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"Study of the behavior of *Lactococcus lacti*s and *Listeria monocytogenes* in competition for food safety in dairy products"

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INDEX

ABSTRACT

Microbial competition is a mechanism that occurs when two or more microbial species compete for ecological niches to support their survival and growth. Different factors can contribute to the outcome of microbial competition, such as molecules exchanged between the competing organisms for the regulation of cell densities and the initial spatial configuration of the microbe–microbe interaction. Specifically, production of compounds that kill or limit the growth of competing strains or species can promote niche monopolization [2]. The released compounds include secondary metabolite antibiotics, bacterial peptides or low-molecular-mass organic compounds. The same happens in food, and it could be possible to explore this 'molecular' communication to improve food safety. In that sense, it is very important to develop tools in the control of bacterial species for a better food safety. The present work focused on the study of microbial competition between *Listeria monocytogenes* (LM) and *Lactococcus lactis* (LAC) monitored through proteomics, peptidomics and metabolomics approach. We study the secretome of these two microorganisms (*Listeria monocytogenes* and *Lactococcus lactis*) alone and in co-culture. In particular, we studied by proteomic analysis the evaluation of proteins secreted by bacteria through one/two-dimensional electrophoresis coupled to mass spectrometry (MALDI TOF). Furthermore, in order to characterize each secretome, label free Shotgun analysis was conducted using nano UPLC-MS system. Furthermore, the secretome of these microorganisms has been studied through first an untargeted proteomics analysis in vitro, followed by validation directly in a system resuming cheese. The objective of the last part of the project has been the monitoring of bacterial competition between through a combination of microbial Imaging mass spectrometry and LC-MS/MS, in order to investigate the metabolic profile of each bacteria in the interacting microbial colonies. In according with obtained preliminary data (one-dimensional and two-dimensional electrophoresis), new data highlighted, during competition, the higher production by *Listeria monocytogenes* of moonlighting protein Enolase (C1KY94) and Glucose 6 Phosphate isomerase (Q71X61), of Septation ring formation regulator EzrA (B8DHE7), involved into cell replication in regulatory mechanisms of cell energetics or metabolism and the lower secretion Endopeptidase P60 (P21171), protein associated with the cell surface and involved in the process of invasion. In parallel, *L. lactis* produced higher amounts of

Secreted 45 kDa protein and switched from lantibiotic Nisin A production to Nisin Z production. In competition with LM, LAC strain investigated produce higher amounts of Secreted 45 kDa protein with peptidoglycan lytic activity and the selective secretion of Nisin Z probably to improve lantibiotic solubility in less acidic environment. Lastly, IMS analysis revealed several interesting compounds during interaction of microbial colonies. At least six compounds are uniquely expressed during the interaction between LM and LAC. Among these, we focused our attention on three compounds: Cyclo-(Leu-leu), Cyclo-(Phe-Tyr), Cyclo-(L-Phe-L-4-Hyp). These compounds are cyclic peptides, isolated by Lactobacilli, with a biological activity]. In particular, they play an important role in bacterial cell to cell communication. Probably, these peptides have a role in inducing of the transcription of gene coding for Nisine. These results could be useful to setup new molecular strategies in the control of bacterial species for a better food safety.

ABSTRACT

La competizione microbica è un meccanismo che si verifica quando due o più specie microbiche competono per la conquista della nicchia ecologica per la loro sopravvivenza e per la loro crescita. Diversi fattori possono influenzare la competizione microbica, come ad esempio lo scambio di molecole tra gli organismi che competono per la regolazione della densità cellulare e la configurazione spaziale dell'interazione microbo-microbo. In particolare, la produzione di molecole in grado di uccidere gli organismi competitor, limitandone la crescita o modulandone il metabolismo potrebbe avere un ruolo chiave nei meccanismi di competizione, antagonismo e di autodifesa nei confronti dei microrganismi competitor per consentire al microrganismo di occupare la nicchia e utilizzarne i nutrienti. Le molecole rilasciate includono metaboliti secondari, peptidi e molecole organiche a basso peso molecolare. Lo stesso accade nei prodotti alimentari, ove potrebbe essere possibile esplorare questa comunicazione 'molecolare' in modo da migliorare la sicurezza alimentare. In questo senso, è molto importante sviluppare strumenti nel controllo delle specie batteriche per una maggiore sicurezza alimentare.

Il presente lavoro è focalizzato sullo studio della competizione microbica tra *Listeria monocytogenes* (LM) e *Lactococcus lactis* (LAC), monitorata attraverso un approccio proteomico, peptidomico e metabolomico. Nella fase sperimentale, si è studiato il secretoma di questi due microrganismi (*Listeria monocytogenes* e *Lactococcus lactis*) nella condizione di monocoltura e in co-coltura. In particolare, i filtrati cellulari dei vari gruppi sperimentali sono stati analizzati mediante elettroforesi mono/bidimensionale accoppiata a spettrometria di massa (MALDI TOF). Al fine di caratterizzare al meglio ciascun secretoma è stata condotta una analisi di proteomica "shotgun" mediante nano UPLC-MS system. Successivamente, il secretoma di questi microrganismi è stato studiato mediante un fine analisi di proteomica "untargeted" direttamente nella matrice alimentare latte, mediante analisi label free-shotgun con le stesse condizioni sperimentali utilizzate per i terreni.

L'obiettivo di questa ultima parte sperimentale del progetto è stato il monitoraggio del meccanismo della competizione batterica attraverso la combinazione di tecniche di spettrometria di massa Imaging e analisi LC-MS/MS, al fine di studiare il profilo metabolico batterico direttamente nelle colonie microbiche interagenti.

In accordo ai dati preliminari ottenuti (elettroforesi mono/bidimensionale), l'analisi shotgun sul filtrato cellulare ha confermato, nella condizione di competizione, una maggiore produzione da parte di *Listeria monocytogenes* delle "moonlighting protein" Enolasi (C1KY94) e Glucosio 6 fosfato isomerasi (Q71X61) e della proteina regolatrice EzrA (B8DHE7), coinvolta nella replicazione cellulare e nei meccanismi di regolazione del metabolismo cellulare, e una minore secrezione della proteina Endopeptidasi P60 (P21171), proteina implicata nella virulenza di *Listeria*.

In parallelo, nella condizione di competizione con LM, *L. lactis* produce una maggiore quantità della proteina Secreted 45 kDa con attività peptidoglicano litica e una secrezione selettiva della nisina Z. Nell'analisi shotgun è stato confermato che *L. lactis* cambia lo spettro della Nisina in competizione con *Listeria* che da Nisina A passa nella sua variante Nisina Z, probabilmente per maggiore solubilità in un ambiente meno acido.

Infine, l'analisi IMS ha rivelato diversi composti interessanti durante l'interazione delle colonie microbiche. Almeno sei composti sono espressi in modo univoco durante l'interazione tra LM e LAC. Tra questi, abbiamo concentrato la nostra attenzione su tre composti principali: ciclo- (Leu-Leu), ciclo- (Phe-Tyr), ciclo- (L-Phe-L-4-Hyp). Questi composti sono peptidi ciclici con attività biologica, isolati da Lattobacilli, e svolgono un ruolo importante nella comunicazione batterica cellula-cellula. Probabilmente, questi peptidi svolgono un ruolo nell'induzione della trascrizione del gene che codifica per la Nisina.

Questi risultati potrebbero essere utile per il set-up di nuove strategie molecolari nel controllo delle specie batteriche per una maggiore sicurezza alimentare

1 INTRODUCTION

The guarantee of food safety during whole shelf-life of products has become the subject of great challenge for food industry due to current trends adopted by the modern consumer. To date, the consumer requires high-quality foods, foods able to have a long shelf-life and all that possibly avoiding the use of chemical compounds. All of these factors underlined the need for reliable techniques to monitor developments in food science and technology and to evaluate the quality and food safety. In order to put these demands into practice, it has become necessary to develop high-throughput methods such as proteomics to accomplish this.

Food safety its might be implicit in the broader concept of food security. Food security involves three process steps: availability of food, overall access to the available food, proper use of the accessed food. Food security, safety, and quality are important for the rising population due to the increasing storage time of food, from vegetables to meat and fruit[1]. In recent years, food safety is an increasingly broadening concept which encompasses mainly three main areas: (1) food quality (food composition); (2) traceability (food origin); (3) food safety per se (absence of allergens, pathogens or other contaminants)[2]. Indeed, food safety is not only a matter of determining the origin of a product, but it is also matter of evaluation of food edibility through biochemical assessment of product purity, both under a chemical and microbiological standpoint.

Proteomics has recently found several applications in the monitoring of food safety. Indeed, several aspects can be monitored through proteomics: food quality, traceability, safety in the view of the improvement of public health, so from traceability to the determination and positive selection of those quality trait that confer resistance to abiotic stress such as cold, osmotic stress. Proteomics, metabolomics methods are presented as effective tools for identification of cellular biomarkers for adaptive behavior of pathogenic microorganisms under different conditions such as cold and heat stress, osmotic, high hydrostatic pressure, and other stress factors.

Another important aspect is the application of proteomics to the assessment of the principle of substantial equivalence between food from genetically modified plants and wild type counterpart. The sensitivity and specificity of the mass spectrometry method based proteomics approaches allows for revealing traces of contaminating agents, such

as *E.coli* bacteria in soybean sprouts [2]. Proteomic integrated approaches offer considerable opportunities to assess production and monitoring of quality and safety of food, and proteome analysis of pathogens and infected food provides reliable information about pathogen activities during infection, outbreak of disease, and recovery period. Pathogen survival and growth on food produce is influenced by number of independent factors such as storage temperature, nature of the product, processing operations and methods, and packaging. The natural microbiota present on products is an additional relevant factor for pathogen survival.

Important applications of proteomics to food quality are focused on the studies of meat and dairy product quality. About these foods, proteomics has been used for the characterization of taste, flavor, and consistency that represents the pure qualitative traits of food products.

As a matter of the fact, it is mandatory for prevention and control of infectious diseases to have facilities that are able to quickly produce reliable, highly specific and sensible tools that allow on one hand and adequate sanitary surveillance and to obtain effective operative tools. Proteomics constitutes a very important approach to integrate with the prevention and control of infection diseases and in particular of the sanitary emergencies and food safety linked to animal health.

In the last few years, microbial proteomics becomes the hard-core junction made by the thematic nodes of sanitary emergencies for human health [1]. Microbial proteomics is opening up new possibilities in the study of disease pathogenesis, in animal welfare, in novel diagnostic and therapeutic markers and in the risk assessment [3]. Microbial proteomics is one of the best tools to control emerging diseases and zoonoses to improve human health and welfare.
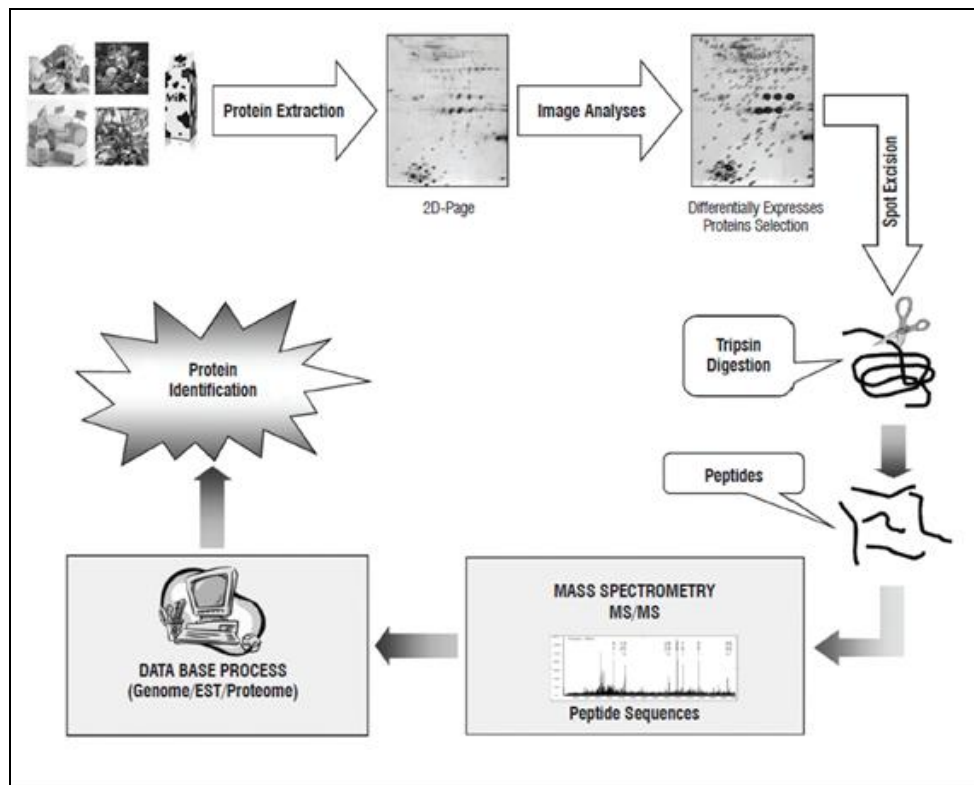
Figure 1 *An experimental workflow illustrating how proteomics can be applied to food safety[3]*

The bacterial competition is a mechanism that tends to eliminate one of the populations from their common habitat, especially when competition is focused on a single resource and when the populations do not otherwise interact[4]. The same happens in food, and it could be possible to explore this social communication to improve food safety.

The choice of *Listeria monocytogenes* is because it causes one of the most serious foodborne diseases. *Listeria monocytogenes* (LM) is a Gram-positive foodborne pathogen that can contaminate many food products, such as meat, milk, cheese, ice cream, raw vegetables, and muskmelon[5].

LM is foodborne pathogen extremely hazardous for human population that usually affects high risk patients such as the elderly, immunosuppressed patients and pregnant women. However, it can also affect people who do not have these risk factors. A peculiar property of *L. monocytogenes* that affects its foodborne transmission is the ability to replicate at low temperatures. The bacteria may therefore grow and accumulate in contaminated food stored in the refrigerator. Indeed, in the spreading of this pathology, milk and dairy products often represent a key point as reservoir for this pathogen[6].

For these reasons, it is mandatory to counteract the *Listeria* growth in food, avoiding the use of chemical compounds. One of the strategies that could be used is based on the selection of specific strains of starter bacteria (*Lactococcus lactis*) able to counteract *Listeria* growth. The use of lactic acid bacteria (LAB) with their inhibitory activities against pathogenic and spoilage microorganisms in food [7, 8] could represent a solution to this problem.

Lactic acid bacteria (LAB) are bacteria naturally present in food, used for the natural fermentation of products because they are capable to preserve the organoleptic and physical characteristic of the food products and confer them a beneficial effects like suppression of growth of pathogens[8]. For these abilities, several LAB strains are used in dairy industry as starter cultures.

It is well known as different strains of lactic acid bacteria (LAB) are able to influence the growth of these pathogens with several mechanisms of action[9].

The first is called "Jameson effect", according to which the population that first reaches the stationary growth phase locks the population competitor prevent it from increasing its concentration[10]. Lactic acid bacteria, present in high concentration, very quickly reach their stationary phase of growth by preventing the growth of pathogenic microorganism, probably in much lower concentrations in the product. High counts of starter bacteria would also implicated in rapid use of nutrient sources (eg. lactose in dairy); competition for nutrients. The influence on growth of pathogens can be production of lactic acid, which causes a decrease in pH enough to inhibit some strains, and also its non-dissociate form causes a reduction of internal pH in sensitive bacteria that produces a collapse in the electrochemical proton gradient resulting in a bacteriostatic or bactericidal effect[11, 12].

In addition to the production of organic acid, such as lactic acid and acetic acid, LAB produce diacetyl, hydrogen peroxide and some polypeptides, called Bacteriocins. Bacteriocins are ribosomally synthesized antimicrobial peptides[13] produced by bacteria which enhance their ability to control food-borne pathogens such as *Clostridium botulinum, Staphylococcus aureus and Listeria monocytogenes*[14, 15].

Nisin A is the first to be discovered [16] and other natural variants of this protein are F, Q, U and Z[17]. The production of bacteriocins by some species of *Lactococcus lactis* could play a key role in pathogens growth inhibition[18].

Another way, documented by scientific studies, is the evaluation of the effect of metabolites of LAB against several bacterial populations. An attractive hypothesis suggests that microbes regulate and optimize their production of such molecules to kill, limit the growth of or modulate the metabolism of potential niche competitors for maximal advantage. Different factors can contribute to the outcome of microbial competition, such as molecules exchanged between the competing organisms for the regulation of cell densities and the initial spatial configuration of the microbe–microbe interaction. Specifically, production of compounds that kill or limit the growth of competing strains or species can promote niche monopolization[19]

The released compounds include secondary metabolite antibiotics, bacterial peptides or low-molecular-mass organic compounds. Specialized metabolites are small molecules that are not directly involved in the normal growth, development, or reproduction of an organism. They play an important role in mechanisms that bacteria used to alter the physiology of neighboring organisms in order to monopolize the ecological niche. These metabolites represent the key components in cell-cell interaction. Therefore, the study of these compounds and molecular pathway directly involved in the production could be useful to fully understand molecular interactions that are the basis of the microbial competition between *Listeria monocytogenes* and *Lactococcus lactis*.

The use of LAB as producers of antimicrobial substances, especially bacteriocins, is a promising advance for the food industry, for improving the safety of food products, extending shelf life and ensuring the health of the consumers. The spectrum of antibacterial activity of LAB strains has the potential to cover a very broad field of application in the food industry.

Therefore, it is desirable to continue to expand our understanding of the effectiveness of the use of naturally occurring antimicrobial molecules the influences that environmental factors have on the implantation and survival of bacteriocinogenic strains and the activity of their bacteriocins in order to quantitatively estimate their efficacy for future applications in food model systems and establish adequate means of application of these bio preservatives. The use of competitive microbiota as a biotechnological tool for food preservation may lead to improve the optimization and quality assurance of food products while at the same time retaining the sensory qualities of the product such as color, flavor, texture and nutritional value.

The objective of this project is the study of the molecular mechanisms of bacterial competition to improve food safety and the quality of the end products through the use of omic tools. In detail, the aim of the work is the monitoring of bacterial competition between *Listeria monocytogenes* and *Lactococcus lactis* in order to highlight mechanisms of bacterial competition involved in this process.

1.1 General characteristics of Listeria monocytogenes

The genus *Listeria* includes six species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria grayi*, *Listeria welshimeri* and *Listeria seeligeri*. Within these six species, only *Listeria monocytogenes* and *L. ivanovii* are pathogens, but only the former is fatal for humans, while the second one regards ungulates[20]. *Listeria monocytogenes* is a Gram-positive, non-spore forming, facultative anaerobic, catalase positive and oxidase negative rod shaped bacterium. It belongs to the Firmicutes and due to its characteristic low percentage of guanine/cytosine bases in its genome, it is closely related to *Bacillus*, *Staphylococcus*, *Streptococcus* and *Clostridium* species. As a matter of fact it belongs to Bacilli class and Bacillales order. Cells are found as a single units, short chains or arrangend in V and Y forms[21] . They have peritrichous flagella, which give them a characteristic tumbling, motility, occurring only between 20 and 25°C. In Brain Heart Infusion (BHI) agar, the colonies are 0.2-0.8 mm in diameter, smooth, punctiform, gray and translucid. *L. monocytogenes* is ubiquitous and widely present in plant, soil, silage, sewage, water and faeces of human and animals. Even if its primary environment is considered to be soil, where it lives as a saprophyte feeding off dead and decaying plant matter[22], *L. monocytogenes* can adapt to live in the cytosol of eukaryotic host cells. Indeed, following its ingestion by a susceptible individual, *L. monocytogenes* is capable of making the transition to a physiological state that promotes bacterial survival and replication in the host cells[23]. As above reported, *L. monocytogenes* contaminates foods because of its ability to survive in food processing plants, where it can resist to several adverse conditions including also environments specifically planned to inhibit bacterial growth. Although its optimum temperature is 37 °C, it is able to grow between -0.4 and 50 °C. Also the pH range is wide (5.6 – 9.0) and it grows in the presence of NaCl concentrations up to 10% and at water activity (Aw) values below 0.93 [21].

1.2 Mechanism of virulence

Considering the prevalence of *L. monocytogenes* in foods and numerous epidemiological data reported in the literature, it is established that the main way of introduction of *L. monocytogenes* in humans is through ingestion of contaminated food. The infectious process evolves in pathology when the bacterium spreads through the intestinal barrier and the blood-borne lymphatic initially reaching the liver, where it multiplies in hepatocytes, and the spleen, so bloodborne reaches the secondary target organs: brain and placenta. Characteristic of *Listeria monocytogenes* is its ability to cross host barriers (intestinal, blood-brain, maternal-fetal) and exceed the bactericidal mechanisms implemented by macrophages as well as to penetrate, by its nature of facultative intracellular pathogen, not necessarily within phagocytic cells (hepatocytes, neurons, etc.).

Several mechanisms are adopted by *Listeria monocytogenes* in order to escape the unfavorable conditions of the environment and survive in the gastric human gut before spreading intra- and inter-cellular. The antacid therapy taken by some individuals and sometimes the same buffering capacity of certain foods, the vehicle of infection, temporarily reducing gastric acidity favorably affect the chances of survival of the pathogen in these districts and have predisposing factors to manifest the disease or establishing the individual condition of asymptomatic carrier [24, 25].

Generally, *L. monocytogenes* adopts other enzymatic mechanisms in order to escape unfavorable condition. The first mechanism adopted by the bacterium in very low pH conditions is the use of glutamate decarboxylase (GAD) system that converts an external glutamic acid molecule to the cell in a gamma-hydroxybutyric acid (GABA), using a proton inside. The final result is to engage a large number of protons decreasing their intracellular concentration, alkalinizing the same time the external medium considered the lower acidity of GABA compared glutamic acid [26]. The second known mechanism called BSH (Bile Salt Hydrolase, hydrolysis of bile salts), is the enzyme system through which *L. monocytogenes* is capable of hydrolyzing the amide bond of the conjugated bile salts. Hence bile acids are released with an emulsifying power lower compared to the first, and ,consequently, with lower bacteriostatic and bactericidal effect[26]

1.2.1 Pathogenesis

The bacterial population that survives in these extreme conditions can affect the host because of its ability to cross the intestinal, the blood brain and fetal-placental barriers[27]. The first mentioned passing is the most important one in listeriosis foods infections . Inside the host cell *L. monocytogenes* has a specific intracellular life cycle [23]:

1) Internalization: it is the first step through which *Listeria* adheres to the surface of a eukaryotic cell and then penetrates into the host cell. During the invasion, a zipper-type mechanism is involved, in which the bacterium gradually sinks into dip-like structures of the host cell surface until it is finally engulfed. Hence, the membrane of the target cell closely surrounds the bacterial cell. The structures, mechanisms, and signal transduction cascades involved in the interaction between *Listeria* and the host cell during phagocytosis are not yet totally elucidated. In literature, some surface proteins such as the internalin A (InlA) and internalin B (InlB), Ami protein, the actin-polymerizing protein ActA, and p60 are recognized as bacterial ligands responsible for adhesion and phagocytosis. About 25 internalins are identified in *Listeria*, and the InlA InlB are the best characterized ones. InlA plays a fundamental role in the invasion of *L. monocytogenes* and in particular allows to enter the human intestinal epithelial cell line Caco-2 by binding the host cell adhesion transmembrane glycoprotein named Ecadherin[28]. The binding between *Listeria* and the E-cadehin activates a complex sequence of events which lead to the depolymerisation of the actin and subsequent envelopment of the bacterium with the membrane of the host cell [29]. Hence *L. monocytogenes* enters the host cell within the phagosomal compartment. InlB allows the bacterium to invade hepatocytes cells by binding to Receptor Tyrosine Kinase (RTK) Met [30]. The RTK Met receptor consists of a single hydrophobic transmembrane-spanning domain, an extracellular N-terminal region, and an intracellular C-terminal region. The link between InlB and the extracellular part of the RTK Met causes the rapid tyrosine phosphorylation via the classical phosphatidylinositol 3 kinase pathway (PI3K) and triggers signaling pathways leading to actin cytoskeleton integration required for internalization of *L. monocytogenes*. Other proteins including Gab1 and CrkII can promote actin polymerisation.

2) Escape from primary phagosome: during the invasion, *L. monocytogenes* is internalized in a primary phagosome, but in order to survive and proliferate it needs to internalize in a primary phagosome, but in order to survive and proliferate it needs to escape from this confinement. Little is known about the characteristics of the *Listeria*-containing vacuolar compartment, but the vacuoles become acidified soon after uptake. About 30 min after its entry, *L. monocytogenes* starts to destroy the phagosome membrane and exits in the cytoplasm. This membrane disruption is mediated by the hemolysin in combination with phospholipases. Hemolysin, or Listeriosin O (LLO), is a 58 kDa protein belonging to a family of cholesterol dependent cytolysins which is encoded by the hly gene and regulated by PrfA, a central temperature sensitive regulator of virulence genes [31]. LLO is activated by thiol reducing agents and is inactivated by the binding of cholesterol [32], and its function is to form pores into the membrane. It plays an important role also in the internalization and host cell interaction. LLO can interfere with host cellular mitochondria in order to preserve *L. monocytogenes* replication by inhibiting the death of host cells or killing agents which are inhibitory to bacterial dissemination[33]. The phospholipases involved in the membrane disruption are: PI-PLC encoded by plcA gene and PC-PLC encoded by plcB [22]. The first one is highly specific for phosphoinositol and glycosyl-PI-anchored proteins, while the second one hydrolyses a great deal of phospholipids[34]. These proteins work synergistically with LLO causing the dissolution of the plasma membrane [35].

3) Intracellular growth: After escaping from the primary phagosome, *L. monocytogenes* actively multiplies in the host cytoplasm with a doubling time of approximately 1 h. Since the environment is permissive, *L. monocytogenes* does not use any stress response mechanism and three metabolic genes (purH, purD, and pyrE, involved in purine and pyrimidine biosynthesis) and an arginine ABC transporter (arpJ) are induced within host cells. The mutation of these genes can be involved in metabolic pathway in order to improve the growth within cells. Indeed, a study indicates that pathogenic *Listeria* spp. may exploit hexose phosphates from the host cell cytoplasm for an efficient intracellular growth [36].

4) Movement and spreading to adjacent cells: intracytoplasmic *L. monocytogenes* is surrounded by a dense cloud, formed by host cell actin filaments, which polymerises to

form an actin tail on one bacterium pole. This tail is composed by two cross-linked actin filaments and let bacterium to move quickly (0.3 mm/s) inside the host cell to infect the new cytoplasm. When bacterium comes into contact with the membrane, push it as a rocket and a sort of finger-like protrusion with a bacterium at the tip is generated. Later this protrusion penetrates in the neighboring cell and is "swallowed". the dissolution of the plasma membrane[35].

5) Escape from secondary phagosome: Inside the new cell, *L. monocytogenes* is in turn engulfed by a second phagosome delimited by a double membrane with the inner membrane originating from the donor cell. *L. monocytogenes* rapidly escapes from the new formed vacuole by dissolving the double membrane, thus reaching the cytoplasm and initiating a new round of intracellular proliferation and direct intercellular spread. The actin-based intracytoplasmic movement and cell-to-cell spread are mediated by the surface protein ActA. ActA id encoded by the ActA gene and is a 639 amino acid, dimerised protein which is formed by three distinct parts [37]. The N terminus is associated with actin assembly and bacterial motility; the central part is responsible for the connection between protein and the bacterial cell wall, while the VCA region interacts with the Arp2/3 complex. Arp2/3 is another protein complex which facilitates the polymerisation of actin [38]. The polymerization involves other proteins such as VAPS and CapZ. These proteins mediate also the evasion of *L. monocytogenes* by the host cell.

A correct evolution of these steps is fundamental for a full *L. monocytogenes* virulence and defects at any point can lead to high attenuation. In Figure 1.2-1 the intracellular cell cycle is reported. Almost all genes reported before, and involved in the invasion, primary phagosomal escape and direct cell to cell transmission, are regulated by the PrfA protein. In particular prfA, plcA, plcB, hly, mpl, actAB and hpt are under the control of this protein. PrfA is a 233 amino acid long, which up-regulates these gene when Listeria is in a host cell and down-regulates them when it lives in the environment. The expression of the PrfA protein is temperature dependent: It is silent at 30°C and maximally expressed at 37°C[39]. In this way PrfA controls the virulence genes at the homeostatic temperature of the host cell.
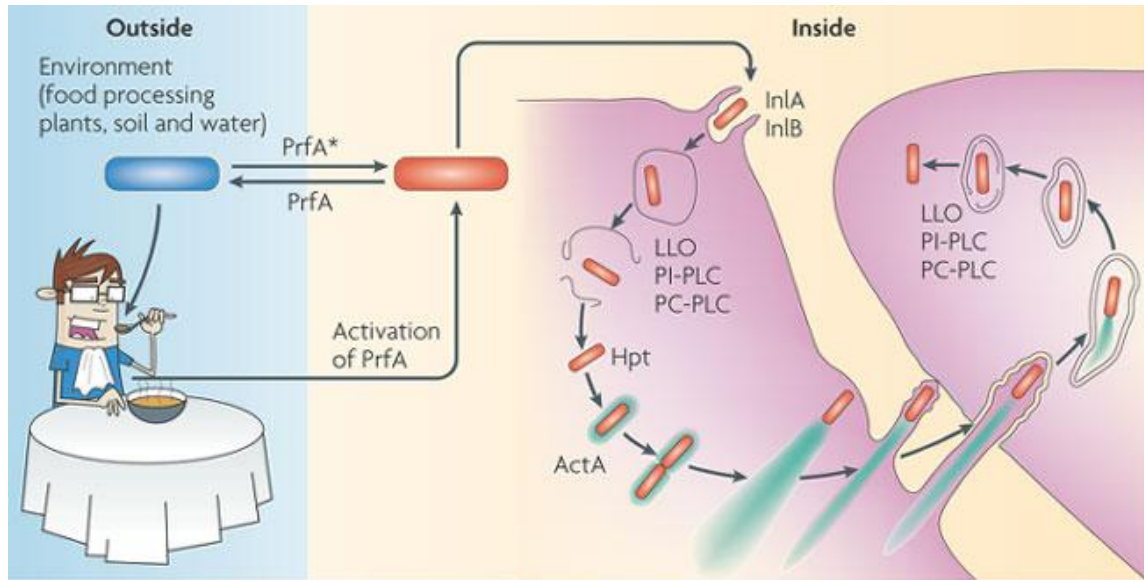
Figure 1.2-1. Intracellular cell cycle of L. monocytogenes [22] (Nancy E. FREITAG et all, 2009).

1.3 Listeria monocytogenes metabolism

L. monocytogenes can live and grow under both aerobic and anaerobic conditions. Most of its metabolic pathways are similar to those of *Bacillus subtilis*, which belongs to the group of low G+C Gram-positive bacteria similarly to *L. monocytogenes*. Nevertheless, there are various significant differences which may be essential for understanding the interference of listerial metabolism with that of the host cells[40]. In aerobic conditions, the respiration takes place and the respiration chains contains (as quinones only) menaquinone, but not coenzyme Q10, also called ubiquinone[40]. Menaquinone derives from a branch of the aromatic amino acids pathway and it functions as a cofactor in the electron transport chain. In aerobic conditions Listeria spp. uses hexoses and pentoses to grow, including maltose, glucose, rhamnose and lactose, but not sucrose[41]. The main metabolic end products in aerobic conditions are lactate (28%), acetate (23%) and acetoin (26%)[42]. Under anaerobic conditions, only hexoses and pentoses support growth. In particular lactate is the major fermentation product (about 79%) thus indicating that the mixed acid fermentation is the major mode of fermentation in *L. monocytogenes*[41]. Other anaerobic end products have been found which include formate (5.4%), ethanol (7.8%), carbon dioxide (2.3%) and acetate (2%)[43]. These results demonstrate that acetoin and lactate are good indicators of aerobic or anaerobic growth. Concerning carbohydrates, glucose and other sugars are preferentially taken up by the bacterium via the phosphotransferase system (PTS). Glucose and other PTS-sugars like fructose, mannose and cellobiose are the preferred carbon sources for *L. monocytogenes* when it grows in minimal liquid media. The study of its genome has revealed an unusually large number of genes (>40) encoding PTS. Unlike the other low G+C Gram-positive bacteria, which have ptsG gene encoding PTS-dependent glucose transporter, the genome of *L. monocytogenes* is incomplete. Despite this deletion, the growth of *L. monocytogenes* is unaffected in minimal media with glucose as the carbon source suggesting that this gene is not involved in the glucose uptake[40]. Mertins et al. (2007) investigated the possibility of a not PTS-dependent glucose uptake, but the ptsH mutant, which did not use the PTS-dependent systems, could not grow in minimal medium using glucose as a carbon source. This finding suggests that the PTS transport is the mainly one responsible for glucose transport. *L. monocytogenes* catabolises glucose

via the glycolytic and the pentose phosphate pathways, but not via the EntnereDoudoroff pathway [40].

The principal glycolysis genes, i.e. gap, pgk, tpi, pgm and eno, used by *L. monocytogenes* are the same as those found in most low G+C Gram-positive bacteria. These genes are down-regulated in minimal medium in favor of an up-regulation of the enzymes involved in the pentose phosphate pathway. This up-regulation indicates the need for an oxidative decarboxylation of glucose by glucose-6-phosphate and the production of CO_2 for the biosynthesis of aromatic amino acids, which are not present in the minimal medium. Joseph et al. (2006) [44] observed a similar down-regulation of glycolysis genes and up-regulation of pentose phosphate pathway when *L. monocytogenes* grows in host cells, perhaps due to a limited availability of PTS sugars. The capability of *L. monocytogenes* to use phosphorylated hexoses (PHs), such as glucose-1-6-phosphate, fructose-6-phosphate, as carbon sources have been observed[45]. The bacterium takes PHs by the host cytosol and transports them into the cell through the hpt transporter. This transporter is under the control of the PrfA virulence regulator, and is highly up-regulated during the internalization of bacterium onto the host cell [46]. *L. monocytogenes* can use also glycerol as a carbon source (Figure 1.3-1). Glycerol is taken up via facilitated transport, phosphorylated by glycerol kinases (encoded by lmo 1034) and then oxidized by glycerol-3-phosphate dehydrogenase (encoded by lmo 1538) to glyceraldehyde-3-phosphate which is finally metabolized by the glycolytic pathway enzymes[40]. The same Authors, instead, excluded amino acids and Acetyl-CoA as carbon fonts. The latter is not used by *L. monocytogenes* due to the lack of the glyoxlyate shunt genes and this also rule out the utilization of fatty acids as a carbon font.
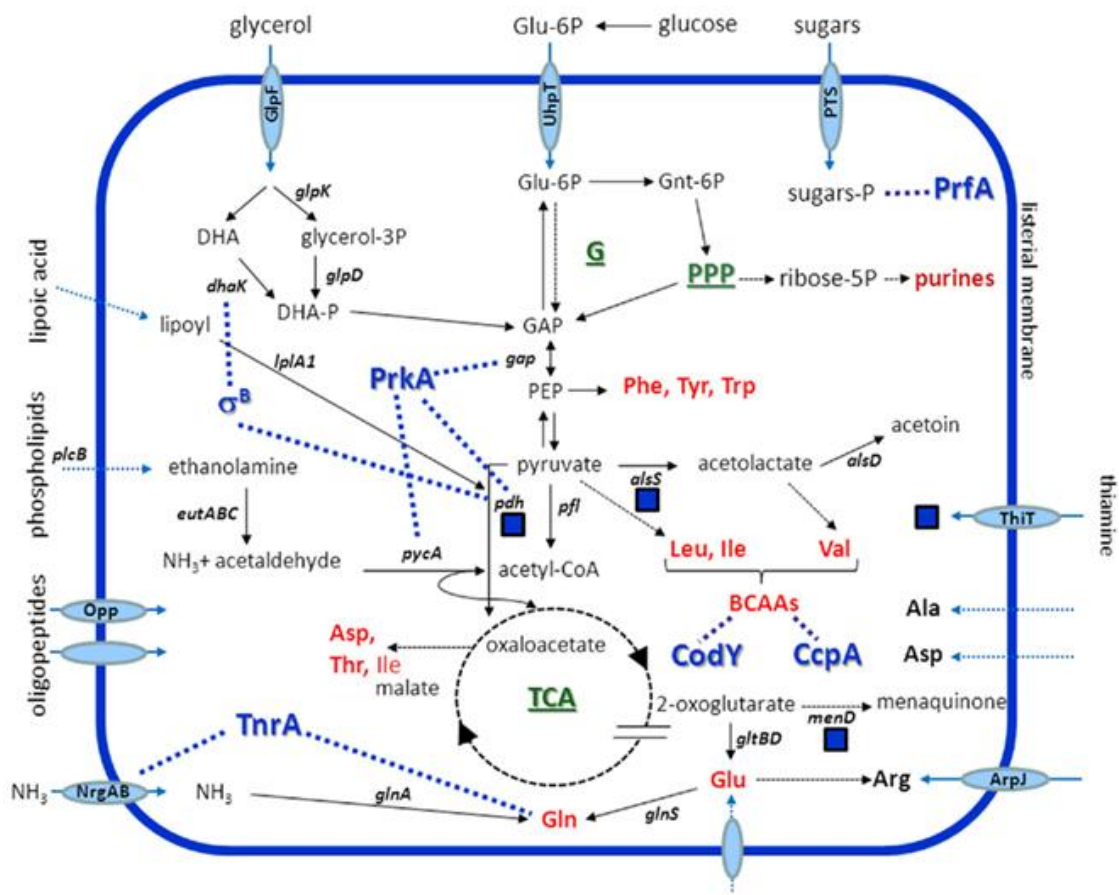
Figure 1.3-1. Simplified view of Listeria metabolic enzymes, transporters, and pathways[47]

(Fuchs et al., 2012).

L. monocytogenes has a regulatory mechanism, called carbon catabolite repression (CCR) which rules prfA virulence regulator and in general the expression of genes associated with secondary carbon sources when the primary carbon sources are available[48]. This regulatory effect allows *L.monocytogenes* to grow optimally in the presence of various carbon sources using those preferential when they are available. Joseph and Goebel (2007) [40] reported that the *L. monocytogenes* metabolism is relieved of CCR control when the bacteria replicate in the host cell cytosol. At the same time the Authors observed an up-regulation of genes encoding an uptake mechanism for phosphorylated hexoses (hpt), oligopeptides and amino acids (lmo 2251) and glycerol (lmo1034, lmo1538). On the basis of these observations it was hypothesized that glucose is not a predominant carbon source inside host cells. Moreover, when glucose or phosphorylated glucose are not available in the environment, an up-

regulation of the genes involved in the pentose phosphate cycle and a down-regulation of those involved in glycolysis was observed. These results suggest that the pentose phosphate cycle is the favorite pathway in the absence of glucose. Concerning nitrogen metabolism, glutamine is the preferential nitrogen source for *L. monocytogenes*. In the absence of this amino acid, especially when the bacterium is inside the host cell cytosol, it is capable to use alternative fonts, such as such ammonium, which is the favorite substitute, arginine and ethanolamine[40, 48]. Inside the *L. monocytogenes* cell, glutamine is converted to glutamic acid by glutamate synthetase (GOGAT) with 2-oxoglutarate (OG) as additional substrate. On the other hand when ammonium is used as an alternative nitrogen source, it is transported in *L. monocytogenes* by the transporter NrgA which is encoded by the ngrAB operon. The transcription of the nrgAB promoter is activated during nitrogen-limited growth by the global regulator TnrA. Ammonium is then incorporated into glutamine, and further to glutamic acid, as above described. This pathway is also observed for Bacillus subtilis. As previously reported, also arginine is a potential nitrogen sources. It is transported inside the cell by a specific arginine ABC transporter (encoded by arpj) and then degraded into citrulline and ammonia by arginine deaminase (encoded by lmo0043-arcA). Citrulline in turn is degraded into a further ammonia molecule and ornithine via the enzymes ornithine carbamoyl transferase (OCT) and carbamoyl carboxy kinase (CCK) encoded by the *L. monocytogenes*-specific arcBCD operon (lmo 0036 and lmo 0039, respectively). Also adenine (to a limited extent) and ethanolamine are two other possible nitrogen sources. The latter is generated through the degradation of phosphatidylethanolamine (PEA), which is an excellent substrate for PlcB, a *listerial* phospholipase C encoded by the PrfA-dependent gene plcB. Ethanolamine is hydrolyzed into ammonia and acetaldehyde by the vitamin B12-dependent ethanolamineammonia lyase encoded by the eutBC genes[40]. Concerning amino acids biosynthesis, Tsai and Hodgson (2003)[49] observed the absence of the genes required for cysteine and methionine biosynthesis. Therefore these amino acids are essential for *L. monocytogenes* which have to absorb them from the environment. Moreover, *L. monocytogenes* lacks also sulphate and nitrate reductases, thus there is a dependency for reduced nitrogen and sulphate sources, which can be gained from cysteine and methionine. However, *L. monocytogenes* is capable of de novo synthesising branched chain amino acids (BCAA), i.e. valine,

isoleucine and leucine, *via* the conventional pathways. Some studies have shown that *L. monocytogenes* has some requirement for them. In particular, the essential precursors of BCAA are pyruvate and threonine (deriving from aspartic acid via oxaloacetate), and their availability is directly or indirectly connected with the citrate cycle that is interrupted in L. monocytogenes due to the lack of 2-oxoglutarate dehydrogenase, which converts alpha-ketoglutarate into succinylCoA. As a result of this incomplete cycle , *L. monocytogenes* is incapable of regenerating oxaloacetate through the Krebs cycle from citrate. Therefore, oxaloacetate is produced by the carboxilation of pyruvate by pyruvate carboxylase, which is encoded by pycA. This step is fundamental for the entrance of Acetyl-CoA into the Krebs cycle and for the synthesis of asparagine, threonine, cysteine andmethionine. Because of the interruption of Krebs cycle, oxaloacetate is also the precursor of malate and succinate[40]. Buzolyova and Somov (1999) observed that pyruvate carboxylase needs CO_2 to produce oxaloacetate[50]. When glucose is the unique carbon source, the oxidative decarboxylation of glucose-6-phosphate, which is the first reaction in the pentose-phosphate pathway, seems to be necessary as suggested by the high induction of the gene for pyruvate carboxylase in *L. monocytogenes*. Some studies have reported that the major source of nitrogen inside the host cell, excluding alanine, asparagine and glutamate which are synthesized de novo, is provided by the host cell, as suggested by the up-regulation of the oligopeptide transporters[50]. The Authors have also observed a down-regulation of the aminoacyl tRNA synthase genes glyS, serS, cysS, alaS, hisS, valS, thrS, ileS, leuS, tyrS, and trpS, as suggested availability of the respective amino acids within the cytosol. *L. monocytogenes* cannot synthesize several vitamin and cofactor such as biotin, lipoic acid, riboflavin and thiamine which are fundamental for its growth. For instance, lipoic acid is an important co-factor of the pyruvate dehydrogenase enzyme (Pdh) complex, which is involved in acetyl CoA formation from pyruvate in the aerobic metabolism[51]. *L. monocytogenes* uses two lipoate ligases in order to absorb lipoic acid from the environment.

1.4 Listeriosis

The disease caused by bacteria belonging to the genus *Listeria* is called listeriosis.

L. monocytogenes is the most pathogenic species for both man and animals. It is a prevalent foodborne disease that affects mostly people over 65 years of age, infants, pregnant women and immunocompromised such as patients with malignancies or under cytotoxic therapy, AIDS patients, diabetics, people with heart valve or kidney or liver disease. In particular pregnant women and those suffering from AIDS have a chance respectively of about three hundred and twenty times higher than of contracting listeriosis compared to a healthy individual (Center for Disease Control and Prevention, 2009). Listeriosis in pregnant women, which may occur at any time during pregnancy, it is generally asymptomatic or begins with a vague symptoms similar to a flu-like syndrome accompanied by chills, headache, muscle and joint pain in the period from 2 to 14 days before spontaneous abortion[23]. The infection is transmitted to the fetus through the placenta causing, depending on the time of infection, miscarriage, premature birth or neonatal sepsis due to a generalized systemic infection known as granulomatosis infantiseptica or neonatal listeriosis. The infection that infants contract during childbirth is transmitted by hiring through the airways or the digestive system via contaminated amniotic fluid or vaginal secretions and can start early with sepsis and respiratory failure and circulatory or (less frequently: 10-15 % of perinatal listeriosis) occur episodes of late listeriosis from one to eight weeks postpartum sepsis and meningitis[23]. Even less frequently (5% of cases) maternal infection is not transmitted to the fetus even in the presence of bacteremia[24]. In young people or adults in predisposing conditions listeriosis it occurs mainly in two main forms:

- Non-invasive form whose pathological manifestations occur within hours of ingestion (12-24 hours) are not unlike those of other food-borne diseases with phenomena such gastrointestinal (diarrhea, vomiting and fever).

- Invasive form as a result of localized or disseminated infection of the central nervous system is manifested by meningitis or meningoencephalitis with headaches, confusion, stiff neck, loss of balance or convulsions, and paralysis of the cranial nerves preceded from an early stage, three variable to ten days, in which patients have fever, headache, vomiting, visual difficulties and general malaise[23]. The average mortality rate for cases

of listeriosis reported amounted on average to a percentage between 20 and 30% that goes up to 38-45% if referred to cases of listeriosis affecting immunocompromised patients or elderly. Despite these figures and the fact that *L. monocytogenes* is ubiquitously widespread and has been isolated in all categories of foods, Listeriosis is a rare disease[25] [52].

As reported in the EFSA report (The Community Summary Report, 2014) regarding the European Community, as it relates to a 30% increase compared with 2013. The EU notification rate was 0.52 cases per 100,000 population. In 2014, 27 MS reported 2.161 confirmed human cases of listeriosis (table 1.4-1 and 2).

Country	2014 National coverage[a]	2014 Data format[a]	2014 Total cases	2014 Confirmed cases & rates Cases	2014 Confirmed cases & rates Rate	2013 Confirmed cases & rates Cases	2013 Confirmed cases & rates Rate	2012 Confirmed cases & rates Cases	2012 Confirmed cases & rates Rate	2011 Confirmed cases & rates Cases	2011 Confirmed cases & rates Rate	2010 Confirmed cases & rates Cases	2010 Confirmed cases & rates Rate
Italy[b]	–	–	52	52	–	128	0.21	112	0.19	129	0.22	157	0.27
Latvia	Y	C	3	3	0.15	5	0.25	6	0.29	7	0.34	7	0.33
Lithuania	Y	C	7	7	0.24	6	0.20	8	0.27	6	0.20	5	0.16
Luxembourg	Y	C	5	5	0.91	2	0.37	2	0.38	2	0.39	0	0.00
Malta	Y	C	1	1	0.24	1	0.24	1	0.24	2	0.48	1	0.24
Netherlands	Y	C	90	90	0.54	72	0.43	73	0.44	87	0.52	72	0.43
Poland	Y	C	86	86	0.23	58	0.15	54	0.14	62	0.16	59	0.16
Portugal[c]	–	–	–	–	–	–	–	–	–	–	–	–	–
Romania	Y	C	5	5	0.03	9	0.05	11	0.06	1	0.01	6	0.03
Slovakia	Y	C	29	29	0.54	16	0.30	11	0.20	31	0.58	5	0.09
Slovenia	Y	C	18	18	0.87	16	0.78	7	0.34	5	0.24	11	0.54
Spain[d]	N	C	161	161	1.15	140	1.00	109	0.93	91	0.78	129	1.11
Sweden	Y	C	125	125	1.30	93	0.97	72	0.76	56	0.60	63	0.67
United Kingdom	Y	C	201	201	0.31	192	0.30	183	0.29	164	0.26	176	0.28
EU Total	–	–	2,174	2,161	0.52	1,868	0.40	1,722	0.38	1,516	0.34	1,663	0.37
Iceland	Y	C	4	4	1.23	1	0.31	4	1.25	2	0.63	1	0.32
Norway	Y	C	29	29	0.57	21	0.42	30	0.60	21	0.43	22	0.45
Switzerland[e]	Y	C	98	98	1.20	64	0.80	39	0.49	47	0.60	67	0.86

(a): Y: yes; N: no; A: aggregated data; C: case-based data; –: no report.
(b): Provisional data for 2014.
(c): No surveillance system.
(d): Sentinel system; notification rates calculated with an estimated population coverage of 30% in 2013–2014 and 25% in 2009–2012.
(e): Switzerland provided data directly to EFSA. The human data for Switzerland also include the ones from Liechtenstein.

Country	National coverage[a]	Data format[a]	2014 Total cases	2014 Confirmed cases & rates Cases	2014 Confirmed cases & rates Rate	2013 Confirmed cases & rates Cases	2013 Confirmed cases & rates Rate	2012 Confirmed cases & rates Cases	2012 Confirmed cases & rates Rate	2011 Confirmed cases & rates Cases	2011 Confirmed cases & rates Rate	2010 Confirmed cases & rates Cases	2010 Confirmed cases & rates Rate
Austria	Y	C	49	49	0.58	36	0.43	36	0.43	26	0.31	34	0.41
Belgium	Y	C	84	84	0.75	66	0.59	83	0.75	70	–	40	0.37
Bulgaria	Y	A	10	10	0.14	3	0.04	10	0.14	4	0.05	4	0.05
Croatia	Y	A	5	4	0.09	0	0.00	0	0.00	–	–	–	–
Cyprus	Y	C	0	0	0.00	1	0.12	1	0.12	2	0.24	1	0.12
Czech Republic	Y	C	38	38	0.36	36	0.34	32	0.31	35	0.33	26	0.25
Denmark	Y	C	92	92	1.64	51	0.91	50	0.90	49	0.88	62	1.12
Estonia	Y	C	1	1	0.08	2	0.15	3	0.23	3	0.23	5	0.38
Finland	Y	C	65	65	1.19	61	1.12	61	1.13	43	0.80	71	1.33
France	Y	C	374	374	0.57	369	0.56	348	0.53	282	0.43	312	0.48
Germany	Y	C	609	597	0.74	463	0.57	414	0.51	331	0.41	377	0.46
Greece	Y	C	10	10	0.09	10	0.09	11	0.10	10	0.09	10	0.09
Hungary	Y	C	39	39	0.40	24	0.24	13	0.13	11	0.11	20	0.20
Ireland	Y	C	15	15	0.33	8	0.17	11	0.24	7	0.15	10	0.22

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[26] Available online: http://www.camcon-eu.net
[27] https://www.food.gov.uk/news-updates/news/2015/14701/campylobacter-survey

Table 1.4- 1 and 2. Reported human cases of listeriosis and notification rates per 100000 in the EU/EEA, by country and year, 2010-2014.

There was a statistically significant increasing trend of listeriosis over 2008-2014. The majority of the countries reported increasing notification rates of listeriosis in 2014 and six MS had statistically increasing trend. Seventeen MS reported 210 deaths due to listeriosis in 2014, which was the highest annual number of deaths reported since 2009. The EU case fatality was 15.0% among the 1,401 confirmed cases with known outcome (Table 1.4-3). Listeriosis infections were most commonly reported in the elderly population with the case fatality peaking at 17.8% in the age group over 65 years old.

Disease	Number of confirmed[a] human cases	Hospitalisation Status available (%)	Hospitalisation Number of reporting MS[b]	Hospitalisation Reported hospitalised cases	Hospitalisation Proportion hospitalised (%)	Deaths Outcome available (%)	Deaths Number of reporting MS[b]	Deaths Reported deaths	Deaths Case-fatality (%)
Campylobacteriosis	236,851	25.4	16	18,303	30.4	73.6	15	25	0.01
Salmonellosis	88,715	32.2	14	9,830	34.4	49.6	15	65	0.15
Yersiniosis	6,625	15.2	12	442	44.0	58.3	14	5	0.13
VTEC infections	5,955	39.9	15	930	39.2	58.6	18	7	0.20
Listeriosis	2,161	38.0	16	812	98.9	64.8	20	210	15.0
Echinococcosis	801	24.0	14	122	63.5	24.6	12	1	0.51
Q- fever	777	NA[c]	NA	NA	NA	51.2	11	1	0.26
Brucellosis	347	62.0	9	142	66.1	41.5	10	0	0.00
Tularaemia	480	47.1	8	92	40.7	49.0	9	0	0.00
Trichinellosis	319	74.6	5	150	63.0	74.9	6	2	0.84
West Nile fever[a]	77	66.2	6	48	94.1	66.2	6	7	13.7
Rabies	3	NA	NA	NA	NA	66.6	3	2	100.0

(a): Exception made for West Nile fever where the total number of cases was included.
(b): Not all countries observed cases for all diseases
(c): NA-not applicable as the information is not collected for this disease.

Table 1.4-3. Reported hospitalization and case-fatality rates due to zoonoses in confirmed human cases in the EU, 2014

In 2014, the non-compliance for different RTE food categories was generally at a level comparable to previous years, with the level of non-compliance highest in fishery products at processing plant (mainly smoked fish). As in previous years and consistent with the results of the EU baseline survey on the prevalence of *L. monocytogenes* in certain RTE foods at retail, the proportion of positive samples at retail was highest in fish products (mainly smoked fish).

In 2014, several MS reported information on *Listeria* in various animal species. Findings of *Listeria* were most often reported in cattle, sheep, goats, pigs and solipeds but *Listeria* was also detected in broilers, cats, dogs, hunted wild boar, foxes, and other wild and zoo animals. *Listeria* is widespread in the environment; therefore, isolation from animals is to be expected and increased exposure may lead to clinical disease in animals. Listeriosis in animals manifests in different forms depending on the species. In sheep the most frequent form is encephalitis, but also abortion and iritis and often death occurs within one day of the onset of symptoms. In cattle the neurological form (with the appearance of brain microabscesses) presents an evolution less acute and animals survive up to two weeks from start of symptoms. Encephalitis can affect animals of any age, but prevails in those under three years of age, although it does not appear before weaning. In young animals the infection occurs mostly in septicemic form (often fatal) with the appearance of necrotic foci in the liver and other abdominal organs. Abortions instead are mostly in late gestation. There are other rarer forms of listeriosis such as pneumonia, endocarditis, and myocarditis. In cattle we have also been described cases of localizations breast with possible etiologic agent elimination through milk even after healing has occurred, thus representing a threat to public health. In equines prevails meninges-encephalic form, while in pigs and poultry has septicemia is followed by symptoms of nervous nature. In humans, the possibility of contracting listeriosis after ingestion of contaminated food is closely related to the simultaneous occurrence of some predisposing factors related mainly to:

- Infective dose

- Features of the pathogenic infecting strain

- Immunological status of the subject

The infectious dose able to determine episodes of listeriosis in an individual can not be quantified with accuracy but only estimated. The values shown are generally those derived from data for the insolation and the *L. monocytogenes* amounts determined in foods responsible for sporadic episodes or outbreaks of listeriosis. Generally it is believed that the infective dose capable of causing disease in humans is between 100-1,000 CFU/g or ml and 1×10^8 CFU / g or ml. Food products have just microbial loads of *L. monocytogenes* are usually below the indicated doses (between 0.04 and 10 CFU/g): are the food intrinsic characteristics (pH, Aw) associated with any heat treatment and the conditions of conservation, as well as the elapsed time between the production and consumption, which affect positively or negatively on the final microbial food itself. The limit of 100 CFU/g as a safety criterion for ready-to-use indicated by the authors of Reg. 2073/05 takes account of these aspects. In relation to the pathogenic characteristics of the infecting strain from studies in vivo and in vitro it has shown that not all strains of *L. monocytogenes* expressing the same degree of virulence [53, 54]. For example at the same conditions it was shown that the "relative virulence", obtained by dividing the number of dead mice for the number of inoculated mice and expressed in percentage, varies between 0 and 100% among different strains all belonging to the species *L. monocytogenes* [55]. Studies directed to understanding these data indicate alterations in the gene sequences of some fundamental pathogenicity factors a possible explanation for this variability, as well as the presence of new factors of pathogenicity whose mechanisms of action are still only partially known[56] [57], including for example the internalin J [58]. Assumed to occur predisposing conditions in terms of infectious dose and pathogenic strain is crucial because it manifests the disease the individual's health status. Belonging to one of the risk groups affects significantly the onset of the disease, but also individuals who do not belong to that defined as immunocompetent are exposed to the same risk to a lesser extent.

In immunocompetent or conditions predisposing ingesting low doses of *L. monocytogenes* may not have any noticeable effect unless the development or enhancement of a protective immune response against the microorganism. Conversely, in the same subjects an oral exposure to high bacterial loads involves the occurrence of the disease in a non-invasive or invasive depending on the virulence of the strain involved. In debilitated or immunocompromised individuals who are therefore not able

to develop an immune response sufficient to limit bacterial multiplication in the liver, the first target organ of *L. monocytogenes*, also the ingestion of low infectious doses can determine the invasive form of the disease. Bacteremia resulting in the massive bacterial growth in hepatocytes and release bacterial cells into the bloodstream often favors the infection of secondary target organs, such as brain or placenta, or causes severe septicemia[23].

1.5 Listeria monocytogenes in Dairy Products: a matter of food safety

Pasteurized milk and the consumption of soft cheeses have been responsible for outbreaks of listeriosis events. *L. monocytogenes* can be isolated from raw milk tanks and dairy products, although with low prevalence[59]. Water samples, used for cheeses washing, have also positive results. After collection, raw milk can easily be contaminated by environmental sources (soil, faeces, silage) or be excreted from the udder of already infected animals. Indeed, it has been observed that cows with mastitis can transfer the pathogen in milk with levels of 10^4-10^5 CFU/ml. Psychotropic characteristics of *L. monocytogenes* and its ability to grow at refrigeration temperatures, have stimulated interest in the study of its behavior in the milk stored at low temperatures. It has been shown that in this matrix the inoculum in raw milk of 10^3 CFU/ml, after 72 hours of storage at 5 °C, undergoes increments of 1 log unit, indicating that the milk cooling provides limited protection against *L. monocytogenes* only ensuring a slowdown in development time[60]. The pasteurized milk has a low contamination level, but still is a substrate adapted to the development during storage. The presence of *L. monocytogenes* in cheeses, in addition to an insufficient thermal consolidation of the starting milk, can be attributed to contamination of surfaces, equipment, water waste and environments for the transformation. *L. monocytogenes* is also characterized by a particular acid-tolerance, influenced by the temperature of incubation and the concentration of salts, but the minimum pH has not yet been well defined. The values of pH of the cheeses are such as to not allow the growth of the pathogen, but only the survival for different times[59]. *L. monocytogenes* survives to the cheese processing and maturation with a microbial charge almost constant. It increases slightly during the manufacture of cheese (cheddar, cottage, italic), focusing in the curd, only to suffer decreases during the maturing process, resulting however detectable at the end of the maturing The pathogen was isolated from soft and semi-soft cheeses, pressed, fresh, semi-hard, from blue cheese, goat, sheep, with contamination levels ranging from 1 to 100 CFU/g product, even if it is possible reach of 10^7-10^8 CFU/g values. Among the Italian cheeses, they are particularly involved and streaked with soft cheese, such as Gorgonzola and Taleggio.

In the soft surface ripening cheeses, such as Camembert, or those blue cheeses like gorgonzola and blue cheese, ripening is characterized by the development of non-acidifying microflora (yeasts, molds, and Micrococcaceae Brevibacteriaceae) that can make the most favorable substrate for the growth of pathogen. In Camembert cheese, *L. monocytogenes* has increased by about 1 log in the first 24 hours of production, remaining more or less constant after 25 days of ripening at 6°C. Subsequently, a rapid increase in the growth occurs, in parallel with the increase of cheese pH. After 56 days of curing, the number of cells in the superficial part of the cheese reaches 1×10^7 CFU/g values. Several studies have focused on *L. monocytogenes* behavior during the production of traditional Mozzarella that involves the use of raw milk inoculated with levels of 10^5 CFU/ml and serum-graft use as starter culture. In the first 100 minutes, L. monocytogenes is multiplied, then a slight reduction has taken place, indications of the presence at the end of the maturation of the curd to the same starting values. After spinning, they have showed 2-3 log reduction in the pathogen, which was still present at levels of 100 to 1000 CFU/g of finished mozzarella. After 24 hours of conservation in the spinning liquid, the pathogen was detectable only after enrichment. The complete disappearance of the pathogen occured only after 48 hours of storage. The pathogen was detected only after enrichment.

1.6 Probiotic LACTIC ACID Bacteria and antimicrobial compounds

Lactic acid bacteria (LAB) are classified as Gram-positive, non-spore forming, non-motile, and rod-and coccus-shaped organisms that can ferment carbohydrates mainly producing lactic acid. Some strains of LAB show attractive therapeutic properties and technological applications, such as proteolytic activity; production of polysaccharides; lactose and citrate fermentation; capacity for adhesion and colonization in digestive mucosa; high resistance to freezing and freeze-drying; production of vitamins; and production of antimicrobial compounds [61-63]. The probiotic LAB could be present in the spontaneous fermentation of different food, and this group is generally recognized as safe[64]. They have been also used as starter cultures, and they have become widespread in the manufacture of fermented vegetables and dairy and meat products [65, 66]. The fermentation depends on the oxidation of carbohydrates and related subproducts to generate end-products. Homofermentative LAB are able to convert available energy source (hexoses) almost completely into lactic acid (over 85%) via pyruvate to produce energy and to equilibrate the redox balance (Figure 1.6-1), whereas heterofermentative LAB degrade hexoses and can lead to the generation of many other metabolites (different organic acids, acetate, acetoin, ethanol, carbon dioxide and aromatic compounds, such as diacetyl and acetaldehyde) (Figure 1.6-2)[67]. In this way, LAB produce volatile substances that contribute to the typical flavor of certain fermented products, such as sourdough (determined by the lactate/acetate ratio), kefir and koumiss (ethanol), butter and buttermilk (diacetyl) and yogurt (acetaldehyde)[68]. The preservative effect of these bacteria is due to the production of one or more active metabolites, such as organic acids (lactic, acetic, formic, propionic and butyric acids), that intensify their action by reducing the pH of the media, and other substances, such as ethanol, fatty acids, acetoin, hydrogen peroxide, diacetyl, antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides and 3-hydroxy fatty acids), bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances (BLIS). However, there are other mechanisms that may be involved in the inactivation or inhibition of the growth of other related species of bacteria and/or pathogens. As a result, a large number of bacteriocins produced by probiotics LAB have been identified, although their potential application as biopreservatives has not been fully developed [69, 70].

GLUCOSE

ATP

1

ADP

Glucose-6-phosphate

Fructose-6-phosphate

Fructose-1,6-diphosphate

2

Glyceraldehyde-3-P ⇌ Dihydroxyacetone-P

2 Pi

2 NAD⁺

3

2 NADH+2H⁺

2 ,3-diphosphoglycerate

2 ADP

2 ATP

2 3-phosphoglycerate

2 2-phosphoglycerate

H₂O

2 Phosphoenolpyruvate

2 ADP

4

2 ATP

2 Piruvate

2 NADH+2H⁺

5

2 NAD⁺

2 LACTATE
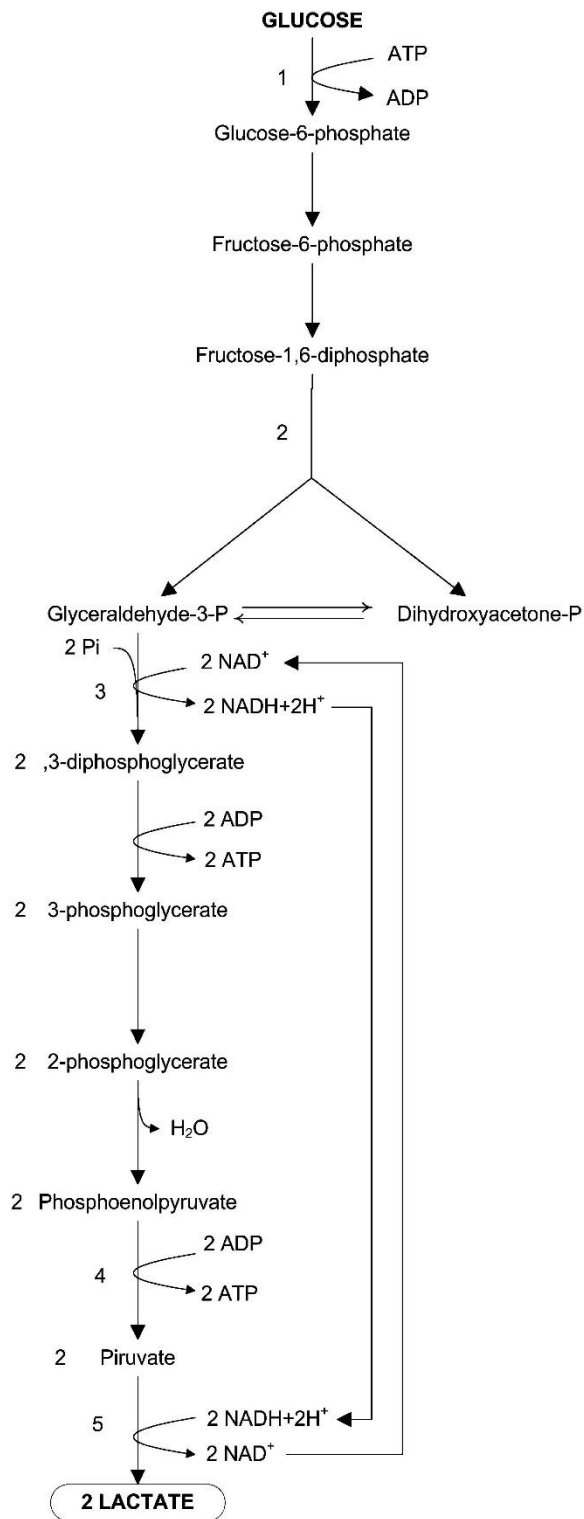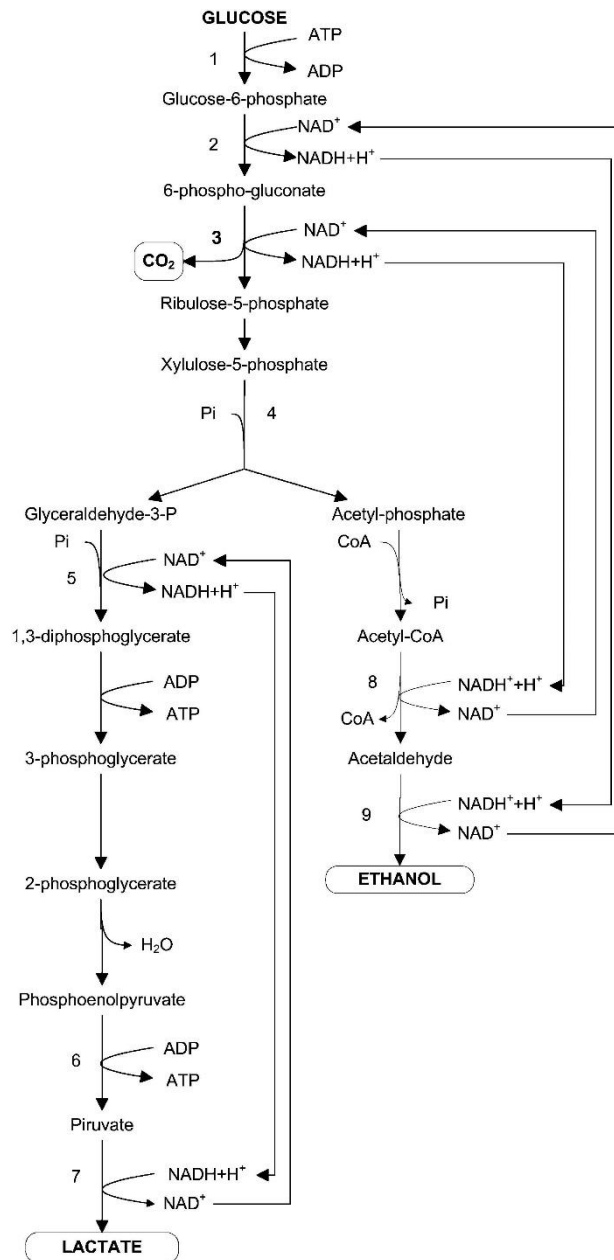
Figure 1.6-1 Homolactic fermentation[71]

Figure 1.6-2. *Heterolactic Fermentation (6-phosphogluconate/phosphoketo¬lase pathway). 1 glucokinase; 2 glucose-6-phosphate dehydrogenase; 3 6-phosphogluconate dehydrogenase; 4 phosphoketolase; 5 glycer¬aldehyde-3-phosphate dehydrogenase; 6 pyruvate kinase; 7 lactate dehydrogenase; 8 acetaldehyde dehydrogenase; 9 alcohol dehydro¬genase)[71]*

1.7 Bacteriocin

Bacteriocins represent a wide group of ribosomally synthesized antimicrobial compounds[72, 73]. Bacteriocins are classified as ribosomal-synthesized peptides, as biologically active proteins or protein complexes with antimicrobial activity against closely related species, and they are produced by different groups of bacteria. Indeed, the first bacteriocin production, discovered in 1925, was found in numerous species of bacteria [74].

In general, these substances are mostly cationic, amphiphilic, membrane-permeabilizing peptides. They have been reported to be unstructured in an aqueous solution, but with the propensity to form α-helical structure when exposed to structure-promoting solvents or membrane-mimicking media[75, 76]. The classification of bacteriocins is based on chemical structure, molecular weight and thermal stability; on that basis four classes of bacteriocins (I, II, III and IV) have been defined [77]. Recently, new classifications of bacteriocins have been proposed. Cotter et al. [13] proposed to divide the bacteriocins into two different categories: the lantibiotics containing lanthionine (Class I) and the non-lanthibiotics (Class II). Class III is reclassified as bacteriolysins, and Class IV has to be withdrawn. The use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatment or in combination with other conventional treatment as part of hurdle technology. It results in more naturally preserved food with better sensorial and nutritional properties. Some genera of LAB produce bacteriocins: *Lactococcus, Streptococcus, Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Carnobacterium, Aerococcus, Oenococcus, Tetragenococcus, Vagococcus and Weisella* [61-63, 78, 79]. Bacteriocins have several properties that make them suitable for use in food preservation. They are generally recognized as safe substances (GRAS); are non-active and non-toxic on eukaryotic cells; become inactivated by digestive proteases so have little influence on the gut microbiota. They are generally thermal resistant (maintaining antimicrobial activity after pasteurization and sterilization) and have a relatively broad antimicrobial spectrum against many foodborne pathogenic and spoilage bacteria. The bactericidal mode of action usually acts on the bacterial cytoplasmic membrane, there is no cross-resistance to antibiotics, and their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation[61].

The application of bacteriocins in food preservation can offer many benefits: provide extra protection during the abuse of temperature conditions; decrease the risk for transmission of foodborne pathogens through the food chain; reduce food losses due to spoilage; reduce chemical preservatives; permit the application of less severe heat treatment without compromising food safety: better preservation of food nutrients and vitamins, as well as sensorial properties of food; permit the marketing of ''novel'' food (less acidic, with a lower salt content and with a higher water content) to satisfy the demands of both the industry and consumers[80, 81]. Researchers have proposed the possibility of incorporating antimicrobial compounds isolated from LAB and bifidobacteria directly into food systems and pharmaceutical products. Certain strategies have been used to incorporate or use biopreservatives in food: direct use of LAB strains with proven antimicrobial activity as starter cultures or starter adjuncts (probiotic concept); use of a biopreservative preparation in the form of a previously fermented product or use of semi-purified, purified or chemically synthesized bacteriocins [82]. Food can be supplemented with ex situ produced bacteriocin preparations, or with the inoculation of a bacteriocin-producer strain under conditions that favor the production of bacteriocins in situ [83, 84]. Several studies have focused on ex situ production of bacteriocins as immobilized preparations, in which the partially purified bacteriocin or the concentrated cultured broth is bound to a carrier [78]. This carrier acts as a reservoir and diffuser of the concentrated bacteriocin molecules to the food ensuring a continuous gradient-dependent supply of bacteriocin. The carrier may also protect the bacteriocin from inactivation by interaction with food components and enzymatic inactivation. Moreover, the precise localized application of bacteriocin molecules on the food surface requires much lower amounts of bacteriocin (compared with the application in the whole food volume), decreasing the processing costs. The production of bacteriocins in situ offers several advantages when compared to ex situ production regarding both legal aspects and costs. The lower cost of biotechnological processes may be highly attractive, especially for small economies and developing countries, where food safety may be seriously compromised[85]. By the application of bacteriocin and/or strains of bacteriocin producer in dairy products, several researchers have demonstrated effectiveness against pathogenic bacteria, such as *Staphylococcus aureus*, *E. coli, Salmonella spp*. and *L. monocytogenes,* in a type of white cheese and

against *L. monocytogenes* in cheeses, such as Camembert [83, 86]. Among the commercial cultures of *Lactobacillus spp.*, the production of an antimicrobial activity against pathogenic microorganisms was observed[87, 88]. Those inhibitory activities of *L. plantarum*, *L. fermentum* and *L. acidophilus* strains originating from Turkish dairy products were due to bacteriocin-like substances, because the neutralization and the catalase treatment of supernatants inhibit the antimicrobial activity of organic acids and hydrogen peroxide against *E. coli* and *Yersinia enterocolitica* and *S. aureus*[82, 89]. In addition, these bacteriocin-like substances are resistant to heat and can be used as biopreservatives. Moreover LAB produced bacteriocin in the temperature range of 4–30°C, and this property could be of interest for applying to refrigerated products[90].

1.8 Regulatory mechanisms of the bacteriocin production

The production of bacteriocin in food must be understood as a dynamic process during which different interactions are always changing, resulting in food preservation[79]. It is regulated by quorum-sensing mechanisms. This process means that the cells present in the environment produce an extracellular auto-inducer molecule by sensing the population density. Once the concentration exceeds a threshold, gene expression is induced[91]. In the case of the lantibiotics nisin and subtilin peptides, the structural peptide functions act as a pheromone that induces its own production to high levels once a cell-density-dependent auto-induction loop is activated [92, 93]. Although Class II bacteriocin acts as a pheromone 144, the presence of another induction peptide that often shows many of the physicochemical properties of bacteriocins can act at low concentrations[94]. The quorum-sensing and inducible bacteriocin production has facilitated the development of systems that allow inducible overexpression of desirable proteins[13]. According to Cotter et al. [13], bacteriocins cannot be grouped based only in their structure, but also on mode of action. Some members of the class I (lantibiotic) bacteriocins, such as nisin have showed a dual mode of action. They can bind to lipid II, the principal transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis of the cell leading to death. Furthermore, they can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads the cell to death. To broaden the use of bacteriocinogenic cultures in food biopreservation, it is important to carefully study the bacteriocin-producing strains of LAB that are well adapted to the particular food environment. It means that the efficacy of bacteriocinogenic cultures as food preservatives should be evaluated for each individual food system[95]. Bacteriocinogenic strains can be used as starter cultures, as adjunct or co-cultures in combination with a starter culture, or as protective cultures (especially in the case of non-fermented foods). The culture will grow during food processing and storage and will produce enough bacteriocin to inhibit the target pathogenic or spoilage bacteria to afford protection.

1.9 Nisin

In 1928, Rogers observed that *L. lactis subsp. lactis* strains had an inhibitory effect on the growth of *Lactobacillus bulgaricus* [96]. In 1947, this bacteriocin was named nisin, or group N inhibitory substance [97]. It is the most characterized bacteriocin produced by LAB; it consists of unusual amino acids lanthionine and methyl-lanthionine, and classified as a class I bacteriocin or lantibiotic[98]. Nisin is *Lactococcus lactis* prototype bacteriocin used as a preservative in the food industry for making dairy products[69, 99].

Nisin is a 34-amino acid antimicrobial peptide, which belongs to the lantibiotic class of bacteriocins (Class I). Lantibiotics are small peptides (< 5 kDa) containing the unusual amino acids lanthionine (Lan), L-methyllanthionine (MeLan) and a number of dehydrated amino acids[100]. Nisin is produced on the ribosome as a prepeptide, which contains 57aminoacid with a 23-residue leader region and 34 residue structural region. The prepeptide is processed through post-translational modifications that lead the dehydration of serine and threonine residues, and cross-linking with cysteine residue[101] to result a biologically active peptide.

The lantibiotics can have multiple mechanisms of action facilitated through the binding of lipid II and insertion into bacterial membranes[102].

The importance of this bacteriocin is due to a wide spectrum of activity against Gram-negative and Gram-positive bacteria including *L. lactis subsp. lactis* and *subsp. cremoris*. *L. bulgaricus*, *S. aureus* and *L. monocytogenes* and prevent the outgrowth of spores of many *Clostridium* and *Bacillus* spp. [78]. The nisin action occurs through disruption of membrane function instigated by formation of pores in the bacterial cell membrane followed by leakage of the cellular material[103]. Nisin is present as two major variants (A and Z), which differ by a single amino acid substituting histidine at position 27 in nisin A and asparagine in nisin Z.

Below the genomic sequences of Nisin A and Nisin Z that differ by single nucleotide are show.

AAAACAGCAA**C**TGTCATTGTAGTATTCACGTAAGC (*Lactococcus lactis* ATCC 11454-**Nisin A**)

AAAACAGCAA**A**TGTCATTGTAGTATTCACGTAAGC (*Lactococcus lactis* CRA 26- **Nisin Z)**

The structural modification has no effect on the antimicrobial activity, but it gives nisin Z higher solubility and diffusion characteristics compared with nisin A, which are important characteristics for food applications [104]. The discovery of nisin brought to the food industry the use of a biopreservative compound produced by LAB. Nowadays, nisin is used in a commercial scale as a food preservative, and research on bacteriocins produced by LAB, searching for novel bacteriocin-producing strains from dairy, meat and vegetable products, and traditional fermented products were carried out [105]. In addition, nisin is licensed as a food preservative (E234) and is recognized as safe [64].

1.10 Lactic Acid bacteria in food safety

The interest on novel biological preservation methods has been increasing over the last few decades. It has been mostly supported by researches indicating that antagonistic microorganisms and their antimicrobial metabolites may have some potential uses as natural preservatives as a way not only to control the growth but also to inactivate undesired microorganisms in food. However, one of the worldwide food industry trends is the necessity to eliminate the use of synthetic chemicals and additives as preservatives. According to the definition of the Food and Agriculture Organization and the World Health Organization (FAO/WHO), probiotics are living microorganisms if, when administered in adequate amounts, confer a health benefit on the host [64, 106, 107]. Biopreservation using lactic acid bacteria (LAB) and/or their antimicrobial metabolites represents an alternative for improving food safety. These antimicrobial properties of LAB are derived from competition for nutrients and the production of one or more antimicrobial active metabolites such as organic acids (principally lactic and acetic acid), hydrogen peroxide and also other compounds, such as bacteriocins and antifungal peptides. The important contribution of probiotic LAB in food preservation has been attracting much attention because of the nutritional qualities of the raw material through an extended shelf life of food and their ability to inhibit spoilage and foodborne pathogens, which is interesting for the food industry [71]. The definition of biopreservation is the extension of shelf life and enhanced safety of food by the use of natural or controlled microbiota and/or antimicrobial compounds[61]. Natural food preservation methods that do not affect the health are considered favorable for consumers, and they should have a smaller impact on food nutritional and sensory properties (as opposed to chemical or physicochemical treatments). At the same time it can reduce processing costs and extend the shelf life of the product; it does not require advanced technological equipment or skills, and therefore, it can be used by undeveloped countries by offering new possibilities 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. Several microorganisms, especially (LAB) with antimicrobial properties, have been commonly associated with food. The use of LAB strains as probiotic and as bioprotective culture in fermented products has also been widely studied.

Besides the acid production, some probiotics LAB strains have the ability to produce a variety of other antimicrobial compounds, as a natural competitor to other microorganisms that share the same niche, such as organic acids, ethanol, hydrogen peroxide, several enzymes and bacteriocins. In the case of dairy products, the main compound metabolized by those cultures is lactose to lactic acid, which lowers the pH value and changes the environment making an unfavorable media for the development of some pathogens and spoilage microorganisms [63, 69, 106]. The LAB as competitive microbiota has a long history of application in fermented foods. Due to their metabolic properties, the LAB is generally employed because of their positive contribution to the flavor, texture and nutritional value in food products, besides their natural antimicrobial properties that extend the product shelf life. Certain probiotic LAB presents the ability to resist acidic conditions and bile salts, and additionally it produces bacteriocins that are active against food pathogens and spoilage microorganisms, contributes to a probiotic culture that may have potential applications for improving the safety of food products [107, 108].

2 EXPERIMENTAL DESIGN

The following part is divided in three chapters, in which it will be described in details the experimental design of the thesis.

Proteomics
- UNTARGETED proteomics analysis in vitro
- VALIDATION directly in a system resuming cheese (milk)

Peptidomics
- Peptides produced by bacteria
- Minimal medium (MDC)

Metabolomics
- Metabolites
- Favorable medium for both bacteria (BHI agar/broth)
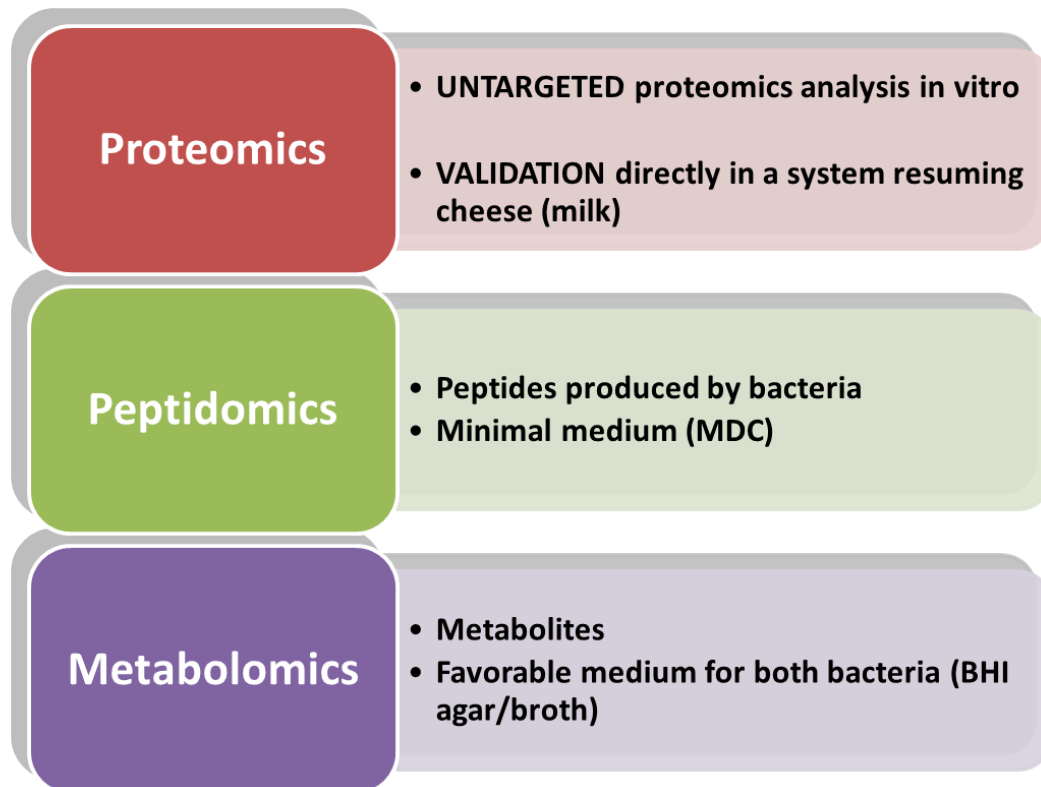
Figure 2-1. Experimental design of the project

3 CHAPTER 1

3.1 Proteomics and Peptidomics

The objective of this first part of project was to improve skills in bacterial cultures, and microbial proteomics. We applied different methods of bioinformatic analysis to study the proteome of cell culture filtrate of *Lactococcus lactis* growing in competition with *Listeria monocytogenes* to highlight the mechanisms of bacterial competition involved in this process.

In collaboration with Istituto Zooprofilattico della Lombardia e dell' Emilia Romagna (IZSLER), that provided us the culture filtrates used for the analysis, we performed the study on the culture filtrates. In particular, in this first part of the project, we have developed the extraction of proteins from medium and we performed proteomic analysis for the evaluation of proteins secreted by bacteria (proteome of the growth media).

On the other hand, in collaboration with Dr Veronique Monnet (INRA, UMR1319 MICALIS, Jouy en Josas, France) we performed the evaluation of extracellular peptidome analysis of separated *Lactococcus lactis* and *Listeria monocytogenes* cultures and of these bacteria growing in competition conditions, in order to characterize peptides potentially involved in gene regulation. In this part we analyzed peptides directly produced by the bacteria in the medium.

3.2 Materials and methods

3.2.1 Bacterial strains and culture condition

Strains *Lactococcus lactis ATCC 11454* and *Listeria monocytogenes ATCC 19115*, obtained from the American type Culture Collection, were provided by IZSLER. They were stored at -80°C in BHI broth containing 20% glycerol.

Both strains were grown in brain heart infusion (BHI) broth at 30°C for 24 hrs. *Listeria monocytogenes* ATCC 19115 preculture (1ml) was inoculted in 100 ml of BHI broth and incubated at 37°C for 24h. Preculture of *Lactococcus lactis* ATCC 11454 (1ml) was inoculted in 100 ml of BHI and incubated at 37°C for 24h. *Listeria monocytogenes* preculture (10^3 ufc/ml) and *Lactococcus lactis* preculture (10^8 ucf/ml) were inoculated in 100 ml of BHI for coculture and incubated at 37°C for 24h. *Listeria monocytogenes* inoculated in BHI after 5 hours of growth of LC ATCC, incubated at 37 ° C.

3.2.2 Supernatant and lyophilized pellets preparation

Cultures were centrifuged (10000 g, 20 min, 4°C) and supernatants were recovered. Supernatant was filtered using PDVF membrane 0,45μm. The pellet was washed in 5 ml of physiological saline, centrifuged at 10000 g for 20 minutes at 4°C and the supernatant was discarded, the pellet was resuspended in 2 ml of physiological saline and lyophilize. For each experimental group, 100 ml of lyophilized cultures medium taken up in 8 ml of H2O. The proteins present in the supernatant were purified and concentrated through precipitation with methanol/chloroform/water. The pellet was solubilized in 6M Urea, 100mMTris pH 7.5

The experimental groups analyzed are the following:

1. 3 BHI (brain heart infusion)

2. 3 BHI with *Lactococcus lactis*

3. 3 BHI with *Listeria* Monocytogenes

4. 3 BHI and *Lactococcus* in co-culture with *Listeria* (LM inoculated in BHI after 5 hours of growth of LC ATCC, incubated at 37 ° C).

3.2.3   Protein assay

Protein concentration was determined by Bradford assay. Optical density was measured at 595 nm using a spectrophotometer (Gene Quant 100, GE Healthcare) and protein concentration was determined against Bovine Serum Albumin (BSA, Thermo Scientific) used as standard. A standard curve was prepared with different BSA concentrations from 1 to 20 ug. The amount of protein was calculated by interpolation of the experimental values with standard proteins to known quantity.

3.2.4   SDS-Page electrophoresis

We proceeded to the analysis of protein profile of the samples through one dimensional electrophoresis optimized for the separation of molecular weights from 200 to 15 kilodaltons.

3.2.5   2D electrophoresis

The samples of each experimental group were analyzed through 2D electrophoresis. For isoelectric focusing (IEF) step, immobilized pH gradient (IPG) polyacrilamide gel strips (GE Healthcare, 7 cm, pH 3-10 NL) and Protean IEF Cell (Bio Rad) were utilized. Prior to IEF, 200 µg of protein sample was dissolved in a solution containing 7 M Urea, 2 M Thiourea, 2% w/v CHAPS, 30 mM DTT, 0.5% w/v Ampholine (pH 3.5-10.0) and 1% w/v bromophenol blue. IPG strips were first actively rehydrated in the presence of the sample at 50 V and 20°C for 17 h. After the rehydration step, paper wicks soaked in water were placed between cathode, anode and gel strip for preventing high voltage to cause burning of the strips. The voltage was gradually increased according to the following protocol: 100 V (4 h), 250 V (2 h), 4000 V (5 h), 4000 V until the cumulative voltage reached 60 kVh. A limitation of current up to 50 µA per gel strip was set. Following IEF, each strip was reduced for 15 min in 5 ml of solution containing 6 M Urea, 2% w/v SDS, 50 mM Tris-HCl buffer, pH 8.8 and 20% v/v Glycerol with 1% w/v DTT added, and then alkylated in 5 ml of same solution with 2.5% w/v of IAA. IPG strips were then washed shortly in 1x running buffer (250 mM Tris-HCl, pH 8.8, 1920 mM Glycine, 1% w/v SDS and MilliQ water), loaded onto 12% w/v polyacrylamide resolving gels along with the protein ladder and fixed with 0.5% w/v agarose gel. Second dimension was carried out in Mini-Protean Tetra system (Bio Rad). In the first step of electrophoresis, until the bromophenol blue front line entered the resolving gel, 8 mA per gel for 15 min

were applied. In the second step, 16 mA per gel were applied until the bromophenol blue front line reached the bottom of the gel. Gels were then removed from the plates, washed three times for 5 min in 100 ml of deionised water and left over night to stain in 100 ml of preheated Coomassie Brilliant Blue G-250 (Sigma-Aldrich)[109].

3.2.6   Image acquisition and statistical analysis

Gel images were acquired using a flatbed scanner (ImageScanner III, GE Healthcare, Uppsala) with a resolution of 600 dpi. Before scanning, gels were washed for 20 s in 70% v/v ethanol and then for 2 min in 100 ml of deionized water. Variations in protein expression between each group were analyzed using the Progenesis SameSpots software (Nonlinear Dynamics, UK), Version 4.5. After evaluating the quality of the images, the module for 2D gel analysis was used to align the images, subtract background, detect, normalize and match spots. At the end, all spots were manually reviewed and selected for excision.

Statistical analysis was performed using the Progenesis Stats module on the log-normalized volumes for all spots. Stats module performs automatically a One-way ANOVA on each spot to evaluate the p value between different groups, p-values under 0.05 were considered statistically significant.

3.2.7   MALDI-TOF MS analysis

Spots were manually excised from 2D gels, destained with washing solution (2,5 M ammonium bicarbonate, 50% v/v ACN and MilliQ water) and dehydrated with 100% v/v ACN. Each spot was reduced with 10 mM DTT (Amersham Bioscience) for 45 min at 56°C and then alkylated for 30 min at 37°C in the dark with 55 mM IAA (Sigma Aldrich). In gel digestion with 0,01 µg/µl porcine trypsin (Promega, Madison, WI) solution for 16 h at 37°C followed. To stop further digestion of peptides by trypsin, 1% v/v TFA in H2O was added. Peptides were desalted and concentrated using C18 ZipTip (Millipore), eluted with α-cyano-4-hydroxycinnamic acid (CHCA) (3,5 mg/ml CHCA, 50% v/v ACN, 0,1% v/v TFA) and spotted on a Ground steel plate (Bruker-Dalonics, Bremen, Germany) previously covered with a layer of 10 mg/ml CHCA (10 mg/ml CHCA in 1:1 EtOH:ACN and 0,001% v/v TFA). Ultraflex III MALDI TOF/TOF spectrometer (Bruker-Daltonics) was used to acquire the spectra in positive reflectron mode. External calibration was carried out with standard peptide calibration mixture (m/z: 1046.5418, 1296.6848, 1347.7354,

1619.8223, 2093.0862, 2465.1983, 3147.4710; Bruker-Daltonics). FlexAnalysis 3.3 software (Bruker-Daltonics) was applied to analyse mass spectra and select moniosotopic peptide masses.

Internal calibration on known trypsin autolysis peaks (m/z: 842.509 and 2211.104) and contaminant ions exclusion of matrix and human keratin peaks was performed and the created peak lists were analyzed by MASCOT v.2.4.1. Following parameters were used for database searching: fixed and variable modifications were defined as carbamidomethylation of cysteins and oxidation on methionins, respectively, up to one missed cleavage was set for trypsin and protein mass tolerance was defined as 50 ppm. Mascot scores above 60 were considered significant in protein identification assignment (p<0,05). Protein identifications were obtained searching against curated databases restricted to Lactococcus lactis subsp. lactis and Listeria Monocytogenes.

In order to confirm the obtained identifications, LIFT mode of the instrument was applied to acquire MS/MS spectra with 4–8 x 103 laser shots using the instrument calibration file. Precursor ions were manually selected for the fragmentation and the precursor mass window was automatically set. Spectra baseline subtraction, smoothing (Savitsky-Golay) and centroiding were operated using Flex-Analysis 3.1 software for each MS/MS spectra acquired. Following parameters were used for database search: maximum one missed cleavage was established, carbamidomethylation of cysteins and oxidation of methionine was set among fixed and variable modifications, respectively, and the mass tolerance was set at 50 ppm for precursor ions and 0.4 Da for fragments. The taxonomy was restricted to Bos taurus. Protein identification confidence interval was set to 95% (p<0.05) so, to be considered correctly identified, peptides had to have an individual identification score above the identity threshold.

3.2.8   Peptidomic Analysis

One strain of *Lactococcus lactis* (ATCC 11454) and one strain of *Listeria monocytogens* (ATCC 19115) have been used in the present study. For the culture of both bacteria we used chemically defined medium (MCD) that does not contained peptides in order to analyze only peptides produced by bacteria in the medium.

We analyzed three different conditions: monoculture of *Listeria*, monoculture of *Lactococcus lactis* and co-culture of *Listeria*+ *Lactococcus lactis*.

After centrifugation, filtration and concentration, samples were analyzed trought HPLC coupled to LTQ orbitrap. LC-MS/MS analysis was performed on the PAPPSO platform (INRA, Jouy-en-Josas, France). An Ultimate 3000 LC system (Dionex) was connected to a linear ion trap mass spectrometer (LTQ, Thermo Fisher) by a nanoelectrospray interface to conduct the separation, ionization and fragmentation of peptides, respectively. Peptidomic data were analyzed by X!Tandem Pipeline software.

3.2.9   Bioinformatics analysis

The genomic sequence of strains has been analyzed for the presence of short genes at the MIGALE plateform (INRA, Jouy-en-Josas, France) using the BactGeneShow program. A gene containing from 48 to 183 bases (peptide from 15 to 60 amino acids) is considered as a short gene (artificial cut off), genes containing more than 183 bases are considered as "normal" genes[110]. Short genes potentially coding for short peptides that are potentially involved in quorum-sensing systems. The threshold that has been used is mainly based on removal of predictions related to genes shorter than 48 bases.

Three steps are fundamental for the construction of the database used for the peptides identification:

1. Extraction of the regions corresponding to coding sequences

2. Reversion of the nucleotidic sequences those are located on the reverse DNA strand

3. Conversion from nucleotides to amino acids.

All these steps are done using bio-informatic scripts that are enclosed in the EMBOSS package.

3.3 Results

All analysis was made on the biological and technical triplicate.

3.3.1   Analysis through SDS-PAGE and mass spectrometry
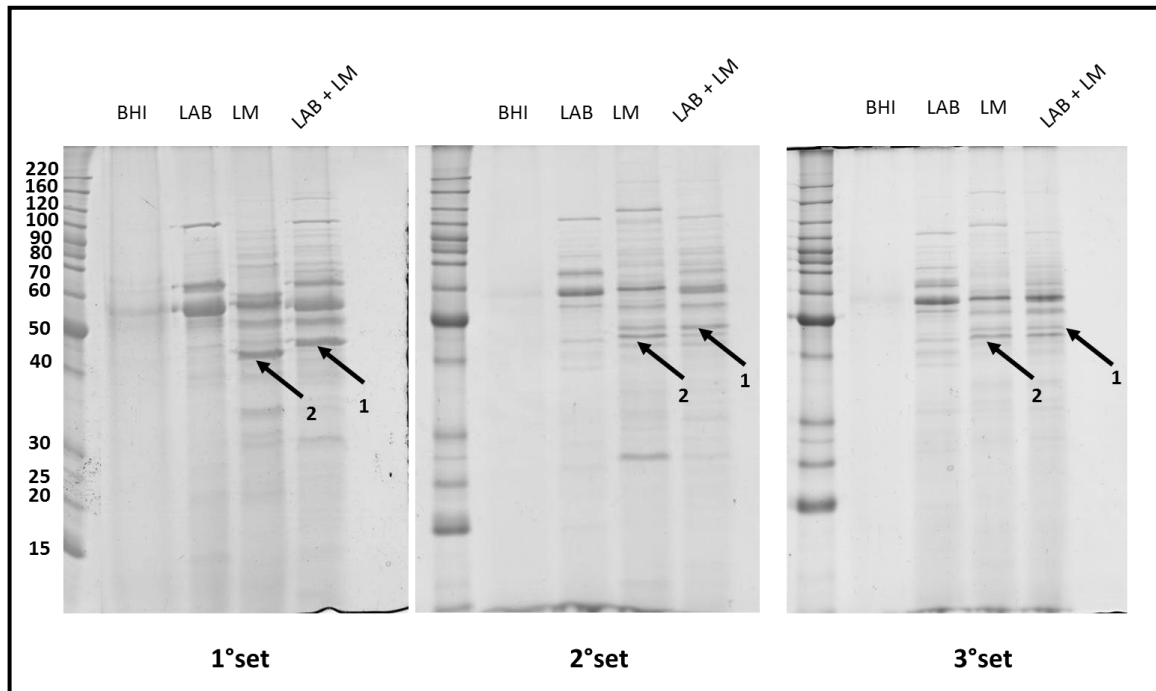
Figure 3.3.1-1. One-dimensional electrophoresis of filtered bacterial growth media . BHI (Brain Heart infusion growth media); LAC (*Lactococcus lactis*); LM (*Listeria monocytogenes*); LM+LAC (bacterial co-culture filtrate). The "sets" representing each biological replicate. 1D electrophoresis analysis is optimized for the separation of molecular weights from 220 to 15 kDa.

In figure 3.3.1-1 the one-dimensional electrophoresis analysis highlighting protein bands analysed through mass spectrometry is shown as representative of each experimental biological replicate group. The data obtained show the presence of a protein band present only in the condition of bacterial competition (BHI + LAB + LM). Arrow 1 and 2 indicate protein bands that show a different expression between the condition of *Listeria monocytogenes* monoculture (LM) and the condition of bacterial competition (LAB+LM).

3.3.2 Analysis through two-dimensional electrophoresis and mass spectrometry

Maps of filtered cell of the various experimental groups were performed through two-dimensional electrophoresis. In figure 3.3.2-1 the two-dimensional maps of the filtered cell of three experimental groups are represented. Image analysis performed showed, as indicated by the arrow in figure 3.3.2-1, the appearance of a spot representing a protein secreted again by one of two bacteria only in conditions of co-culture (panel a). In the panel b and c, as indicated by the arrow, spot representing 2942 is not present.
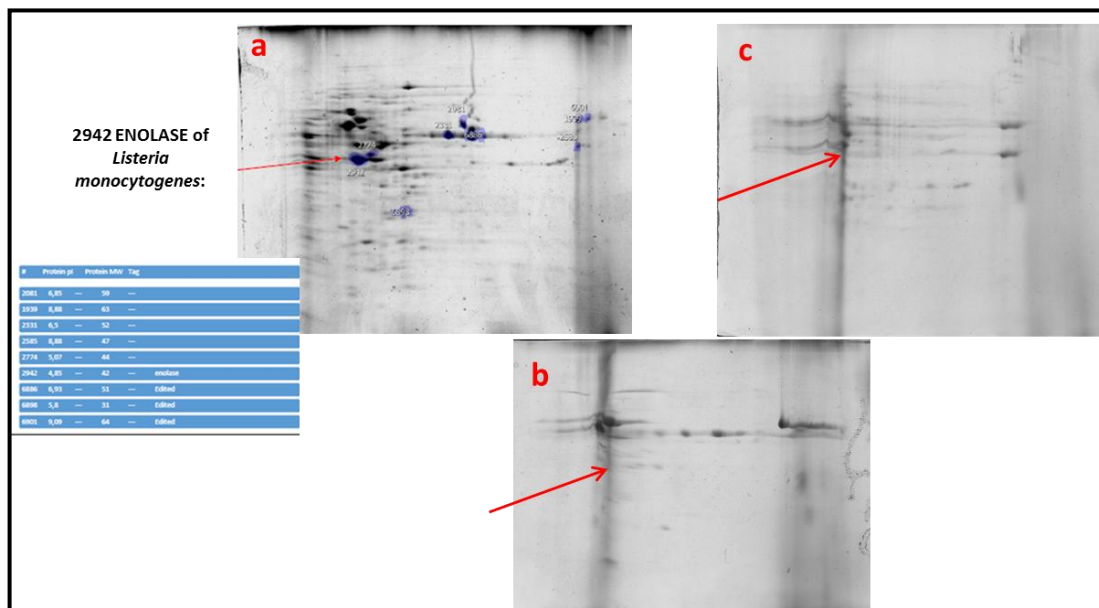
Figure 3.3.2-1. Maps of the 2DE of filtered bacterial growth media of three experimental groups - Filtered cell co-cultures of the two bacteria (Lab+LM,panel a); filtered monoculture of LAB (panel b) and filtered cell monoculture of LM (panel C)

The protein derived from the band obtained by one-dimensional gel and from the spot by 2D gel was analysed by MALDI TOF analysis. The protein expressed only in the condition of co-culture was identified as Enolase of *Listeria monocytogenes* (gi|46908628, NCBI database).

3.3.3 Peptidomics Analysis
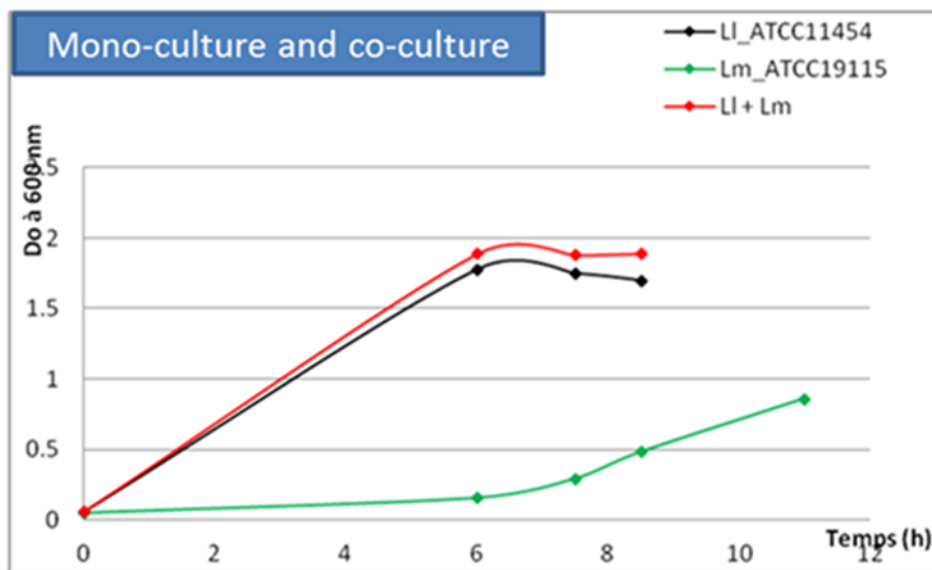
Figure3.3.3-1 Growth curves of monoculture (*Listeria ATCC 19115, Lactococcus lactis ATCC 11454*) and co-culture (*Listeria monocytogenes ATCC 19115-Lactococcus lactis ATCC 11454*). Culture was stopped at OD_{600} of 1.0, corresponding to the exponential growth phase.

In Figure 3.3.3-1 growth curves of monoculture and coculture of *Listeria monocytogenes* and *Lactococcus lactis* with different strains are shown.

Database searching, performed by X! Tandem Pipeline, allowed the identification of peptides that accumulate in the medium during the growth of the strains. About 957 peptides were identified for the LM ATCC 19115 monoculture, 2350 for *Lactococcus lactis* ATCC 11454 and 1440 for the mixed culture. 957 peptides derive from 115 proteins for the monoculture of *Listeria monocytogenes* ATCC 19115; 2350 peptides from 110 proteins for the monoculture of *Lactococcus lactis* ATCC 11454 and 1440 peptides derive from 115 proteins identified in mixed culture (**Figure 3.3.3-2**).

Figure 3.3.3-2 Representative distribution of proteins identified in Monoculture (*Listeria monocytogenes* ATCC 19115, *Lactococcus lactis ATCC 11454*) and in co-culture (*Listeria-Lactococcus*). In the red circle, the number of proteins identified only in coculture condition.

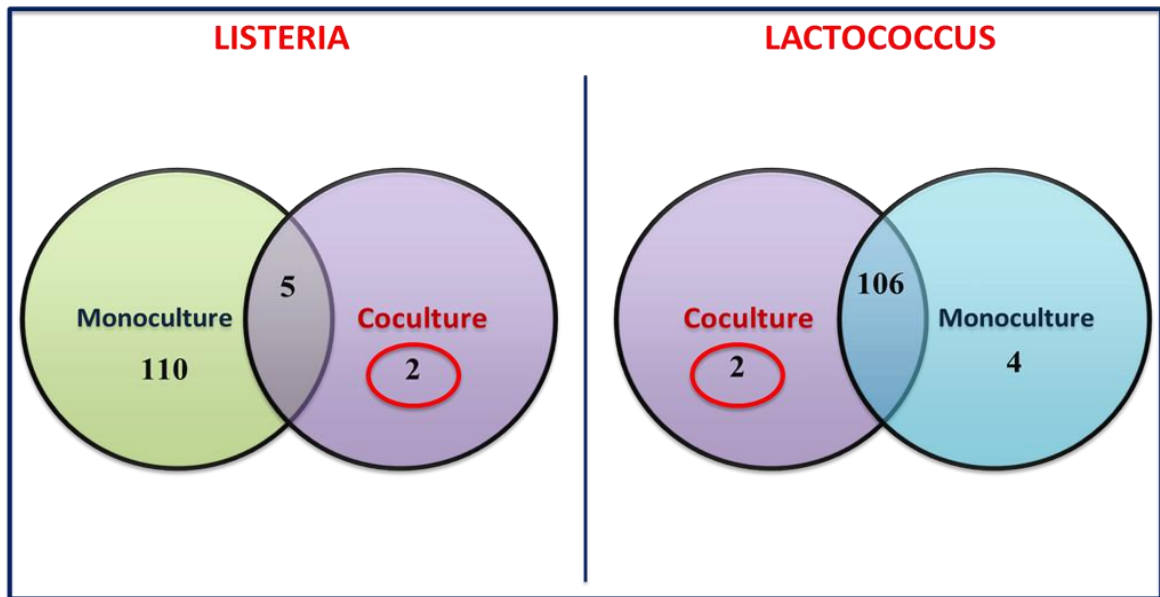| Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_50S ribosomal protein L2|Cytoplasmic	-10,77275658	9	13	5	-	3
ATCC_11454_F0F1 ATP synthase subunit C|Extracellular, Secreted via minor pathways (bacteriocin) (no CS)	-19,13783073	38	7,3	13	-	4
ATCC_11454_ (ABC transporter substrate-binding protein	-157,9499359	50	59,9	55	-	32
ATCC_11454_recombinase A|Cytoplasmic	-15,20877838	9	41,3	4	-	4
ATCC_11454_ ABC transporter substrate-binding protein	-620,2020874	89	59,6	317	-	139
ATCC_11454_peptidyl-prolyl cis-trans isomerase|Membrane, N-terminally anchored (No CS)	-4,920818806	10	29,9	2	-	2
ATCC_11454_gamma-aminobutyrate permease	-22,81734085	10	51,8	6	-	6
ATCC_11454_oligopeptide ABC trasporter ATP binding protein|Cytoplasmic	-4,221848488	6	38,8	4	-	2
ATCC_11454_oligopeptide ABC trasporter ATP binding protein|Cytoplasmic	-11,87245464	7	34,8	4	-	4
ATCC_11454_ amino acid permease	-16,05438042	11	49,9	10	-	6
ATCC_11454_DNA-directed RNA polymerase subunit alpha|Cytoplasmi	-13,19205952	4	34	4	-	3
ATCC_11454_50S ribosomal protein L30|Cytoplasmic	-9,563837051	21	6,1	4	-	2
ATCC_11454_30S ribosomal protein S5|Cytoplasmic	-10,19942093	21	17,5	4	-	4
ATCC_11454_glycerophosphodiester phosphodiesterase]	-100,8089447	14	76,7	37	-	15
ATCC_11454_superoxide dismutase|Cytoplasmic	-6,912218571	10	23,2	3	-	3
ATCC_11454_|carbon starvation protein|Membrane, Multi-transmembrane	-14,46248341	4	85,1	8	-	4
ATCC_11454_ beta-lactamase	-75,20846558	32	50,3	27	-	18
ATCC_11454_transport permease|Membrane, Multi-transmembrane	-14,755723	10	26	4	-	4
ATCC_11454_30S ribosomal protein S9|Cytoplasmic	-5,706969261	9	13,6	3	-	3
ATCC_11454_glutamate synthase subunit beta|Cytoplasmic	-9,819014549	2	52,2	2	-	2
ATCC_11454_|glutamate synthase large subunit|Cytoplasmic	-23,7961998	3	164,2	7	-	7
ATCC_11454_branched-chain amino acid aminotransferase|Cytoplasmic	-5,207608223	5	36,8	4	-	2
ATCC_11454_ D-alanine transfer protein DltD	-102,35923	48	48,6	46	-	23
ATCC_11454_FtsW1|Membrane, Multi-transmembrane	-10,40230465	3	46	3	-	3
ATCC_11454_pyruvate kinase|Cytoplasmic	-21,3377533	6	54,1	7	-	6
ATCC_11454_ RTX toxin	-35,34415817	11	69,5	10	-	8
ATCC_11454_carbamoyl phosphate synthase large subunit|Cytoplasmic	-10,40726852	1	117,4	5	-	4
ATCC_11454_N-acetylmuramidase|Extracellular, secreted	-25,14151764	28	23,6	7	-	5
ATCC_11454_|transporter|Membrane, Multi-transmembrane	-36,43174744	18	43,3	14	-	13
ATCC_11454_penicillin-binding protein 1B|Membrane	-40,01389694	15	86,8	22	-	12
ATCC_11454_tyrosyl-tRNA synthetase|Cytoplasmic	-22,69204903	14	47,1	9	-	6

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_oxidoreductase|Cytoplasmic	-13,8986578	15	36,5	5	-	5
ATCC_11454_asparaginyl-tRNA synthetase|Cytoplasmic	-12,95002651	7	50,7	5	-	4
ATCC_11454_peptide-binding protein	-103,0604858	38	65,9	31	-	23
ATCC_11454_ N-acetylmuramoyl-L-alanine amidase	-51,55121994	3	53,6	23	-	7
ATCC_11454_fructose-bisphosphate aldolase|Cytoplasmic	-17,89045906	5	31,9	7	-	7
ATCC_11454_|myosin-cross-reactive antigen|Membrane, N-terminally anchored (No CS)	-24,38909721	13	67	8	-	7
ATCC_11454_molecular chaperone DnaK|Cytoplasmic	-5,711303711	3	64,8	2	-	2
ATCC_11454_D-alanyl-D-alanine carboxypeptidase|Membrane, N-terminally anchored (No CS)	-4,106792927	14	27,4	2	-	2
ATCC_11454_amino acid ABC transporter substrate-binding protein	-108,8238373	46	30,5	29	-	19
ATCC_11454_phosphopentomutase|Cytoplasmic	-7,69389677	5	45,7	3	-	3
ATCC_11454_transcription regulator|Membrane, N-terminally anchored (No CS)	-10,71955872	7	33,6	3	-	3
ATCC_11454_proline dipeptidase|Cytoplasmic	-6,598255157	2	39,8	2	-	2
ATCC_11454_ valyl-tRNA synthetase	-9,61783886	3	100,4	3	-	3
ATCC_11454_transcription regulator|Cytoplasmic	-12,19585991	12	10,8	2	-	2
ATCC_11454_ Putative uncharacterized protein yedB	-41,91228104	23	36,7	11	-	8
ATCC_11454_ membrane protein	-25,69314003	10	56,6	12	-	5
ATCC_11454_cold shock protein E|Cytoplasmic	-69,72196198	68	7	19	10	13
ATCC_11454_hypothetical protein|Membrane, N-terminally anchored (No CS)	-5,045757294	7	28,8	2	-	2
ATCC_11454_UDP-N-acetylglucosamine 1-carboxyvinyltransferase|Cytoplasmic	-3,886056662	2	45,7	2	-	2
ATCC_11454_ (ABC transporter)	-76,96114349	24	85	47	-	19
ATCC_11454_dTDP-4-keto-6-deoxyglucose-3, 5-epimerase|Cytoplasmic	-10,11278629	15	22,2	4	-	4
ATCC_11454_x-prolyl-dipeptidyl aminopeptidase|Cytoplasmic	-11,56288433	3	87,6	3	-	3
ATCC_11454_arginyl-tRNA synthetase|Cytoplasmic	-18,26686668	5	62,8	6	-	5
ATCC_11454_|amino acid ABC transporter substrate binding protein|Extracellular, Lipid anchored	-35,35427094	16	30,9	18	-	7
ATCC_11454_glyceraldehyde 3-phosphate dehydrogenase|Cytoplasmic	-41,43119049	11	25	12	-	10
ATCC_11454_transporter|Membrane, Multi-transmembrane	-11,43475628	7	17,2	3	-	3
ATCC_11454_fumarate reductase	-210,8803406	69	52,7	95	-	56
ATCC_11454_thiamine biosynthesis lipoprotein|Extracellular	-39,8343544	26	39,6	13	-	9
ATCC_11454_50S ribosomal protein L27|Cytoplasmic	-13,32505894	20	10	4	-	4
ATCC_11454_phosphate ABC transporter substrate-binding protein|Extracellular, Lipid anchored	-15,44869709	9	30,5	9	-	3

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_Lasergene_maturation protein|Extracellular, Lipid anchored	-22,03201675	18	33,7	11	-	4
ATCC_11454_manganese ABC transporter substrate-binding protein	-22,38810349	22	34,9	8	-	6
ATCC_11454_glycerol-3-phosphate acyltransferase PlsX|Cytoplasmic	-7,545155048	6	34,5	2	-	2
ATCC_11454_ Nisin immunity protein	-78,19429779	58	27,7	42	-	23
ATCC_11454_peptidase	-79,39823151	21	74,6	31	-	21
ATCC_11454_ nisin biosynthesis two-component system, sensor histidine kinase NisK	-13,74126625	7	50,5	4	-	3
ATCC_11454_ lantibiotic ABC transporter permease	-7,78188467	12	27,5	4	-	3
ATCC_11454_ Basic membrane protein A	-118,1277466	45	36,5	35	-	23
ATCC_11454_(lysine specific permease	-235,8040466	28	51,7	65	-	52
ATCC_11454_glutamine ABC transporter permease and substrate binding protein|Membrane, Multi-transmembrane	-54,88779449	8	78,2	36	-	14
ATCC_11454_30S ribosomal protein S20|Cytoplasmic	-19,55498695	35	8,3	7	-	5
ATCC_11454_30S ribosomal protein S10|Cytoplasmic	-3,83505249	10	11,7	2	-	2
ATCC_11454_1-deoxy-D-xylulose-5-phosphate synthase|Cytoplasmic	-12,53276253	2	57,9	3	-	3
ATCC_11454_transglycosylase	-52,34898376	13	20,5	28	-	8
ATCC_11454_penicillin-binding protein|Membrane, N-terminally anchored (No CS)	-23,44273758	8	83,3	5	-	5
ATCC_11454_616|prolyl-tRNA synthetase|Cytoplasmic	-8,44009304	1	69,1	3	-	2
ATCC_11454_[3749-4285]hypothetical protein|Cytoplasmic	-16,72498894	6	20,5	5	-	3
ATCC_11454_hypothetical protein|Membrane, N-terminally anchored (No CS)	-43,07733536	45	18,7	15	-	11
ATCC_11454_outer membrane lipoprotein precursor|Membrane, N-terminally anchored (with CS)	-15,4644537	18	31,1	7	3	4
ATCC_11454_outer membrane lipoprotein precursor|Membrane, N-terminally anchored (No CS)	-34,79339218	26	31,2	14	6	7
ATCC_11454_ferrichrome ABC transporter substrate binding protein|Extracellular, Lipid anchored	-16,28508377	13	34,2	4	-	4
ATCC_11454_ penicillin-binding protein 2A	-101,8434525	21	74,3	27	-	17
ATCC_11454_|zinc ABC transporter substrate binding protein|Extracellular, Lipid anchored	-29,08574486	23	30	16	-	7
ATCC_11454_hypothetical protein|Cytoplasmic	-5,279840946	9	17,7	2	-	2
ATCC_11454_autolysin|Cell wall associated (LysM domain)	-21,94146729	14	46,5	10	-	5
ATCC_11454_endo-1,4-beta-xylanase D|Membrane, N-terminally anchored (No CS)	-38,67086029	13	41,6	14	-	5
ATCC_11454_ permidine/putrescine ABC transporter substrate-binding protein	-32,58127594	19	40,5	14	-	7
ATCC_11454_hypothetical protein|Membrane, Multi-transmembrane	-24,66345978	27	10,8	10	-	6
ATCC_11454_30S ribosomal protein S1|Cytoplasmic	-17,9473381	9	44,6	5	-	5

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_dipeptidase PepV|Cytoplasmic	-16,70692444	5	51,8	10	-	7
ATCC_11454_ copper transporter	-8,059882164	6	77,4	2	-	2
ATCC_11454_hypothetical protein|Membrane, Multi-transmembrane	-10,87575722	5	26,3	4	-	3
ATCC_11454_|glutamine synthetase|Cytoplasmic	-9,494850159	5	49,6	2	-	2
ATCC_11454_|glutamate or arginine ABC transporter substrate binding protein|Extracellular, Lipid anchored	-15,30856514	16	28,4	7	-	3
ATCC_11454_|glyceraldehyde 3-phosphate dehydrogenase|Cytoplasmic	-41,43119049	8	35,9	12	-	10
ATCC_11454_hypothetical protein|Membrane, N-terminally anchored (with CS)	-30,82732964	25	35,3	10	-	7
ATCC_11454_glucokinase|Cytoplasmic	-6,573000908	7	33,7	4	-	3
ATCC_11454_deoxynucleoside kinase|Cytoplasmic	-15,47118664	11	24,6	4	-	4
ATCC_11454_exported serine protease|Membrane, N-terminally anchored (No CS)	-21,69059753	9	41,5	8	-	5
ATCC_11454_|hypothetical protein|Extracellular, Lipid anchored	-33,56362152	19	36,6	14	-	10
ATCC_11454_ Nisin biosynthesis protein NisB	-116,957695	20	103,8	44	-	34
ATCC_11454_ lantibiotic nisin-A	-273,5541687	43	5,9	716	-	37
ATCC_11454_|transcription regulator|Cytoplasmic /TMH start AFTER 60	-31,89652252	14	52,1	11	-	6
ATCC_11454_signal peptidase I|Membrane, N-terminally anchored (No CS)	-19,87844086	22	23,5	10	-	3
ATCC_11454_elongation factor G|Cytoplasmic	-8,620876312	4	77,8	3	-	3
ATCC_11454_hypothetical protein|Membrane, Multi-transmembrane	-19,39291573	10	39	13	-	5
ATCC_11454_D-alanyl-D-alanine carboxypeptidase	-35,75728989	21	46,8	13	-	9
ATCC_11454_MULTISPECIES: hypothetical protein [Lactobacillales]	-25,91507149	27	20,8	13	-	6
ATCC_11454_rod shape-determining protein MreC|Membrane, N-terminally anchored (with CS)	-45,94273758	27	31,3	14	-	8
ATCC_11454_ AAA-ATPase, putative	-9,290730476	1	83,9	2	-	2
ATCC_11454_ mucus-binding protein	-52,85584259	12	30,5	31	-	7

Figure 3.3.3-3 *Proteins identified in medium from Lactcococcus lactis monoculture.*

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_ oligopeptide ABC transporter substrate binding protein|Extracellular, Lipid anchored	-90,75102234	35	59,9	39	-	21
ATCC_11454_ recombinase A|Cytoplasmic	-9,121133804	8	41,3	3	-	3
ATCC_11454_ ABC transporter substrate-binding protein	-385,2743835	72	59,6	168	-	86
ATCC_11454_ amino acid permease	-16,95748329	9	49,9	13	-	6
ATCC_11454_|DNA-directed RNA polymerase subunit alpha|Cytoplasmic	-13,3391819	4	34	4	-	3
ATCC_11454_ 50S ribosomal protein L30|Cytoplasmic	-9,95039463	21	6,1	3	-	2
ATCC_11454_ glycerophosphodiester phosphodiesterase	-71,66625977	13	76,7	26	-	12
ATCC_11454_Lasergene_penicillin-binding protein 2B|Membrane, N-terminally anchored	-5,585026741	2	60,2	3	-	2
ATCC_11454_elongation factor Tu|Cytoplasmic	-8,530413628	15	21	3	-	3
ATCC_11454_Lasergene_ carbon starvation protein|Membrane, Multi-transmembrane	-7,753501415	1	85,1	3	-	2
ATCC_11454_ beta-lactamase	-26,34361649	21	50,3	14	-	9
ATCC_11454_D-alanine transfer protein|Membrane, N-terminally anchored	-65,02615356	28	48,6	30	-	13
ATCC_11454_ pyruvate kinase|Cytoplasmic	-13,9910593	6	54,1	5	-	4
ATCC_11454_ RTX toxin	-22,57942963	11	69,5	10	-	7
ATCC_11454_carbamoyl phosphate synthase large subunit|Cytoplasmic	-6,911863804	1	117,4	3	-	2
ATCC_11454_ transporter|Membrane, Multi-transmembrane	-17,53790855	9	43,3	7	-	6
ATCC_11454_ penicillin-binding protein 1B|Membrane, N-terminally anchored	-33,08220673	13	86,8	15	-	9
ATCC_11454_|tyrosyl-tRNA synthetase|Cytoplasmic	-18,95018005	10	47,1	7	-	5
ATCC_11454_ asparaginyl-tRNA synthetase|Cytoplasmic	-7,494850159	4	50,7	2	-	2
ATCC_11454_ peptide-binding protein	-63,33303452	28	65,9	26	-	17
ATCC_11454_ N-acetylmuramoyl-L-alanine amidase	-39,82132721	3	53,6	16	-	7
ATCC_11454_ fructose-bisphosphate aldolase|Cytoplasmic	-3,414539337	4	31,9	2	-	2
ATCC_11454_ myosin-cross-reactive antigen|Membrane, N-terminally anchored (No CS)	-10,87305737	7	67	5	-	4
ATCC_11454_ molecular chaperone DnaK|Cytoplasmic	-5,360015869	3	64,8	3	-	2
ATCC_11454_ amino acid ABC transporter substrate binding protein|Extracellular, Lipid anchored	-68,94965363	40	30,5	20	-	13
ATCC_11454_ Putative uncharacterized protein yedB	-32,14206696	17	36,7	7	-	6
ATCC_11454_ bifunctional acetaldehyde-CoA/alcohol dehydrogenase|Cytoplasmic	-21,39892387	3	98	9	-	7
ATCC_11454_|aminopeptidase N|Cytoplasmic	-6,583359241	2	95,2	2	-	2
ATCC_11454_ cold shock protein E|Cytoplasmic	-31,81031418	56	7	9	-	6

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
ATCC_11454_ hypothetical protein|Membrane, N-terminally anchored (No CS)	-5,505845547	7	28,8	2	-	2
ATCC_11454_ UDP-N-acetylglucosamine 1-carboxyvinyltransferase|Cytoplasmic	-4,440572262	2	45,7	2	-	2
ATCC_11454_ hypothetical protein|Membrane, Multi-transmembrane	-50,14336395	14	85	40	-	12
ATCC_11454_ |50S ribosomal protein L28|Cytoplasmic	-4,034327984	38	7,1	3	-	2
ATCC_11454_hypothetical protein|Membrane, N-terminally anchored (No CS)	-38,72576141	28	31,5	11	-	8
ATCC_11454_ |HU like DNA-binding protein|Cytoplasmic	-16,62662697	44	9,6	5	-	3
ATCC_11454_ hypothetical protein|Cell Wall, LPxTG Cell-wall anchored	-46,71979904	9	160,9	18	-	13
ATCC_11454_amino acid ABC transporter substrate binding protein|Extracellular, Lipid anchored	-15,04762077	9	30,9	8	-	4
ATCC_11454_glyceraldehyde 3-phosphate dehydrogenase|Cytoplasmic	-25,88830948	10	25	7	-	7
ATCC_11454_ |transporter|Membrane, Multi-transmembrane	-7,563837528	15	17,2	3	-	3
ATCC_11454_ fumarate reductase flavoprotein subunit|Extracellular, Lipid anchored	-141,0642548	62	52,7	70	37	38
ATCC_11454_thiamine biosynthesis lipoprotein|Extracellular, Lipid anchored	-30,80196571	27	39,6	11	-	10
ATCC_11454_ 50S ribosomal protein L27|Cytoplasmic	-8,292770386	20	10	3	-	3
ATCC_11454_ phosphate ABC transporter substrate-binding protein|Extracellular, Lipid anchored	-13,40318298	9	30,5	6	-	3
ATCC_11454_Nisin immunity protein	-69,8260498	41	27,7	28	-	17
ATCC_11454_ peptidase	-34,15182495	11	74,6	16	-	10
ATCC_11454_nisin biosynthesis two-component system, sensor histidine kinase NisK	-5,14266777	7	50,5	4	-	2
ATCC_11454_lantibiotic ABC transporter permease	-6,443697453	6	27,5	2	-	2
ATCC_11454_ Basic membrane protein A	-71,2654953	31	36,5	19	-	14
ATCC_11454_ lysine specific permease	-191,8108368	32	51,7	58	-	47
ATCC_11454_ glutamine ABC transporter permease and substrate binding protein|Membrane, Multi-transmembrane	-44,40297318	6	78,2	26	-	10
ATCC_11454_ 30S ribosomal protein S20|Cytoplasmic	-11,30629921	16	8,3	5	-	4
ATCC_11454_ hypothetical protein|Cytoplasmic	-2,973058224	3	67,7	2	-	2
ATCC_11454_phage infection protein|Membrane, Multi-transmembrane	-7,585026741	1	99,4	2	-	2
ATCC_11454_ 1-deoxy-D-xylulose-5-phosphate synthase|Cytoplasmic	-11,50584507	2	57,9	3	-	3
ATCC_11454_Lasergene_contig254.fasta[4889-6739] prolyl-tRNA synthetase|Cytoplasmic	-8,047692299	1	69,1	3	-	2
ATCC_11454_Lasergene_ outer membrane lipoprotein	-12,86799717	10	31,1	6	1	3

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
precursor|Membrane, N-terminally anchored (with CS)						
ATCC_11454_ outer membrane lipoprotein precursor|Membrane, N-terminally anchored (No CS)	-39,87060165	26	31,2	14	5	7
ATCC_11454_ ferrichrome ABC transporter substrate binding protein|Extracellular, Lipid anchored	-23,38294029	18	34,2	8	-	6
ATCC_11454_|penicillin-binding protein 2a|Membrane, N-terminally anchored (No CS)	-41,62255859	13	74,3	10	-	8
ATCC_11454_zinc ABC transporter substrate binding protein|Extracellular, Lipid anchored	-21,40572739	15	30	11	-	6
ATCC_11454_autolysin|Cell wall associated (LysM domain)	-23,46354485	14	46,5	9	-	5
ATCC_11454_endo-1,4-beta-xylanase D|Membrane, N-terminally anchored (No CS)	-28,7718811	12	41,6	7	-	4
ATCC_11454_dipeptidase PepV|Cytoplasmic	-7,699143887	4	51,8	3	-	3
ATCC_11454_ |glyceraldehyde 3-phosphate dehydrogenase|Cytoplasmic	-25,88830948	7	35,9	7	-	7
ATCC_11454_ |N-acetylmuramidase|Cell wall associated (LysM domain)	-54,36312103	37	30,1	17	-	14
ATCC_11454_Lantibiotic nisin-Z	-199,5661316	43	5,9	548	-	26
ATCC_11454_ transcription regulator|Cytoplasmic /TMH start AFTER 60	-9,265760422	8	52,1	3	-	3
ATCC_11454_ D-alanyl-D-alanine carboxypeptidase|Membrane, N-terminally anchored (No CS)	-27,49382019	14	46,8	10	-	6
ATCC_11454_rod shape-determining protein MreC|Membrane, N-terminally anchored (with CS)	-42,15868378	21	31,3	9	-	7
ATCC_11454_AAA-ATPase, putative	-8,547906876	1	83,9	2	-	2
ATCC_11454_ (mucus-binding protein)	-94,56162262	29	39,6	48	-	22

Figure 3.3.3-4 *Proteins identified of Lactococcus lactis in medium from co-culture with*

Listeria monocytogenes

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
[gene=lmo2196] [protein=hypothetical protein] [protein_id=NP_465720.1]	-295,9704285	65	62,4	144	-	73
[gene=lmo0275] [protein=hypothetical protein] [protein_id=NP_463806.1]	-223,1373444	69	29,9	105	-	52
[gene=lmo2156] [protein=hypothetical protein] [protein_id=NP_465680.1]	-83,05193329	80	13	90	-	21
[gene=lmo0412] [protein=hypothetical protein] [protein_id=NP_463941.1]	-102,827858	36	31,2	90	-	22
[gene=pflB] [protein=pyruvate formate-lyase] [protein_id=NP_464931.1]	-150,5036011	35	83,7	80	-	45
[gene=lmo0355] [protein=fumarate reductase subunit A] [protein_id=NP_463885.1]	-105,3512955	53	54,3	65	-	29
[gene=lmo0135] [protein=peptide ABC transporter substrate-binding protein] [protein_id=NP_463668.1]	-135,5366669	48	58,2	63	-	28
[gene=lmo1634] [protein=bifunctional acetaldehyde-CoA/alcohol dehydrogenase] [protein_id=NP_465159.1]	-124,9096298	30	94,5	58	-	36
[gene=lmo0049] [protein=hypothetical protein] [protein_id=NP_463582.1]	-50,7807312	83	6,1	56	-	13
[gene=lmo0927] [protein=hypothetical protein] [protein_id=NP_464452.1]	-84,45317841	20	74,6	51	-	18
[gene=lmo0181] [protein=sugar ABC transporter substrate-binding protein] [protein_id=NP_463712.1]	-113,7911835	36	46,5	48	-	29
[gene=gap] [protein=glyceraldehyde-3-phosphate dehydrogenase] [protein_id=NP_465982.1]	-71,51019287	19	36,2	41	-	22
[gene=pflA] [protein=pyruvate formate-lyase] [protein_id=NP_465441.1]	-90,11711121	24	85,3	39	-	33
[gene=iap] [protein=invasion associated secreted endopeptidase] [protein_id=NP_464110.1]	-62,30820084	15	50,2	36	-	13
[gene=cydA] [protein=cytochrome D ubiquinol oxidase subunit I] [protein_id=NP_466240.1]	-66,47289276	28	52,7	36	-	18
[gene=atpE] [protein=ATP synthase F0F1 subunit C] [protein_id=NP_466057.1]	-94,17996216	47	7,1	33	-	13
[gene=tcsA] [protein=CD4+ T cell-stimulating antigen, lipoprotein] [protein_id=NP_464913.1]	-80,00270844	47	38,3	30	-	20
[gene=lmo1333] [protein=hypothetical protein] [protein_id=NP_464858.1]	-52,69665146	14	17,8	28	-	8
[gene=lmo0847] [protein=glutamine ABC transporter] [protein_id=NP_464373.1]	-37,95451736	3	53,2	24	-	6
[gene=lmo0723] [protein=metyl-accepting chemotaxis protein] [protein_id=NP_464250.1]	-40,08582687	13	65,7	24	-	12
[gene=lmo2637] [protein=hypothetical protein] [protein_id=NP_466160.1]	-40,94929123	39	32,6	21	-	12
[gene=lmo1585] [protein=peptidase] [protein_id=NP_465110.1]	-50,85908508	27	36,6	20	-	13
[gene=spl] [protein=peptidoglycan lytic protein P45] [protein_id=NP_466028.1]	-52,13805008	28	42,6	20	-	10

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
[gene=rpmE2] [protein=50S ribosomal protein L31] [protein_id=NP_466071.1]	-24,67240143	54	9,2	18	-	10
[gene=flgE] [protein=flagellar hook protein FlgE] [protein_id=NP_464224.1]	-59,86499786	20	42,7	18	-	12
[gene=lmo2754] [protein=D-alanyl-D-alanine carboxypeptidase] [protein_id=NP_466276.1]	-31,09364128	4	48	17	-	7
Lm_EGDe|NC_003210.1_cdsid_NP_463717.1 [gene=lmo0186] [protein=hypothetical protein] [protein_id=NP_463717.1]	-35,23162842	25	44,4	16	-	9
[gene=lmo0178] [protein=xylose repressor] [protein_id=NP_463709.1]	-43,02509689	11	44,9	16	-	9
[gene=lmo0644] [protein=hypothetical protein] [protein_id=NP_464171.1]	-22,10790443	2	69,1	15	-	2
[gene=lmo0625] [protein=hypothetical protein] [protein_id=NP_464152.1]	-25,31043053	12	26,5	15	-	5
[gene=lmo1666] [protein=peptidoglycan-linked protein] [protein_id=NP_465191.1]	-42,83107758	6	184,3	15	-	10
[gene=atpB] [protein=ATP synthase F0F1 subunit A] [protein_id=NP_466058.1]	-17,47401047	5	26,9	14	-	4
[gene=lmo1303] [protein=cell division suppressor] [protein_id=NP_464828.1]	-28,76209641	33	11,8	14	-	5
[gene=lmo0415] [protein=endo-1,4-beta-xylanase] [protein_id=NP_463944.1]	-27,24845886	12	52,4	14	-	7
[gene=rplS] [protein=50S ribosomal protein L19] [protein_id=NP_465312.1]	-14,73954678	17	13	13	-	3
[gene=rplQ] [protein=50S ribosomal protein L17] [protein_id=NP_466128.1]	-26,08486938	19	15,1	13	-	6
[gene=rpmA] [protein=50S ribosomal protein L27] [protein_id=NP_465065.1]	-9,909993172	25	10,5	12	-	4
[gene=lmo1249] [protein=hypothetical protein] [protein_id=NP_464774.1]	-31,84598732	30	9,1	12	-	7
[gene=rpsI] [protein=30S ribosomal protein S9] [protein_id=NP_466119.1]	-20,36712456	36	14,3	11	-	6
[gene=rplA] [protein=50S ribosomal protein L1] [protein_id=NP_463780.1]	-19,96768951	12	24,4	11	-	6
[gene=rpsM] [protein=30S ribosomal protein S13] [protein_id=NP_466131.1]	-19,20896149	25	13,6	10	-	6
[gene=lmo1527] [protein=preprotein translocase SecDF] [protein_id=NP_465052.1]	-30,62588882	7	82,5	10	-	8
[gene=lmo0152] [protein=peptide ABC transporter substrate-binding protein] [protein_id=NP_463685.1]	-18,30381012	8	61,9	10	-	6
[gene=rpsE] [protein=30S ribosomal protein S5] [protein_id=NP_466138.1]	-30,73596001	21	17,4	10	-	7
[gene=flaA] [protein=flagellin] [protein_id=NP_464217.1]	-29,05342484	10	30,3	10	-	6
[gene=ftsL] [protein=cell division protein FtsL] [protein_id=NP_465564.1]	-12,87863731	28	13,6	9	-	6

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
[gene=fliP] [protein=flagellar biosynthesis protein FliP] [protein_id=NP_464203.1]	-18,53342819	8	28,6	9	-	3
[gene=lmo0130] [protein=5'-nucleotidase] [protein_id=NP_463663.1]	-23,37744522	5	82,4	9	-	5
[gene=eno] [protein=phosphopyruvate hydratase] [protein_id=NP_465978.1]	-22,45306206	14	46,4	9	-	7
[gene=tuf] [protein=elongation factor Tu] [protein_id=NP_466175.1]	-24,21827507	13	43,2	9	-	6
[gene=lmo2518] [protein=LytR family transcriptional regulator] [protein_id=NP_466041.1]	-21,2093277	12	39	8	-	5
[gene=fliF] [protein=flagellar MS-ring protein FliF] [protein_id=NP_464240.1]	-25,99409485	8	59,9	8	-	4
[gene=lmo1438] [protein=penicillin-binding protein] [protein_id=NP_464963.1]	-19,81234169	10	79,8	8	-	6
[gene=lmo0217] [protein=DivIC protein] [protein_id=NP_463748.1]	-14,54817677	13	14,9	8	-	4
[gene=lmo1318] [protein=hypothetical protein] [protein_id=NP_464843.1]	-20,40206718	7	46,6	8	-	7
[gene=murC] [protein=UDP-N-acetylmuramate--L-alanine ligase] [protein_id=NP_465130.1]	-20,69671059	10	49,9	7	-	6
[gene=lmo1216] [protein=N-acetylmuramoyl-L-alanine amidase] [protein_id=NP_464741.1]	-12,40818787	8	36,2	7	-	5
[gene=rpsH] [protein=30S ribosomal protein S8] [protein_id=NP_466141.1]	-20,84308815	21	14,6	7	-	4
[gene=dltD] [protein=DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid] [protein_id=NP_464496.1]	-22,93544769	12	48,4	7	-	4
[gene=lmo1250] [protein=antibiotic resistance protein] [protein_id=NP_464775.1]	-22,78182411	13	44,1	6	-	6
Lm_EGDe|NC_003210.1_cdsid_NP_463711.1 [gene=lmo0180] [protein=sugar ABC transporter permease] [protein_id=NP_463711.1] [location=180052..180900]	-27,79893494	11	31,5	6	-	6
[gene=murA] [protein=UDP-N-acetylglucosamine 1-carboxyvinyltransferase] [protein_id=NP_466049.1]	-18,48212242	12	45,9	6	-	5
Lm_EGDe|NC_003210.1_cdsid_NP_465743.1 [gene=lmo2219] [protein=foldase] [protein_id=NP_465743.1] [location=2306833..2307714]	-19,46470642	13	32,6	6	-	4
Lm_EGDe|NC_003210.1_cdsid_NP_463581.1 [gene=lmo0048] [protein=sensor histidine kinase AgrB] [protein_id=NP_463581.1] [location=51775..52389]	-9,187602043	5	23,3	6	-	3
[gene=cysK] [protein=cysteine synthase] [protein_id=NP_463754.1]	-19,67457771	9	32,1	6	-	4
[gene=lmo2638] [protein=NADH dehydrogenase] [protein_id=NP_466161.1]	-15,77337646	10	70,3	6	-	6
[gene=hly] [protein=listeriolysin O precursor] [protein_id=NP_463733.1]	-5,825649261	6	58,6	6	-	2
[gene=lmo0441] [protein=D-alanyl-D-alanine carboxypeptidase] [protein_id=NP_463970.1]	-25,44439316	9	74,5	6	-	5
[gene=lmo2229] [protein=penicillin-binding protein] [protein_id=NP_465753.1]	-14,31568813	9	77,7	6	-	4

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
[gene=lmo0098] [protein=PTS mannose transporter subunit IID] [protein_id=NP_463631.1]	-9,323287964	13	33,3	5	-	4
[gene=lmo0319] [protein=phospho-beta-glucosidase] [protein_id=NP_463849.1]	-12,50440025	4	53,5	5	-	3
[gene=lmo1240] [protein=hypothetical protein] [protein_id=NP_464765.1]	-11,48085499	9	19,7	5	-	4
[gene=rplP] [protein=50S ribosomal protein L16] [protein_id=NP_466148.1]	-6,537003517	7	16,1	4	-	2
[gene=fbaA] [protein=fructose-1,6-bisphosphate aldolase] [protein_id=NP_466079.1]	-8,38111496	7	30	4	-	3
[gene=rpmB] [protein=50S ribosomal protein L28] [protein_id=NP_465341.1]	-4,939302444	17	6,9	4	-	2
[gene=glnA] [protein=glutamine synthetase] [protein_id=NP_464824.1]	-13,95155811	4	50,3	4	-	3
[gene=pbpA] [protein=penicillin-binding protein 2A] [protein_id=NP_465416.1]	-12,78728008	5	90,7	4	-	4
[gene=dltA] [protein=D-alanine--poly(phosphoribitol) ligase subunit 1] [protein_id=NP_464499.1]	-9,501207352	5	58	4	-	4
[gene=lmo2192] [protein=peptide ABC transporter ATP-binding protein] [protein_id=NP_465716.1]	-15,23062229	7	36,5	4	-	2
[gene=lmo0724] [protein=hypothetical protein] [protein_id=NP_464251.1]	-14,99782372	7	26,7	4	-	4
[gene=lmo2504] [protein=cell wall-binding protein] [protein_id=NP_466027.1]	-12,13197041	10	46,9	4	-	4
[gene=lmo0132] [protein=inosine 5-monophosphate dehydrogenase] [protein_id=NP_463665.1]	-9,717785835	4	54,9	4	-	3
[gene=lmo2362] [protein=amino acid antiporter] [protein_id=NP_465885.1]	-13,57740974	4	55	4	-	3
[gene=lmo1068] [protein=hypothetical protein] [protein_id=NP_464593.1]	-17,40723038	5	30,7	4	-	4
[gene=lmo0462] [protein=hypothetical protein] [protein_id=NP_463991.1]	-6,977571487	8	16,8	4	-	2
[gene=lmo0560] [protein=glutamate dehydrogenase] [protein_id=NP_464088.1] [location=complement(598968..600344)]	-15,11139393	3	49,1	4	-	4
[gene=lmo0778] [protein=hypothetical protein] [protein_id=NP_464305.1]	-10,13140297	24	13,5	4	-	3
[gene=rpsS] [protein=30S ribosomal protein S19] [protein_id=NP_466151.1]	-6,799423218	13	10,4	3	-	2
[gene=rpsK] [protein=30S ribosomal protein S11] [protein_id=NP_466130.1]	-9,243211746	19	13,7	3	-	3
[gene=rpoA] [protein=DNA-directed RNA polymerase subunit alpha] [protein_id=NP_466129.1]	-11,32975483	4	34,8	3	-	2
[gene=lmo1511] [protein=hypothetical protein] [protein_id=NP_465036.1]	-5,432526588	9	26,7	3	-	3
[gene=plcA] [protein=phosphatidylinositol-specific phospholipase c] [protein_id=NP_463732.1]	-14,35971737	8	36,2	3	-	3

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques
[gene=guaB] [protein=inosine-monophosphate dehydrogenase] [protein_id=NP_466280.1]	-8,721868515	2	52,4	3	-	3
[gene=pnpA] [protein=polynucleotide phosphorylase] [protein_id=NP_464856.1]	-5,70005703	2	79,4	3	-	3
[gene=lmo0727] [protein=glucosamine--fructose-6-phosphate aminotransferase] [protein_id=NP_464254.1]	-10,67985439	4	65,6	3	-	3
[gene=lmo2360] [protein=transmembrane protein] [protein_id=NP_465883.1]	-6,301934242	3	97	3	-	3
[gene=rplO] [protein=50S ribosomal protein L15] [protein_id=NP_466136.1]	-9,649751663	7	15,7	3	-	2
[gene=fus] [protein=elongation factor G] [protein_id=NP_466176.1]	-10,64550781	1	76,7	3	-	2
[gene=lysS] [protein=lysyl-tRNA synthetase] [protein_id=NP_463759.1]	-6,953520775	6	57,3	3	-	3
[gene=lmo1067] [protein=GTP-binding elongation factor] [protein_id=NP_464592.1]	-10,44733143	2	68,6	3	-	3
[gene=fliQ] [protein=flagellar biosynthesis protein FliQ] [protein_id=NP_464204.1]	-7,398374557	13	10,1	2	-	2
[gene=rplF] [protein=50S ribosomal protein L6] [protein_id=NP_466140.1]	-5,909389496	12	19,3	2	-	2
[gene=rplN] [protein=50S ribosomal protein L14] [protein_id=NP_466145.1]	-7,568636417	11	13,1	2	-	2
[gene=atpC] [protein=ATP synthase F0F1 subunit epsilon] [protein_id=NP_466051.1]	-5,806875229	9	14,6	2	-	2
[gene=gltX] [protein=glutamyl-tRNA synthetase] [protein_id=NP_463768.1]	-5,41702795	2	55,9	2	-	2
[gene=rplV] [protein=50S ribosomal protein L22] [protein_id=NP_466150.1]	-2,776763678	16	12,8	2	-	2
[gene=serS] [protein=seryl-tRNA synthetase] [protein_id=NP_466269.1]	-4,113509178	2	49	2	-	2
[gene=rpmI] [protein=50S ribosomal protein L35] [protein_id=NP_465309.1]	-7,142667294	17	7,6	2	-	2
[gene=lmo1499] [protein=hypothetical protein] [protein_id=NP_465024.1]	-5,690370083	5	40	2	-	2
[gene=lmo0516] [protein=encapsulation protein CapA] [protein_id=NP_464044.1]	-19,31875801	3	53,2	2	-	2
[gene=asnC] [protein=asparaginyl-tRNA synthetase] [protein_id=NP_465420.1]	-2,919373512	2	48,9	2	-	2
[gene=lmo1384] [protein=hypothetical protein] [protein_id=NP_464909.1]	-5,905878544	3	36,3	2	-	2
[gene=pbpB] [protein=penicillin-binding protein 2B] [protein_id=NP_465563.1]	-5,267606258	1	81,7	2	-	2

Figure 3.3.3-5 *Proteins identified in medium from Listeria monocytogenes monoculture*

Description	log(E value)	Coverage	MW	Spectra	Specific uniques	Uniques

[gene=lmo0355] [protein=fumarate reductase subunit A] [protein_id=NP_463885.1]	-20,3242588	14	54,3	12	4	5
[gene=iap] [protein=invasion associated secreted endopeptidase] [protein_id=NP_464110.1]	-4,973058224	3	50,2	2	-	2
[gene=lmo1067] [protein=GTP-binding elongation factor] [protein_id=NP_464592.1]	-2,973058224	3	68,6	2	-	2
[gene=lmo2156] [protein=hypothetical protein] [protein_id=NP_465680.1]	-18,921978	42	13	15	-	3
[gene=lmo2196] [protein=hypothetical protein] [protein_id=NP_465720.1]	-9,789146423	8	62,4	3	-	3
[gene=rpoA] [protein=DNA-directed RNA polymerase subunit alpha] [protein_id=NP_466129.1]	-13,3391819	4	34,8	4	-	3
[gene=rpsH] [protein=30S ribosomal protein S8] [protein_id=NP_466141.1]	-13,86889458	27	14,6	6	3	4

Figure 3.3.3-6 *Proteins identified of Listeria monocytogenes in medium from co-culture with*

Lactococcus lactis

3.4 Discussion

The most significant result obtained through the proteomics analysis (one-dimensional and two-dimensional electrophoresis) of filtered cell is the expression of enolase of *L. monocytogenes* in the condition of co-culture. Enolase increases by at least 30 percent during the co-culture. The enolase is an intermediate glycolytic enzyme, particularly it converts 2-phosphoglycerate in phosphoenolpyruvate. Regarding its role during the co-culture, interesting information underline how the protein secreted into the co-culture medium could serve as a signal extracellular. Infact it is known that cell membrane proteins can act as signals for uptake of nutrients in the medium. Furthermore, enolase belongs to a group "moonlight proteins" which are proteins, normally cytosolic, secreted on the wall under certain conditions (mainly to increase the virulence or improve the adhesion to a host) and released subsequently in the medium.

A moonlighting protein is a single protein that has multiple functions that are not due to gene fusions, multiple RNA splice variants or multiple proteolytic fragments. Moonlighting proteins do not include families of homologous proteins if the different functions are performed by different proteins in the protein family. They also do not include proteins that have multiple cellular roles but use the same biochemical function in each role. A single protein with multiple functions might seem surprising, but there are actually more than 100 examples of proteins that 'moonlight'.

Moonlighting proteins are highly conserved cytoplasmic proteins, present on the bacterial surface that can have multiple functions depending on the type of cell in which they are expressed. A large number of works have shown that these proteins, which lack of transposed signal sequences to the cell surface and anchoring to the membrane mechanisms, are located on the surface of microbial pathogens[111] and are mostly involved in metabolic pathways and mechanisms in response to cellular stress. Among the different bacterial Moonlighting proteins now identified, it is increasingly clear that most play an important role in bacterial virulence[112]. Moreover, in some pathogens which cause caries, enolase is a molecular target of fluoride ions[113]. That is could suggest to monitor the fluoride ions concentration in processing facilities.

Considering the peptidomic analysis, a very important result obtained is the identification of the protein Elongation factor Tu of *Lactococcus lactis* which increases in condition of competition. Elongation factor Tu also belongs to the group of moonlighting

proteins, in fact these proteins was recently identified as an actor in adhesion to epithelial cells at the surface of *Lactobacillus johnsonii*. Another interesting result, obtained from peptidomic analysis, is related to the identification of Nisin Z. In fact, peptides derived fron Nisin Z of *Lactococcus lactis* were detected in condition of competition. On the contrary, peptides derived from Nisin A of *Lactococcus lactis* were detected in *Lactococcus lactis* monoculture. So, in this manner, Lac in competition swiches from Nisin A to Nisin Z production. It is a very interesting result because there is no evidence in the literature that describes this change of Nisin production. Infact, in general each strain of LAB owns genetic information related only one protein variant Nisin so usually each strain is capable to produce one variant of Nisin active against a wide range of Gram positive bacteria[31].

4 CHAPTER 2

4.1 Untargeted and targeted proteomics analysis: validation in milk

A strain of *Lactococcus lactis subsp lactis ATCC 11454* has been used in this work to study antimicrobial activity against *Listeria monocytogenes* through the study of each secretome in microbial competition. *Listeria monocytogenes* strain plays an important role in the field of food Safety because continues to be a relevant pathogen in dairy products, as several recent listeriosis outbreaks were linked to cheeses [114, 115]

In order to highlight these mechanisms of microbial competition, the secretome of these microrganisms has been studied through a proteomic approach of the secretome. The present work focused on a first untargeted proteomics analysis in vitro, followed by validation directly in a system resuming cheese.

4.2 Materials and methods

Strain of *Lactococcus lactis ATCC 11454* and strain *of Listeria monocytogens (ATCC 19115*), were cultivated in appropriate medium cultures (BHI), alone and also in competition. Filtrated cultures (SECRETOME) were lyophilized and resuspended for proteomics analysis. Shotgun analysis on each secretome was performed on nano UPLC-MS system (Waters Corp., Manchester, UK).

4.2.1   Bacterial strains and culture condition

Strains Lactococcus lactis ATCC 11454 and Listeria monocytogenes ATCC 19115, obtained *from the American* type Culture Collection, were provided by IZSLER. They were stored at -80°C in BHI broth containing 20% glycerol.

Both strains were grown in brain heart infusion (BHI) broth at 30°C for 24 hrs. 1 ml of *Listeria monocytogenes* ATCC 19115 preculture (1ml) was inoculted in 100 ml of BHI broth and incubated at 37°C for 24h. 1 ml of preculture Preculture of *Lactococcus lactis* ATCC 11454 (1ml) was inoculted in 100 ml of BHI and incubated at 37°C for 24h. 0.5 ml of *Listeria monocytogenes* preculture (10^3 ufc/ml) and 0.5 ml of *Lactococcus lactis* preculture (10^8 ucf/ml) were inoculated in 100 ml of BHI for coculture and incubated at 37°C for 24h. Listeria monocytogenes inoculated in BHI after 5 hours of growth of LC ATCC, incubated at 37°C.

4.2.2   Supernatant and lyophilized pellets preparation

Cultures were centrifuged (10000 g, 20 min, 4°C) and supernatants were recovered. Supernatant was filtered using PDVF membrane 0.45µm. The pellet was washed in 5 ml of physiological saline, centrifuged at 10000 g for 20 minutes at 4°C and the supernatant was discarded, the pellet was resuspended in 2 ml of physiological saline and lyophilized. For each experimental group, 100 ml of lyophilized cultures medium taken up in 8 ml of H_2O. The proteins present in the supernatant were purified and concentrated through precipitation with methanol/chloroform/water. The pellet was solubilized in 6 M Urea, 100mMTris pH 7.5

The experimental groups analyzed are the following:

1. 3 BHI (brain heart infusion)

2. 3 BHI with *Lactococcus lactis*

3. 3 BHI with *Listeria Monocytogenes*

4. 3 BHI and *Lactococcus* in co-culture with Listeria (LM inoculated in BHI after 5 hours of growth of LC ATCC, incubated at 37 ° C).

4.2.3   Protein assay

Protein concentration was determined by Bradford assay. Optical density was measured at 595 nm using a spectrophotometer (Gene Quant 100, GE Healthcare) and protein concentration was determined against Bovine Serum Albumin (BSA, Thermo Scientific) used as standard. A standard curve was prepared with different BSA concentrations from 1 to 20 ug. The proteins were quantified with Spectophotometer using the method of Bradford with reading of the samples in triplicate at 595nm. The amount of protein was calculated by interpolation of the experimental values with standard proteins to known quantity.

4.2.4   Protein digestion

Reduction and alkylation of proteins were obtained by adding 100 mM DTT (1 h at 37°C) and 200 mM iodoacetamide (1 h at RT). Protein samples were digested with 1:20 (w/w) sequence grade trypsin (Promega, Madison, WI, USA) at 37°C overnight. Reactions were stopped by adding 0.1% (v/v) TFA.

4.2.5   Chromatography and mass spectrometry

0.6 µg of digested proteins were loaded on nanoACQUITY UPLC System (Waters Corp., Milford, MA) coupled to a Q-Tof Premier mass spectrometer (Waters Corp., Manchester, UK). Saccharomyces cerevisiae Enolase (ScEnolase) digestion was added to samples as an internal standard. Tryptic peptides were trapped and desalted onto a Symmetry C18 5 µm, 180 µm x 20 mm precolumn (Waters Corp.) and subsequently separated using a NanoEase BEH C18 1.7 µm, 75 µm x 25 cm nanoscale LC column (Waters Corp.)

operating at 35°C. Peptide separation was obtained by a gradient of 3–40% B over 150 min at a flow rate of 250 nL min-1, followed by a gradient of 40–90% B over 5 min and a 15 min rinse with 90% B (phase A: water with 0.1% formic acid; phase B: 0.1% formic acid in acetonitrile). The Q-Tof Premier mass spectrometer operated in ''Expression Mode'' switching between low (4 eV) and high (15–40 eV) collision energies with a scan time of 0.8 s over 50–1990 m/z mass range.

All analysis was made on the biological and technical triplicate.

4.2.6   Database search

LC-MS/MS data were processed using ProteinLynx Global-Server (version 3.0.2 PLGS, Waters). Protein identifications were obtained with the embedded ion accounting algorithm of PLGS software searching against *Listeria* or *Lactococcus* or *Listeria monocytogenes + Lactococcus lactis* database (UniprotKB/Swiss-Prot Protein Knowledgebase) to which the sequence of ScEnolase (P00924) was appended. Parameters for the database search were: automatic tolerance for precursor ions, automatic tolerance for product ions, minimum 3 fragment ions matched per peptide, minimum 7 fragment ions matched per protein, minimum 2 peptide matched per protein, 1 missed cleavage, carbamydomethylation of cysteines and oxidation of methionines as fixed and variable modifications. The false positive rate (FPR) of the identification algorithm was set under 1%.

4.2.7   Protein expression profiling

Quantitative analysis was performed by the PLGS dedicated tool adding to each sample as internal standard digestion of Enolase from Saccharomyces cerevisiae (100 fmol) according to Silva JC et al[28]. Identified proteins were normalized against P00924 entry (ScEnolase) while the most reproducible peptides for retention time and intensity deriving from ScEnolase digestion were used to normalize the EMRTs table. The list of normalized proteins was screened according to the following criteria: protein identified in at least 2 out of 3 runs of the same sample; proteins with a 0 < P > 0.05 or 0.95 < P > 1, and proteins with a ratio of expression level +/- 0.30 on a natural log scale.

4.2.8   Validation

The experimental conditions are exactly the same used in culture media experiments: *Listeria monocytogenes* and *Lactococcus lactis (ATCC 11454)* as monocultures and both in coculture growing in the fluid milk.

In order to better investigate the milk protein profile, all samples were depleted from caseins as described in [116] ,depleted samples (milk, milk with *Listeria monocytogenes*, *Lactococcus lactis* with milk, milk with *Listeria monocytogenes and Lactococcus lactis*) were subjected to a proteomic analysis. In particular, the "label free shotgun analysis" was conducted by nano UPLC-MS system (Waters), in which 0.6 µg of each sample were separated by chromatography coupled to mass spectrometry. Each analysis was performed in triplicate biological and technical, processed and analyzed using the software PLGS (version 3.0.2, Waters).

4.3 Results and Discussion

In this work the strain of *Lactococcus lactis ATCC 11454* has been used to study its antimicrobial activity against *Listeria monocytogenes* through the study of each secretome in microbial competition. *L. monocytogenes* strain plays an important role in the field of food Safety because *L. monocytogenes* continues to be a relevant pathogen in dairy products. In order to highlight these mechanisms of microbial competition, the secretome of these microorganisms has been studied through a proteomic approach of the secretome. The present work focused on an untargeted label free shotgun analysis in vitro followed by validation in in a system resuming cheese-making (milk).

4.3.1   Expression analysis by nLC-MS^e

In table 4.3.1_1 the quantitative analysis of differentially expressed proteins of *Listeria monocytogenes* in BHI monoculture (A) and in coculture (B) with *Lactococcus lactis ATCC 11454*, identified by Label-free nUPLC-MS/MS, is shown.

Accession[a]	Description	PLGS Score	Amount (fmol)	St dev
C1KVG6	Aspartate tRNA ligase OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN aspS PE 3 SV 1	44.52	7.07	0.01
C1KY94	Enolase OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN eno PE 3 SV 1	147.86	69.01	0.15
P21171	Probable endopeptidase p60 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN i	72.54	254.40	0.57
Q71WB8	Elongation factor G *Listeria monocytogenes* serotype 4b (strain F2365)	68.74	n.q	n.q
Q71XG7	Tryptophan--tRNA ligase *Listeria monocytogenes* serotype 4b strain F2365	62.83	n.q	n.q
Q720K2	Uracil DNA glycosylase 2 OS *Listeria monocytogenes* serotype 4b strain F2365 GN ung2 PE 3 SV 1	63.53	26.19	0.34
Q8Y9X7	Uracil DNA glycosylase 1 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN ung	99.33	11.56	0.18
Q8YAA3	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN	1731.26	326.21	4.76

Table A

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
B8DHE7	Septation ring formation regulator EzrA OS *Listeria monocytogenes* serotype 4a strain HCC23 GN ezrA	75.36	10.88	0.09
C1KY63	Glycine cleavage system H protein OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN gcvH P	52.26	97.50	0.05
C1KY94	Enolase OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN eno PE 3 SV 1	375.00	177.94	0.65
C1KYI0	50S ribosomal protein L11 OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN rplK PE 3 SV 1	1051.00	125.68	0.53
C1KYI3	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN rplL PE 3 S	2020.00	406.79	0.04
C1L2V9	DNA mismatch repair protein MutS OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN mutS PE	162.00	36.41	0.00
G2JZ74	D alanine aminotransferase OS *Listeria monocytogenes* serotype 1 2a strain 10403S GN dat PE 3 SV 1	104.00	15.88	0.03

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
Q71X61	Glucose 6 phosphate isomerase OS *Listeria monocytogenes* serotype 4b strain F2365 GN pgi PE 3 SV 1	188.56	27.29	0.11
Q8Y4L2	Glycine cleavage system H protein OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD	89.04	n.q	n.q
P21171	Probable endopeptidase p60 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN i	72.54	n.q	n.q
Q71WB8	Elongation factor G *Listeria monocytogenes* serotype 4b (strain F2365)	68.74	n.q	n.q
Q71XG7	Tryptophan--tRNA *ligase Listeria monocytogenes* serotype 4b strain F2365	62.83	n.q	n.q
Q8Y9X7	Uracil DNA glycosylase 1 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN ung	63.53	10.36	0.25
Q8YAA3	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN	1731.26	313.86	3.82

Table B

Table 4.3.1_1. **A)** quantitative analysis of differentially expressed proteins identified in medium from *Listeria monocytogenes* monoculture **B)** and medium from co-culture with *Lactococcus lactis*. a SwissProt/UniprotK accesion number; the terms nq means below instrumental detection limit.

In figure 4.3.1-1 data obtained by PLGS analysis are shown, comparing monoculture vs coculture of *Listeria monocytogenes*
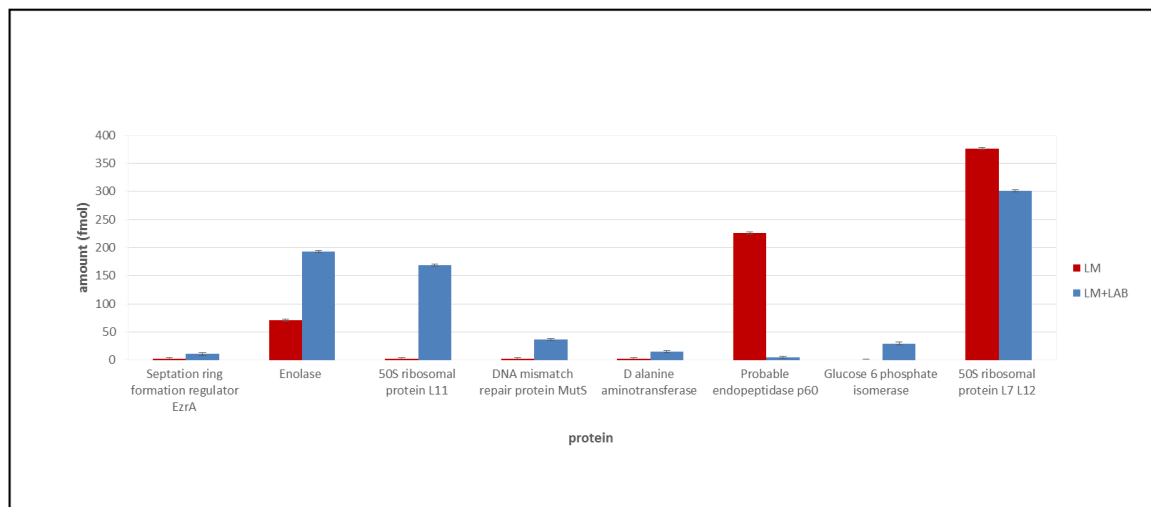
Figure 4.3.1-2 Some of differentially expressed proteins of *Listeria monocytogenes* in BHI coculture with *Lactococcus lactis* identified by Label-free nUPLC-MS/MS

In according with obtained preliminary data, obtained data highlighted, during competition, the higher production by *Listeria monocytogenes* of moonlighting protein Enolase (C1KY94) and Glucose 6 Phosphate isomerase (Q71X61) , of Septation ring formation regulator EzrA (B8DHE7), involved into cell replication in regulatory mechanisms of cell energetics or metabolism and the lower secretion Endopeptidase P60 (P21171), protein associated with the cell surface and involved in the process of invasion.

Among these, the most significant result obtained is that Enolase increases by at least 30 percent during the co-culture. The enolase is an intermediate glycolytic enzyme; particularly it converts 2-phosphoglycerate in phosphoenolpyruvate. Regarding its role during the co-culture, interesting information underline how the protein secreted into the co-culture medium could serve as extracellular signal. Infact it is known that cell membrane proteins can act as signals for uptake of nutrients in the medium. Furthermore, enolase belongs to a group "moonlight proteins" which are proteins, normally cytosolic, secreted on the wall under certain conditions (mainly to increase the virulence or improve the adhesion to a host) and released subsequently in the medium [29]. Major groups of proteins that moonlight in bacterial virulence include the

following: the metabolic enzymes of the glycolytic pathway; enzymes of other metabolic pathways such as the glyoxylate cycle, and molecular chaperones and protein-folding catalysts. Among the most commonly identified moonlighting virulence functions of bacterial proteins are adhesion and modulation of leukocyte activity[112].

Moreover, in some pathogens which cause caries, enolase is a molecular target of fluoride ions[113]. A number of studies have shown that fluoride inhibits glycolysis and reduces acid production[117]. This antimicrobial effect of fluoride has been suggested by some to be due to the inhibition of the glycolytic enzyme enolase.

That is could suggest to monitor the fluoride ions concentration in processing facilities.

In Table 4.3.1-2 the quantitative analysis of differentially expressed proteins of *Lactococcus lactis ATCC 11454* in BHI monoculture and in coculture with *Listeria monocytogens*, identified by Label-free nUPLC-MS/MS, are show.

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
P13068	Lantibiotic nisin A OS *Lactococcus lactis* subsp lactis GN spaN PE 1 SV 1	600.4	56.00	2.07
P22865	Secreted 45 kDa protein OS *Lactococcus lactis subsp cremoris* strain MG1363 GN usp45 PE 1 SV 3	214.8	nq	nq
Q9CG42	50S ribosomal protein L7 L12 OS *Lactococcus lactis subsp lactis* strain IL1403 GN rplL PE 3 SV 1	735.7	12.68	0.03
Q9CI64	DNA binding protein HU OS *Lactococcus lactis subsp lactis* strain IL1403 GN hup PE 3 SV 1	876.2	nq	nq

Table A

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
D2BNK8	Uncharacterized protein OS *Lactococcus lactis* subsp lactis strain KF147 GN usp PE 4 SV 1	456	n.q	n.q
F2HKA4	Nisin NisinA OS *Lactococcus lactis subsp lactis* strain CV56 GN nisA PE 4 SV 1	2568	n.q	n.q
H5SXJ3	GTNG 0265 lantibiotic antimicrobial peptinisin Ade OS *Lactococcus lactis* subsp lactis IO 1 GN nisA	2804	n.q	n.q
P22865	Secreted 45 kDa protein OS *Lactococcus lactis* subsp cremoris strain MG1363 GN usp45 PE 1 SV 3	452	20.96	3.46
P29559	Lantibiotic nisin Z OS *Lactococcus lactis* subsp lactis GN nisZ PE 1 SV 1	2864	768.47	0.51
Q45RP0	Lantibiotic nisin A Fragment OS *Lactococcus lactis* subsp lactis GN nis PE 4 SV 1	2427	n.q	n.q

Table B

Table 4.3.1-2. A) quantitative analysis of differentially expressed protein identified in medium from *Lactococcus lactis* monoculture B) and medium from co-culture with *Listeria monocytogenes*. a SwissProt/UniprotK accesion number; the terms nq means below instrumental detection limit.

In figure 4.3.1-3 data obtained by PLGS analysis are shown comparing monoculture vs coculture of *Lactococcus lactis ATCC 11454*.
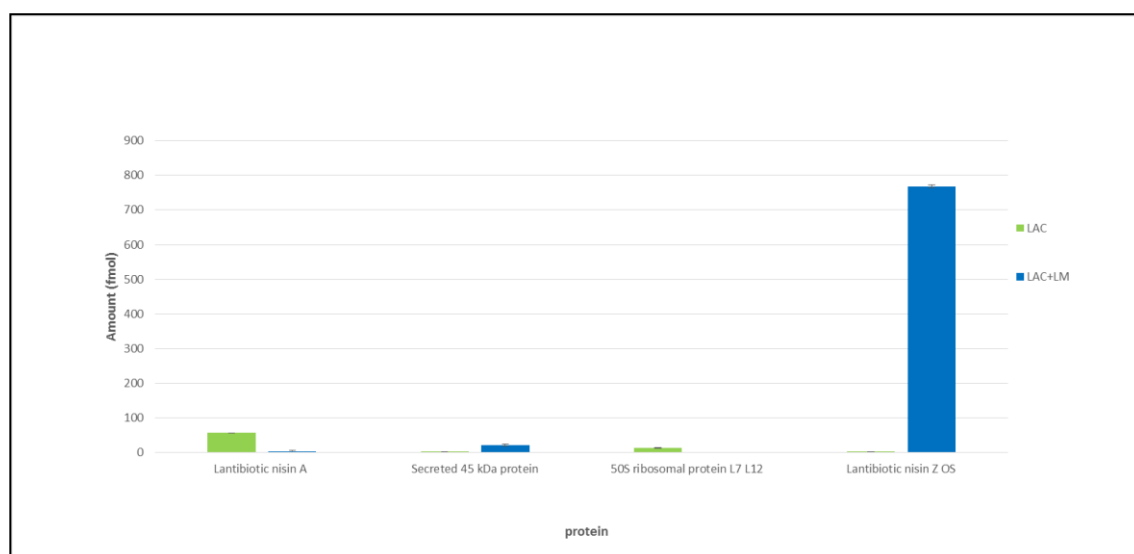
Figure 4.3.1-3 Some differentially expressed proteins of *Lactococcus lactis* in BHI coculture with *Listeria monocytogenes* identified by Label-free nUPLC-MS/MS.

The analysis shotgun showed, as important result, that *L. lactis* produced higher amounts of Secreted 45 kDa protein (P22865), protein with hypothetical peptidoglycan lytic activity, and switched from lantibiotic Nisin A (P13068) production to Nisin Z (Q7DH25) production. It is a very interesting result, already obtained from the peptidomics analysis, because there is no evidence in the literature..

Nisin is a prototype bacteriocin produced by *Lactococcus lactis*, used as a preservative in the food industry for making dairy products,. Nisin A is the first to be discovered[23] and other natural variants of this protein are F, Q, U and Z[24].In general each strain of *LAB* owns genetic information related only one protein variant Nisin so usually each strain is capable to produce one variant of Nisin active against a wide range of Gram positive bacteria.

Nisin Z differs from Nisin A by just one amino acid residue at position 27 because of a single nucleotide substitution. Nisin A contains histidine, and nisin Z asparagine. This residue allows to nisin Z a improved solubility at higher pH values respect to nisin A[31].

So, in competition with *Listeria monocytogenes*, the investigated *L. lactis* strain produce higher amounts of Secreted 45 kDa protein with peptidoglycan lytic activity and the selective secretion of NisinZ in order to enhance lantibiotic solubility in less acidic environment. The demonstrated features of this L. lactis strain through that proteomics approach may help in the additives-free listeriosis prevention.

4.3.2   Validation

It has been applied label-free proteomics strategy to evaluate the expression profile of candidate proteins in milk. In Table 4.3.2-1 and Table 4.3.2-2 the relative quantitative analysis of differentially expressed proteins respectively of *Listeria monocytogenes* and *Lactococcus lactis ATCC 11454* in milk monoculture and in coculture, identified by Label-free nUPLC-MS/MS, are show.

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
B8DF04	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serotype 4a strain HCC23 GN rplL PE 3 SV 1	168,63	313.86	0.22
C1KYI3	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serotype 4b strain CLIP80459 GN rplL PE 3 S	162,26	406.787	0.16
P21171	Probable endopeptidase p60 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN i	126,99	10.88	0.05
Q724G1	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serotype 4b strain F2365 GN rplL PE 3 SV 1	107,26	313.86	0.23
Q8YAA3	50S ribosomal protein L7 L12 OS *Listeria monocytogenes* serovar 1 2a strain ATCC BAA 679 EGD e GN	137,57	125.67	0.16
Q71XG7	Tryptophan--tRNA ligase *Listeria monocytogenes* serotype 4b strain F2365	73,83	10.36	0.05

Table 4.3.2-1A

Accession[a]	Description	PLGS Score	Amount (fmol)	St dev
B8DDD3	Glycine cleavage system H protein OS *Listeria monocytogenes* serotype 4a strain HCC23 GN gcvH PE 3	42,42	97.49	0.15
B8DF07	50S ribosomal protein L11 OS *Listeria monocytogenes* serotype 4a strain HCC23 GN rplK PE 3 SV 1	816,03	300.74	0.07
Q02129	ATP phosphoribosyltransferase *Listeria monocytogenes* serotype 4a strain HCC23 GN rplK PE 3 SV 1	818,28	14.96	0.08
Q71X61	Glucose 6 phosphate isomerase OS *Listeria monocytogenes* serotype 4b strain F2365 GN pgi PE 3 SV 1	88,56	80.73	0.05

Table 4.3.2-1B

Table 4.3.2-1A)Relative quantitative analysis of differentially expressed proteins identified in milk from *Listeria monocytogenes* monoculture Table 4.3.2-1B) and milk from co-culture with *Lactococcus lactis* a SwissProt/UniprotK accession number; the terms nq means below instrumental detection limit.

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
P13068	Lantibiotic nisin A OS *Lactococcus lactis* subsp lactis GN spaN PE 1 SV 1	62,50	23.7	0.1
Q02129	ATP phosphoribosyltransfer ase *Lactococcus lactis* subsp lactis strain IL1403 GN usp45	818,28	12.4	0.33
D2BPR6	NADH dehydrogenase OS *Lactococcus lactis* subsp lactis strain IL1403 GN usp45	660,72	52.4	0.14

Table 4.3.2-2A)

Accessiona	Description	PLGS Score	Amount (fmol)	St dev
Q9CDJ1	Putative uncharacterized protein usp45 OS *Lactococcus lactis subsp lactis* strain IL1403 GN usp45	660,72	20.4	0.23
D2BNK8	Uncharacterized protein OS *Lactococcus lactis subsp lactis* strain KF147 GN usp PE 4 SV 1	660,72	n.q	n.q
P29559	Lantibiotic nisin Z OS *Lactococcus lactis subsp lactis* GN nisZ PE 1 SV 1	49,04	168.42	0.08

Table 4.3.2-2B)

Table 4.3.2-2A) Relative quantitative analysis of differentially expressed proteins identified in milk from *Lactococcus lactis* monoculture and (*4.3.2-2B)*) milk from co-culture with *Listeria monocytogenes.* a SwissProt/UniprotK accession number; the terms nq means below instrumental detection limit.

Among these, we selected, for their importance, glucose 6-phosphate isomerase of LM (Figure 4.3.2-1) and Nisin A-Z of LAC (Figure 4.3.2-2). Glucose 6 Phosphate isomerase increases during the condition of competition; it belongs to a "moonlighting proteins"group, proteins associated with pathogen higher virulence and proteolytic activity. Moreover, shotgun analysis in milk confirmed that, during competition, LAC ATCC switched from Nisin A production to Nisin Z. In according with obtained preliminary data, the validation in the milk of candidate's proteins confirms the hypothesis regarding the adaptation of LM and LAC ATCC in competition. LAC ATCC strain used the selective secretion of NisinZ in order to enhance lantibiotic solubility in less acidic environment.
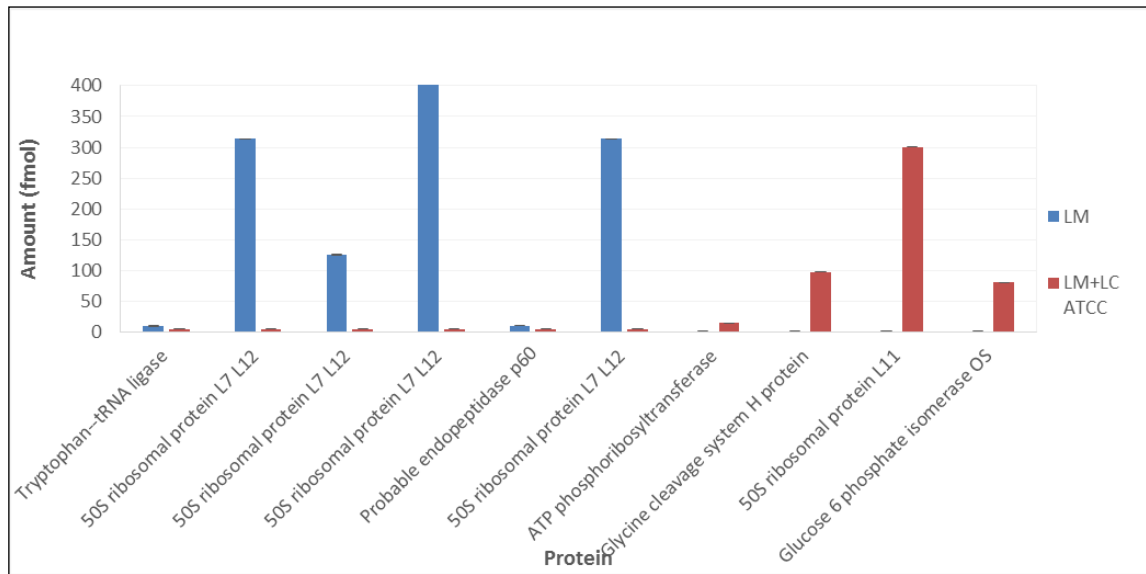
Figure 4.3.2-1 Differentially expressed proteins of *Listeria monocytogenes* in milk coculture with *Lactococcus lactis* identified by Label-free nUPLC-MS/MS
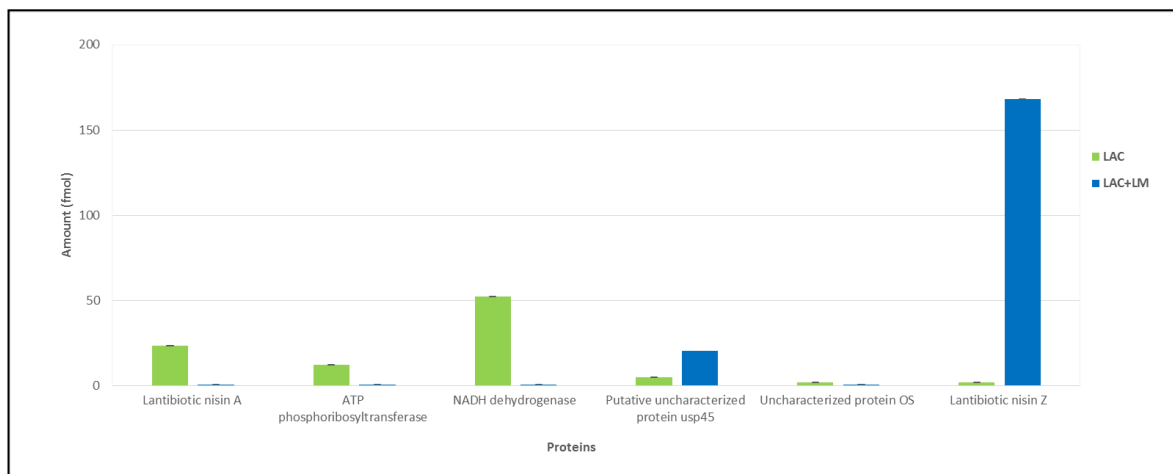
Figure 4.3.2-2 Differentially expressed proteins of *Lactococcus lactis* in milk coculture with Listeria mnocytogenes identified by Label-free nUPLC-MS

5 CHAPTER 3

5.1 Microbial Imaging mass spectrometry

In order to fully understanding molecular interaction pathways that are the basis of the microbial competition between Listeria and *Lactococcus*, we will perform, as next step of this project, the metabolic profiling of each bacteria in the context of interacting microbial colonies. In fact, an attractive hypothesis suggests that microbes regulate and optimize their production of such molecules to kill, limit the growth or modulate the metabolism of potential niche competitors for maximal advantage. Traditionally, individual microbial metabolites have been targeted using bioactivity-guided fractionation. More recent "omics" technologies, such as metabolomics, begin to capture molecules on a global scale, but do not distinguish between molecules which are always present and molecules with changing spatial distributions, disregarding the spatial localization of the molecules within a phenotype and the multifaceted chemical exchange within and between microbial cell populations. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging mass spectrometry (IMS) applied directly to microbes on agar-based medium captures global information about microbial molecules, allowing for direct correlation of chemotypes to phenotypes. IMS technique is capable of simultaneously detecting a wide range of discrete chemical signals without the need for chemical tags or labels, this will allow us to probe the native chemical environment of each microbial colony and maybe identify novel metabolites that were previously undetected by other analytical means. The use of imaging mass spectometry, alone or in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS) in order to investigate the metabolic profile of each bacteria in the interacting microbial colonies. To realize these aims, I went to the Lab directed by prof Pieter Dorrestein in University of California, San Diego (http://dorresteinlab.ucsd.edu/Dorrestein_Lab/Welcome.html) for six month during my exchanged period, where I learnt to use high throughput instrumentation and I had the possibility to use this techniques applied to my specific samples.

5.2 Materials and methods

5.2.1   Culturing and sample preparation for IMS

Strains *Lactococcus lactis* ATCC 11454 and *Listeria monocytogenes* ATCC 19115, obtained from the American type Culture Collection, were stored at -80°C in BHI (brain heart infusion) broth containing 20% glycerol. Bacteria were streaked on petri dish containing BHI agar and incubated at 30°C for 24h. Big colony was picked up and it was grown in 5ml of BHI broth at 30°C for 24 hrs. For IMS experiment colony growth was initiated by streaking 5 ul overnight growths of *Listeria monocytogenes* and Lactococus lactis at different cell densities (OD Listeria <OD Lacto) on the prepared agar petri dish. Bacterial colonies were allowed to grow for 48 h at 30° C before transferring the co-culturing experiment to a MSP 96 MALDI anchor plate (Bruker, Daltonics)[25]. All analysis were made on the biological and technical triplicate

5.2.2   Spray Technique

The sample was dried at 40°C overnight and transferred to the enclosed spray chamber of a Bruker ImagePrep device (Bruker Daltonics, Billerica, MA, USA). The plate was covered with matrix by spraying 2.5 mL of a matrix solution in 60 consecutive cycles in a nitrogen atmosphere. Each cycle comprised three steps: 2 s spraying, 10 s incubation times, and 40 s of active drying using nitrogen gas. After 30 cycles, the sample was turned by 180° to avoid inhomogeneous matrix deposition. Matrix solutions were of 20 mg/mL dissolved in ACN/MeOH/H_2O (70:25:5, v/v/v) with a 1:1 mixture of α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5- dihydroxybenzoic acid (DHB).

5.2.3   MALDI IMS

All data were collected on an UltraFlex Speed MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) equipped with a Nd:YAG smartbeam2 laser (Bruker Daltonics). Samples were dried in vacuo for 30 min prior to the analysis [8].

5.2.4   Data analysis

The datasets were analysed by using the FlexImaging software. FlexImaging (Bruker-Daltonics) was used to set up and control the acquisition. Spatial resolution was set to

500 μm. For each section the acquisition was performed in reflectron positive mode. All reflectron acquisitions were performed in the range of 100–6000 Da with voltages of 25 and 21.7 kV for the first and second ion extraction stages, respectively and 9 kV for the lens, 26.3 kV for reflector 1, 13.8 kV for reflector 2, and a laser power of 30%. One hundred shots per spectrum were accumulated for the reflectron mode (20 shots random walk) at a frequency of 500 Hz. Standard signals of 1–6 kDa were used to calibrate for the reflectron mode. After acquisition, the data were analyzed using the FlexImaging software. The resulting mass spectrum was filtered manually in 0.5–3.0-absorbance unit increments with individual colors assigned to the specific masses. Ions of interest were identified by the use of tandem mass spectrometry.

5.2.5   Molecules identification by LC MS/MS

5.2.5.1 *Extraction Procedure and LC MS/MS analysis*

General chemical extraction of the samples was performed sliced into small pieces (0.1 cmx0.1cm) and extracted with 10 mL of methanol plus 0.1% formic acid. The solvent was separated from agar pieces by filtration and concentrated in vacuo. Extracts were resuspended in 1 mL of methanol and centrifuged prior to analysis LC-MS/MS. Mass spectrometry was performed using a Bruker Daltronics Maxis qTOF mass spectrometer equipped with a standard electrospray ionization source. The mass spectrometer was tuned by infusion of Tuning Mix ES-TOF (Agilent Technologies, Santa Clara, CA,USA) at a 3 μL/min flow rate. For accurate mass measurements, a wick saturated with hexakis (1H,1H,3H-tetrafluoropropoxy)phophazene ions (Synquest Laboratories, Alachua, FL, USA; m/z 922.0098) located within the source was used for lock mass internal calibration. Samples were introduced by a Thermo Scientific UltraMate 3000 Dionex ultraperformance liquid chromatograph (UPLC) using a 20-μL injection volume. Methanol/FA extracts were separated using a Phenomenex (Torrance, CA, USA) Kinetex 2.6 μm C18 (30×2.10 mm) UPLC column. A linear water acetonitrile gradient (from98:2 to 2:98 water/acetonitrile) containing 0.1% formic acid was utilized. The flow rate was 0.5 mL/min. The mass spectrometer was operated in data-dependent positive ion mode, automatically switching between full-scan MS and MS/ MS acquisitions. Full-scan MS spectra (m/z 50 to 2000) were acquired, and the top 10 most intense ions in a particular scan were fragmented using collision-induced dissociation at 35 eV for +1 ions and 25 eV

for +2 ions in the collision cell. All analysis were made on the biological and technical triplicate

5.2.5.2 Data Analysis

Visualization of ion intensity was optimized to highlight differences between samples being compared. Structural verification of ions putatively identified in IMS was performed manually by comparing the exact mass from the LCMS/ MS. LC-MS/MS data analysis was performed using Bruker Daltronics DataAnalysis v4.1 (Build 362.7). Lockmass internal calibration using hexakis(1H,1H,3H-tetrafluoropropoxy) phosphazene ions (Synquest Laboratories; m/z 922.0098) was applied. Extracted ion chromatograms (EICs) using the exact mass of a metabolite of interest were created. The MS/MS spectra from these EICs were manually compared with previously reported data.

5.2.5.3 Database search

The MS/MS spectra are searched against GNPS spectral libraries (Global Natural Products Social molecular network), seeding putative node matches in the molecular networks.Comparing the MS2 spectra of the unknown metabolite with a library of MS2 spectra generated from structurally characterized metabolites. Herein, this comparison is based upon the similarity cosine scoring of MS/MS spectra. Networks are visualized online in-browser or exported for third-party visualization software such as Cytoscape[26]. GNPS is an open-access knowledge base for community-wide organization and sharing of raw, processed and identified tandem mass (MS/MS) spectrometry data[27].GNPS has the largest collection of publicly accessible natural product and metabolomics MS/MS data sets and is the only infrastructure where public data sets can be reanalyzed together and compared with each other.To date, GNPS has made 272 public GNPS data sets openly available, which comprise >30,000 MS runs with ~84 million MS/MS spectra. Parameters used for the database search were: parent mass tolerance (Parent mass peak tolerance) set to 2 Da; Ion Tolerance (MS2 peak tolerance) 0.5 Da, Min Pairs Cos (Cosine score threshold to make a match) 0.5; Min Matched Peaks (Minimum matched peaks to make a match) set to 6. Library search spectral is based on the comparison upon the similarity cosine scoring of MS/MS spectra.

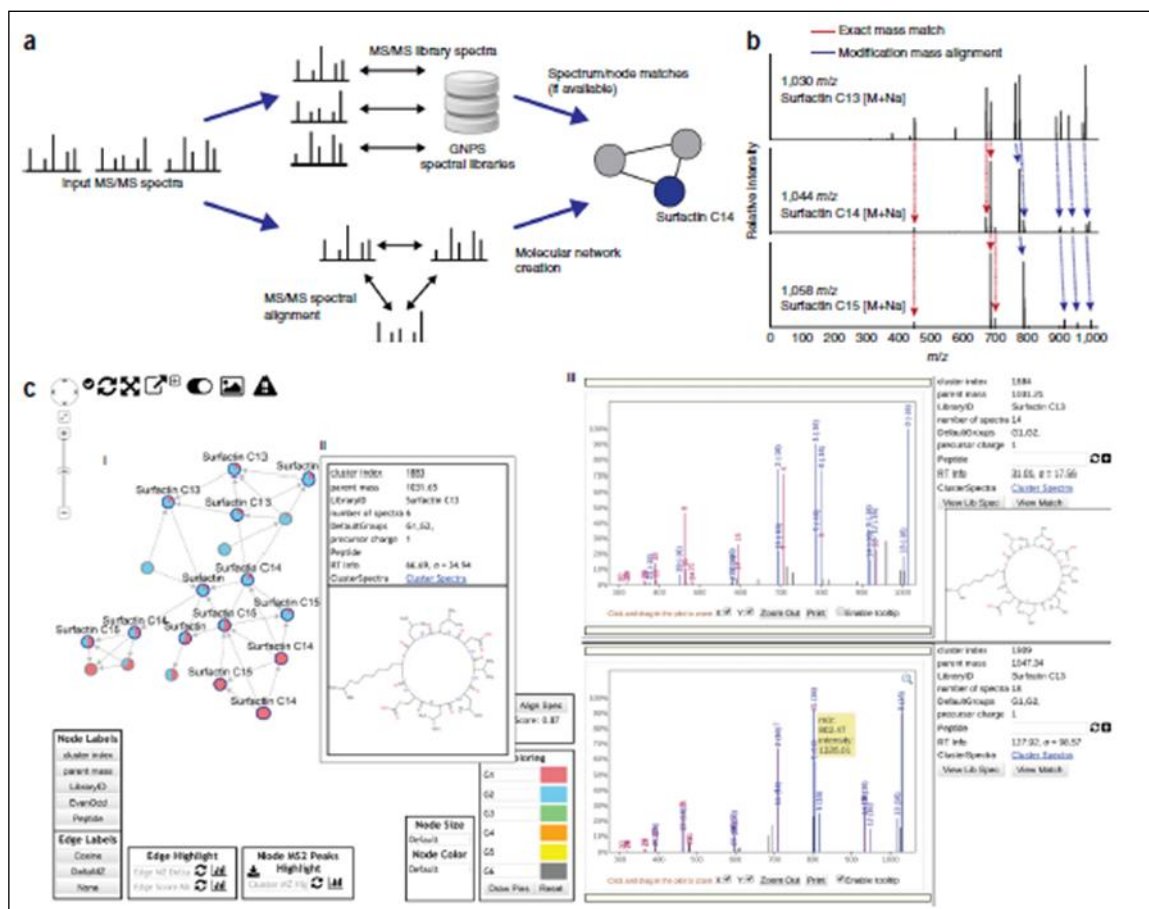### 5.2.5.4 Molecular network creation and visualization

Figure5.2.5.4-1 Molecular network creation and visualization.

In figure 5.2.5.4-1 molecular networks are show. Molecular networks are constructed from the alignment of MS/MS spectra to one another. Edges connecting nodes (MS/MS spectra) are defined by a modified cosine scoring scheme that determines the similarity of two MS/MS spectra with scores ranging from 0 (totally dissimilar) to 1 (completely identical). MS/MS spectra are also searched against GNPS spectral libraries, seeding putative node matches in the molecular networks. Networks are visualized online in-browser or exported for third-party visualization software such as Cytoscape31. (b) An example alignment between three MS/MS spectra of compounds with structural modifications that are captured by modification-tolerant spectral matching used in variable dereplication and molecular networking. (c) In-browser molecular network visualization enables users to interactively explore molecular networks without requiring any external software. To date, >11,000 molecular networks have been

analyzed using this feature. Within this interface, (i) users are able to define cohorts of input data and correspondingly, nodes within the network are represented as pie charts to visualize spectral count differences for each molecule across cohorts. (ii) Node labels indicate matches made to GNPS spectral libraries. These matches provide users a starting point to annotate unidentified MS/MS spectra within the network. (iii) To facilitate identification of unknowns, users can display MS/MS spectra in the right panels by clicking on the nodes in the network, giving direct interactive access to the underlying MS/MS peak data. Furthermore, alignments between spectra are visualized between spectra in the top right and bottom right panels to gain insight as to what underlying characteristics of the molecule could elicit fragmentation perturbations [27].

5.3 Statistical analysis

A molecular network was created using the online workflow at GNPS. The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra . Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks.

Parameter	Value
PAIRS_MIN_COSINE	0.7
ANALOG_SEARCH	0
tolerance.PM_tolerance	2.0
tolerance.Ion_tolerance	0.5
MIN_MATCHED_PEAKS	6
TOPK	10
CLUSTER_MIN_SIZE	2
MAXIMUM_COMPONENT_SIZE	100
MIN_PEAK_INT	0.0
FILTER_STDDEV_PEAK_INT	0.0
RUN_MSCLUSTER	on
FILTER_PRECURSOR_WINDOW	1
FILTER_LIBRARY	1
WINDOW_FILTER	1
SCORE_THRESHOLD	0.7
MIN_MATCHED_PEAKS_SEARCH	6
MAX_SHIFT_MASS	100.0

Table 5.3-1 Parameters table

5.4 Results and Discussion

Specialized metabolites are chemical compounds with a low molecular weight produced by many microorganisms growing on substrates. These compounds are not essential for microorganism growth but their natural productions have certain significant. They could play an important role in mechanisms of competition, antagonism and self-defence mechanisms against other living organisms to allow the microorganism to occupy the niche and utilize the food. These metabolites are key components in cell-cell interactions as quorum sensors, virulent factor and natural product. Traditionally, individual microbial metabolites have been targeted using bioactivity-guided fractionation. More recent "omics" technologies, such as metabolomics, begin to capture molecules on a global scale, but do not distinguish between molecules which are always present and molecules with changing spatial distributions, disregarding the spatial localization of the molecules within a phenotype and the multifaceted chemical exchange within and between microbial cell populations. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) imaging mass spectrometry (IMS) applied directly to microbes on agar-based medium captures global information about microbial molecules, allowing for direct correlation of chemotypes to phenotypes. IMS technique is capable of simultaneously detecting a wide range of discrete chemical signals without the need for chemical tags or labels, this will allow us to probe the native chemical environment of each microbial colony and maybe identify novel metabolites that were previously undetected by other analytical means[118].

In this work, we have chosen to study mediators of bacterial interaction using the advance technology IMS in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

In figure 5.4-1 we report the average reflectron positive mode MALDI-TOF MS spectra of colonies of *Listeria monocytogenes* and *Lactococcus lactis* growing alone versus the microbial interaction between *L. monocytogenes* and *Lactococcus lactis*.

In the mass spectrum obtained from colonies the signals are much concentrated in the low mass range, in agreement with the production of molecules with a low molecular weight by bacteria in order to counteract other living organisms.
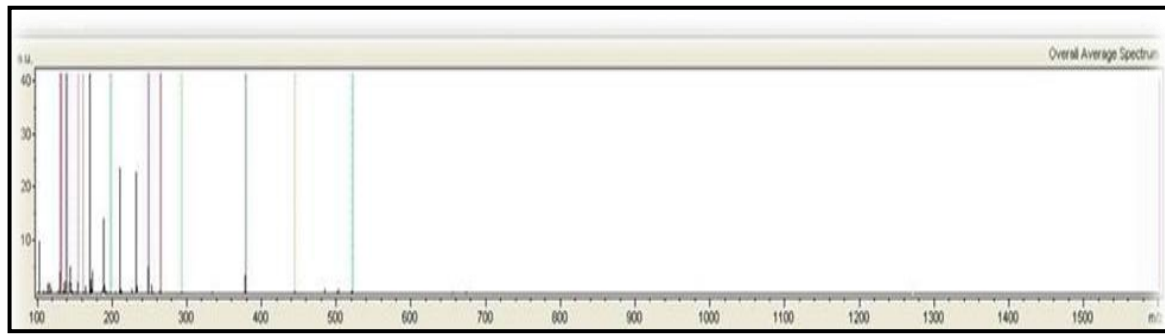
Figure 5.4-1 Avarage reflectron positive mode MALDI-TOF MS spectra of live colony IMS of microbial interaction between LM and LAC. Colours peaks represent signals m/z selected for the analysis IMS

IMS data analysis was performed using Bruker Daltronics FlexImaging v3.0. Visualization of ion intensity was optimized to highlight differences between samples being compared. We focused our attention on signals that were specific for condition of microbial competition in order to identify molecules that could be involved in this mechanism. In figure 5.4-2A is reported the optical image of colonies of *Listeria monocytogenes* (LM) and *Lactococcus lactis* (LAC) growing alone (ctrl) and the interaction between *Listeria monocytogens* and *Lactococcus lactis*. In figure 5.4-2B, C and D show the localization of signals at m/z = 250.33, m/z = 334.37 and m/z = 284.08 on colonies of each bacteria. All three signals are specific for the interaction between *Listeria monocytogenes* and *Lactococcus lactis* and they are more abundant in the bottom part of the colony of *Lactococcus lactis*, the part of the colony that is closest to the colony of *Listeria monocytogenes*. Signals have a specific localization in the colony of *Lactococcus lactis*.
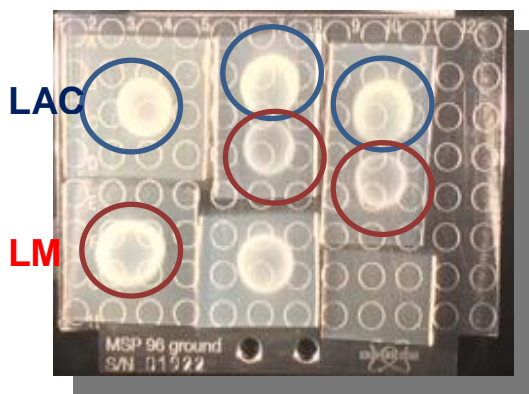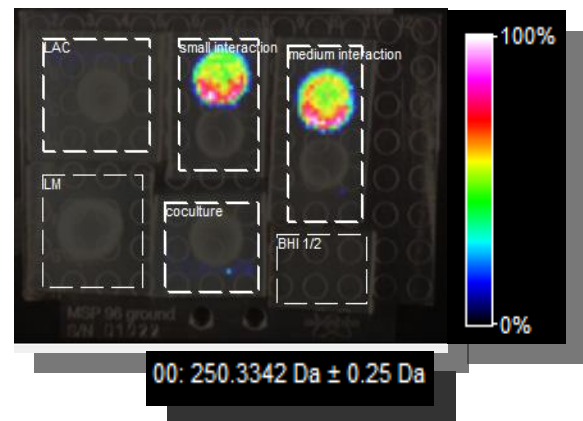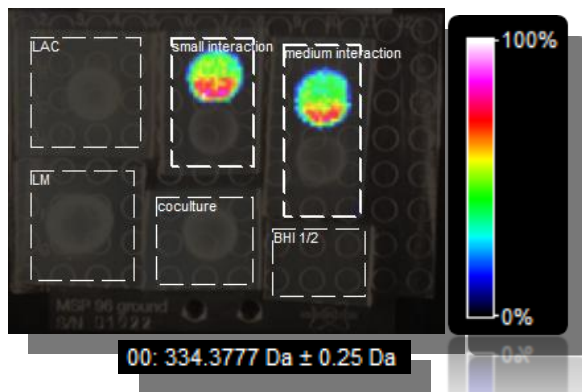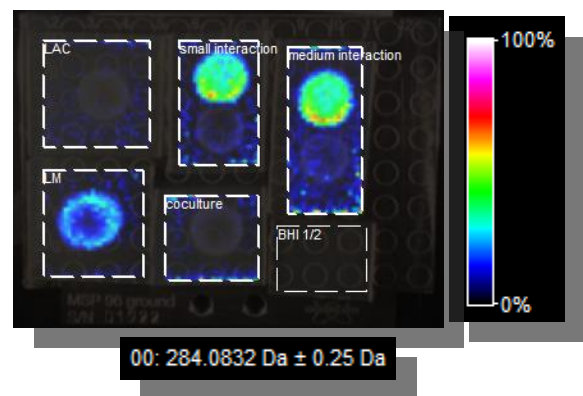
Figure 5.4-2 A) the optical image of colonies of *Listeria monocytogenes* (LM) and *Lactococcus lactis* (LAC) growing alone (ctrl) and the interaction between Listeria monocytogens and *Lactococcus lactis*. We evaluated two different distance of interaction (0.5-1 cm) ; **B,C,D)** Microbial IMS images of selected signals. Ion intensity colour scaling indicates that the highest naturally released molecule is located at the bacterial interface.

In order to identify these signals, structural verification of ions putatively identified in IMS was performed manually by comparing the exact mass from the LC MS/ MS. The MS/MS spectra are searched against GNPS spectral libraries (Global Natural Products Social molecular network).

GNPS is a data-driven platform for the storage, analysis, and knowledge dissemination of MS/MS spectra that enables community sharing of raw spectra, continuous annotation of deposited data, and collaborative curation of reference spectra (referred to as spectral libraries) and experimental data (organized as data sets). GNPS provides the ability to analyze a data set and to compare it to all publicly available data. At present 221,083 MS/MS spectra from 18,163 unique compounds are used for searches in GNPS. GNPS can be used for molecular networking, a spectral correlation and visualization approach that can detect sets of spectra from related molecules, even when the spectra themselves are not matched to any known compounds[119]. Molecular networks are constructed from the alignment of MS/MS spectra to one another. Edges connecting nodes (MS/MS spectra) are defined by a modified cosine scoring scheme that determines the similarity of two MS/MS spectra with scores ranging from 0 (totally dissimilar) to 1 (completely identical). In table 5.4-1 compounds identified by GNPS are showed.
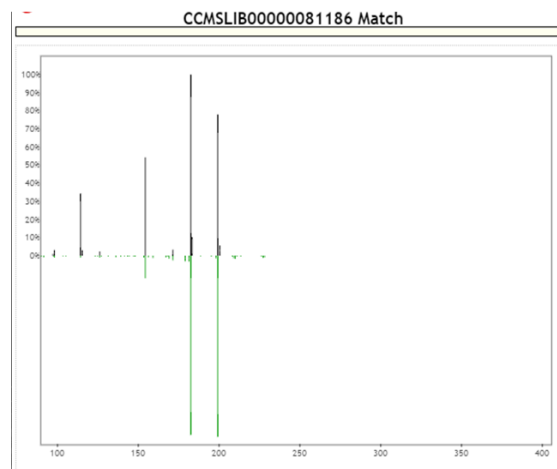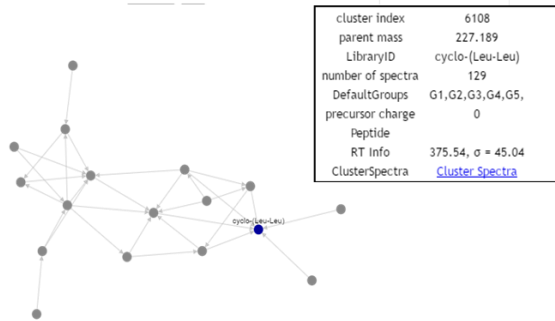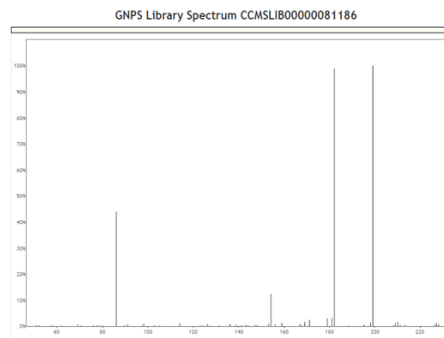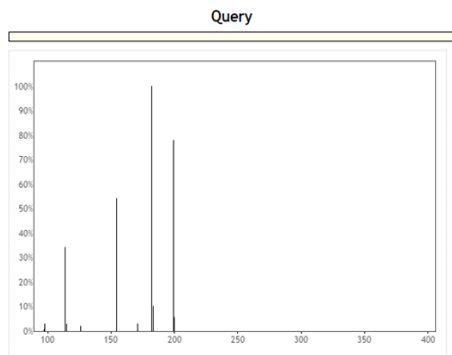
Compound_Name	Library Class	Cosine	MZErrorPPM	MassDiff	LibMZ	Instrument	IonMode	PI	Ion Source
			-	-	-				
Massbank:FIO00055 gamma-Dodecalactone	Bronze	0.80	11	0.002	199.17	LC-ESI-QTOF	Positive	Putative Massbank Match	ESI
Massbank:FIO00435 Dexamethasone	Bronze	0.85	4882	1.920	393.21	LC-ESI-QTOF	Positive	Putative Massbank Match	ESI
Massbank:KNA00379 L-Serine|L-2-Amino-3-hydroxypropionic acid|L-3-Hydroxy-alanine|Serine	Bronze	0.86	4651	1.820	391.28	LC-ESI-ITFT	Positive	Putative Massbank Match	ESI
Massbank:MT000040 Glycodeoxycholic acid|glycodeoxycholate	Bronze	0.77	3974	1.789	450.10	LC-ESI-IT	Positive	Putative Massbank Match	ESI
Peptide with possible PPQVV seq, Possibly media derived	Bronze	0.74	143	0.147	1025.57	qTof	Positive	P.Dorrestein	LC-ESI
ReSpect:PM014406 Cyclo(Phe-Tyr)	Bronze	0.83	459	0.143	311.00	QIT	Positive	Putative ReSpect Match	ESI
ReSpect:PM014407 Cyclo(Phe-Leu)	Bronze	0.84	877	0.229	261.00	QIT	Positive	Putative ReSpect Match	ESI
ReSpect:PM018191 Tryptophan	Bronze	0.95	3	0.001	205.10	Q-TOF	Positive	Putative ReSpect Match	ESI
Ursodiol	Bronze	0.81	107	0.040	375.29	qTof	Positive	Sirenas	LC-ESI
cyclo(L-Phe-L-4-Hyp)	Bronze	0.82	168	0.044	261.12	qTof	Positive	Keyzers	DI-ESI
cyclo-(Leu-Leu)	Bronze	0.80	66	0.015	227.17	Ion Trap	Positive	CCLiaw, YLYang	LC-ESI
cyclo-(Val-Phe)	Bronze	0.88	16	0.004	247.14	Ion Trap	Positive	CCLiaw, YLYang	LC-ESI

Table 5.4-1. View all compounds identified by GNPS database. The table showed the library matches, metadata is associated with the library spectra including the compound name, a CAS number if it is a commercial compound and a PUBMED ID correlating to the published data. The score shown correlates to the cosine scoring function where 1 is an exact match.
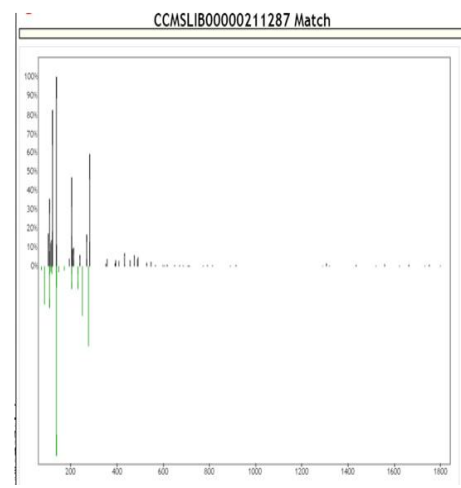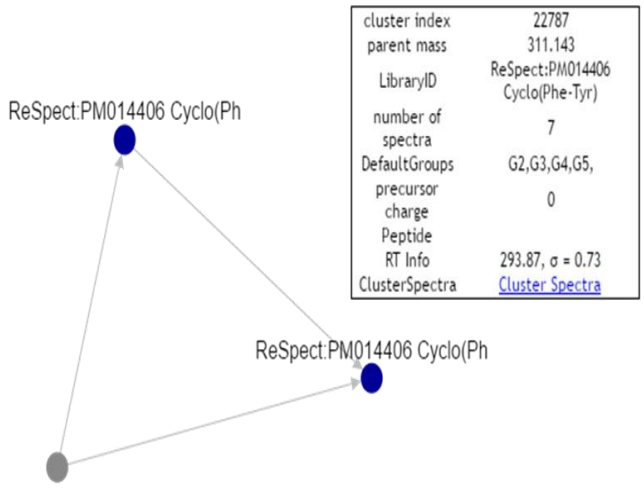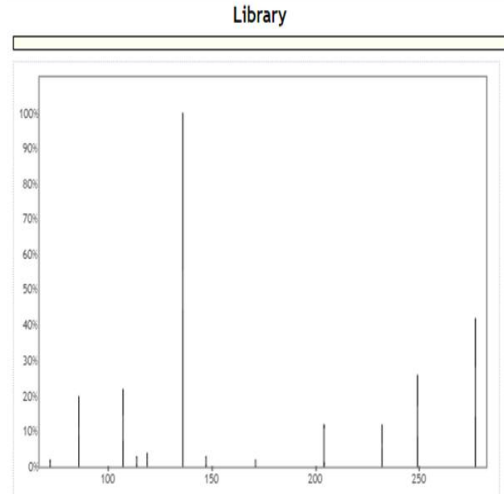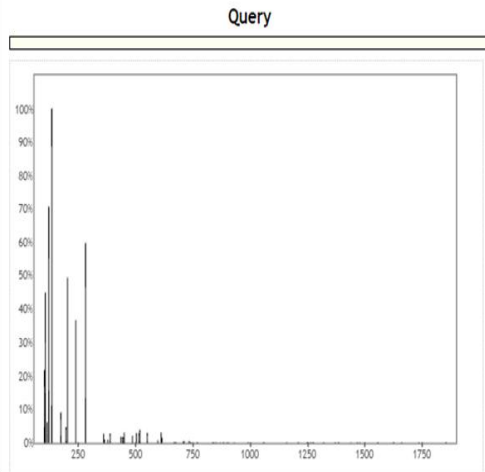
Among results obtained, comparing the exact mass from the LC MS/ MS with ions putatively identified in IMS, we focused our attention on three compounds that are specific of the bacterial interaction and also with a relevant biological function.

The signal m/z 250.33 (261.00m/z +Na$^+$) corresponds to Cyclo-(Leu-leu); the signal m/z 334.37 (311.00 m/z+Na$^+$) to Cyclo-(Phe-Tyr) and the signal m/z 284.08 (261.12m/z+Na$^+$) to Cyclo-(L-Phe-L-4-Hyp). Interestingly, the mass difference between variant found by IMS analysis and LC MS/MS is approximately 23 for each three signals , which could correlate to the addition of Sodium.

In figure 5.4-3 molecular networks of the three compounds identified, constructed from the alignment of MS/MS spectra.
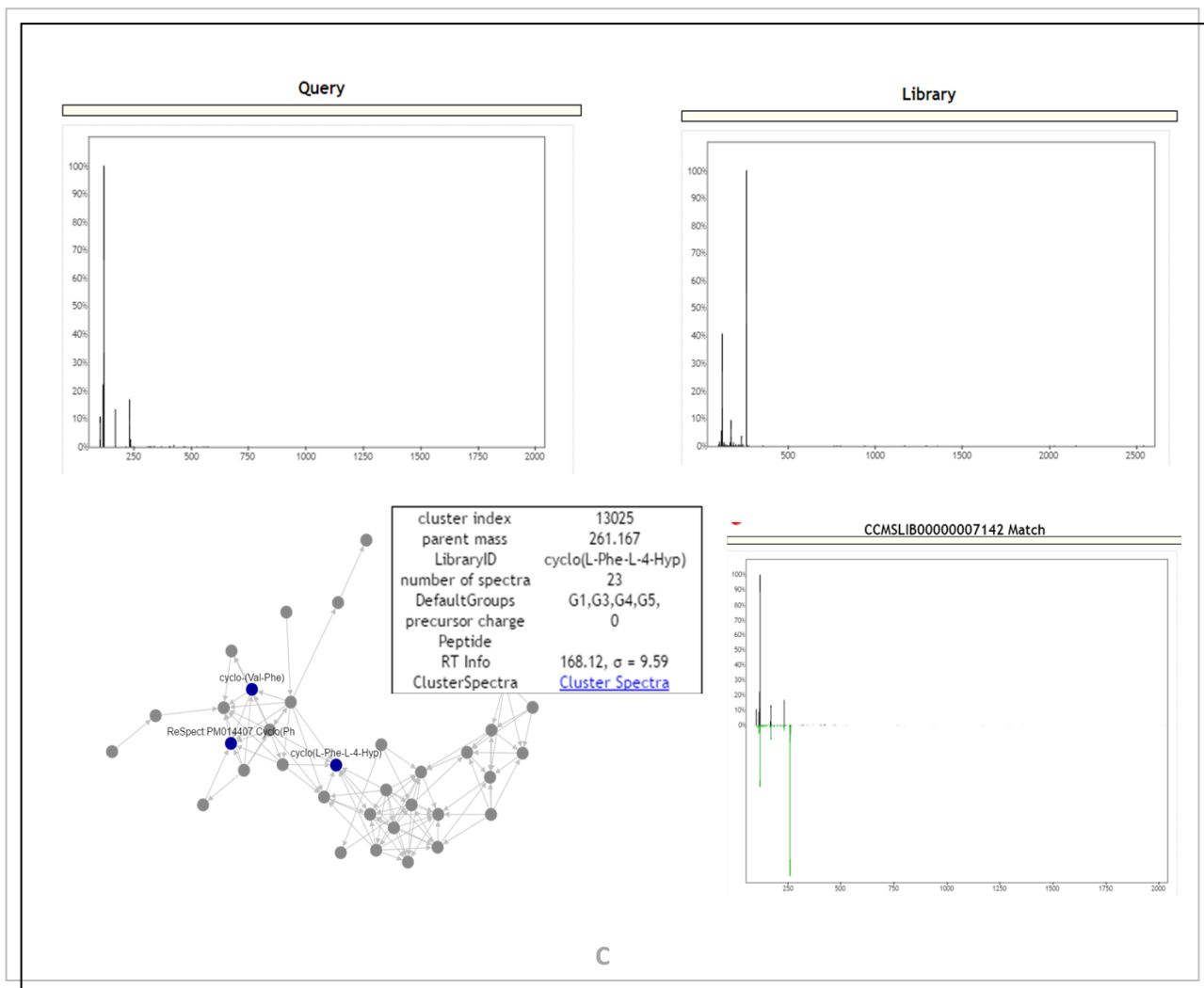
Query

GNPS Library Spectrum CCMSLIB00000081186

cluster index	6108
parent mass	227.189
LibraryID	cyclo-(Leu-Leu)
number of spectra	129
DefaultGroups	G1,G2,G3,G4,G5,
precursor charge	0
Peptide	
RT Info	375.54, σ = 45.04
ClusterSpectra	Cluster Spectra

CCMSLIB00000081186 Match

A

Query

Library

cluster index	22787
parent mass	311.143
LibraryID	ReSpect:PM014406 Cyclo(Phe-Tyr)
number of spectra	7
DefaultGroups	G2,G3,G4,G5,
precursor charge	0
Peptide	
RT Info	293.87, σ = 0.73
ClusterSpectra	Cluster Spectra

ReSpect:PM014406 Cyclo(Ph

ReSpect:PM014406 Cyclo(Ph

CCMSLIB00000211287 Match

B

Figure 5.4-3 *Molecular network creation and visualization A) Cyclo-(Leu-leu);B) Cyclo-(Phe-Tyr); C) Cyclo-(L-Phe-L-4-Hyp). Node labels indicate matches made to GNPS spectral libraries. To facilitate identification of unknowns, users can display MS/MS spectra in the right panels by clicking on the nodes in the network, giving direct interactive access to the underlying MS/MS peak data.*

These compounds are cyclic peptides, isolated by Lactobacilli, with a biological activity[4]

The first compound belongs to the family of Diketopiperazines. Diketopiperazines are smallest cyclic peptides known with different bacterial function: antibiotic biosynthesis, production of virulence factors, exopolysaccharide biosynthesis, bacterial aggregation, plasmid conjugal transfer and transition into the stationary phase[120]. They represent the novel family of signalling compounds identified in cell-free supernatants of several

gram-positive cultures. The precise role played by diketopiperazines in bacterial cell-to-cell communication has yet to be established, but their potential to act as auto-inducer antagonists, preventing bacterial biofilm formation[121].

Cyclo-(Phe-Tyr) is a compound produced by Lactobacillus plantarum CRL 778 for the first time [122]. It is an active secondary metabolites with lysozyme activity, phagocytic actvity and bactericidal activity[123].

The third compound is Cyclo-(L-Phe-L-4-Hyp) produced by L. Plantarum[124]. Cyclic dipeptides possess antibacterial activities. Due to their chiral, rigid, and functionalized structures, they bind to a large variety of receptors with high affinity, giving a broad range of biological activities.

There are evidence in the literature that these cyclic peptides identified are involved in bacterial cel-to cell communication but their potential role role played in the mechanism has to be yet established[125].

Our hypothesis is that these cyclic peptide identified could mediate the quorum sensing. Quorum sensing is a cell density-dependent signal transduction system, which controls a variety of the physiological behaviour in bacteria, such as virulence, conjugation, biofilm formation, motility and antibiotic production. During quorum sensing, bacteria produce and secrete the small signal molecules outside the cell to recognize the population density. In particular, it was documented that, Gram+ bacteria use small peptide (cyclic peptide) to interact, to communicate, and to regulate gene expression in a coordinated manner in response to different environments[126]. In gram-positive bacteria, quorum sensing is involved in the regulation of genetic competence in Bacillus subtilis[127] and Streptococcus pneumonia[128], virulence and biofilm formation in Staphylococcus aureus[129], and conjugation in Enterococcus faecalis[130] and in the production of antimicrobial peptides, including bacteriocins and lantibiotics, in lactic acid bacteria[131].

So, in agreement with the literature and with our previous studies, these cyclic peptides, identified with imaging mass spectrometry, could have a role in antibiotic production and in particular in inducing of the transcription of gene coding for Nisin, the bacteriocin produceded by *Lactococcus lactis*.

In this part of the project microbial competition between *Listeria monocytogenes* and *Lactococcus lactis* was monitored by combination of IMS and LC MS/MS in order to

investigate the metabolic profile of each bacteria colony in the interacting microbial colonies. Several interesting compounds, uniquely expressed during interaction of microbial colonies, were obtained, as it has been discussed above. So, Imaging Mass Spectrometry could be well validated method to investigate "online molecular conversation between bacteria" to increase knowledge and strategy in food safety, and could be consider a valid tool to use directly on food.

6 Conclusion

Most environments host an amazingly diverse collection of microbial species.
Microbial species exist in constant competition with one another for suitable ecological niches to support their survival and growth. The populations eject into environment compounds that kill or limit the growth of competing strains or species can promote niche monopolization. The microbial competition is a mechanism that tends to eliminate one of the populations from their common habitat, especially when competition is focused on a single resource and when the populations do not otherwise interact. The same happens in food, and it could be possible to explore this social communication to improve food safety.

There are no doubts as to whether that food safety is essential for food security and food quality. Food safety and quality represent a global importance, particularly because it affects the health but also economy and trade due to the strict rules for food exportation. Indeed, some countries such as the US, Russia, China, require for the export of animal products certification 'Listeria free'; this represents a major economic loss for Italian industries of typical products. So, it is mandatory for prevention and control of infectious diseases to have facilities that are able to quickly produce reliable, highly specific and sensible tools that allow on one hand and adequate sanitary surveillance and to obtain effective operative tools such as proteomics.

Proteomics represents a real challenge in this field, because it is able to produce rapid methods to investigate the modification or the presence or absence of targeted proteins in complex food including raw materials and matrices.

Proteomics should guide us to functional proteomics in food. Proteomics, metabolomics methods are presented as effective tools for identification of cellular biomarkers for adaptive behavior of pathogenic microorganisms under different conditions such as cold and heat stress, osmotic, high hydrostatic pressure, and other stress factors. Proteins are fundamental and integral food components, both nutritionally and functionally. Thus, the application of proteomics technologies will contribute to the following research areas of food science and technology, which includes evaluation of safety, body distribution and metabolism of food ingredients, detection and control of food spoilage and the presence of pathogenic microorganisms. Furthermore, proteomes of

certain food (wheat, fish) can be used to identify the origin of a particular food or its quality during the food processing. Proteome and/or metabolome of starter cultures in fermentation processes (beer, cheese, etc.) can be also used to predict the quality of the fermented end-product.

These results that we obtained will be useful for developing new biological strategies to control the bacteria for undesirable bacterial activities in cheese and dairy products. We expect that our proteomic investigation of dairy products will support food industries to produce safe and quality food, to ensure food safety during whole shelf-life of products in order to solve the problem of growth of potential pathogens in products further increase the quality of this product.

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