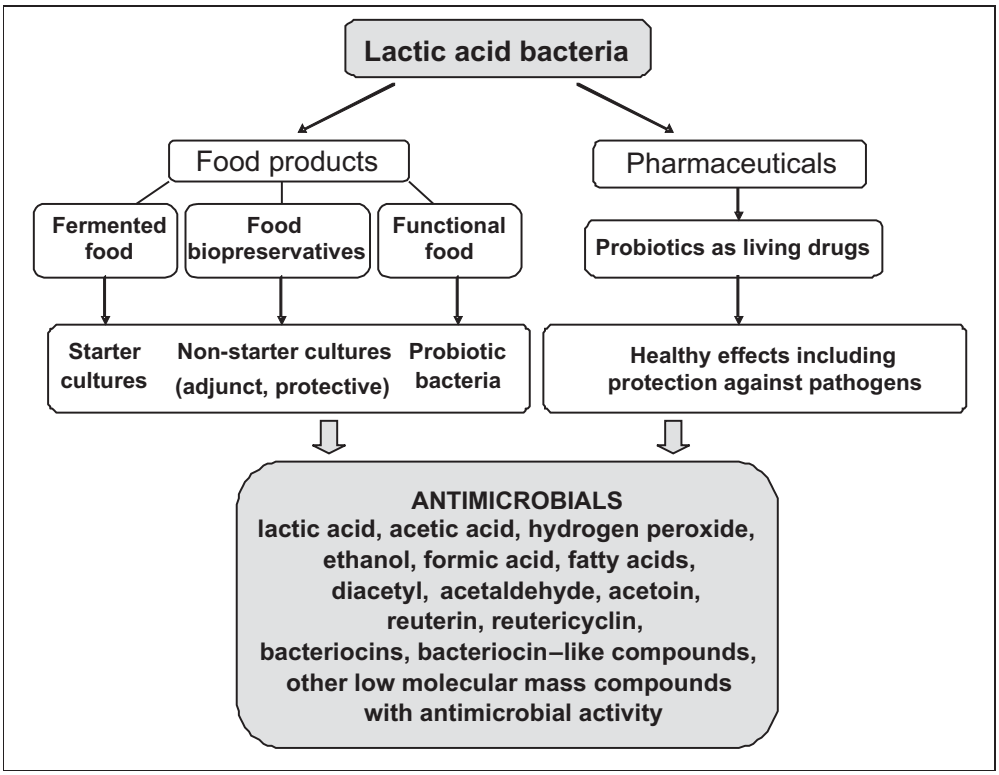

Figure 1.2 Industrial potential of antimicrobials from lactic acid bacteria

1.3.1 Antimicrobials from Lactic Acid Bacteria

The metabolites of LAB with antimicrobial activity are accumulated in their environment at the levels and proportions that depend on the species of LAB and chemical composition of the growth media. Fermentation of hexoses by lactic acid bacteria is characterized by homofermentative production of lactic acid or by heterofermentative production of equimolar amounts of lactate, acetate/ethanol and carbon dioxide. Pentoses are fermented by many heterofermentative and homofermentative LAB in the same way since phosphoketolase of homofermentative LAB is generally inducible by pentoses. Fermentation of pentoses yields the equimolar amounts of lactic and acetic acid.

Most of heterofermentative species have flavoprotein oxidases, which catalyse the reduction of oxygen, resulting in the accumulation of hydrogen peroxide. During heterofermentations, products such as formic acid, acetoin, acetaldehyde and diacetyl, which possess antimicrobial activity, can be accumulated. Malic, lactic and citric acid can be further metabolised to other antimicrobial products such as acetic acid, formic acid and CO_2 (Lindgren and Dobrogosz, 1990).

1.3.1.1 Organic acids

The most important and best characterised antimicrobials produced by LAB are lactic and acetic acid. The amount and type of acids produced during fermentation influence the subsequent microbial activity in the fermented material. Acetic acid, for example, is more antagonistic against yeasts compared to lactic acid. Some oxidative yeasts are able to utilize organic acids as a carbon and energy source and consequently cause spoilage through deacidification in fermented, especially plant material where they are naturally present (Daechel et al., 1987). The inhibitory effect of organic acids is mainly caused by undissociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol and interferes with essential metabolic functions. The toxic effects of lactic and acetic acid include the reduction of intracellular pH and dissipation of the membrane potential (Kashket, 1987; Lorca and de Valdez, 2009).

1.3.1.2 Hydrogen peroxide

Antimicrobial activity of hydrogen peroxide is attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell proteins (Lindgren and Dobrogosz, 1990). In raw milk, hydrogen peroxide produced by lactic acid bacteria can, after being catalysed by lactoperoxidase, oxidise endogenous thiocyanate. The oxidized intermediary products are toxic to different bacteria (Daechel, 1989). Hydrogen peroxide production has been considered as the main metabolite of LAB that could protect against urogenital infections, especially in the case of bacterial vaginosis (Reid, 2008).

1.3.1.3 Diacetyl, acetaldehyde and acetoin

Heterofermentative LAB produce active acetaldehyde by decarboxylation of pyruvate. This product then condenses with pyruvate, forming a-acetolactate and it is converted by a-acetolactate synthases to diacetyl. The product of decarboxylation of a-acetolactate and reduction of diacetyl is acetoin (Collins et al., 2009; Jyoti et al., 2003). Diacetyl (2,3-butanedione) is best known for the buttery aroma that it imparts to fermented dairy products, but this property as well as high concentration needed to provide preservation of food limit the use of diacetyl as food preservative. Similarly, an acetaldehyde, usually present in fermented dairy products in concentrations smaller than necessary for

inhibition of undesired microorganisms, also plays a role in controlling the growth of contaminants, together with other antimicrobial metabolites of lactic acid bacteria (Vanderbergh, 1993).

1.3.1.4 Carbon dioxide

The influence of carbon dioxide on product preservation is twofold. Namely, except for its own antimicrobial activity, it creates an anaerobic environment by replacing the existent molecular oxygen. The antifungal activity of CO_2 is due to the inhibition of enzymatic decarboxylations and to its accumulation in the membrane lipid bilayer resulting in dysfunction in permeability (Lindgren and Dobrogosz, 1990).

1.3.1.5 Reuterin and reutericyclin

Selected isolates of *Lactobacillus reuteri* produce two compounds, reuterin and reutericyclin, both active towards Gram-positive bacteria. Reutericyclin is a tetramic acid derivative and reuterin is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of b-hydroxypropionaldehyde with a broader spectrum of inhibitory activity, including Gram-negative bacteria, fungi and protozoa (Kuleasan and Çakmakçi, 2002; Gänzle and Vogel, 2003; Leroy et al., 2006).

1.3.1.6 Other low molecular mass antimicrobials

Other low molecular mass compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, moulds and yeasts have been described, including antifungal cyclic dipeptides, phenyllactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids (Ström et al., 2002; Sjögren et al., 2003; Valerio et al., 2004). Niku-Paavola *et al.* (Niku-Paavola et al., 1999) discovered new types of antimicrobial compounds produced by *Lactobacillus plantarum* (benzoic acid, methylhydantoin and mevalonolactone) active against fungi and some Gram-negative bacteria.

1.3.1.6.1 Fatty acids

Using the bioassay-guided isolation protocol described above, we discovered several 3-hydroxylated fatty acids with antifungal activity from *Lactobacillus plantarum* MiLAB

14 (Sjögren et al., 2003). No previous reports on the antifungal activity of hydroxylated fatty acids produced by LAB are available. The 3-hydroxylated fatty acids from MiLAB 14 were all isolated from the supernatant.

Lipolytic LAB can produce significant amounts of antimicrobial fatty acids that also contribute to the sensory quality of fermented foods (Earnshaw, 1992). Rao and Reddy (1984) found several fatty acids from cultures of LAB in fermented milk. While investigating straight- chained fatty acids, Woolford (1975) observed that antimicrobial activity increases with chain length. Caprylic (C8) acid and longer fatty acids are generally the most effective (except undecanoic, C11). Woolford (1975) noted that acids longer than 10 carbons were difficult to solve in water solutions. Baird-Parker (1980) also concluded that the antimicrobial activity of organic acids generally increased with chain length, but due to low solubility in water, aliphatic acids longer than C10 or C11 were not as effective as antimicrobial compounds. Kabra (1983) on the other hand found that fatty acids with 12–16 carbons were most effective and exhibited detergent-like properties. We observed that a hydroxylated fatty acid with 12 carbons had the strongest antifungal activity (Sjögren et al., 2003).

Bergsson et al., (2001) investigated the effect of fatty acids and mono-glycerides on the growth of *C. albicans*. They found that when yeast cells were treated with 10 mM of the fatty acids, it was only capric (C10) and lauric (C12) acid that inhibited the yeast, which agrees with the data of Woolford (1975). Corsetti et al. (1998) discovered that a *Lactobacillus sanfranciscensis* isolate from sourdough produced a mixture of organic acids with antimould activity. Caproic (C6) acid played a key role, but propionic, butyric and valeric acids also contributed to the inhibitory effect.

We found that the hydroxylated fatty acids had strong antifungal activity against a broad spectrum of yeasts and moulds (Sjögren et al., 2003). Yeasts were generally more sensitive to the fatty acids than moulds. The minimum inhibitory concentrations (MIC) of the hydroxylated fatty acids against moulds and yeasts ranged between 10 and 100 μg/mL (Sjögren et al., 2003). This could be compared with standard antifungal drugs, e.g. amphotericin B that inhibits fungal growth at concentrations in the μg/mL range (McGinnis and Rinaldi, 1991).

Production of hydroxylated fatty acids followed bacterial growth, indicating that they do not result from cell lysis (Sjögren et al., 2003). The metabolic role of these

hydroxylated fatty acids is not clear, and their potential antifungal activities in natural ecosystems are not known.

1.3.1.6.2 Phenyllactic acid

Lavermicocca et al. (2000) reported the production of phenyllactic acid and 4-hydroxy-phenyllactic acid from *L. plantarum* 21B, a sourdough isolate with antifungal activity against several species of filamentous fungi, Gram-positive and Gram-negative bacteria. Phenyllactic acid has also been identified from culture supernatants of *L. plantarum* MiLAB 393 (Ström et al., 2002), *L. coryniformis* strain Si3, and strains of *Pediococcus pentosaceus* and *L. sakei* (Magnusson et al., 2003). Phenyllactic acid is only active against yeasts and moulds at mg mlK1 concentrations. However, this metabolite will most certainly contribute to the overall antifungal effect in synergy with other compounds produced by LAB. Indeed, in sourdough bread started with *L. plantarum* 21B the onset of growth of the mould Aspergillus niger was delayed 7 days, compared to bread started with a *Lactobacillus brevis* that did not produce phenyllactic acid (Lavermicocca et al., 2000).

1.3.1.6.3 Bacteriocins of lactic acid bacteria

Some of LAB produce bacteriocins, antibacterial proteinaceous substances with bactericidal activity against related species (narrow spectrum) or across genera (broad spectrum of activity) (Rogelj and Bogovic-Matijasic, 1994; Cotter et al., 2005). Bacteriocin biosynthesis is a desirable characteristic for strain selection as it serves as an important mechanism of pathogen exclusion in fermented foods as well as in the gastrointestinal environment.

Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by many Gram-positive and Gram-negative bacteria; however, those produced by food grade LAB have received considerable attention due to their potential application in food industry as natural preservatives (biopreservatives). LAB bacteriocins are small antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocins (Klaenhammer, 1988; De Vuyst and Vandamme, 1994; Chen and Hoover, 2003). There are several proposed bacteriocin classifications divided into 3 or 4 classes:

(*i*) lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose biologically inactive prepeptides are subjected to extensive post-translational modification; (*ii*) small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocins like or *Listeria*-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb) and circular bacteriocins (class IIc); and (*iii*) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III) (Cotter et al., 2005; Klaenhammer, 1988; De Vuyst and Leroy, 2007). Some authors (Klaenhammer, 1993; Nes et al., 1996) also proposed (*iv*) class IV bacteriocins that require non-proteinaceous moieties (lipid, carbohydrate) for their activity (Cotter et al., 2005; De Vuyst and Leroy, 2007; Klaenhammer, 1993; Nes et al., 1996).

1.3.2 The protective culture approach

LAB represent the microbial group most commonly used as protective cultures, as they are present in all fermented foods and have a long history of safe use (Franz et al., 2010; Schillinger et al., 1996). Safety for the consumers is an aspect of great importance, in particular for foods which are not cooked before consumptions, but also for other types of foods since cross-contaminations, both at the retail and consumer level, are possible. The absence of pathogenic traits should be demonstrated for cultures suggested for use in foods (Maragkoudakis et al., 2009). Beside safety, protective cultures should guarantee the absence of detrimental effects on the target food; since LAB may contribute to spoilage in several types of foods, it is essential to study their effect on food texture and quality, with particular emphasis on the nutritional value of the product (Castellano et al., 2010). Furthermore, the capability of surviving to industrial processing conditions is of great importance for industries producing protective cultures at the large scale (Santini et al., 2010). LAB have historically been used as preserving agents in a number of fermented foods, as reviewed by many Authors (Caplice and Fitzgerald, 1999; Giraffa et al., 2010; Settanni and Moschetti, 2010). However, the role of LAB as protective cultures has also been evidenced in several non-fermented foods. This section will focus on recent applications of protective cultures to non-fermented foods, including meat, plant and seafood products, aimed at the increase of microbial safety and quality.

1.3.2.1 Meat products

Meat and meat products are excellent substrates for microorganism growth (Galvez et al., 2010). Refrigeration is the technology of choice to extend the shelf-life of retailed meat, often applied in combination with vacuum-packaging. Refrigeration can contribute to the selection of spoilage psycotrophic bacteria, mainly Enterobacteriaceae, *Pseudomonas* spp. and *Brochothrix thermosphacta* (Katikou et al., 2005). Moreover, some mesophilic species, such as *Salmonella* spp. and pathogenic *E. coli*, are capable of growing in slightly temperature-abused refrigerated foods and seriously compromise the safety of the meat. In addition, *Listeria monocytogenes* has caused several outbreaks in recent years linked to meat products. Although several studies have focused on the in vitro selection and characterization of LAB strains to be used as protective cultures on meat, there has been relatively little application on meat products, which mainly regards chicken meat, beef meat and ham. The work of Maragkoudakis et al. (2009) is the first successful application of live protective LAB on chicken meat. Two strains (*Enterococcus faecium* PCD71 and *Lactobacillus fermentum* ACADC179) were applied to raw chicken meat, resulting in a reduced growth rate of *L. monocytogenes* and *Salmonella enteriditis*. Interestingly, the selection of the strains was performed among 600 LAB of food origin, with regards to desirable functional properties such as antimicrobial activity against the target pathogens and spoilage microorganisms, survival to food processing and gastrointestinal tract conditions and basic safety properties. Moreover, no spoilage effect and reduction of the nutritional values were evidenced. The bacteriostatic effect against *Listeria* has been ascribed to the action of bacteriocins; however, the Authors concluded that a complex array of factors not yet completely elucidated could be involved in the antimicrobial action. Protective cultures have a long description of application on sliced beef meat mainly against spoilage bacteria (Galvez et al., 2010). *Lactobacillus sakei* and *Lactobacillus curvatus* of meat origin are the most common applied strains (Castellano et al., 2010). *L. sakei* CETC 4808, known to produce bacteriocin-like molecules, was successfully applied against spoilage bacteria on the surface of vacuum-packaged sliced beef without affecting chemical and sensory quality (Katikou et al., 2005). *L. curvatus* CRL705 strain was inoculated on the surface of vacuum-packaged refrigerated beef steaks stored for 60 days; the strain became the dominant population and was able to control the growth of spoilage microorganisms naturally present on the meat (Castellano et al., 2010). Tissue

degradation was delayed with respect to non-inoculated samples and sensory alterations could not be appreciated. In addition, the same strain was potentially active against *Listeria* spp. strains due to the action of a specific bacteriocin. Protective cultures have also been used for shelf-life prolongation of cooked meat products such as ham. *L. sakei* 10A, isolated from turkey meat, possessed antagonistic activity against *Leuconostoc mesenteroides* and *B. thermosphacta* (Vermeiren et al., 2006).

1.3.2.2 Vegetables and fruits

The increasing importance of minimally processed vegetables and fruits, such as pre-washed and pre-cut salads, and prepared fruit salads, has initiated many studies for microbial safety of these products, which are sold in a ready-to-use form and do not generally contain preservatives (Trias et al., 2008). However, the high humidity as well as the high number of cut surfaces with a resultant release of nutrients can provide ideal conditions for microbial growth, including pathogens. Classical treatments, employing chlorine or ozone, very often fail to remove pathogens (Trias et al., 2008). An indigenous *Pseudomonas putida* strain was found to possess relevant pathogen antagonistic efficacy as well as a favorable effect on the quality of the inoculated, packaged and stored lettuce (Wei et al., 2006). The strain possessed no risk potential; it can be applied post-harvest or at a process step in the production line anteceding the final washing. However, the majority of the strains applied as protective cultures in vegetable and fruit are LAB. Three *L. mesenteroides* strains, isolated from fresh fruit and vegetables (Trias et al., 2008) have been applied as bioprotective cultures in wounded Golden Delicious apples and Iceberg lettuce leaf cuts and found to reduce the amount of *Salmonella enterica* serovar *Typhimurium* and *E. coli* and to completely inhibit the growth of *L. monocytogenes* without sensory or visual modifications of the product. A *L. curvatus* strain possessing antimicrobial activity against *L. monocytogenes* was isolated from non-fermented and not heat processed refrigerated pickles and used as biopreservative agent (Reina et al., 2005).

1.3.2.3 Seafoods

Biopreservation is of extreme interest to ensure safety and quality of minimally processed seafood, whose demand has sensibly increased in recent years (Calo-Mata et al., 2008). Salt or sugar are often added to reduce the water activity (aw) and a mild processing, such as cold-smoking, is frequently applied. Nevertheless, spoilage or pathogenic microorganisms can grow on these foods. The major microbial risks associated with seafood are *Clostridium botulinum* type E and *L. monocytogenes*. Whereas *C. botulinum* type E can be adequately controlled by the combination of salt and low temperatures, *L. monocytogenes* can grow at 0°C and tolerate low aw usually lethal for bacteria. Safety and spoilage control of seafood can be improved by applying protective cultures, mainly LAB, as reviewed by Leroi (2010). Some LAB strains are known to secrete active bacteriocins also at high salt concentration and low temperatures, both in aerobic and anaerobic atmospheres (Tomé et al., 2008). In spite of the high number of in vitro studies, very few commercial applications have appeared in seafood products, as the organoleptic and nutritional quality of the food is often compromised and several bacteria that gave in vitro promising results proved to be ineffective in products (Leroi, 2010). *Carnobacterium divergens* V41 strain was applied to sterile cold-smoked salmon co-inoculated with a mixture of *L. monocytogenes* strains (Brillet et al., 2005). In samples possessing a high initial natural microbiota (>10^4 e 10^5 CFU/g), inoculated and autochthonous LAB quickly became dominant over potentially spoilage and pathogenic bacteria. The anti-listerial activity of 3 LAB strains used individually or as co-cultures was assayed on cold-smoked salmon artificially contaminated with *L. innocua* and stored under vacuum at 4°C (Vescovo et al., 2006). The association of *Lactobacillus casei* T3 and *Lactobacillus plantarum* PE2 was the most effective, probably due to a competition mechanism against the pathogen. Ready-to-eat seafoods such as cooked and peeled shrimps are highly susceptible to the colonization of pathogens and spoilage bacteria. The growth of these microorganisms can be contrasted by psycotrophic LAB, which are capable of delaying the sensory spoilage of the products, beside inhibiting the growth of *L. monocytogenes* and *Staphylococcus aureus.*

1.3.3 Phenyllactic acid

Phenyllactic acid (PLA) has broad and effective antimicrobial activity against both bacteria and fungi and can therefore be employed and developed as a new type of natural antiseptic agent to extend the shelf life of food and feed (Dieuleveux et al., 1998; Lavermicocca et al., 2003; Schnürer and Magnusson, 2005). It is also a useful precursor for the synthesis of many important drugs, including Danshensu (3,4-dihydroxyphenyllactic acid) which can inhibit platelet aggregation and coronary artery disease, hypoglycemic reagents, protease inhibitors, and anti-HIV reagents (Zhou et al., 2005; Yang et al., 2010; Budt et al., 1991; Kano et al., 1998; Urban and Moore, 1992; Morita and Mori, 1996). Because of its wide use in food and pharmaceutical industries, PLA production has attracted the attention of biotechnologists.

Chemical and biotechnological routes have been developed for PLA production. The chemical transformation strategy has some disadvantages, including a complex technology route, excessive by-products, and environmental pollution (Xiao et al., 2010). Regarding the requirement for environmental protection and sustainable development, biotransformation has emerged as a powerful strategy for the production of this valuable compound.

Phenyllactic acid was rarely reported except for the medicine plants, and it was only found in honey and sourdough (Kuś et al., 2014; Isidorov et al., 2015). In fact, has been commonly found in honey, a food product characterized by very hard environmental features and populated by specific population of lactic acid bacteria (Tuberoso et al., 2011). Moreover, the PLA presence and its antimicrobial activity was widely studied in sourdoughs (Van der Meulen et al., 2007; Ryan et al., 2009). In detail, in honey, its content is commonly much higher than that of other phenolic acids. So, it has even been suggested as a chemical marker for some honeys (Tuberoso et al., 2011).

PLA was proven to be a regular metabolite from lactic acid bacteria (LAB) and was produced by a wide range of LAB species (Valerio et al., 2004). The microbial biotransformation based on metabolic engineering is one of avail- able biological approaches for production of PLA (Vermeulen et al., 2006; Zheng et al., 2013).

In the last two decades, several microorganisms, including Geotrichum candidum, propionibacteria, and lactic acid bacteria (LAB) were found to be PLA producers (Lind

et al., 2005; Magnusson et al., 2003; Ström et al., 2002; Thierry and Maillard, 2002; Valerio et al., 2004).

Many studies have focused on the ability of LAB to produce PLA because LAB have GRAS (generally recognized as safe) status. LAB, especially *Lactobacillus* strains, yielded PLA at the low level of 0.05–0.57 mM via the phenylalanine (Phe) metabolic pathway (Li et al., 2008; Vermeulen et al; 2006).

1.3.3.1 PLA biosynthesis pathway

The PLA biosynthesis pathway in LAB is well characterized (Mu et al., 2012b). 3-Phenyllactic acid production is a side pathway of phenylalanine metabolism, in which phenylalanine is transaminated to phenylpyruvic acid by aminotransferase and phenylpyruvic acid is further reduced to PLA by a dehydrogenase (Figure 1.3). Phenylalanine supplementation may thus increase PLA biosynthesis. Conversely, the transamination reaction of phenylalanine is catalyzed by aromatic aminotrans- ferase, which has a broad substrate spectrum, including tyrosine (Yvon et al., 1997). Therefore, tyrosine inhibits the transamination of phenylalanine and tyrosine supplementation thus decreases PLA production. Many studies have shown that tyrosine supplementa- tion strongly inhibits PLA production, but it did not completely inhibit the PLA production of LAB strains in flask fermentation (Valerio et al., 2004; Mu et al., 2012a).

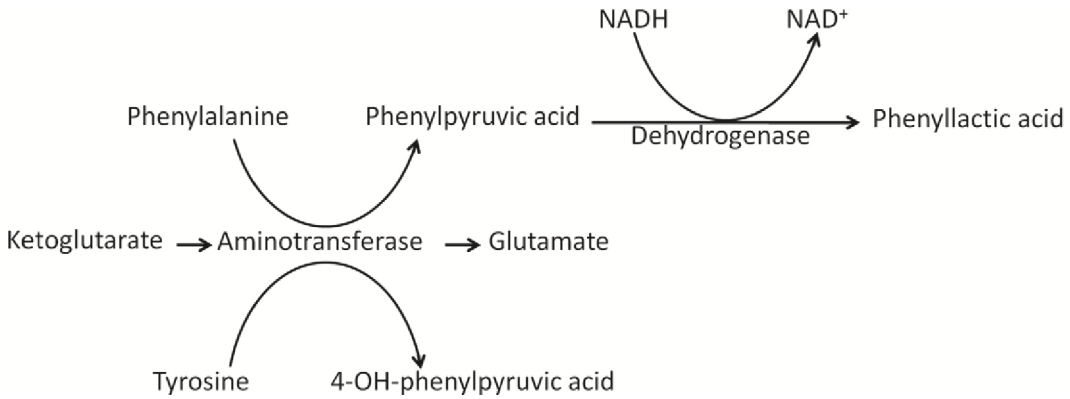

Figure 1.3. Possible pathway of 3-phenyllactic acid (PLA) production by *Pediococcus pentosaceus* SK25 in fermented milk.

The transamination reaction in the Phe pathway was the bottleneck for PLA formation (Vermeulen et., 2006), and the use of phenylpyruvic acid (PPA) as a substitute substrate led to a 14-fold increase in PLA production (Li et al., 2007). PPA showed obvious

inhibitory effects in the biotransformation process, and therefore, fed-batch fermentation could be conducted for producing a high amount of PLA. It should be noted that PPA powder must be dissolved beforehand because of its slow dissolution rate at the biotransformation temperature (Li et al., 2007). PLA concentration was significantly increased with the application of this strategy (Mu et al., 2009).

PLA is the acknowledged reduction product of PPA, but the enzymes responsible for this reaction remain unclear. In previous studies, hydroxyisocaproate dehydrogenase (HicDH), phenyllactic acid dehydrogenase (PLDHase), and D-lactate dehydrogenase (D- LDH) of Lactobacillus were assumed to be involved in PLA production from PPA.

1.3.3.2 PLA antimicrobial activity

Phenyllactic acid (**PLA**) is an organic acid with broad-spectrum antimicrobial activity (Mu et al., 2012b). It inhibits not only, but also foodborne pathogenic bacteria, including *Listeria monocytogenes* (Dieuleveux and Gueguen, 1998; Ning et al., 2017), *Staphylococcus aureus*, and *Escherichia coli* O157:H7 (Ohhira et al., 2004). Lavermicocca et al. (2000) reported first the production of PLA by *Lactobacillus plantarum* that was effective against the main genera that affect the baked goods: *Penicillum*, *Aspergillus* and *Fusarium*. In subsequent year, several LAB have been screened for their antifungal potential and their ability to produce PLA (Prema et al., 2010; Ryan et al., 2011; Gerez et al., 2013).

In addition, the antimicrobial potential of PLA could be enhanced when the organic acids co-existed due to the synergistic effect (Rodríguez et al. 2012).

Due to its broad inhibitory activity against a variety of foodborne microorganisms, PLA has interesting potential as an antimicrobial agent in the food industry. Laboratory research has suggested that PLA could be produced in fermented foods using LAB as starter, such as sourdoughs (Van der Meulen, et al., 2007; Ryan et al., 2009). During the sourdough fermentation process, fungal growth was significantly delayed in the presence of PLA-producing LAB strain, *Lactobacillus plantarum* 21B, in coculture with *Saccharomyces cerevisiae* (Lavermicocca et al., 2000).

1.4 Antimicrobial activity and mode of action

The antimicrobial activity of phenolic compounds -occurring in vegetable foods and medicinal plants-, organic acids as well as other microbial compounds has been extensively investigated against a wide range of microorganisms.

The range of natural substances and their action against bacteria may achieve values that only inhibit the bacterial growth (bacteriostatic) or may be used at either high concentrations or are inherently more aggressive and their action results in a decline in the number of bacterial cells (bactericide). The bacteriostatic action has a reversible character since, after neutralization of the agent, the microbial cells will recover their reproductive capacity. In contrast, the bactericidal effect has a permanent effect; as even after the neutralization of the agent, the microbial cells are not capable of growth and reproduction (Bloomfield et al., 1991).

Usually, the antimicrobial action is determined by using microbial populations and not individual cells. In these circumstances, we are dealing with a dynamic situation: some cells are reproducing whereas others may have already been dead and, for this reason, sometimes the difference between the microbiostatic and microbiocidal values is difficult to establish.

The determination of these effects on microbial growth is based on the growth curve analysis done under standard conditions; this means that the agent is absent and the nutritional, temperature and atmospheric conditions are optimal for the microorganism under study. Generally, the discontinuous system of microbial growth is adopted. In Figure 1.4 (A and B) the characteristic growth curves under a (A) or microbiocide (B) effect are shown.

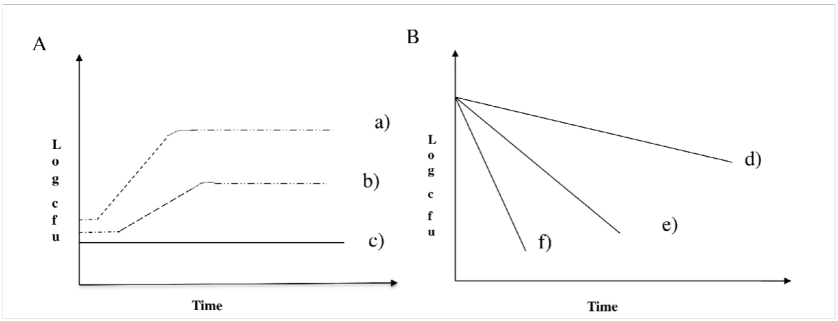

Figure 1.4. A. Growth curves. a) normal growth curve b) and c) inhibitory effect (bacteriostatic); B. Survival curves, d, e and f) bactericidal effect with increasing concentrations from d to f (adapted from Bloomfield (1991)).

The normal growth curve is represented under a) and can be divided in:

- the exponential phase during which cell division occurs according to a logarithmic or exponential relationship;
- the stationary phase during which the total number of viable cells remains constant. This phase occurs due to several factors such as nutrient exhaustion, oxygen, decrease in pH value and toxic products accumulation.
- the bacteriostatic effect may be understood as an increase of the lag phase accompanied by a decline on the specific growth rate ( ) which may be partial, as in b) or total as in c).

The kinetics of the bactericidal effect is usually determined from the survival (dead) curves (Fig. 3 B). The characteristic survival curve is represented by d), the curves of the type e) and f) are associated with an increased death rate.

Different types of tests for evaluation of the antimicrobial activity of natural substances *in vitro* are in use and the selection of each technique seems to be done according to several characteristics, namely technical demand and cost. Usually three types of methods are distinguished: diffusion, dilution and the bioautographic techniques (Burt, 2004; Kalemba and Kunicka, 2003; Scorzoni et al., 2007). The agar diffusion is one of most frequently used and is characterized by a great simplicity and cost-effectiveness. In this technique, several reservoirs of the natural substance can be employed. The most common technique uses filter paper disks (Kim et al., 1995; Chorianopoulos et al., 2007) or stainless steel cylinders which are distributed on the agar medium surface, and also holes punched into the agar medium may be used as reservoirs of the natural substance (Baratta et al., 1998; Dorman and Deans, 2000). In any case, the natural substance quantity and the reservoir diameter are crucial parameters.

The reservoir containing the natural substance to be evaluated, and after being in contact with the inoculated medium and the required incubation period, then the diameter of the transparent zone around the reservoir (inhibition zone) is measured. This method was first designed to evaluate the antibiotic properties from crude extracts. Whereas the agar diffusion method may be considered very precise on the essential oil antimicrobial activity determination, several less favourable aspects can be pointed out, such as the volatile characteristics of the essential oil components will result, on their loss, simultaneously with the solvent during incubation, whereas the less soluble compounds may not diffuse appropriately across the culture medium (Burt,

2004; Kalemba et al., 2003; Scorzoni et al., 2007; Janssen et al., 1987). The parameters to consider include the disk or cylinder/hole diameter, the quantity of the oil and the solvent or emulsifier used. This last factor seems to differ significantly between studies and several substances have been used, including ethanol (Deans and Svoboda, 1989; Sivropoulou et al., 1996; Ouattara et al., 1997; Marino et al., 2001; Tullio et al., 2006; Valero et al., 2006; Becerril et al., 2007; Burt et al., 2007); Tween-20 (Hammer et al., 1999; Pol and Smid, 1999; Griffin et al., 2000; Tzortzakis and Economakis, 2007); Tween-80 (Cosentino et al., 1999; Mourey and Canillac, 2002; Hood et al., 2003; Knowles et al., 2005), methanol (Rasooli et al., 2006; Bajpai et al., 2007), dimethyl sulfoxide (DMSO) (Hili et al., 1997; Firouzi et al., 1998; Blanc et al., 2006). Particular importance must be given to the use of safe concentrations of solvent or emulsifier in order to not disturb microbial growth. Another aspect to take in account is to have a negative control (such as sterile water or solvent) and a positive control (usually a reference antibiotic) in each assay.

The degree of the essential oil activity is revealed by the size of inhibition zone that is expressed by the diameter of the referred inhibition zone (in mm or cm) and usually the diameter of the disc/hole/cylinder is included. Due to the simple nature of this assay and the reduced amount of natural substance required, the use of this technique is generally recommended for the evaluation of numerous natural substances, and highlight the ones that present the highest activity allowing them to be subjected to more in depth characterization. This technique is also used to determine the susceptibility of a significant range of microbial species to a particular natural substance. However, this technique is less suitable for quantification purposes, such as the determination of the MIC and MBC values.

The techniques that require a homogeneous dispersal in water (dilution method; in agar or in liquid medium) are usually applied in order to determine the values of Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) through growth curve analyses by comparison with the culture grown in the absence of the natural substance (control culture). The MIC and MBC parameters are largely used in the evaluation of the natural substance antimicrobial activity but significantly differences have been found on their precise definition (Burt, 2004; Lambert et al., 2001; López et al., 2007). Other than this controversial aspect constituting an obstacle to the appreciation of the different studies, it seems that standardization is required.

The dilution method in agar or liquid medium is used for both bacteria and fungi. The volumes of culture broth supplemented with different natural substance concentrations vary substantially but presently the tendency is to use reduced volumes varying from 1-5ml (Valero et al., 2006; Rasooli et al., 2006; Dimitrijević et al., 2007) to 10ml (Ghalfi et al., 2006). The use of methods based on microdilutions is more intense (Burt and Reinders, 2003; Chorianopoulos et al., 2007; Mann and Markham, 1998; Lambert et al., 2001; Shapiro et al., 1994; Avato et al., 1997) and appear to be very appropriate for the determination of MIC and CMB values. The efficacy of the antimicrobial activity when this method is applied both using tubes or microplates is verified by the change on optical density (OD) (Kalemba and Kunicka, 2003), by colorimetry (Hammer and Carson, 1999; López et al., 2007; Chandrasekaran and Venkatesalu, 2004; Feng and Zheng, 2007; Dimitrijević et al., 2007) or by viable determination (Sivropoulou et al., 1997), the latter being a very demanding technique. A combination of agar dilution and viable counts using the drop method (Chen et al., 2003) may constitute a good alternative technique for bacteria that have a reduced growth in broth. Because growth is measured by change in OD it is crucial to assure that no changes in the OD are due to the natural substance itself or the dispersing agent in use. In case this is not possible, the viable count must be done.

The use of methods based on microdilutions is mainly recorded in the determination of the susceptibility to antibiotics and they supply an important amount of information principally at present where the need for new and effective antimicrobial agents is very challenging, especially for natural products such as essential oils and plant extracts. Additionally, these methods can rapidly discriminate the resistant strains that emerge at very high frequency even among the foodborne pathogens (White et al., 2002; Mayrhofer et al., 2004).

Other non-conventional methods such as the microatmosphere, bioautographic and impedance or conductance measurements can also be used in the evaluation of antimicrobial activity of the natural substances (Burt, 2004; Kalemba and Kunicka, 2003).

1.4.1 Target Sites in Antimicrobial Action

The target site and mode of action of most natural substance is still not well understood, especially in yeast. Commercial applications of essential oils would benefit from deeper

insight into the mode of action behind individual compounds, as this could facilitate the exploitation of, e.g., synergistic combinations with more powerful antimicrobial properties.

Many different techniques have been applied to elucidate the mode of action of essential oils and their constituents. After establishing the killing or inhinibition activity of a compound, an array of experiments can be performed to identify how a compound interacts with the cell to cause the observed effects. In this context, it is important to distinguish between experiments that identify the target site from those that eluicisate the mode of action. The site of action refers to the part of the cell which interacts with the compound, e.g., the cell membrane, cell wall, or intracellular proteins, enzymes, nucleic acids, or metabolites. The mode of action, however, yields more elaborate knoledge about the molecular mechanisms or intermolecular interactions behind the inhibition or killing effects. An overview of methods adressing the site or mode of action of antimicrobial compounds is following provided.

1.4.1.1 Locating the site of action

High-resolution microscopy, such as electron microscopy or atomic force microscopy (AFM), can reveal the most extreme consequences of exposure to an antimicrobial compound, i.e., deformation of cells occuring from lysis or from damages to the cell wall. An advantage of TEM is that ultra-thin cross sections can reveal ultrastructural changes in the interior of the cell. Scanning electron microscopy (SEM) and AFM only image the cell suface. AFM has one important advantage over electron microscopy, in that it allows measurements in liquid under physiological conditions, avoiding difficult sample preparation and the artifacts associated herewith (Alessandrini and Facci, 2005). A limitation of both AFM and electron microscopy is, however, that specific cellular structures must be identified according to morphology unless some form of labeling can be applied. While antibodies conjugated to metal nanoparticles have been used with TEM in few studies (Romero et al., 2010), no labeling techniques have been reported for SEM and AFM. It is, however, possible to combine AFM with optical microscopy and thus take advantage of the numerous options for fluorescent labeling of biomolecules.

An important site of action is the cell membrane, and indeed, many essential oil constituents have been prosed to act on the bacterial membrane. Interaction of

antimicrobial compounds with the membrane can affect the transport of nutrients and ions, the membrane potential, and the cell. These effects are investigated by measuring the efflux of intracellular ions like K^+ and H^+ (Ultee et al., 1999; Lambert et al., 2001). Efflux of small ions is not necessarily indicative of complete loss of membrane function, and can be observed in viable cells where growth is inhibited because the cell uses energy for repair or survival rather than cell proliferation (Bouhdid et al., 2010). Effects on the cell membrane that lead to cell death is more accurately predicted by detecting the efflux of larger molecules like ATP or carboxyfluorescein diacetate (cFDA) after esterase reaction (Xu et al., 2008), or by influx of large polar organic DNA-binding stains like ethidium bromide (Lambert et al., 2001) and propidium iodide (Bouhdid et al., 2010). It should be pointed out that it is always good practise to validate the observed effects by combining several techniques. Monitoring the release of calcein encapsulated in membrane vesicles can, for example, be used as a complimentary technique to confirm the membrane as the site of action (Miron et al., 2000).

If no effects are observed on cell structure and membrane functionality, it is assumed that the site of action is intracellular. The target can be proteins and enzymes is general, or it can be esential cellular processes involved in biosynthesis or energy generation. An intracellular site of action can for example be determined by incorporation of radioactively labeled substrates used in particular biosynthesis pathways (Schneider et al., 2010). Lack of or decreased incorporation is then taken as an indication of the process being affected by the antimicrobial compound. For example, radiolabeled nucleotides or amino acids can be used to detect if DNA replication or protein synthesis takes place, respectively (Schneider et al., 2010).

Some compounds have multiple sites of action, and in that case, it can be difficult to pinpoint which one is ultimately responsible for cell death. For example, a compound that affects membrane permeability will also affect the membrane potential and thereby energy generation by respiration. It is thus difficult to distinguish direct effects on energy-generating processes from the indirect effect a permeable membrane has on these processes. At sublethal concentrations, changes to the transcriptome and proteome during exposure can reveal how the cell responds to the compound, and upregulation of genes involved in certain metabolic or biosynthesis pathways can be indicative of which cell structures or processes that are affected (Burt et al., 2007; Rao et al., 2010).

1.4.1.2 Elucidating the mode of action

The probably most comprehensive approach to investigate the mode of action of a particular compound is to perform random transposon mutagenesis in order to search for mutations that compensate for the antimicrobial effect of a particular compound. In this way, it is possible to identify the mode of action of compounds that interact specifically with, e.g., a single enzyme or with particular proteins or lipids in the membrane (Shapira and Mimran, 2007; Van Hoang et al., 2011). The approach is, however, not suited for investigating antimicrobial compounds that act simultaneously on several components in the cell, as a single mutation is unlikely to facilitate compensation for the antimicrobial effect on the cella s a whole.

Antimicrobial compounds that act on the membrane can cause depolarization or increased permeability through various mechanisms. For example, some antimicrobial peptides form pores (Cotter et al., 2005; Fantner et al., 2010) while other compounds, such as certain essential oil constituents, have a fluidifying effect on the membrane (Trombetta et al., 2005; Cristani et al., 2007). Membrane properties like lipid packing can be investigated in membrane vesicles by LAURDAN staining combined with spectrofluorometry (Nielsen and Rios, 2000), and membrane fluidity can be ivestigated directly in bacteria by differential scanning calorimetry (Trombetta et al., 2005) or fluorescense anisotropy measurements of DPH using a spectrofluorometer (Liao et., 2010). AFM imaging has also in recent years allowed for the high-resolution visualization of native membranes on a solid support. Structural changes resulting from the integration of an antimicrobial compound into the membrane can thus be visualized directly (Brasseur et al., 2008), and the effect on membrane rigidity can be quantified by AFM force spectroscopy (Sullan et al., 2010). Functionalizing the AFM tip with the antimicrobial compound of interest furthermore allows investigation of interaction forces between the compound and its target. This approach was, for example, used to map binding events of vancomycin on the surface of bacteria and confirmed that binding occured at the site of cell wall synthesis in dividing cells (Gilbert et al., 2007).

1.4.1.3 Bacterial cell targets

The number of studies conducted on the action mechanisms of plant essential oils has been increasing (Dorman and Deans, 2000; Burt et al., 2007; Rasooli et al., 2006;

Lambert et al., 2001; Rhayour et al., 2003; Turgis et al., 2009). There are still enormous differences when comparing the high number of studies on natural substance characteristics and their components, to the number of studies performed on the investigation of the specific targets of the antimicrobial action of natural substances and their components. Acquiring knowledge about the cell targets of natural substances and their components is crucial in order to understand which cell targets are affected. The survival of the pathogen in a food matrix or in a living tissue depend on this knowledge or the host infection process can be impaired. Ultimately a proper application system can be elaborated based on more accurate information.

The antimicrobial actions of natural substances are linked to their hydrophobicity resulting in increased cell permeability and consequent leaking of cell constituents (Dorman and Deans, 2000; Lambert et al., 2001; Turgis et al., 2009; Ultee et al.). It is important to comprehend that a disturbed cell structure may affect other cellular structures in a cascade type of action (Carson and Mee, 2002).

1.4.1.3.1 Cell wall and membrane disturbance

The evaluation of the loss of cell constituents contributes to elucidate the severity of the cell membrane damage and a significant number of studies have used this approach to clarify the antibacterial action of natural substances (Bouhdid et al., 2009; Cox et al., 2000; Carson and Mee, 2002) and they indicate that the tested natural compound effect the bacterial cell on the same target, the cytoplasmic membrane.

Bouhdid et al (Bouhdid et al., 2009) investigated the cellular damage by *Origanum compactum* EO on *Pseudomonas aeruginosa* ATCC 27853 and *Staph. aureus* ATCC29213 by evaluating the cell viability, potassium leakage using flow cytometry and transmission electron microscopy. The treatment of *Ps. aeruginosa* at the MIC value and at 1.5x of the MIC value resulted in a series of physiological injuries, namely the growth was totally inhibited, the respiratory activity significantly diminished, the cell membrane permeability was affected and the membrane potential failed. By contrast, the cell damage in *Staph. aureus* was not so pronounced at the MIC value, in particular both the membrane potential and the permeability were not significantly affected, but when the MIC value was increased to 1.5x the viability and the membrane potential go through a significant reduction. The differences registered between the two

bacteria are mainly due to differences in the membrane and cell wall composition and structure (Bouhdid et al., 2009).

The observation done by scanning electron microscopy (SEM) of the two Gram negative foodborne bacteria, *E. coli* 0157:H7 strain EDL 933 and *Salmonella enterica* subsp *enterica* serovar Typhi strain ATCC 19430 when exposed to mustard EO (allyl isothiocyanate is the main component) evidence an imperfect and unfinished cell shape (Turgis et al., 2009). The treatment of *E. coli* 0157:H7 with Spanish oregano causes alterations on the cell wall, the presence of white spots or holes on the cell wall were observed (Gaunt et al., 2005). However, the use of Spanish oregano against *L. monocytogenes* cells did not caused the production of white spots or holes, but the production of an imperfect *L. monocytogenes* cell (Oussalah et al., 2006). It seems evident that *E. coli* 0157:H7 cells treated with Chinese cinnamon EO were able to keep the energy sufficiently high to repair and maintain the cell surface which was apparently not damaged. This type of injury can be related with the differences on the cell wall structure. Such differences on cell damage were also verified with other bacterial pathogens, namely *Bacillus subtilis* (strain APL 87/35) and *E. coli* (strain APL 87/1) cells treated with oregano and clove EOs (Rhayour et al., 2003). *E. coli* cells treated with both EOs showed a more evident damage: holes at cell surface whereas in *B. subtilis* the damage just resulted on cell surface malformation (Rhayour et al., 2003).

Enriched thymol EO such as *Thymus erocalyx* and *T. x-porlock* (thymol content ranged from 63.8 to 31.7%, respectively) can cause injury to *L. monocytogenes* by disrupting or inducing the formation of a very thick and rough cell wall and even at lowest thyme oil concentration used the disruption of the cell membrane and lack of cytoplasm was observed (Rasooli et al., 2006).

The treatment of *Staph. aureus* with *Inula graveolens* (rich in bornyl acetate (43.3%) and borneol (26.2%)) and *Santolina corsica* (rich in myrcene (34.6%) and santolina triene (13.5%)) EOs at MIC values (5 mg/ml) for both EOs) produces invaginations of the cytoplasmic membrane accompanied by the formation of a thicker cell wall and aggregation of the cytoplasmic contents (Guinoiseau et al., 2010).

A possible indirect action of EOs on the membrane is the secretion of toxins. This aspect is particularly important to the control of *S. aureus* and *B. cereus*. The exposure of *B. cereus* to carvacrol resulted on inhibition of diarrheal toxin production (Ultee and Smid, 2001) and the use of Oregano at 0.3 and 15 µl/ml completely abolish the enterotoxin production of *S. aureus* (De Souza et al., 2010). Structural modifications

and energy limitation may explain the inhibition of toxin production. The secretion of toxins may be prevented by modifications in the bacterial membrane due to the attachment of the essential oil that may disturb the phospholipid bilayer with consequences to the trans-membrane transport process limiting in this way the release of toxins to the contiguous environment (Okubo et al., 1989). By other hand the limitation of intracellular ATP and proton motive force will restrict the secretion of toxins.

Combined treatments may also act synergistically degenerating the bacterial cells. The cells of *Ps. flourescens* treated with *C. citratus* oil vapour and negative air ions experience a complete relapse, the cytoplasmic material is spilled out of the cells whereas the cells treated with negative air ions alone only experience a restricted cell surface deformation (Tyagi and malik, 2010).

1.4.1.3.2 Zeta potential

The charge properties of the cell surfaces can play a vital role in the microbial homeostasis and resistance to antimicrobial agents (Ferreira et al., 2011). Under physicological conditions, bacterial cells have normally negative surface charge, due to presence of anionic groups (e.g. carboxyl and phosphate) in their membranes (Gilbert et al., 1991; Lerebour et al., 2004; Palmer et al., 2007). However, the magnitude of the charge varies from species to species and can be influenced by various conditions, namely age of the culture, ionic strength and pH (Ahimou et al., 2002; Palmer et al., 2007).

The surface charge of cells is frequently determined based on their zeta potential, which is calculated from the mobility of cells in the presence of an electrical field under defined pH and salt concentrations. Zeta potential measurements demostrated that after phenolic acid exposure, the cells become less negatively charged.

1.4.1.3.3 Leakage of cytoplasmic material

According to Carson et al. (2002), marked leakage of cytoplasmic material is considered indicative of gross and irreversible cytoplasmic membrane damage. Some phenolic products are recognized as having membrane active properties against microorganisms, causing leakage of cell costituents (Johnston et al., 2003). These

products could diffuse through the cytoplasmic membrane, increasing its permeability and, consequently, leakage of bacterial cell constituents including proteins, nucleic acids, and inorganic ions such as potassium or phosphate. Additionally, it was found by Ota et al. (2011) that ferulic acid, *p*-coumaric acid and caffeic acid, affects the cell membrane structure by rigidity and alteration of the dynamics of phospholipid chains. Moreover, Tamba et al. (2007) verified that (-)-epigallocatechin gallate induced large pore formation in lipid membranes resulting in leakage of the fluorescent probe calcein. The concomitant phenomena after the pore formation were the decrease in the diameter of vesicles and their transformation into small lumps due to the attractive interaction between neighboring lipid membranes. These authors also concluded that the binding of this flavonoid to the external monolayer of the lipid membranes increases its membrane area decreasing the intermembrane distance that induces an increase in its surface pressure. Other authors also referred the strong interaction between polyphenols and lipid membranes (Sun et al., 2009; Yu et al., 2011). These phenomena explain the antimicrobial activity of this compound. Kajiya et al. (2004) in a study performed with (+)-catechin derivatives demonstrated that the activity of these phytochemicals depended on the number of hydroxyl groups on the B-ring. On the other hand, the affinity for lipid bilayers was aumented with elongation of the alkyl chain lengths of the derivatives. Similar to the referred studies we can hypothesize that the antibacterial activity of phenolic acids is associated with both the affinity for the lipid bilayer and the disruption of the membrane structure. Also, the phenolic acid–lipid interaction can help to explain the higher susceptibility of the Gram-negative bacteria. In fact, the lipid content of the cell walls of the Gram-negative bacteria is substantially higher than that of the Gram-positive cell wall.

1.4.1.3.4 ATP production

The disruption of the cell membrane by any antimicrobial agent, including the EOs and phenolic compounds will compromise a series of vital functions, namely the energy for conversion processes, nutrient processing, synthesis of structural macromolecules, and the secretion of many growth key enzymes. The production of ATP in prokaryotes occurs both in the cell wall and in the cytosol by glycolysis. Therefore, it is expected that alterations on intracellular and external ATP balance will be affected due to the action of the phenolic compounds on the cell membrane. The correlation between the

intracellular and extracellular ATP concentration has been found (Turgis et al., 2009; Helander et al., 1998; Oussalah et al., 2006). ATP losses are supposed to occur through the disturbed membrane (Turgis et al., 2009; Oussalah et al., 2006). The treatment of *E. coli* 0157:H7 strain EDL 933 and *Salmonella enterica* subsp *enterica* serovar Typhi strain ATCC 19430 with mustard EO causes a significant loss of intracellular ATP, in particular when the essential oil is used at the determined MIC value (0.2%, v/v) (Turgis et al., 2009). The addition of carvacrol at 2mM or 1mM results on a decrease of intracellular ATP to 0 after 10 and 14 min., respectively (Ultee et al., 1999). The use of oregano EO at 0.020 and 0.025% (w/v) against *L. monocytogenes* cause a decrease on intracellular ATP (Caillet and Lacroix, 2006) and at 0.010% and 0.013% (w/v) also produces a decline on the intracellular ATP content of *Staph. aureus* (Caillet et al., 2009). In both *L. monocytogenes* and *Staph. aureus* a combined treatment with oregano EO and irradiation caused a more significant reduction on the intracellular ATP quantity (Caillet and Lacroix, 2006; Caillet et al., 2009).

Other intracellular events may contribute to the intracellular ATP decrease accomplished with a minor ATP release, namely the intracellular ATP may suffer a significant reduction by hydrolysis which can be due to the loss of inorganic phosphate across the compromised high permeable membrane (Turgis et al., 2009; Oussalah et al., 2006; Abee et al., 1994) or in virtue of the efforts made by the cell to recover the electrochemical gradient by proton extrusion driven by the ATP as an increased hydrolysis is established. This last mechanism was verified when *L. monocytogenes* cells were eliminated by the treatment with the bacteriocin pediocin PA-1 (Chen and Montville, 1995).

1.4.1.3.5 Protein synthesis

Burt et al. (Burt et al., 2007) first reported the action of EO components on protein synthesis. The EO components, carvacrol and *p*-cymene induced the synthesis of heat shock proteins (HSPs) when bacterial cells were treated with these two EO components (Burt et al., 2007). The HSPs are molecular chaperones involved in the different processes of assembly and release of newly synthesized polypeptides that, in general, increases when bacterial cells contact with toxic substances or other stress conditions. The cells of *E. coli* O157:H7 incubated overnight in the presence of carvacrol at 1mM produced significant amounts of heat shock protein 60 (HSP60) (GroEL) and the

synthesis of flagellin is inhibited resulting in non-motile cells. In contrast, *p*-cymene at 1mM or 10mM did not affect the production of HSP60 or flagellar synthesis (Burt et al., 2007). The approach to evaluate the effect of EO or its components on protein synthesis that can contribute with a more comprehensive view is the proteomic approach, namely the use of two-dimensional electrophoresis (2-DE) coupled with MALDI-TOF MS and this type of approach as used in the study of Di Pascua et al. (Di Pasqua et al., 2010) to evaluate the modifications on the protein expression of *Salmonella enterica* ser. Thompson treated with a sub lethal concentration of thymol. Di Pascua et al. (Di Pasqua et al., 2010) found that *Salmonella* cells treated with 0.01% over expressed a set of molecular chaperone proteins, namely DnaK, GroEL, HTpG and the Trigger factor Tf, outer membrane associated proteins (OmpX and two OmpA) and proteins involved directly or indirectly in the citrate metabolism and ATP synthesis were also affected evidencing the action of thymol as a large-scale stressor and acting in different pathways.

1.4.1.3.6 pH disturbance

The pHin in bacterial cells exposed to EOs and phenolic coumponds has been monitored and a significant reduction has been found (Turgis et al., 2009; Oussalah et al., 2006). The pH homeostasis may be impaired by the action of EOs on the membrane that loses its capacity to block protons (Lambert et al., 2001; Turgis et al., 2009; Oussalah et al., 2006). In the study of Turgis et al. (Turgis et al., 2009) a significant decrease on the intracellular pH (pHin), the initial pHin (no EO) changed from 6.23 to 5.20 for *E. coli* 0157:H7 and from 6.59 to 5.44 for *S. Typhi* when the bacterial cells were treated with the MIC value of the mustard EO.

The pHin of *E. coli* 0157:H7 was affected by the use of the Spanish oregano at 0.025% (v/v), Chinese cinnamon at 0.025% (v/v) and savory EOs at 0.05% but the *E. coli* 0157:H7 pHin was more affected by Chinese cinnamon EO. At concentration 0.025% (v/v) the Chinese cinnamon EO caused a decrease of the pHin from 7.25 ± 0.20 to 5.16 ± 0.05 whereas the Spanish oregano at this same concentration caused a decrease from 7.25 ± 0.20 to 6.68 ± 0.37 (Oussalah et al., 2006). This effect is similar to the mustard EO described by Turgis et al. (Turgis et al., 2009).

The addition of oregano EO to *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultures caused a rapidly pH gradient dissipation (Knoles et al., 2005). In *B. cereus* the

addition of 0.25mM to 0.5mM of carvacrol causes a decrease in the pH gradient and at 1mM the pH gradient is completely dissipated (Ultee et al., 1999).

The maintenance of the pHin at appropriate levels to achieve the various crucial cellular processes (DNA transcription, protein synthesis and enzymatic activity) is critical when the cell is exposed to severe injury (Patterosn et al., 1997; Kneen et al., 1998; Iwani et al., 2002). Even the neutrophile *E. coli* is capable to overcome acid stress and do this by activating four different acid resistance mechanisms (Foster, 2004; Iver et al., 2003). These systems function on the basis of amino acid decarboxylases (glutamate, arginine and lysine) and antiporters1. The reduction of the pHin by the EO treated cells is compared to the action of weak organic acids, namely benzoic in the yeast *Saccharomyces cerevisae*, as the weak acids also EOs are lipophilic and as weak acids it is possible that due to their lipid permeability a subsequent release of protons occurs (Turgis et al., 2009; Hirshfield et al., 2003; Ricke, 2003; Carpenter and Broadbent, 2009). If the intracellular proton release goes over cytoplasmic buffering capacity or the capacity of proton efflux systems, the intracellular pH value starts to decrease and vital cellular functions may be broken (Booth, 1985; Axe and Bailey, 1995).

1.4.1.3.7 Intracytoplasmic changes

In the study conducted by Becerril et al. (Becerril et al., 2007) *E. coli* cells treated with oregano EO exhibited intracytoplasmic changes, where coagulated material appeared in specific areas located to the cell wall and apical ends. When *E. coli* cells were treated with cinnamon EO, the periplasmic space showed significant changes, in particular they became larger and irregular. The investigators also noted the absence of fimbriae in the altered (larger) periplasmic space. *Staph. aureus* cells treated either with oregano or cinnamon EO exhibited the same cell malformations as *E. coli* but in a less pronounced manner.

1.4.1.3.8 DNA

Once the bacterial DNA is physically attached to the bacterial cell membrane it is expected that EO may act on DNA and this fact has been used to measure the genotoxicity and antimutagenic effects of EOs and other agents (Ames et al., 1973; Laouer et al., 2009; Burdock and Carabin, 2008; Mezzoug et al., 2007). The most used

tests are the Ames test (Ames et al., 1973) and the SOS-Chromotest (Quillardet et al., 1982). The Ames test is based on the use of different sets of *Salmonella enterica* subspecies *enteric*a serovar Typhimurium strains (four strains) that have different mutations in the histidine operon, becoming auxotrophic for histidine. The strains sets have a deletion on the *uvrB* region of the chromosome removing the DNA repair system (Maloy et al., 1994). The other mutations are on *gal*[2] and *rfa*[3] that affect, to different levels, the polysaccharide side chain of the lipopolysscharide (LPS) that covers the bacterial surface. These mutations confer a high rough appearance to the bacterial cells and such bacteria are highly permeable and fully nonpathogenic. The TA1535 set (TA 1535, TA1536, TA1537 and TA1538) which have mutation on *rfa* and *uvrB* is the most susceptible to mutagenesis, so is subject to regular testing for mutagens and carcinogens, *in vitro*. The TA1975 set (TA 1975, TA1976, TA1977, TA1978) only have the *rfa* mutation, so it is suggested to analyze the effect of the repair system on mutagenesis and eradication. Quillardet et al. (Ames et al., 1973) mounted a colorimetric assay based in the SOS response (Maloy et al., 1994) and called it SOS-chromotest. This test is based on an operon fusion that putted the lacZ[4] under the control of the *sfiA* gene[5] (*sfiA*::*lacZ*) in *E. coli* K-12 (denominated PQ37 *uvrA* strain). The mutagenic activity of an agent at a given concentration C(R(C)) can be expressed by the ration of β-galactosidase activity to alkaline phosphatase activity. In this strain, alkaline phosphatase synthesis is constitutive and is not inducible by DNA damaging agents being determined in simultaneously with β-galactosidase. The SOS induction factor I(F)=R(C)/R(0) in which R(0) is the mutagenic activity calculated in the absence of the agent. The mutagenic and anti-mutagenic effects are useful whenever the safety aspect of the EO use is required and several studies are covering this aspect (Mezzoug et al., 2007; Burdock and Carabin, 2008; De Martino et al., 2009).

1.4.1.3.9 Quorum sensing

Bacteria produce and use small signalling molecules to evaluate their external environment and their internal physiological status i.e. to cell-cell communication (quorum sensing) modulating their populations. These molecules are generally known by autoinducers. The Gram-negative bacteria use acyl homoserine lactones (HSLs) whereas the Gram-positive bacteria use modified oligopeptides (Camilli and Bassler, 2006). Quorum sensing (QS) is involved in biofilm production, motility, swarming,

stress resistance and virulence (Kjelleberg and Molin, 2002). The participation of QS on so many essential aspects of the bacterial life makes this process an interesting target to control infections, diminish antimicrobial resistance and food spoilage (March and Bentley, 2204). The investigation of the anti-QS activity of EOs or its components is in progress (Niu et al., 2006; Brackman et al., 2008; Khan et al., 2009; Szabó et al., 2010; Brackman et al., 2011).

The effect of cinnamaldehyde on transcription of two HSLs, the 3-oxo-C6 and the 3-oxo-C12-HSL was evaluated by using a green fluorescent protein bioreporter system and the effect on the bioluminescence mediated by the 3-hydroxy- C4-HSL and the autoinducer-2 (AI-2) of *Vibrio harveyi* was followed by using two bioluminescent reporters *V. harveyi* strains (BB886 and BB170) (Niu et al., 2006). At 200 μmol/L cinnamaldehyde reduced in 70% the transcription of LuxR66 led by the PluxI promotor, which is induced by the 3-oxo-C6-HSL. In contrast, the effect of cinnamaldehyde on LasR6, whose transcription is lead by PlasR promoter and induced by 3-oxo-C12-HSL was not significant (Niu et al., 2006). The exposure of *V. harveyi BB886* (the bioluminescence of this strain is led by 3-hydroxy-C4-HSL) to 60μmol/l of cinnamaldehyde resulted in a 55% reduction of bioluminescence and the near 60% of the bioluminescence of the BB170 strain (mediated by AI-2) was reduced at 100μmol/L (Niu et al., 2006). Virulence of *V. harveyi* to *Artemia* shrimp can be reduced by the use of cinnamaldehyde and its derivative 2-NO_2-cinnamaldehyde when used in combination (Brackman et al., 2008). Using the nematode model *Caenorhabditis elegans* Brackman and colleagues (Brackman et al., 2011) demonstrated the efficacy of 3,4-dichloro-cinnamaldehyde on the reduction of the virulence of *V. anguillarum*, *V. harveyi*, *V. vulnificus* by mainly affecting the DNA ligand ability of LuxR.

The EO of rose, geranium, lavander, rosemary and clove seem to be very effective on as QS inhibitors whereas orange and juniper EO seem to have no anti-QS properties (Khan et al., 2009; Szabó et al., 2010).

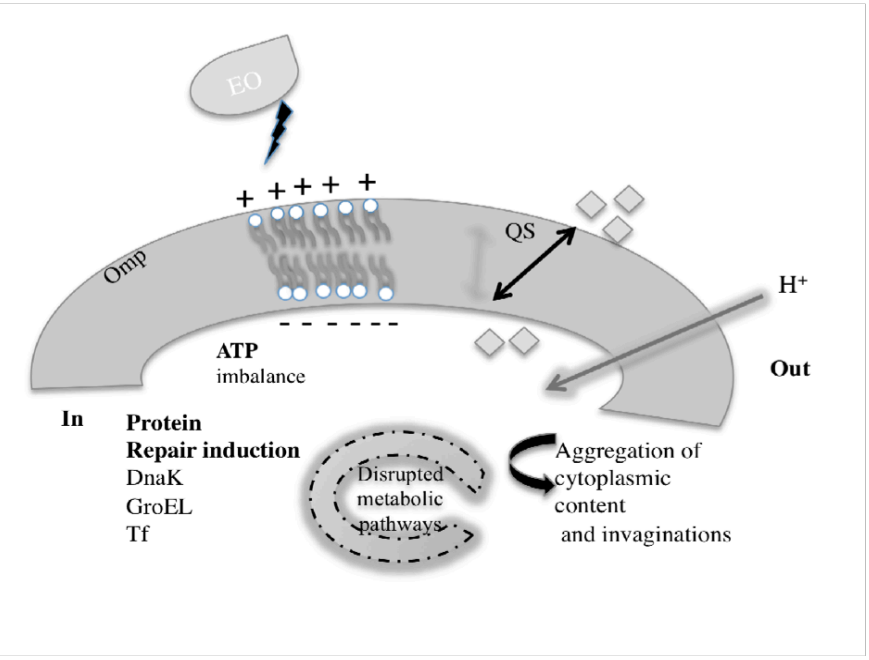

Figure 1.5. Identified bacterial cell structures and cellular processes disrupted by the action of EOs or their components. Omp (Outer membrane protein), QS (quorum sensing). EO treated cells are more permeable to protons, experience an ATP imbalance and induce the synthesis of chaperones. Metabolic pathways can be injured.

1.4.2 Antimicrobial mechanisms of natural compounds

1.4.2.1 Lactic acid

Lactic acid is the major LAB metabolite, causing pH reductions that inhibit many microorganisms (Eklund, 1989). The undissociated, more hydrophobic form of the acid diffuses over the cell membrane and dissociates inside the cell, releasing H^+-ions that acidify the cytoplasm (Axelsson, 1990; Piard and Desmazeaud, 1991). In addition to the pH effect, the undissociated acid collapses the electro-chemical proton gradient, causing bacteriostasis and finally death of susceptible bacteria (Eklund, 1989). Therefore, the main toxic effects of lactic include the reduction of intracellular pH and dissipation of the membrane potential (Kashket, 1987; Lorca and de Valdez, 2009).

As an effective antibacterial, lactic acid has a broad spectrum of both gram-positive bacteria and gram-negative bacteria (Qiao et al., 2008).

Lactic acid, the characteristic fermentation product of LAB may reduce pH to a level where putrefactive (e.g. *clostridia* and *pseudomonads*), pathogenic (e.g. *salmonellae* and *Listeria* spp.) and toxinogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum*) will either be inhibited or destroyed. Moreover, the undissociated acid, on account of its fat solubility (Brown and Booth, 1991), will diffuse into the bacterial cell, thereby reducing the

intracellular pH and slowing down metabolic activities. Growth of *Escherichia coli* (e.g.> is inhibited at pH 5.1 by lactic acid, as compared to pH 4.5 in presence of hydrochloric acid (Gudkow, 1987). The rapid reduction of pH below 5.3 during raw sausage fermentation is sufficient to inhibit growth of *salmonellae* (Schillinger and Liicke, 1988) and *Staphylococcus aureus* (Hechelmann et al., 1988).

Salmonella and *E. coli* exposure to lower concentration of lactic acid (0.25%) exhibited higher susceptibility, compared with that of *Listeria*. The type of microorganisms and their cell membrane structure and composition could also play an important role in the susceptibility to antimicrobials (Borges et al., 2013; Hayrapetyan et al., 2008). In general, *S. Enteritidis*, *E. coli* and *L. monocytogenes* could be completely inactivated after exposure to 0.5% lactic acid for 2 h.

The cell wall was generally not the sole antibacterial target, while cell membrane was the principle action site for organic acids as previously reported (Wang et al., 2014), due to the disruptive action of organic acid on cytoplasmic membrane by altering the hydrophobic property and polarity. In this respect, Wang et al. (2015) demonstrated that lactic acid induced the damage of membrane permeability and integrity (Wang et al., 2015). However, the antibacterial mechanism of organic acid may be different, since the antibacterial activity was not only dependent on the pH produced by organic acid, but also was closely related with their chemical structures (Hismiogullari et al., 2008).

1.4.2.2 Phenolic compounds

The antimicrobial activity of phenolic compounds occurring in vegetable foods and medicinal plants has been extensively investigated against a wide range of microorganisms. Among polyphenols, flavan-3-ols, flavonols, and tannins received most attention due to their wide spectrum and higher antimicrobial activity in comparison with other phenolic compounds, and due to the fact that most of them are able to suppress a number of microbial virulence factors (such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins) and show synergism with antibiotics. The antimicrobial properties of certain classes of polyphenols have been proposed either to develop new food preservatives (Rodríguez et al., 2010), due to the increasing consumer pressure on the food industry to avoid synthetic preservatives, or to develop innovative therapies for the treatment of various microbial infections (Jayaraman et al., 2010; Saavedra et al., 2010), considering the increase in microbial resistance against conventional antibiotic therapy.

Considering flavan-3-ols, the antibacterial activity of catechins has been known since the 1990s, when it was demonstrated that these compounds, largely present in oolong tea and, above all, green tea (*Camellia sinensis*), inhibited the in vitro growth of several bacterial species, such as *Vibrio cholerae*, *Streptococcus mutans*, *Campilobacter jejuni*, *Clostridium perfringes*, and *Escherichia coli* (Borris, 1996; Sakanaka et al., 1992; Diker et al., 1991; Ahn et al., 1991; Isogai et al., 1998).

More recently, it was demonstrated that some tea catechins, such as (-)-gallocatechin-3-gallate, (-)-epigallocatechin-3-gallate, (-)-catechin-3-gallate, and (-)-epicatechin-3-gallate, are active at nanomolar levels against some other food-borne pathogenic bacteria, such as *Bacillus cereus*. Most of these compounds were found to be more active than antibiotics, such as tetracycline or vancomycin, at comparable concentrations: this suggested that the tested tea catechins could exert a positive effect against gastrointestinal diseases (Friedman et al., 2006).

Among tea catechins, epigallocatechin gallate (EGCG) has received the most attention and has been investigated more thoroughly in its antibacterial, antiviral, and antifungal activities. As far as the antibacterial activity is concerned, 56 clinical isolates of *Helicobacter pylori*, a urease producing gastric pathogen that may contribute to the formation of ulcers and gastric cancer in humans, including 19 isolates, highly resistant to metronidazole and/or clarithromycin, were used to determine in vitro EGCG sensitivity. The minimum inhibitory concentration (MIC) required to inhibit the growth of 90% of organisms was found to be 100 mg/ml. It is interesting to underline that those clinical isolates which were highly resistant to antibiotics also showed a similar EGCG sensitivity (Yanagawa et al., 2003).

As reported above, tea catechins are active against *E. coli*. In particular, EGCG at sub-MIC (25 mg/mL) did not affect *E. coli* O157:H7 growth rate, but showed significant antipathogenic effect because it decreased some virulence factors such as biofilm formation and bacterial swarm motility (Lee et al., 2009).

Also, EGCG antiviral activity was discovered in the 1990s. EGCG was found to prevent infection caused by the flu virus by binding to the viral hemagglutinin, thereby preventing the attachment of viral particles to the target receptor cells (Nakayama et al., 1993). Other studies showed that modifications of viral membrane properties contributed to tea catechin's antiviral effect against the flu virus while, at the same time, structure–activity studies showed that the 3-galloyl side chain potentiates the parent catechin molecule antiviral activity. In fact, both EGCG and epicatechin gallate (ECG)

were found to be 10–15 times more active against the flu virus than epigallocatechin (EGC) (Song et al., 2005). Other investigations confirmed EGCG antiviral activity against adenovirus and enterovirus infections (Weber et al., 2003; Ho et al., 2009).
EGCG also exhibited variable time-dependent and concentration-dependent fungicidal activities. Several fungi, including *Candida albicans*, proved sensitive to this compound, suggesting that flavan-3-ols may be useful in the treatment of *C. albicans* superinfections of the oral cavities, intestine, and vagina, which may result from an excessive use of antibiotics (Hirasawa et al., 2004).

1.4.2.2.1 Flavonol antimicrobial activity

As far as flavonols are concerned, we can see a remarkable activity against several Gram-positive bacteria, such as *Staphylococcus aureus*, *Lactobacillus acidophilus*, and *Actinomyces naeslundii* and Gram-negative bacteria, such as *Prevotella oralis*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*, probably due to different mechanisms of action, among which the most convincingly identified is the aggregatory effect on all bacterial cells (Cushnie et al., 2007).
It is worth mentioning the following investigation which reported that rhamnetin, myricetin, morin, and quercetin showed high activity against Chlamydia pneumoniae, an obligate intracellular Gram-negative pathogen, which is a common cause of acute upper and lower respiratory infections, including pharyngitis, sinusitis, and pneumonia. In this study, the pretreatment of a human cultured cell line (HL cells), which is conventionally used in *C. pneumoniae* cultivation, with flavonols, decreased the infectivity of *C. pneumoniae* by 50% compared to the percentage seen in untreated controls, at polyphenol concentrations ranging from 0.5 to 50 mM (Alvesalo et al., 2006). When the compounds were continuously present in cell cultures, infectivity was clearly lower, varying from 0 to 32%. All compounds also decreased the infective yields, and the most chlamydiosidic compound was found to be rhamnetin, which killed *C. pneumoniae* at the tested concentrations. As the opportunity to use polyphenols as therapeutic agents is often limited by their bioavailability (Manach et al., 2004), it is interesting to highlight that due to their hydrophobicity, flavonols are capable of penetrating cell phospholipid membranes, and therefore they are also able to exert their antibacterial activity inside the cell. Moreover, rhamnetin resulted to be more active

than quercetin and morin, probably because of the methoxy group in the A-ring, which makes this molecule more hydrophobic (Alvesalo et al., 2006).

Recent investigations also pointed out the fungicidal activity of flavonols. It was shown that propolis, which is recommended worldwide for external topical use as it relieves various types of bacterial and fungal dermatitis, possessed antifungal activity (against *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*), and the main responsible agents for this activity were identified as flavonols (galangin, izalpinin, and rhamoncitrin) (Agüero et al., 2010). Among the other propolis that polyphenols found active, were flavanone (pinocembrin and pinostrobin) and chalcones (2,4-dihydroxychalcone and 2,4-dihydroxy-3-methoxychalcone), that other studies reported to show antimicrobial activity (Avila et al., 2008; Batovska et al., 2009).

1.4.2.2.2 Tannin antimicrobial activity

Tannins are subclassified into proanthocyanidins (condensed tannins) and gallotannins and ellagitannins (hydrolyzable tannins).

The proanthocyanidins occur in fruits, barks, leaves, and the seeds of many plants. They are dimers, oligomers, and polymers of catechins that are bound together by links between C4 and C8 (or C6) and are composed of a myriad of oligomeric products that differ first, in region and stereochemical configuration of the flavanol interlinkages, second, in the phenolic hydroxylation pattern, and third, in the configuration of the hydroxylated C-ring C3 center of the flavan-3-ol building block. These oligoflavanols are further subdivided into two basic types, A-type and B-type, which are characterized by the occurrence of either a double or a single linkage connecting two flavanol units (Quideau et al., 2011). These differences in the chemical structures make investigations directed towards their biological properties, or to their structure–activity relationships, quite challenging. The most studied proanthocyanidins are those derived from berries that inhibit the growth of several pathogenic bacteria, such as uropathogenic *E. coli*, cariogenic S. mutans, and oxacillin-resistant *S. aureus* (Côté et al., 2010). The cranberry proanthocyanidins, consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, were found to be active against the reported pathogenic bacteria. Several mechanisms could explain the effect of the A-type proanthocyanidin in the bacterial growth inhibition, such as the destabilization of the cytoplasmic membrane, the permeabilization of the cell membrane, the inhibition of extracellular

microbial enzymes, direct actions on microbial metabolism, or the deprivation of the substrates required for microbial growth, especially essential mineral micronutrients such as iron and zinc (via proanthocyanidin chelation with the metals), whose depletion can severely limit bacterial growth (Heinonen, 2007; Dixon et al., 2005).

Besides antibacterial activity, proanthocyanidins showed antiviral effects against influenza A virus and type-1 herpes simplex virus (HSV). In this case the mechanism of action seems to consist in preventing the entry of the virus into the host cell, which is the first critical step in primary HSV-1 infection (Geschera et al., 2011).

Gallotannins and ellagitannins derived from the metabolism of the shikimate-derived gallic acid (3,4,5-trihy-droxybenzoic acid) follow through various esterification and phenolic oxidative coupling reactions to yield numerous (near 1000) monomeric and oligomeric polyphenolic galloyl ester derivatives of sugar, mainly D- glucose (Quideau et al., 2011). The antimicrobial activity of hydrolysable tannins is well known. Ellagitannins, the main phenolic compound of the Rubus and Fragaria genus (raspberry, cloudberry, and strawberry) show very interesting properties because they inhibit, to different extents, the growth of selected Gram-negative intestinal bacteria (strains of *Salmonella*, *Staphylococcus*, *Helicobacter*, *E. coli*, *Clostridium*, *Campylobacter*, and *Bacillus*), but they are not active against Gram-positive beneficial probiotic lactic acid bacteria (Puupponen-Pimia et al., 2001). Unfortunately, *Listeria monocytogenes*, a common bacterium found in the environment and associated with animals that may cause meningitis, sepsis, or abortion, is not affected by these berry compounds (Puupponen-Pimiä et al., 2005).

As far as gallotannins are concerned, penta-O-galloylglucose, hexa-O-galloylglucose, hepta-O-galloylglucose, octa-O-galloylglucose, nona-O-galloylglucose, and deca-O-galloylglucose isolated from mango kernels showed antibacterial activity against food-borne bacteria. Gram-positive bacteria were generally more susceptible than Gram-negative, in fact the MICs against *Bacillus subtilis*, *B. cereus*, *Clostridium botulinum*, *C. jejuni*, *L. monocytogenes*, and *S. aureus* were 0.2 mg/mL or less; enterotoxigenic *E. coli* and *Salmonella enterica* were inhibited by 0.5–1 mg/mL. Also, in this case, lactic acid bacteria exhibited strong resistance (Engels et al., 2011).

The activity of gallotannins is attributable to their strong affinity for iron and it is also related to the inactivation of membrane-bound proteins.

Considering the antifungal activity of ellagitannins, the discovery of this property derives from the observation that the durability of hardwoods, such as oaks and chest-nuts is thought to owe much to the deposition of ellagi-tannins which are able to precipitate protein and/or remove metal cofactors through their strong affinity for metal ions, acting as a microbial barrier. A recent investigation showed that ellagitannins isolated from Ocotea odorifera, a medicinal plant commonly used in Brazil, have potent activity against *Candida parapsilosis* at a concentration level of 1.6 mM (Yamaguchi et al., 2011). Ellagitannins possess anti- viral activities, in particular against HIV infection (Martino et al., 2004; Notka et al., 2004) and manifest inhibitory effects on HSV-1 and/or HSV-2 replication, as well as Epstein-Barr virus (Notka et al., 2004). Ellagitannins activity against Herpes virus seems due to a marked inhibitory effect on the replication of both HSV-1 and HSV-2, including acyclovir-resistant strains, with acyclo-vir being the first effective specific drug against the Herpes virus made available (Ito et al., 2007).

1.4.2.2.3 Phenolic acids antimicrobial activity

Phenolic acids have one functional carboxylic acid and are hydroxylated derivatives of benzoic (e.g., gallic, protocatechuic, and *p*-hydroxybenzoic acids) and cinnamic acids (e.g., caffeic, *p*-coumaric, and ferulic acids) (Robbins, 2003; Stalikas, 2007; Wang et al., 2011). Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring, and the type of substituents, cause significant changes on the properties of the phenolic products (Robbins, 2003; Sroka and Cisowski, 2003; Stalikas, 2007). The site and the number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, and are the principal structural features influencing the antioxidant capacity of phenolics, with evidence that increasing hydroxylation results in toxicity increase. In addition, some authors have found that more highly oxidized phenols have higher antimicrobial properties (Cowan, 1999; Samy and Gopalakrishnakone, 2010; Scalbert, 1991). The hydroxycinnamic acids have significantly higher antioxidant activity than the hydroxybenzoic acids, which can be attributed to the presence of the CH=CH-COOH group in the hydroxycinnamic acids and the COOH group in the hydroxybenzoic acids. The presence of the CH=CH-COOH groups in hydroxycinnamic acids provide higher

H-donating capability and subsequent radical stabilization than the carboxylate group in hydroxybenzoic acids (Kim et al., 2006; Rice-Evans et al., 1996).

Phenolic acids show weaker antimicrobial activity in comparison with flavonoids; nevertheless, some investigations are worth mentioning. Some phenolic acids (gallic, caffeic, and ferulic acids) showed antibacterial activity against Gram-positive (*S. aureus* and *L. monocyto*genes) and Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*). These compounds were found to be more efficient against the reported bacteria than conventional antibiotics such as gentamicin and streptomycin. Differently, chlorogenic acid showed no activity against Gram-positive bacteria (Saavedra et al., 2010).

Considering another nonflavonoid class of compounds, lignans, a recent investigation showed that the hexane extract obtained from Aristolochia taliscana roots, a plant used in traditional Mexican medicine, contains neolignans, among which Licarin A was found to be the most active, with MICs ranging from 3.12 to 12.5 μg/ml against four mono-resistant variants and 12 clinical isolates of *Mycobacterium tuberculosis* strains (León-Dìaz et al., 2010). These results confirm previous investigations on lignans biological properties (Saleem et al., 2005) and suggest that these compounds represent a potentially active agent to fight tuberculosis, a pathology that, in recent years, has become more of a worldwide concern as one-third of the world's population is currently infected with *M. tuberculosis*.

1.4.2.3 Bacteriocins

Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by many Gram-positive and Gram-negative bacteria; however, those produced by food grade LAB have received considerable attention due to their potential application in food industry as natural preservatives (biopreservatives). LAB bacteriocins are small antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocins (Klaenhammer et al., 1988, De Vuyst et al, 1994, Chen et al., 2003). There are several proposed bacteriocin classifications divided into 3 or 4 classes: (*i*) lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose biologically inactive prepeptides are subjected to extensive post-translational modification; (*ii*) small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocins like or *Listeria*-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb) and circular bacteriocins (class IIc); and (*iii*) bacteriolysins or

large, heat-labile, lytic proteins, often murein hydrolases (class III) (De Vuyst et al., 2007)). Some authors (Klaenhammer et al., 1993; nes et al., 1996) also proposed (*iv*) class IV bacteriocins that require non-proteinaceous moieties (lipid, carbohydrate) for their activity.

Bacteriocins that are produced by LAB can be of broad or narrow spectrum, but in general, the activity is directed against low G+C Gram-positive species (Klaenhammer et al., 1988). The antibacterial spectrum includes spoilage organisms and foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Wide ranges of mode of action have been described for bacteriocins, such as enzyme activity modulation, inhibition of outgrowth of spores and formation of pores in cell membrane. Most bacteriocins interact with anionic lipids that are abundantly present in the membranes, and consequently initiate the formation of pores in the membranes of susceptible cells (Chen et al., 2003; Moll et al., 1999). However, generalized membrane disruption models cannot adequately describe the mode of action of bacteriocins. Rather, specific targets seem to be involved in pore formation and other activities. For the nisin and epidermin family of lantibiotics, the membrane-bound cell wall precursor lipid II has been identified as target (Héchard and Sahl, 2002). Most of class II bacteriocins dissipate the proton motive force (PMF) of the target cell via pore formation (Venema et al., 1995). The subclass IIa bacteriocin activity depends on a mannose permease of the phosphotransferase system (PTS) as a specific target. The subclass IIb bacteriocins (two-component) also induce dissipation of the PMF by forming cation- or anion-specific pores; specific targets have not yet been identified. Finally, subclass IIc comprises miscellaneous peptides with various modes of action such as membrane permeabilisation, specific inhibition of septum formation and pheromone activity (Klaenhammer et al., 1988).

1.4.2.3.1 Factors affecting bacteriocin efficiency

The activity of bacteriocins produced by different LAB is not uniform and constant and depends on the chemical composition and physical conditions of food; it mainly depends on pH and is reduced by bacteriocin binding to food components, adsorption to cell or protein, activity of proteases and other enzymes (Schillinger et al., 1996). A correlation between nisin degradation and extent of proteolysis in pasteurized cream was found by Phillips et al. (1983). Buyong et al. (1998) ascribed the reduction in pediocin activity from 64,000 to 2,000 U/g after six months of maturation of Cheddar cheese to the action of proteases and peptidases. NaCl at certain concentrations can reduce the growth of LAB and consequently the production of bacteriocins, besides protecting the target bacteria such as *L. monocytogenes* from their action (Hugas et al., 2002). Sarantinopoulos et al. (2002) observed reductions in bacteriocin activity and *E. faecium* FAIR-E 198 growth rate after addition of 2% NaCl to MRS broth. Nilsen et al. (1998) ascribed

this phenomenon to the interference of NaCl in the production factor binding the inductor to the receptor.

Aside from interacting with food components, bacteriocins may be adversely affected by processing and storage conditions such as pH and temperature of the product. According Drosinos et al. (2005), the optimal pH for bacteriocin production (5.5) does not match that for microbial growth (6.5). Because of their maximum stability under acidic conditions, nisin activity is increased when used in acidic foods. Therefore, effective applications of nisin require that the pH of food is less than 7 to ensure satisfactory solubility, stability during processing and storage period (Hernandez et al., 1993). Leroy and De Vuyst (1999) reported that bacteriocin activity decreases with increasing temperature owing to increased activity of proteases.

The inhibitory efficiency of bacteriocins is also related to the level of food contamination by the target organism. If the initial contamination is too high, bacteriocin activity is low and unable to prevent the development of contaminating microorganisms. Rilla et al. (2004) investigated the action of *Lc. lactis* subsp. *lactis* IPLA 729 against *S. aureus* at two different concentrations, specifically $1.8x10^4$ and $7.2x10^6$ CFU/mL: after 24 h of incubation, they did not detect *S. aureus* in the more dilute sample, while the other showed a still high count ($5.0x10^4$ CFU/mL).

1.5 Incorporation of antimicrobials in food systems

Antimicrobials in foods can be added in different forms to control the growth of pathogenic and spoilage microorganisms. These can be either added directly or through slow release from packaging materials. In this section, we highlight three major methods of incorporating antimicrobials in food systems. These methods could play an important role in reducing harmful microorganisms, thus extending product shelf-life.

1.5.1. Direct applications

Researchers have demonstrated the antimicrobial activity of different natural compounds against a wide range of pathogenic microorganisms. There have been a number of studies conducted in culture media and tested in food products. These antimicrobial compounds have been directly applied in food systems either in the form of a powder or a liquid. However, only a few natural antimicrobials have found practical application in the food industry and their use in foods as preservatives is often limited due to the strong smell and taste they impart to these foods. In addition, natural antimicrobial solubility in complex food matrices is another limitation (Soković et al., 2010).

Budka and Khan (2010) demonstrated the effect of EOs from basil, thyme, and oregano against *B. cereus* in rice-based foods. Carvacrol (EO of oreganum and thyme) at 0.15 mg/g inhibited the growth of *B. cereus* on rice (Ultee et al., 2000). Freshly ground garlic at a concentration of 1% was shown to reduce the Salmonella count when added to mayonnaise (Leuschner and Zamparini, 2002). The antimicrobial activitiy of phenolic compounds from several plant species has been shown to inhibit *S. aureus* in chicken soup (Hadzifejzovi et al., 2013). Yuste and Fung (2002) reported 6 log CFU/ml reductions of *L. monocytogenes* in pasteurized apple juice with 0.1 e 0.3% (w/v) of ground cinnamon after 1h of incubation, and no further growth of the microorganism occurred during 7 days of storage. Cava Nowak et al., (2007) evaluated the efficacy of EOs of cinnamon bark, cinnamon leaf, and clove against *L. monocytogenes* in semi skimmed milk incubated at 7°C for 14 days. They observed that the MIC was 500 ppm for cinnamon bark EO and 3000 ppm for the cinnamon leaf and clove EOs. These results indicated the possibility of using these EOs in milk beverages as natural antimicrobials. Similarly, Smith-Palmer et al., (2001) reported the inhibition of *L.*

monocytogenes and *S. Enteritidis* in both low fat and full fat cheese in the presence of 1% clove, cinnamon, thyme, and bay oil. The antimicrobial effect of rosemary extract against *L. monocytogenes* was assessed by Munõz et al. (2009). When rosemary extract at 0.1 ml/100 ml concentration obtained by using the super critical fluid extraction method was tested against *L. monocytogenes* at 30°C in broccoli juice and incubated for 30 days, a bactericidal effect was observed.

The use of natural antibacterial compounds such as extracts of spices, herbs, and EOs, has been reported in literature to improve the shelf-life of meat. The shelf-life of meat based products increased when products were dipped in thyme and oregano oil at 0.1 and 0.3% (Karabagias et al., 2011). The combination of thyme EO at 0.6% with nisin at 1000 IU/g significantly decreased the population of *L. monocytogenes* in minced beef during storage at 4 and 10 °C (Solomakos et al., 2008). A synergistic effect of rosemary extracts with pre-freezing was shown to reduce *C. jejuni* populations by more than 2.0 logs in chicken meat (Piskernik et al., 2011). Xi et al., (2011) investigated the effect of cranberry powder against *L. monocytogenes* growth in meat model system. The results showed a 2-4 log CFU/g reduction in bacterial population at concentrations of 1-3% when compared to the control sample treated with nitrite. This showed a possibility of using natural ingredients such as cranberry powder instead of sodium nitrite to enhance the antibacterial quality and shelf-life of naturally cured meat.

Overall, plant extracts could be used as natural antimicrobial additives to prolong the shelf-life of foods. However, the level of these preservatives required for sufficient inhibition of microorganisms in foods may be considerably higher in comparison to laboratory media. Because this higher concentration may negatively impact the organoleptic properties of food, the use of natural compounds in combination with other natural preservatives or with other technologies could produce synergistic effects against foodborne pathogens.

1.5.2. Edible films and coatings

In recent years, food-packaging industries have shown an interest in edible films and coatings from natural antimicrobials. Edible films also improve food quality by providing barriers to moisture, and oxygen, and could serve as a barrier to surface-contaminating microorganisms (Cao et al., 2009; Jang et al., 2011; Joerger, 2007). In addition, edible films and coatings help reduce environmental concerns created by

conventional plastic packaging. Various approaches have been proposed and demonstrated for the use of edible films and coatings to deliver antimicrobial compounds to a variety of food surfaces, including fruits, vegetables, and meat products (Devlieghere et al., 2008).

Ayala-Zavala et al. (2013) demonstrated the antimicrobial activity of an edible film formulated with cinnamon leaf oil that can be useful in preserving the quality of fresh-cut peaches. Similarly, Raybaudi-Massilia et al. (2008) reported the reduction of *E. coli* O157:H7 population by >4 logs on fresh cut Fuji apples with cinnamon, clove, and lemongrass oils at 0.7%, or their active compounds, cinnamaldehyde, eugenol, and citral, at 0.5% incorporated into alginate films. In another study, the addition of grapefruit seed extract to the rapeseed proteinegelatin film inhibited the growth of *E. coli* O157:H7 and *L. monocytogenes* in strawberries (Jang et al., 2011). The antimicrobial activity of the polypropylene/ethylene-vinyl alcohol copolymer (PP/EVOH) films with 5% oregano EO against pathogenic microorganisms *E. coli*, *S. enterica* and *L. monocytogenes* and natural microflora was recently investigated by Muriel-Galet et al. (2012) on packaged salads. The author's findings showed a reduction in spoilage flora as well as inhibition of the growth of pathogens on contaminated salads. The antimicrobial activity of apple-based edible films containing plant antimicrobials (cinnamaldehyde and carvacrol) was also investigated by Ravishankar et al. (2009). These films were shown to be effective against *S. enterica* and *E. coli* O157:H7 on poultry, and against *L. monocytogenes* on ham.

Similarly, chitosan based films have proven to be very effective in food preservation. The shelf-life of carrot sticks coated with chitosan was evaluated. An edible coating containing 0.005 mg/mL chitosan applied to carrot sticks under modified atmospheric packaging over 12 days at 4 °C was shown to maintain quality and prolong the shelf-life (Simões et al., 2009). The antimicrobial effects of edible chitosan films containing nisin, peptide P34, and natamycin were investigated by Cé et al. (2012) against *L. monocytogenes*, *B. cereus*, *S. aureus*, *E. coli*, *S. Enteritidis*, *C. perfringens*, *Aspergillus phoenicis*, and *Penicillium stoloniferum*. These chitosan films were effective in controlling microbial growth in minimally could thus be a feasible method for the biopreservation of food. Chitosan was also shown to possess a film-forming property, to decrease water vapor and oxygen transmission, diminish respiration rate, and increase the shelf-life of fruit (Jiang and Li, 2001). Incorporation of chitosan-coated films with green tea extracts (4%) inhibited the growth of *L. monocytogenes* on ham steak during

storage at room and refrigerated temperature (Vodnar, 2012). Similarly, Leleu et al. (2011) reported the use of chitosan coatings to reduce bacterial contamination of egg contents resulting from trans-shell penetration by *S. Enteritidis* and other bacteria, such as *Pseudomonas spp.*, *E. coli*, and *L. monocytogenes*.

The antibacterial activity of soy protein edible films incorporated with oregano or thyme EOs was tested on fresh ground beef patties at 4 °C. Films with 5% of EOs significantly inhibited the growth of *Pseudomonas* spp. and coliform counts (Emiroglu et al., 2010). Increased concentrations of catechin in a film used during storage of sausages resulted in a decrease in *E. coli* O157:H7 and *L. monocytogenes* populations (Ku et al., 2008). Nisin-incorporated polymer films have shown to control the growth of undesirable bacteria, thereby extending the shelf-life and enhancing the microbial safety of meats (Cutter et al., 2001). The effectiveness of active films using antibacterial peptides of *Bacillus licheniformis* Me1 against *L. monocytogenes* in dairy products was recently demonstrated by Nithya et al. (2013). Their results showed that antimicrobial peptide from films diffused slowly into the foodmatrix (paneer) during the storage period, thereby extending the shelf-life of the product.

The incorporation of antimicrobials in food packaging such as films and coatings could prevent surface growth in foods where a large portion of spoilage and contamination occurs. This approach also reduces the addition of larger quantities of antimicrobials that are usually incorporated into the bulk of the food. The gradual release of an antimicrobial compound from packaging films and coatings to the food surface could have an advantage over direct application of antimicrobials in food systems. Franssen et al., (2003) reported that food packaging prepared from edible antimicrobial coatings containing polypeptides, such as lysozyme, peroxides, and lactoferrins have been shown to extend the shelf-life of food products and make them safer for human consumption, in addition to providing physical protection for the food. These studies suggest that the food industry and consumers could use these films and coatings to control surface contamination by foodborne pathogenic microorganisms.

1.5.3. Nanoparticles

Nanotechnology has been developing rapidly as one of the most significant technological advances of our time. Nanoscience and nanotechnology have already been applied in various fields including medicine and the food industry (Sozer and Kokini,

2009). In the last few years, the application of nanotechnology to food safety has attracted the attention of many researchers due to its considerable potential for the development of antimicrobial delivery systems (Zou et al., 2012). This technology could be used to improve antimicrobial stability and could be applied directly or as a coating or packaging in different food systems to inhibit the growth of foodborne pathogens. Applications of nanotechnology to deliver antimicrobials have been reported in several studies. However, the study of nanoparticles as antimicrobials in food models is very limited due to the complexity of food components. Some of the recent studies that have been effectively applied in food models using natural compounds are discussed in this section.

Zou et al. (2012) demonstrated the potential use of liposomal nanoparticles for enhancing the antimicrobial efficacy of nisin against *L. monocytogenes* and *S. aureus* in food systems. The antimicrobial activity of free nisin and nisin loaded solid lipid nanoparticles was also studied by Prombutara et al. (2012). The results of their study showed stable and longer antimicrobial activity of nisin loaded nanoparticles against *L. monocytogenes* DMST 2871 compared to free nisin, indicating that the nisin was released from nanoparticles throughout the storage period. In raw and cooked chicken meat systems, naturally occurring phenolic compounds delivered by nanoparticles were proven to be more effective against *S. typhimurium* and *L. monocytogenes* at much lower concentrations than when delivered individually without nanoparticles (Ravichandran et al., 2011). These findings demonstrate the potential for nanoparticles to be used for food safety applications such as the delivery of phenolic compounds for pathogen reduction.

EOs are widely used natural compounds of plant origin. However, the poor water-solubility of EOs makes it difficult to incorporate them into foods and reduces antimicrobial action (Weiss et al., 2009). Therefore, a higher concentration of EOs is required to achieve higher antimicrobial efficacy which could alter the sensory properties of foods. In a recent study, Shah et al., (2012a, 2012b) reported a nanodispersion method to overcome this challenge. A follow-up study by Shah et al. (2012b) reported improved antimicrobial activity of nanodispered eugenol against *E. coli* O157:H7 and *L. monocytogenes* in bovine milk. Thymol-containing nano-dispersions are also effective against pathogens in food applications. Shah et al. (2012a) also investigated the efficacy of thymol dispersed in whey protein isolate and maltodextrin nanocapsules to inhibit *E. coli* O157:H7 and *L. monocytogenes* in apple

cider and 2% reduced fat milk. More recently, Xue et al. (2013) demonstrated the higher antilisterial activity of nanoemulsified thymol in milk compared to free thymol. In this study, authors reported the reduction of *L. monocytogenes* from ~5 log CFU/ml to below the detection limit in 6h by nanoemulsified thymol in skim and 2% fat milk. In full fat milk, the bacterial population was reduced to undetectable limits after 48 h of incubation at room temperature. In all tested food systems, nano-encapsulated EOs were more evenly distributed even at higher concentrations above the solubility limit than free EOs, thus resulting in higher antimicrobial efficacy.

The application of antimicrobial compounds that have been widely applied to microbial control in food products and processing environments has met with several limitations including undesirable flavor, low solubility, and instability (Zou et al., 2012). The efficacy of such antimicrobial properties is exhausted due to interactions with food components (proteins and lipids), inactivation by enzymatic degradation or uneven distribution of antimicrobial compounds within the complex food systems (Prombutara et al., 2012). Nanoscale antimicrobial delivery systems could enhance the efficacy of antimicrobials by improving their solubility and dispersibility and thus improve the quality of food products. Nanotechnology is also being developed in the areas of food packaging. The incorporation of nanomaterials into food packaging has been shown to improve food quality in fresh fruits and vegetables, bakery products and confectionery by protecting food from moisture, lipids, gases, off-flavors and odors (Sozer and Kokini, 2009). Despite all of the potential applications, nanotechnology is still a new subject in the field of food safety. The specific properties and characteristics of nanomaterial used in food applications need to be carefully examined for any potential health risks.

1.5.4 Perspectives and limitations in the application of essential oils and phenolic compounds in food

A range of essential oil components have been accepted by the European Commission for their intended use as flavorings in food products and include linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene, all of which are considered to present no risk to the health of the consumer. The United States Food and Drug Administration (FDA) also classifies these substances as generally recognized as safe (GRAS). The crude essential oils classified as GRAS by FDA include amongst

others clove, oregano, thyme, nutmeg, basil, mustard, and cinnamon. There are regulatory limitations on the accepted daily intake of essential oils or essential oil components, so before they can be used in food products, a daily intake survey should be available for evaluation by FDA.

Despite the demonstrated potential of essential oils and their constituents in vitro, their use as preservatives in food has been limited because high concentrations are needed to achieve sufficient antimicrobial activity. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as fat (Cava-Roda et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010), starch (Gutierrez et al., 2008), and proteins (Cerrutti and Alzamora, 1996; Kyung, 2011). Furthermore, the antimicrobial potency of essential oil constituents also depends on pH (Juven et al., 1994), temperature (Rattanachaikunsopon and Phumkhachorn, 2010), and the level of microbial contamination (Somolinos et al., 2010). Extrapolation of results from in vitro tests to food products is thus difficult at best, and a lower performance of the antimicrobial compound must be expected. For example, Cilantro oil had significant antibacterial activity at 0,018% in vitro, but when applied to a ham model, even 6% cilantro oil had no antimicrobial activity (Gill et al., 2002). Before being added to food products, it is therefore useful to investigate how essential oils or their constituents interact with food components in vitro. Food matrix interactions with the essential oils or their constituents can be investigated by measuring the growth of microrganssms in culture medium containing a range of concentrations of fat, protein, or starch as well the antimicrobial compound of interest. Such experiments have been performed using a so-called food model media (Gutierrez et al., 2009), and can be used to provide quick answers to which kind of food products the compound in question can be used in.

The intense aroma of essential oils, even low concentrations, can cause negative organoleptic effects exceeding the threshold acceptable to consumers (Lv et al., 2011). Having to increase the concentration of essential oils to compensate for their interactions with food matrix components is therefore highly unfortunate and limits their application to spicy foods where the accpetable sensory threshold is relatively high. Different strategies can be used to circumvent this problem. One option is to use essential oils in active packaging rather than as an ingredient in the product itself. Essential oils can be encapsulated in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food surface or to the headspace of packages

of, e.g., fruit, meat, and fish (Pelissari et al., 2009; Sànchez-Gonzàles et al., 2011). Sachets that release volatile essential oils into the headspace environment are simply placed within an enclosed food package (Ahvenainen, 2003). The advantage of incorporating volatile components of essential oils in films or edible coatings is that the diffusion rate of the agents away from the food product can be reduced, thereby maintaining the active compounds in the headspace or no the product surface for extended periods of time (Phillips and Laird, 2011; Sànchez-Gonzàles et al., 2011). A way to minimize organoleptic effects of essential oils into nanoemulsions. This approach increases the stability of volatatile components, protecting them from interacting with the food matrix, and increases the antimicrobial actvity due to increased passive cellular uptake (Donsí et al., 2011).

Lowering the concentration of essential oils without compromising their antimicrobial activity can also be obtained by applying them in combination with other antimicrobial compounds that provide a synergistic effect. Synergies are known to occur for essential oil combinations, and it is therefore a field with countless opportunities to find potent antimicrobial blends, which may be the key to implementing essential oils in food preservation without simultaneous organoleptic effects.

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CHAPTER 2

OBJECTIVES

PhD research aims to facilitate the development of green strategies for the control of undesirable microorganisms in food products. In the last two decades, much attention has been focused on "green strategies" that using microorganisms or their antimicrobial compounds can assure shelf-life extension and the control of more and more dangerous microorganisms such as *Listeria monocytogenes*. So far, several screening processes are developed in order to select the most appropriate effective strains to be used as protective cultures. *Lactobacillus plantarum* is surely the most versatile and widespread species and the production of 3-phenyllactic acid seem the most effective and interesting bio-preservative compounds. However, these screening programmes are labour intensive and a large number of strains isolated from different food matrices are assessed, thereby requiring more expensive investments in order to avoid unsatisfactory results. In addition, until now, several issues, such as the high minimal inhibitory concentration levels, the stability of antimicrobial compounds, the knowledge of action mode, as well as the relation between microbial growth and compound formation kinetics, still remain unclear. These findings call for a more simplified and useful approach when searching for new protective strains, taking into account that food stress conditions strongly influence the development of specific microbial strains. It would be extremely interesting to ascertain the effect of different environments on the selection of strains able to exert antimicrobial activities. Nevertheless, the correlation between strain resistance to stress conditions and the ability to produce antimicrobial effects was poorly investigated. Therefore, the present PhD research looks at the correlation between the *Lb. plantarum* strain isolated from hard environments and the ability to assure inhibitory action against undesirable microorganisms as well as the ability to produce a specific antimicrobial compound (3-phenyllactic acid). With the above-mentioned aim, this research focuses on the antimicrobial activity expressed by Lb. plantarum isolated from five different sources.

In addition, the present PhD research focuses the attention on the effects of growth phase on the 3-phenyllactic acid (PLA) formation by *Lb. plantarum*. Moreover, the cultivation conditions that are able to assure the highest PLA levels are investigated. In fact, the enhancement and the improving of PLA formation represents an actual and crucial topic to assure a better PLA development in food industry. PLA formation could be linked to stress response mechanisms performed by *Lb. plantarum*. However, no information on relation between LAB stress response and PLA production is available in literature as well as very few information is reported on the relation between microbial growth phase and PLA formation. Even if the prevailing opinion in scientific community believes that PLA formation is related to LAB growth arrest has not been explained the linkage to metabolic pathways involved in stationary phase. Moreover, little information, if not any, can be also found on the optimal pH condition of PLA metabolic pathway in *Lactobacillus* species.

Finally, the anti-*Listeria* mechanism of 3-phenyllactic acid was investigated. For this purpose, the antimicrobial effect of PLA is evaluated to different pH and, hence, the PLA anti-*Listeria* is compared with those expressed by the lactic acid and the better studied hydroxybenzoic and hydroxycinnamic acids.

CHAPTER 3

ANTIMICROBIAL ACTIVITY OF *LACTOBACILLUS PLANTARUM* STRAINS ISOLATED FROM DIFFERENT ENVIRONMENTS

3.1 Introduction

In the last two decades, great attention has been focused on the food biopreservation (Stiles, 1996; Rodgers, 2001; Corsetti et al., 2008; Saris, 2014), a "*bio-strategy*" that assure the extension of shelf-life and food safety through the use of microorganisms or their antimicrobial compounds (Lucke, 2000). Lactic acid bacteria (LAB) should be considered as the ideal choice for application as protective cultures in food products (Maragkoudakis et al., 2009). Their antagonism refers to inhibition through competition for nutrients or to production of one or more antimicrobial active metabolites (Ray and Bhunia, 2008). The long tradition of using in food substantiated with scientific understanding on their antimicrobial and enhanced health effects suggest them as perfect alternatives to chemical preservatives. The use of LAB for their antimicrobial properties is well known and has been extensively studied (Leroy and De Vuyst, 2004; Corsetti et al., 2014; Sorrentino et al., 2013). LAB have been reported to produce wide range of microbial growth inhibiting substances. Selected LAB strains or the bioactive compounds purified from the culture medium can be exploited as efficient alternatives for food preservation. *Lactobacillus plantarum* is the most versatile and widespread species among LAB. In fact, it's found in different food-matrices and environments ranging from vegetables, dairy, and meat products, to the human gastrointestinal (GI) tract. Some strains of *Lb. plantarum* are known for their ability to produce several natural antimicrobial substances, such as bacteriocins, BLIS, phenyllactic acid, organic acids and hydrogen peroxide. The versatility of *Lb. plantarum* is linked to its natural genomic architecture (Siezen et al., 2011) and is at the root of its success in the

industrial applications not only as starter culture but also as bio-protective agent (Sorrentino et al., 2013, Sakaridis et al., 2012). In this last field, the *in vitro* screening of bacterial protective properties is labour intensive and a large number of strains isolated from different food matrices is required. *Lactobacillus* strains isolated from different sources as plant material, food products or human and animal can be screened for their antimicrobial activity and it is very important to screen a large number of strains in easy, rapid and reliable way. A simplified approach may be useful in the search of new protective strains, taking into account that food stress conditions strongly influence the development of specific microbial strains (Ricciardi et al., 2012; Filannino et al., 2014; Heunis et al., 2014). Therefore, it would be very interesting to ascertain the effect of different environments on the selection of strains able to exert antimicrobial activities. Nevertheless, the correlation between strain resistance to stress conditions and the ability to produce antimicrobial effects was poorly investigated. The present research seeks more in depth knowledge of the relation between antimicrobial properties of *Lb. plantarum* strains and their source of isolation. With the above-mentioned aim, the antimicrobial activity expressed by *Lb. plantarum* isolated from five different sources was evaluated and the nature of the compounds determining the inhibition was investigated.

3.2 Materials and methods

3.2.1 Producer and indicator strains

One hundred and ten *Lb. plantarum* strains (producers), belonging to the Department of Agriculture Environment and Food (DIAAA), were isolated from different fermented foods (sourdoughs, wines, cheese, fermented sausages and honey). The main features of food matrices, as well as the number of *Lb. plantarum* strains isolated from each source, are reported in Table 3.1.

Table 3.1. Producer strains and food matrices

Number of strains	Short IS	Strains	Isolation source	Features of isolation source				References
				pH	aw	lcohol (%vol)	NaCl %	
17	C_	C_11; C_12; C_21; C_25; C_29-30; C_35-36; C_43; C_54; C_56; C_63; C_66; C_68; C_71; C_74; C_78	cheese (Caciocavallo)	5.55 - 5.75	0.96-0.97	n.d.	1.9 - 2.2	Coppola et al., 2003
13	FS_	FS_8; FS_14; FS_22; FS_24; FS 28; FS 32; FS 36; FS 39;	fermented sausage (Soppressata)	5.75 - 5.80	0.94 - 0.97	n.d.	2.8 - 3.1	Coppola et al., 1998
9	FS_	FS_CV11; FS_CV21; FS_CV25; FS_CV28; FS_CV30; FS_IV2; FS_IV29; FS_IV38; FS_IV87	fermented sausage (Ventricina)	5.15 - 5.18	0.93 - 0.94	n. d.	3.0 - 3. 5	Tremonte et al., 2007; Pannella, 2010
5	W_	W_A1-A5	red wine (Aglianico)	3.71 - 3.88	n.d.	13.6	n.d.	Testa et al., 2014
12	W_	W_M2; W_M5; W_M11-12; W_M14; W_M16-20; W_M23;	red wine (Montepulciano)	3.60 - 3.80	n.d.	11.8 - 13.5	n.d.	Testa et al., 2014
2	W_	W_P2; W_P5	red wine (Piedirosso)	3.62 - 3.65	n.d.	12.4 - 12.8	n.d.	Testa et al., 2014
3	W_	W_P16; W_P18; W_P19	red wine (Pentro d'Isernia)	3.66 - 3.77	n.d.	11.3 - 11.6	n.d.	Testa et al., 2014
3	W_	W_R1; W_R2; W_R4	red wine (Rosso Molise)	3.62	n.d.	12.5	n.d.	Testa et al., 2014
5	W_	W_T1; W_T4; W_T13-14; W_T17	red wine (Tintilia)	3.66	n.d.	14	n.d.	Testa et al., 2014
6	W_	W_TA1; W_TA4-8	red wine (Taurasi)	3.68	n.d.	14.2	n.d.	Testa et al., 2014
4	H_	H_BB1-4	honey (bee bread)	3.5 - 3.8	0.57	n.d.	n.d.	DiAAA collection
6	S_	S_9-10; S_20; S_24; S_29; S_33	sourdough from Campania Region	3.7 - 4.0	0.98	n.d.	0.8 - 1.2	Pannella, 2010
18	S_	S_B1; S_D2; S_D3; S_L4; S_M1; S_M2; S_M3; S_M4; S_N1-N2; S_Q1-Q4; S_R1-R4	sourdough from Molise Region	3.6 - 4.1	0.97 - 0.98	n.d.	0.7 - 1.1	Reale et al., 2011
7	S_	S_J14; S_J22; S_J35; S_SEP11; S_SEP16; S_W1-W2	sourdough from Molise Region	3.6 - 4.2	0.98	n.d.	0.9 - 1.0	Reale et al., 2005

All the strains were tested for their antimicrobial activity against 33 undesirable microbial strains (indicators), listed in Table 3.2.

Table 3.2. Indicator strains

Species	Strains	Origin	Collection	Cultivation	References
Lactobacillus brevis	A4, B2	sourdough	DIAAA	MRS broth, 28 °C	REALE *et al*. 2011
Lb. casei	SERB108, SERB69	wine	DIAAA	MRS broth, 28 °C	SORRENTINO 2010
Listeria innocua	ATCC 33090	-	DSMZ	BHI, 37 °C	
Brochotrix thermosphacta	DSM 20171^T	-	DSMZ	Corin broth, 28 °C	
Clostridium sporogenes	DSM 795^T	-	DSMZ	RCM, 28 °C	
Pseudomonas fluorescens	DSM 50090^T	-	DSMZ	Nutrient broth, 28 °C	
P. fragi	DSM 3456^T	-	DSMZ	Nutrient broth, 28 °C	
P. putida	DSM 291^T	-	DSMZ	Nutrient broth, 28 °C	
Acetobacter aceti	DSM 3508^T	-	DSMZ	MYP broth, 28 °C	
A. aceti	111, 111E, ASRT, ASC	winegar	DIAAA	MYP broth, 28 °C	PANNELLA 2010
A. pasteurianus	DSM 3509^T	-	DSMZ	MYP broth, 28 °C	
A. tropicalis	DSM 15551^T	-	DSMZ	MYP broth, 28 °C	
Gluconacetobacter hansenii	DSM 5602^T	-	DSMZ	MYP broth, 28 °C	
Ga. hansenii	194BV, ASAC4, ASR, ARLA, AC1, 141A	wine	DIAAA	MYP broth, 28 °C	PANNELLA 2010
Ga. hansenii	203B1	fruit	DIAAA	MYP broth, 28 °C	PANNELLA 2010
Ga. liquefaciens	DSM 5003^T	-	DSMZ	MYP broth, 28 °C	
Gluconobacter oxydans	146B, AC6	wine	DSMZ	MYP broth, 28 °C	
Penicillium spp.	T1, T2, T3, T4, T5	black truffle	DIAAA	MYP broth, 28 °C	SORRENTINO *et al.* 2013

Producer and indicator strains were propagated twice for 16 h at 28°C in proper media before their use.

3.2.2 Detection of antimicrobial activity exerted by growing cells

The spot-on-the-lawn technique was performed against each indicator to detect growing cells (GC) of producers having inhibitory properties. The method used was described by Moraes et al., (2010), and the presence of a distinguishable inhibition zone around the spots, evaluated after 24 h incubation at 28°C, was considered as positive antagonistic effect. The degree of inhibition was defined as low (5 mm < ∅ <15 mm), moderate (15 mm ≤ ∅ < 25 mm), strong (25 mm ≤ ∅ < 35 mm), and very strong (35 mm ≤ ∅ < 45 mm). A calibrated-densitometer (GS-800, Bio-Rad, Hermles CA, USA) was used for imaging acquisition and Adobe Photoshop CS6 Extended software was used for the measurement of clearing zones. Each experiment was carried out in triplicate.

3.2.3 Detection of antimicrobial activity exerted by cell free supernatants

The antimicrobial activity of cell free supernatants (CFS) was detected by the agar well diffusion assay described by Moraes et al. (2010), following the modifications of Tremonte et al. (2007; 2010). After 24-48 h of incubation at 28°C, dishes were investigated for zones of inhibition. Inhibition halos were normalized using the following formula:

$$\text{Inhibition Score (IS)} = \frac{\text{diameter inhibition halo (mm)}}{\text{diameter well (mm)}}$$

On this basis, the antimicrobial effect was considered as low (1 < IS < 3), moderate (3 ≤ IS < 5), strong (5 ≤ IS < 7), and very strong (7 ≤ IS < 9).

Dishes inoculated with each indicator strain and without CFS were used as control. To detect the presence of acids or proteins with inhibitory effect produced by *Lb. plantarum*, the agar-well diffusion assay was also performed including two additional tests:

1) nCFS: filter-sterilized CFS of each *Lb. plantarum* strain, neutralised with 1N NaOH (Sigma-Aldrich, St. Louis, MO) up to pH 7;

2) pCFS: filter-sterilized CFS of each *Lb. plantarum* strain added with α-

chymotrypsin, proteinase K, and trypsin (Moraes et al., 2010) to a final concentration of 1 mg/mL each. All proteases were supplied by Sigma-Aldrich. Each experiment was carried out in triplicate.

3.2.4 Effect of pH influence of CFS in the inhibitory process

According to the results of the agar well diffusion assay, the CFS from 23 producer strains and 3 indicator strains (*Brochothrix thermosphacta* DSM 20171^T, *Pseudomonas fluorescens* DSMZ 50009^T and *Listeria innocua* DSM 20649^T) were used. In detail, 12 CFS were screened *vs Ps. fluorescens*, 20 *vs B. thermosphacta* and 7 *vs L. innocua* (some CFS were used in all the 3 tests, other in only 1 test). For this purpose, overnight cultures of each indicator strain were centrifuged (8000 rpm for 15 min at 4°C), the pellet was washed with PBS and then suspended in Muller-Hinton (MH) medium. The experimental test was set up by adding 3 mL of each CFS to 3 mL of MH containing the indicator strain. To establish the role of low pH (characterizing those CFS with inhibitory activity) on the inhibitory process, the CFS, having a proper pH value, was also neutralised (CFSn) to pH 6.5 with NaOH 1N. As a control, a test was performed by adding 3 mL of MRS (the medium used for the growth of producers) to 3 mL of inoculated MH (the medium used for the growth of indicators). After incubation for 50 h at the proper temperatures allowing the growth of the indicators (28°C for *B. thermosphacta* and *Ps. fluorescens* and 37°C for *L. innocua*), the absorbance (OD) at 620 nm was determined with a microplate reader (Multiskan FC, Thermo Scientific). The inhibitory activity of CFS or of CFSn on indicator strains was expressed by the following ratios:

$$\text{rCFS} = \frac{OD\ t_{50}^{(CFS)} - OD\ t_{0}^{(CFS)}}{OD t_{50}^{(CONTROL)} - OD t_{0}^{(CONTROL)}}\ ;\ \text{rCFSn} = \frac{OD\ t_{50}^{(CFSn)} - OD\ t_{0}^{(CFSn)}}{OD t_{50}^{(CONTROL)} - OD t_{0}^{(CONTROL)}}$$

where $OD\ t_{50}^{(CFS)}$ and $OD\ t_{0}^{(CFS)}$ represent the absorbance values of indicator cultures added with CFS at 50 h and at 0 h of incubation, respectively, and $OD\ t_{50}^{(CFSn)}$ and $OD\ t_{0}^{(CFSn)}$ represent the absorbance values of indicator cultures added with CFSn at 50 h and at 0 h of incubation, respectively.

The following ranges were arbitrarily considered to define the inhibitory activity of both

CFS and CFSn: 0.0-0.2, very strong; 0.2-0.4, strong; 0.4-0.6, moderate; 0.6-0.8, low; 0.8-1.0, very low-absent.

3.2.5 Assessment of the survival of indicators in different conditions

On the basis of previous results, the assays were performed using *Lb. plantarum* H_BB1 as producer, and *B. thermosphacta*, *Ps. fluorescens* and *L. innocua* as indicators.

Each test was performed using CFS, CFSn (prepared as above), or MRS acidified (MRS-AL) with lactic acid (88-92% extra pure, Riedel-de Haen, USA) at the proper pH value of each CFS. Five mL of each medium were then added to 5 mL of MH containing each indicator strain. As control, 5 mL of MRS were added to 5 mL of each indicator in MH. Bacterial suspensions were incubated at 28-37°C for 48 h, and the survival of each indicator strain was assessed by plate counts in proper media at 4 h regular time intervals. Experimental data were then modelled with the software DMFit (Web Edition) in order to construct models of the kinetic parameters.

The concentration of total lactic acid in the CFS from producer strains was determined by enzymatic assay (Megazyme Kit), following the manufacturer's procedure.

3.2.6 Screening of phenyllactic acid (PLA)-producing *Lb. plantarum* strains

The content of PLA in CFS was determined by HPLC analysis according to Armaforte et al. (2006). The assay was performed using CFS of ten *Lb. plantarum* (W_TA8, W_T4, W_TA5, H_BB1, H_BB2, H_BB3, H_BB4, S_20, FS_IV29, C_56), which were showed different intensity of antimicrobial activity in previous assays.

In order to obtain CFS, LAB cultures were propagated twice for 24 h at 28 °C in 10 mL of MRS broth. An aliquot (200 µL) of activated cultures were then inoculated into fresh sterile MRS broth (20 mL) and allowed to grow at 28 °C without shaking for 24 h. Finally, cells were removed by centrifugation (8000 rpm for 15 min at 4°C; Centrifuge 5415 R; Eppendorf, Germany), and the CFS obtained were filter-sterilized (Filter Unit Red rim FP 30⁄0.2 CA-S, 0.22µm pore size; Schleider & Schuell, Germany).

A Varian ProStar 230 instrument (Mulgrave, AUS) supplied with UV–VIS detector set to 210 nm and a column Kinetex 5u C18 100A (150 mm x 4.6 mm) (Phenomenex, USA) were used for determination of PLA. The mobile phases were acetonitrile (eluent

A) and 0.005 N H2SO4 (eluent B) at the following gradient (A/B): 0-3 min 25/75%; 4-6min 50/50%; 8-12 min 100/0 %. PLA concentration was quantified through a corresponding standard (Sigma-Aldrich Co, St. Louis, MO, USA) calibration curve derived from a plot of area counts versus concentration. Analytical assay was carried out in three replicate.

3.2.7 Statistical analysis

Mean values and standard deviations were determined with the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA). Calculation of similarities in the profiles of antimicrobial activity of producers, as well as of antimicrobial susceptibility of indicators, were obtained with the software Genesis through a hierarchical cluster analysis based on the Euclidean distance metric and the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm. Data were shown in a pseudo-heat map with producer strains reported in the rows and indicator strains in the columns.

3.3 Results and Discussion

3.3.1 Antimicrobial activity expressed by *Lb. plantarum* growing cell

The spot-on-the-lawn test evidenced different effects of *Lb. plantarum* growing cells (GC) *vs* undesirable microorganisms. As general consideration, the antimicrobial activity expressed by *Lb. plantarum* was strain-dependent, confirming what reported by other Authors (Engelhardt et al., 2015). In fact, out of 110 tested GC, 42 produced a strong or a strong/moderate inhibitory activity against all the assayed Gram-negative bacteria, except for *A. pasteurianus* type strain. Thirty-six of them were isolated from wines, 4 from honey and only 2 from sourdoughs. The remaining 29 GC of strains isolated from sourdough and those isolated from fermented sausages showed a moderate or a low inhibitory activity *vs* Gram-negative bacteria. GC of *Lb. plantarum* strains isolated from cheese had no or low antimicrobial activity. Among Gram negative bacteria, acetic acid bacteria (except *A. pasteurianus* type strain) showed the highest sensitivity to the action of *Lb. plantarum* GC.

The assay *vs* Gram-positive bacteria showed 20 GC (16 from wine- and 4 from honey-strains) with a strong inhibitory action. Other 57 GC (31 from sourdough strains, 21 from wine strains and 5 from fermented sausage strains) produced a strong inhibition *vs C. sporogenes* and *B. thermosphacta* type strains, while 33 GC (16 from fermented sausages and 17 from cheese) had no or low inhibition. Generally, *B. thermosphacta* and *C. sporogenes* showed the highest sensitivity, while a moderate inhibition *vs L. innocua* type strain was observed.

Results obtained *vs* moulds evidenced that the majority of *Lb. plantarum* GC (82%) were unable to inhibit *Penicillium* spp. Only 9 GC (5 from wine- and 4 from honey-strains) showed a very strong inhibitory activity, while 10 GC (9 from wine- and 1 from sourdough- strains) caused a moderate or a low inhibition.

3.3.2.1 Antimicrobial activity expressed by *Lb. plantarum* cell free supernatants against Gram-negative strains

The agar well diffusion assay was used to evaluate the activity of *Lb. plantarum* cell free supernatants (CFS), as well as to assess the involvement of proteinaceous and/or acid compounds in the inhibitory process (nCFS and pCFS). Overall, CFS exerted a lower antimicrobial activity on both bacteria and moulds than that exhibited by growing

cells (Figure 3.1). Results also highlighted that a considerable number of CFS exerted a remarkable antimicrobial activity *vs* several indicator strains. In detail, producer strains were grouped into 6 clusters according to the inhibitory action expressed by their CFS against Gram-negative strains (Figure 3.1 A).

Cluster A grouped 5 CFS, 3 from wine- and 2 from honey- strains, producing a strong inhibitory activity against all the assayed Gram-negative bacteria, with the exception of *Acetobacter pasteurianus* type strain. Cluster B grouped 21 CFS - 19 from wine- and 2 from honey- strains - showing a strong or a moderate antimicrobial activity *vs* numerous indicator strains and no action *vs A. pasteurianus*. Eleven CFS (9 from wine- and 2 from sourdough-strains), grouped in cluster C, strongly inhibited *A. aceti* and *A. tropicalis* type strains, exerted a moderate inhibition against *Gluconoacetobacter hansenii* strains, and showed a low action *vs* the other assayed indicators. Forty CFS, grouped in cluster D (5 from wine-, 6 from fermented sausage- and 29 from sourdough-strains), showed a strong or a moderate inhibition *vs A. aceti* and *A. tropicalis* type strains, and a low inhibition *vs* all the other bacteria grouped in cluster 1, 2 and 3. The remaining 33 CFS, all resulting from cheese- and fermented sausage- strains, were grouped in clusters E and F and were characterized by a moderate, low, or no inhibitory action against all the assayed Gram-negative bacteria.

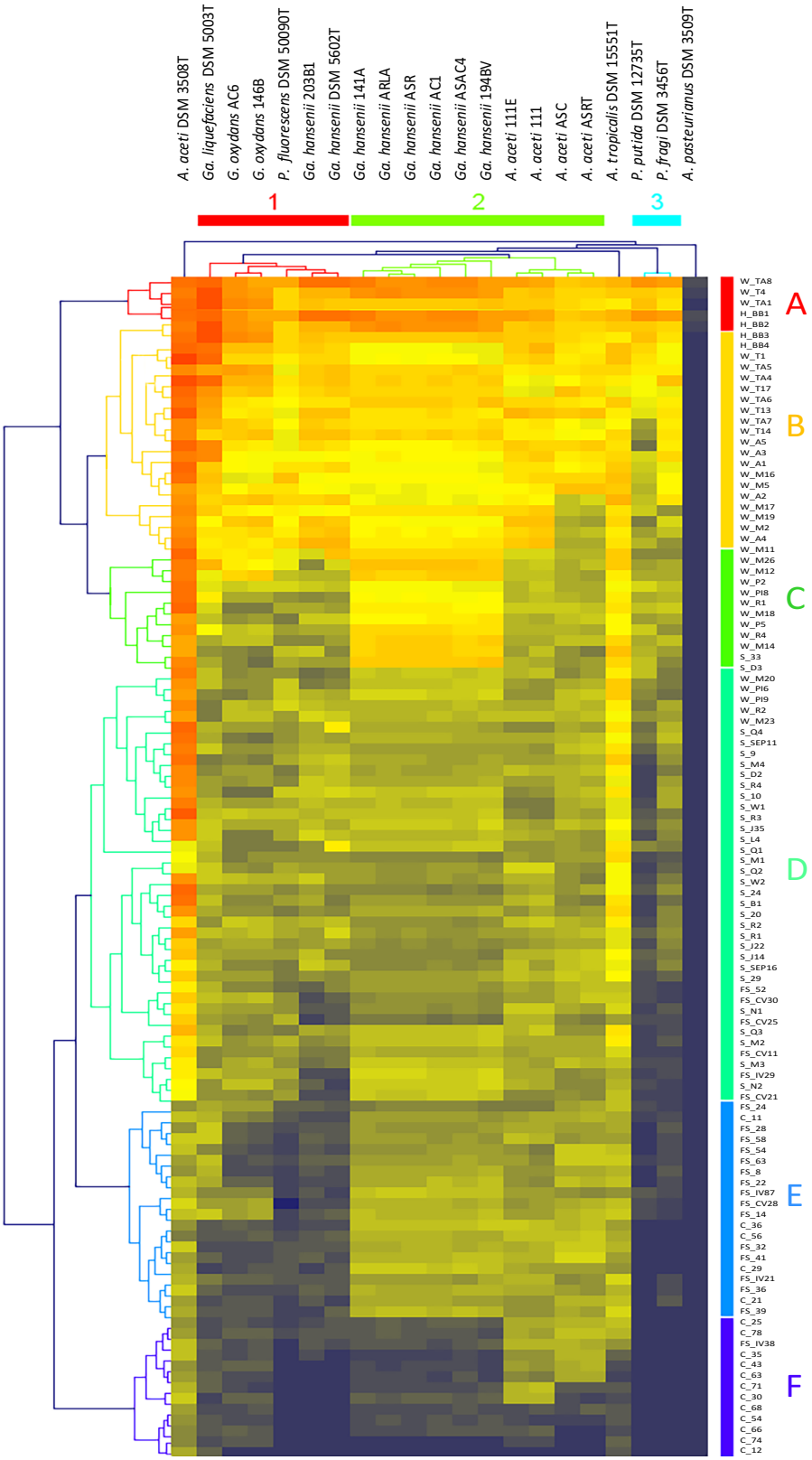

Figure 3.1A. Heat map, using Hierarchical clustering and Euclidean distance (Software Genesis), obtained by the analysis of results from the agar well diffusion assay conducted on 110 *Lb. plantarum* cell free supernatants (CFS) *vs* Gram-negative strains.

2.3.2.2 Antimicrobial activity expressed by *Lb. plantarum* cell free supernatants against Gram-positive strains

The results of the CFS activity against Gram-positive bacteria are reported in Figure 3.1 B.

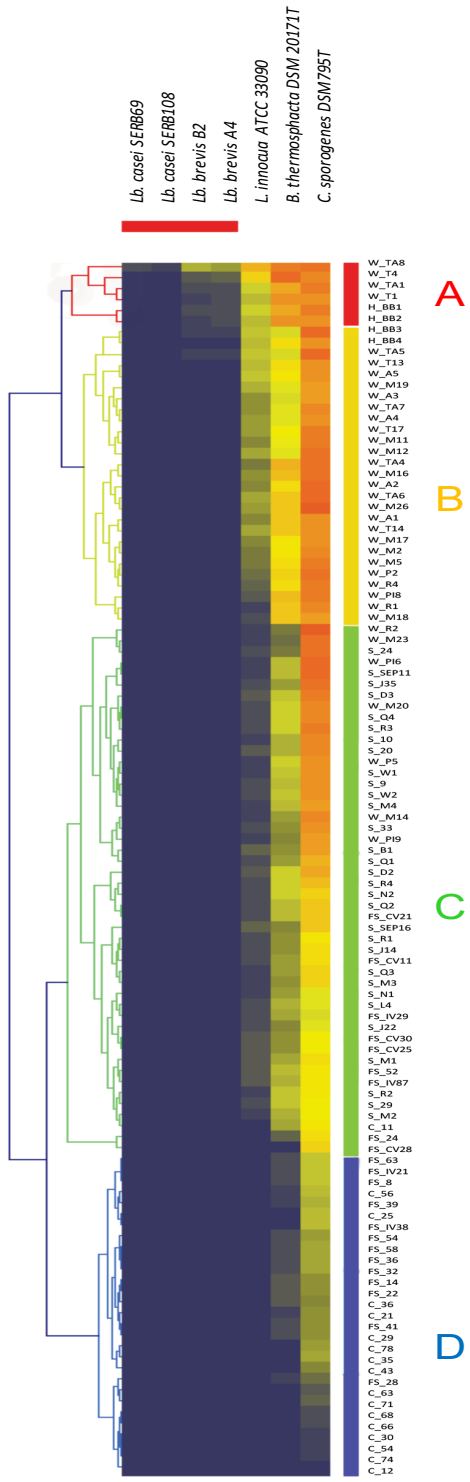

Figure 3.1B. Heat map, using Hierarchical clustering and Euclidean distance (Software Genesis), obtained by the analysis of results from the agar well diffusion assay conducted on 110 *Lb. plantarum* cell free supernatants (CFS) *vs* Gram-positive strains.

Producer strains were grouped into 4 clusters. Cluster A was composed by 6 CFS (4 from wines- and 2from honey- strains) showing the largest *spectrum* of inhibition and the highest inhibitory activity. Twenty-seven CFS (25 from wines- and 2from honey-strains) were grouped in cluster B and caused a strong or a very strong inhibition against *Listeria innocua*, *Clostridium sporogenes* and *Brochothrix thermosphacta*. Cluster C collected 48 CFS of *Lb. plantarum* strains isolated from all the different investigated matrices, and having a lower *spectrum* of antagonism than that showed by CFS from clusters A and B. This datum was particularly marked *vs B. thermosphacta* and *C. sporogenes* type strains. The lowest *spectrum* and intensity of antimicrobial activity was detected for 29 CFS belonging to cluster D. This last cluster grouped the CFS of strains isolated only from fermented sausages and cheese.

3.3.2.3 Antimicrobial activity expressed by *Lb. plantarum* cell free supernatants against moulds

The behaviour of indicator CFS *vs* moulds is reported in Figure 3.1C.

As general consideration, tested moulds showed the lowest sensitivity to the CFS than that highlighted *vs* both Gram-positive and Gram-negative bacteria. Only 8 CFS, 4 from wine- and 4 from honey-strains, produced a strong inhibitory activity against *Penicillium* spp. The CFS of the remaining 102 strains exhibited a moderate, a low or no inhibitory activity.

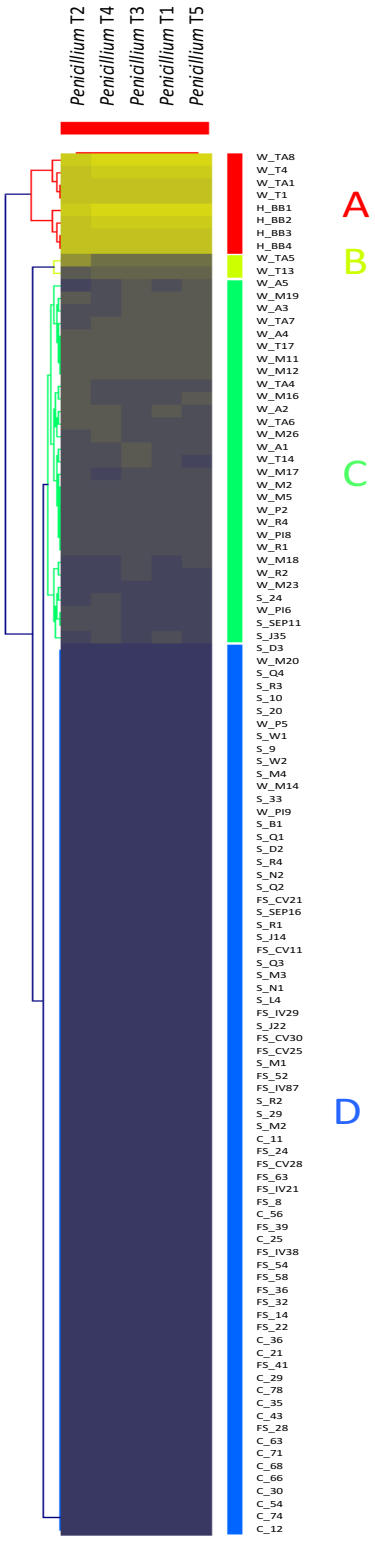

Figure 3.1C. Heat map, using Hierarchical clustering and Euclidean distance (Software Genesis), obtained by the analysis of results from the agar well diffusion assay conducted on 110 *Lb. plantarum* cell free supernatants (CFS) *vs* moulds.

Previous assays were repeated with neutralised CFS (data not shown). In this case, no inhibitory activity was recorded for 90 producer strains. This evidence suggests that the

antimicrobial activity of almost all assayed *Lb. plantarum* strains was mainly due to the low pH of supernatants.

However, 9 nCFS of *Lb. plantarum* strains, five isolated from wine and four isolated from honey, evidenced the ability to inhibit all (producer strains W_TA8 and H_BB1) or more (producer strains W_T4, W_TA1, W_T1, W_TA5, H_BB2, H_BB3 and H_BB4) (Figure 3.2).

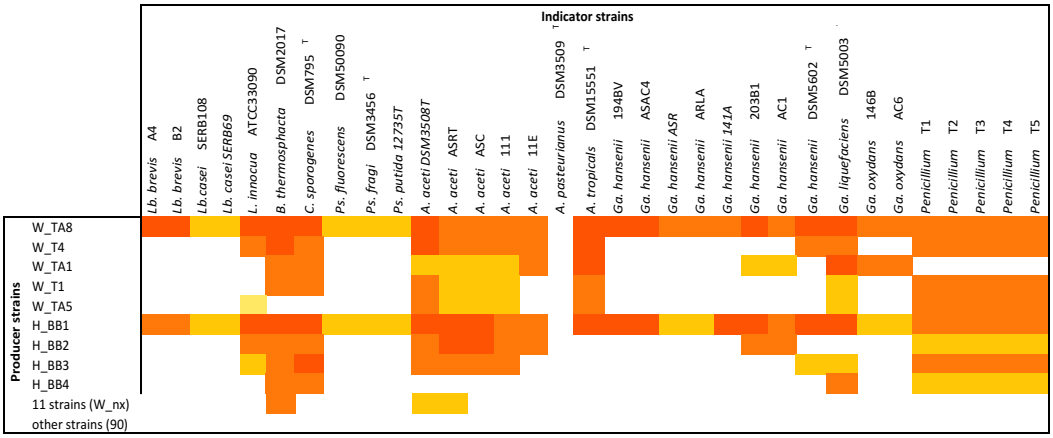

Figure 3.2. *Lb. plantarum* nCFS and pCFS *vs* indicators

Remarkably, the same antimicrobial activity persisted also when the CFS was exposed to proteinases (pCFS).

3.3.3 Influence of pH on CFS inhibitory action assayed in culture-broth

On the basis of these last results combined with those from heat map analyses, 21 producer strains were selected to evaluate in culture-broth the inhibition effects expressed by cell free supernatant (CFS) and by neutralized cell free supernatant against strains belonging to *Ps. fluorescens*, *B. thermosphacta* and *L. innocua*.

Data reported in Figure 3.3 highlights the inhibitory action exerted by 12 CFS or CFSn *vs Ps. fluorescens*. Results evidenced that the CFS and the CFSn from three strains (W_T13, W_TA5 and W_T1) produced a low inhibition action against *Ps. fluorescens*. While CFS from the other strains showed a very strong (W_TA1, W_T4, W_TA8 and H_BB1), a strong (H_BB2) or a moderate (H_BB3, H_BB4, W_TA6; W_TA4) inhibitory activity. However, also in these cases the inhibition appeared generally lower (low or very low) when the CFSn was used, evidencing that the inhibitory activity for the majority of the strains was essentially due to the low pH of the CFS.

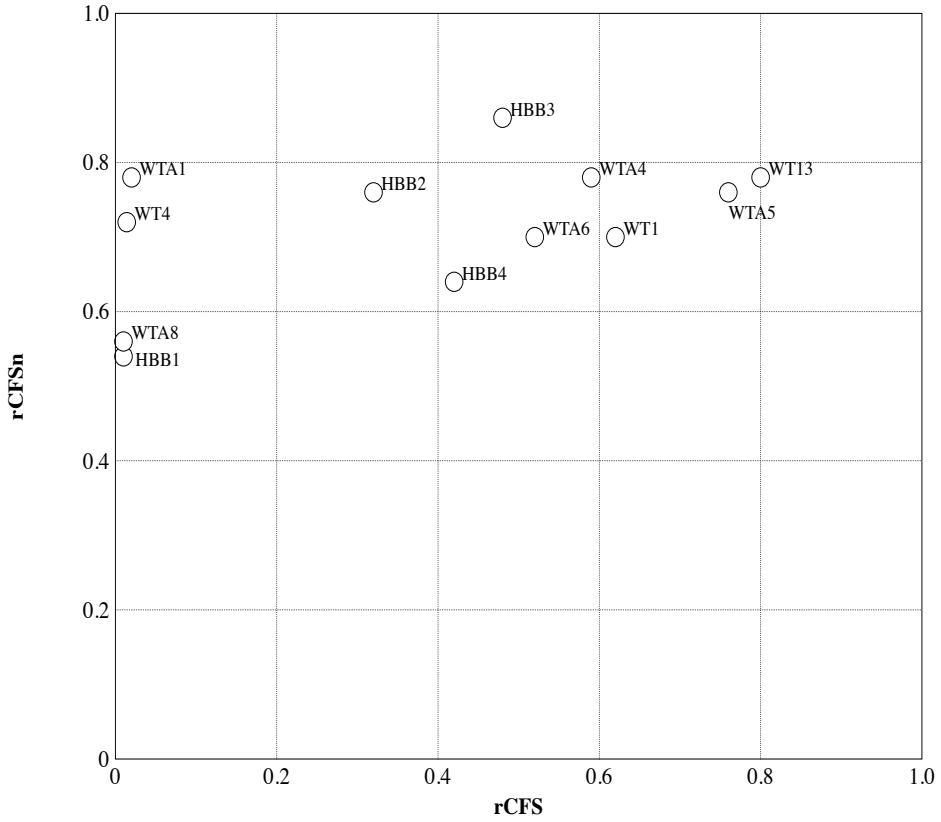

Figure 3.3. Inhibitory activity exerted by 12 CFS and 12 CFSn *vs Ps. fluorescens*. rCFS and rCFSn are ratios calculated through the formula reported in the section of Materials and Methods. Ranges: 0.0-0.2, very strong inhibition; 0.2-0.4, strong inhibition; 0.4-0.6, moderate inhibition; 0.6-0.8, low inhibition; 0.8-1.0, very low-absent inhibition.

Only two strains (W_TA8 and H_BB1) produced a relevant inhibitory effect (moderate) also when the neutralized CFS (CFSn) were used.

The results concerning the inhibitory effect of 20 CFS and 20 CFSn *vs B. thermosphacta* are reported in Figure 3.4.

CFSs from 15 *Lb. plantarum* (W_A1, W_M26, W_TA4, H_BB4, H_BB3, H_BB2, W_T1, W_TA1, W_T4, H_BB1, W_TA8, W_TA6, W_M16, W_T17 and W_A5) evidenced a very strong antimicrobial activity *vs B. thermosphacta*; however, the effect was different when the CFSn was used. In detail, the activity expressed by 8 CFSn (W_T4, H_BB4, W_T1, H_BB1, W_TA8, H_BB3, W_TA1, H_BB2) was slightly lower than that produced by the corresponding CFS, and they were considered as strong inhibitors.

The inhibitory effect of the remaining 7 CFSn was strongly lower than that previously described. In fact, a moderate inhibition was produced by CFSn from 4 strains (W_TA4, W_M26, W_M16 and W_TA6) and a low inhibition was evidenced by other

CFSn from 3 strains (W_A1, W_A5 and W_T17). In this case, it can be assumed that the inhibitory effect of CFS was mainly due to the low pH.

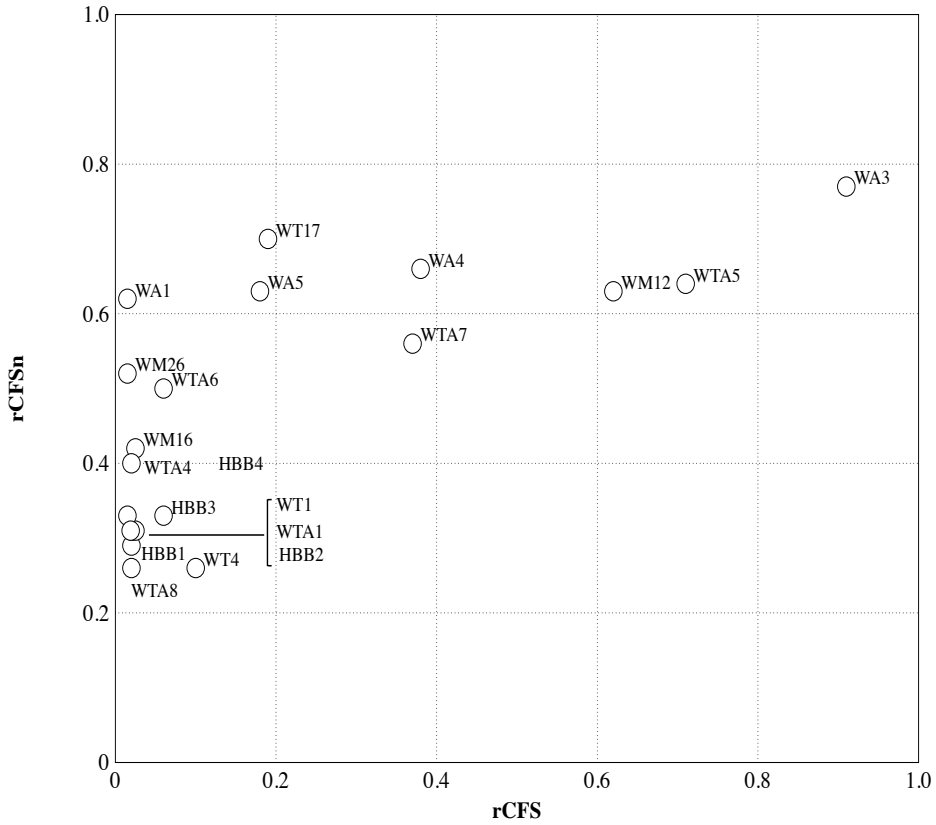

Figure 3.4. Inhibitory activity exerted by 20 CFS and 20 CFSn *vs B. thermosphacta*. rCFS and rCFSn are ratios calculated through the formula reported in the section of Materials and Methods. Ranges: 0.0-0.2, very strong inhibition; 0.2-0.4, strong inhibition; 0.4-0.6, moderate inhibition; 0.6-0.8, low inhibition; 0.8-1.0, very low-absent inhibition.

The antimicrobial activity expressed by the remaining 5 strains was of lesser interest. In detail, 2 CFS (W_TA7 and W_A4) showed a strong inhibitory activity. The corresponding CFSn showed a moderate (W_TA7) or low (W_A4) inhibition. Other 3 CFS and CFSn (W_M12, W_TA5 and W_A3) showed a low inhibition.

Finally, the inhibitory effect of CFS and CFSn from 7 strains (H_BB1, H_BB2, HBB_3, H_BB4, W_TA8, W_TA5, W_T4) *vs L. innocua* is reported in Figure 3.5. The results evidenced that the effects of CFSn was substantial similar to that expressed by the CFS. In fact, a low inhibition was evidenced by both CFS and CFSn from the strain WTA5. CFS from W_TA8 and W_T4 showed a strong and a moderate inhibition action when used as CFS and CSFn respectively. Likewise, the inhibitory action (strong) exhibited from CFSn from H_BB2, H_BB3, H_BB4 was only slightly lower than that (very strong) produced by the correspondent CFS. Lastly, the effect produced by the

strain HBB_1 was very strong when used as both CFS and CFSn.

The results achieved by the evaluation of the antimicrobial effects expressed by CFS and the correspondent neutralized CFS support the hypothesis that the inhibition was due to the production of extracellular compounds having neither acid (such as lactic acid, that represent the principal extracellular metabolites produced by *Lb. plantarum*) nor proteinaceous nature. Other Autors (Garofalo et al., 2012) stated that the antimicrobial activity of same *Lactobacillus* strains, including strains of *Lb. plantarum*, was due to the combination of lactic acid and one or more hitherto unknown, non-proteinaceous compounds which are active only at low pH. The results obtained in our study evidenced that the inhibitory effect produced by certain *Lb. plantarum* strains also remains at higher pH values.

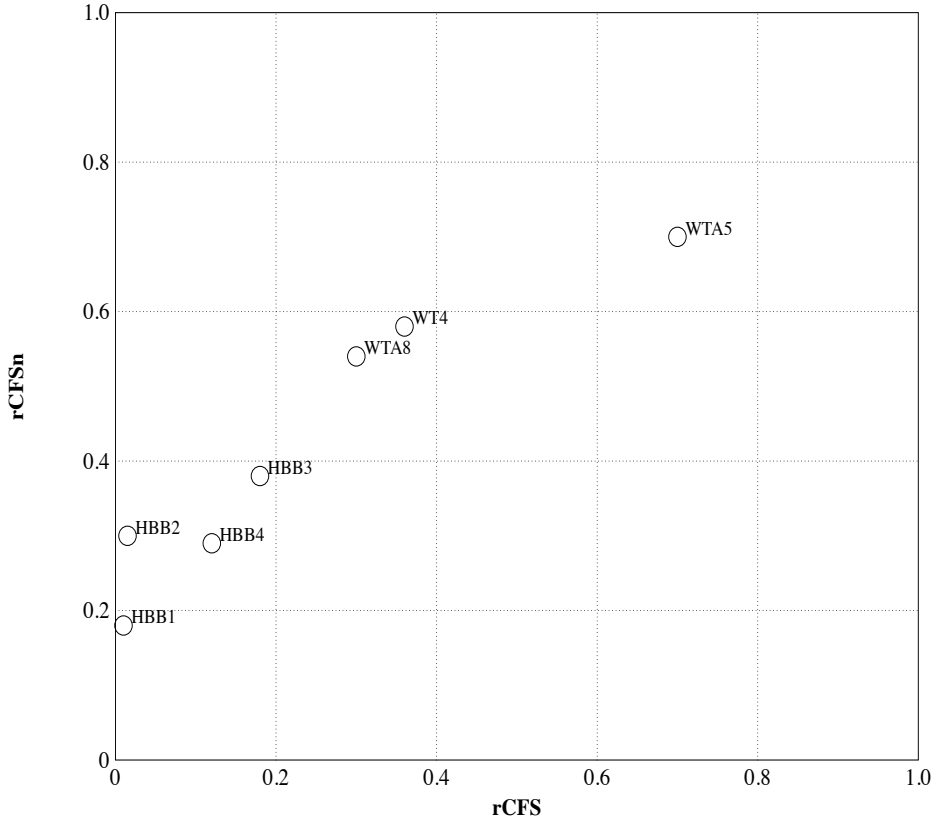

Figure 3.5. Inhibitory activity exerted by 2 CFS and 2 CFSn *vs L. innocua*. rCFS and rCFSn are ratios calculated through the formula reported in the section of Materials and Methods. Ranges: 0.0-0.2, very strong inhibition; 0.2-0.4, strong inhibition; 0.4-0.6, moderate inhibition; 0.6-0.8, low inhibition; 0.8-1.0, very low-absent inhibition.

Therefore, the comparison between the inhibitory effects produced by CFS and lactic acid could be providing more information on the antimicrobial compound. Moreover, in order to better appreciate differences between lactic acid and CFS the most lactic acid

resistant strains among the indicators should be chosen. *L. innocua* strains are well known for their acid stress resistance. In fact, *L. innocua* is frequently found in several foodstuffs (Kovacevic et al., 2012; Jami et al., 2014; Ebner et al., 2015; Melo et al., 2015) also in those characterized by pH values lower than 5.0 (CAC, 2009). Acid stress response in *Listeria* species has been the subject of several investigations, which documented the induction of a number of molecular mechanisms involving the F_1F_0-ATPase complex, the arginine deaminase (ADI), the glutamate decarboxylase (GAD) pathways (Cotter et al., 2001; Ryan et al., 2009; Karatzas et al., 2012) as well as a new type of universal stress protein (Tremonte et al. 2016).

3.3.4 Effect of *Lb. plantarum* CFS and lactic acid on *L. innocua* behaviour

On the basis of the previous results the strain H_BB1 was choice as producer. Figure 3.6 shows the survival of *L. innocua* in the presence of the CFS and the CFSn from *Lb. plantarum* H_BB1.

As expected, the behaviour of *L. innocua* showed an increase in the control batch with a maximum specific growth rate of 0.04 h^{-1} (Table 3.3). While, a strong reduction was observed when the CFS or the lactic acid was added to the culture-broth. However significant differences were observed also between these two last batches.

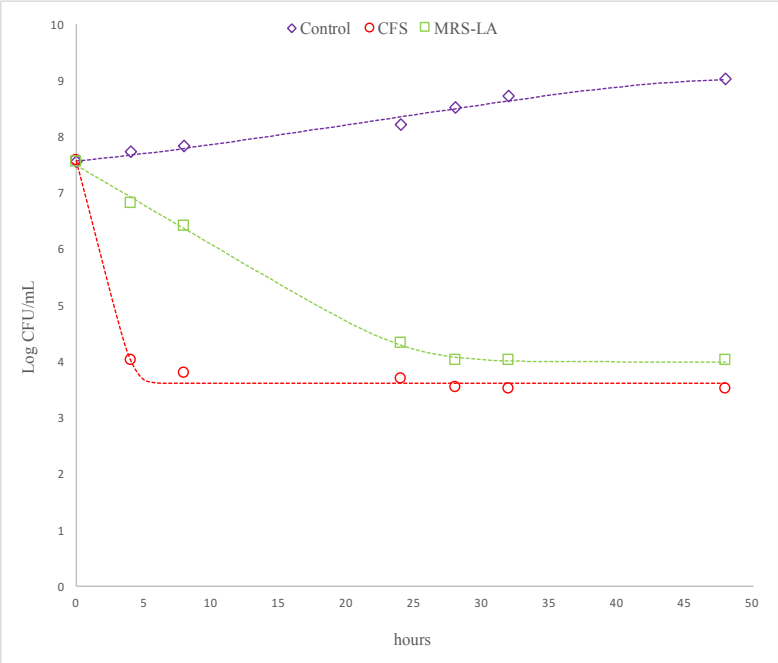

Figure 3.6 Survival of *L. innocua* in presence of cell free supernatant (CFS) from *Lb. plantarum* H_BB1, in presence of MRS acidified with

lactic acid (MRS-LA) and in presence of MRS (control). Symbols represent the experimental date and the curves represent the D-model.

The CFS from *Lb. plantarum* H_BB1 produced a reduction of *L. innocua* of approximately 4.0 Log CFU/mL already after 4 hours of incubation with a specific death rate of -0.93 h^{-1}. Whereas, in presence of lactic acid (batch MRS-LA), *L. innocua* undergone a reduction of 3.5 Log CFU/mL only after 24 hours of incubation showing very low values of maximum specific death rate (μ_{max} of about -0.14 h^{-1}). In fact, this value was significantly lower than that recorded in the presence of CFS (-0.93 h^{-1}). This finding evidenced that the inhibitory effect of CFS from *Lb. plantarum* H_BB1 against *L. innocua* was due to the synergic presence of more than one inhibitory substance. In fact, the lactic acid cannot be the only compound with growth inhibition properties against *L. innocua*, very likely showing synergism with other compounds from LAB metabolism.

Table 3.3 Survival kinetic parameters of *L. innocua* in presence CFS from *Lb. plantarum* H_BB1, in presence of MRS acidified with lactic acid (MRS-LA) and in presence of MRS (control).

	y_0 (Log CFU/mL)	**y_end** (Log CFU/mL)	**μ_{max}** (h^{-1})	**R-square**	**SE of Fit**
Control	7.6 ± 0.1a	9.1 ± 0.2a	0.04 ± 0.01a	0.968	0.099
CFS	7.6 ± 0.1a	3.6 ± 0.1b	0.93 ± 0.06b	0.993	0.121
MRS-LA	7.5 ± 0.1a	4.0 ± 0.1b	·0.14 ± 0.01c	0.998	0.077

Lactic acid in the batches CFS and MRS-LA was determined. Even if the CFS and the MRS-LA were characterized by the same pH values, the results evidenced that the concentration of lactic was higher than the batch MRS-LA. Therefore, in addition to lactic acid the CFS might possess also another compound of acid nature. Phenyllactic acid (PLA) is a phenolic acid, likely produced from phenylpyruvate via the action of lactate dehydrogenase. In detail, PLA is a by-product of phenylalanine metabolism in LAB, where the first step involves its transamination by a non-specific aminotransferase. The α-amino group is then transferred to a suitable acceptor such as α- ketoglutarate, yielding phenyl pyruvic acid (PPA) and the corresponding amino acid. Finally, PPA can then be reduced by hydroxyl acid dehydrogenases to PLA (Mu et al., 2012). In recent years, great attention was focused on this metabolite due to its antimicrobial properties. In fact, PLA is known as an antimicrobial compound with a wide activity spectrum against yeast such as *Candida* spp., *Rhodotorula* spp., and

against moulds including *Aspergillus* and *Penicillium* species (Valerio et al., 2004; Prema et al., 2010). In addition, several Authors has been showed that PLA was able to inhibit both Gram-positive and Gram-negative bacteria (Ning et al., 2017).

3.3.5 Screening of phenyllactic acid (PLA)-producing *Lb. plantarum* strains

Data reported in Table 3.4 evidenced that in the cells free supernatant from all the assayed strains (able to produce antimicrobial effects) significant levels of PLA was found. Moreover, the results highlighted significant differences among the assayed strains showing that PLA production is strain-dependent. This finding is in accordance with those exposed by author Authors (Ryan et al., 2009). However, for the first time, a relation between PLA-producing strains and isolation environment of the strains. Strains H_BB1 and H_BB2 have recorded the highest PLA-producing ability reaching levels of about 129.93 and 128.21 mg/mL respectively, in the cell-free supernatant after 24 h incubation.

Table 3.4 PLA production *Lb. plantarum* strains

***Lb. plantarum* strains**	**Isolation source**	**PLA (mg/mL)**	**pH**	**References**
W_TA8	red wine (Taurasi)	99.32±0.02a	3.68	Testa et al., 2014
W_T4	red wine (Tintilia)	98.88±0.02a	3.66	Testa et al., 2014
W_TA5	red wine (Taurasi)	90.69±0.01b	3.68	Testa et al., 2014
H_BB1	honey (bee bread)	129.93±0.01c	3.50	DiAAA collection
H_BB2	honey (bee bread)	128.21±0.01c	3.53	DiAAA collection
H_BB3	honey (bee bread)	112.94±0.02d	3.62	DiAAA collection
H_BB4	honey (bee bread)	116.66±0.02d	3.57	DiAAA collection
FS_IV29	fermented sausage (Ventricina)	49.84±0.03c	5.15	Tremonte et al., 2007; Pannella, 2010
C_56	cheese (Caciocavallo)	31.67±0.02f	5.55	Coppola et al., 2003
S_20	sourdough from Campania Region	88.41±0.03g	4.00	Pannella, 2010

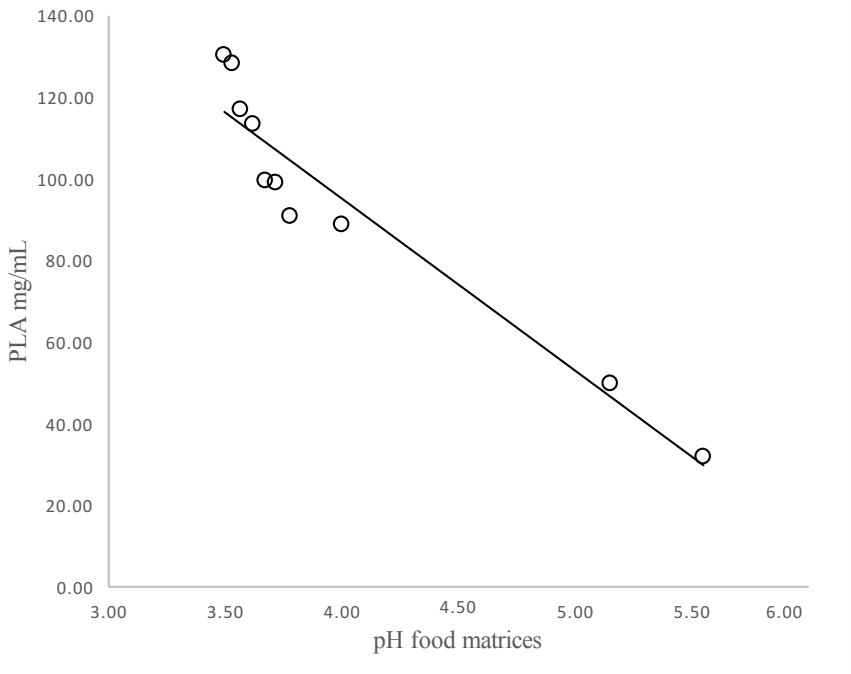

Figure 3.7. Correlation between PLA product (mg/mL) and pH values of source (food matrices) of isolation

The analysis of these data clearly suggests the existence of a relation (Figure 3.7) between the antimicrobial properties expressed by assayed strains and their isolation source depending on the specific physic-chemical features of each isolation source (see Table 3.1).

In fact, those environments characterised by harsh conditions (high ethanol levels, low pH and high sugar levels), such as wines and honey, harboured a higher number of antagonistic strains than other fermented matrices (e.g. cheese, sourdoughs or fermented sausages). This fact could be due to selective pressures more accentuated in wines and honey than in the other investigated food matrices. Consequently, a relation between the antimicrobial activity expressed by strains of *Lb. plantarum* and their isolation environment was also discovered. The relation between environmental conditions and antagonistic properties of *Lb. plantarum* is further strengthened by examining the results of the antimicrobial activity expressed by strains isolated by the same matrice, still having different physico-chemical features. In fact, strains from wines with higher ethanol content (e.g. Taurasi and Tintilia, Table 3.1) evidenced a stronger antimicrobial activity than those isolated from wines characterised by lower ethanol content (e.g. Pentro d'Isernia and Montepulciano, Table 3.1).

In conclusion, data reported in this study indicate that specific food conditions are able to influence the occurrence of certain strains able not only to respond to specific adverse

conditions, but also to compete with other bacterial populations. A similar remark was made by Cao et al. (2013), which found an association between antibacterial activity in *Bacillus amyloliquefaciens* and the presence of gene expression that is crucial for bacterial cells to adapt to environmental stress.

Surely, the most important scientific enrichment produced by this study is ascribable to results highlighting that the choice of the source of isolation could be an important preliminary tool for the individuation of antagonistic strains. However, the correlation between *Lb. plantarum* PLA formation ability and their isolation sources would lead to open new frontiers in understanding the PLA formation process.

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CHAPTER 4:

EFFECT OF GROWTH PHASES AND CULTIVATION CONDITION ON PHENYLLACTIC ACID PRODUCTION BY *LACTOBACILLUS PLANTARUM*

4.1 Introduction

Lactobacillus plantarum strains are known for their ability to produce numerous natural antimicrobial substances (Prema et al., 2010; Rumjuankiat et al., 2015). As highlighted in literature, great attention was also focused on a specific metabolite represented by the phenyllactic acid (PLA), a phenolic acid characterized by an interesting antimicrobial activity (Valerio et al., 2004; Dallagnol et al., 2011; Mu et al., 2012; Corsetti et al., 2014; Russo et al., 2017). PLA inhibits not only food-spoiling fungi (Valerio et al., 2004; Schwenninger et al., 2008) but also food-borne pathogenic bacteria, including *Listeria monocytogenes* (Ohhira et al., 2004; Dieuleveux et al., 1998). Moreover, the antimicrobial activity of PLA is greater than other organic acid such as acetic acid, lactic acid (Gerez et al., 2009), and its smell is realy compatible with food product. Its amphiphilic properties and the hydrophobic group-benzene ring could play a key role in the antimicrobial activities. In addition, due to its good hydrophilicity, PLA diffuses in foodstuffs better than other preservative agents. Thus, PLA is a promising candidate for the development of food preservative.

However, this compound is produced in low amounts that do not reach the minimum concentration necessary to inhibit the microbial growth (Vermeulen et al., 2006; Ryan et al., 2009). In fact, the antimicrobial activity of PLA producer strains could be also justified as a synergic effect among different compounds (Corsetti et al., 1998; 2014; Schnürer and Magnusson, 2005). Many LAB have been used to produce PLA showing maximum PLA production of about 0.1 g L^{-1} (Valerio et al., 2004; Strom et al., 2005).

PLA is a by-product of phenylalanine metabolism in LAB, where the first step involves its transamination by a non-specific aminotransferase. The α-amino group is then transferred to a suitable acceptor such as α-ketoglutarate, yielding phenyl pyruvic acid (PPA) and the corresponding amino acid. Finally, PPA can then be reduced by hydroxyl acid dehydrogenases to PLA (Mu et al., 2012). Some Authors (Vermeulen et al., 2006) have been reported that the rate-limiting step in PLA formation was represented by Phenylalanine (Phe) transamination. Li et al. (2007; 2008) reported that the blockage due to Phe could be overcome using PPA as substrate and an increase of 14-fold in PLA content was obtained. However, it must also be considered that aminotransferase enzyme is active with other amminoacids, such as tryptophan, methionine and leucine (Yvon et al., 1997; Rijnen et al., 1999). Therefore, this enzyme catalyses the transference of ammonium from an R-amino group to a keto-acid acceptor being alpha-ketoglutarate (a-KG) the favourite acceptor in most LAB. Because of this, the bioavailability of a-KG becomes a limiting factor for all transamination reactions and amino acid catabolism (Yvon et al., 1998; Rijnen et al., 2000). The amino acid degradation in LAB may be increased by glutamate dehydrogenase (GDH), enzyme that catalyses the reversible oxidative deamination of glutamate (Glu) to a-KG and ammonium (Rijnen et al., 2000; Tanous et al., 2002). Thus, the a-KG formation through GDH activity would be an indirect way to increase PLA. Moreover, Dallagnol et al. (2011) stated that the synthesis of PLA by *Lb. plantarum* can be improved with higher amounts of Phe. On the other hand, the same Authors reported that production of both PLA can be increased by cometabolism of glucose with Citrate. Finally, in order to increase PLA production, statistical experimental design techniques were also applied. In detail, Mu et al., (2009) putting in place an optimal medium to produce PLA evidenced that using corn steep liquor as the main nitrogen source instead of peptone in MRS as the main nitrogen source giving a maximum yield of 2.30 g L^{-1}.

Therefore, the enhancement and the improving of PLA formation represents an actual and crucial topic to assure a better PLA development in food industry. Results reported in the previous chapter has been evidenced that PLA production by *Lb. plantarum* could be also positively influenced by environment pressures such as low pH values. On the bases of these last statement, PLA formation seems to be linked to stress response mechanisms performed by *Lb. plantarum*. However, no information on relation between LAB stress response and PLA production is available in literature as well as very few information is reported on the relation between microbial growth phase and PLA

formation. Even if the prevailing opinion in scientific community believes that PLA formation is related to LAB growth arrest (Coloretti et al., 2007; Cortés-Zavaleta et al., 2014) has not been explained the linkage to metabolic pathways involved in stationary phase. In addition, the discovering of PLA presence also in the first hour of fermentation (Valerio et al., 2004) makes further nebulous the relation between PLA production and LAB growth phase. Little information, if not any, can be also found on the optimal pH condition of PLA metabolic pathway in *Lactobacillus* species.

In this light, the research reported herein focused the attention on effect of growth phase on the PLA formation by *Lb. plantarum* H_BB1. Moreover, cultivation conditions able to assure highest PLA levels, was investigated.

4.2 Materials and methods

4.2.1 Microorganism

The strain *Lb. plantarum* H_BB1 was previously isolated from honey (bee bread), and belonging to Food Microbiology Culture Collection of the DIAAA (Dept. of Agricultural, Environmental and Food Science, University of Molise), was used. The strain was maintained at -80 °C and propagated twice in MRS broth (Oxoid, Milan, Italy) at 28°C prior of use.

4.2.2 Growth conditions

Three batch fermentations were carried out at 28 °C in Erlenmeyer flaks containing 500 mL of MRS broth at initial pH of 6.5, 4.0 and 3.5. For this purpose, 1% of overnight culture was inoculated into two sterile MRS broth previously acidified with chloride acid (HCl) 4M at pH 4.0 and 3.5. A fermentation in MRS broth without HCl adding was performed as control.

4.2.3 Growth kinetic parameters, Cell dry weight and pH evaluation

During the fermentation period of each batch, samples were collected at regular time interval and assayed to evaluate the growth kinetic parameters, cell dry weight and pH values. In detail, microbial growth was detected by viable cell counts method in MRS agar plate.

The maximum specific grow rate (μ_{max}), lag phase, initial load values (*y_0*) and final load values (*y_end*), were estimated with the D-model of Baranyi and Roberts (1994) using the software DMFit web edition.

Cell dry weight (CDW) was determined gravimetrically. For this purpose, 10 mL of cell cultures were collected by centrifugation at 5500 rpm for 8 min at 4°C. The pellet was washed with PBS1x, re-suspended in the same buffer and filtered through nitrocellulose filters (pore size, 0.45 μm, previously dried at 105°C for 2h). Filters were dried at 105°C for 24 h and CDW was calculated based on differences between the initial and final filter weights.

Finally, the pH values were determined by pH-meter (Hanna Instruments HI 2002-02 edge).

4.2.4 D-glucose consumption and lactic acid production

The consumption of D-glucose and the D- and L-lactic acid production were evaluated during the entire fermentation time.
In detail, residual D-glucose, D-lactic and L-lactic acid concentration were determined enzymatically using an automated chemistry analyzer (iCubio iMagic-M9 r-biopharm AG) in accordance with the manufacturer instructions.

4.2.5 3-phenyllactic acid production curves

PLA levels in cell free supernatants were determined by high-performance liquid chromatography (HPLC) according to Armaforte et al. (2006). For this purpose, a Varian ProStar 230 instrument (Mulgrave, AUS) supplied with UV–VIS detector set to 210 nm and a column Kinetex 5u C18 100A (150 mm x 4.6 mm) (Phenomenex, USA) were used. The mobile phases were acetonitrile (eluent A) and 0.005 N H_2SO_4 (eluent B) at the following gradient (A/B): 0-3 min 25/75%; 4-6min 50/50%; 8-12 min 100/0 %. PLA concentration was quantified through a corresponding standard (Sigma–Aldrich Co, St. Louis, MO, USA) calibration curve derived from a plot of area counts versus concentration. Analytical assay was carried out in three replicates.

4.2.6 Statistical analysis

Three independent replicates of each experiment were performed, and their results were expressed as mean values ± standard deviation. Analysis of variance (ANOVA) and the Tukey's pairwise comparisons were performed to test differences in *Lb plantarum* behaviour and to describe relation between growth conditions and metabolites production.

4.3 Results and discussion

4.3.1 PLA production

The production of PLA by *Lb. plantarum* H_BB1 was investigated in MRS broth. The results are reported in Figure 4.1 and showed that detectable PLA levels were found already after two hours. However, the maximum specific PLA production rate was observed between the 8^{th} and the 11^{th} hour of fermentation. The comparison between PLA behaviour and growth curves evidenced that the PLA accumulation begins immediately after the end of the lag phase and reached the highest levels between the exponential and the stationary phase. As far as I know, these results show for the first time that the PLA production is strictly related to the growth and to the exponential phase of *Lb. plantarum*. So far, the prevailing scientific opinion retain that PLA is related to the stationary phase and the highest production was detected to the end of this phase. In detail, Cortés-Zavaleta et al. (2014) reported that PLA appeared after 48 hours of fermentation -corresponding to the middle of stationary phase- and progressively increased thereafter. Also, Vermeulen et al. (2006), studying the growth kinetic and PLA production in *Lb. plantarum*, stated that PLA formation proceed when growth has ceased highlighting that the conversion is not related to the exponential phase. However, some Authors have expressed doubts in the relation between the stationary growth phase and the PLA formation. In detail, Coloretti et al. (2007) studying antifungal activity of *Lactobacillus* discovered the presence of PLA in early fermentation phase. Moreover, Valerio et al. (2004) even if had found the maximum production of PLA after 72 hours, evidenced detectable PLA levels already after 6 hours of incubation.

The results obtained in the present study suggest that the PLA could be assume new metabolic meanings. In fact, the accumulation from the beginning of the exponential phase highlight typical behaviour of a primary metabolite. On the other hand, the highest production rate between the exponential and stationary phase suggests that PLA production could be assume a key role in the acid stress response. In fact, in the transition between exponential and stationary phase, pH showed values similar to the pKa of lactic acid (Figure 4.1).

As evidenced by several Authors (Li et al., 2008; Dallagnol et al., 2001) NADH and its oxidation to NAD^+ are essential for the enzymatic production of PLA from PPA. The

resulting NAD^+ regeneration represent a beneficial strategy for *Lb. plantarum* to react to acid stress condition during growing phase (Pieterse et al., 2005).

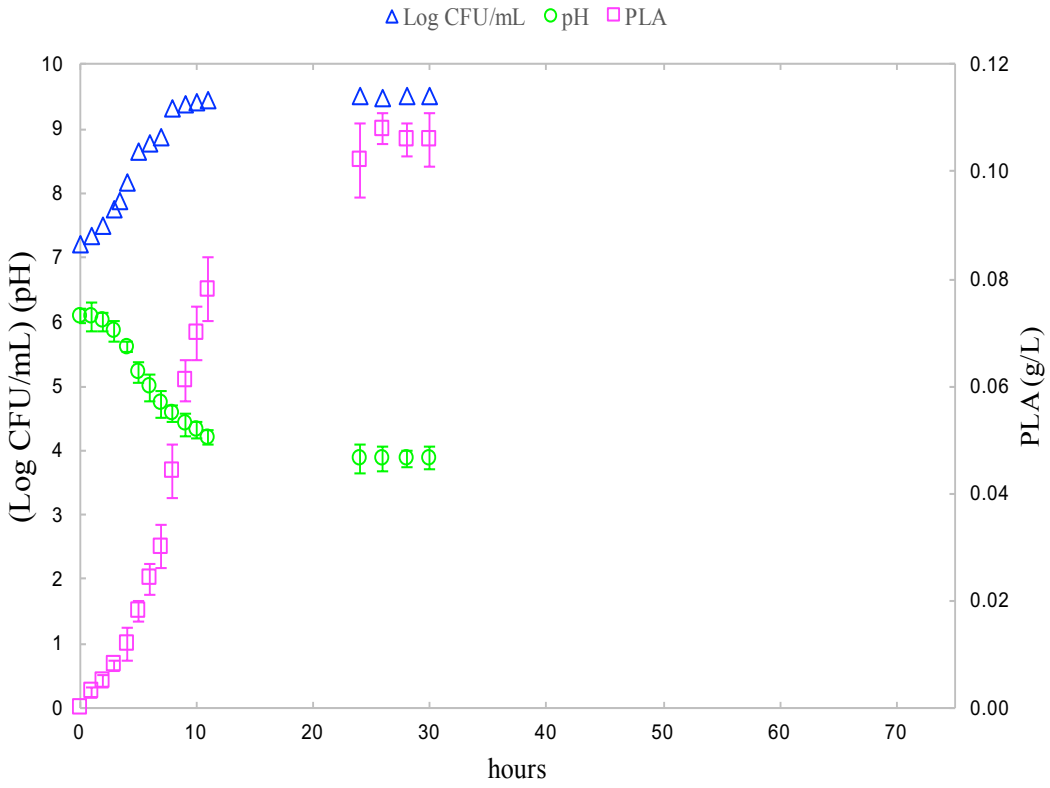

Figure 4.1 Behaviour of PLA formation, pH values and microbial growth of *Lb. plantarum* H_BB1 in control conditions (MRS broth)

On the basis of the above reported statements, the evaluation of ecological factors on the PLA formation process appear essential. In detail, sub-lethal pH could positively affect some metabolic pathways in *Lb. plantarum.* The interaction between lactic acid bacteria strains and their environments has gained increase interest in the last decade (Redon et al., 2005; Di Cagno et al., 2007; Filannino et al., 2014), as it is essential to achieve an optimal production of PLA (Rodriguez et al., 2012; Cortés-Zavaleta et al., 2014).

4.3.2 Effect of cultural conditions on the growth and primary metabolites production

Growth kinetics of *Lb. plantarum* H_BB1 cultivated in MRS or in pre-acidified MRS are illustrated in Figure 4.2.

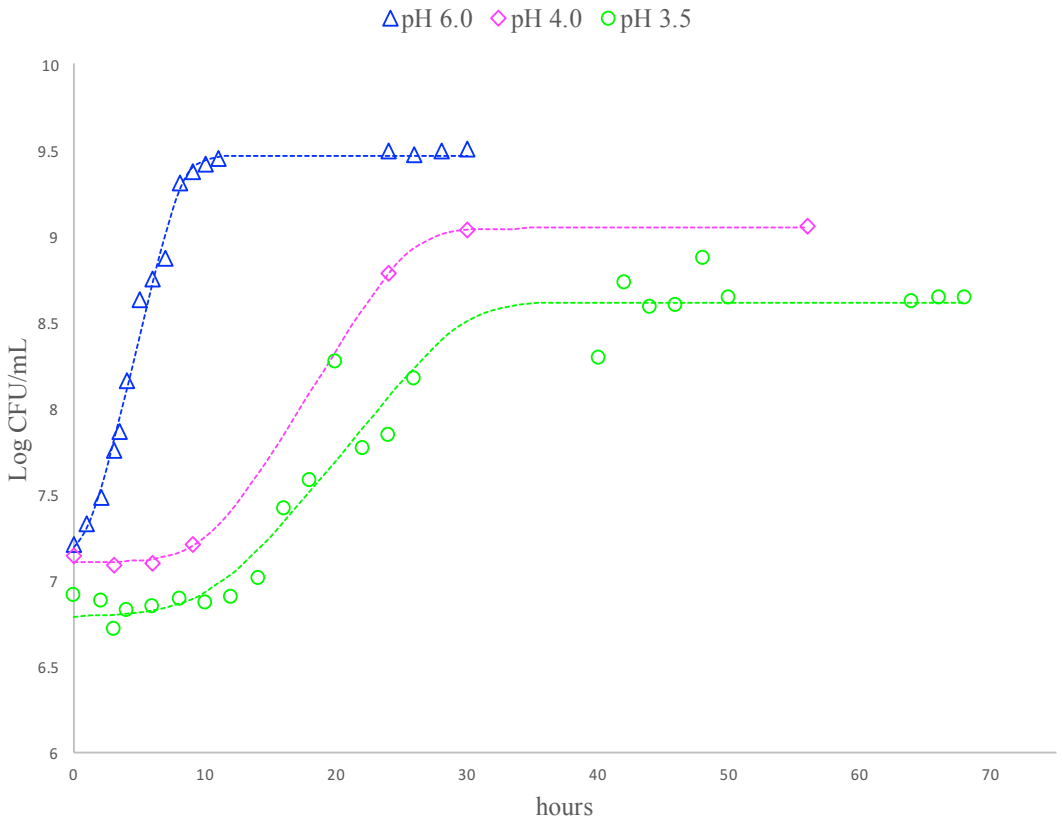

Figure 4.2. Growth kinetic curves of *Lb. plantarum* H_BB1 cultivated in MRS (triangle), in MRS pre-acidified to pH 4.0 (diamond) or to pH 3.5 (circle).

The statistical analysis highlighted that the different cultural conditions affected the growth parameters of tested strain (Table 4.1). In detail, the strain evidenced a very short lag phase (1.1 h) and a maximum specific growth rate (μ_{max}) of about 0.3 h^{-1} when cultivated in MRS broth with initial pH of about 6.0. Conversely, MRS pre-acidified with HCl to pH 4.0 or 3.5 caused an increase ($P<0.05$) in the lag phase and a strong decrease in the μ_{max} values. No significant differences ($P>0.05$) in lag phase and μ_{max} values were detected between the two batches pre-acidified at different pH values (pH 4.0 or pH 3.5). Whereas, significant differences were found in final count levels (y_end) among the different samples. Specifically, the highest values were reached by the strains cultivated in control conditions (MRS with initial pH 6.0) while the lowest were observed in the MRS culture-broth previously acidified to pH 3.5. Intermediate values were detected when the strain was cultivated in MRS pre-acidified to pH 4.0.

Table 4.1. Growth kinetic parameters of *Lb. plantarum* H_BB1 cultivated in MRS (triangle), in MRS pre-acidified to pH 4.0 (diamond) or to pH 3.5 (circle)

	y_0 (Log CFU/mL)	Lag (h)	y_end (Log CFU/mL)	μ_{max} (h^{-1})	R-square	SE of Fit
pH 6.0	7.2 ± 0.1a	1.1 ± 0.4a	9.5 ± 0.0a	0.31 ± 0.01a	0.991	0.082
pH 4.0	7.1 ± 0.0a	10.1 ± 0.9b	9.0 ± 0.0b	0.12 ± 0.01b	0.948	0.182
pH 3.5	6.9 ± 2.1b	10.5 ± 2.1b	8.6 ± 0.1c	0.09 ± 0.02b	0.999	0.032

These results obtained in this study confirmed the data reported in literature (Pieterse et al., 2005) highlighting a strong tolerance to acid conditions in the assayed *Lb. plantarum* strain. The acid stress resistance of strains belonging to *Lb. plantarum* has been widely reported and elucidated in literature (McDonald et al., 1990; Cotter and Hill, 2003). Singular and specific strategies were often implemented by *Lb. plantarum* strains in response to acid stress. Russel and Diez-Gonzalez (1997) reported that *Lb. plantarum* strains react to stress acidity by lowering the intracellular pH. In fact, *Lb. plantarum* can grow at intracellular pH values as low as 4.6-4.8 (Mc Donald et al., 1990). On the other hand, Heunis et al. (2014) reported that acid-stressed *Lb. plantarum* strains produced specific compounds and supplied the cell with energy to ensure their survival. In detail, the production and/or accumulation of basic compounds, more specifically ammonia, seem to be a central strategy to survive.

4.3.3 Effect of cultural conditions on primary metabolites production

The residual glucose behaviour, the lactic acid production and the evolution of pH values during the growth of *Lb. plantarum* H_BB1 were reported in Figures 4.3-4.5. Regardless to the cultural conditions, a decrease in glucose content was observed concurrently to the exponential growth phase and to the beginning of stationary phase. Consequently, the highest glucose consumption was detected during the first ten hours in the control fermentation batch (Figure 4.3) and within the 30^{th} and the 40^{th} hour in the other two fermentation batches pre-acidified to pH 4.0 (Figure 4.4) or to pH 3.5 respectively. However, differences in residual glucose content were observed among the batches. In detail, the glucose was completely metabolized in control condition while a partial consumption was detected both in the batch pre-acidified at pH 4.0 and in those pre-acidified to pH 3.5. The predominant metabolites biosynthesized during the fermentation were the D-lactate and L-Lactate, which were formed as the end-product of glycolysis and secreted in the culture medium.

Surprisingly, regardless to growth capacity and amount of glucose consumption, no significant differences in D-lactic acid final accumulation were detected among the batches. Only the batch pre-acidified at pH 3.5 showed a L-lactic production significant lower than that observed in the other batches. The resulting increase in extracellular lactic acid produced a decrease of pH values highlighting significant differences among the different batches. A decrease in pH values more than two units was observed in the control batch (Figure 4.3), while a decrease of only about 0.8 or 0.5 units was detected in the batches pre-acidified to pH 4.0 or to pH 3.5 respectively (Figures 4.4-4.5).

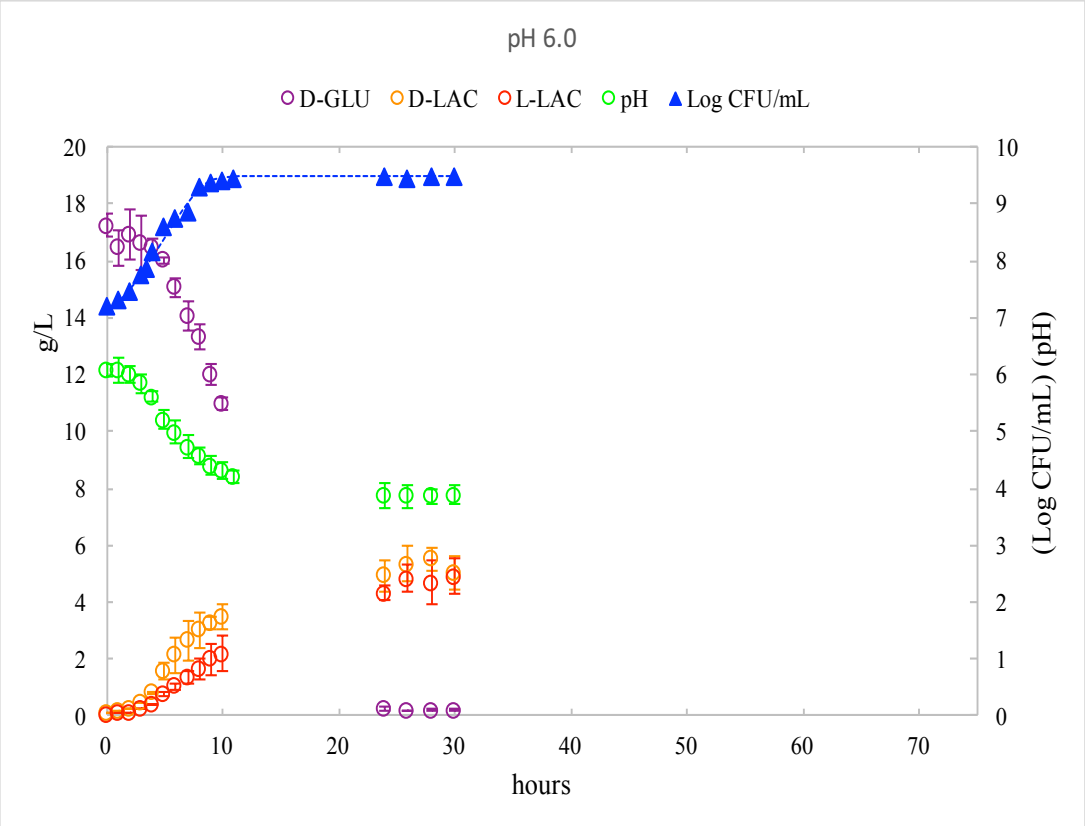

Figure 4.3 Residual glucose behaviour, the lactic acid production the evolution of pH values

Due to their low initial pH, the pre-acidified batches showed growth and metabolic parameters lower than those detected in the control condition. In fact, in these batches, the lactic acid, because its p*K*a (3.86) value, was predominantly found as undissociated lactic acid. It is widely known that the undissociated form, diffusing across the cell membrane towards the more alkaline cytosol produce the highest inhibitory effect on bacterial growth (Shelef et al., 1994; Axe and Bailey, 1995). Pieterse et al. (2005) studying the effect of dissociated and undissociated lactic acid

on *Lb. plantarum* evidenced that dissociated lactate affected the expression of a relatively low numbers of genes. Whereas, the gene expression and the transcription profile of the cultures were strongly influenced in presence of the undissociated form. In detail, not only genes encoding stress protein but also genes associated to other enzymes and stress protein were found. Therefore, undissociated lactic acid in *Lb. plantarum* induces several changes in metabolic activities and a more general stress response.

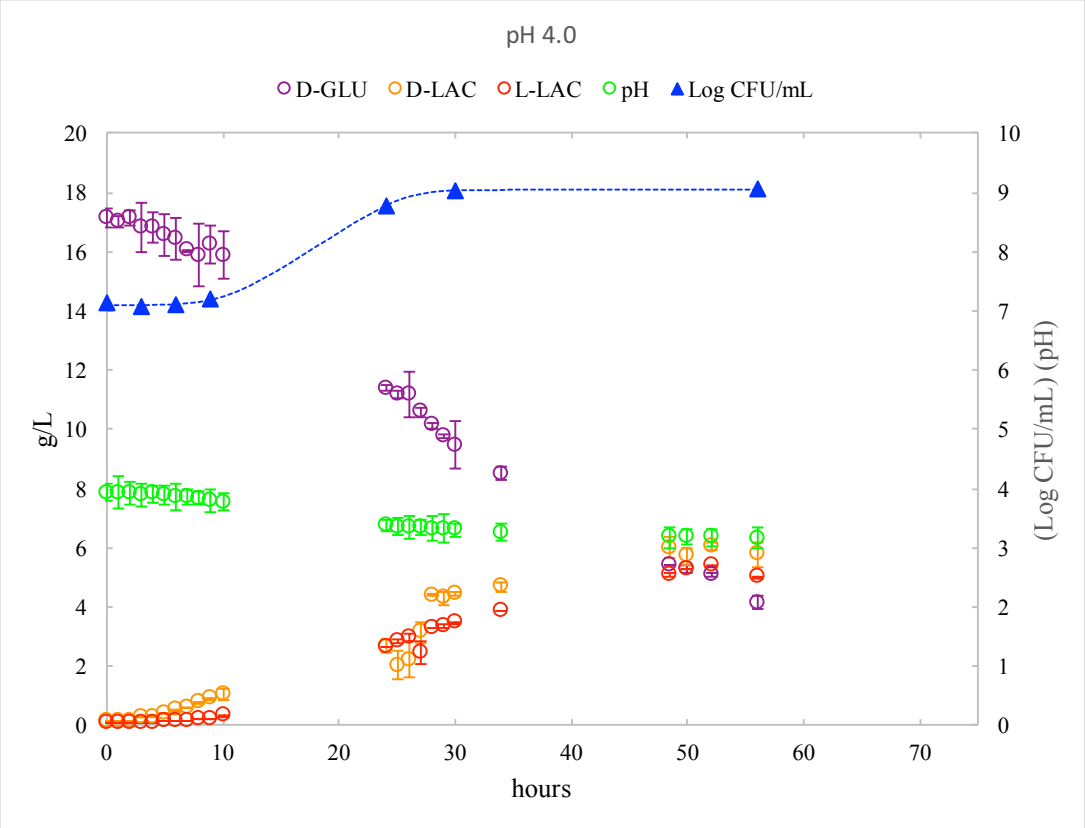

Figure 4.4 Residual glucose behaviour, the lactic acid production the evolution of pH values

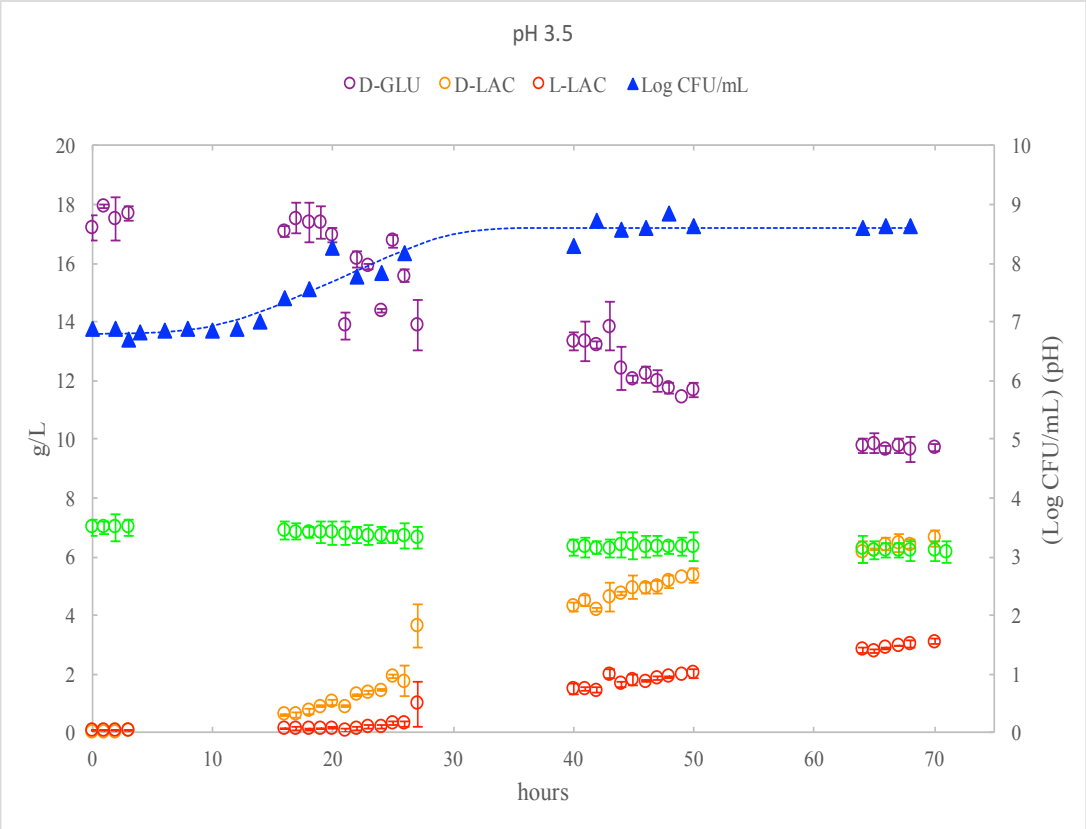

Figure 4.5 Residual glucose behaviour, the lactic acid production the evolution of pH values

It's well-known that low extracellular pH values and the high presence of undissociated lactic acid lead to the pyruvate accumulation and to low NAD^+ levels (as a consequence of end-product inhibitory) with consequently inhibitory effects on the growth (Ferain et al. 1996). Strategies that aim to avoid pyruvate accumulation or to NAD^+ regeneration could produce beneficial effects on *Lb. plantarum* growth.

4.3.4 Effect of cultural conditions on PLA production

The production of PLA by *Lb. plantarum* H_BB1 was also investigated MRS pre-acidified to pH 4.0 and to pH 3.5.

The behaviours of PLA formation, pH and microbial growth in MRS pre-acidified to pH 4.0 or to pH 3.5 are reported in Figure 4.6-4.7 respectively. The results clearly highlighted a relation between the PLA formation and the exponential growth phase. In details, when the strain was cultivated in MRS pre-acidified to pH 4.0 the highest specific PLA production rate was appreciated during the transition lag-exponential phase and during the late exponential phase.

While in MRS pre-acidified at pH 3.5 a constant increase was detected during the entire exponential phase.

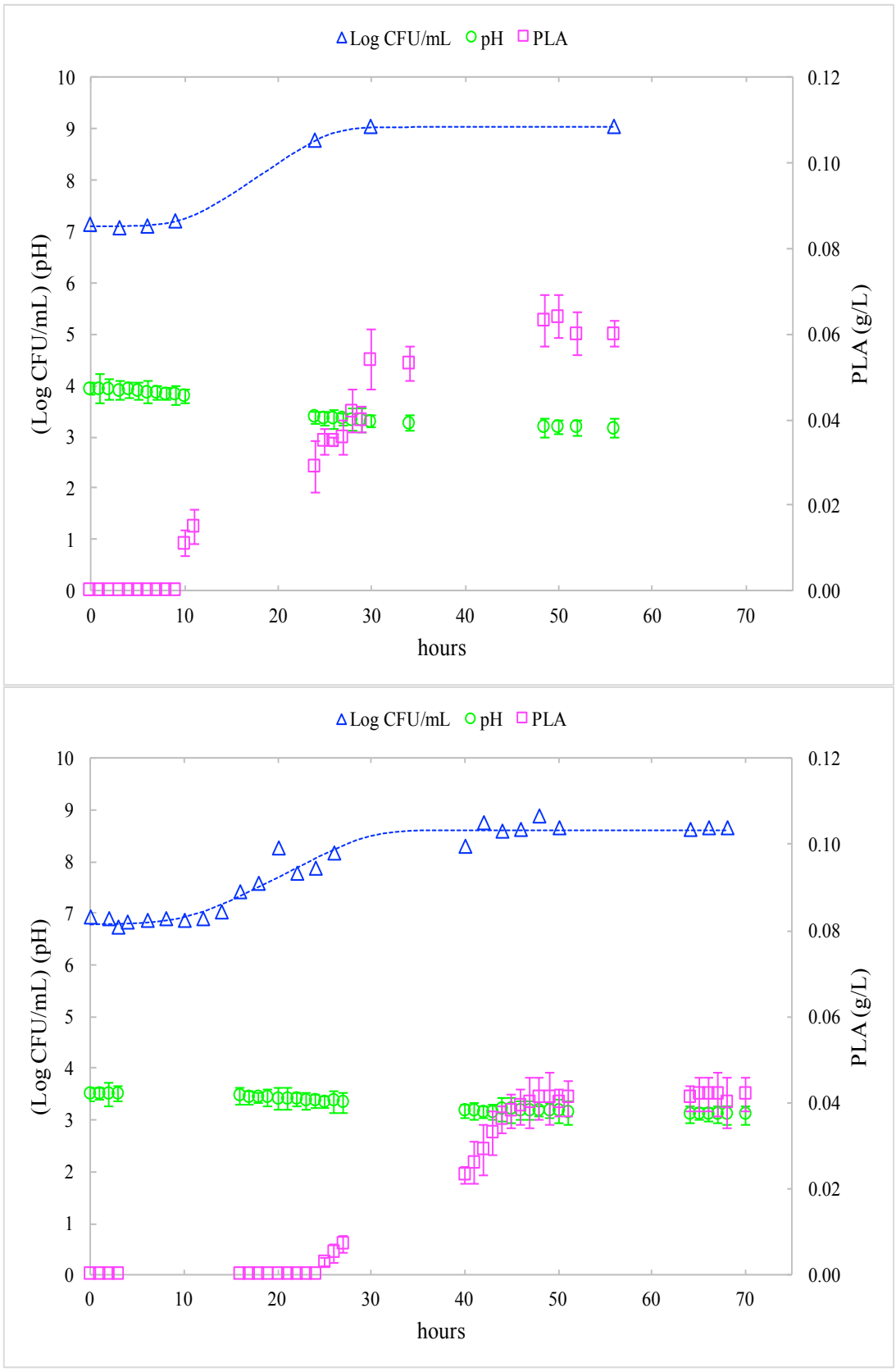

Figure 4.6-4.7 Behaviour of PLA formation, pH values and microbial growth of *Lb. plantarum* H_BB1 in MRS pre-acidified to pH 4.0 or to pH 3.5

So, it is fair to assume that the metabolic pathway involved in PLA formation is tied to the energetic metabolism of growing cells. Key reactions of PLA formation, such as the regeneration of NAD^+ levels, the transamination reaction (where the α-ammino group is transferred to a keto acid acceptor) and the deamination reactions with NH_3 and amino acceptor regeneration, found different linkages with typical metabolic activities of growing cells. The results evidenced that in no case the PLA formation should be related to cell growth arrest. Whereas, its formation could be representing an adaptation response of growing cells to acid stress. This last statement is well represented in the Figure 4.8 where the behaviour of ratio between PLA (g/L) and biomass (g/L) levels was reported. Significant difference in ratio values was found among the different cultural conditions. The highest values were recorded when the strain was cultivated in MRS pre-acidified to pH 4.0. While the lowest ratio values were detected for the strain cultivated in normal condition. In detail, in the batch pre-acidified to pH 4.0 an increase in ratio values of about 40% respect to the control batch was revealed. Also in the batch, pre-acidified to pH 3.5, were detected ratio values higher than that revealed in the control batch. However, the behaviour registered in the batch to pH 3.5 was less performing than that highlighted in the batch pre-acidified to pH 4.0. So, sub-optimal pH (4.0) enhanced the PLA formation by a specific *Lb. plantarum* strain. Several Authors (Bron et al., 2012; Filannino et al., 2014) reported that the acidic environment altered the transcriptomic profile in *Lb. plantarum* during the late exponential phase, which positively reflected on several metabolism pathways. In detail, Filannino et al. (2014) stated that these pathways include D-alanine and histidine metabolism, as well as aromatic amino acid synthesis (phenylalanine, tyrosine and tryptophan, which are involved in the up-regulation of the shikimate pathway). Moreover, the same Authors reported that these pathways may also be involved in redox balancing and NAD^+ regeneration.

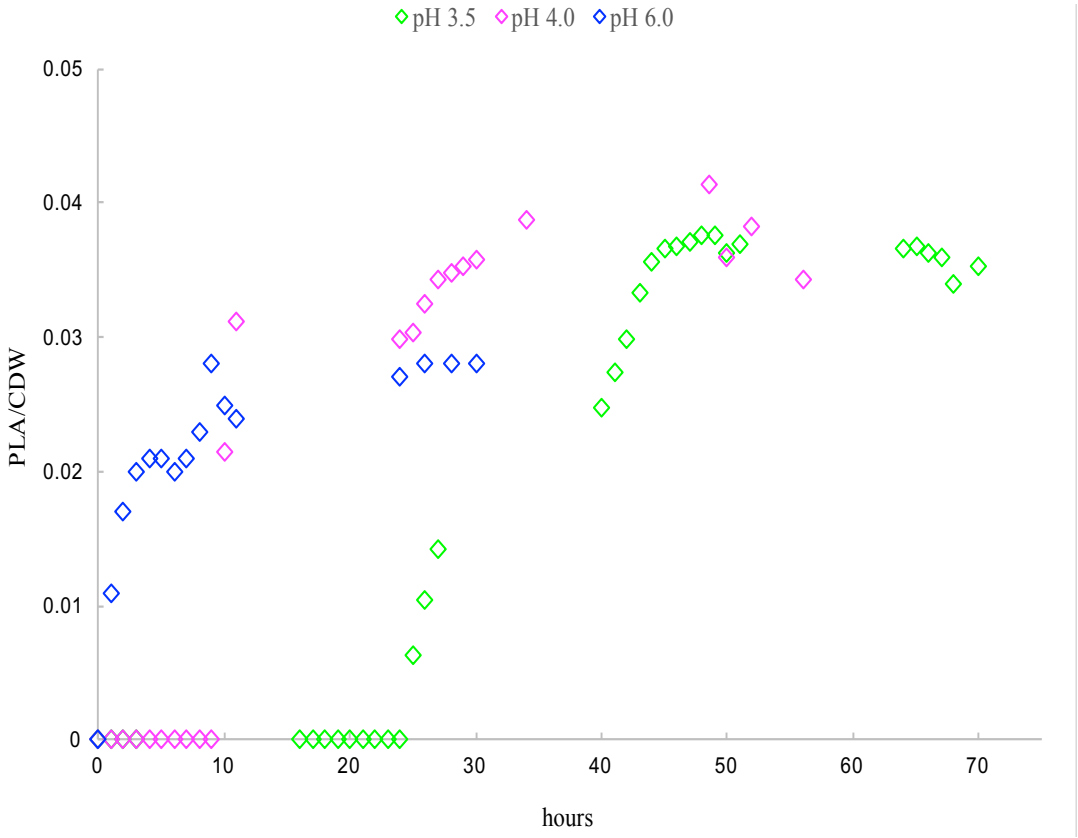

Figure 4.8 Behaviour of ratio between PLA (g/L) and biomass (g/L)

On the basis of the above reported results, the PLA could be considered a "primary-like metabolite" of *Lb. plantarum* in sub-optimal pH conditions. This fact may open new horizons in the development of a new optimal design for the maximum PLA production.

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CHAPTER 5:

ANTI-*LISTERIA* MECHANISM OF 3-PHENYLLACTIC ACID

5.1 Introduction

3-Phenyllactic acid (PLA) has been reported as an antimicrobial compound able to produce an interesting anti-*Listeria* activity (Dieuleveux et al., 1998; Manu, 2012; Liu et al., 2017; Ning et al., 2017). The antagonistic effect expressed by PLA against *Listeria* was defined for the first time by Dieuleveux et al. (1998), which had identified PLA in *Geotrichum candidum* cultures. Thanks to this specific antimicrobial-activity, the PLA is really becoming an interesting preservative compound in food industry. Though is not the most dangerous bacteria, *L. monocitogens* represents the most feared bacteria in the food industry. Many reports have demonstrated the interaction between *L. monocytogenes* and food matrices, showing the high adaptive capacity of these bacteria to survive in extreme environmental conditions (Gandhi and Chikindas, 2007). Organic acids, such as the lactic acid, have long been used as food additives and preservatives for inhibiting the microbial growth and much of their inhibitory mechanism can be attributed to the pH (Jo et al., 2007). Protons are released from un-dissociated molecules in the cytoplasm to decrease the intracellular pH, inhibiting essential microbial metabolic reactions (Olasupo et al., 2004). Therefore, the antimicrobial activity is ascribed to the ability to freely cross the cytoplasmic membrane (Brul and Coote, 1999) and to the ability to induce damage of membrane permeability (Wang et al., 2015). Unfortunately, in the case of several food types, sub-lethal pH values may induce resistance mechanisms to acid stress, which make the *Listeria* cells more resistant to severe acid conditions (Gandhi and Chikindas, 2007). In literature has been widely described the *Listeria* acid stress resistance. One of the most important and well-studied systems in acid resistance of *L. monocytogenes* is the glutamate decarboxylase (GAD) system (Cotter et al., 2001). An extracellular glutamate molecule is imported by an antiporter in exchange for an intracellular γ-aminobutyrate (GABA) molecule. Each molecule of glutamate is decarboxylated by a decarboxylase to produce a molecule of GABA. During this process, a proton is consumed and as result an

increase of the cytoplasmic pH is produced. These events protect the cell against the acidic environmental conditions (Feehily and Karatzas, 2013). Moreover, more recently a new universal stress protein involved in acid stress response in *Listeria* has been discovered (Tremonte et al., 2016). On the basis of these evidences, the individuation of an alternative anti-*Listeria* compounds and the investigation of its mode of action represent a crucial step in quality and safety preservation of food. To date, antimicrobial activity of PLA, including also anti-*Listeria* ability, was well recognized. Whereas, little information is available on PLA anti-*Listeria* mechanism. In fact, only one in-depth report (Ning et al., 2017) has been published highlighting the membrane as the principal target site. However, in a preliminary and less recent study (Dieuleveux et al., 1998) the cell wall was as main action site. It might to assume that the PLA mode of action is similar to lactic acid. Nevertheless, considering the chemical structure of PLA could be also hypothesize an action mode different from the lactic acid and more similar to phenolic acids.

Therefore, the present research aimed to evaluate the antimicrobial effect of PLA to different pH. Moreover, the PLA anti-*Listeria* has been compared with those expressed by the lactic acid and the better studied hydroxybenzoic and hydroxycinnamic acids.

5.2 Materials and methods

5.2.1 Chemicals

Phenyllactic (PLA, 3-phenyllactic acid), a benzoic acid represented by gallic acid (GA, 3,4,5-trihydroxybenzoic acid) and two hydroxycinnamic acid such as ferulic (FA, 3-methoxy-4-hydroxycinnamic acid) and caffeic acid (CA, 3,4-dihydroxycinnamic acid) were obtained from Sigma-Aldrich. For each phenolic compound, stock solution was prepared by dissolving an appropriate amount in dimethyl sulfoxide (DMSO) at a final concentration of 100 mg/mL. The stock solution was sterilized by filtration through a Millipore filter, and it was stored at 4°C before use. Moreover, to comparative purpose an organic acid represented by lactic acid (Sigma, Italy) was used.

5.2.2 Bacterial strains and growth media

Listeria innocua DSM 20649^T was used as model microorganism for antimicrobial tests. The strain, stored at -80°C in 20% (v/v) glycerol, was activated by transferring 20 μL in 10 mL of Mueller Hinton Broth (Oxoid) and incubated for 18-24 h at 37°C.

5.2.3 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PLA

MIC and MBC values of PLA and of other phenolic acids (gallic, ferulic and caffeic acid) on *Listeria innocua* cells, were evaluated. For this purpose, the effect of a range of concentrations (between 0.35 and 30.00 mg/mL) of each phenolic compound was tested on *Listeria innocua* DSM20649 using the macrobroth dilution method. In detail, the effect was evaluated on cells inoculated at final concentration of about 10^5 CFU/mL in Mueller–Hinton broth (MHB) and incubated at optimal growth temperature (37°C). Tube of MHB without phenolic compound and inoculated with *L. innocua* cells as above described was used as control. After 24 h, the turbidity of each tube was evaluated at 600 nm using a spectrophotometer (Bio-spectrometer basic, Eppendorf-Italy). The MIC was considered as the lowest dose where no increase in optical density (600 nm) was observed (CLSI, 2012). Samples (100 μl) from clear tubes were plated on Mueller-Hinton agar (MHA) plates. The MBC was defined as the lowest concentration that can completely kill the bacteria. The studies were conducted in triplicate.

5.2.4 Decimal reduction time

The antimicrobial activity of all phenolic compounds (PLA, GA, FA and CA) against *L. innocua* cells was evaluated by measuring the reduction in numbers (Log CFU/mL) over 48h as described by Carson et al. (2002) with some modifications. Briefly, bacterial suspensions were prepared by centrifuging 150mL MHB overnight cultures (grown 37°C for 15h) at 8000 rpm for 10 min at 4°C. The pellets were washed gently using MES buffer, and then resuspended in 150 mL of MES buffer in order to obtained a final cell density of about 10^5 or 10^8 CFU/mL. The *L. innocua* MES suspension was divided into three aliquots each of which was treated as follows:

- MIC: each phenolic compound was added at the MIC concentration to 50 mL of *L. innocua* suspension;
- MBC: each phenolic compound was added at the MBC concentration to 50 mL of *L. innocua* suspension;
- Control: cell suspension without phenolic compound added.

Moreover, cells suspended in MES buffer, added with lactic acid (PC Laboratory Reagents, Malaysia), at the same concentration of the PLA, was used as other control.
The pH of each suspension was maintained at values of about 5.5.
At regular time intervals (2h) aliquots of 1 mL were removed from the suspension and then were serially diluted and plated on MHA. Finally, the plates were incubated at 37°C for 48h before enumeration. Three replicates were made for each experiment. The experimental data were used to estimate the death kinetic parameters through the D-model of Baranyi and Roberts (1994) using the software DMFit (Web Edition).

5.2.5 Bacterial surface charge: zeta potential

The zeta potential of bacterial suspensions containing PLA at the 2xMIC concentration, in ultrapure water (pH 6), was determined using a Nano Zetasizer (Malvern Instruments). Cell suspensions, without phytochemical, were used as controls. The zeta potential was measured by applying an electric field across the bacterial suspensions. The experiments were repeated at least three times.

5.2.6 Loss of cellular content

The release of cell constituents into the supernatants was measured according to the method described by Rhayour et al. (2003). Cells from the working culture (15 mL) were collected by centrifugation (5500 rpm for 15 min), washed three times, and resuspended in MES buffer. Cell suspension was incubated at 37 °C for 5 h in the presence of PLA at MBC concentration or without PLA (control). Then, 10 mL samples were taken and filtered through a 0.22 μm-pore-size filter (Carson et al., 2002). The concentration of the constituents released was determined by UV absorption measurements of each filtrate using an UV-spectrophotometer (Bio-spectrometer basic, Eppendorf-Italy) at 260 nm.

5.2.7 Statistical analysis

Three independent replicates of each experiment were performed, and their results were expressed as mean values ± standard deviation. Analysis of variance (ANOVA) and the Tukey's pairwise comparisons were performed.

5.3 Results and discussion

5.3.1 Minimum Inhibitory Concentrations and Minimum Bactericide Concentrations

Listeria innocua was chosen as the indicator to investigate the antimicrobial mechanism of PLA. *Listeria innocua* is regarded as a non-pathogenic indicator for the presence of *Listeria monocytogenes* in foods (Rosimin et al., 2016). In the last years, great attention was focused on these two species which had showed several mechanisms of acid stress adaptation (Tremonte et al., 2016). So far, only two reports (Dieuleveux et al., 1999; Ning et al., 2017) have been investigated the antimicrobial activity of PLA on *L. monocytogenes*. Dieuleveux et al. (1999) reported that cell wall represents the action target of PLA, whereas the recent results published by Ning et al. (2017) suggested that PLA can damage the integrity of cytoplasmic membrane.

The results obtained in the present study also confirm the MIC values detected by Ning et al. (2017) regard to *L. monocytogenes* (1.25 mg/mL). In detail, the macro-dilution assay carried out to pH 5.5 or to pH 5.0 highlighted that MIC value for *L. innocua* was 0.94 and 0.47 mg/mL respectively. Other Authors (Lavermicocca et al., 2003; Perma et al., 2010; Cortés-Zavaleta et al., 2014), studying the effect of PLA on fungi, reported MIC values much higher than that revealed in the present study against *L. innocua*. This finding evidenced that PLA, which has been considered for many years an antifungal metabolite (Lavermicocca et al., 2003; Vermeulen et al., 2006), is able to inhibit also bacteria cells. Moreover, very low concentrations were required to produce anti-*Listeria* activity. MIC values of about 0.47 or 0.94 mg/mL appear compatible whit the maximum PLA production revealed in *Lb. plantarum* cultures. In fact, as reported in literature (Rodrìguez et al., 2012; Cortéz-Zavaleta et al., 2014; Corsetti et al., 2014), and also detected in the present PhD research (chapter 3 and 4), *Lb. plantarum* strains produced up to 0.12 or 0.23 mg/mL and this production level could be increased two or even tenfold when specific cultural strategies were applied (Mu et al. 2009; Dallagnol et. al. 2011; Zhang et al., 2014). So, a resolution to the gap between PLA required to assure antimicrobial activity and the PLA levels detected in fermentation batches seems possible. This gap has for a long time a serious obstacle to apply PLA producing bacteria as protective or as anti-*Listeria* cultures in food characterized by neutral or sub-acid pH.

In the present study, the relation between pH values and anti-*Listeria* activity of PLA was clarified. In Figure 5.1 were reported the MIC values detected at different pH.

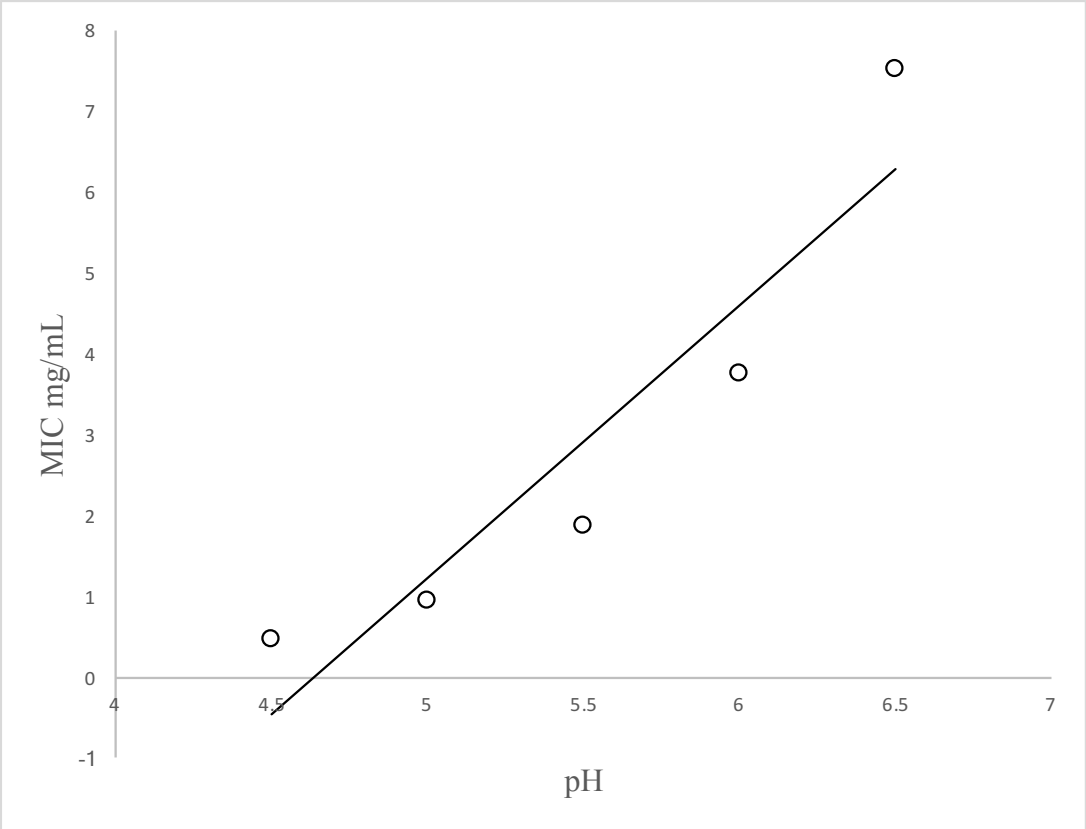

Figure 5.1 Correlation between anti-*Listeria* activity of PLA (MIC) and pH values.

A relation between MICs and pH values was found and a significant decrease in PLA anti-*Listeria* activity was detected at the highest pH values tested. In detail, PLA anti-*Listeria* activity was shown pH dependent highlighting the typical behaviour of activities of other weak acid preservatives and organic acid (lactic acid, citric acid, acetic acid, etc.). Therefore, it's possible to assume that the PLA (pKa 3.46) mode of action is related to its undissociated form able to cross the microbial membrane (Wang et al., 2015).

5.3.2 Effect of Phenyllactic and Lactic Acid on *L. innocua* decay

In order to understand the PLA mode of action, the anti-*Listeria* effect produced by PLA at pH 5.5 was compared to the effect explicated by lactic acid to the same pH value. Lactic acid was choice because its antimicrobial mechanism is well known. The cell membrane is the main antibacterial target for lactic (Wang et al., 2015).

The survival kinetic curves of *L. innocua* in presence of PLA (2 x MIC), of LA used at same concentration of PLA as well as the kinetic curves of the bacterium without preservative agents addition (control condition) are reported in Figure 5.2. The corresponding survival kinetic parameters (shoulder, maximum death rate and y_end values) are reported in Table 5.1. As expected, a significant decrease ($P<0.05$) of *L. innocua* cells was observed during the incubation time in presence of preservative agents (PLA and LA), while a behaviour substantially constant or a weakly decreasing was detected in the control condition.

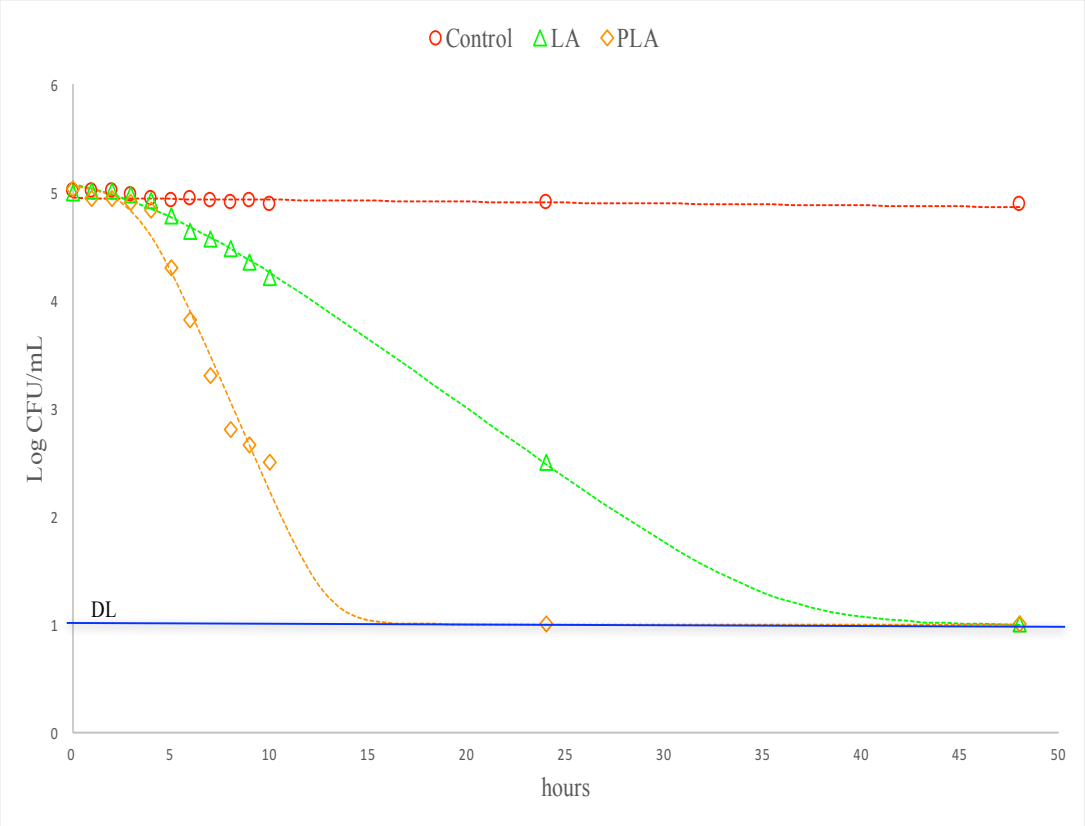

Figure 5.2 Survival of *L. innocua* in MSE buffer containing PLA (2xMIC), LA at same concentration of PLA or without preservative agents (Control). Symbols represent the experimental date and the curves represent the D-model. DL is the detection limit.

Table 5.1 Survival kinetic parameters estimated in *L. innocua* after exposure PLA (2xMIC), LA at same concentration of PLA or without preservative agents (Control).

	y_0 (Log CFU/mL)	Shoulder (h)	y_end (Log CFU/mL)	μ_{max} (h^{-1})	R-square	SE of Fit
Control	5.0 ± 0,1a	0.0 ± 0.0a	4.9 ± 0.0a	0.00 ± 0.01a	0.304	0.036
PLA	5.1 ± 0.1a	3.3 ± 0.6b	1.0 ± 0.1b	-0.43 ± 0.04b	0.987	0.036
LA	5.1 ± 0.1a	4.1 ± 0.5c	1.0 ± 0.1b	-0.13 ± 0.01c	0.998	0.049

In detail, the behaviour of *L. innocua*, in absence of preservative agents, was described by a linear model, whereas the survival in presence of LA or PLA was well represented by a complete Baranyi and Robert model (shoulder + linear decrease + asymptote). However, significant differences were also detected between the two kinetic curves describing the effect of PLA and LA on *L. innocua*. PLA was able to induce maximum death rate values (-0.43 h^{-1}) significantly higher respect to the LA which produced μ_{max} values of about -0.13 h^{-1}. Moreover, a higher resistance (shoulder) in *L. innocua* strains was appreciated in presence of LA than that in presence of PLA.

On the basis of these results, it's possible to assert that antimicrobial action of PLA was substantially different from LA. The different and more efficacious effect produced by PLA could be due to the its amphiphilic properties resulting from the hydrophobic group-benzene ring and hydrophilic group-carboxy in its chemical structure. These properties would allow an interaction with lipid and protein of membrane cytoplasmic as well as an interaction with genomic materials. Therefore, a comparison between PLA and more studied phenolic acids (hydroxybenzoic and hydroxycinnamic) should be investigated.

5.3.3 Effect of phenolic acids on *L. innocua* decay

To better understand the antimicrobial effect of PLA on *L. innocua*, further experiments were conducted using three phenolic compounds (gallic, ferulic and caffeic acid), widely studied. The mechanism of action expressed by hydroxybenzoic acid (gallic acid, GA) and hydroxycinnamic acid (ferulic and caffeic acids, FA and CA) against different undesirable microorganisms, including both Gram negative and Gram positive bacteria, was already study by several Authors (Guitiérrez-Larrainzar et al., 2012; Borges et al., 2013; Fernandez-Alvarez et al., 2014; Chen et al., 2017). On the basis of the result reported by these Authors, hydroxybenzoic and hydroxycinnamic acids seem to led to irreversible changes in membrane properties (charge, intra and extracellular permeability), to a decrease of negative surface charge, as well as to rupture or pore formation in the cell membranes with consequent leakage of intracellular constituents.

Figures 5.3-5.4 report survival kinetic curves of *L. innocua* in presence of PLA or phenolic acid (GA, FE, CA) used at MIC or MBC concentration (Table 5.2) respectively. The corresponding survival kinetic parameters (shoulder, maximum death rate and y_end values) are reported in Table 5.3-5.4.

Table 5.2 MIC and MBC values of PLA, GA, CA, and FA on *Listeria innocua* cells in MH broth at pH 5.5.

	PLA	GA	CA	FA
MIC (mg/mL	0.94	1.25	1.88	2.50
MBC (mg/mL)	1.88	2.50	5.00	5.00

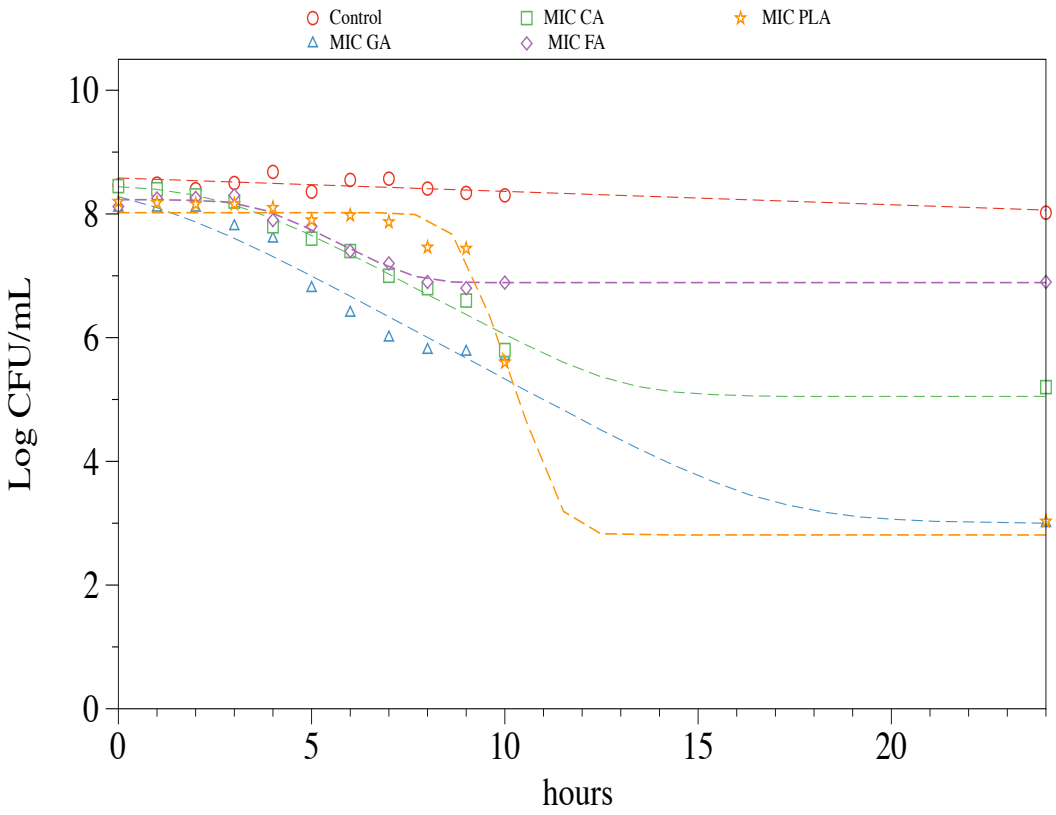

Figure 5.3 Survival of *L. innocua* in MSE buffer containing GA, CA, FA and PLA at MIC concentration or without preservative agents (Control). Symbols represent the experimental date and the curves represent the D-model.

As expected, a linear model well describes the slight and constant decrease of *L. innocua* in control conditions. Regardless to the type of phenolic acid, when used at MBC concentration a completely decay in *L. innocua* count levels was observed. Whereas, the use of phenolic compounds at MIC concentration produce a reduction of about 1 or 5 log UFC/mL depending on the type of phenolic acid.

Table 5.3 Survival kinetic parameters estimated in *L. innocua* after exposure GA, CA, FA and PLA at MIC concentration or without preservative agents (Control).

	y_0 (Log CFU/mL)	Shoulder (h)	y_end (Log CFU/mL)	μ_{max} (h^{-1})	R-square	SE of Fit
Control	8.5 ± 0.1	6.8 ± 0.2	7.9 ± 0.1	-0.02 ± 0.01	0.777	0.105
GA MIC	8.3 ± 0.1	1.3 ± 0.1	3.0 ± 0.2	-0.34 ± 0.05	0.977	0.262
FA MIC	8.2 ± 0.1	3.5 ± 0.4	6.9 ± 0.1	-0.32 ± 0.04	0.978	0.090
CA MIC	8.4 ± 0.1	2.8 ± 0.7	5.1 ± 0.1	-0.33 ± 0.03	0.985	0.147
PLA MIC	8.0 ± 0.1	8.7 ± 0.2	2.8 ± 0.2	-1.86 ± 0.41	0.986	0.236

In detail, the highest inhibition was appreciated in presence of PLA and GA which produced a strong inhibition showing a reduction about 5 Log CFU/mL already at 12th and 18th hour of incubation, respectively. However, PLA was able to induce maximum death rate values (-1.86 h^{-1}) significantly higher respect to the GA which produced -μ_{max} values of about -0.34 h^{-1}. Moreover, significant differences between GA and PLA was found also in the shoulder parameters which were estimated in 1.3 hours in presence of GA and reached 8.7 hours when the PLA was used. The effects produced by the hydroxycinnamic acids (CA and FE) were less performing respect both PLA and GA. The use of caffeic and ferulic acids produced maximum specific death rate values in *L. innocua* decay similar to those observed in presence of gallic acid. However, the highest shoulder values detected in presence of hydroxycinnamic acids than that registered in the batch added with GA leads a lower decay of *L. innocua*.

Significant differences among the batches were appreciated also when the phenolic acid was used at MBC concentrations. Albeit in all cases a complete decay in *L. innocua* was observed within the 6th hour significant differences were found both in shoulder and in maximum specific death rate.

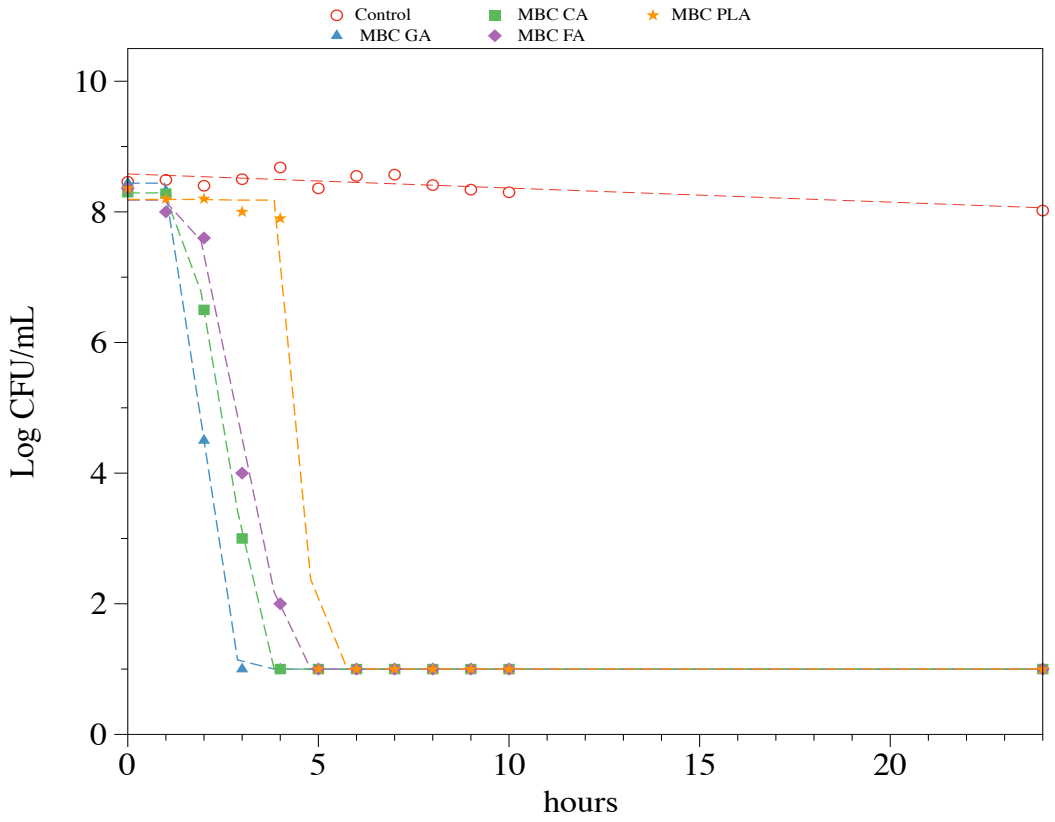

Figure 5.4 Survival of *L. innocua* in MSE buffer containing GA, CA, FA and PLA at MBC concentration or without preservative agents (Control). Symbols represent the experimental date and the curves represent the D-model. DL is the detection limit.

As reported in Table 5.4 the use of hydroxycinnamic and hydroxybenzoic acids produced in the assayed strain very low shoulder values, ranging from 0.97 (GA) to 1.70 (FA), and maximum death rate values ranging from -3.82 to -2.80 h^{-1}.

Table 5.4 Survival kinetic parameters estimated in *L. innocua* after exposure GA, CA, FA and PLA at MBC concentration or without preservative agents (Control).

	y_0 (Log CFU/mL)	Shoulder (h)	y_end (Log CFU/mL)	μ_{max} (h^{-1})	R-square	SE of Fit
Control	8.5 ± 0.1a	6.8 ± 0.2a	7.9 ± 0.1a	-0.02 ± 0.01a	0.777	0.105
GA MBC	8.4 ± 0.1a	1.0 ± 0.0b	1.0 ± 0.0b	-3.82 ± 0.01b	1.000	0.001
FA MBC	8.2 ± 0.2a	1.7 ± 0.1c	1.7 ± 0.1c	-2.80 ± 0.09c	0.994	0.234
CA MBC	8.3 ± 0.3a	1.5 ± 0.2c	1.5 ± 0.3c	-3.50 ± 0.01d	1.000	0.005
PLA MBC	8.2 ± 0.2a	4.0 ± 0.0d	1.0 ± 0.0b	-6.91 ± 0.02e	0.999	0.085

The use of PLA at MBC concentration induced in the assayed strain the shoulder extension and a strong increase in maximum specific death rate.

The data set generated from both MIC an MBC survival test highlighted that the GA and the PLA showed the most performing anti-*Listeria* activity. However, the differences between PLA and GA in death kinetic parameters suggest that PLA

produces anti-*Listeria* activity through a specific mechanism somewhat different from those usually adopted by other phenolic compounds. Antimicrobial activity of phenolic acids involves several mechanisms of action such as permeability destabilization or the rupture of cytopalsmatic membrane as well as enzymes inhibition through nonspecific interaction (Ota et al., 2011; Borges et al., 2013). It's possible to suppose that PLA utilize more than one of these pathways but differently from the other phenolic compounds.

5.3.4 Effect of phenolic acids on surface charge and loss of cellular content

Membrane has been proposed as one of the most important action targets of phenolic compounds (Hayrapetyan et al., 2012; Borges et al., 2013). Therefore, information in surface charge change or in loss of cellular content could provide important clarification on antimicrobial mechanisms. Due to the presence of anionic groups (e.g., carboxyl and phosphate), the surface charge of bacterial cells is generally negative. Data obtained by zeta potential measurement (Figure 5.5) showed the charge change in the assayed *L. innocua* strain after exposure to the different phenolic compounds. Zeta potential measurements demonstrated that after phenolic acids exposure, the cells become more ($P<0.05$) negatively charged when exposed to PLA. While in presence of other phenolic compounds (GA, CA, FE) no variation in charge was detected. This result confirm data reported in literature (Borges et al., 2013) which attributed no effect to hydroxycinnamic (FA) and hydroxybenzoic (GA) in Gram-positive charge change.

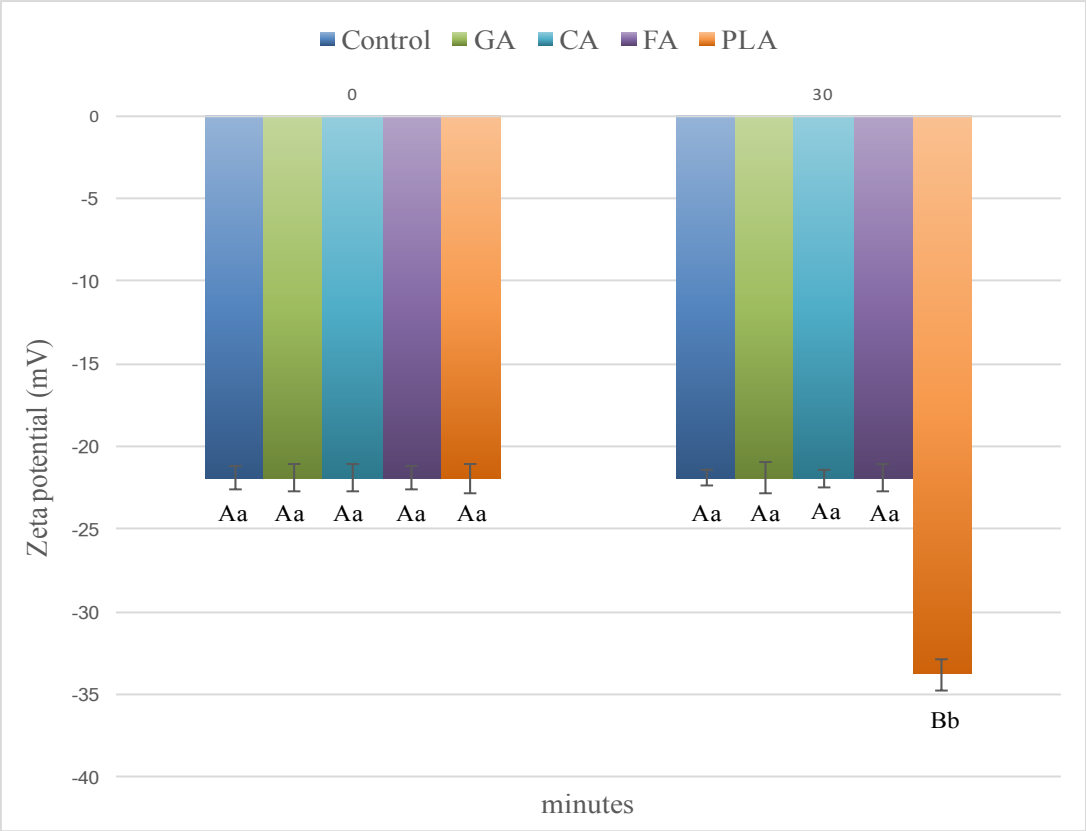

Figure 5.5 Zeta potential of *L. innocua* cells exposed to ultrapure water containing GA, CA, FA and PLA at 2xMIC concentration or without phenolic compounds (Control).

On the other hand, surprising results obtained by PLA use. In fact, so far change in charge surface by phenolic acid was observed only in Gram negative bacteria. This fact may open new horizon in the understanding of PLA anti-*Listeria* mechanism. It's possible to suppose that the PLA anti-*Listeria* action is also associated with the affinity with cell surface and the interaction PLA-cell surface could contribute to the damage of cellular structures. The rupture of cellular structures was also supported by the results of the cellular content loss (Figure 5.6).

The release of cell constituents was determined by the measurement of the absorbance at 260nm of the filtrates of *L. innocua* cultures. The treatment with PLA at 2xMIC concentrations induced a significant ($P < 0.05$) increase in cell constituent's release.

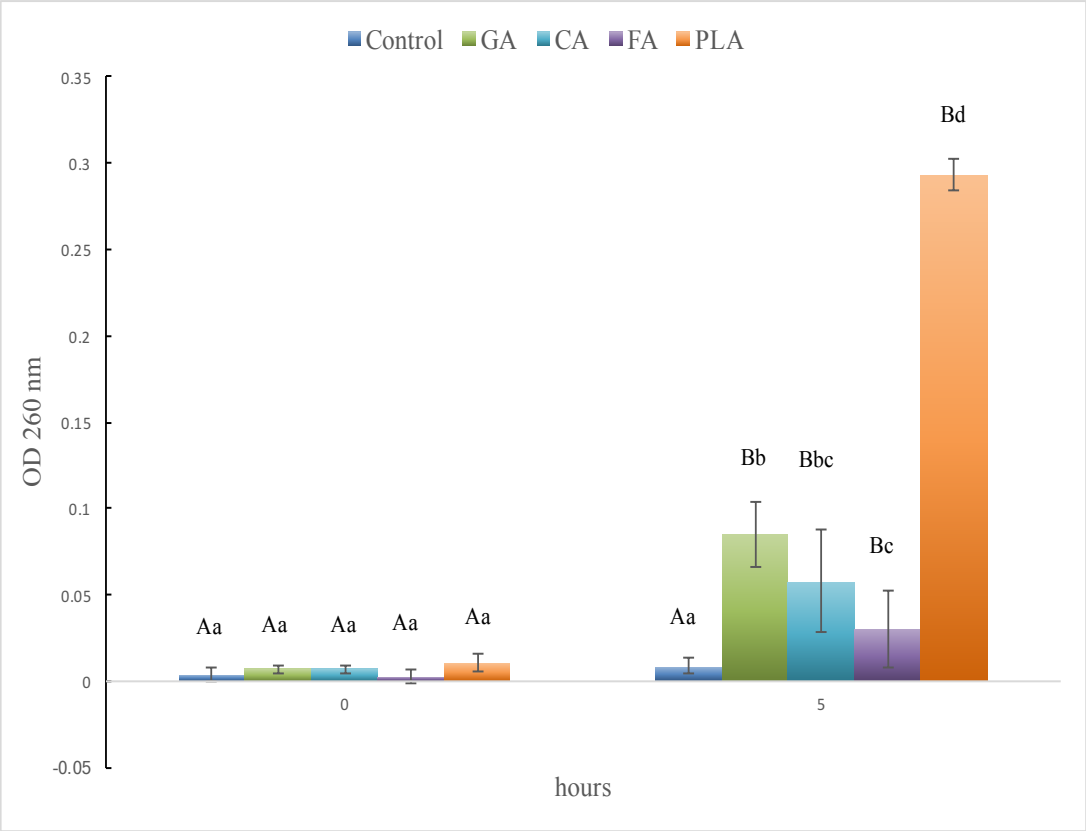

Figure 5.6 Cellular content loss of *L. innocua* to ultrapure water containing GA, CA, FA and PLA at 2xMIC concentration or without phenolic compounds

Whereas, when *L. innocua* cultures were exposed to the 2xMIC concentrations of the other phenolic acids (GA, FE and CA) only a weak variation in OD values was detected. This finding integrated with results from zeta potential demonstrate that at pH 5.5 PLA strongly interact with the surface of *Listeria innocua* strain promoting membrane damage, release of intracellular content and the consequent cell death.

Therefore, the results might help in explaining the differences in anti-*Listeria* mechanism of phenolic compounds. Hydroxybenzoic and hidroxycinnamic acid seem to induce an alteration in membrane permeability without causing its rupture. Whereas, PLA having the main targets in cellular surface and in cytoplasmic membrane, leads to a severe rupture of the cellular structures. All these evidences contribute to the enrichment of scientific knowledge in anti-*Listeria* mechanism of PLA and evidenced that PLA effectiveness is superior than that expressed by other preservative acids.

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CHAPTER 6:

CONCLUSIONS

In the preliminary step, the PhD research, analyzing the advancements in control strategies based on natural compounds and living organisms and/or their antimicrobial products, highlighted that so far, several issues such as the high minimal inhibitory concentration levels, the stability of antimicrobial compounds, the knowledge of action mode, as well as the relation between microbial growth and compound formation kinetics, still remain unclear, making the individuation of a simplified screening procedure necessary. In this light, the PhD research preliminarily focuses the attention on the effects of different environments on the selection of strains able to exert antimicrobial activities and to produce specific antimicrobial compounds such as 3-phenillactic acid (PLA). The results highlighted significant differences among the assayed strains showing that PLA production is strain-dependent. In addition, for the first time, a relation between PLA-producing strains and isolation environment of the strains was highlighted. In fact, those environments characterised by harsh conditions (high ethanol levels, low pH and high sugar levels), such as wines and honey, harboured a higher number of antagonistic strains than other fermented matrices (e.g. cheese, sourdoughs or fermented sausages). This could be due to selective pressures which are more accentuated in wines and honey than in the other food matrices researched.

The most important scientific enrichment produced by first step of PhD activities is attributable to results highlighting that the choice of the source of isolation could be an important preliminary tool for the individuation of antagonistic strains. However, the correlation between *Lb. plantarum* PLA formation ability and their isolation sources would lead to opening new frontiers in understanding the PLA formation process. PLA formation seems to be linked to stress response mechanisms performed by *Lb. plantarum*. However, no information with regards the LAB stress response and PLA production is available in literature and little information is reported on the relation between the microbial growth phase and PLA formation. Even if the prevailing opinion in the scientific community believes that PLA formation is related to LAB growth arrest, the linkage to metabolic pathways involved in its stationary phase has not been clarified. Little information, if any, can be found on the optimal pH condition of PLA metabolic pathway in the *Lactobacillus* species.

The present study, comparing the PLA behavior with growth curves of *Lactobacillus*, highlighted that the PLA accumulation begins immediately after the end of the lag phase and reached the highest levels between the exponential and the stationary phase. As far as I know, these results show, for the first time, that the PLA production is strictly related to the growth and to the exponential phase of *Lb. plantarum*. More specifically, the results obtained in the present study suggest that the PLA could assume new metabolic meanings. In fact, the accumulation from the beginning of the exponential phase highlight typical behaviour of a primary metabolite. On the other hand, the highest production rate between the exponential and stationary phase suggests that PLA production could assume a key role in the acid stress response. In fact, in the transition between exponential and stationary phase, pH showed values similar to the *pKa* of lactic acid. On the basis of the above reported statements, the evaluation of ecological factors on the PLA formation process appear essential. More precisely, sub-lethal pH could positively affect some metabolic pathways in *Lb. plantarum*. The results suggest that the metabolic pathway involved in PLA formation is tied to the energetic metabolism of growing cells. Key reactions of PLA formation, such as the regeneration of NAD^+ levels, the transamination reaction (where the α-ammino group is transferred to a keto acid acceptor) and the deamination reactions with NH_3 and amino acceptor regeneration, found different linkages with typical metabolic activities of growing cells. The results evidenced that, in no way, could the PLA formation be related to cell growth arrest. Whereas, its formation could represent an adaptation response of growing cells to acid stress. In fact, evaluating the behaviour of ratio between PLA (g/L) and biomass (g/L) levels, the highest performances were detected when the strain was cultivated in MRS pre-acidified to pH 4.0. On the basis of the above reported results, the PLA could be considered a "primary-like metabolite" of *Lb. plantarum* in sub-optimal pH condition and may open new horizons to the development of an advanced optimal design for maximum PLA production. The results of this study have evidenced that PLA is able to inhibit bacteria cells and very low concentrations were required to produce anti-*Listeria* activity. MIC values of about 0.47 or 0.94 mg/mL appear compatible with the maximum PLA production revealed in *Lb. plantarum* cultures. In fact, this PhD research, recognizes that *Lb. plantarum* strains produced up to 0.12 or 0.23 mg/mL and this production level could be increased two or even tenfold when specific cultural strategies were applied. Therefore, a resolution to the gap between PLA required to assure antimicrobial activity and the PLA levels detected in fermentation batches seems possible. This gap has long proven to be a serious obstacle when applying PLA producing bacteria as protective or as anti-*Listeria* cultures in food characterized by neutral or sub-acid pH. Moreover, the relation between pH values and anti-*Listeria* activity of PLA was clarified. A relation between MICs and pH values was found and a significant reduction in PLA anti-*Listeria* activity was detected at the highest pH values tested.

Regarding to the understanding of PLA mode of action, useful information was obtained by the comparison of anti-*Listeria* effect produced by PLA at pH 5.5 with those explicated by lactic acid to the same pH value. In detail, the results evidenced that the antimicrobial action of PLA was substantially different from lactic acid. The different and more successful effect produced by PLA must be due to its amphiphilic properties resulting from the hydrophobic group-benzene ring and hydrophilic group-carboxy in its chemical structure. These properties would allow an interaction with the lipid and protein in cytoplasmic membrane as well as an interaction with genomic materials. Studying the anti-listeria action of PLA and other phenolic acids (hydroxybenzoic and hydroxycinnamic), differences between PLA and GA in death kinetic parameters were detected. These evidences suggest that PLA produces anti-*Listeria* activity through a specific mechanism which is somewhat different from those usually adopted by other phenolic compounds. Antimicrobial activity of phenolic acids involves several mechanisms of action such as permeability destabilization or the rupture of the cytoplasmic membrane as well as enzymes inhibition through nonspecific interaction. It is possible to hypothesize that PLA utilizes more than one of these pathways but differently from the other phenolic compounds. It is possible to surmise that the PLA anti-*Listeria* action is also associated with the affinity with cell surface and the interaction PLA-cell surface could contribute to the damage of cellular structures. Hydroxybenzoic and hydroxycinnamic acid seem to induce an alteration in membrane permeability without causing its rupture. Whereas, PLA having the main targets in cellular surface and in cytoplasmic membrane, leads to a severe rupture of the cellular structures. All these evidences contribute to the enrichment of scientific knowledge in the anti-*Listeria* mechanism of PLA and highlighted that PLA effectiveness is superior to that expressed by other preservative acids.