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Antimicrobial Peptides against *Listeria monocytogenes*: omic approaches and potential biotechnological applications

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SUMMARY

ABBREVIATIONS

AMPs: antimicrobial peptides

CLPs: cyclic lipopeptides

NRPS: nonribosomal peptides

MLSA: multilocus sequence analysis

LFQ: label-free quantification

FDR: false discovery rate

PCA: principal component analysis

PLS-DA: partial least squares discriminate analysis

VIP: variable importance in projection

PCC: peripheral cell component

FIGURES LIST

ABSTRACT

Listeria monocytogenes poses a threat especially to people with weak immune systems, children, and pregnant women. Nisin is employed in food industries to contrast Gram-positive bacteria outbreaks, which could endanger the health of consumers. However, the research of new antimicrobial compounds (AMPs) has become pivotal to contrast the always more frequent detection of *L. monocytogenes* nisin resistance. In this doctoral dissertation, the *Bacillus velezensis* P34, producer of fengycin cyclic lipopeptides as possible alternative AMPs, to use against *L. monocytogenes*, was studied. *Bacillus velezensis* P34 whole genome was investigated because of the presence of antimicrobial compound gene clusters, and the secreted compounds were identified as fengycin A and B and, in smaller quantities, bacillomycin L through mass spectrometric analysis. Proteomics analysis was carried out to study the "proteosurfactome" including moonlight protein of *L. monocytogenes* treated with a sub-lethal concentration of nisin detecting the inhibition of the virulence process besides the biofilm promotion as a bacterial resistance strategy. An additional proteomic study permitted, for the first time, to detect the physiological reaction on *L. monocytogenes* treated with the AMPs secreted by *Bacillus velezensis* P34; in the latter, a biotechnological application, specifically a liposome nanoencapsulation, was evaluated. Among the main results of this latter work, strong deregulation of the manganese transporters was observed with intense regulation of others metals transporters for the metal ions homeostasis, besides a strong downregulation of proteins related with the main virulence factors Prfa, σB and virR Moreover, an additional proteomic and lipidomic comparison study was performed, nisin and fengycin AMPs were considered in the experimental drawing of this latter. The strong downregulation of proteins and, quantitatively, the variation of membrane lipidome fatty acids strongly suggested the

inhibition of biofilm when treated with a sub-lethal concentration of fengycin lipopeptides; whereas a group of membrane proteins was investigated for their action on the maintaining of the possible membrane resistance to AMPs. The results suggested the promotion of biofilm multicellular organization as the main concern when sub-lethal concentration of nisin was used, besides to that the *L. monocytogenes* responses to the CLPs synthesized by *Bacillus velezensis* P34 may represent an important start for deeper studies involving the antimicrobial action of fengycin to use against Gram-positive bacteria, including multiresistant strains.

Keywords: Antimicrobial Peptides, Resistance, Nisin, Fengycin, Pathogen, Biofilm, Virulence

RESUMO

A *Listeria monocytogenes* representa uma ameaça, especialmente para pessoas com sistema imunológico fraco, crianças e mulheres grávidas. A Nisina é utilizada na indústria alimentar para comparar surtos de bactérias Gram-positivas que podem pôr em risco a saúde dos consumidores. No entanto, a pesquisa de novos compostos antimicrobianos (AMPs) tornou-se fundamental para contrastar a detecção cada vez mais frequente da resistência à nisina por *L. monocytogenes.* Neste trabalho, *Bacillus velezensis* P34 produtor de fengicina um lipopeptídeos cíclicos foi estudado como possível alternativa de AMPs para uso contra *L. monocytogenes*. O genoma completo de *B. velezensis* P34 foi investigado quanto à presença de aglomerados de genes de compostos antimicrobianos. Os compostos secretados foram identificados como fengicina A e B, e em menores quantidades bacilomicina L, por meio de análise de espectrometria de massas. A análise proteômica foi realizada para estudar o "proteosurfactoma", incluindo a proteína moonlight de *L. monocytogenes* tratada com uma concentração subletal de nisina, detectando a inibição do processo de virulência, além da promoção do biofilme como estratégia de resistência bacteriana. Um estudo proteômico adicional permitiu pela primeira vez a reação fisiológica em *L. monocytogenes* tratada com os AMPs secretados por *Bacillus velezensis* P34. Neste estudo, uma nanoencapsulação em lipossomas foi avaliada como aplicação biotecnológica. Dentre os principais resultados deste último trabalho, foi observada forte desregulação dos transportadores de manganês com intensa regulação de outros transportadores de metais para a homeostase de íons metálicos, além de forte regulação negativa de proteínas relacionadas com os principais fatores de virulência Prfa, σB e virR. Além disso, foi realizado um estudo de comparação proteômica e lipidômica adicional, no qual os AMPs de nisina e fengicina foram considerados nesta pesquisa. A forte regulação

negativa de proteínas e a variação quantitativa dos ácidos graxos do lipidoma da membrana sugeriram fortemente a inibição do biofilme quando tratado com uma concentração subletal de lipopeptídeos de fengicina; enquanto um grupo de proteínas de membrana foi investigado por sua ação na manutenção da possível resistência de membrana aos AMPs. Os resultados sugeriram a estrutura multicelular do biofilme como a principal preocupação quando pequenas quantidades de nisina podem ser utilizadas, além de que o estudo da ação de compostos secretados de *Bacillus velezensis* P34 sobre *L. monocytogenes* pode representar um importante ponto de partida para estudos mais aprofundados de fengicina para uso contra Gram-bactérias positivas, incluindo cepas multirresistentes.

Palavras-chave: Peptídeos Antimicrobianos, Resistência, Nisina, Fengicina, Patógeno, Biofilme,Virulência

1 INTRODUCTION

1.1 Pathogenic bacteria in food

The majority of people will, at least once during their lives, have contact with pathogens present in food. The pathogenic microorganisms living in food were named foodborne pathogens. In general, pathogenic microorganisms include bacteria able to generate diseases in a host (such as humans). For the World Health Organization (WHO), every year, almost 1,5 billion people fall ill eating contaminated food. 420,000 of these die, including 125,000 children considered with high risk of contracting and overcoming foodborne disease (WHO, 2019). Foodborne diseases are an important cause of morbidity and mortality, as well as a significant impediment to socioeconomic development worldwide. The report resulting from a WHO initiative to estimate the global burden of foodborne diseases provides a global estimate of thirty-one foodborne hazards, causing 32 diseases, and the majority of these was caused by bacteria (WHO, 2015). Food contamination by microorganisms can also cause food deterioration, in addition to causing illness in the consumer. During the microbial spoiling, metabolism production of substances determining undesirable sensorial alteration of the food usually appear, making it unacceptable for the consumer (GRAM *et al*., 2002; BURKEPILE *et al.*, 2006; RAWAT, 2015). Spoilage of food continues to occur despite technological advances. Considerable economic losses are due to microbial spoilage, resulting in final products with an inadequate shape or appearance (PETRUZZI *et al.*, 2017). Another important aspect to consider was related to food losses, relating it to the amount of resources provided for food production wasted in vain, including the greenhouse gas emission employed for food production (FAO, 2011). In addition to pathogens and spoilage microbes, food can host

beneficial microorganisms. These latter microorganisms generally include the group of lactic acid bacteria (LAB), employed for the production of fermented food such as: cheese, yogurt, beer, bread, etc., but also microbes that help the food conservation due to the ability to produce substances with antimicrobial properties, such as antimicrobial peptides, acids and alcohols (BOURDICHON *et al.*, 2012; SIEDLER, BALTI AND NEVES, 2019).

1.2 *Listeria monocytogenes*

For a long time, the genus *Listeria*, including rod-shaped, facultative anaerobic and Gram-positive bacteria, was thought to contain only eight species and two subspecies, but with the development of whole genome sequencing technologies, today, the genus is composed by 17 species (WELLER *et al.*, 2015). Among the species of this genus, only two species, namely *L. monocytogenes* and *L. ivanovii*, were defined as pathogenic to humans and animals respectively (ALLERBERGER AND WAGNER, 2010). In this work, the attention was directed to *L. monocytogenes*, which is among the leading causes of death from foodborne illness. There are 13 serotypes associated with the vast majority of foodborne infections caused by *L. monocytogenes*. Soil, water and plant material were the main source where these bacteria have commonly been found; humans and animals can contract the bacteria in a saprophytic style of life via many possible routes from many sources (WEIS AND SEELIGER, 1975).

Listeria strains are Gram-positive bacteria widespread in the environment. *L. monocytogenes* is a foodborne pathogen and its control in food production facilities requires constant focus by risk managers. *L. monocytogenes* causes a foodborne illness that, in most cases, appears through febrile disease, but, in some cases, causes systemic listeriosis with severe symptoms, characterized by high hospitalization and fatality rates (BUCHANAN *et al.*, 2017). This bacterium is able to build biofilms on abiotic surfaces,

improving the resistance to dehydration and sanitizers (BERNBOM *et al.,* 2011). Moreover, unlike many other common bacteria that cause foodborne diseases, *L. monocytogenes* can survive and multiply at low temperatures, usually found in refrigerators (WHO, 2018a).

The infective dose of *L. monocytogenes* is undetermined so far, but it is believed to vary with the strain and susceptibility of the host, and the food matrix involved also may affect the dose-response relationship. In milk that was not previously pasteurized, it is likely that fewer than 1,000 cells may cause disease in susceptible individuals. However, the infective dose may vary widely and depends on a variety of factors (FDA, 2012). The nonphatogenic *L. monocytogenes* can switch into a pathogen style of life, following its ingestion by susceptible humans or animals; this context play a fundamental role in the regulation of the key virulence factor PrfA, holding the main function in the transaction from the saprophytic stage to the virulent intracellular stage (FREITAG *et al.,* 2009). In fact, the bacterium, inside human and animal hosts, is capable of making the transition to a physiological state that promotes bacterial survival and replication in host cells through the PrfA virulence factor action (FREITAG 2006). Inside immune-compromised individuals and pregnant women, the bacterium is capable of causing systemic infections that lead to meningitis, encephalitis, and, in the case of pregnant women, infection of the developing fetus, which can lead to abortion, stillbirth or neonatal infections (BECROFT *et al.,* 1971; DREVETS AND BRONZE, 2008). *L. monocytogenes,* one of the most well-studied bacterial pathogens, has, in fact, become a paradigm for the study of host–pathogen interactions and bacterial adaptation to mammalian hosts (BIERNE AND COSSART, 2006).

1.3 *Listeria monocytogenes* in Food

L. monocytogenes bacteria employ diverse strategies to survive against environmental stressors, including low temperature (SWAMINATHAN *et al.*, 2007), low pH (VERMEULEN *et al.*, 2007), and high salinity (RIBEIRO *et al.*, 2014). In food processing plants, for the ability of *L. monocytogenes* to survive to the mentioned environmental stresses that normally serve to limit bacterial growth, attention must be paid to the design of food-processing equipment and to the effectiveness of the cleaning and disinfecting procedures in factories (THEVENOT, DERNBURG AND VERNOZY-ROZAND, 2006). However, for *L. monocytogenes*, all the precautions procedures have been proved to be insufficient. In some cases, the pathogen may persist in food industry equipment and premises. Scientific studies have paid more attention to the *Listeria* abilities, such as: adhesion, biofilm forming ability, resistance to desiccation, acid and heat, tolerance to increased sub-lethal concentration of disinfectants, or resistance to lethal concentrations (CARPENTIER AND CERF, 2011). Therefore, unconventional technologies, such as high hydrostatic pressure, ultrasound, and microwave, have started to be thought of as efficient commercial sterilization methods in destroying *L. monocytogenes*. However, the composition and characteristics of foods, processing conditions, together with the *L. monocytogenes* resistance (in species level) against these advanced processes, have affected the efficiency against this foodborne pathogen (BAHRAMI *et al.*, 2020). Moreover, the foodborne pathogen *L. monocytogenes* is a concern in food safety because of its ability to form biofilm and to persist in the food industry (COLAGIORGI *et al.*, 2017). To enhance the safety and shelf-life of food products, the use of non-thermal treatments that do not increase the temperature of food products for bacterial inactivation is receiving attention. In this regard, the use of free or encapsulated form of natural antimicrobial compounds singly or in combination with other technologies have been

attractive for researchers (BAHRAMI *et al.,* 2019). Based on the quantitative risk characterisation of *L. monocytogenes* in various ready-to-eat food categories, the first risk is represented more by heat-treated meat than smoked and gravad fish and soft and semi-soft cheese. *L. monocytogenes* has been found in many raw and ready-to-eat (RTE) food products, representing the main cause of listeriosis, since they can be eaten without further decontaminating treatment (EFSA, 2018; FAO/WHO, 2004). Studies have shown that *Listeria* has the second-highest value after meat, while fresh seafood represent an important concern (DILLON *et al.,* 1992). *L. monocytogenes* in seafood products has been detected with relatively high incidence in ready-to-eat cold smoked salmon and cooked fish products (EMBAREK, 1994). Recently, in an examination of coliform bacteria study in raw seafood categories, *L. monocytogenes* was found in 18.71% of the samples examined, representing a seriously risky seafood in the light of these results (DUMEN *et al.,* 2020). In this context, food safety concerns may be represented by the presence of *L. monocytogenes* multidrug resistant isolated by ice retail in fish markets of Kerala, India (BASHA *et al.,* 2019).

Hypervirulent clones of *L. monocytogenes*, strongly associated with dairy products and meat, were the results of adaptation to food processing environments, with a higher prevalence of stress resistance and benzalkonium chloride tolerance genes and a higher survival and biofilm formation capacity in presence of sub-lethal benzalkonium chloride concentrations (MAURY *et al.,* 2019). The environment of animal growth represents a potentially important reservoir for *L. monocytogenes* (ESTABAN *et al.,* 2009). Unpasteurized milk illustrates the correlation between the animals and the possible contamination of dairy products inside the food processing factories (LINNAN *et al.,* 1988). Dairy products, in particular soft cheeses, pose a major concern to the dairy industry

and public health authorities as they are a source of listeriosis outbreaks. Cheeses offer a suitable environment for the survival and growth of *L. monocytogenes*, allowing this pathogen to display tolerance responses that can favour its presence in cheese and persistence in dairy processing plants (MELO, ANDREW AND FALEIRO, 2015). The presence of *L. monocytogenes* on plant materials is likely due to contamination from decaying vegetation, animal faeces, soil, surface, river and canal waters, or effluents from sewage treatment operations (BEUCHAT, 1996). Healthy life styles include the essential role represented by raw or minimally processed fruits and vegetables; because of that, these can be potentially important sources of food-borne human pathogenic bacteria. However, *L. monocytogenes* outbreaks based on the consumption of fresh produce have been reported in lower numbers compared to those derived from other food sources (HOFMANN *et al.*, 2014). Food-borne outbreaks in the EU shows that *L. monocytogenes* is the most relevant pathogen associated with blanched frozen vegetables; however, the listeriosis risk associated lower than any of the evaluated ready-to-eat food categories, even if the risk was 3,600 times greater for blanched frozen vegetables consumed uncooked rather than cooked (EFSA, 2020). Packaged salads are also an emerging concern for *L. monocytogenes* contamination, having caused a multistate outbreak in 2016 in the United States (CDCP, 2016).

1.4 Listeriosis

Pathogens are classified as bacteria capable of causing damage in the host via specific virulence factors that encompass the production of toxins, features allowing attachment to and invasion of epithelial cells and components essential for their viability (CASADEVALL AND PIRODSKY, 1999). After the initial isolation and description in 1926, the *L. monocytogenes* has been shown to be of worldwide prevalence, being

associated with serious diseases in a wide variety of animals, including humans. Ingestion is the most common form of listeriosis transmission, being the previous mainly reported in products such as meats, ready-to-eat foods, dairy products, seafood and vegetables (ALLEN *et al.*, 2016; BUCHANAN *et al.*, 2017). The infection resulting from eating foods contaminated with *L. monocytogenes* can cause two types of syndromes, namely, non-invasive syndromes (mild form of the disease) and invasive syndromes (severe form of the disease). In the non-invasive syndrome, the bacterium is limited to the gastrointestinal tract, and this occurs more in healthy individuals, manifesting itself a few days after the infection, whereas in the invasive form, the bacteria reach other organs besides the gastrointestinal tract, affecting more the susceptible group, with the incubation period varying from days to 3 months. In most cases, however, the signs appear between one to two weeks, with a mortality rate between 20% and 30% (WHO, 2018A; ZHU, GOONERATNE AND HUSSAIN, 2017). The severe forms of Listeriosis are easily recognized, such as encephalitis, abortion and septicaemia. The invasion of peripheral nervous cells and rapid entry into the brain was postulated as a unique characteristic of its virulence (LOW AND DONACHIE, 1997). *L. monocytogenes* is a ubiquitous, intracellular pathogen that has been implicated within the past decade as the causative organism in several outbreaks of foodborne disease. The organism has a multi-factorial virulence system, playing a crucial role in the organism's ability to multiply within host phagocytic cells and to spread from cell to cell (FABER AND PETERKIN, 1991). *L. monocytogenes* is, indeed, capable of making the transition into a pathogen life style following its ingestion by susceptible humans or animals. Recent studies suggest that *L. monocytogenes* mediates its saprophyte-to-cytosolic-parasite transition through the careful modulation of the activity

of a virulence regulatory protein known as PrfA, using a range of environmental cues that include available carbon sources (FREITAG, PORT AND MINER, 2009).

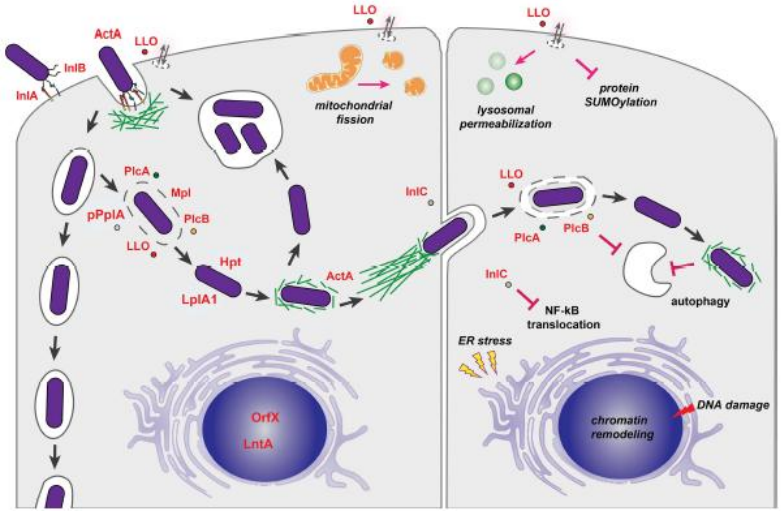
1.5 *Listeria monocytogenes* cell infection cycle and virulence process

The intracellular life style is critical in *L. monocytogenes*, the virulence mechanism (Fig.1) includes the ability to escape from extracellular host defence mechanisms, including the complement or antibodies, and hinders detection by patrolling cell populations, e.g., neutrophils; in macrophages, cytoplasmic translocation allows the escape from degradative components of the phagocytic cascade, while providing access to a "Trojan horse" host cell population that can safely transport bacteria to distant locations within the infected organism (PIZARRO-CERDA AND COSSART, 2019).

L. monocytogenes has evolved sophisticated mimicries to exploit the host cell, including the entry step, where the internalin action was pivotal and the following harnesses actin machinery to enable intracellular and intercellular spread (HAMON *et al.,* 2006). For the intracellular stage of life, *L. monocytogenes*' pivotal role has been attributed to the interaction between the superficial proteins Internalin A and B (InlA, InlB) and the cellular ligand. The domain leucine-rich repeats of InlA interact with the host cellular receptor E-cadhein (MENGAUD *et al.,* 1996; LECUIT *et al.,* 1997) with a species-specific manner (LECUIT *et al.,* 1999). Also, for the InlB, the leucine-rich repeats are critical for cell invasion (BRAUN *et al.,* 1998; BRAUN *et al.,* 1999) and bind the hepatocyte growth factor receptor Met (SHEN *et al.,* 2000). This interaction activates an intracellular signalling cascade that permits the fagocitosys of the pathogen inside a membrane-bound compartment. The latter vacuole structure is rapidly disrupted by secretion of a pore-forming toxin (listeriolysin O [LLO]) (GAILLARD *et al.,* 1987; BIRMINGHAM *et al.,* 2008; KORTEBI *et al.,* 2017) and two phospholipases (PlcA, PlcB) activated by

metalloprotease Mpl (MENGAUD *et al.*, 1991A; VAZQUEZ-BOLAND *et al.*, 1992; MENGAUD *et al.*, 1991B; BITAR *et al.*, 2008). *L. monocytogenes* secretes a pheromone, pPplA, that triggers the production of an unknown factor that cooperates with LLO in facilitating vacuolar disruption (XAYARATH *et al.*, 2015). Once the bacterial cells translocate into the host cell cytoplasm, the pathogens activate several bacterial metabolic pathways that favour both the uptake of cellular resources and bacterial proliferation. At this point, several strategies to escape cytoplasmic innate immune responses are activated including the actin polymerization by bacterial surface protein (ActA), which also allows the spread into the neighbouring cells, favouring cytoplasmic motility and avoidance of autophagosomes (BIRMINGHAM *et al.*, 2007). These secondary infected cells host the pathogen *L. monocytogenes* within a double-membrane compartment; also, in this case, disruption takes place through action of the proteins LLO and PlcA, PlcB (CAMILI *et al.*, 1993; MARQUIS *et al.*, 1995; SMITH *et al.*, 1995; GRÜNDLING *et al.*, 2003). Extracellular LLO is able to modulate different cellular functions, including mitochondrial fission (STAVRU AND COSSART, 2011), lysosomal permeabilization (MALET *et al.*, 2017), protein SUMOylation (RIBET *et al.*, 2010), ER stress (PILLICH *et al.*, 2012), DNA damage (SAMBA-LOUAKA *et al.*, 2014), and chromatin remodelling (HAMON *et al.*, 2007).

Figure 1: *Listeria monocytogenes* virulence process during the intracellular lifestyle



Source: PIZZARRO-CERDA AND COSSART, 2019

1.6 Key virulence Factors

The completion of each stage of the *L. monocytogenes* life cycle depends on the orchestrated activity of specialized bacterial factors, which were detected as controlled by a specific set of regulators. Dozens of molecules deployed by *L. monocytogenes* promote its cell infection cycle, in this context some virulence factors and modulators assume an important role, including the bacterial resistance and evasion to host defence mechanisms (CAMEJO *et al.*, 2011). Emerging evidence suggests that the shift from the extra host environments (saprophytic) to the host gastrointestinal tract and, subsequently, to intracellular environments (pathogen) stage of life *L. monocytogenes* requires regulatory interplay between σ^B and PrfA at transcriptional, posttranscriptional, and protein activity levels (GABALLA *et al.*, 2019).

1.6.1 Prfa key virulence factor

PrfA directly regulates a small set of protein-encoding genes directly acting in the intracellular virulence process (DE LAS HERAS *et al.,* 2011). The PrfA dependent virulence genes include the following: hly, which encodes the cholesterol-activated pore-forming toxin listeriolysin O (LLO); plcA, which encodes a phosphatidylinositol phospholipase; plcB, which encodes a lecithinase with broad substrate specificity; and mpl, which encodes a metal-dependent protease involved in PlcB maturation (CAMAJO *et al.,* 2011). Moreover, the PrfA boxes have also been detected upstream from a number of genes that encode internalins, which facilitate host cell invasion in the entry step. Specifically, a PrfA box has been identified both upstream from inlAB and inlC (PIZARRO-CERDÀ *et al.,* 2012). However, inlAB and inlC are also regulated by other non-PrfA-dependent promoters, including a σB-dependent promoter (KIM *et al.,* 2005). Host-derived glutathione (GSH) as a cofactor role is required for the full activation of PrfA (RENIERE *et al.,* 2015), the major role in *L. monocytogenes* virulence was given to the PrfA regulon. *L. monocytogenes,* to ensure the success of each infection process, must tightly control the expression of each PrfA-regulated gene both temporally and spatially. Unregulated activation of PrfA can exert an undue burden on the cell, as shown by the reduced growth rate and lower fitness of a strain expressing constitutively active PrfA (BRUNO *et al.,* 2010; MARR *et al.,* 2006).

1.6.2 Sigma(σ) B regulon

To survive in stress conditions, *L. monocytogenes* has to modulate the gene transcriptions adapting to environmental conditions. The pathogen possesses many regulatory proteins to modulate gene expression. To contrast the environmental stress, a pivotal role was detected by σB regulon (GULDIMAN *et al.,* 2017; DOREY *et al.,* 2019). The genome of *L. monocytogenes* encodes up to five σ factors, the housekeeping σ A and 4 alternative

factors: σ B, σ C, σ L, and σ H (O'BYRNE AND KARATZAS, 2008). *L. monocytogenes* includes the ability to survive exposure to severe environmental conditions, including: low temperature, acid stress, high osmolarity, and gastrointestinal factors. A large set of genes were involved actively in response to this stress. In general, the stress response in *L. monocytogenes* was defined under the control of the alternative sigma factor σ B (GULDIMANN *et al.*, 2016; VAN SCHAIK AND ABEE, 2005). A total of hundred genes have been shown to be codified under transcriptional control of σ B since its discovery (ABRAM *et al.*, 2008A, B; RAENGPRADUB *et al.*, 2008; TOLEDO-ARANA *et al.*, 2009 LIU *et al.*, 2017). In contrast to PrfA, which has shown pivotal role into the host cell environment, σB plays a major role in *L. monocytogenes* survival in nature, in the built environment (CHATURONGAKUL *et al.*, 2008; NICAOGÁIN AND O'BYRNE, 2016), and in the digestive tract but is less essential within the host cytoplasm (KAZMIERCZAK *et al.*, 2006; GAHAN AND HILL, 2005). *L. monocytogenes* and *B. subtilis*, expression of the σB regulon, occur when cells encounter stress, and an unregulated expression of σB greatly reduces cell growth (GULDIMANN *et al.*, 2017; DOREY *et al.*, 2019).

1.6.3 Additional regulons

Among these regulatory factors, the VirR regulon, known to act against environmental stress, has shown a role on regulating the expression of genes necessary for resistance to antimicrobial agents and virulence (GRUBAUGH *et al.*, 2018; CHAKRAVARTY AND MASSÈ, 2019; LEBRETON AND COSSART, 2017). Subsequently, regarding the infection of the host cell, *L. monocytogenes* upregulates the transcription of other factors necessary for productive infection. In addition to PrfA and σ B to survive under changing conditions, *L. monocytogenes* can sense the intracellular host environment partially through the global regulator CodY (LOBEL *et al.*, 2012). Specifically, the role of CodY

extends to repressing σ B expression under conditions of nutrient excess, while repressing other genes involved in metabolism, motility, and virulence (LOBEL AND HERSKOVITS, 2016).

1.7 *L. monocytogenes* antibiotic resistance

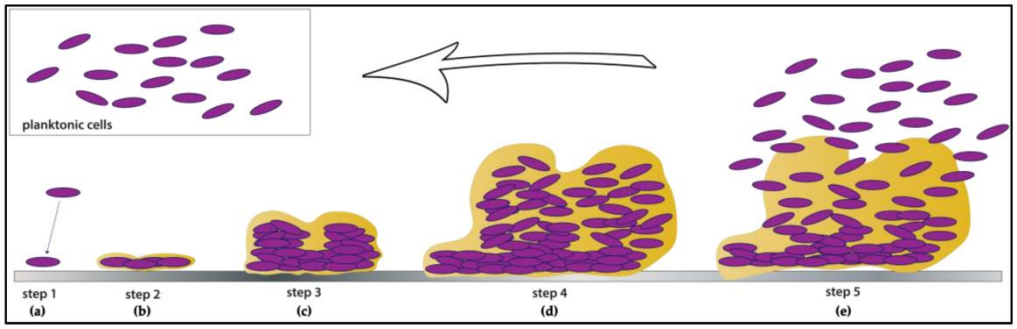
The first multidrug-resistant *L. monocytogenes* strain was described in 1988 by a strain isolated from a patient in France who was suffering from meningoencephalitis (POYART-SALMERON *et al.*, 1990). *L. monocytogenes* is widely susceptible to clinically relevant classes of antibiotics that are active against Gram-positive bacteria, however, a prevalence of resistant strains was estimated at 1.27% among isolates from humans (MORVAN *et al.*, 2010). Studies considering environmental isolates have reported an increased rate of resistance to one or several clinically relevant antibiotics. In particular, the prevalence of antimicrobial resistance genes in *L. monocytogenes*, isolated from dairy farms, was detected (CONTER *et al.*, 2009). Diverse factors in food processing chains and environments – including extensive or sub-inhibitory antibiotics use, horizontal gene transfer, exposure to environmental stresses, biofilm formation, and presence of persisted cells – play crucial roles in the development of antibiotic resistance by *L. monocytogenes* (OLAIMAT *et al.*, 2018). In this context, a study revealed that, from a total of 336 Listeria isolates, strain coming from ready-to-eat meat products displayed higher overall antimicrobial resistance (31.3%) than those from the environment (13.4%). Listeria resistances were observed with higher rate in *L. inocua* compared to *L. monocytogenes* isolates. However, all the strains assayed were sensitive to the preferred antibiotics used to treat listeriosis (GOMEZ *et al.*, 2014). In a study that compared the antimicrobial rate in a group of *L. monocytogenes* food isolated with other studies around the world, food isolated resistant strains were more prevalent in Germany than others, including the EU, the USA

and China, showing similar stocks. The multidrug-resistance in strains predominantly belonged to serotype IV (59%) (NOLL, KLETA AND DAHOUK, 2018). Others studies have shown how most of the *L. monocytogenes* strains from food and food-processing environments were susceptible to the antibiotics commonly used in veterinary and human listeriosis treatment. However, considering the fact that *L. monocytogenes* is slowly becoming antibiotic resistant, a continued surveillance of emerging antimicrobial resistance to ensure effective treatment of human listeriosis is needed (CONTER *et al.*, 2009). A recent study has shown how *L. monocytogenes* species are specialized in small specific niches, reducing the possibility of coexistence with potential donors of antibiotic resistance. However, occasional increases in population sizes can derive from the selection of the species based on acquired resistance to antibiotics, biocides, heavy metals or by a natural tolerance to extreme conditions (BAQUERO *et al.*, 2020). In food industries, sanitizing agents are commonly used to contrast eventual bacteria presence. Laboratory conditions simulating a food processing environment permitted to detect *L. monocytogenes* resistance to the peroxide sanitizer as well as others (quaternary ammonium compounds and chlorine); the biofilm was considered fundamental in this resistance process (PAN *et al.*, 2006). The reduction to a sublethal concentration of sanitizer was detected as the main concern in food industries. *L. monocytogenes* biofilm contributes with the sanitizers' concentration reduction to subinhibitory levels, reaching the bacterial cells. Also, the acquisition of resistance mechanisms in *L. monocytogenes* was already reported. However, the recommended sanitizers doses overcome the problem, although the possible sanitizers environment dilution and biodegradation after the application with the microorganisms have to be controlled, exposing the subinhibitory concentrations (MARTÍNEZ-SUÁREZ *et al.*, 2016).

1.8 Biofilm

Biofilm is a microbial association or community attached to various surfaces and environments, including food and food processing surfaces, where bacteria find an ideal environment for biofilm formation as a sufficient nutrient availability for microbial growth and attachment. Therefore, these specialized structures become a challenge in food safety and human health (ABEBE, 2020). *L. monocytogenes* in food industry can persist for a long time in different locations of a processing facility, especially in the areas difficult to clean and disinfect, representing a concern in food safety (COLAGIORGI *et al.,* 2017; BERRANG *et al.,* 2005; CHAITIEMWONG *et al.,* 2010). Schematic representation of the biofilm development stages (Fig. 2) report the development of *L. monocytogenes* biofilm in five different steps (VASUDEVAN, 2014; COLAGIORGI *et al.,* 2017). The first step involves planktonic cells' reversible attachment to surfaces; the second step includes the adherence of cells, the beginning of formation of a monolayer, and the production of extracellular matrix; the following third step involves the self-production of extrapolymeric matrix by the cells and the continuation of growth with the formation of multilayered microcolonies; in the fourth step, the cells become irreversibly attached to the surface and embedded in the matrix with the presence of a "mature" biofilm; in the last stage of biofilm formation, cells are able to detach from the biofilm and return in planktonic form, ready to colonize new surfaces.

Figure 2: *Listeria monocytogenes*'s biofilm cyclic organization: from the attachment to the planckotonic cells liberation



Sources: COLAGIORGI *et al.*, 2017; VASUDEVAN, 2014;

Concretely, *L. monocytogenes* was well-known for forming biofilm of structured bacterial cells communities embedded in a self-produced matrix of extracellular polymeric substances (EPSs), characterized by an altered gene expression and, as consequence, phenotype (DONLAN, 2002). Specifically in *L. monocytogenes*, a significant role in the process of biofilm formation was ascribed to flagella, biofilm-associated proteins (BapL), SecA2, and cell-cell communication (GUILBAUD *et al.*, 2015, RENIER *et al.*, 2014), as well as extracellular DNA (eDNA) (HARMSEN, LAPPANN, KNOCHEL, & MOLIN, 2010). A summary of molecular determinants of biofilm formation by *L. monocytogenes* has been reported (KOCOT AND OLSZEWKA, 2017). Recently, the role of the virulence genes was reported; in particular, the sessile *Listeria monocytogenes* cells expressed reducing levels of the *lap*, *inlA*, *hly*, *prfA*, and *sigB* with consequent reduction of adhesion, invasion, translocation, and cytotoxicity in the cell culture model comparing them with the planktonic cells. The virulence of *L. monocytogenes* isolated by biofilm aggregations was temporarily attenuated but appeared upregulated and fully restored with the time (BAI *et al.*, 2021).

In *L. monocytogenes*, the biofilm-forming ability act as resistance mechanisms against even sanitizing agents**,** through, for example, the employment of disinfectant benzalkonium chloride at concentrations close to the minimum inhibitory (RODRIGUEZ-

MELCON *et al.*, 2019; PAN *et al.*, 2006, VAN DER VEEN AND ABEE, 2011). As a consequence, a deeper insight into the biofilm formation process was then required to enable adopting best hygienic practices against *L. monocytogenes* and, resultantly, ensuring food safety (KOCOT AND OSZENWSKA, 2017). Moreover, in this foodborne pathogen, the biofilm formation was detected influenced by various stress conditions. Physical conditions such as the temperature play an important role in favouring biofilm formation (DI BUONAVENTURA *et al.*, 2008; MOLTZ *et al.*, 2006), as well as the nature of adhesion surface, including its hydrophobicity (MIDELET *et al.*, 2006). Persister cells of *L. monocytogenes* isolated from food environments were able to attach food processing surfaces and build biofilm (ALESSANDRIA *et al.*, 2010). In this context, the diffuse use of the natural antimicrobial nisin as a natural food preservative can represent a concern, considering that nisin has been observed to create a population of persister cells (WU *et al.*, 2017).

1.9 Antimicrobial Peptides

Due to an increase in drug-resistant infections, the discovery of new compounds as presented a serious challenge to antimicrobial therapies. The failure of the most potent antibiotics to kill multidrug-resistant bacteria emphasizes the urgent need to develop other control agents. The discovery and development of antimicrobial peptides (AMPs) represent a growing class of natural and synthetic peptides with a wide spectrum of targets, including viruses, bacteria, fungi, and parasites (BAHAR AND REN, 2013). AMPs are evolutionarily conserved peptides with small molecular weight, usually positively charged and having both hydrophobic and hydrophilic sides that permit these molecules to be soluble in aqueous environments yet also enter lipid-rich membranes (IZADPANAH AND GALLO, 2005). Frequently, these cationic AMPs can bind and interact with the negatively

charged bacterial cell membranes, leading to the change of the electrochemical potential on bacterial cell membranes, inducing cell membrane damage and the permeation of larger molecules such as proteins, destroying cell morphology and membranes, eventually resulting in cell death. Furthermore, AMPs have been demonstrated to have their own advantages over the traditional antibiotics with a broad-spectrum of antimicrobial activities, even overcoming bacterial drug-resistance (LEI *et al.*, 2019). AMPs, indeed, were considered as possible candidates for the design of new antimicrobial agents not only because of their natural antimicrobial properties but also for their low propensity for development of resistance by microorganisms (WANG *et al.*, 2016). In addition to their activity on bacterial membranes, antimicrobial peptides were reported to be able to directly and indirectly modulate responses of the innate immune system. The increasing interest for the potential biomedical applications led to the possibility of including AMPs in therapeutics and as a food preservative (MOR, 2000). Therefore, besides the direct antimicrobial function of AMP, these small peptides have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells, influencing diverse processes, such as cell proliferation, immune induction, wound healing, cytokine release, chemotaxis, and protease-antiprotease balance (KOCZULLA AND BALS, 2003). Natural antimicrobials, known as host defence peptides or antimicrobial peptides, defend host organisms against microbes, but most have modest direct antibiotic activity. Improved variants have been developed using simple molecule design and optimization strategies, including computer-assisted design strategies, have been tested clinically (FJELL *et al.*, 2012). Despite the increased interest, the adoption of peptide antimicrobials in clinical practice is still limited as a consequence of several factors, including side effects, problems with bioavailability, and high production costs, which, combined with a rigorous

regulatory environment, have retarded the clinical translation of antimicrobial peptides as potential therapeutic agents. Fortunately, existing computational and experimental tools attempt to ease the preclinical and clinical development of antimicrobial peptides as novel therapeutics (MAGANA *et al.,* 2020). Also, AMPs in food application seems to still be the cause of concerns, including the integrity and stability of these molecules in real food systems. To overcome these problems, the incorporation of AMPs into food packaging has also been studied to ensure AMPs controlled release, limiting the direct exposure with food components that can lead to different reactions and formation of new unknown compounds that remains a challenge in this field. Moreover, further studies may be needed to define the conditions of use, such as type of foods, proposed use level, the temperature range of use, and method of application in order to get official approval for food applications (LEON MADRAZO AND SEGURA CAMPOS, 2020). The development of a bacterial antibiotic-resistant has been observed as collateral sensitivity to antimicrobial peptides with high frequency, whereas cross-resistance is relatively rare. These advances allowed the identification of antimicrobial peptide–antibiotic combinations, enhancing antibiotic activity against multidrug-resistant bacteria and slowing down, once more, the evolution of resistance (LAZAR *et al.,* 2018). However, if AMPs are to be used clinically, it is crucial to understand their natural biology in order to reduce the risk of collateral harm and avoid the crisis of resistance now facing conventional antibiotics (LAZZARO, ZASLOFF AND ROLFF, 2020).

1.10 Antimicrobial peptides in food industry

Antimicrobial compounds secreted by bacteria might be utilized for extending shelf life in the food industry, among other applications (ZHAO AND KUIPERS, 2016). Bacteria produce diverse types of antimicrobial peptides (AMPs), including ribosomally

synthesized, also known as bacteriocins (CHIKINDAS *et al.*, 2018), and non-ribosomally synthesized AMPs, e.g., cyclic lipopeptides (CLPs) (FIRA *et al.*, 2018). Already used in agro-food areas, bacteriocins are peptides, ribosomes naturally synthesized, produced by both Gram-positive and Gram-negative bacteria, which will allow these bacteriocin producers to survive in a highly competitive polymicrobial environment. Bacteriocins exhibit antimicrobial activity with variable spectrum depending on the peptide, which may target several bacteria (SIMONS, ALHANOUT AND DUVAL, 2020). In this context, common bacterial species that produce bacteriocins are called lactic acid bacteria (LAB), considered in many cases probiotics. Bacteriocins have achieved great importance in different food biopreservative areas, such as: dairy products, meat products, fish products, etc. (ABDULHUSSAIN KAREEM AND RAZAVI, 2020). Beside the bacteriocins, others antimicrobial peptides have received great attention for their potential benefits to extend the shelf-life of food-products, showing antibiofilm activity on stainless steel surface, and, for this reason, may be a promising eco-friendly sanitation agent against *Listeria* on abiotic surfaces (PALMIERI *et al.*, 2018). The use of AMPs in food preservation constitutes a promising alternative and offers many benefits, such as reducing the use of chemical preservatives, reducing food losses due to spoilage, and development of health-promoting food supplements (AHMED AND HAMMAMI, 2019).

1.10.1 NonRibosomal Peptides (Cyclic Lipopeptides)

Bacillus strains are able to produce CLPs, collectively grouped into the surfactin, fengycin, and iturin families. Surfactin group is the most studied microbial surfactant due to its antimicrobial, antiviral and antitumor properties, moreover it helps on mobility; indeed, *Bacillus* strains unable to produce surfactin do not show swarming motility (JADEJA *et al.*, 2019). Fengycin was the first potent antifungal lipopeptide discovered

(VANITTANAKOM AND LOEFFLER, 1986), although it has been recently confirmed that fengycin reduce *Staphylococcus aureus* presence in human intestine by inhibiting quorum sensing, through which *S. aureus* responds to their population density by altering gene regulation (PIEWNGAM *et al.,* 2018). Another group of Bacillus CLPs comprise the iturin family, similar to other bacterial lipopeptides, represented by different isomers that show broad-spectrum of antimicrobial activity (MAGET-DANA AND PEYPOUX, 1994; FALARDEAU *et al.,* 2013). Iturin lipopeptides component family include iturin, bacillomycin D, bacillomycin F, bacillomycin L, mycosubtilin, and mojavensin.

1.10.2 Bacteriocins (Nisin)

Therefore, bacteriocins are post-translationally modified peptides widely disseminated among different bacterial clades (ABRIOUEL *et al.,* 2011). Bacteriocins include the lantibiotic nisin allocated into the class I, an antimicrobial peptide produced by *Lactococcus lactis* strains mostly known for its activity against Gram-positive bacteria (GHARSALLAOUI *et al.,* 2016). Nisin was discovered in 1928 and granted the "generally recognized as safe" (GRAS) status by the US Food and Drug Administration (FDA) in 1988; nowadays, it is employed by more than 48 countries in various food applications (MÜLLER-AUFFERMANN *et al.,* 2015). Nisin has been largely employed as a food preservative in dairy products and its high antimicrobial efficiency has been documented. However, there are some reports on *L. monocytogenes* strains with acquired tolerance or resistance to the bacteriocin nisin (WU *et al.,* 2018).

1.11 Nanotechnology in food applications

Nanotechnology is usually referred to the engineering of material structures with sizes ranging from 1-100 nm to suit the need for different sectors. The employment of

nanotechnology in all areas of the food sector showed a great potential, ensuring among the others an increase in the shelf life of food (AIGBOGUN *et al.*, 2017). The rapid development of nanotechnology has permitted the transformations of traditional food through the introduction of smart and active packaging for improving food quality and safety (HE, DENG AND HWANG, 2019). The incorporation of nanotechnology in food industries has, indeed, improved food security, thus increasing shelf life and inhibiting microorganism growth among the other positive effects (NURFATIHAH AND SIDDIQUEE, 2019). The advantages coming from food application nanotechnologies were considered pivotal to overcome the reduction of antimicrobial efficacy observed in food applications, as the encapsulation of antimicrobials into liposomes possibly protect and enhance the efficacy and stability of some compounds (LOPES AND BRANDELLI, 2018). However, despite the important nanotechnology benefits in food application, emerging concerns for example regarding the accumulation of nanostructured materials in human bodies were reported. Therefore, safety and health concerns, as well as regulatory policies, must be considered in all the processes parting from the production to the consummation of nano-processed food products (BAIPAI *et al.*, 2018).

1.11.1Liposome nano-encapsulation

Nano-encapsulation is a distinct process, consisting on the entrapment of a substance within another transport material in nanometres scale, between 1 nm and 1.000 nm (FATHI, MARTIN AND MCCLEMENTS, 2014; BRANDELLI, LOPES AND BOELTER, 2017). Nanoscale lipid bilayers, or nanoliposomes, are generally spherical vesicles formed by the dispersion of phospholipid molecules in a water-based medium by energy input. Due to their bi-compartmental structure, which consists of lipidic and aqueous compartments, these nanocarrier structures in food permit to encapsulate

hydrophobic and hydrophilic molecules along with bioactive compounds and food ingredients, supplementary systems for therapeutic purpose (SUBRAMANI AND GANAPATHYSWAMY, 2020; ZARRABI *et al.*, 2020). On the strength of the versatility, bioactivity, non immunogenicity, and nontoxicity of bioactive nanoliposomes, their utilization in food has strong potential, especially for the development of continuous modes of liposome production, commercial availability of sources of phospholipids and easy-to-prepare liposomal dispersions (DEMIRCI *et al.*, 2017). Nanoliposome encapsulation of antimicrobials was already considered pivotal to overcome stability issues and undesirable interaction with food components (PINILLA AND BRANDELLI, 2016).

1.12 Mass Spectrometry and Omics Technologies

Since DNA sequencing methods and the advent of new generation sequencing of high-throughput methodologies having become fundamental in various scientific fields, these latter strategies permitted, among other things, the development of the mass spectrometry (MS) instrumentation discovered in the late 1980s, which permitted to separate and detect ions into gas phase after a previous molecule ionization using different techniques (CANAS *et al.*, 2006). Mass spectrometry has become an essential analytical tool for identification of expressed proteins by a cell or tissue; this methodology was named proteomics (GYGI AND AEBERSOLD, 2000). In addition to the proteomics investigations, it was observed that the mass spectrometry methods have vast potential also as a tool for metabolomics studies, giving a comprehensive and quantitative analysis of a wide array of metabolites in biological samples. Metabolomics investigations have turned into a challenge for the understanding of phenotypic changes in an organism in response to genetic, nutritional, toxicological, environmental, and pharmacological influences (DETTMER *et al.*, 2007). Therefore, the advent of mass spectrometry technologies inside

the omic technologies permitted the development of proteomics and metabolomics; these latter methodologies have been pivotal for the detection and quantification of proteins and metabolites from different tissues in untargeted or targeted manner (GIROLAMO *et al.*, 2013).

1.12.1Microbial Proteomics

Proteomics is a commonly recognised and adopted OMIC approach to identify the global change in bacterial proteins with the objective of understanding the molecular mechanisms of bacterial pathogenesis, drug action, and identification of disease outcome determinants (GAGARINOVA *et al.*, 2017; MIAO *et al.*, 2018; SHI *et al.*, 2019). Moreover, proteomics is recognised as one of the "omic technologies" that can give light of antimicrobial compound "mode of action" (FREIBERG *et al.*, 2004), besides providing information about resistance to antibiotics (MONTEIRO *et al.*, 2016).

1.12.2 Microbial Lipidomics

Lipids play a pivotal role in all biological systems and act in numerous structural and functional roles including the providing of structural molecules for forming cellular membrane bilayers (VAN MEER, VOELKER AND FEIGENSON 2008). Since the advent of the mass spectrometry-based lipidomics, studies have been performed to study mammalian and other eukaryotic systems. Recently, there has been a growing rise in the exploration of bacterial lipidomics. Bacterial lipids can offer information about the maintenance of the bacteria structure and protection from the surrounding environment, including the antibiotic susceptibility (APPALA *et al.*, 2020). Considering that the cationic AMPs offer a promising therapeutic alternative to conventional antibiotics, there is a strong need to improve our understanding about the AMP mechanism of action.

Lipidomics can be considered new approaches for studying the bacterial membrane (RASHID, VELEBA AND KLINE, 2016). Lipidomic profile changes among bacterial species, presenting antimicrobial resistance. Some of the changes depending on the specific fatty acid compositions were observed. As a consequence, the characteristic changes in individual lipid species could be used as biomarkers to identify and underlay resistance mechanisms and for evaluating potential therapies (HINES *et al.,* 2017). Membrane disruption caused by AMPs and the changes in the structural properties that microbial membranes undergo in response to AMPs were investigated by lipidomics techniques (HEWELT-BELKA *et al.,* 2016;LEE *et al.,* 2019).

2 General Objective

Study of the different *L. monocytogenes* responses to Antimicrobial Peptides (AMPs), including the nano-encapsulation into phosphatidylcholine liposomes as biotechnological application, through the use of omic approaches.

2.1 Specific Objectives

- Characterization and identification of the bacterial genome of AMPs secreted by the *Bacillus velezensis* P34;
- Proteomic profile study of *L. monocytogenes* responses to the lipopeptide P34 (fengycin and small quantities of bacillomycin), including nanoliposome encapsulation;
- Proteomic profile of *L. monocytogenes* superficial proteins responses to the commercial nisin bacteriocins;
- Proteomic and lipidomic profiles of *L. monocytogenes* responses to both CLPs mostly fengycin and nisin.

3 ARTICLES DESCRIPTION

The aim of this Ph.D. thesis was the molecular strategies study involved in *L. monocytogenes* responses to antimicrobial peptides (AMPs) used as stressor agents. Specifically, nisin (commercial AMP for the food industry) and cyclic lipopeptides (CLPs) produced by *Bacillus* P34, a bacteria isolated from a freshwater fish gut, were employed. In addition of this thesis was the study and investigate the possible biotechnological application of these compounds against *L. monocytogenes* through "omics" science methodologies. The materials and methods section of this Ph.D. thesis has been reported in the form of scientific articles brefly described in the following section.

The previous named *Bacillus* P34 bacterium have been studied by our group, in this work the interest was directed on the genome annotation, focusing on the production of antimicrobial compounds. I carried out a phylogenetic analysis for identification of the bacterial species as *Bacillus velezensis* P34. HPLC analysis permitted the quantification of the principal components of the antimicrobial products secreted by the *Bacillus* species. These components were identified as cyclic lipopeptides (CLPs), mostly fengycin and iturin. Moreover to identify the CLPs isomers produced by *B. velezensis* P34 Maldi-Tof MS and LC-MS analyses were carried out. This first paper was titled "Diversity of cyclic antimicrobial lipopeptides from *Bacillus* P34 revealed by functional annotation and comparative genome analysis" and has been published by the journal *Microbiological Research* (IF: 3.970)

Proteomics analysis of *L. monocytogenes* cultures treated with sub-lethal concentration of CLPs peptides produced by *B. velezensis* P34 after purification, were performed. Moreover a nano-encapsulation of the purified lipopeptides P34 (mostly fengycin and small

quantities of Bacillomycin L) inside nanoliposomes bilayers constituted by a dried lipidic film of soybean purified phosphatidylcholine was carried out. The bacterial treatments included the lipopeptides P34 free (F-P34) and P34 encapsulated into nanoliposomes (N-P34), while empty nanoliposomes (NE) and fresh buffer were used as controls. These treatments were injected into the bacteria culture after 6h from the *Listeria* inoculation, than an additional hour of inoculation was carried out to stress the bacteria. The total protein extraction of *L. monocytogenes* and orbitrap LC-MS analyses were carried out. Statistical analysis of the proteomics database coming from the analysis permitted to detect two main process modifications by the comparison between treatments and control samples. Specifically, reduction or a probable inhibition of virulence because of the AMPs action on membrane protein complexes: PTS (phosphotransferase systems) system and oligopeptide transporters working negatively up to the key virulence factor PrfA. Another group of proteins included into the paper results section were involved up to the metal ions homeostasis process. These latter proteins included membrane ion transporters as the manganese metal ions transporters, already considered possible important antimicrobial target in other Gram-positive bacteria. In this context to maintain the metal ions homeostasis, upregulation of ferric ion metal transports systems to replace the manganese transport across the membrane were shown. This second paper was titled "Proteomic analysis of *Listeria monocytogenes* exposed to free and nanostructured antimicrobial lipopeptides" and has been published by the journal *Molecular Omics* (IF: 3.743).

Moreover an addition study including a set of proteomics analysis, applying a sublethal dose of antimicrobial peptide produced by bacteria on *L. monocytogenes* was included. The commercial bacteriocin "nisin" was used as a model antimicrobial peptide acting on bacterial surface. Particularly, focus was gave on the surfaceproteomics because of the key

function of this peripheral proteins on the main molecular process considered possible target of new antimicrobial compounds, beside to be evolved the *Listeria* pathogenicity. Superficial proteins extraction and Orbitrap LC-MS analysis were carried out. The obtained results demonstrated how the bacteria protect itself by this specific antimicrobial stressor forming biofilm. On the other hand, a drastic reduction of the main proteins acting on the *Listeria* virulence process were detected. The article was titled "Nisin influence on the expression of *Listeria monocytogenes* surface proteins" and has been published by the *Journal of Proteomics* (IF:3.509).

In conclusion a comparison study including a set of proteomic and lipidomic analysis applying sublethal doses of antimicrobial peptides considered in this thesis work, respectively nisin and CLPs, mostly fengycin, both produced by bacteria on *L. monocytogenes* was realized. Lipids and proteins extraction followed by mass spectrometry were carried out. Statistical and elaboration of the lipidomic and proteomics databases coming from the analysis permitted to detect two main process modification. The obtained results revealed inhibition of the *L. monocytogenes* biofilm by the fengycin lipopeptides treated samples in comparison with the promotion of this multicellular organization by the nisin treated samples. Moreover, a set of proteins were highlighted for their action in supporting the membrane resistance against both the AMPs. The article was titled "Lipidomic and proteomic analysis of Listeria monocytogenes reveals modulation of biofilm formation by exposure to antimicrobial peptides" and has been submitted to the *Cellular and Molecular Life Sciences* (IF: 9.261).

3.1 First Scientific Article:

Diversity of cyclic antimicrobial lipopeptides from *Bacillus* P34 revealed by functional annotation and comparative genome analysis

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Diversity of cyclic antimicrobial lipopeptides from *Bacillus* P34 revealed by functional annotation and comparative genome analysis

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Abstract

Cyclic lipopeptides (CLPs) from *Bacillus* strains have demonstrated a wide range of bioactivities making them interesting candidates for different applications in the pharmaceutical, food and biotechnological industries. Genome sequencing, together with phylogenetic analysis of the *Bacillus* sp. P34, isolated from a freshwater fish gut, showed that the bacterial strain belongs to the *Bacillus velezensis* group. *In silico* investigation of metabolic gene clusters of nonribosomal peptide synthetases (NRPS) revealed the genetic elements associated with the synthesis of surfactin, fengycin and iturin family component bacillomycin. Further, an assay was conducted to investigate the production of CLPs in the presence of heat inactivated bacterial cultures or fungal spores. Maximum fengycin concentration was observed at 24 h (2300−2700 mg/mL), while maximum iturin amounts were detected at 48 h (250 mg/mL) in the presence of heat-inactivated spores of *Aspergillus niger*. Heat-inactivated cells of *Listeria monocytogenes* caused a reduction of both fengycin and iturin amounts. The production of fengycins A and B and the iturin family component bacillomycin L was confirmed by mass spectrometry analyses. This study reinforces the potential of *B. velezensis* P34 as a valuable strain for biotechnological production of CLPs recognized as important antimicrobial substances.

Keywords: Antimicrobial peptides, Iturin, Fengycin, Genomics, Lipopeptides

1. Introduction

Enormous expectative has been given to new bacterial genomes and other molecular analysis on the increasing amount of information about known and novel bioactive molecules. The research on dynamics of bacterial populations, considering the interplay of bacteria with their environment, with other organisms and with hosts (e.g. gut microbiota, plant microbiota) is gaining importance, and certainly involve antimicrobials of all sorts (Tracanna et al., 2017; Lasa and Solano, 2018).

Bacillus is one of the most studied bacterial groups, and it has been recognized as one of the biggest antimicrobial producers, including about 90 different types of peptides recognized for their antimicrobial as well as cytostatic or cytotoxic activities (Coutte et al., 2017). *Bacillus* species are easy to handle and grow in the laboratory using common culture media, with a higher productivity of antimicrobial compounds. Among various antimicrobials produced by *Bacillus*, several strains are known to produce three main different families of cyclic lipopeptides (CLPs), namely surfactins, iturins and fengycins (or plipastatins). These lipopeptides are typically produced and secreted at the end of the exponential growth phase or during the stationary phase, through a complex enzymatic mechanism of biosynthesis catalyzed by non-ribosomal peptide synthetases (NRPSs) (Jacques, 2011; Fira et al., 2018). These antimicrobial substances are chemically characterized as cyclic oligopeptides containing D-amino acid residues linked to a β-hydroxy fatty acid. The fatty acid chain can be linear or branched, varying in length (typically C6-C18) and in the degree of oxidation (Cochrane and Vederas, 2016). The amphiphilic character of CLPs seems to be important for biological activity, which render them ideal biosurfactant properties. Hence, many CLPs are crucial for bacterial swarming motility and biofilm formation (Banat et al., 2010). Many species of the genus *Bacillus* are

able to secrete CLPs, such as *Bacillus subtilis* (Vanittanakom and Loeffler, 1986), *Bacillus pumilus* (Kalinovskaya et al., 2002) *Bacillus licheniformis* (Peypoux et al., 1999), *Bacillus velezensis* (Palazzini et al., 2016) and *Bacillus amyloliquefaciens* (Mnif and Ghribi, 2015). Fengycin was initially described as a potent antifungal lipopeptide (Vanittanakom and Loeffler, 1986), although it has recently been confirmed that fengycins reduce *Staphylococcus aureus* population in human intestine by inhibiting quorum sensing (Piewngam et al., 2018). Surfactin is one of the most powerful biosurfactants, contributing to biofilm formation and providing *Bacillus* strains of swarming motility (Jadeja et al., 2018). Surfactin is the most studied *Bacillus* lipopeptide due to its antimicrobial, antiviral and antitumor properties. Another group of *Bacillus* CLPs comprises the iturin family, similarly to other bacterial lipopeptides this group is represented by different isomers that show a broad-spectrum antifungal activity (Maget-Dana and Peypoux, 1994; Falardeau et al., 2013). The iturin family of lipopeptides includes iturins A–E, bacillomycin D, bacillomycin F, bacillomycin L, mycosubtilin and mojavensin. These are cyclic heptapeptides, where the first three amino acids (Asn, Tyr, Asn) of the heptapeptide are conserved among all family members while the latter four may vary among each family component. *Bacillus velezensis* was considered the only species able to produce bacillomycin L, besides iturin A and bacillomycin D (Dunlap et al., 2019). Competition and/or cooperation mechanisms among bacterial communities dominate the intraspecific and interspecific relationships, with the main objective of utilization of space and resources. The secretion of molecules to kill or impair neighboring cells is one of the mechanisms attributed to bacteria for competition for these resources in different niches (Hibbing et al., 2010; Ilinskaya et al., 2017; Lasa and Solano, 2018). In this context, increased antimicrobial activity of *B. amyloliquefaciens* LBM 5006 has been demonstrated

when this strain was cultivated with intact or thermally inactivated cells of other bacteria (Benitez et al., 2011). In addition, the expression of essential genes related to the biosynthesis of iturin and fengycin was stimulated in *B. amyloliquefaciens* P11 incubated with inactivated cells of *S. aureus* (Leães et al., 2016), suggesting that an external stimulus by the alien bacteria improves the secretion of antimicrobial molecules. Although antimicrobial CLPs from *Bacillus* have been widely studied, new variants of these lipopeptides were recently discovered (Ait Kaki et al., 2020), probably as a result of genetic mutations (Bóka et al., 2019). Thus, the genome analysis of novel *Bacillus* strains obtained from alternative sources might help the identification of new substances with potential biotechnological applications, once minor changes in the DNA sequence may influence biological properties. A group of *Bacillus* strains isolated from the gut of Piau-com-pinta (*Leporinus* sp.), a teleost fish from the Amazon River, has been investigated for their antimicrobial activities. Among them, *Bacillus* sp. P34 was shown to produce peptides with broad antimicrobial spectrum against Gram-positive bacteria and viruses (Motta et al., 2007; Silva et al., 2014). The objective of this study was to investigate the *Bacillus* sp. P34 genome by searching for gene clusters associated with production of antimicrobial substances, particularly CLPs. From the identification of secondary metabolite gene clusters, the production of antimicrobial compounds was also investigated. In this case, *Bacillus* sp. P34 was cultivated with heat-inactivated cells of a Gram-positive bacterium (*Listeria monocytogenes*) and spores from a filamentous fungus (*Aspergillus niger*). The culture supernatants from *Bacillus* sp. P34 under these conditions were evaluated for antimicrobial activity against bacterial pathogens as well as for CLPs production.

2. Materials and methods

2.1. Microorganisms and maintenance

Bacillus sp. P34 (GenBank CP040378) was recovered from the collection of the Laboratory of Applied Microbiology and Biochemistry (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) and used as antimicrobial peptide producer. *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923 from American Type Culture Collection (ATCC, Manassas, VA, USA) were used as indicator strains. *Aspergillus niger* IFL5 from the culture collection of the Laboratory of Food Toxicology (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) was also included in this study.

All bacterial strains were stored at −21 °C in brain-heart infusion (BHI; Merck KGaA, Darmstadt, Germany) containing 20 % (v/v) glycerol or BHI agar plates and propagated in the same medium at 37 °C before use. The fungus was maintained on potato dextrose agar (PDA; Oxoid, Wesel, Germany) slants covered with mineral oil at 4 °C and subcultured periodically. The working isolate was grown onto PDA agar plates.

2.2. Genome sequencing, annotation and comparison

Bacillus P34 total DNA was extracted with phenol-chloroform following usual procedures and purified using a Genomic DNA Clean & Concentrator (Zymo Research, Irvine, CA, USA). In order to improve the coverage of P34 genome DNA sequencing, DNA fragment libraries were prepared with one ng of DNA using a Nextera XT DNA sample preparation kit. Samples were sequenced using an Illumina® MiSeq System to create both 2 × 250 and 2 × 150 paired-end reads with the Illumina v2 reagent kit (Illumina, San Diego, CA, USA), followed by quality-based read trimming. After quality checking with FastQC (Andrews, 2010), reads were assembled using A5-miseq (Coil et al., 2015) and SPAdes (Bankevich et

al., 2012) programs. The best assembly was chosen based on Quast 4.0 (Gurevich et al., 2013) statistics, considering the number of contigs, the mean contig length, the N50, the number of contigs greater than 1 Kb, and the maximum contig length. Mauve was used for contig ordering (Darling et al., 2004). The closest bacterial genome according to the NCBI BLAST algorithm, *B. amyloliquefaciens* subsp. *plantarum* FZB42, was used as reference in the alignments. After, the ordered contigs were concatenated as one unique sequence, using the Union tool from the Emboss package, and the sequence was submitted to the RAST server (Aziz et al., 2008) for automatic gene annotation. Genes of interest had their annotation refined manually., The BRIG software (Alikhan et al., 2011) was used for graphic comparison among the closest bacterial genomes and P34 sequence. The genome sequence and gene annotation are available at the NCBI and EMBL databases under the accession number CP040378.

2.3. Phylogenetic analysis

Multilocus Sequence Analysis (MLSA) is a bioinformatics methodology able to define species and subdivisions within species for poorly studied or newly discovered taxa (Gevers et al., 2005). A phylogenetic analysis was carried out using MEGA X software (Kumar et al., 2018) with the aim of defining to which species P34 strain belongs. The species selected for the MLSA analysis were: *Bacillus amyloliquefaciens* FZB42, *Bacillus amyloliquefaciens* DSM7 and *Bacillus subtilis* 168 as reference bacterial species; the following species were chosen for the complete BLAST genome similarity *Bacillus velezensis* LABIM40, *Bacillus velezensis* TrigoCor1448, *Bacillus velezensis* CC09, *Bacillus amyloliquefaciens* B15, *Bacillus amyloliquefaciens* LL3, *Bacillus*

amyloliquefaciens XH7 and *Bacillus amyloliquefaciens* IT-4. A total of 5 housekeeping genes encoding core metabolic enzymes (Charlebois and Doolittle, 2004) were selected for the MLSA, and their nucleotide sequences were obtained from NCBI database. The following genes were considered for MLSA analysis: chaperone protein (*dnaK*), recombinase A (*recA*), RNA polymerase beta subunit (*rpoB*), RNA polymerase sigma 70 factor (*rpoD*), tryptophan synthase beta chain (*trpB*).

In-silico genome-to-genome comparison was made using the Genome to Genome Distance Calculator (GGDC) trying to compare it with the MLSA results. The GGDC analysis uses the concept of DNA-DNA hybridization (DDH) and Genome Blast Distance Phylogeny enriched with more comprehensive empirical database and by considering a broader range of numerical data transformations and statistical models (Meier-Kolthoff et al., 2013). In GGDC, the organism distances were defined by the recommended formula Identities/HSP (High-scoring Segment Pairs) from BLAST. The distances values were transformed to values analogous to DDH using a Generalized Linear Model (GLM) and results are reported as logistic regression of the GLM, with a probability ≥70 % for same species and with probability ≥79 % for the same sub species (Meier-Kolthoff et al., 2014).

2.4. Genome mining for secondary metabolites

Secondary metabolite gene clusters were identified by the online tools *antiSMASH* (Blin et al., 2013) and online *NP.searcher* for identification of metabolite gene clusters (Li et al., 2009), both methods using the complete nucleotide sequence of *Bacillus* sp. P34 genome. In general, a given gene was considered present in the genome of *Bacillus* sp. P34 when more than 65 % identity with the reference sequence(s), and more than 85 % of coverage was observed.

A genetic comparison of the core iturin genes was performed, using Bacillus species recognized as producers of different iturin family components (Dunlap et al., 2019). Both nucleotide alignment and phylogenetic tree were build through MEGA X software (Kumar et al., 2018), to comprehend the genetic product similarity.

The evaluation of key genes for lipopeptide synthesis was represented by *sfp* and *yczE*, coding for phosphopantheteine transferase and the integral membrane protein, respectively (Chen et al., 2007, 2009). A gene comparison between *Bacillus* P34 and *B. amyloliquefaciens* FZB42 was carried out to verify possible modifications and similarities using MEGA X software (Kumar et al., 2018).

2.5. Production of antimicrobial activity

A pre-inoculum of *Bacillus sp.* P34 was prepared through cultivation in BHI broth during 24 h at 37 °C and 180 rpm. A quantity of 2% (v/v) of the pre-inoculum was added to each 50 mL BHI broth, following by incubation at 30 °C for 6, 24 and 48 h with shaking at 180 rpm. All cultivations were performed in triplicate. The viable cells enumeration was determined by spread plate using 100 μL of serial 10-fold dilutions onto BHI agar plates, incubated at 37 °C for 24 h.

Production of antimicrobial compounds by Bacillus P34 was investigated by adding heat-inactivated cells of *L. monocytogenes* ATCC 7644 or spores of *A. niger* IFL5. For this purpose, 2% of inactive cultures (cells or spores) suspension was added into the BHI broth containing *Bacillus* sp. P34 before the incubation.

Thermally inactivated cells of *L. monocytogenes* were obtained following the method described by Leães et al. (2016) and diluted in 10 mM phosphate buffer pH 7.0 to a final concentration of 108 CFU/mL. Heat-inactivated spores of *A. niger* were obtained from a culture on PDA plates and suspended with 10 mM phosphate buffer pH 7.0. Spores counting was performed in a Neubauer chamber to obtain a suspension at 108 spores/mL, autoclaved at 121 °C for 30 min. A flow chart of these steps is detailed in the Supplementary Fig. S1. Thus, three different bacterial cultures were carried out in triplicate: the negative control, represented by the culture of Bacillus sp. P34 in 50 mL BHI (Treatment 1); culture of Bacillus sp. P34 with inactivated cells of *L. monocytogenes* (Treatment 2); and Bacillus sp. P34 grown with heat-inactivated spores of *A. niger* (Treatment 3).

After 6, 24 and 48 h incubation, a previous screening of the antimicrobial activity from the three treatments was made against *L. monocytogenes* and *S. aureus*. Samples were also taken for quantification of lipopeptides.

2.6. Determination of antimicrobial activity

The micro-dilution method was used for the antimicrobial activity evaluation (Mayrhofer et al., 2008). Activity was determined as the equivalent of the last dilution giving an inhibition zone and indicated as activity units (AU) per milliliter (Motta and Brandelli, 2002). All the aliquots were filtered with 0.22 μm membrane, and 20 μL were applied on discs (6 mm) onto BHI agar plates inoculated with a swab submerged in suspensions of indicator strains (L. monocytogenes and S. aureus) corresponding to a 0.5 McFarland turbidity standard. Plates were incubated at 37 °C for 24 h and inhibition zones were measured.

2.7. Lipopeptide extraction and analysis

Triplicate samples obtained from the bacterial cultures were tested for the ability to produce lipopeptides, more specifically iturin, surfactin and fengycin. The cell cultures were separated by centrifugation at 10,000 g for 15 min at 4 °C and the supernatants were subjected to extraction process of the extracellular lipopeptides. Extraction was performed by the addition of n-butanol (1:4, v/v) into the bacterial supernatant, after 2 min vortex samples were left resting overnight. The organic phase was completely evaporated at 30 °C (Yazgan et al., 2001). The final extracts were dissolved in 1 mL methanol and filtered before high performance liquid chromatography (HPLC) analyses. Samples were dissolved in acetonitrile:water (40:60, v/v) containing 0.1 % formic acid and filtered before mass spectrometry analysis.

2.7.1. HPLC analysis of lipopeptides

Quantitative determination of lipopeptides was performed using a HPLC system model E2695 (Waters, Milford, MA, USA) with a photodiode array detector (Waters, model PAD-2998) and using a C18 column (XBridgeTM Shield RP 18; 5 μm; 4.6 mm x 150 mm, Waters).

Iturin and surfactin determination was performed as previously reported (Veras et al., 2016). The conditions used for iturin A analysis were: a mobile phase of 0.1 % acetic acid:acetonitrile (60:40, v/v) at a constant flow of 0.6 mL/min with column oven temperature set to 20 °C; 20 μL of injection volume and the wavelength used was 230 nm. The following conditions were employed for surfactin analysis: 0.1 % trifluoroacetic

acid:methanol (10:90, v/v) as mobile phase; isocratic flow rate at 0.3 mL/min and 40 µL of injection volume. Surfactin peaks were detected at 210 nm (Veras et al., 2016). Fengycin quantification was realized using 0.1 % trifluoroacetic acid:acetonitrile (4:6, v/v) as mobile phase and elution rate set at 1 mL/min. Finally, the elution was detected at 220 nm absorbance and the injection volume used was 20 µL (Wei et al., 2010). Iturin A (≥ 90 %), surfactin (≥98.0 %) and fengycin (≥90 %) standards were purchased from Sigma (St. Louis, MO, USA) and each compound was dissolved in methanol. Different concentrations from these solutions were prepared (20, 40, 60, 80, 100, 120 and 140 mg/L) in order to develop calibration curves using the same chromatographic conditions described above. The retention time was 8 min for iturin A; 10.5, 12 and 13 min for surfactin isomers (C-13, C-14 and C-15), respectively and 9.6 min for fengycin. Linear regression equations were obtained with the standard solution signals of iturin A, surfactin isomers and fengycin. Quantification of the lipopeptides was performed by correlating the peak areas of the sample extracts with the standard curves.

2.7.2. Mass spectroscopy analysis of lipopetides

MALDI-TOF mass spectra were recorded on a Bruker Autoflex Speed (Bruker Daltonik, Germany) instrument operating in positive reflectron mode. Ion source 1 was set to 19.0 kV and source 2–16.70 kV, with lens and reflector voltages of 8.40 kV and 21.00 kV, respectively. Samples were spotted on an MTP 384 target plate ground steel BC and dried at room temperature. The matrix used was HCCA (α-cyano-4-hydroxy-cinnamic acid) in TA30 10.0 mg/mL (mixing 1 µL of sample with 1 µL of matrix solution). The instrument was calibrated with the commercial mixture Peptide Calib Standard mono (Bruker). Laser

excitation was at 355 nm, typically at 60 % of 150 mJ maximum output, and 2000 shots were accumulated.

2.7.3. HPLC-ESI-MS/MS analysis

The fragmentation data of the specific masses based on MS/MS were analyzed in detail to determine the presence of the mass fingerprint of each compound related to the compounds detected through MALDI-TOF MS analysis. The samples were analyzed for lipopeptides detection following a previously established method (Debois et al., 2014). A Shimadzu HPLC (Tokyo, Japan) connected to a diode array detector (SPD-M20A) and a quadrupole mass spectrometer (Q-TOF) analyzer with an electrospray ionization source (ESI) (Bruker Daltonics, MicrOTOF-Q III model, Bremen, Germany) was used. The compounds separation employed an X-Bridge RP 18 (150 mm x 4.6 mm) column with 5 μm packing. Elution was performed through constant flow rate of 0.6 mL/min using a gradient of acetonitrile in H2O both acidified with 0.1 % (v/v) formic acid as follows: one min at 30 %, from 30 % to 95 % in 3.4 min and maintained at 95 % for 2 min.

2.8. Statistical analysis

Data were expressed as means ± SD of triplicate samples. Lipopeptides quantification results were subjected to analysis of variance ANOVA by using the software SAS for Windows version 9.0 (SAS Inc., Carry, NC, USA). Significant differences were considered at 95 % confidence level by using the Tukey test.

3. Results and discussion

3.1. Phylogenetic analysis

The MLSA analysis using selected housekeeping genes allowed to know more about the position of *Bacillus* sp. P34 inside the *B. amyloliquefaciens* group even if it is coming from a different source than those enunciated by Fan et al. (2017). The bacterium *B. amyloliquefaciens* FZB42 resulted the nearest species among those selected by the analysis. The phylogenetic tree was constructed considering the reference genomes of *B. subtilis* 168 and *B. amyloliquefaciens* DSM7 as comparison for the other nine similar species selected for complete genome similarity (Fig. 1). In the last two decades, several changes have been reported about the nomenclature of the closely species of the *Bacillus* genus. This was related to the way of classification including the classic taxonomical parameters such as morphology, physiological characteristics, G + C content, and phylogenetic analysis through 16S rRNA gene sequencing. In this time interval, different species division have been considered and a complete species subdivision between *B. amyloliquefaciens* and *B. velezensis* has been recently reported (Rabbee et al., 2019). The phylogenetic analysis allowed to classify *Bacillus* P34 as *B. velezensis*, indeed the phylogenetic tree (Fig. 1) showed a closer relation of housekeeping genes with *B. amyloliquefaciens* subsp. *plantarum* FZB42, included inside the *B. velezensis* group (Dunlap et al., 2016).

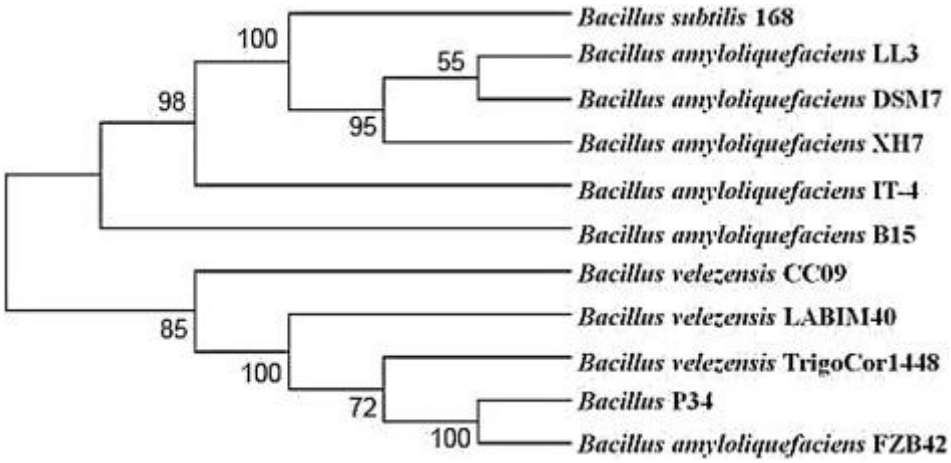

Fig. 1. Phylogenetic analysis of *Bacillus* P34 based on multilocus sequence analysis of selected housekeeping genes. The evolutionary history was realized using the Neighbor-Joining method and the distances were computed with the Maximum Composite Likelihood method. Next to the branches were allocated the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary tree was based on the alignment of the five selected housekeeping genes.

Moreover, to strengthen and confirm the data obtained from MLSA analysis, the virtually based DNA-DNA hybridization was carried out by calculating the GGDC, showing that all genomes were considered part of the same subspecies because of DDH > 79 % (Supplementary Table 1). Specifically, the species highlighted in the same clade group of *Bacillus* P34 from MLSA have shown a DDH > 95 % (*B. amyloliquefaciens* FZB42, *B. velezensis* LABIM40, *B. velezensis* TrigoCor1448, *B. velezensis* CC09). Meantime the genome of *B. subtilis* 168, *B. amyloliquefaciens* DSM7, *B. amyloliquefaciens* LL3 and *B. amyloliquefaciens* XH7 were contemplated as different bacterial species group of the query *Bacillus* P34, because of DDH < 70 %.

3.2. Genome comparison and annotation

Once established the closest genetically related species with *Bacillus* P34 genome, a RAST comparison was performed using the nearest associated *B. amyloliquefaciens* subsp. *plantarum* FZB42 (Table 1). Furthermore, comparison of the 11 complete genomes was carried out using the BLAST Ring Image Generator (BRIG) tool (Fig. 2), considering *Bacillus* P34 genome as central reference. The rings of the single genomes were packed considering the species relation derived from MLSA analysis, and interesting results were possible to deduce considering the annotation of the genome areas corresponding to secondary metabolites gene clusters. Highlighted macrolactin and difficidin areas shown different similarity allowing to divide the bacteria into two groups, the 4 strains most phylogenetically distant shown a low percentage value of similarity, probably related with the absence of those two gene clusters.

Table 1. General genome characteristics of the *Bacillus* P34 compared with the phylogenetic nearest *Bacillus amyloliquefaciens* FZB42 with RAST.

Characteristics	***Bacillus* sp. P34**	***B. amyloliquefaciens* FZB42**
Size (bp)	3,930,678	3,918,589
G + C content (%)	46.6	46.5
Number of contigs (with PEGs)	1	1
Number of subsystems	341	414

Characteristics	***Bacillus* sp. P34**	***B. amyloliquefaciens* FZB42**
Number of coding sequences	4061	3863
Number of RNAs	132	110

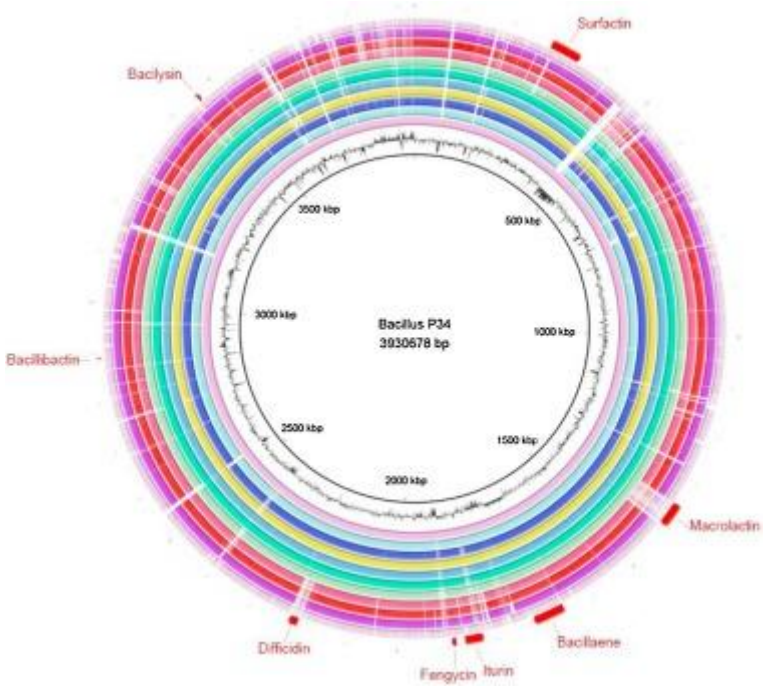

Fig. 2. BRIG ring comparisons of *Bacillus* P34 and the other bacteria, respectively from the internal to the external ring: *B. amyloliquefaciens* FZB42, *B. velezensis* TrigoCor1448, *B. velezensis* LABIM40, *B. velezensis* CC09, *B. amyloliquefaciens* B15, *B. amyloliquefaciens* IT-45, *B. amyloliquefaciens* XH7, *B. amyloliquefaciens* LL3, *B. amyloliquefaciens* DSM7 and *B. subtilis* 168. BLAST matches of the *Bacillus* strains are shown as concentric colored rings on a sliding scale according to percentage identity (100 %, 75 %, or 50 %). GC content and also shown. Red headlines

shown the antimicrobial secondary metabolites found in the reference *Bacillus* P34 genome.

3.3. Gene clusters for antimicrobial compounds

Using antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) it was possible to identify 10 gene clusters, and those with high similarity with known compounds were emphasized (Table 2). The gene clusters of nonribosomal peptide synthetases (NRPS) associated with the synthesis of CLPs investigated in this work, namely surfactin, fengycin and iturin family component bacillomycin, were detected. However, the bacillomycin gene cluster was not present as an individual cluster but hosted inside of the fengycin gene cluster (Fig. 3). This occurs because the genes present on a contig occasionally do not match the antiSMASH criteria for gene cluster revelation (Blin et al., 2013). Genome annotation allowed to identify all the core biosynthetic gene translators for the enzymatic machinery indispensable for the non-ribosomal process of CLPs synthesis, and the enzymatic protein sequences of *ituA, fen* and *srfA* were checked by BLAST. *Bacillus* P34 genome include the several antimicrobial gene clusters and those with similarity higher than 75 %, their respective biosynthesis machinery and bioactivities are listed in Table 2. Products computed by the program NP.searcher, using the *Bacillus* P34 genome as query, revealed that one modular NRPS, one mixed modular NRPS/PKS, two trans-AT PKS, and one non-mevalonate terpenoid *mep* gene were found in the genome. These have shown a certain relationship degree with the resulting data from antiSMASH.

Table 2. Gene clusters found in *Bacillus* P34 genome using antiSMASH software considering synthesis pathways, the most similar known gene clusters, the bioactivity, and the similarity with *Bacillus amyloliquefaciens* FZB42 gene clusters.

Type	Most similar known cluster	Bioactivity	Similarity
transAT-PKS	Macrolactin	Antibacterial	100 %
NRPS	Surfactin	Surfactant	82 %
transAT-PKS-NRPS	Fengycin	Antifungal	100 %
transAT-PKS-NRPS	Bacillaene	Antibacterial	100 %
Other	Bacilysin	Antibacterial	100 %
PKS-NRPS	Difficidin	Antibacterial	100 %
NRPS	Bacillibactin	Siderophore	100 %

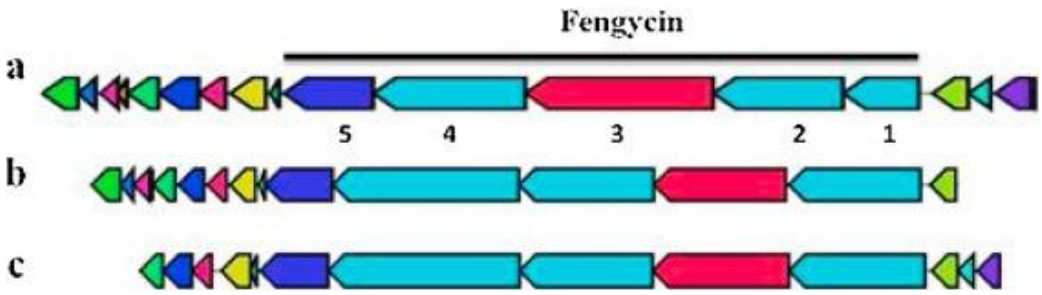

Fig. 3. Fengycin gene cluster of the query sequence *Bacillus* P34 (a) with the highlighted the core biosynthetic genes represented by (1) *fenA*,(2) *fenB,* (3) *fenC,* (4) *fenD,* (5) *fenE*; (b) fengycin and (c) Plipastatin.

3.3.1. Fengycin cluster

Fengycin gene cluster in *Bacillus* P34 genome showed 100 % similarity for both fengycin and plipastatin by antiSMASH analysis. One important difference between these two molecules reside in the peptide moiety at the position of the D-tyrosine, which is encoded by the gene 2 (*fenB*) of fengycin operon and by the gene 4 (*ppsD* or *fenD*) of plipastatin operon (Fig. 3). The L and D isomeric forms of tyrosine related to the presence of a Gln residue instead of a Glu at position 8 were found to constitute the differences between fengycin and plipastatin (Wang et al., 2004). Hussain (2019) studied the differentiation between fengycin and plipastatin based on the position of tyrosine epimerization domain in the second and fourth genes of the cluster. Thus, the tyrosine epimerization domain was investigated using degenerate primers of the tyrosine epimerization on *fenB* and *fenD* *Bacillus* P34 sequences obtained from antiSMASH annotation. Results of the BLAST analysis revealed the presence of the tyrosine epimerization domain in the fourth gene *fenD* with a size of 1641 bp, according with the *B. amyloliquefaciens* FZB42 sequence size, and absence of the domain on the *fenB*.

3.3.2. Iturin and surfactin clusters

Bacillus P34 iturin operon was represented by four open reading frames (ORFs) called *MCAT, bmyA, bmyB, bmyC*. The operon showed 100 % similarity with bacillomycin and mycosubtilin and 80 % similarity with iturin A gene clusters of *B. amyloliquefaciens* FZB42 (Fig. 4). A phylogenetic tree was built after gene alignment, showing the closest phylogenetic relationship with the species producing bacillomycin L (Supplementary Fig. S2).

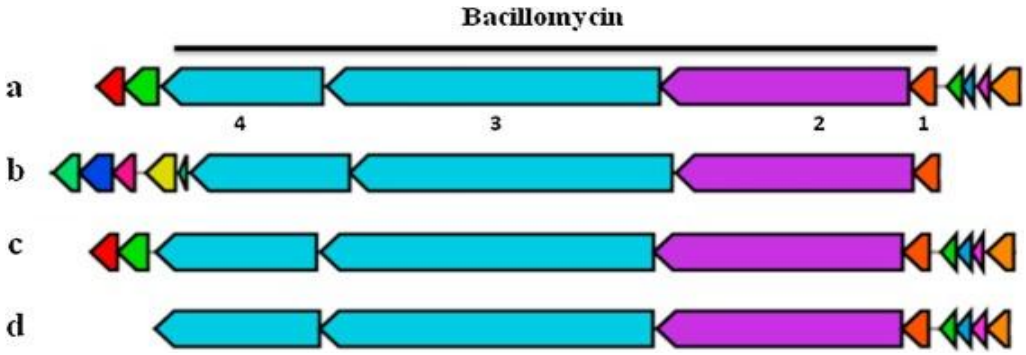

Fig. 4. Bacillomycin gene cluster of the query sequence *Bacillus* P34 (a) with the core biosynthetic genes constituted by (1) MCAT (2) *bmyA*, (3) *bmyB*, (4) *bmyC* under the black lines; (b) mycosubtilin and (c) bacillomycin gene clusters with 100 % similarity, (d) iturin cluster with 80 % similarity.

The last gene cluster for lipopeptide biosynthesis reported was surfactin and four ORFs involved in its production were found from the query sequence *Bacillus* P34. The results of antiSMASH analysis demonstrated a similarity of 82 % with the surfactin cluster of *B. amyloliquefaciens* FZB42 (Fig. 5).

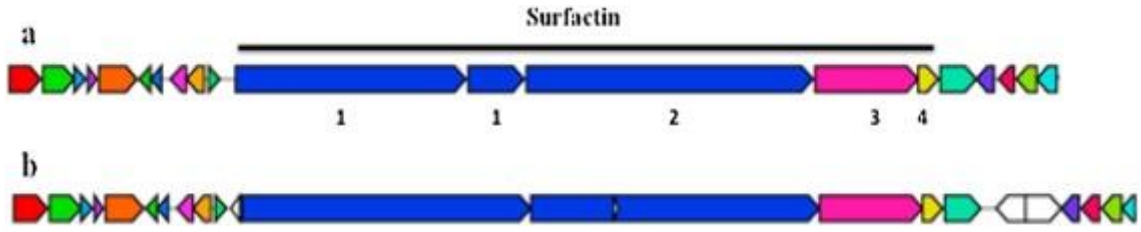

Fig. 5. Surfactin gene cluster of *Bacillus* P34 (a) with the core biosynthetic genes cluster of the surfactin A: (1) *srfAA*, (2) *srfAB*, (3) *srfAC*, (4) *srfAD* under the black line. (b) Surfactin gene cluster with 82 % similarity with the query sequence.

3.3.3. Essential genes for CLP synthesis

The sequence alignment of *yczE* and *sfp* genes from *Bacillus* P34 and *B. amyloliquefaciens* FZB42 clearly shown high similarity for the *sfp* gene (98 % identical pairs), while low similarity was observed for *yczE* gene (31 % identical pairs), as detailed in the Supplementary Table S3.

In *B. amyloliquefaciens* FZB42, *yczE* is the essential gene for secreting the antibiotic lipopeptide bacillomycin D, and *yczE* deletion mutants were able to produce surfactin and fengycin but were defective on bacillomycin D production (Koumoutsi et al., 2007). Recently, a genomic study on *B. velezensis* CMT-6 demonstrated that deletion or insertion may happen in *yczE* gene not affecting the production of these three lipopeptides (Deng et al., 2019). On the other hand, the reference strain *B. subtilis* subsp. *subtilis*168 contains the surfactin and plipastatin operons but is unable to produce these lipopeptides due to a non-functional *sfp* gene that results a truncated protein product (Nakano et al., 1988; Coutte et al., 2010). However, transgenic line of strain 168 with corrected *sfp* copy (Coutte et al., 2010) and *B. subtilis* strains with a single base deletion in the *sfp* gene produce active phosphopantetheine transferase that enables them on lipopeptide synthesis (Bóka et al., 2019).

3.4. Bacillus P34 growth

Bacillus P34 was grown in the presence of heat inactivated microbial cells or spores in order to observe the effect on antimicrobial activity. A slight variation in cell counts was observed among the three different treatment (Fig. 6). Indeed, only after 6 h from inoculation, during the exponential phase, the main difference among the collected data was observed, whereas similar CFU/mL values were detected at 24 h and 48 h. During the exponential phase the treatment 1 showed almost 1 log difference with the other two

treatments. Plateau or stationary phase was observed at 24 h in all treatments with similar number of viable cells at around 8.5×10^8 CFU/mL. A predictable reduction of viable cell counts was observed in all treatments at 48 h, suggesting the start of death phase.

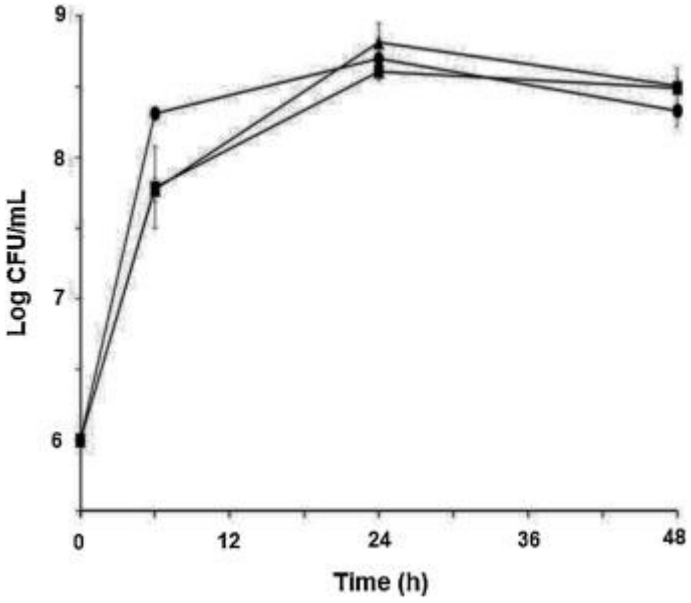

Fig. 6. Viable cell counts of *Bacillus* sp. P34 during cultivation on BHI broth at 30 °C: control (Treatment 1, circles); with heat-inactivated cells of *L. monocytogenes* (Treatment 2, squares; with heat-inactivated spores of *A. niger* (Treatment 3, triangles). The viable cells enumeration was done by spread plate of culture dilutions on BHI agar at 37 °C for 24 h.

3.5. Antimicrobial activity and lipopeptides quantification

The antimicrobial activity detected from the culture supernatants showed results correlated with the quantitative determination of lipopeptides, mostly for iturin and fengycin (Fig. 7). However, under normal conditions (without inactivated microbial cells), the supernatant demonstrates a maximum antimicrobial activity after 48 h from the initial inoculation, showing 500 and 700 AU/mL for *L. monocytogenes* e *S. aureus*, respectively. In presence

of inactivated cells of bacteria or fungi the maximum antimicrobial activity was detected 24 h after the inoculation (with similar values to the above cited control). The surfactin was only detected in early cultivation time of the treatments 2 and 3 at concentrations below 30 mg/L. In fact, under the culture conditions of this work, the bioactivity was essentially represented by fengycin and bacillomycin L. Indeed, when produced simultaneously, surfactin and fengycin may co-aggregate to form inactive complexes (Cawoy et al., 2015).

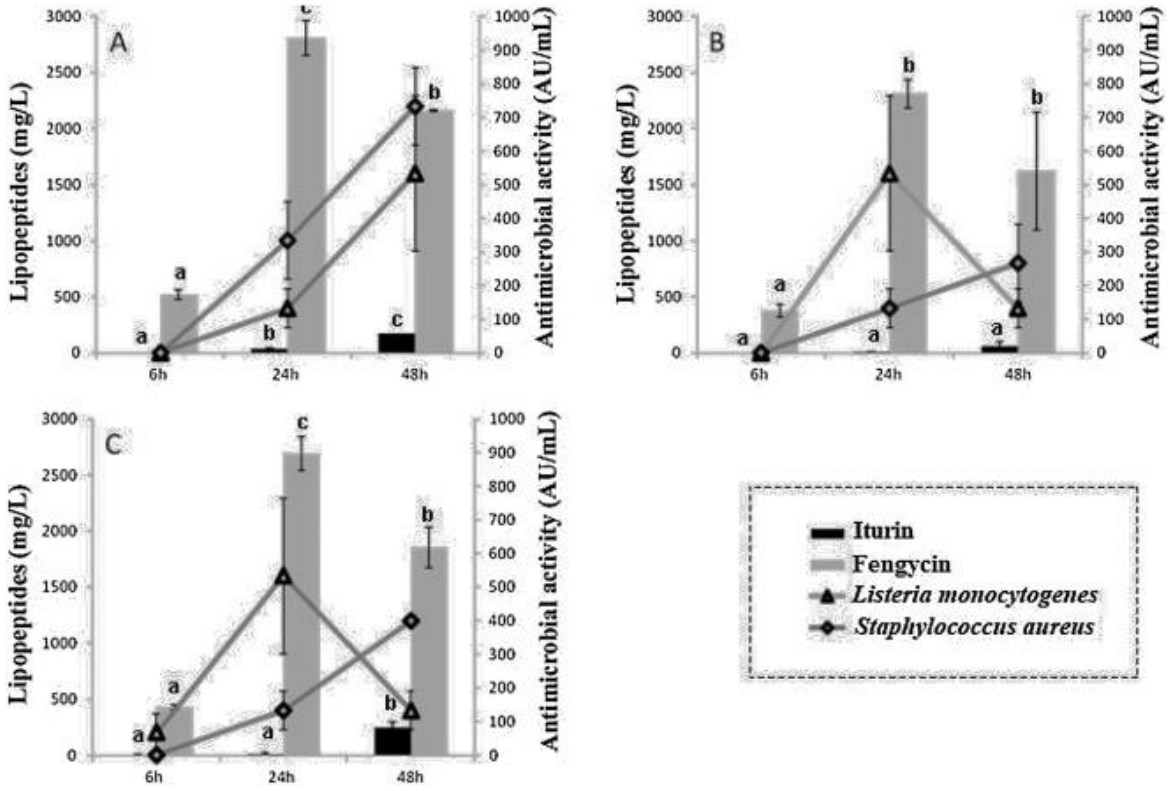

Fig. 7. Antimicrobial activity and cyclic lipopeptides production by *Bacillus* P34 in BHI medium with detection times 6 h, 24 h and 48 h from inoculation. **A** Control (Treatment 1); **B** Heat-inactivated cells of *L. monocytogenes* (Treatment 2); **C** Heat-inactivated spores of *A. niger* (Treatment 3). Different lower case letter over lipopeptide bars of each treatment indicate significant differences ($p < 0.05$).

A similar pattern of lipopeptides production was observed for the different treatments (Fig. 7). The quantification of fengycin showed maximum values at 24 h (2300−2700 mg/L), following by a reduction at 48 h. This decrease is possibly related with the cell autolysis at prolonged cultivation times, releasing hydrolytic enzymes that could degrade this peptide. *Bacillus* are recognized as producers of proteases and lipases, which have been described as inactivating enzymes of the antimicrobial activity of P34 (Motta et al., 2007b). On other hand, the iturin concentration increased in all the treatments during cultivation. Indeed, higher iturin quantity was detected for the treatment 3 with inactivated fungi spores, while lower amounts were by the treatment 2 with bacterial cell debris. As reported in previous studies, the transcription of essential genes involved in iturin and fengycin synthesis can be stimulated in *Bacillus* cultured with bacterial and fungal debris (Leães et al., 2016).

A constant increase of the antimicrobial activity was observed in the control (BHI, treatment 1) with greater values at 48 h on both *L. monocytogenes* and *S. aureus*. These results were more than 2-fold higher as compared with the other two treatment. The aliquots of 6 h cultivation showed no detectable antimicrobial activity. Differential results of antimicrobial activity were observed when *Bacillus* P34 was cultivated with thermally inactivated cells and spores (treatment 2 and 3, respectively). The aliquots obtained at 24 h demonstrated greater antimicrobial activity on *L. monocytogenes* as compared with the treatment 1. Lastly, a drastic reduction in antimicrobial activity was observed at 48 h. Antimicrobial activity against *S. aureus* showed a different trend, in fact the antimicrobial activity increased with the cultivation time, although the antimicrobial activities of the control (treatment 1) were two times greater. These results, in general, can be related with the quantity of fengycin and iturin detected by HPLC analysis.

Among *Bacillus* lipopeptides, the antibacterial activity of surfactin has been more extensively investigated (Ongena and Jacques, 2008). However, some previous studies have addressed the effects of iturins and fengycins on pathogenic bacteria (Mora et al., 2015; Cochrane and Vederas, 2016). Specifically, the antibacterial activity of iturin has been demonstrated against *S. aureus* (Ongena and Jacques, 2008; Son et al., 2016), while the activity of fengycin against *L. monocytogenes* has been detected in other studies (Lee et al., 2016; Perez et al., 2017). Components of the iturin family and fengycin showed dose-dependent antifungal activity and antagonistic effect on *S. aureus* (Ma et al., 2012), which could be the explanation for the antimicrobial activity observed against *S. aureus*. Thus, the synergistic interaction between bacillomycin L and fengycin may play a fundamental role on the antimicrobial activity as previously shown against Gram-negative and Gram-positive uropathogenic bacteria (Moryl et al., 2015). However, a surfactin-like effect on membrane phospholipids was demonstrated for fengycin at high concentrations, underlying a possible mechanism of antibacterial activity (Deleu et al., 2005). The antimicrobial activity against *L. monocytogenes* observed in this work could be associated with the amounts of fengycin in the medium, as the antimicrobial activity was reduced when fengycin concentration was below 2000 mg/L (Fig. 7B,C). However, the production of other antimicrobials by *Bacillus* P34 can not be ruled out at this point.

This specific relationship between lipopeptides and species-specific antimicrobial activity could be an interesting platform for future studies. The different behavior observed for lipopeptides production and antimicrobial activity could be related to specific environmental conditions created in the culture medium. Different works have shown that species pair can either upregulate or suppress its antibiotic production depending on the identity of a neighboring species (Abrudan et al., 2015; Kelsic et al., 2015). Competition

conditions by bacteria can occur for the overlapping of the metabolic ecological niche and the bacterial species compete for the same alimentary resources (Ghoul and Mitri, 2016), sometimes for the near phylogenetically species within a community it might be more probable (Mitri and Foster, 2013; Zelezniak et al., 2015).

3.6. Mass spectroscopy analysis

Mass spectroscopy analysis was carried out on the samples that show antimicrobial activity associated with iturin and fengycin HPLC quantification. A similar mass spectrum was observed for the samples. In particular, iturin was detected between *m/z* 1000 and 1100, excepting for the treatment with heat inactivated *L. monocytogenes* cells at 24 h, whereas fengycin was detected in all samples between *m/z* 1450 and 1550 (Fig. 8).

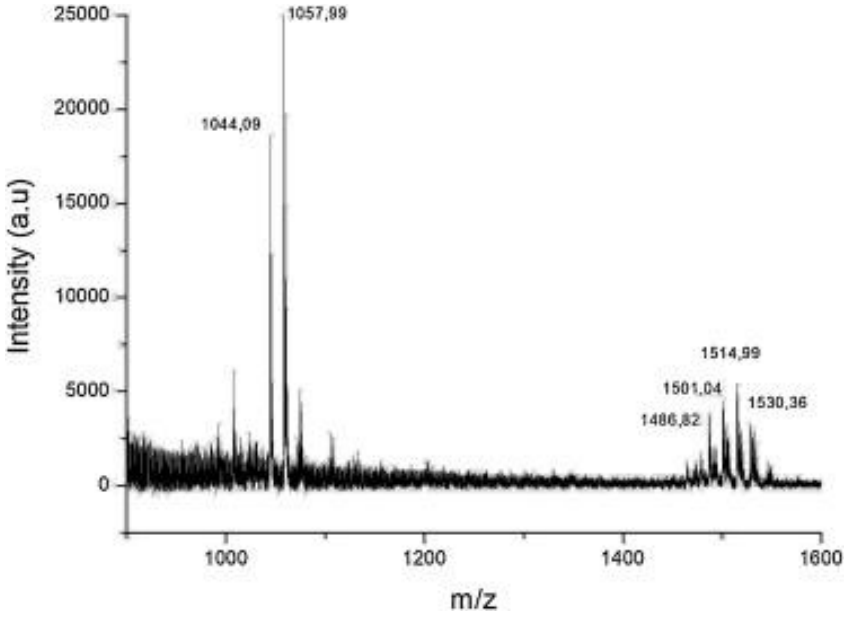

Fig. 8. Typical MALDI-TOF spectrum of lipopeptides extracted from *Bacillus* sp. P34 cultures. Iturin/bacillomycin peaks are observed between *m/z* 1000-1100 and fengycin at *m/z* 1450-1550.

The most representative fengycin peaks were at *m/z* 1486, 1501, 1515 and 1530 (Supplementary Table S2). MS-MS spectra of major components showed ions detected at *m/z* 1080, 966, 384 and *m/z* 1108, 994, 984 (Supplementary Fig. S3), suggesting the production of fengycin A and B, respectively (Ait Kaki et al., 2020). On the other hand, the most representative iturin components were observed at *m/z* 1044 and 1058, corresponding to $[M + Na]^+$ part of the bacillomycin L (Supplementary Table S2). Bacillomycin L fingerprint was obtained from MS-MS spectra of $[M+H]^+$ ions *m/z* 1021 and 1035 (Supplementary Fig. S4) as reported elsewhere (Aktuganov et al., 2014; Dunlap et al., 2019).

In the three treatments, lipopeptides were secreted as different isomers, the main iturin component, bacillomycin L, with chain lengths of C14 and C15, whereas fengycin isomers presented chain lengths of C15 and C16.

4. Conclusions

The experimental data obtained in this work allowed to identify the strain P34 as *Bacillus velezensis*. The genome of this bacterium contains several gene clusters associated with the production of antimicrobial compounds, including CLPs like fengycin, bacillomycin and surfactin. In the conditions studied, considerable amounts of fengycin and bacillomycin L were detected, while the presence of heat-inactivated spores of *A. niger* improved the iturin component secretion in the medium. On contrast, the addition of heat-inactivated cells of *L. monocytogenes* caused a reduction of CLPs production. The antimicrobial activity was associated with the fengycin and bacillomycin quantities detected in the culture broth, close dose-dependent manner. This work provides strong evidence on the biotechnological

potential of *B. velezensis* P34 for production of CLPs recognized for their broad spectrum of antimicrobial activity.

Declaration of Competing Interest

Authors declare no conflicts of interest regarding this manuscript.

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Appendix A. Supplementary data

The following is Supplementary data to this article:

Microbiological Research

Supplementary Material

Diversity of cyclic antimicrobial lipopeptides from *Bacillus* P34 revealed by functional annotation and comparative genome analysis

Paolo Stincone, Flávio Fonseca Veras, Jamile Queiroz Pereira, Fabiana Quoos Mayer, Ana Paula Muterle Varela, Adriano Brandelli

Supplementary Figure S1

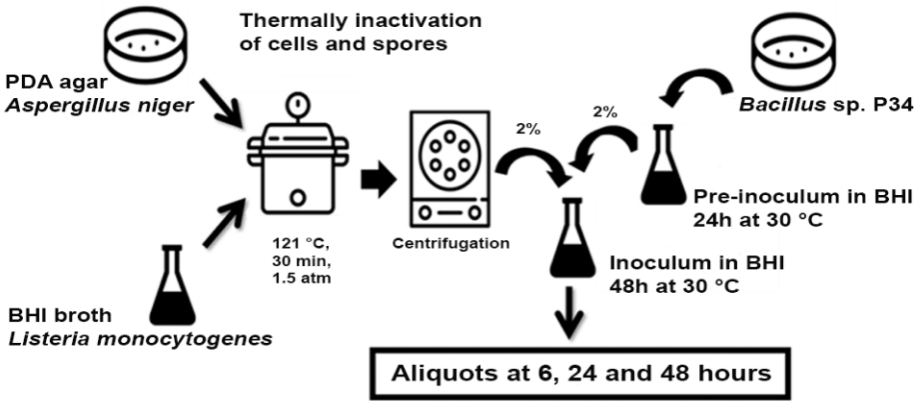

Figure S1. Schematic illustration of the cultures preparation considering the thermally inactivation procedure used for *Listeria monocytogenes* cells and *Aspergillus niger* spores added to the *Bacillus* P34 cultures.

Supplementary Table S1

Table S1. Genome to Genome Distance Calculator (GGDC) among *Bacillus* P34 and the species genomes compared in the work. The data reported are derived from the application of the formula 2 of the method considered.

Query genome	Reference genome	DDH	Model C.I.	Bootstrap C.I.	Distance	Prob. DDH ≥ 70%
Bacillus_P34	Bacillus_velezensis_CC09	88.1	[85.6-90.2%]	88-88.1%	0.0142	95.09
Bacillus_P34	Bacillus_amyloliquefaciens_B15	87.8	[85.2-89.9%]	87.8-87.8%	0.0146	94.97
Bacillus_P34	Bacillus_amyloliquefaciens_DSM7	56	[53.2-58.7%]	56-56%	0.059	38.34
Bacillus_P34	Bacillus_amyloliquefaciens_FZB42	90.1	[87.8-92%]	90.1-90.1%	0.012	95.82
Bacillus_P34	Bacillus_amyloliquefaciens_IT-45	80.9	[78-83.5%]	80.9-81%	0.0222	91.29
Bacillus_P34	Bacillus_amyloliquefaciens_LL3	55.5	[52.7-58.2%]	55.5-55.5%	0.06	36.62
Bacillus_P34	Bacillus_amyloliquefaciens_TrigoCor1448	90.1	[87.8-92%]	90.1-90.1%	0.012	95.84
Bacillus_P34	Bacillus_amyloliquefaciens_XH7	55.5	[52.8-58.2%]	55.5-55.5%	0.0599	36.78
Bacillus_P34	Bacillus_subtilis_168	20.8	[18.6-23.2%]	20.8-20.8%	0.2112	0
Bacillus_P34	Bacillus_velezensis_LABIM40	92.3	[90.3-94%]	92.3-92.3%	0.0096	96.51

Supplementary Table S2

Table S2. The main *m/z* peaks detected for *Bacillus* sp. P34 samples with the respective lipopeptide assignment.

Mass peak (*m/z*)	Assignment				BHI [a]		BHI + Lm		BHI + An		References
	Isomer	Lipopeptide	Ion	Amino acid configuration	24 h	48 h	24 h	48 h	24 h	48 h	
1044.4	C14	Iturin (Bacillomycin L)	[M+Na]+	Gln4, Pro5, Ser6,Asn7	+	+	+	-	+	+	(Aktuganov et al., 2014; Dunlap et al., 2019)
1057.5	C15	Iturin (Bacillomycin L)	[M+Na]+	Gln4, Pro5, Ser6,Asn7	+	+	+	-	+	+	
1075.5	C16	Iturin	[M+H]+	Asn1	-	-	-	-	+	-	
1463.8	C16	Fengycin A	[M+H]+	Ala6	-	-	-	-	-	+	(Ait Kaki et al., 2020)

1472.3	C15	Fengycin A	[M+Na]+	Ala6	-	-	-	-	-	+
1486.2	C16	Fengycin A	[M+Na]+	Ala6	+	+	+	+	+	+
1501.4	C15	Fengycin B	[M+Na]+	Val6	+	+	+	+	+	+
1515.2	C15	Fengycin B	[M+K]+	Val6	+	+	+	+	+	+
1530.2	C16	Fengycin B	[M+K]+	Val6	+	+	+	+	+	+

a Samples obtained from *Bacillus* P34 growing on BHI, BHI + heat inactivated cells of *L. monocytogenes* or BHI + heat inactivated spores of *A. niger*. Symbols (+) or (-) represent the presence or absence of the m/z peak in the correspondent samples.

Supplementary Figure S2

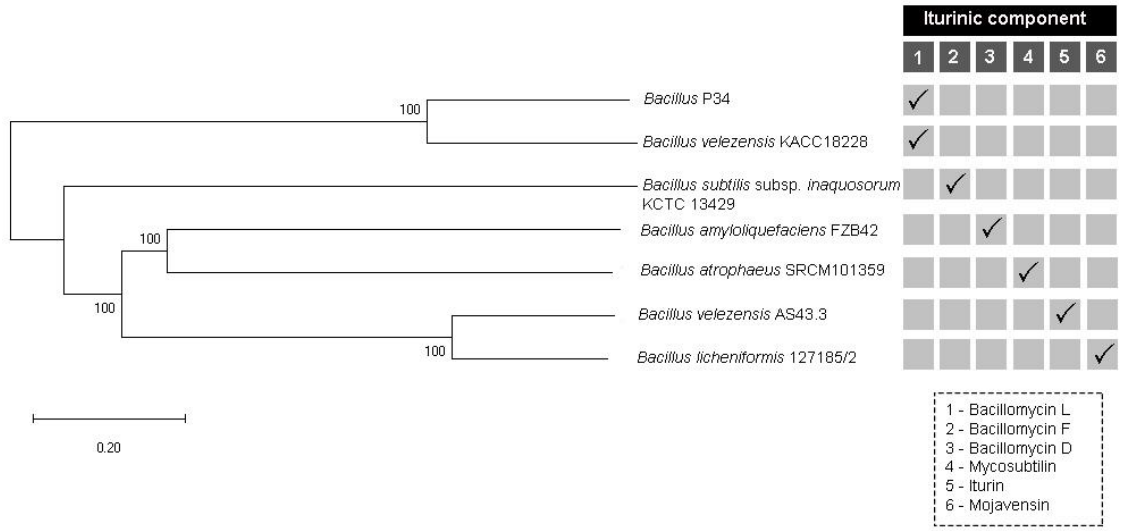

Figure S2. Neighbor-joining phylogenetic tree (gene tree) of Bacillus group, constructed using core DNA sequences of iturin synthesis. Bootstrap phylogenetic method was applied with N of 1050 replication. The scale bar corresponds to 0.20 nucleotide substitutions per site.

Supplementary Table S3

Table S3. The nucleotide pair frequencies calculated using MEGA X software considering the genes *sfp* and *yczE* from *Bacillus* P34 and *B. amyloliquefaciens* FZB42 gene comparisons.

	Gene	
	Sfp	***yczE***
Identical Pairs	662	195
Transitional Pairs	11	153
Transversional Pairs	2	282
Total	675	630

Supplementary Figure S3

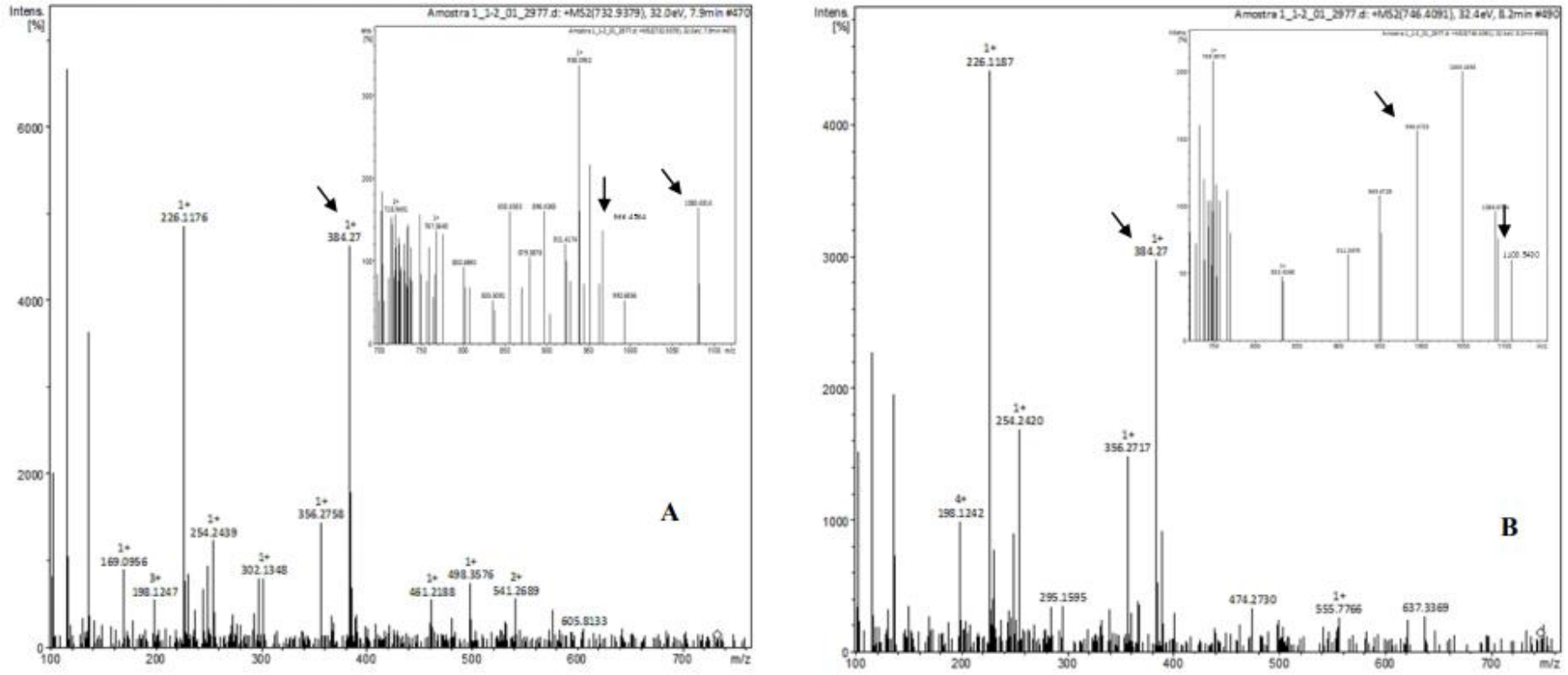

Figure S3. MS-MS spectra of Bacillus P34 fengycin products. (A) Fengycin A with [M+H] + m/z 732.41 and diagnostic ions m/z 1080, 966, 384. (B) Fengycin B with [M+H] + m/z 946.41 and diagnostic ions m/z 1108, 994, 384.

Supplementary Figure S4

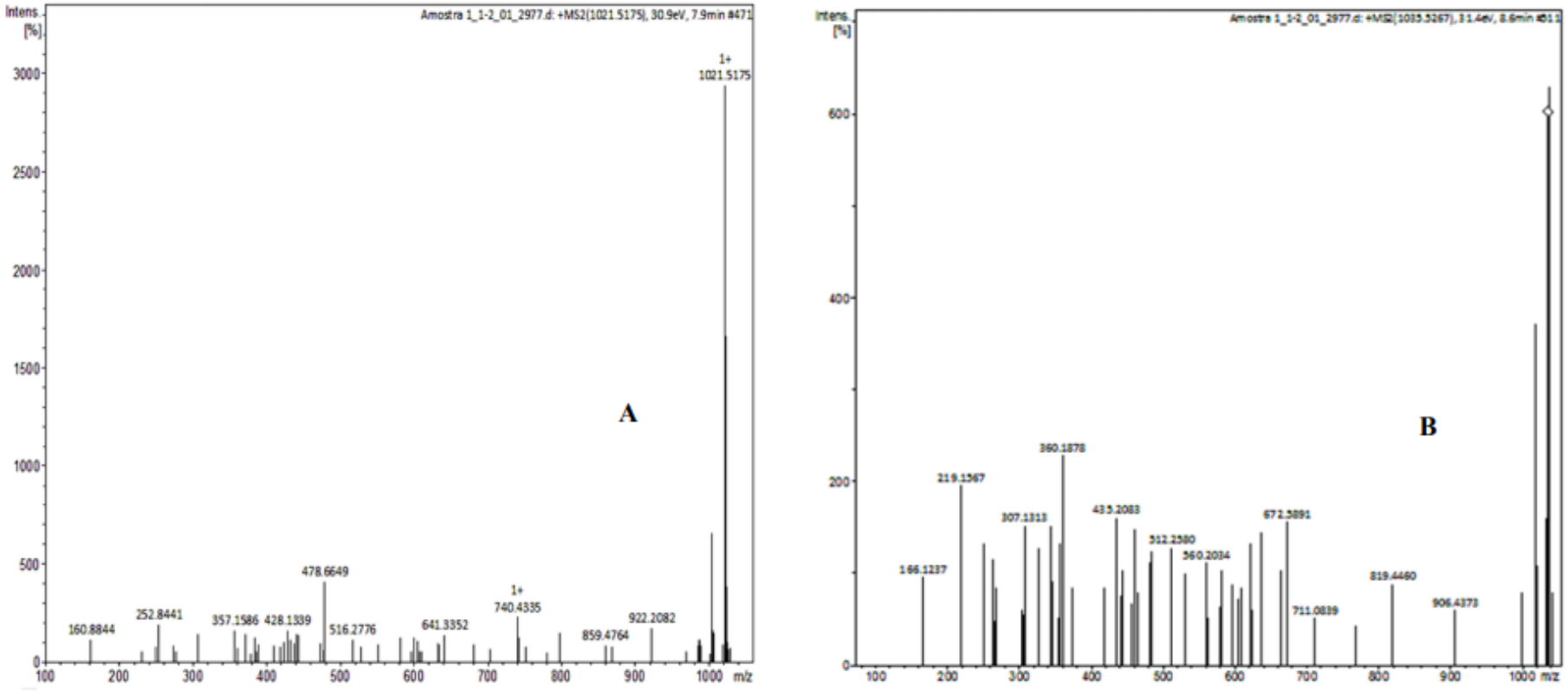

Figure S4. MS-MS spectra of the *Bacillus* P34 iturin component bacillomycin L with [M+H] + m/z 1021.51 (A) and 1035.52 (B).

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3.2 Second Scientific Article

Proteomic analysis of *Listeria monocytogenes* exposed to free and nanostructured antimicrobial lipopeptides

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Proteomic analysis of *Listeria monocytogenes* exposed to free and nanostructured antimicrobial lipopeptides

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Abstract

In this work, the effect of antimicrobial lipopeptide P34 on *Listeria monocytogenes* was evaluated for the first time through a proteomics approach. Bacteria were treated with sublethal doses of peptide P34 (F-P34) and P34 encapsulated into nanoliposomes (N-P34), while empty nanoliposomes (NE) and fresh buffer were used as controls. The proteomic analyzes allowed to highlight two groups of proteins differentially expressed: a group of proteins regulated in response to both free and encapsulated P34, and a second group of proteins exclusively regulated in the treatment with encapsulated P34. The antimicrobial peptide P34 caused a significant downregulation of proteins associated with the transport of manganese and upregulated proteins related with iron transport in *L. monocytogenes*. In addition, the modulation of phosphoenolpyruvate phosphotransferase systems (PTS) for sugar transport was observed, with the upregulation of fructose PTS and downregulation of other PTS and enzymes related with glycerol metabolism. The sugar and oligopeptide transporters regulated by antimicrobial action may influence the key virulence factor PrfA, reducing the pathogenicity of this microorganism.

Keywords: lipopeptide; Listeria monocytogenes; PTS; PrfA; nanoencapsulation; proteomic analysis

Introduction

Listeria monocytogenes is one of the most important foodborne pathogens. This bacterium is recognized for its survival capacity under conventional food processing and storage conditions, including low temperatures, high salt concentration, and acidic pH.[1] According to their virulence performance, strains of this bacterium have been divided into two groups where the virulence has been strictly correlated with the isolation source.[2] Thus, it seems the heterogeneity of *L. monocytogenes* virulence is related to ecological niches and food preparation conditions. In the virulence process, PrfA is a regulatory protein able to activate a set of key virulence factors during host infection, this activation comes from integration of several environmental cues that signal the transition between these two contrasting lifestyles, saprophytic-parasitic.[3] The role of σB on the resistance of L. monocytogenes to environmental stress has been largely documented, including the ability to survive under both saprophytic and hostassociated stresses.[4,5] Therefore, sB regulon works in the gastrointestinal context coordinating the general stress response in *L. monocytogenes* detecting possible menaces and regulating the transcription of PrfA, indispensable for the following spread into the host cell cytoplasm.[6] However, the genetic transcription regulation for the intrinsic stress resistance in *L. monocytogenes* includes eight different regulators, in addition to the already mentioned PrfA and sB, the VirR regulator is also involved in the bacteria resistance to stress.[7] The use of natural preservatives, such as bacteriocins and other antimicrobial peptides (AMPs), has been proposed as an effective alternative to control foodborne pathogens, including *L. monocytogenes*. Nisin is a bacteriocin produced by Lactococcus lactis subsp. lactis with strong antimicrobial activity against Gram-positive bacteria.[8] This bacteriocin has been employed in the food industry as a preservative approved by regulatory agencies in several countries. Although nisin is recognized for its antilisterial activity, the emergence of tolerant and resistant strains pressures the research for new sources of AMPs.[9] Most AMPs

act on peripheral cellular components, although some intracellular bacterial targets have not been excluded. Several peripheral cellular components are essential for bacterial life, including membrane proteins involved in the uptake of carbon sources and metal ions. These membrane components play a fundamental role in the pathogenic process that communicates information from the external environment, followed by virulence intracellular responses.[10,11] Thus, the knowledge on the bacterial targets that are affected by AMPs needs to be better explored for possible use in substitution or combination with current antimicrobials. The antimicrobial lipopeptide P34, produced by a *Bacillus* strain isolated from a freshwater fish gut, shows effective inhibitory activity against *L. monocytogenes.* [12] This peptide was characterized as a fengycin-like peptide and its mode of action was investigated causing extensive damage on the cell envelope of *L. monocytogenes*. [13] Further studies revealed the absence of toxicity of peptide P34 on eukaryotic cells in vitro and in the mice model.[14,15] The recent genomic analysis of *Bacillus* P34 allowed the identification of gene clusters for non-ribosomal peptide synthetases (NRPSs) and the detection of fengycin and bacillomycin L through mass spectrometry analysis.[16] The widespread use of AMPs is limited because their low bioavailability, which has been related with their susceptibility to proteolysis, short shelf life in the circulatory system, and low permeability across barriers.[17] The activity of AMPs can be lost during food applications due to process conditions or undesirable interactions with components of food matrix. Nanotechnologies can surmount these problems by encapsulating AMPs into nanostructures, protecting and improving their efficacy and stability in several applications.[18] In this regard, further studies with the lipopeptide P34 showed that its liposome encapsulation maintains activity against *L. monocytogenes.* [19] However, the responses at molecular level to this peptide either free or encapsulated have not been described yet. Proteomics is one of the ‘‘omics technologies’’ that can give light on the mechanisms of action of antimicrobial compounds.[20] In this work, a proteomics approach was

used to compare the protein profiles of *L. monocytogenes* treated with the lipopeptide P34. In this study, P34 was used in a sub-lethal concentration to study the influence on proteins related to bacterial physiology and pathogenicity, similarly to previous studies employing nisin as the antimicrobial peptide.[21,22] Moreover, the modification of bacterial proteome in response to the peptide encapsulated into nanoliposomes was also investigated.

Results

Production and encapsulation of peptide P34

The production of fengycin peptides by *Bacillus velesensis* P34 was carried out as described previously.[16] The obtained product showed 51 200 activity units (AU) per mL. Thus, dilutions were performed to achieve the sub-lethal concentration of 100 AU mL^{-1} on *L. monocytogenes* ATCC 7644, as demonstrated by a previous work.[13]

The same concentration amount of bacterial peptide was encapsulated (N-P34), for the treatment of *L. monocytogenes* cultures. The empty and P34-loaded liposomes showed respective particle size of 145 and 160 nm, and zeta potential values of −17 and −27 mV, essentially as reported in our previous studies.[19] The liposomes presented an encapsulation efficiency of 98% for peptide P34.

Effect of free and encapsulated P34 on *L. monocytogenes*

The effect of sub-lethal concentration of free and encapsulated P34 on the viable cell counts was investigated. The treatments were added to the BHI medium at 6 h of *L. monocytogenes* incubation, and after 60 min, reduced bacterial counts were observed for both free and encapsulated P34 treatments as compared with the control and empty nanoliposomes (Fig. 1). A 2.4 log CFU mL^{-1} reduction was observed for the treatment with free P34, while the encapsulated P34 caused a smaller decrease in viable counts after 60 min incubation. At

24 h incubation, an additional decrease in viable counts was observed for encapsulated P34 indicating a sustained release of the peptide.

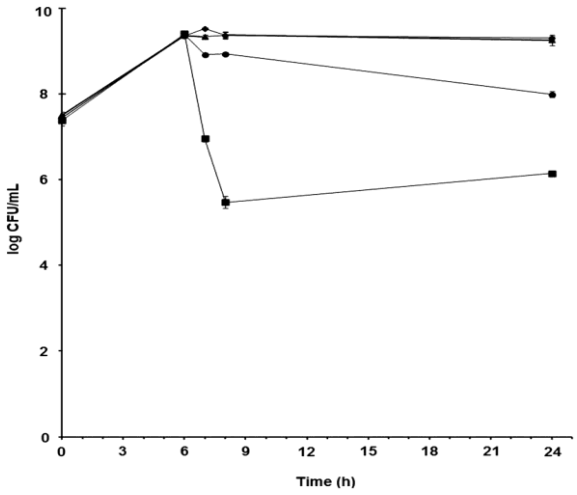

Fig. 1 Bacterial growth curve of *Listeria monocytogenes* during cultivation on BHI. Control (triangles); with empty nanoliposome NE (diamond); with nanoliposome carrying P34 (circles); with free P34 (squares). The viable cells enumeration was done by spread plate of culture dilutions on BHI agar at 37 °C for 24 h

Proteomic analysis

A total of 950 proteins were detected by the mass spectrometry analysis, and after elimination of eventual false positive proteins showing LFQ intensity detected only in ≤50% of the samples, 811 proteins were submitted to the bioinformatics analysis. The principal component analysis (PCA) of the protein groups showed the effect of the peptide P34 and nanoencapsulation on the *L. monocytogenes* proteome (Fig. 2A). The groups treated with free peptide P34 (F-P34) and encapsulated P34 (N-P34) were clearly separated from the control groups treated with empty nanoliposomes (NE) and fresh buffer (Control). These results suggest that the treatment with sub-lethal dose of P34 had a different effect on the physiological responses of *L. monocytogenes* in comparison with other treatments. In addition, it was possible to observe a separation between the groups F-P34 and N-P34

showing the influence of the peptide encapsulation on the response of *L. monocytogenes* (Fig. 2A).

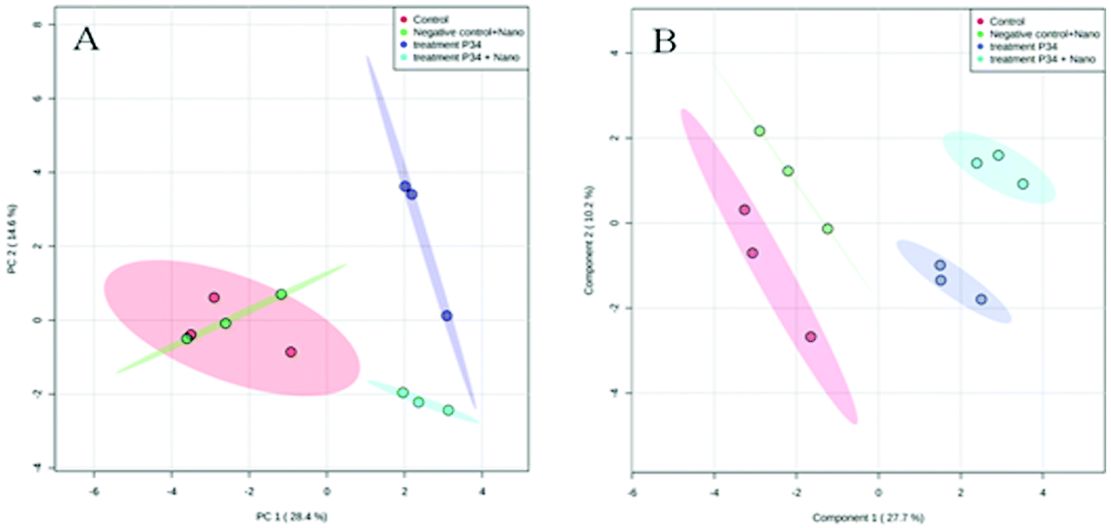

Fig. 2 (A) Principal Component Analysis (PCA) and (B) Partial Least Squares – Discriminant Analysis (PLS-DA) in proteome samples of *L. monocytogenes* treated with free peptide P34 (blue), encapsulated peptide P34 (magenta), empty nanoliposomes (green) and control (red).

PLS-DA (Fig. 2B) was used to identify proteins differentially expressed related to the treatment of *L. monocytogenes* with free (F-P34) and encapsulated peptide P34 (N-P34). Thus, 159 differentially expressed proteins were found in the proteome with VIP >1.0 in the first component of PLS-DA (Table S1, ESI†). Cross-validation showed high performance for the PLS-DA models with $R^2 > 0.86236$ and $Q^2 > 0.62043$ (Fig. S1, ESI†).

Among the 159 proteins with VIP score ≥ 1, reported on the heatmap (Fig. 3), more than 70% were found as proteins with differential expression among the groups, namely samples treated with P34 (F-P34 and N-P34), control samples (NE and Control). This can be observed in the Venn diagram (Fig. 4), where it is emphasized the number of proteins exclusively regulated in the treatment with N-P34.

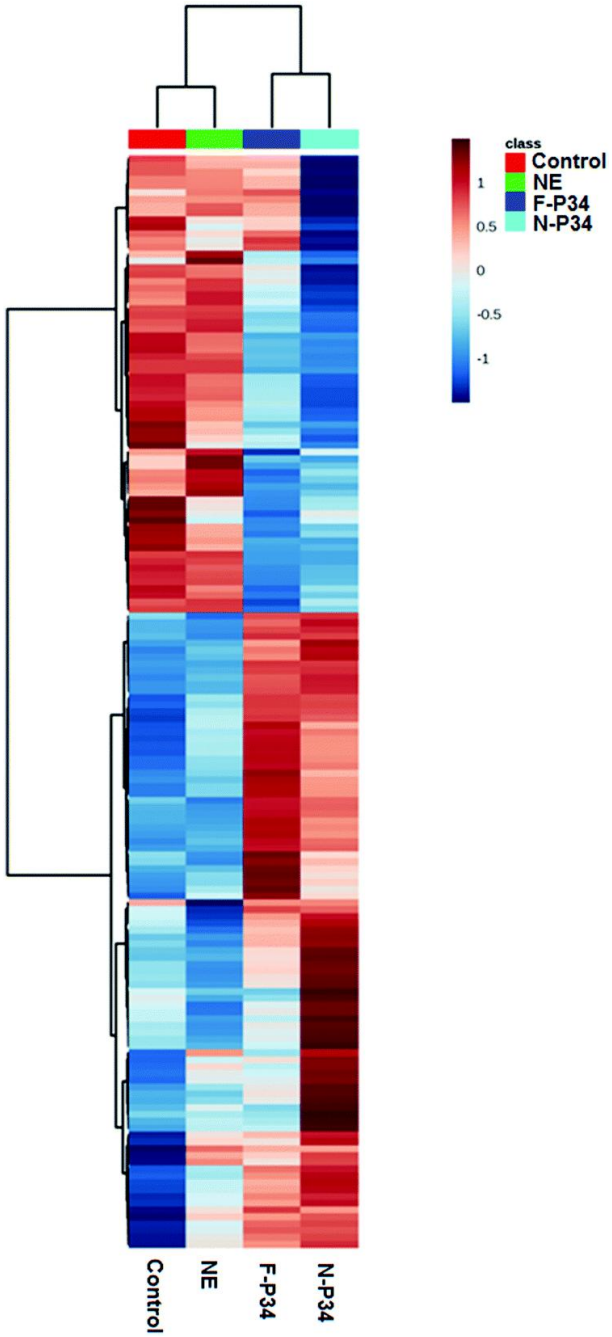

Fig. 3 Heatmap of the top 159 proteins identified by PLS-DA statistical analysis with VIP score ≥ 1. Samples are represented in columns, each column represent a triplicate samples of *Listeria monocytogenes* total proteome cultures respectively of samples withou any treatment (red), samples with empty nanoliposome (green), samples with free peptide P34 (blue), samples with nanoencapsulated peptide P34 (turquoise). Each colored cell on the map corresponds to a triplicate concentration value for the 159 proteins, represented in rows. The

two-colour scale ranging from the lowest intensity (*z*-score of −2) in blue to the highest intensity (*z*-score of +2) in red were used.

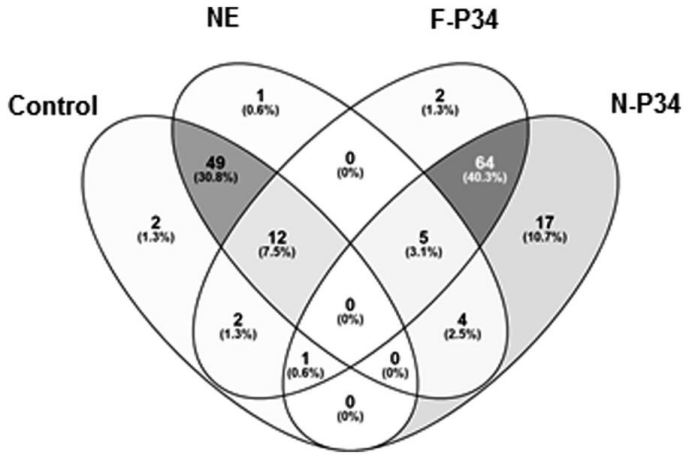

Fig. 4 Venn diagram reporting all the validated upregulated proteins. The overlapping regions or intersections represent the number of proteins common to neighboring treatments. Control: untreated *Listeria monocytogenes*; NE: *Listeria monocytogenes* with empty nanoliposome cultures; F-P34: *Listeria monocytogenes* cultures with subletal concentration of P34. N-P34: *Listeria monocytogenes* cultures with nanoliposome encapsulating P34.

The Venn diagram permitted to create two groups of proteins selected for comparative proteomics study. Within a first group all the proteins differentially expressed by the P34 action (free and encapsulated) were included, raising the degree of VIP score (VIP ≥ 2) to reduce the number of proteins. In this case, a total of 21 downregulated proteins and 16 upregulated proteins were detected (Table S2, ESI†). A second group included those proteins exclusively regulated in samples treated with encapsulated P34 (Table S3, ESI†). Indeed, statistical analysis permitted the identification of 17 proteins exclusively upregulated in bacterial cells treated with the liposome-encapsulated P34. Moreover, another group of 12 proteins was found in significantly lower amounts in N-P34 samples as compared with all other treatments. This group includes phospholipase C, Lmo1710 (flavodoxin-like protein) and Lmo1306 (UPF0154 membrane protein), with VIP score >2.0.

Protein ontology of peptide P34 treatments

Protein molecular functions and biological process were investigated for the two protein groups considered in this work, using CELLO2GO (Tables S4 and S5, ESI†). Downregulated proteins from samples treated with both free and encapsulated P34 showed, among the proteins selected with VIP score ≥2, mainly proteins with molecular functions associated with ion binding (31.2%) and oxidoreductase (13.3%) activities, while all other molecular functions detected shown percentages <10%. Analyzing the protein groups involved with biological processes, most proteins were involved with carbohydrate metabolic process (11.4%) and with catabolic process (10.1%). Among the upregulated proteins, the main molecular functions were ion binding (23.8%) and oxidoreductase activities (19%), and the biological processes involving this protein group were transport (26.8%), homeostatic process (23.2%), response to stress (14.3%), and other lower percentages were not reported. When the protein ontology was evaluated for the group of proteins exclusively downregulated in encapsulated P34-treated samples, the main molecular functions were ion binding (33.6%) and oxidoreductase activity (16.9%). Biological processes showing the highest percentages for these downregulated proteins were response to stress (14.9%) and biosynthetic process (10%). Upregulated proteins in relation to all other treatments showed in molecular function: ion binding (21.1%), oxidoreductase activities (10.8%). While the main protein in biological process detected were small molecule metabolic process (12%), cellular nitrogen compound metabolic process (12%), and biosynthetic process (11%).

Proteins related to stress tolerance

The key virulence regulator σB act in the gastrointestinal environment during the saprophytic phase of the *L. monocytogenes* in response to diverse acting stress and translate hundreds protein genes working to contrast these stresses, beside to that also VirR regulon work to contrast stress acting specifically on the bacteria cell envelope. A group of proteins regulated by these key virulence factors was individuated in our set of data (Table 1). The group of

genes regulated by the σB regulon includes *lmo0592*, *lmo1257*, *lmo2785* (*kat*) and *lmo0685*, whereas the expression of *dltD* is under the VirR regulation. Lmo2785 protein or catalase acts also on peroxide stress process being under the regulation of the σB together with PerR and OhrR.[23] The Lmo0685 protein working as σB inhibitor was upregulated, suggesting a negative modulation of this latter stress-related regulon. Indeed, all proteins included in this group work on stress tolerance response and were differentially regulated in samples subjected to P34 treatments.

Table 1 Detected proteins related to the σB regulon and stress tolerance

Protein name	VIP Score	Regulation	Treatment[a]	Gene name	Activity
Lmo0592 protein	2.64	Down	F-P34, N-P34	*lmo0592*	Response to stress
Lmo1257 protein	4.82	Down	F-P34, N-P34	*lmo1257*	Response to stress
Lmo2785 protein (catalase)	2.89	Down	F-P34, N-P34	*lmo2785(kat)*	Response to stress[b]
Lmo0685 protein	2.77	Up	F-P34, N-P34	*lmo0685*	Response to stress
DltD	2.14	Up	F-P34, N-P34	*dltD*	Response to stress[c]

a Triplicate treatments with free peptide P34 (F-P34); triplicate treatments with nanoencapsulated peptide P34 (N-P34). b Protein associated with the peroxide resistance activity. c Protein associated with key virulence regulator VirR.

Proteins related with PrfA virulence factor

Different proteins related with PrfA, a key transcriptional activator protein involved in the *L. monocytogenes* virulence process, have been identified as differentially expressed upon treatment with both free and encapsulated P34 (Table 2). Among these, membrane proteins associated with carbohydrates and oligopeptides transport were detected. Only FruA and FruB, related to fructose-specific phosphoenolpyruvate phosphotransferase system (PTS), were upregulated, while other PTS associated with lactose/cellobiose (coded by *lmo1719* and *lmo2373*) were downregulated in all the samples treated with P34. In

addition, mannose-specific PTS component IIAB (*lmo0096*) was exclusively downregulated in N-P34 treatments (Fig. 5). The listerial proteins coded by *lmo0152* and *lmo0135* were also downregulated in samples treated with P34 (Table 2). These proteins are structurally related to OppA, involved in active transport of oligopeptides across the cytoplasmic membrane.

Table 2 Detected proteins involved on PrfA regulation including membrane transporters of carbohydrates and oligopeptides. Metabolic proteins involved in glycerol metabolism were reported for the connection with PrfA

Protein name	**VIP score**	**Regulation**	**Treatment**[a]	**Gene name**	**Activity**
Lmo0135 protein	3.49	Down	F-P34, N-P34	*lmo0135*	Oligopeptides transport
Lmo0152 protein	2.33	Down	F-P34, N-P34	*lmo0152*	Oligopeptides transport
Lmo1719 protein	2.52	Down	F-P34, N-P34	*lmo1719*	Sugar transporter
Lmo2373 protein	2.39	Down	F-P34, N-P34	*lmo2373*	Sugar transporter
Pyruvate formate-lyase	3.99	Down	F-P34, N-P34	*pflB*	Metabolic
Glycerol kinase	1.36	Down	N-P34	*glpK*	Metabolic
Glycerol-3-phosphate dehydrogenase	1.58	Down	N-P34	*glpD*	Metabolic
Phospholipase C	2.24	Down	N-P34	*plcB*	Metabolic
Lmo0096 protein	1.49	Down	N-P34	*lmo0096*	Sugar transporter
FruA protein	3.94	Up	F-P34, N-P34	*fruA*	Sugar transporter
Tagatose-6-phosphate kinase	2.19	Up	F-P34, N-P34	*fruB*	Sugar transporter

a Triplicate treatments with free peptide P34 (F-P34); triplicate treatments with nanoencapsulated peptide P34 (N-P34).

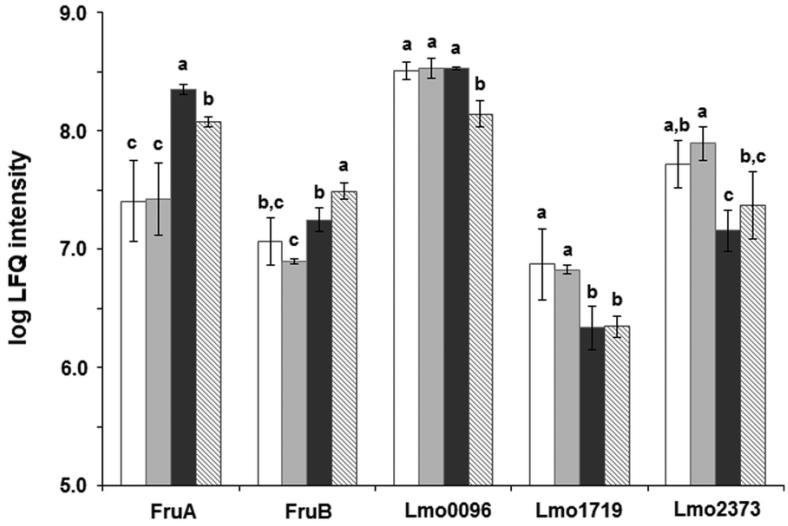

Fig. 5 Variation of the log LFQ intensity of PTS proteins according to the different treatments of *Listeria monocytogenes*. (White bars) *L. monocytogenes* untreated control; (pale gray) *L. monocytogenes* treated with empty nanoliposomes; (dark gray) *L. monocytogenes* treated with free P34; (dashed) *L. monocytogenes* treated with nanoencapsulated P34. The treatments were carried out in triplicate and analyzed by one-way analysis of variance (ANOVA) followed by Tukey HSD test ($P \leq 0.05$). Significant difference among the treatments are marked with different letters above the bars for each protein.

Analysing the downregulated proteins in the N-P34 samples, two metabolic proteins directly involved in the use of glycerol as carbon source were detected, namely glycerol kinase (GlpK) and glycerol 3-phosphate dehydrogenase (GlpD). The enzyme pyruvate formate lyase was also detected as downregulated with high VIP score (Table 2). This enzyme is important to regulate the anaerobic glucose metabolism and may have a role in anaerobic glycerol utilization.

In addition, phospholipase C, encoded by *plcB* gene, was also detected as downregulated in N-P34 samples. This protein is associated with the PrfA-modulated virulence process in *L. monocytogenes*, as the production of phospholipase C (lecithinase) provides the ability to breakdown the membrane vacuoles after initial host cell invasion and after cell-to-cell spread during infection.[24]

Proteins related with metal ions homeostasis

A common host strategy to combat the invading pathogen is the suppression of Fe^{2+}, Mn^{2+} and Zn^{2+} carried out by the neutrophils calprotectin from the innate immunity system. Despite the starvation in the host organism, some pathogens are still capable to cause infections. Fundamental in this context is a dedicated high affinity transport system for these metallic ions. The proteins differentially regulated by the exposure of *L. monocytogenes* to peptide P34 are reported in Table 3.

Table 3 Detected proteins involved on metals ions homeostasis

Protein name	VIP score	Regulation	Treatment[a]	Gene name	Activity
Heme-degrading monooxygenase	2.83	Up	F-P34, N-P34	*isdG*	Iron transport
Lmo1959 protein	3.29	Up	F-P34, N-P34	*lmo1959*	Iron transport
Catalase	2.89	Down	F-P34, N-P34	*kat*	Peroxide resistance
DNA protection during starvation protein	5.41	Down	F-P34, N-P34	*dps*	Peroxide resistance
Lmo2184 protein	1.94	Up	N-P34	*lmo2184*	Iron transport
Lmo0541 protein	2.19	Up	N-P34	*lmo0541*	Iron transport
Lmo0366 protein	1.72	Up	N-P34	*lmo0366*	Iron transport
Manganese-binding lipoprotein MntA	4.25	Down	F-P34, N-P34	*mntA*	Manganese transport
Manganese transport system ATP-binding protein MntB	3.58	Down	F-P34, N-P34	*mntB*	Manganese transport

a Triplicate teatments with free peptide P34 (F-P34); triplicate treatments with nanoencapsulated peptide P34 (N-P34).

Two components of the ATP-binding cassette MntABC were detected with higher VIP score among the proteins negatively regulated in *L. monocytogenes* treated with peptide P34, specifically, the Mn^{2+} transporter proteins coded by the genes *mntA* and *mntB*. The most common Mn^{2+} transporters in bacteria, MntH is expressed in a culture medium with availability of Mn^{2+} while MntABC is induced by Mn^{2+} limitation.[25]

On contrary, different proteins associated with iron absorption were upregulated in *L. monocytogenes* exposed to peptide P34 (Table 3). The FhuD lipoprotein codified by the gene *lmo1959* is a transporter working on the ferric ion uptake.[26] Although the FhuD lipoprotein shown more affinity for ferric siderophores, it also bound other iron complexes, including hemin.[27] Another protein detected as upregulated was the product of the gene *isdg*. This cytoplasmic protein is able to bind the heme component and cleaves its tetrapyrrol ring structure releasing the iron.[28]

An additional group of tree proteins related to iron uptake were exclusively detected as upregulated by *L. monocytogenes* treated with encapsulated peptide P34 (Table 3). Among these, a membrane protein encoded by the gene *lmo0541* is also under the regulation of the ferric uptake regulator (Fur) indicating a probable activity in iron transport. The Lmo0541 protein worth attention among those with unknown function belonging to the Fur regulon. Moreover, this protein shares 31% identity and 49% similarity with *L. monocytogenes* FhuD, which was upregulated in all treatments with peptide P34. Another upregulated protein was the *lmo2184* gene transcript, a transporter described as part of a gene cluster that includes the sortase B enzyme and SvpA protein that acts for attachment to peptidoglycan components.[29] The last protein exclusively upregulated on encapsulated P34 treatment that is associated with iron transport was the ferrous ion permease lipoprotein encoded by the *lmo0366* gene.

Finally, the *dps* and *kat*, gene products of the PerR regulon were highly downregulated upon treatment with peptide P34 (Table 3). PerR is a Fur homolog that functions as the central regulator of the inducible peroxide stress response. Metal uptake should be tightly regulated with oxidative stress response because the ability of some metal ions, in particular Fe^{2+}, to react with hydrogen peroxide producing harmful hydroxyl radicals. On contrast, the peptide P34 elicited on *L. monocytogenes* a stimulus to transport iron while repressing some oxidative stress responses.

Discussion

The antimicrobial activity of peptide P34 has been essentially associated to the fengycin lipopeptide, while bacillomycin L occurs as a minor component.[16] It has been suggested that fengycin auto-aggregates by inserting their acyl chains into model membrane bilayers, which is related to membrane damage.[30] The fengycin binding and aggregation disturbs phospholipid membranes, playing a key role in cell disruption, which appears to be dependent on the type of membrane with optimal action on fungal than bacterial membranes.[31] However, bacterial membranes are affected by fengycin in a dose dependent manner,[32] in addition to the possible antimicrobial synergistic effect generated by the presence of bacillomycin L.[33] Thus, bacterial cells are expected to elicit specific stress responses when exposed to sub-lethal doses of antimicrobial lipopeptides, as demonstrated by the proteomic approach developed in this work.

The results of this study showed a first group of five proteins related to σB and VirR factors, detected as differentially regulated by treatments with peptide P34 in comparison with the controls (Table 1). σB positively regulates the transcription of hundreds of proteins associated with stress response, including gastrointestinal stresses, mostly during the saprophytic phase of *L. monocytogenes* life cycle.[5] The positive action of σB in *L. monocytogenes* stress tolerance was also shown against nisin and antibiotics commonly used to combat

listeriosis.[34] In this regard, previous studies reported that the proteins translated by *lmo1257*, *lmo2785* (*kat*) and *lmo0592* genes were positively regulated by σB under stress conditions in *Listeria*.[35,36] However, these proteins were downregulated in cells treated with P34, while Lmo0685 protein, positively regulated in P34 treatments, work as a σB inhibitor.[37] These results support the hypothesis that peptide P34 may be related with negative modulation of σB regulon.

The VirR virulence regulator acts on the transcription of genes involved in cell envelope stress defenses, including an important role in opposing cationic antimicrobial peptides.[38–40] Specifically, the protein DltD was strongly upregulated by VirR in *L. monocytogenes* treated with nisin at sub-lethal concentration.[41] Thus, the upregulation of DltD protein detected in P34 treatments may be related to a response against the stress caused by the antimicrobial on the cell envelope, as the peptide P34 causes pore formation and membrane damage in *L. monocytogenes*.[13]

Moreover, this study showed differential regulation of PTS proteins associated with sugar transport in *L. monocytogenes* treated with peptide P34, which could be associated with virulence response into host cells. PTS represent the main carbohydrate transporters used by *L. monocytogenes*, which has specific systems for glucose, fructose, mannose, cellobiose and other carbohydrates.[42] The PTS is a complex protein kinase system involved in several secondary regulatory functions and these protein systems are important components for virulence regulation in *L. monocytogenes*.[43] Therefore, the transport of non-phosphorylated sugars (fructose, glucose-mannose-cellobiose) seems to modulate negatively the main virulence factor PrfA due to the non-phosphorylated state of the EIIA component from the PTS, not allowing the release of PrfA (Fig. 6). On the other hand, glycerol and sugars 6-phosphate were detected as a carbon substrates independent of PTS, determining the

maintenance of phosphorylated EIIA with the enhancement of virulence promotion by PrfA, this situation usually occurs inside the host cells.[44,45]

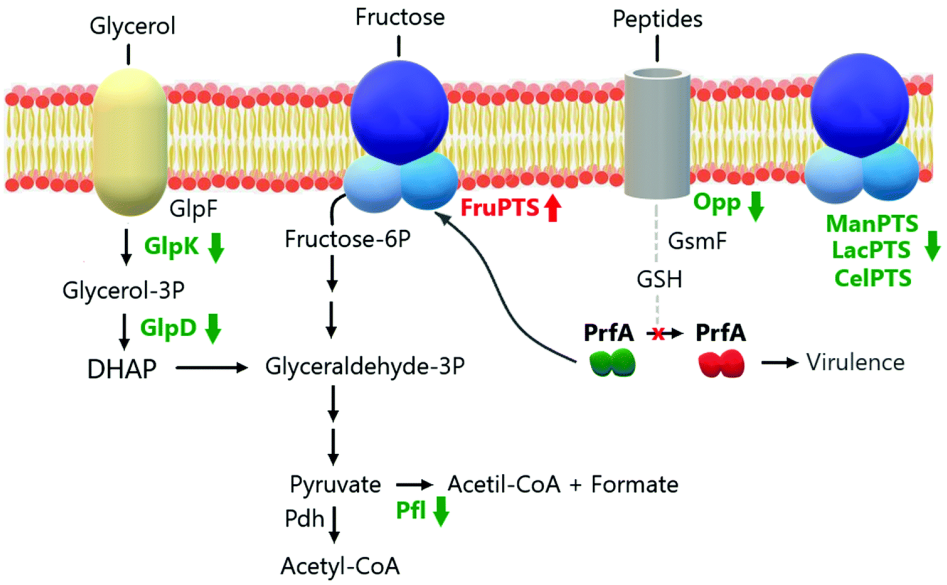

Fig. 6 Schematic representation of the influence of peptide P34 on the regulation of proteins related to the virulence factor PrfA. Upregulated proteins are represented in red and downregulated proteins in green.

Fructose is one of the main carbon sources for *L. monocytogenes*, and the proteomics data obtained in this study allowed to detect the proteins coded by *fruA* and *fruB* genes among the upregulated proteins from P34 treated samples. Although this system is understudied in *L. monocytogenes*, the well-conserved *fru* operon permits comparison with other low-GC Gram-positive bacteria.[46] Studies on a mutant strain for the fructose-specific PTS permease IIA suggest a role of this protein in the virulence of *L. monocytogenes*. Compared to the wild-type parental strain, the majority of the virulence genes including the major virulence regulator *prfA* were upregulated in the deletion mutants, indicating that this PTS negatively regulated the virulence gene expression.[10] Thus, the overexpression of *fruA* and *fruB* suggests that *L. monocytogenes* virulence could be reduced when exposed to peptide P34.

On the other hand, the proteins coded by the genes *lmo1719* and *lmo2373*, respectively the subunits EIIA and EIIB of other PTS involved in the lactose/cellobiose uptake,[47] were among

the proteins downregulated in samples treated with P34. A downregulation of the mannose-specific PTS system IIAB component encoded by the gene *lmo0096*,[48] was exclusively observed in treatments with encapsulated P34 (N-P34). Indeed, the mannose PTS, as major sugar uptake system in bacteria, has been studied as bacteriocin receptor/target protein in *L. monocytogenes*.[49,50] Previous studies have suggested that bacteriocins can act on this PTS disrupting the proton motive force.[51] The downregulation may be related to resistance strategy against the antimicrobial being the mannose PTS the docking molecule that helps the antimicrobial to reach the plasma membrane, as recently observed through molecular analysis.[52] Thus, these sugar transporters are considered as possible targets for the development of new antimicrobials because of their exclusive presence in prokaryotes.[10]

The downregulated proteins encoded by *lmo0152* and *lmo0135* are structurally related to OppA, involved in active transport of oligopeptides across the cytoplasmic membrane. *L. monocytogenes* contains a multidomain protein (termed GshF) that carries out complete synthesis of glutathione.[53] The uptake of cysteine peptides by Opp transporters can provide cysteine substrate for GshF, increasing glutathione synthesis. Great importance has been given to the PrfA functional positive regulation provided by the cofactor glutathione.[54] Glutathione binds PrfA promoting prolific interaction with the target DNA for the transcription of virulence genes.[55] Thus, downregulation of Opp proteins involved in cysteine transport might indirectly act by obstructing PrfA-mediated virulence (Fig. 6).

Beside the treatments with peptide P34 on the differentially regulated specific sugar PTS, results from the treatment with N-P34 underlines a repression of the virulence, in agreement with the upregulation of FruAB, through repression of the glycerol metabolic pathway with downregulation of glycerol-3-phosphate dehydrogenase and glycerol kinase. In this context is interesting the quite high differentially negative expressed pyruvate formate lyase (Pfl) by the

P34-treated samples. Pfl is important to regulate anaerobic glucose metabolism, and plays a significant role in the anaerobic layer of a biofilm.[56]

The master virulence factor PrfA modulation plays a fundamental role on the passage through the saprophytic (outside of the host cells) to the parasite intracellular pathogenesis.[44] In previous studies, inhibition of PrfA was demonstrated when glucose or other glycolytic carbohydrates were used as the major carbon source,[42] condition in which the virulence activity could be unproductive for listerial growth, while PrfA activity was higher when glycerol was used as the main carbon source.[57,58] Indeed, there is a complex crosstalk between PrfA and carbohydrate metabolism in *L. monocytogenes*,[3] and the effect of P34 on expression of PTS proteins may have influence on the PrfA-mediated virulence. However, as PrfA activation is not favoured in BHI medium future studies are needed to confirm this hypothesis.

Another important group of proteins with modified regulation by exposure to peptide P34 was related with the metal ions homeostasis. Metal-binding proteins play a fundamental role during the invasion of *L. monocytogenes* in the host. Bacterial proteins that carry transition metals, such as manganese (Mn), iron (Fe), and zinc (Zn) are essential to the host invasion process due to their role in facilitating the structure and function of proteins.[59] A strong repression of two fundamental proteins involved on the manganese uptake specifically MntA and MntB was observed in samples treated with both free and encapsulated peptide P34. A recent study carried out with other Gram positive bacteria, *Staphylococcus aureus*, showed the crucial role of MntABC protein cluster for growth in both manganese limitation and oxidative stress conditions, proving the importance of this lipoprotein on the *S. aureus* virulence.[11] The MntABC transporter was considered a possible antibiotic target, since *in vivo* studies on murine models infected by methicillin-resistant *S. aureus* (MRSA)

genetically inactivated on the various transporter components shown strong attenuated infection.[60]

On the other hand, upregulation of iron transporters was shown by *L. monocytogenes* treated with peptide P34, probably as a compensation response to the reduction of manganese uptake caused by downregulation of manganese transporters as recently demonstrated.[61] Thus, a kind of compensation may occur to reduce as much as possible the limitation of microelements into the bacterial cells. *L. monocytogenes* controls the ferric uptake regulator (*fur*) transcription by the iron concentration into the cells.[62] If an imbalance of ferric ions takes place, ABC-transporters are transcripted by the (i) *fhu* operon for ferric hydroxamate siderophores absorption; (ii) through iron extraction from eukaryotic binding proteins (transferrin, lactoferrin, ferritin, hemoglobin); and (iii) employing sortase-anchored proteins SrtB and the *hup* operons, detected acting on the transport across membrane. In addition, membrane reductases for catecholate ferric siderophores were studied, but unknown membrane transporters for this ferric compounds has been yet discovered.[25,26,63] The ABC iron transporter encoded by *lmo2184* includes sortase B and peptidoglycan attachment protein in the gene cluster. Although sortase-anchored proteins work in correlation with iron deprivation in Gram-positive bacteria,[28] it seems that these proteins do not play an indispensable role in iron utilization by *L. monocytogenes*.[25] The lipoprotein transporter encoded by *lmo0366* gene is positively correlated with the presence of PrfA. However, its presence in *L. innocua*, where the general virulence genes are absent, may indicates a secondary surface function that is not exclusively associated with the pathogenic lifestyle of *L. monocytogenes* but becomes more important during the infection process.[64]

In the intracellular context, the peroxide resistance regulator (PerR) known as a repressor cluster of the Fur regulon[65] appeared repressed. The ferritin-like protein and catalase, encoded respectively by *dps* and *kat*, are under regulation of PerR and were strongly downregulated in

all the treatments with peptide P34. Fur regulon negatively act on PerR, with *kat* and *dps* contributing with the inhibition of virulence bacterial processes.[66] As demonstrated in *Bacillus subtilis*, PerR-mediated repression of most target genes can be elicited by either manganese or iron, while repression of *perR* and *fur* is selective for manganese. These studies indicate that repression of PerR regulon genes by either manganese or iron requires PerR and is generally independent of Fur.[67] Evidence from *L. monocytogenes* FrvA (Lmo0641), an ortholog of *B. subtilis* Fur- and PerR-regulated Fe^{2+} efflux ATPase, indicates that Fur is involved in Fe^{2+} efflux during *L. monocytogenes* infection.[68] Thus, the regulation of metal ion transport can greatly influence the transcriptional response of the various PerR regulon genes and virulence of *L. monocytogenes*.

Free peptide P34 causes rapid membrane damage in *L. monocytogenes*,[13] while liposomes may be adhered but not to fuse with *L. monocytogenes* after 1 h, suggesting that liposomes provide a controlled release of P34.[19] Thus, it could be expected that free and encapsulated P34 would elicit different responses on target bacteria. In this work, a group of proteins differentially regulated in *L. monocytogenes* cultures treated with encapsulated P34 is described for the first time (Table S3, ESI†). Among these, the protein Lmo0096 (MptA), a PTS component with a potential role in regulation of PrfA activity,[69] was downregulated as discussed above. In *L. monocytogenes*, spontaneous development of resistance to class IIa bacteriocins has been associated with deficient synthesis of the MptA subunit of a mannose-specific PTS,[70] suggesting that the regulation of the Man-PTS pathway is an important resistance mechanism in *L. monocytogenes*.[71] In addition, the upregulation of the Lmo2167 protein, included into the metallo-beta-lactamase family, always regulated by the σB factor[72] may be a general bacterial resistance strategy against the antimicrobial treatment. The downregulated membrane protein Lmo1306 is similar to YneF protein of *Bacillus subtilis*, a

putative membrane-bound diguanylate cyclase (NCBI accession NP_416039.1). This enzyme is involved in the biosynthesis of cyclic-di-GMP, a bacterial second messenger regulating biofilm formation, motility and virulence, which affect pathogenesis of many bacteria.[73] In *L. monocytogenes*, cyclic-di-GMP signaling pathway seems to induce an exopolysaccharide responsible for cell aggregation and improved tolerance to disinfectants and dehydration, which may contribute to survival in foods.[74] Cyclic-di-GMP is also described as an effector of lifestyle change, as recently demonstrated for the predatory bacterium *Bdellovibrio bacteriovorus*.[75] Moreover, the downregulation of the phospholipase C protein directly involved in the host cell-to-cell listeria spread,[24] indicate a correlation between the protein pattern and the decreased virulence when the pathogen was treated with encapsulated P34.

In conclusion, this work presents a first proteomic study about the impact of free and nanoencapsulated peptide P34 on *L. monocytogenes* proteome. The action of antimicrobial peptide P34 on *L. monocytogenes* permitted to detect three fundamental target processes. A reduction or a possible inhibition of virulence starting by the saprophytic life phase with diverse under regulated proteins related to the σB activity. In addition, a reduction of the virulence could be also related to the effect on surface proteins involving both PTS systems and oligopeptide transporters working negatively on the key virulence factor PrfA, fundamental in the switch to the parasitic phase of *L. monocytogenes*. Moreover, a clear impairment of proteins involved in the manganese ion transport across the membrane, considered as an important antimicrobial target in other Gram-positive bacteria, was observed. In this context, upregulation of iron metal transport systems appears to replace the manganese deficit inside bacterial cells. Proteins involved in glycerol metabolism and iron transport exclusively regulated in response to encapsulated P34 indicate differential response of *L. monocytogenes* to nanostructured antimicrobial.

Experimental section

Production and partial purification of the peptide P34

Bacillus velesensis P34 was recovered from a stock at −20 °C in brain-heart infusion (BHI; Merck KGaA, Darmstadt, Germany) containing 20% (v/v) glycerol. The strain was pre-cultivated in BHI for 24 h at 30 °C and then cultivated in the same conditions to produce the antimicrobial lipopeptide P34, which was subjected to a partial purification as described previously.[12,16] The antimicrobial activity was monitored by the serial twofold dilution method, and expressed as activity units (AU) per milliliter.[76]

Liposome encapsulation

Preparation of nanoliposomes was carried out using the thin film methodology.[19] The dried lipid film was prepared using the purified soybean phosphatidylcholine (PC ≥ 94%) Phospholipon 90G® provided by Lipoid (Ludwigshafen, Germany). The aqueous phase containing the peptide P34 in 10 mM phosphate buffer (pH 7.0) was added and the system was heated at 60 °C for 2 min, followed by 1 min under vigorous agitation and 1 min interval. The solution was then subjected to ultrasound for size reduction of the lipocapsules using a probe-type ultrasound (Unique OF S500, frequency 20 kHz, power 250 W) for 5 cycles of 1 min with interval of the same time. Lastly, the liposomes were filtered using a 0.22 μm membrane (Sartorius, Göttingen, Germany) before to be introduced in the bacterial cultures.

Liposomes were subjected to ultrafiltration (Ultracel YM-10 membrane, Millipore) at 10 000 g for 20 min. The amount of free P34 was determined in the filtrate by HPLC and the encapsulation efficiency was determined as described previously.[77] Particle size and zeta potential (ζ) analyses were carried out after dilution of the formulations in ultrapure water using a Zetasizernano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany).

L. monocytogenes treatment with peptide P34

L. monocytogenes ATCC 7644 was pre-cultured in BHI agar and used to prepare a bacterial suspension with an OD_{600} of 0.150 in saline solution. This suspension (500 μL) was inoculated into 50 mL BHI broth to achieve 10^7 CFU mL^{-1} and incubated in a rotary shaker at 37 °C and 150 rpm. The viable cells enumeration was determined by spread plate using 100 μL of serial 10-fold dilutions onto BHI agar plates, incubated at 37 °C for 24 h.[76] After 6 h cultivation of *L. monocytogenes*, peptide P34 was added to the culture and samples were incubated for additional 1 h before protein extraction. In this study, four different treatments were performed with the *L. monocytogenes* strain using the free P34 peptide (F-P34), the P34 encapsulated into nanoliposomes (N-P34) and as controls, the empty nanoliposomes (NE) and the bacterium *L. monocytogenes* without any treatment (C). Treatments were performed with 100 AU mL^{-1} of peptide P34, previously demonstrated as sub-lethal concentration for *L. monocytogenes* ATCC 7644 in liquid culture.[13] Thus, *L. monocytogenes* was submitted to 60 min incubation with the peptide P34. For this last incubation period a total of 0.5 mL of phosphate buffer (pH 7.0) were added, because used for dissolution of the peptide P34. Three biological replicates were performed for each treatment.

Total protein extraction

The protein extraction methodology carried out to the samples was previous reported,[78] with minor modifications. Briefly, each culture medium from the triplicate samples were centrifuged at 10 000 *g* for 15 min at 4 °C. The collected pellets were washed 3 times with 2 mL of PBS buffer pH 7.4 and suspended in Tris–HCl buffer pH 7.5 containing 10 μL of Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL,USA). The solutions were lysed with probe-type ultrasound (Unique OF S500, frequency 20 kHz, power 250 W) for 5 cycles of 30 seconds each with intervals of 1 min on ice. The lysed cells were centrifuged at

10 000 *g* for 20 min and the supernatants were frozen at −80 °C. Samples were lyophilized before mass spectrometry analysis.

Protein digestion and desalinization

Total protein quantification was performed by Bradford method, using a standard curve of bovine serum albumin. The digestion of proteins was performed by the following methodology.[79] A sample quantity of 100 μL were treated with 100 μL of 8 M urea to obtain a final solution of 4 M urea, the proteins were reduced with dithiothreitol (5 mM for 25 min at 56 °C) and alkylated with iodoacetamide (14 mM for 30 min at room temperature protected from light). Digestion of proteins were performed diluting urea with 50 mM ammonium bicarbonate to a final concentration of 1.6 M, lastly 1 mM of calcium chloride was added to the samples and incubating for 16 h at 37 °C (2 μg of trypsin).[79] The enzymatic reaction was stopped with 2% (v/v) of trifluoroacetic acid. Desalination was performed employing C18 stage tips using water and 0.1% formic acid to wash the peptides from the salt residues.[80] Finally, the elution of the samples were performed using a 60% (v/v) acetonitrile and 0.1% (v/v) formic acid. The samples were dried in a vacuum concentrator and stored at -20 °C for successive analysis by LC-MS/MS.

LC-MS/MS Orbitrap analysis

The samples were reconstituted in 100 μL of 0.1% (v/v) formic acid ultrapure sterile water, 4.5 μL of the resulted peptide mixture was analysed in LTQ Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer coupled to nanoflow liquid chromatography on an EASY-nLC system (Proxeon Biosystems, West Palm Beach, FL, USA) with a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90% (v/v) acetonitrile gradient in 0.1% (v/v) formic acid in an analytical PicoFrit Column (20 cm × ID 75 μm, 5 μm particle size) (New Objective Inc., Woburn, MA, USA) at a flow rate of 300 μl

min^{-1}. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275 °C.

Proteomic analysis

Raw data were processed using MaxQuant v1.3.0.3 software,[81] and MS/MS spectra were searched against *L. monocytogenes* UniProt database using the Andromeda search engine.[82] Statistical analysis of 12 samples of the 4 treatment groups were carried out using MetabolAnalyst (version 4.0, http://www.metaboanalyst.ca/). Among all the features analysed those that represented more than 50% values missing were removed and estimation of the remaining missing value using k-nearest neighbour (KNN). PCA and PLSDA analysis was calculated by maximizing the co-variance between the matrix data set and the class labels. One of the important measures achieved was the variable importance projection (VIP) score. Thus, each protein received a VIP value, for the follow analysis only the proteins with VIP score ≥1 where identified and characterized through UNIProt (https://www.uniprot.org/). These latter proteins were used to construct a heatmap and protein regulations were highlighted in each group. Venn diagram was constructed using Venny 2.1 for categorizing proteins uniquely upregulated in a specific condition.[83]

Additionally, proteins were characterized on basis of their structural and functional annotations using enrichment analysis of gene ontology (GO) with E-value 0.001 using CELLO2GO.[84]

Conflicts of interest

Authors declare no conflicts of interest.

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Molecular Omics

Supplementary material

Proteomic analysis of *Listeria monocytogenes* exposed to free and nanostructured antimicrobial lipopeptides

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Supplementary Table S1

Table S1. PLS-DA values used to identify differentially expressed proteins related to the exposure of *L. monocytogenes* to empty nanoliposome, free and nano-encapsulated peptide P34, with the VIP threshold > 1.0 in the first component of PLS-DA.

Protein name	Gene name	VIP Score		
		Comp. 1	Comp. 2	Comp. 3
DNA protection during starvation protein	*dps*	5.41	5.18	5.14
Aldehyde-alcohol dehydrogenase	*lmo1634*	5.16	4.84	4.81
Lmo1257 protein	*lmo1257*	4.83	4.57	4.54
Manganese-binding lipoprotein MntA	*mntA*	4.26	4.29	4.26
Pyruvate formate-lyase	*pflB*	3.99	3.84	3.81
FruA protein	*fruA*	3.95	3.80	3.78
Manganese transport system ATP-binding protein MntB	*mntB*	3.58	3.74	3.71
Lmo0135 protein	*lmo0135*	3.49	3.27	3.27
Dihydrolipoyl dehydrogenase	*lmo1371*	3.49	3.29	3.27
Lmo1959 protein	*lmo1959*	3.29	3.14	3.13
Peptide chain release factor 3	*prfC*	3.16	2.96	2.94
Translation initiation factor IF-1	*infA*	2.92	2.80	2.78
Catalase	*kat*	2.89	2.71	2.70
Uncharacterized protein Lmo0216	*lmo0216*	2.86	2.68	2.67
Heme-degrading monooxygenase	*isdG*	2.84	2.67	2.65
Lmo0685 protein	*lmo0685*	2.77	2.60	2.58
Cysteine synthase	*cysK*	2.77	2.62	2.60

UDP-N-acetylenolpyruvoylglucosamine reductase	*murB*	2.77	2.66	2.64
Lmo0663 protein	*lmo0663*	2.65	2.50	2.49
Lmo0592 protein	*lmo0592*	2.64	2.63	2.63
UPF0145 protein lmo0208	*lmo0208*	2.60	2.46	2.44
Pseudouridine synthase	*lmo1949*	2.58	2.42	2.40
Lmo1719 protein	*lmo1719*	2.53	2.37	2.36
Bifunctional protein FolD	*folD*	2.40	2.27	2.27
Lmo2373 protein	*lmo2373*	2.39	2.27	2.26
ATP-dependent Clp protease proteolytic subunit	*clp*	2.37	2.23	2.24
DNA-directed RNA polymerase subunit omega	*rpoZ*	2.34	2.23	2.21
Lmo0152 protein	*lmo0152*	2.33	2.20	2.19
Lmo0066 protein	*lmo0066*	2.32	2.18	2.17
Pyridoxal 5-phosphate synthase subunit PdxT	*pdxT*	2.30	2.16	2.14
UPF0356 protein lmo1028	*lmo1028*	2.24	2.10	2.09
Phospholipase C	*plcB*	2.24	2.16	2.16
50S ribosomal protein L35	*rpmI*	2.21	2.07	2.07
Lmo0541 protein	*lmo0541*	2.19	2.16	2.17
Tagatose-6-phosphate kinase	*fruB*	2.19	2.06	2.05
Lmo2752 protein	*lmo2752*	2.16	2.03	2.01
Lmo2152 protein	*lmo2152*	2.15	2.11	2.10
Protein DltD	*dltD*	2.14	2.03	2.01
Lmo2167 protein	*lmo2167*	2.10	1.97	1.97
Ribulose-phosphate 3-epimerase	*lmo1818*	2.10	2.00	1.99
30S ribosomal protein S21	*rpsU*	2.09	1.96	1.96
Lmo1079 protein	*lmo1079*	2.09	1.96	1.94
Lmo1946 protein	*lmo1946*	2.05	1.92	1.91
Deoxyuridine 5-triphosphate nucleotidohydrolase	*lmo1691*	2.04	2.02	2.00
Lmo1812 protein	*lmo1812*	2.00	1.87	1.86
50S ribosomal protein L32-1	*rpmF1*	1.99	1.92	1.93
Lmo0955 protein	*lmo0955*	1.96	1.98	1.97
1-phosphatidylinositol phosphodiesterase	*plcA*	1.95	1.98	2.02
Lmo2184 protein	*lmo2184*	1.95	2.05	2.05
Lmo1710 protein	*lmo1710*	1.88	2.10	2.10
Glucose-1-phosphate thymidylyltransferase	*lmo1081*	1.87	1.77	1.76
UPF0173 metal-dependent hydrolase lmo1577	*lmo1577*	1.80	1.70	1.69
Transcription termination factor Rho	*rho*	1.78	1.67	1.66
dTDP-4-dehydrorhamnose 3,5-epimerase	*lmo1082*	1.77	1.67	1.66
Lmo1510 protein	*lmo1510*	1.74	1.74	1.73
Lmo2433 protein	*lmo2433*	1.73	1.71	1.70
Single-stranded DNA-binding protein 1	*ssb1*	1.73	1.62	1.61
Lmo0982 protein	*lmo0982*	1.73	1.68	1.67
Lmo0366 protein	*lmo0366*	1.73	1.66	1.67
Lmo1017 protein	*lmo1017*	1.72	1.65	1.66
Lmo2209 protein	*lmo2209*	1.71	1.61	1.60

tRNA-specific 2-thiouridylase MnmA	*mnmA*	1.70	1.59	1.59
L-lactate dehydrogenase 1	*ldh1*	1.68	1.63	1.63
Serine acetyltransferase	*cysE*	1.67	1.87	1.86
Lmo0335 protein	*lmo0335*	1.66	1.56	1.55
Lmo2569 protein	*lmo2569*	1.65	1.60	1.60
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	*murA2*	1.64	1.60	1.60
ATP synthase gamma chain	*atpG*	1.64	1.57	1.56
ATP-dependent DNA helicase	*pcrA*	1.63	1.54	1.53
50S ribosomal protein L36	*rpmJ*	1.63	1.58	1.57
UPF0154 protein lmo1306	*lmo1306*	1.60	2.29	2.28
Translation initiation factor IF-3	*infC*	1.60	1.50	1.49
Lmo0900 protein	*lmo0900*	1.60	1.79	1.79
HTH-type transcriptional regulator MntR	*mntR*	1.58	1.57	1.56
Lmo0774 protein	*lmo0774*	1.58	1.50	1.49
Lmo2168 protein	*lmo2168*	1.58	1.56	1.55
Glycerol-3-phosphate dehydrogenase	*glpD*	1.58	1.49	1.48
Putative peptidoglycan bound protein LPXTG motif	*lmo0842*	1.57	1.91	1.90
Signal peptidase I	*lmo1271*	1.57	1.48	1.47
Lmo0579 protein	*lmo0579*	1.53	1.44	1.44
10 kDa chaperonin	*groS*	1.53	1.44	1.45
Lmo1529 protein	*lmo1529*	1.50	1.40	1.44
Ribosome hibernation promoting factor	*hpf*	1.49	1.41	1.42
Lmo0096 protein	*lmo0096*	1.49	1.54	1.53
FolC protein	*folC*	1.48	1.52	1.52
Purine nucleoside phosphorylase DeoD-type	*deoD*	1.47	1.42	1.42
Putative zinc metalloprotease Lmo1318	*lmo1318*	1.46	1.36	1.35
30S ribosomal protein S13	*rpsM*	1.44	1.34	1.33
Lmo2208 protein	*lmo2208*	1.44	1.48	1.46
Lmo1283 protein	*lmo1283*	1.43	1.34	1.35
Uncharacterized protein Lmo1967	*lmo1967*	1.42	1.52	1.51
Lmo2199 protein	*lmo2199*	1.41	1.32	1.32
Lmo0848 protein	*lmo0848*	1.40	1.43	1.42
30S ribosomal protein S18	*rpsR*	1.40	1.31	1.33
50S ribosomal protein L18	*rplR*	1.40	1.31	1.30
Threonine--tRNA ligase	*thrS*	1.39	1.30	1.30
Regulatory protein Spx	*spxA*	1.37	1.91	1.90
Lmo1515 protein	*lmo1515*	1.37	1.32	1.31
50S ribosomal protein L21	*rplU*	1.37	1.29	1.28
UPF0637 protein lmo1065	*lmo1065*	1.36	1.29	1.29
Glycerol kinase	*glpK*	1.36	1.29	1.31
Lmo0257 protein	*lmo0257*	1.35	1.30	1.29
Lmo0193 protein	*lmo0193*	1.35	1.29	1.28
Diaminopimelate epimerase	*dapF*	1.35	1.26	1.26
Glutathione peroxidase	*lmo0983*	1.34	1.27	1.27
Aminomethyltransferase	*gcvT*	1.34	1.44	1.44

Lmo0847 protein	*lmo0847*	1.33	1.32	1.32
Lmo2232 protein	*lmo2232*	1.32	1.24	1.26
FADprotein FMN transferase	*lmo2636*	1.31	1.28	1.28
30S ribosomal protein S12	*rpsL*	1.30	1.24	1.23
Thiol peroxidase	*tpx*	1.30	1.24	1.23
Lmo0185 protein	*lmo0185*	1.29	1.22	1.21
Initiation-control protein YabA	*lmo0164*	1.29	1.48	1.47
tRNA uridine 5-carboxymethylaminomethyl modification enzyme MnmG	*mnmG*	1.29	1.20	1.20
Lmo1887 protein	*lmo1887*	1.28	1.27	1.27
Formate--tetrahydrofolate ligase	*fhs*	1.28	1.22	1.22
Aspartate--tRNA ligase	*aspS*	1.28	1.21	1.20
Glucose-6-phosphate 1-dehydrogenase	*zwf*	1.27	1.23	1.22
Uncharacterized RNA methyltransferase lmo1751	*lmo1751*	1.26	1.30	1.29
NADPH dehydrogenase	*namA*	1.26	1.22	1.21
Lmo0734 protein	*lmo0734*	1.25	1.17	1.17
50S ribosomal protein L31 type B	*rpmE2*	1.24	1.16	1.16
Lmo1094 protein	*lmo1094*	1.23	1.17	1.17
50S ribosomal protein L20	*rplT*	1.23	1.15	1.14
Lmo0084 protein	*lmo0084*	1.23	1.16	1.15
Lmo1820 protein	*lmo1820*	1.22	1.20	1.19
Lmo2696 protein	*lmo2696*	1.19	1.11	1.12
Metal-dependent carboxypeptidase	*lmo1886*	1.18	1.15	1.15
ATP synthase subunit delta	*atpH*	1.17	1.10	1.10
Lmo2193 protein	*lmo2193*	1.17	1.14	1.13
Lipoate--protein ligase	*lmo0931*	1.17	1.24	1.23
AlsS protein	*alsS*	1.17	1.10	1.09
Lmo1431 protein	*lmo1431*	1.17	1.10	1.09
tRNA pseudouridine synthase A	*truA*	1.16	1.14	1.14
CydA protein	*cydA*	1.15	1.09	1.09
Lmo1415 protein	*lmo1415*	1.15	1.08	1.07
Lmo1727 protein	*lmo1727*	1.14	1.13	1.12
Lmo2700 protein	*lmo2700*	1.14	1.11	1.11
Lmo1030 protein	*lmo1030*	1.14	1.08	1.08
Alanine dehydrogenase	*lmo1579*	1.13	1.07	1.06
Lmo1620 protein	*lmo1620*	1.13	1.07	1.06
GTP cyclohydrolase 1	*folE*	1.13	1.18	1.17
CydD protein	*cydD*	1.13	1.08	1.08
Probable transcriptional regulatory protein lmo1535	*lmo1535*	1.12	1.09	1.08
Probable tautomerase lmo2564	*lmo2564*	1.12	1.05	1.04
Lmo2537 protein	*lmo2537*	1.11	1.07	1.06
GTP-sensing transcriptional pleiotropic repressor CodY	*codY*	1.10	1.03	1.03
Pseudouridine synthase	*lmo1843*	1.10	1.05	1.04
Lmo2370 protein	*lmo2370*	1.10	1.05	1.04

30S ribosomal protein S14 type Z	*rpsZ*	1.10	1.03	1.02
tRNA-dihydrouridine synthase	*lmo0227*	1.09	1.13	1.12
Bifunctional protein GlmU	*glmU*	1.09	1.03	1.03
L-lactate dehydrogenase 2	*ldh2*	1.09	1.03	1.03
DNA polymerase III subunit gamma/tau	*dnaX*	1.09	1.08	1.07
Lmo1005 protein	*lmo1005*	1.08	1.05	1.04
Citrate synthase	*citZ*	1.08	1.01	1.00
30S ribosomal protein S2	*rpsB*	1.07	1.03	1.03
Lmo1019 protein	*lmo1019*	1.07	1.02	1.01
Recombination protein RecR	*recR*	1.07	1.01	1.01

Supplementary Figure S1

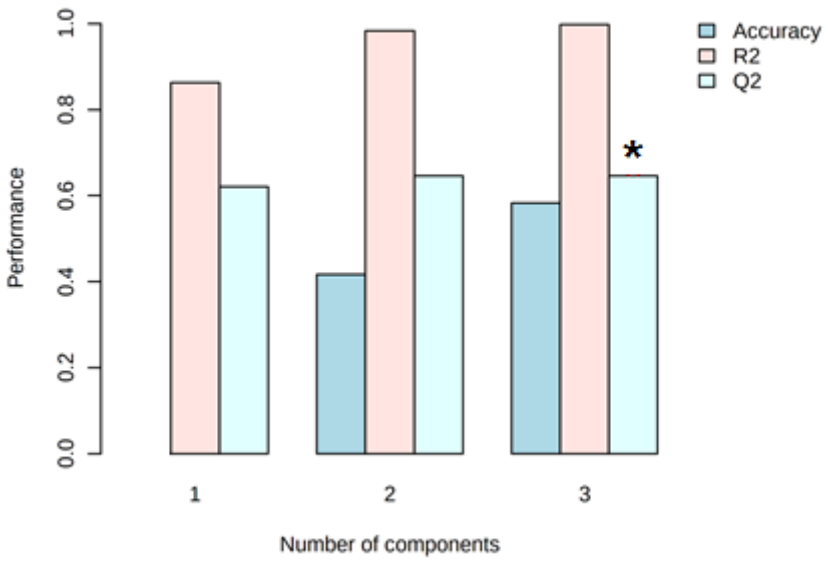

Figure S1. Relation between components and performance of accuracy, R^2 and Q^2. The red * indicate the highest value for the Q^2 parameter among the components.

Supplementary Table S2

Table S2. List of proteins with VIP ≥2 differentially regulated in the treatments with peptide P34. These proteins were used as base for the proteomics study of this work.

Protein name	**VIP score**	**Gene name**	**Protein regulation**
Lmo0135 protein	3.49	*lmo0135*	Down
Catalase	2.89	*kat*	Down
Aldehyde-alcohol dehydrogenase	5.16	*lmo1634*	Down
Pyruvate formate-lyase	3.99	*pflB*	Down
Manganese-binding lipoprotein MntA	4.25	*mntA*	Down
Manganese transport system ATP-binding protein MntB	3.58	*mntB*	Down
Translation initiation factor IF-1	2.91	*infA*	Down
Peptide chain release factor 3	3.15	*prfC*	Down
DNA protection during starvation protein	5.41	*dps*	Down
Lmo1257 protein	4.82	*lmo1257*	Down
Lmo0592 protein	2.64	*lmo0592*	Down
Lmo0663 protein	2.65	*lmo0663*	Down
50S ribosomal protein L35	2.21	*rpmI*	Down
Lmo2152 protein	2.15	*lmo2152*	Down
Pyridoxal 5'-phosphate synthase subunit PdxT	2.30	*pdxT*	Down
Lmo1028 protein	2.24	*lmo1028*	Down

Lmo0208 protein	2.59	*lmo0208*	Down
DNA-directed RNA polymerase subunit omega	2.34	*rpoZ*	Down
Lmo2373 protein	2.39	*lmo2373*	Down
Lmo1719 protein	2.52	*lmo1719*	Down
Lmo0152 protein	2.33	*lmo0152*	Down
Dihydrolipoyl dehydrogenase	3.48	*lmo1371*	Up
Lmo0685 protein	2.77	*lmo0685*	Up
FruA protein	3.94	*fruA*	Up
Uncharacterized protein Lmo0216	2.85	*lmo0216*	Up
Heme-degrading monooxygenase	2.83	*isdG*	Up
Lmo1959 protein	3.29	*lmo1959*	Up
UDP-*N*-acetylenolpyruvoylglucosamine reductase	2.77	*murB*	Up
Lmo1079 protein	2.09	*lmo1079*	Up
Ribulose-phosphate 3-epimerase	2.10	*lmo1818*	Up
Lmo2752 protein	2.16	*lmo2752*	Up
Protein DltD	2.14	*dltD*	Up
30S ribosomal protein S21	2.09	*rpsU*	Up
Deoxyuridine 5'-triphosphate nucleotidohydrolase	2.04	*lmo1691*	Up
Tagatose-6-phosphate kinase	2.18	*fruB*	Up
Lmo1946 protein	2.05	*lmo1946*	Up
Lmo0066 protein	2.32	*lmo0066*	Up

Supplementary Table S3

Table S3. Exclusively regulated protein by nanoencapsulated peptide P34. The list proteins with VIP score ≥1.

Protein name	VIP score	Gene name	Protein regulation
Lmo0366 protein	1.73	*lmo0366*	Up
30S ribosomal protein S18	1.40	*rpsR*	Up
Lmo2184 protein	1.95	*lmo2184*	Up
Lmo0579 protein	1.53	*lmo0579*	Up
Thiol peroxidase	1.30	*Tpx*	Up
Lmo0541 protein	2.19	*lmo0541*	Up
Lmo1094 protein	1.23	*lmo1094*	Up
dTDP-4-dehydrorhamnose 3,5-epimerase	1.77	*lmo1082*	Up
Lmo1620 protein	1.13	*lmo1620*	Up
Lmo1510 protein	1.74	*lmo1510*	Up
Lmo2168 protein	1.58	*lmo2168*	Up
Lmo0185 protein	1.29	*lmo0185*	Up
Lmo2696 protein	1.19	*lmo2696*	Up
GTP cyclohydrolase 1	1.17	*folE*	Up
Pseudouridine synthase	1.10	*lmo1843*	Up

Lmo2167 protein	2.10	*lmo2167*	Up
Lmo1005 protein	1.08	*lmo1005*	Up
Phospholipase C	2.24	*plcB*	Down
Glycerol-3-phosphate dehydrogenase	1.58	*glpD*	Down
Lmo0084	1.23	*lmo0084*	Down
Lmo1710 protein	2.09	*lmo1710*	Down
50S ribosomal protein L36	1.62	*rpmJ*	Down
Glycerol kinase	1.36	*glpK*	Down
Lmo0096 protein	1.49	*lmo0096*	Down
10 kDa chaperonin	1.53	*groS*	Down
AlsS protein	1.17	*alsS*	Down
Ribosome hibernation promoting factor	1.49	*Hpf*	Down
Uncharacterized protein Lmo1967	1.42	*lmo1967*	Down
UPF0154 protein Lmo1306	2.28	*lmo1306*	Down

Supplementary Table S4

Table S4. Protein regulation of free and nanoencapsulated P34 proteins group and the exclusively proteins expressed by the treatment with P34 nanoencapsulated according to molecular functions assigned by CELLO2GO algorithm. Molecular functions assigned with smaller percentages in both protein groups are not detailed.

Molecular function	**Exclusively N-P34**		**F-P34 and N-P34**	
	Upregulation	Downregulation	Upregulation	Downregulation
Ion binding	21.1%	33.6%	23.9%	20.1%
Oxidoreductase activity	10.8%	16.9%	10.8%	10.2%
Hydrolase activity	8.3%	8.6%	13.1%	3.4%
Kinase activity	2%	8.3%	4.9%	3.6%
Transmembrane transport	-	-	5.1%	10.5%

Supplementary Table S5

Table S5. Protein regulation of free and nanoencapsulated P34 proteins group and the exclusively proteins expressed by the treatment with P34 nanoencapsulated according to biological process assigned by CELLO2GO algorithm. Biological processes assigned with smaller percentages in both protein groups are not detailed.

Biological process	**Exclusively N-P34**		**F-P34 and N-P34**	
	Upregulation	Downregulation	Upregulation	Downregulation
Small molecule metabolic process	12%	7.9%	6.5%	5.3%
Cellular nitrogen compound metabolic process	12%	-	4.5%	6.6%
Biosynthetic process	11%	10%	10.6%	9.5%
Response to stress	8.3%	14.9%	5.9%	7.1%
Transport	7.4%	8.3%	12%	14.6%
Homeostatic process	7.4%	-	15.4%	3.3%

3.3 Third Scientific Article

Nisin influence on the expression of *Listeria monocytogenes* surface proteins

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Nisin influence on the expression of *Listeria monocytogenes* surface proteins

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Abstract

In this work, a comparative analysis of the peripheral cell component (PCC) proteins of *Listeria monocytogenes* was carried out. The study was conducted on two set of samples consisting of bacteria treated with sub-lethal concentration of nisin and untreated bacteria as control. PCC proteins were extracted by Tris-Urea-EDTA treatment and then subjected to trypsin digestion and mass spectrometry analysis. The whole cell proteome was analyzed through label-free quantitative proteomics approach. Proteomic analysis was carried out using OrbiTrap Mass Spectrometer coupled to nanoflow liquid chromatography. The treatment with sub-lethal nisin concentration resulted in 62 up regulated and 97 down regulated proteins compared to untreated samples. Using PSORTb 3.0, 19 and 18 surface proteins were detected among the up regulated and down regulated proteins, respectively. Proteins related with increased biofilm formation by *L. monocytogenes*, such as moonlight proteins of the pyruvate dehydrogenase complex and flagellin-related proteins, were identified as up regulated surface proteins. Proteins associated with virulence of *L. monocytogenes*, including listeriolysin O, internalin B and actin assembly-inducing protein, were detected among the down regulated proteins. To confirm proteomics data, increased production of biofilm was experimentally confirmed in nisin-treated cells through crystal violet method.

Biological significance

Proteosurfaceomics can be defined as the "omics" science applied to the proteins of the peripheral cell component (PCC). The surface proteins of *Listeria monocytogenes*, an important foodborne pathogen were investigated after treatment with nisin, a bacteriocin approved as a natural food preservative by regulatory agencies. Recent cases of nisin

tolerance by *Listeria* spp. were documented, and deeper studies on the molecular process behind the bacterial survival may help in both understanding the development of tolerance process and comparing nisin effect with other antimicrobial compounds.

Keywords: Antimicrobial peptides, Biofilm, Lantibiotics, Proteomics, Virulence proteins

1. Introduction

Listeria monocytogenes is a Gram-positive bacterium, facultative anaerobic, resistant to high salt concentration, low temperature, and low pH conditions. It is a clinically relevant microorganism, contaminating food products that can cause gastrointestinal infections and, in some cases, enter into the bloodstream causing severe conditions, such as miscarriages and central nervous system infections [1]. This bacterium is able to build biofilms on abiotic surfaces, improving the resistance to dehydration and sanitizers [2]. Biofilm formation can be an effective bacterial strategy to survive against environmental stressors, including low temperature [3], low pH [4], and high salinity [5].

The use of bacteriocins, such as nisin, is one of the strategies used by the food industry to prevent biofilm formation on the surfaces in direct contact with foods [6]. Nisin is an antimicrobial peptide produced by *Lactococcus lactis* strains and it is mostly known for its activity against Gram-positive bacteria [7]. This bacteriocin can kill up to 90% of Gram-positive bacteria, frequently common in food industry. The lantibiotic nisin was discovered in 1928 and its benefits as a natural antimicrobial for effective food preservation was indicated by the Food and Agriculture Organization (FAO)/World Health Organization (WHO). Nisin granted the "generally recognized as safe" (GRAS) status by the US Food and Drug Administration (FDA) in 1988 and today it is implemented by more than 48 countries in various food applications [8]. Nisin has been largely employed as food preservative in dairy products and its high antimicrobial efficiency in cottage cheese not sterilized and contaminated with *Listeria* spp. strains was documented [9].

However, a major challenge on nisin utilization as food preservative will impose over the following years. Since nisin has been largely used in the food industry, it has been raising questions about its efficiency against nisin-tolerant or nisin-resistant strains, which can restrict its food applications. In this regard, there are some reports

about *L. monocytogenes* strains with acquired resistance to nisin [10,11]. Many nisin tolerance mechanisms can be acquired by either modifying cellular metabolism or forming a complex biofilm that can help bacterial cells to survive. In the first case, it was already reported that nisin can induce different metabolic alterations, but the role of surface proteins was not assessed, even though nisin resistance mechanisms changed bacterial cell wall and membrane composition. About biofilm formation, some evidence pointed out that nisin and other lantibiotics are able to disrupt biofilms formed by *L. monocytogenes*, although the mechanism is not yet elucidated [12, 13, 14, 15].

Proteosurfaceome can be defined as the omics science studying the proteins present on the bacterial surface, with very important role on bacterial communication, including in biofilm formation and bacteria-host communication. The use of bioinformatics tools is commonly relevant in proteosurfaceome studies to help the identification of surface proteins with multifunctional action, called moonlight proteins [16]. Moonlight proteins include a class of proteins with multifunctional physiological or biochemical action that are predominantly intracellular, but perform a second function in other cellular locations, typically on the cell surface. Their different functions can be performed simultaneously and/or change as a response to environmental perturbations [17].

Studies on surface proteins may contribute on the identification of molecular bacterial strategies of adherence, other pathogenic and resistance mechanisms of *L. monocytogenes* as well [18]. Moreover specifically, after *L. monocytogenes* adhesion to the potential mammalian host a set of virulence gene proteins are transcribed permitting the invasion and spread of the bacterial pathogen [19]. Therefore, this study aims to give light to the *L. monocytogenes* responses under a stressful condition represented by the nisin treatment at sub-lethal concentration. A proteosurfaceome approach was performed directing the

attention on two different process, observed by the presence of nisin up to the bacterial target, namely the virulence and biofilm arrangement.

2. Materials and methods

2.1. Strain, culture conditions and nisin susceptibility tests

Listeria monocytogenes ATCC 7644 was used throughout this study. Pre-cultured colonies in Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) agar were used to prepare a bacterial suspension in saline solution corresponding to a 0.5 McFarland turbidity standard (approximately 10^8 CFU/mL). One milliliter of this suspension was inoculated in 100 mL BHI broth (Oxoid) to achieve 10^6 CFU/mL and incubated in a rotary shaker at 37 °C and 150 rpm. After 6 h cultivation, nisin was added to the culture and samples were incubated for additional 1 h before protein extraction. Nisin (Chrisin®) was provided by Chr. Hansen A/S (Hørsholm, Denmark). According to the manufacturer, the formulation contains 2.5% (*w*/w) pure nisin. Nisin stock solution (1 mg/mL) was prepared by diluting Chrisin® in 0.01 M HCl and stored at 4 °C. The working concentrations were obtained by dilution of the nisin stock solution in 10 mM sodium phosphate buffer (pH 7.0).

The sub-lethal nisin concentration was determined through the microbial growth evaluation [20]. In this experiment, the tested concentrations were 1.0, 0.3, 0.2 and 0.1 μg/mL, added to each culture at 6 h incubation. Samples were collected at 0, 6 and 7 h incubation, and the viable cell counts were determined in triplicate on BHI agar plates.

Control and nisin-treated samples were performed as three biological replicates. In this work, the triplicate cultures no treated with the antimicrobial nisin was considered as control, while treatment the triplicate samples treated with 0.1 μg/mL of nisin, which exerted less antimicrobial effect, and then the concentration selected.

2.2. Protein extraction

Bacterial cells (about 7.5 log CFU/mL) were recovered by centrifugation at 7000 *g* for 6 min at 10 °C, followed by 3 washing cycles with 10 mM phosphate buffered saline (PBS) pH 7.4 to remove medium and cell debris. Pellets were suspended in 300 μL of 8 M Urea, 40 mM Tris base and 5 mM EDTA followed by incubation at 25 °C for 30 min under gentle agitation [21]. Finally, samples were centrifuged at 11,000 *g* for 6 min and the supernatant was filtered with a 0.22 μm membrane and stored at −20 °C. Protein extracts from different biological replicates were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). Samples were run in 12% polyacrylamide gels and proteins bands were visualized by staining with Commassie Brilliant blue G-250. Protein quantification was carried out through the Bradford method.

2.3. Protein digestion and desalting

The protein digestion was carried out as described previously [22]. Samples were treated with 8 M urea, followed by protein reduction with 5 mM dithiothreitol for 25 min at 56 °C, and then alkylation with 14 mM iodoacetamide for 30 min at room temperature and protected from any light sources. Protein digestion was performed by diluting urea with 50 mM ammonium bicarbonate to a final concentration of 1.6 M. Finally, 1 mM calcium chloride was added to the samples for trypsin (20 μg/μL, Promega, Madison, WI, USA) digestion for 16 h at 37 °C [22]. The enzyme reaction was halted with 2% (*v*/v) trifluoroacetic acid. Desalinization was performed using C18 reverse phase stage tips [23] eluted with 60% (v/v) acetonitrile and 0.1% (v/v) formic acid. Samples were dried in a vacuum concentrator and stored at −20 °C for further analysis by LC-MS/MS.

2.4. LC-MS/MS analysis

Samples reconstituted in 3.0 μL (~3 μg of peptide mixture) of 0.1% (*v*/v) formic acid were analyzed in LTQ Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer coupled to nanoflow liquid chromatography on an EASY-nLC system (Proxeon

Biosystems, West Palm Beach, FL, USA) with a Proxeon nanoelectrospray ion source. Peptides were separated by a 2–90% (*v*/v) acetonitrile gradient in 0.1% (v/v) formic acid in an analytical PicoFrit Column (20 cm × ID75 μm, 5 μm particle size) (New Objective Inc., Woburn, MA, USA) at a flow rate of 300 mL min^{-1} over 65 min. The nanoelectrospray voltage was set to 2.2 kV, and the source temperature was 275 °C. All instrument methods were set up in the data dependent acquisition mode. The full scan MS spectra (*m/z* 300–1600) were acquired in the Orbitrap analyser after accumulation to a target value of 1×10^6. The resolution in the Orbitrap was set to $r = 60{,}000$ and the 20 most intense peptide ions with charge states ≥2 were sequentially isolated to a target value of 5000 and fragmented in the linear ion trap using low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1000 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 60 s, and a repeat count of 1. An activation q = 0.25 and activation time of 10 ms were used.

2.5. Proteomics data analysis

Raw data were processed using MaxQuant v1.3.0.3 software [24], and MS/MS spectra were searched against *L. monocytogenes* UniProt database using the Andromeda search engine [25]. Selected proteins with valid label-free quantification (LFQ) intensity values present in ≥50% of the samples were considered for the statistical analysis. False discovery rate (FDR) coming from the application of univariate analysis *t*-test were calculated for each feature. MetaboAnalyst 3.068 was used for statistical analysis. A Volcano plot was created displaying information about fold change in protein expression on the *x*-axis versus the significance expressed as the negative logarithm of the corrected *P* value on the *y*-axis [26]. Furthermore, two other data-driven analytical approaches were used to visualize differences between the two group of samples: the Principal Component Analysis (PCA) was carried out the variation grade between the control and the nisin-treated samples; and also the Partial Least Squares

Discriminate Analysis (PLS-DA) was performed and important features were selected with a threshold of VIP (Variable Importance in Projection) ≥ 1.0 and used for the hierarchical clustering heatmap analysis. All the proteins with ≥1.0 VIP score were identified using Uniprot [27] and regrouped in down and up regulated proteins as reported on the heatmap. The two protein categories, up and down regulated, were submitted to String v11 Database for constructing protein-protein interactions [28] and the networks were visualized with Cytoscape (www.cytoscape.org/). The surface proteins were predicted through the subcellular localization carried out by PSORTb (version 3.0) [29] and the functional annotations using analysis of gene ontology (GO) was performed with CELLO2GO [30]. Moonlight proteins were identified using the MoonProt 2.0 [31].

2.6. Effect of sub-lethal nisin concentration on biofilm formation

The biofilm assay was performed to verify the effect of sub-lethal dose of nisin on biofilm formation by *L. monocytogenes*. The assay was performed by the crystal violet technique as described elsewhere [31]. Briefly, a fresh culture was prepared in BHI to achieve a concentration of 10^6 CFU/mL by aerobic incubation for 6 h at 37 °C. An aliquot of this suspension was separated as untreated culture, and nisin was added to the remaining culture to reach a working concentration of 0.1 μg/mL. Then, 200 μL of control or nisin-treated cultures were placed into 9 wells each of 96-well microplate, and incubated at 37 °C for 24 h. After incubation, the medium was discarded and the microplate was washed three times with 10 mM sodium phosphate buffer (pH 7) to remove planktonic cells. The adhered cells were fixed by adding methanol for 20 min, the solvent was removed and the microplate was dried overnight at room temperature. The adhered bacteria were stained with 2% (*w*/*v*) violet crystal for 15 min, and then washed with water to remove the free dye. Finally, 95% ethanol was added to the wells for 30 min to extract the dye and quantify adherent bacteria. Results were obtained by reading the optical density of the solution using a microplate reader

(SpectraMax M2e) at 570 nm. The interpretation was made by comparing the optical density value of wells containing *L. monocytogenes* treated with nisin (ODNis) with the optical density values of the wells containing untreated *L. monocytogenes* (ODCont). The effect of nisin on biofilm formation was considered inductive when the ODNis>ODCont, inhibitor when ODNis<ODCont, and without effect when ODNis = ODCont [32]. Data were expressed as means ± standard deviations of five replicated samples. Biofilm OD quantification results were subjected to analysis of variance ANOVA by using the software SAS for Windows version 9.0 (SAS Inc., Carry, NC, USA). Significant differences were considered at 95% confidence level by using the Tukey test.

3. Results

3.1. Determination of sub-lethal nisin concentration

In this study, the sub-lethal nisin concentration was determined as a preliminary step to obtain viable stressed cells for protein extraction and proteomic analysis. Nisin was evaluated at concentrations ranging from 0.1 to 1.0 µg/mL during the exponential growth phase. The concentration of 0.1 µg/mL caused no significant decrease of viable cells, while microbial counts were reduced by more than 2 log CFU/mL with the other concentrations tested (Fig. 1). Consequently, this concentration was selected for subsequent tests.

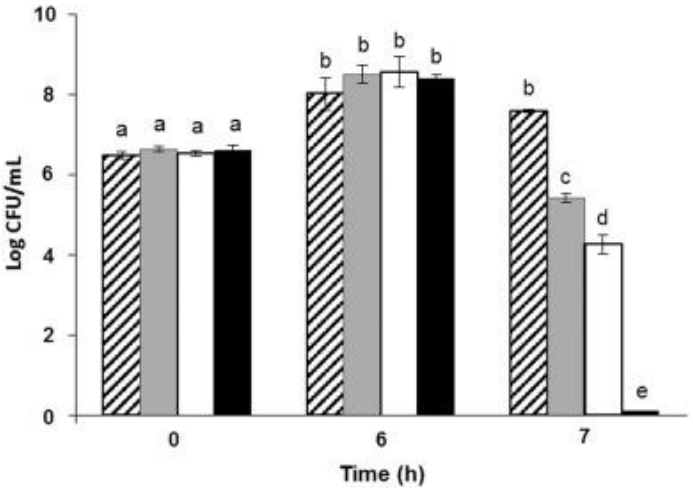

Fig.1. Definition of the sub-lethal nisin concentration in *Listeria monocytogenes* cultures. Viable cells were counted at 0, 6, and 7 h from the inoculation time, in cultures with: 0.1, 0.2, 0.3, 1 μg/mL concentrations of nisin added at 6 h. 0.1 μg/mL (dashed bars); 0.2 μg/mL (grey bars); 0.3 μg/mL (white bars); 1 μg/mL (black bars). Values are the means ± standard deviations of three independent experiments.

Proteins extracted from *L. monocytogenes* cultures were analyzed by SDS-PAGE and thereafter subjected to LC-MS/MS analysis for proteomic studies. The protein extraction protocol exhibited good yield and reproducibility, as the electrophoretic protein profiles were very similar among triplicate biological samples (Supplementary Fig. S1).

3.2. Proteomics data analysis

Mass spectrometry analysis permitted to detect a total of 847 LFQ (label free quantified) protein intensities among the control and treated samples. Before the statistical analysis, data filtering was carried out considering only the protein intensities present in at least 3 samples, which represent half of the total number of samples. Thus, only 721 LFQ protein intensities were used for proteomics data analysis. The Volcano plot permitted to identify 57 proteins with significant differences ($P \leq .05$) in their amounts (Fig. 2). In this case, the peripheral cell proteins aldehyde-alcohol dehydrogenase (*aad*), internalin B (*inlB*) and peptide ABC transporter substrate-binding protein (*lmo2196*) associated with the virulence process and the flagellar hook protein FlgE (*lmo0697*) involved in biofilm formation were comprised among the significant ones. Futher statistical analysis included the PCA and PLS-DA analysis showing the clear discrimination between control and nisin-treated samples as two separate groups (Fig. 3A, B). VIP scores were calculated and only 159 proteins resulted with VIP ≥1.0 (Supplementary Table S1). These 159 proteins were used to construct a Heatmap graph (Fig. 4A), each sample of the two triplicates were reported considering the proteins and their expression rates. In the treated samples, a total of 97 proteins were down regulated, while 62 proteins were up regulated. Minor differences were observed within the sample components

of each triplicate. Protein networks with both up and down regulated proteins were created considering the protein-protein relation and their VIP scores (Fig. 4B, C). The nodes size variation corresponds to the VIP score of each identified protein. The flagellar proteins flaA (flagelin A) and lmo0697 (flagellar hook protein FlgE), and a cluster of oxidorreductase enzymes lmo1961 (ferredoxin-NADP reductase 1), lmo2390 (ferredoxin-NADP reductase 2), lmo1371 (dihydrolipoyl dehydrogenase) and a putative amino acid transporter lmo0848 were observed in the upregulated group. The network of down regulated proteins showed clusters including enzymes associated with energy metabolism such as lmo1634 (aldehyde-alcohol dehydrogenase), lmo0355 (fumarate reductase subunit A) and pflA (pyruvate formate-lyase-activating enzyme), and ribosomal proteins rpsS, rpsP, rpsH, rpsG (30S ribosomal proteins S19, S16, S8 and S7 respectively) and infA (translation initiation factor IF-1).

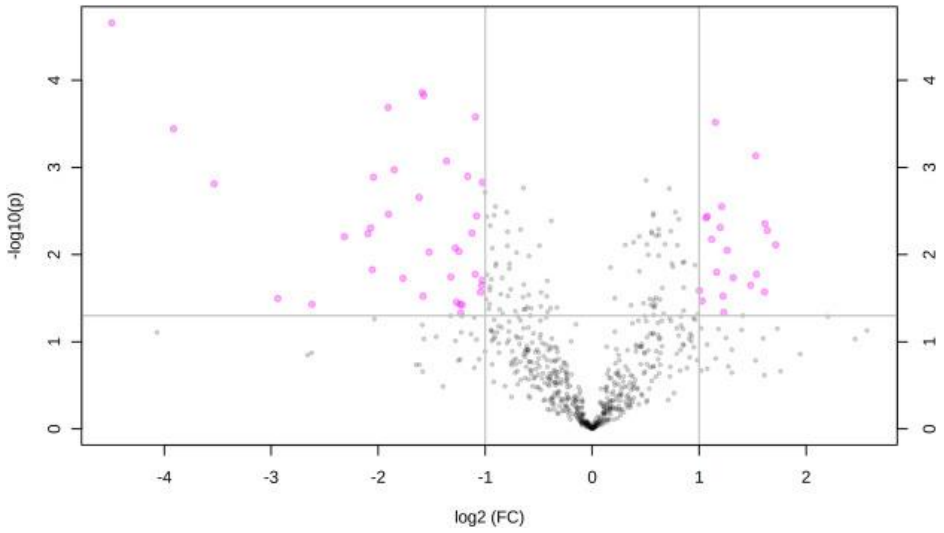

Fig. 2. Volcano plot created by the comparison Treatment/Control groups, combining the log fold change (FC) and *t*-tests expressed as -log10 (*P*-value). The horizontal line represents the adjusted *P*-value threshold ≤0.05. The vertical lines represent the fold change threshold. Proteins selected as significantly different are highlighted as pink dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

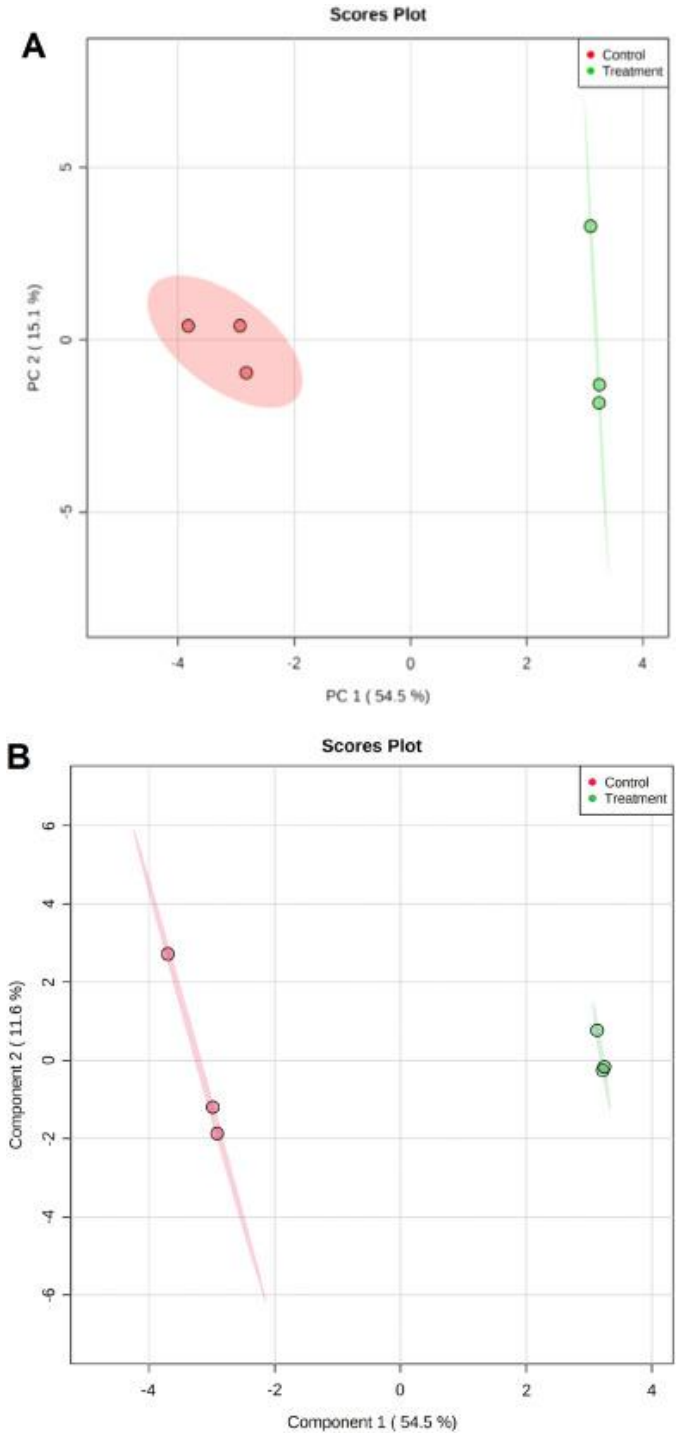

Fig. 3. (A) Principal Component Analysis (PCA) and (B) Partial Least Squares - Discriminant Analysis (PLS-DA) based on the LFQ intensities of the protein groups. 2D score plots displayed the 95% confidences regions. Red circles correspond to the negative control (absence of nisin) triplicate samples, while green circles correspond to the nisin-treated triplicate samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

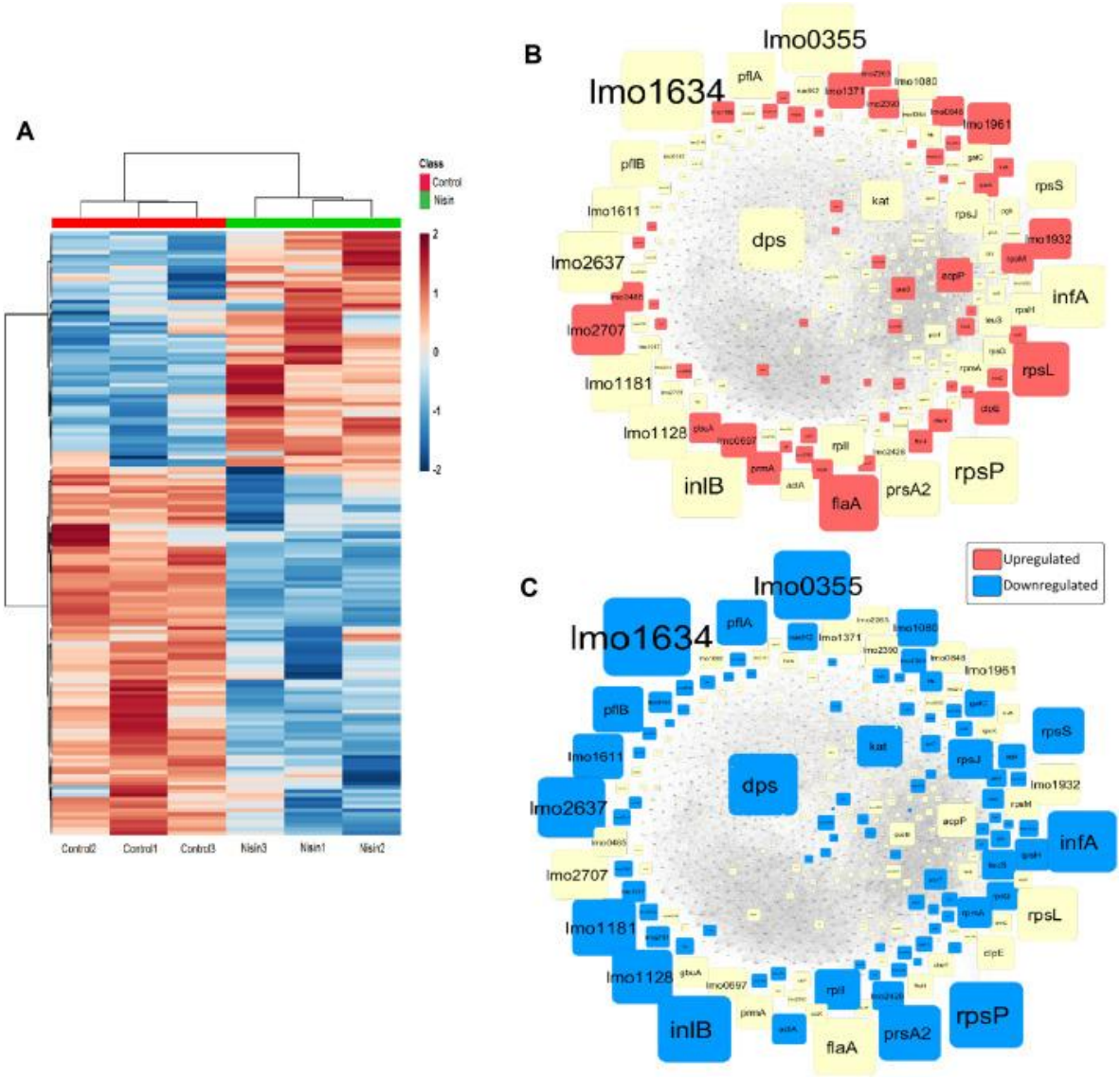

Fig. 4. Overview of identified proteins and their interactions. (A) Heatmap of the top 159 proteins identified by PLS-DA statistical analysis with VIP score ≥ 1. Samples are represented in columns, as the triplicate samples treated with sub-lethal nisin concentration (green) and triplicate control samples (red). Each colored cell on the map corresponds to a concentration value for the 159 proteins, represented in rows. The two-colour scale ranging from the lowest intensity (*z*-score of −2) in blue to the highest intensity (*z*-score of 2) in red were used. A heatmap with detailed list of respective proteins is presented as supplementary material. (B,C) Protein-protein interaction networks generated on STRING v11 database based on the identified proteins, where the upregulated proteins were highlighted in red (B) and downregulated proteins in blue (C). The higher VIP scores are depicted in these networks by a bigger node size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Surface proteins

Among the 159 selected proteins, differentially classified by the regulation rate (62 up regulated and 97 down regulated), the bacterial surface proteins were detected using PSORTb 3.0. Particularly, 19 surface proteins were allocated among the up regulated proteins, corresponding to 30.6% of the total (Supplementary Table S2). Among the proteins included into the up regulated group, 12 (19.35%) were cytoplasmic membrane (CM) proteins, 4 (6.46%) with extracellular (EX) localization, and 3 (4.84%) with not determined (ND) localization. The 18 surface proteins detected into the down regulated group correspond to 18.6% of the total down regulated proteins (Supplementary Table S3). These proteins were classified as follows: 8 (9.28%) were CM proteins, 4 (4.12%) were cell wall proteins (CW), 4 (4.12%) EX proteins, and 2 (2.15%) were proteins with ND localization.

All these protein subgroups (Cell Wall, Cytoplasmatic Membrane, Extracellular and Not Determined) were included in a bigger group named Peripheral Cellular Compartment (PCC).

3.4. Molecular functions involving PCC proteins

Protein molecular functions were investigated for the two PCC protein groups considered in this work, using CELLO2GO with *E*-value of 0.001 (Fig. 5). The main molecular functions associated with the group of up regulated PCC proteins were: hydrolase activity (17.5%), ion binding (13.9%), transmembrane transporter activity (10.4%), ATPase activity (9.6%), structural molecular activity (5.3%), oxidoreductase activity (4.4%).

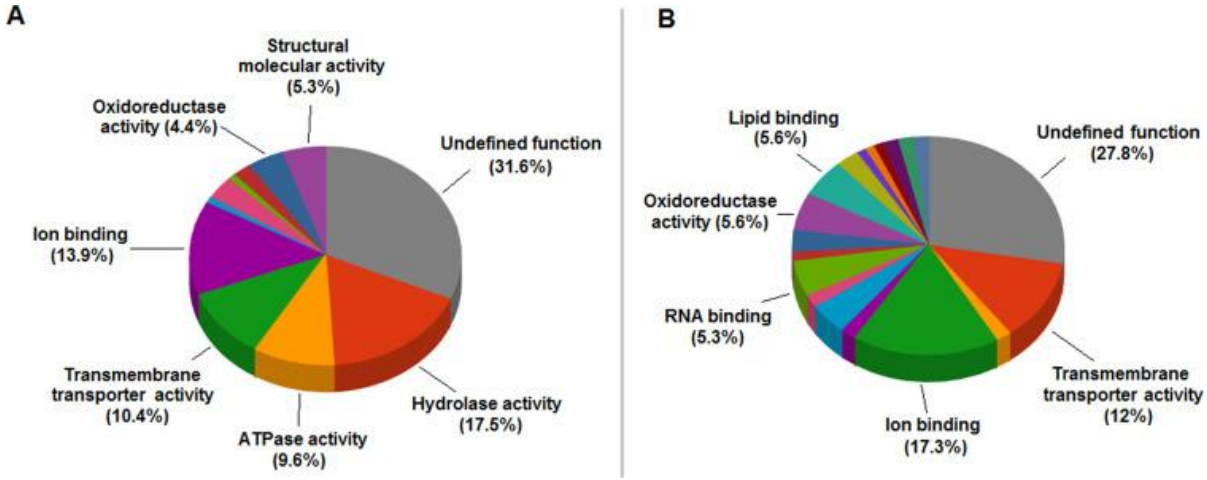

Fig. 5. Pie chart of up regulated (A) and down regulated (B) PCC proteins according to molecular functions assigned by CELLO2GO algorithm. Molecular functions assigned with smaller percentages (>5%) are not detailed.

Regarding the molecular functions related to the down regulated PCC proteins, ion binding (17.3%), transmembrane transporter activity (12%), lipid binding (5.6%), oxidoreductase activity (5.6%) and RNA binding (5.3%) were among the more represented in the group (Fig. 5).

3.5. Nisin influence on biofilm formation

A group of differentially expressed proteins identified as surface proteins by PSORTb 3.0 can be associated with biofilm formation by *L. monocytogenes* (Table 1). These include proteins of the pyruvate dehydrogenase complex, also classified as moonlight proteins, and cell motility proteins. Considering that biofilm formation may be induced by the presence of antimicrobial substances at concentrations below the minimum inhibitory concentration, the influence of sub-lethal dose of nisin (0.1 μg/mL) on biofilm formation by *L. monocytogenes* was investigated. In this assay, the OD values resulting from crystal violet method were 3.12 ± 0.63 and 1.65 ± 0.32 for nisin-treated and control cells, respectively (Fig. 6A). The values measured for nisin-treated cells were significantly higher ($P < .05$) as compared with controls, in agreement with the formation of aggregates in the bottom of microplates after incubation (Fig. 6B).

Table 1. Detected proteins involved on biofilm process promotion with the respective VIP score, PCC distribution and gene name.

Protein name[a]	VIP score	PCC[b]	Cellular process	Gene
Pyruvate dehydrogenase beta subunit	1.36	CM	Moonlight	*pdhB*
Dihydrolipoyl dehydrogenase	2.11	CM	Moonlight	*lmo1371*
Flagellar hook protein FlgE	2.08	EX	Cell motility	*lmo0697*

Protein name[a]	VIP score	PCC[b]	Cellular process	Gene
Flagellin	2.94	EX	Cell motility	*flaA*
FruA protein	1.22	CM	Transport	*fruA*

[a]All listed proteins showed upregulation, excepting FruA that was downregulated.

[b] Peripheral cell component (PCC) distribution: CM, cytoplasmatic membrane; EX, extracellular; CW, cellular wall; NA = not available.

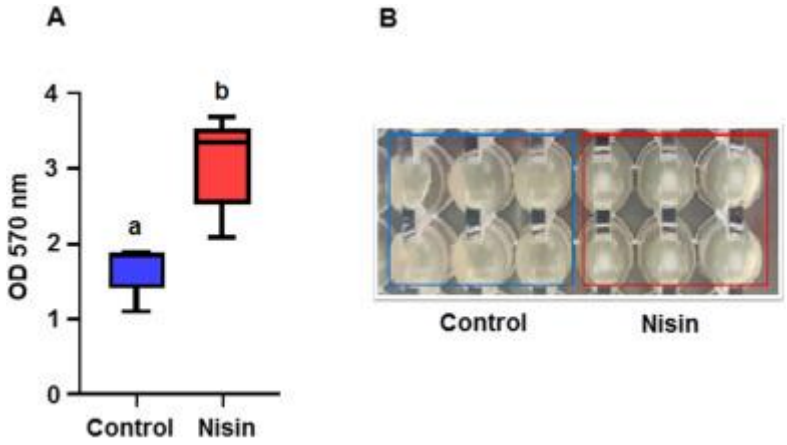

Fig. 6. (A) Boxplot of the spectrophotometer detected abs at 570 nm by both samples: negative control in blue and nisin treatment (0.1 μg/mL) in red. Different lower case letter over the plot of each treatment indicate significant differences ($P < .05$). (B) Microplate wells with unnoticed biofilm formation (control, left panel), while visible biofilm formation was observed in the nisin-treated samples (right panel), after 24 h incubation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Nisin influence on virulence-associated proteins

Among nisin-treated samples, some PCC proteins detected as down regulated were identified as involved in the virulence of *Listeria* spp. (Table 2). These proteins, encoded by the genes *inlB, prsA, llo* or *hly, actA, lmo2196, lmo0135* and *lmo1634*, contribute to the virulence process of *L. monocytogenes*. Among the identified proteins, membrane transporters that take part of the virulence process, the toxic protein listeriolysin O, and proteins involved in adhesion and invasion to host, such as internalin B and actin assembly-inducing protein. In

addition, the moonlight protein acetaldehyde-alcohol dehydrogenase, also recognized as *Listeria* adhesion protein (LAP), was identified at down regulated rate in nisin-treated cells.

Table 2. Detected proteins involved on reduction of virulence with the respectively VIP score, PCC distribution and gene name.

Protein name [a]	VIP score	PCC [b]	Cellular process	Gene
Foldase protein PrsA 2	2.90	CM	Folding	*prsA2*
Actin assembly-inducing protein	1.93	CM	Virulence	*actA*
Listeriolysin O	1.36	EX	Virulence	*hly*
Internalin B	4.32	CW	Virulence	*inlB*
Peptide ABC transporter substrate-binding protein	1.34	CW	Transporter	*lmo2196*
ABC transporter substrate-binding	1.19	CW	Transporter	*lmo0135*
Aldehyde-alcohol dehydrogenase	6.82	NA	Moonlight	*aad*

[a] All listed proteins showed downregulation.

[b] Peripheral cell component (PCC) distribution: CM, cytoplasmatic membrane; EX, extracellular; CW, cellular wall; NA = not available.

4. Discussion

The results obtained from proteosurfaceome and bioinformatics analysis of the identified proteins, highlight the propensity of *L. monocytogenes* cells, in the applied condition of antimicrobial stress, to produce biofilm and reduction of virulence.

The pre-treatment of surfaces with bacteriocins was suggested as a realistic way to prevent pathogenic biofilm settlement and it has been seen as a safe and environmentally-friendly antimicrobial method to mitigate post-processing food contamination [33].

In *L. monocytogenes* as well as in other bacteria, biofilms are complex systems, and the bulk

biofilm includes active bacteria, inert biomass and extracellular polymeric substances (EPS) [34]. The EPS secreted by *L. monocytogenes* includes proteins, polysaccharides, and extracellular DNA (eDNA), being proteins present in large quantities among the EPS components [35]. Abiotic factors such as growth substrates and temperature [35] together with strain-specific features, serotype and genotype impact on *L. monocytogenes* EPS production [36]. As the cells remain attached and protected by the EPS layer, sessile bacteria can resist to disinfection procedures increasing the risks of contamination. In this work, samples treated with sub-lethal concentration of nisin have shown a tendency to form biofilms and present a pool of PCC proteins with differential expression that may be associated with biofilm assembly. Among the proteins possibly involved in biofilm formation, two moonlight proteins were identified through the Moonprot 2.0 software. Metabolic proteins can be inferred contributing on the build of the bacterial biofilm. Some of the identified PCC proteins of the up regulated group have an important role in the pyruvate dehydrogenase complex (PDC) that occupies a key position in the oxidation of glucose by linking the glycolytic pathway to the oxidative pathway of the Krebs cycle, with irreversible oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2.

The moonlight protein pyruvate dehydrogenase beta subunit (PdhB) has been already detected on the bacterial surface of some bacteria, as a fibrinogen binding protein. Its fibrinogen-binding activity can promote strong interactions with fibrinogen promoting bacterial aggregation and biofilm formation as well as inhibiting neutrophil phagocytosis [37]. In fact a previous work demonstrated the action of moonlight proteins with PCC involvement, important role was represented for the biofilm formation as bacterial protection against harsh environments [38], which could be represented by the sub-lethal nisin concentration present in the culture medium. Dihydrolipoyl dehydrogenase (DLD) is another of the three main component of the PDC. This protein has been already identified as one of

the components with highest expression rate during biofilm formation [39,40], however based in our bibliographic research the elucidation of DLD contribution in biofilm growth is not clear yet. These proteins related with energy production, under stress factors (subjected to chemical/antimicrobial stressors) as the sub-lethal nisin activity, work as resistance mechanism as were reported in different studies considering different microorganisms, including *L. monocytogenes* [41], *Vibrio parahaemolyticus* [42], tellurite-resistant *E. coli* [43], and toluene-resistant *Pseudomonas putida* [44].

Flagellin (FlaA) was previously detected among the most up regulated proteins of *L. monocytogenes* treated with 1 μg/mL nisin, with a VIP score > 3 [45]. Besides the FlaA protein, another up regulated protein in the present work was the FlgE protein. Both these flagella components are described as biofilm-associated proteins. FlaA protein was also up regulated in *L. monocytogenes* exposed to ethanol [41]. Probably bacterial cells use this strategy to obstruct bacteriocin activity by forming a bacterial aggregate that limits the exposed area to the sub-lethal nisin concentration [46].

Moreover the down regulated fructosidase (FruA) enzyme has been demonstrated to be involved on inhibition of biofilm formation [47] performing the hydrolysis reaction from fructan to fructose but also act as sucrose hydrolyse activity.

L. monocytogenes strains are able to form biofilms on different surfaces including stainless steel, aluminium, rubber, polypropylene, among others [6,48,49]. Recent studies shown that the application of antimicrobial substances at concentrations below the minimum inhibitory concentration may induce biofilm development by some bacteria [50]. In this work, the exposition of *L. monocytogenes* to sub-lethal dose of nisin confirmed the increased biofilm formation and regulation of proteins involved in this phenomenon. Induction of biofilm formation by *L. monocytogenes* has already been reported when exposed to sub-lethal doses of other antimicrobial substances [51,52]. In addition, it was suggested that sub-lethal doses

may cause changes in bacterial cell surface that increase their adhesion capability [50]. The nisin concentrations used in food products has been exhaustively reviewed, and nisin amounts used in some food systems such as skim milk (≤ 0.5 μg/mL), cheese slurry (1 μg/mL), homogenized whole milk (≤ 12.5 μg/mL) and meat emulsions (0.25 to 2.5 μg/mL) may result in incomplete inhibition of target bacteria [53]. In this context, the exposition to sub-minimal inhibitory concentration of nisin in skim milk was already correlated with the fast onset of resistance in *Streptococcus thermophilus* [54]. Although the sub-lethal concentration would be strain-specific, the exposure of *L. monocytogenes* to lower nisin concentrations might stimulate biofilm synthesis together with other resistance strategies. The findings of this study indicate that exposure to sub-lethal concentrations of nisin can stimulate biofilm formation by *L. monocytogenes*, which could be a serious hazard since different studies demonstrated that this ecosystem provides high tolerance to antimicrobial compounds [55,56].

Furthermore, some down regulated PCC proteins identified in nisin-treated samples are associated with the virulence process of *Listeria*. Two important virulence factors of *L. monocytogenes*, namely listeriolysin O (Hly or LLO) and the foldase protein PrsA, were both down regulated in the nisin-treated samples [57]. PrsA is a membrane-bound foldase, expressed ubiquitously in Gram-positive bacteria and required for the folding, a crucial protein organization step to achieve the correct conformation and become active. Thus, many surface and secreted proteins that are potentially folded by PrsA may be influenced by its down regulation, indicating that PrsA may indirectly affect the bacterial pathogenesis [58].

After entry in the host cells, listeriolysin O (LLO) help the bacteria to escape from cytosolic vacuoles for finally start the replication process. Thus, reduced concentration of LLO will compromise the early virulence process into the host cells. A recent study showed a decrease in LLO levels when *L. monocytogenes* was exposed to the ethanol extract of *Eucalyptus*

camaldulensis leaf at sub-lethal concentration, although the reason for this bacterial response is still unknown [59]. In addition, the surface protein iternalin B (InlB) was identified among the down-regulated proteins with the highest VIP-score recorded in this work. This protein has an essential role in the virulence process, with the main function related with bacterial adherence to the eukaryotic cells [60]. The obtained data also revealed a down regulation of the motility-inducing surface actin protein (actA), another protein involved in the *Listeria* virulence inside the host cells facilitating cell to cell spread [61].

Some membrane oligopeptide substrate-binding proteins participating in the virulence process of *L. monocytogenes* were also down regulated. The oligopeptide OppA transport system (gene *lmo2196*) is required for bacterial growth at low temperatures and intracellular survival, helping the bacteria to escape from macrophages at the early stages of infection [62]. The second substrate-binding component of oligopeptide transport system is the cysteine transport associated protein CtaP (gene *lmo0135*), which is involved in bacterial adherence to the host cell [63]. Finally, another metabolic protein acting on cellular surface is aldehyde-alcohol dehydrogenase (*lmo1634*). Although PSORTb 3.0 did not detect this protein among the PCC proteins, a deeper investigation within the down regulated protein group using Moonprot and Uniprot permitted to identify this bi-functional protein acetaldehyde-CoA/alcohol dehydrogenase. In a previous study, it was demonstrated the fundamental role in *L. monocytogenes* virulence by interacting with the host cell Hsp60 and promoting the bacterial adhesion to human intestinal cells [64].

Therefore, through the differential expression of PCC proteins identified in this study, it could be hypothesized that the treatment with sub-lethal concentration of nisin may weaken the virulence process of *L. monocytogenes*. The downregulation of proteins reported in Table 2 could be a potential target for new antimicrobial compounds to be used synergistically or in place of nisin. Virulence of *Listeria* strains can be under different genetic regulation

depending on the food matrix or other environments [65]. An overlapping of the stress response and virulence may be affirmed in *L. monocytogenes*, otherwise impossible may be the bacterial life in the gastrointestinal tract, probably higher virulence help the bacteria to survive these stressful conditions [66]. Four transcriptional regulators were found as controlling *L. monocytogenes* virulence: PrfA, σ^B, CodY, and VirR [67]. The stressful condition represented by the sub-lethal concentration of nisin probably act at least on one of these regulators, explaining the decrease of diverse virulence factors found in this work. Further studies on PrsA foldase would be important because it could influence folding, and therefore function, of many peripheral proteins.

5. Conclusion

In this work, peripheral proteins (PCC) of *L. monocytogenes* were investigated with focus on the regulation of protein expression in response to sub-lethal concentration of nisin. Considering that *Listeria* resistance have been detected in some food isolated strains, the results are relevant to provide a better understanding on the bacterial response to lantibiotic peptides, with the perspective of identifying bacterial proteins associated with mechanisms of resistance or tolerance. Statistical and bioinformatics analysis provided the possibility to identify a group of PCC proteins allocated among the 159 proteins detected with significant difference between treatment and control samples. Proteins associated with increasing biofilm formation and reduction of virulence are among the most up regulated and down regulated proteins, respectively. Although different works highlighted biofilm formation when bacteria were stressed by antimicrobials, the mechanisms involved in biofilm induction by sub-lethal concentration of nisin or other antimicrobials have not yet been fully established. The proteomics results may provide a new light in this direction also considering the potential contribution of moonlight proteins in this context.

Declaration of Competing Interest

None to declare.

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Appendix A. Supplementary data

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Supplementary material

Nisin influence on the expression of Listeria monocytogenes surface proteins Paolo Stincone, Kendi Nishino Miyamoto, Palmira Penina Raúl Timbe, Isadora Lieske, Adriano Brandelli

Supplementary Figure S1

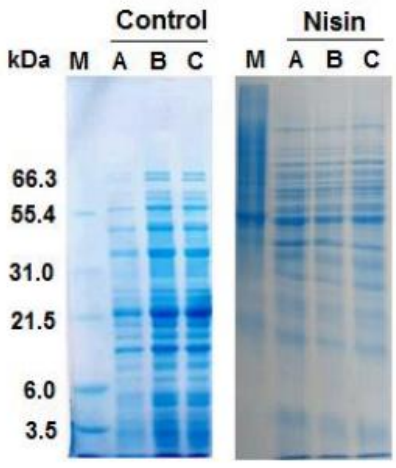

Figure S1. SDS-PAGE analysis of triplicate biological samples of surface proteins of control and nisin-treated cells. Lanes A, B, C correspond to samples loaded at 40 μg/lane and stained with Commassie blue G-250; M = molecular weight marker.

Table S1. PLS-DA values used to identify differentially expressed proteins related to the exposure of *L. monocytogenes* to nisin with the VIP threshold > 1.0 in the first component of PLS-DA.

Protein Group	**Comp. 1**	**Comp. 2**	**Comp. 3**
trQ8Y6Q0Q8Y6Q0_LISMO Aldehyde-alcohol dehydrogenase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1634 PE3 SV1	6.8204	6.7999	6.7997
trQ8YA11Q8YA11_LISMO Lmo0355 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0355 PE4 SV1	4.8485	4.8359	4.8358
trQ92CE7Q92CE7_LISMO Lmo1257 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1257 PE4 SV1	4.7675	4.7531	4.7529
spQ8Y699RS16_LISMO 30S ribosomal protein S16 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsP PE3 SV1	4.3218	4.3094	4.3093
spP0DQD2INLB_LISMO Internalin B OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNinlB PE1 SV1	4.3205	4.308	4.3078
spQ7AP54HBP2_LISMO Hemin/hemoglobin-binding protein 2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNhbp2 PE1 SV1	3.6735	3.6683	3.6682
spQ8Y8G1DPS_LISMO DNA protection during starvation protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNdps PE1 SV1	3.5115	3.5024	3.5023
spP65110IF1_LISMO Translation initiation factor IF-1 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNinfA PE3 SV1	3.444	3.4367	3.4367
trQ8Y436Q8Y436_LISMO Lmo2637 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2637 PE4 SV1	3.1738	3.1655	3.1654
trQ8Y7U0Q8Y7U0_LISMO Lmo1181 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1181 PE4 SV1	2.9848	2.9838	2.9837
spQ02551FLAA_LISMO Flagellin OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNflaA PE3 SV2	2.9442	2.9357	2.9356

spQ8Y557PRSA2_LISMO Foldase protein PrsA 2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNprsA2 PE3 SV1	2.9037	2.9017	2.9016
trQ8Y7Z1Q8Y7Z1_LISMO Lmo1128 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1128 PE4 SV1	2.8647	2.8565	2.8565
spP66372RS12_LISMO 30S ribosomal protein S12 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsL PE3 SV1	2.8632	2.8555	2.8555
trQ8Y3X2Q8Y3X2_LISMO Lmo2707 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2707 PE4 SV1	2.7146	2.7118	2.7117
spP66484RS19_LISMO 30S ribosomal protein S19 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsS PE3 SV1	2.5526	2.5448	2.5448
trQ8Y786Q8Y786_LISMO Pyruvate formate-lyase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpflB PE4 SV1	2.5256	2.5182	2.5181
spP0A442PFLA_LISMO Pyruvate formate-lyase-activating enzyme OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpflA PE3 SV1	2.5178	2.5103	2.5102
trQ8Y6S2Q8Y6S2_LISMO Lmo1611 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1611 PE3 SV1	2.5121	2.5045	2.5044
spQ8Y5U4FENR1_LISMO Ferredoxin--NADP reductase 1 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1961 PE3 SV1	2.374	2.3703	2.3703
spQ8Y3P9CATA_LISMO Catalase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNkat PE3 SV1	2.3365	2.3298	2.3297
spQ8YAR2RL9_LISMO 50S ribosomal protein L9 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrplI PE3 SV1	2.3365	2.3296	2.3295
trQ8Y5X2Q8Y5X2_LISMO Lmo1932 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1932 PE4 SV1	2.3189	2.3644	2.3644
spP66330RS10_LISMO 30S ribosomal protein S10 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsJ PE3 SV1	2.296	2.2904	2.2903

trQ8Y838Q8Y838_LISMO Lmo1080 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1080 PE4 SV1	2.1879	2.1817	2.1816
trQ8Y7B5Q8Y7B5_LISMO Dihydrolipoyl dehydrogenase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1371 PE3 SV1	2.1069	2.1005	2.1005
trQ92DV7Q92DV7_LISMO Flagellar hook protein FlgE OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0697 PE3 SV1	2.0799	2.075	2.0749
spP63439ACP_LISMO Acyl carrier protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNacpP PE3 SV1	2.0664	2.0848	2.085
trQ8Y8B1Q8Y8B1_LISMO ATP-dependent protease OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNclpE PE3 SV1	2.0488	2.0425	2.0425
spP0DJO9PRMA_LISMO Ribosomal protein L11 methyltransferase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNprmA PE3 SV1	2.0394	2.0385	2.0384
spP66623RS8_LISMO 30S ribosomal protein S8 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsH PE3 SV1	1.9853	1.9794	1.9793
spP66125RL27_LISMO 50S ribosomal protein L27 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpmA PE3 SV1	1.9448	1.9392	1.9392
spP33379ACTA_LISMO Actin assembly-inducing protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNactA PE1 SV1	1.9313	1.9383	1.9383
spP66383RS13_LISMO 30S ribosomal protein S13 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsM PE3 SV1	1.9178	1.9135	1.9134
trQ7AP76Q7AP76_LISMO GbuA protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNgbuA PE4 SV1	1.9178	1.917	1.917
spQ8Y6M4SYL_LISMO Leucine--tRNA ligase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNleuS PE3 SV1	1.9178	1.9123	1.9123
trQ8Y8P8Q8Y8P8_LISMO Lmo0848 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0848 PE4 SV1	1.9043	1.8996	1.8995

trQ8Y9P0Q8Y9P0_LISMO Lmo0485 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0485 PE4 SV1	1.9043	1.8985	1.8985
spQ8Y4P5FENR2_LISMO Ferredoxin--NADP reductase 2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2390 PE3 SV1	1.8908	1.8854	1.8853
trQ8Y4L1Q8Y4L1_LISMO Lmo2426 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2426 PE3 SV1	1.8638	1.8611	1.8611
spP66611RS7_LISMO 30S ribosomal protein S7 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsG PE3 SV1	1.8368	1.8332	1.8332
trQ8Y8T3Q8Y8T3_LISMO Lmo0811 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0811 PE4 SV1	1.8368	1.8312	1.8311
trQ8Y515Q8Y515_LISMO Lmo2263 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2263 PE4 SV1	1.799	1.7937	1.7937
spP65770NADK2_LISMO NAD kinase 2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNnadK2 PE3 SV1	1.7963	1.7955	1.7954
spP58817GATC_LISMO Aspartyl/glutamyl-tRNAAsn/Gln amidotransferase subunit C OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNgatC PE3 SV1	1.7828	1.7796	1.7796
trQ8Y8V1Q8Y8V1_LISMO Lmo0791 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0791 PE4 SV1	1.7017	1.6969	1.6968
spQ8Y4I2PGK_LISMO Phosphoglycerate kinase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpgk PE3 SV1	1.6882	1.6834	1.6834
spP66352RS11_LISMO 30S ribosomal protein S11 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsK PE3 SV1	1.6612	1.659	1.659
trQ8Y7C9Q8Y7C9_LISMO Lmo1354 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1354 PE3 SV1	1.6342	1.6293	1.6292
trQ8YAC6Q8YAC6_LISMO ATP-dependent zinc metalloprotease FtsH OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNftsH PE3 SV1	1.6342	1.6302	1.6301

trQ926Y9Q926Y9_LISMO Inosine-5-monophosphate dehydrogenase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNguaB PE1 SV1	1.6342	1.63	1.63
spP0A4H5CHEY_LISMO Chemotaxis protein CheY OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNcheY PE3 SV1	1.6207	1.6286	1.6286
trQ8Y767Q8Y767_LISMO Ribonuclease J OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrnj PE3 SV1	1.6004	1.5985	1.5985
trQ8Y6J2Q8Y6J2_LISMO Lmo1692 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1692 PE4 SV1	1.5802	1.5757	1.5757
trQ8Y3Q3Q8Y3Q3_LISMO Lmo2781 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2781 PE4 SV1	1.5667	1.5693	1.5694
trQ8Y897Q8Y897_LISMO Lmo1017 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1017 PE4 SV1	1.5532	1.5499	1.5499
trQ8Y9N8Q8Y9N8_LISMO Lmo0487 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0487 PE4 SV1	1.5532	1.552	1.5521
spQ8Y5G1PDXT_LISMO Pyridoxal 5-phosphate synthase subunit PdxT OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpdxT PE3 SV1	1.5396	1.5369	1.5368
trQ8YAV6Q8YAV6_LISMO DNA gyrase subunit A OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNgyrA PE3 SV1	1.5146	1.5145	1.5144
trQ8Y695Q8Y695_LISMO Signal recognition particle protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNffh PE3 SV1	1.5126	1.5104	1.5104
spQ8Y7K9CLPX_LISMO ATP-dependent Clp protease ATP-binding subunit ClpX OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNclpX PE3 SV1	1.5126	1.5081	1.5081
spQ927L2RL22_LISMO 50S ribosomal protein L22 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrplV PE3 SV1	1.4991	1.4948	1.4948
spO32823TRXB_LISMO Thioredoxin reductase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNtrxB PE3 SV1	1.4721	1.4677	1.4676

trQ8Y3K6Q8Y3K6_LISMO Lmo2829 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2829 PE4 SV1	1.4721	1.4689	1.4689
spQ8Y6Y6MINC_LISMO Probable septum site-determining protein MinC OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNminC PE3 SV1	1.4721	1.4694	1.4694
trQ8Y7I0Q8Y7I0_LISMO Glutamine synthetase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNglnA PE3 SV	1.4181	1.4137	1.4137
spQ8Y444RL6_LISMO 50S ribosomal protein L6 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrplF PE3 SV1	1.4046	1.4023	1.4023
spQ8Y708SYH_LISMO Histidine-tRNA ligase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNhisS PE3 SV1	1.4046	1.4006	1.4006
spQ92C24RS15_LISMO 30S ribosomal protein S15 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsO PE3 SV1	1.4046	1.4004	1.4003
spQ8Y4C1ATPB2_LISMO ATP synthase subunit beta 2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNatpD2 PE3 SV1	1.3911	1.3872	1.3872
trQ8Y7F8Q8Y7F8_LISMO Lmo1323 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1323 PE4 SV1	1.3911	1.3885	1.3885
trQ8Y463Q8Y463_LISMO Lmo2592 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2592 PE4 SV1	1.3785	1.382	1.3819
trQ8Y970Q8Y970_LISMO Lmo0663 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0663 PE4 SV1	1.3776	1.3735	1.3734
trQ8Y9H5Q8Y9H5_LISMO Lmo0553 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0553 PE4 SV1	1.3776	1.4125	1.4126
spP13128TACY_LISMO Listeriolysin O OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNhly PE1 SV1	1.3641	1.3606	1.3606
trQ8Y864Q8Y864_LISMO PdhB protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNPdhB PE4 SV1	1.3641	1.3599	1.3599

trQ8Y8P0Q8Y8P0_LISMO UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNmurF PE3 SV1	1.3641	1.3627	1.3626
spQ8YAE0YDJC_LISMO Carbohydrate deacetylase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0191 PE3 SV1	1.3641	1.3601	1.3601
spQ9RQI6CLPP_LISMO ATP-dependent Clp protease proteolytic subunit OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNclpP PE1 SV1	1.3506	1.3487	1.3487
trQ7AP52Q7AP52_LISMO Lmo2196 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2196 PE4 SV1	1.3371	1.333	1.3329
trQ8YA01Q8YA01_LISMO Lmo0366 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0366 PE4 SV1	1.3371	1.336	1.336
trQ8Y8W8Q8Y8W8_LISMO Lmo0774 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0774 PE4 SV1	1.3236	1.3204	1.3204
spQ8YAB1SYC_LISMO Cysteine--tRNA ligase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNcysS PE3 SV1	1.3236	1.3195	1.3195
spQ927L6RS17_LISMO 30S ribosomal protein S17 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpsQ PE3 SV1	1.3236	1.3322	1.3322
trQ8Y419Q8Y419_LISMO Lmo2658 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2658 PE4 SV1	1.3168	1.314	1.314
spP66207RL322_LISMO 50S ribosomal protein L32-2 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpmF2 PE3 SV1	1.3101	1.308	1.3081
trQ8Y559Q8Y559_LISMO Lmo2217 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2217 PE4 SV1	1.3101	1.3068	1.3068
trQ929L9Q929L9_LISMO Lmo2152 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2152 PE4 SV1	1.3101	1.3131	1.3131
trQ8Y741Q8Y741_LISMO Lmo1481 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1481 PE4 SV1	1.3006	1.297	1.297

trQ927Y2Q927Y2_LISMO Ribosome hibernation promoting factor OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNhpf PE1 SV1	1.283	1.2824	1.2824
trQ8Y5S6Q8Y5S6_LISMO Lmo1979 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1979 PE4 SV1	1.2784	1.2794	1.2793
trQ8Y5M6Q8Y5M6_LISMO Pyridoxal phosphate homeostasis protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2031 PE3 SV1	1.2746	1.2724	1.2724
trQ8Y3P3Q8Y3P3_LISMO Lmo2792 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2792 PE4 SV1	1.2695	1.2671	1.267
trQ8Y435Q8Y435_LISMO Lmo2638 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2638 PE4 SV1	1.2695	1.2661	1.266
trQ92D14Q92D14_LISMO Lmo1008 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1008 PE4 SV1	1.2695	1.2854	1.286
spQ8Y6Z5Y1535_LISMO Probable transcriptional regulatory protein lmo1535 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1535 PE3 SV1	1.2689	1.2656	1.2656
spP33380LDH1_LISMO L-lactate dehydrogenase 1 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNldh1 PE3 SV2	1.256	1.2533	1.2532
trQ92AP5Q92AP5_LISMO Lmo1763 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1763 PE4 SV1	1.256	1.2522	1.2522
trQ8Y6W1Q8Y6W1_LISMO Pyruvate kinase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpykA PE3 SV1	1.2425	1.2388	1.2388
spQ8Y7N5IXTPA_LISMO dITP/XTP pyrophosphatase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1239 PE3 SV1	1.2425	1.2388	1.2388
spQ8Y7Q1SYFB_LISMO Phenylalanine--tRNA ligase beta subunit OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpheT PE3 SV1	1.2425	1.2395	1.2394
trQ8Y6G5Q8Y6G5_LISMO Lmo1722 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1722 PE4 SV1	1.2375	1.2397	1.2398

spP0A357CSPB_LISMO Cold shock-like protein CspLB OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNcspLB PE3 SV1	1.2155	1.2118	1.2119
trQ8Y4U6Q8Y4U6_LISMO FruA protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNfruA PE4 SV1	1.2155	1.2121	1.2121
trQ8Y9Y0Q8Y9Y0_LISMO Lmo0387 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0387 PE4 SV1	1.2155	1.2119	1.2119
spP0A355CSPA_LISMO Cold shock-like protein CspLA OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNcspLA PE1 SV1	1.202	1.2037	1.204
spP67234Y1796_LISMO UPF0109 protein lmo1796 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1796 PE3 SV1	1.202	1.2007	1.2006
spP0A3L1IF3_LISMO Translation initiation factor IF-3 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNinfC PE3 SV1	1.1885	1.1859	1.1859
trQ8Y4E0Q8Y4E0_LISMO Cell division ATP-binding protein FtsE OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNftsE PE3 SV1	1.1885	1.1849	1.1849
spQ8Y5M7SEPF_LISMO Cell division protein SepF OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNsepF PE3 SV1	1.1885	1.185	1.185
spQ8Y641MSRB_LISMO Peptide methionine sulfoxide reductase MsrB OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNmsrB PE3 SV1	1.1885	1.1858	1.1858
trQ8YAJ0Q8YAJ0_LISMO Lmo0135 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0135 PE1 SV1	1.1885	1.1923	1.1923
spQ927L7RL14_LISMO 50S ribosomal protein L14 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrplN PE3 SV1	1.1885	1.1855	1.1854
trQ7AP53Q7AP53_LISMO Lmo2193 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2193 PE3 SV1	1.175	1.1723	1.1723
spQ8Y6U0THII_LISMO Probable tRNA sulfurtransferase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNthiI PE3 SV1	1.175	1.1719	1.172

trQ8YAB9Q8YAB9_LISMO tRNA-dihydrouridine synthase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0227 PE3 SV1	1.175	1.1722	1.1722
spP0A438PTHP_LISMO Phosphocarrier protein HPr OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNptsH PE3 SV1	1.1615	1.1579	1.1579
trQ8Y3P4Q8Y3P4_LISMO Partition protein ParB homolg OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNparB PE3 SV1	1.1615	1.1586	1.1586
trQ8Y7N4Q8Y7N4_LISMO Phosphoesterase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1240 PE3 SV1	1.1615	1.1594	1.1594
spQ927L9RL5_LISMO 50S ribosomal protein L5 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrplE PE3 SV1	1.1615	1.1593	1.1592
trQ8Y4R2Q8Y4R2_LISMO Lmo2372 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2372 PE4 SV1	1.148	1.1445	1.1445
trQ92AT9Q92AT9_LISMO Lmo1719 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1719 PE4 SV1	1.148	1.1446	1.1446
spQ8Y420DGTL2_LISMO Deoxyguanosinetriphosphate triphosphohydrolase-like protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2657 PE3 SV1	1.1412	1.1395	1.1394
spP0A485RL31B_LISMO 50S ribosomal protein L31 type B OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpmE2 PE3 SV1	1.1345	1.1318	1.1318
spQ8Y3N3RSMG_LISMO Ribosomal RNA small subunit methyltransferase G OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrsmG PE3 SV1	1.1345	1.1323	1.1323
trQ8Y9Z7Q8Y9Z7_LISMO Lmo0370 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0370 PE4 SV1	1.125	1.1216	1.1215
trQ8Y681Q8Y681_LISMO Ribulose-phosphate 3-epimerase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1818 PE3 SV1	1.121	1.1255	1.1255
trQ8Y6T3Q8Y6T3_LISMO Catabolite control protein A OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNccpA PE4 SV1	1.121	1.1183	1.1182

spQ927M5RL30_LISMO 50S ribosomal protein L30 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpmD PE3 SV1	1.121	1.1204	1.1204
spP0DJM3GRPE_LISMO Protein GrpE OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNgrpE PE3 SV1	1.1075	1.1043	1.1043
trQ8Y4I7Q8Y4I7_LISMO Lmo2452 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2452 PE4 SV1	1.1075	1.106	1.106
spQ8Y601PANB_LISMO 3-methyl-2-oxobutanoate hydroxymethyltransferase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpanB PE3 SV1	1.1075	1.1057	1.1056
spQ8Y8A0DAPEL_LISMO N-acetyldiaminopimelate deacetylase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1012 PE3 SV1	1.1075	1.1079	1.1079
trQ8Y8C6Q8Y8C6_LISMO Lmo0982 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0982 PE3 SV1	1.1075	1.1041	1.1041
spP65927PYRH_LISMO Uridylate kinase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpyrH PE3 SV1	1.094	1.0933	1.0933
trQ7AP59Q7AP59_LISMO Lmo1601 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1601 PE4 SV1	1.094	1.0927	1.0927
trQ8Y6D1Q8Y6D1_LISMO Lmo1757 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1757 PE1 SV1	1.094	1.0931	1.0931
trQ8Y834Q8Y834_LISMO dTDP-4-dehydrorhamnose reductase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1084 PE3 SV1	1.094	1.0956	1.0956
trQ8YA10Q8YA10_LISMO Lmo0356 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0356 PE4 SV1	1.094	1.0917	1.0916
trQ8YAM0Q8YAM0_LISMO Lmo0098 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0098 PE4 SV1	1.094	1.091	1.0911
spQ8YAD1PTH_LISMO Peptidyl-tRNA hydrolase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpth PE3 SV1	1.091	1.0889	1.0889

spQ8Y553Y2223_LISMO UPF0342 protein lmo2223 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2223 PE3 SV1	1.0805	1.0797	1.0797
trQ8Y6L6Q8Y6L6_LISMO Lmo1668 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1668 PE4 SV1	1.0805	1.0772	1.0772
trQ8Y749Q8Y749_LISMO Cytidine deaminase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1463 PE3 SV1	1.0805	1.0808	1.0808
spP66166RL29_LISMO 50S ribosomal protein L29 OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNrpmC PE3 SV1	1.0669	1.0648	1.0647
trQ8Y5V3Q8Y5V3_LISMO Diaminopimelate decarboxylase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlysA PE3 SV1	1.0602	1.0613	1.0615
trQ8Y5M5Q8Y5M5_LISMO Cell division protein FtsZ OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNftsZ PE3 SV1	1.0534	1.0503	1.0503
trQ8Y8D9Q8Y8D9_LISMO Lmo0965 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0965 PE4 SV1	1.0534	1.051	1.051
spQ8Y640MSRA_LISMO Peptide methionine sulfoxide reductase MsrA OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNmsrA PE3 SV1	1.0419	1.0552	1.0552
trQ8Y3Y7Q8Y3Y7_LISMO Lmo2692 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2692 PE1 SV1	1.0399	1.0384	1.0384
trQ8Y581Q8Y581_LISMO Lmo2192 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo2192 PE3 SV1	1.0399	1.0389	1.0389
trQ8Y5Z5Q8Y5Z5_LISMO Lmo1908 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1908 PE4 SV1	1.0399	1.038	1.0381
spQ8Y495PYRG_LISMO CTP synthase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNpyrG PE3 SV1	1.0264	1.0235	1.0235
spQ8Y571GPMA_LISMO 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNgpmA PE3 SV1	1.0264	1.0241	1.0241

trQ8Y8K3Q8Y8K3_LISMO Lmo0898 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0898 PE4 SV1	1.0264	1.0233	1.0233
trQ8Y6S4Q8Y6S4_LISMO Lmo1609 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo1609 PE4 SV1	1.0129	1.015	1.0149
spQ8Y7A7ACYP_LISMO Acylphosphatase OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNacyP PE3 SV1	1.0129	1.0129	1.0129
spQ8Y8N0CSHA_LISMO ATP-dependent RNA helicase CshA OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNcshA PE2 SV1	1.0129	1.0102	1.0102
trQ8Y937Q8Y937_LISMO Lmo0704 protein OSListeria monocytogenes serovar 1/2a strain ATCC BAA-679 / EGD-e OX169963 GNlmo0704 PE4 SV1	1.0129	1.01	1.01

Supplementary Table S2

Table S2. Surface proteins upregulated: protein name, VIP score, cellular component, biological and functional process involved and gene name.

Protein name	VIP score	Cellular component	Biological process	Molecular function	Gene
GbuA protein	1.92	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity	*gbuA*
Nitroreductase	1.47	CM	N/A	N/A	*lmo2829*
ATP-binding cassette domain-containing protein	1.04	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity	*lmo2192*
ATP-dependent zinc metalloprotease	1.63	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Peptidase activity, hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, protein binding, small conjugating protein binding	*ftsH*
Cell division ATP-binding protein	1.19	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity, protein transporter activity	*ftsE*
ATP synthase subunit beta 2	1.39	CM	Transport, biosynthetic process, cellular nitrogen compound metabolic process, transmembrane	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding,	*atpD*

			transport, small molecule metabolic process.	transmembrane transporter activity	
ABC transporter ATP-binding protein	1.18	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity	*lmo2193*
Pyruvate dehydrogenase beta subunit	1.36	CM	Carbohydrate metabolic process, generation of precursor metabolites and energy, catabolic process, small molecule metabolic process, biosynthetic process, lipid metabolic process, sulfur compound metabolic process.	Oxidoreductase activity, ion binding	*pdhB*
Amino acid ABC transporter ATP-binding protein	1.90	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity	*lmo0848*
YtxH domain-containing protein	1.31	CM	N/A	N/A	*lmo2217*
ABC transporter ATP-binding protein	1.15	CM	Transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process.	Hydrolase activity, ATPase activity, ion binding, organic cyclic compound binding, drug binding, transmembrane transporter activity, protein transporter activity	*lmo2372*
Dihydrolipoyl dehydrogenase	2.11	CM	Carbohydrate metabolic process, generation of precursor metabolites and energy, catabolic process, homeostatic process, cellular nitrogen compound metabolic process, small molecule metabolic process, transport, cofactor methabolic process.	Oxidoreductase activity, ion binding, organic cyclic compound binding, transmembrane transporter activity	*lmo1371*

Flagellar hook protein FlgE	2.08	EX	Locomotion, cell motility	Structural molecule activity, hydrolase activity	*lmo0697*
Flagellin	2.94	EX	Locomotion, cell motility, pathogenesis.	Structural molecule activity, protein binding	*flaA*
Lmo2692 protein	1.04	EX	N/A		*lmo2692*
Acylphosphatase	1.01	EX	Cellular protein modification process	Hydrolase activity	*acyP*
Lmo0370 protein (Alkylphosphonate utilization protein)	1.13	ND	N/A	N/A	*lmo0370*
Lmo1601 protein (YtxH domain-containing protein)	1.09	ND	N/A	N/A	*lmo1601*
Cell division protein SepF	1.19	ND	Cell cycle, cellular component assembly, cell division	N/A	*sepF*

Supplementary Table S3

Table S3. Surface proteins down regulated: protein name, VIP score, cellular component, biological and functional process involved and gene name.

Protein name	VIP score	Cellular component	Biological process	Functional process	Gene
FruA protein	1.22	CM	Transport	Transmembrane transporter activity, kinase activity, ion binding	*fruA*
Alpha/beta hydrolase	2.86	CM	N/A	N/A	*lmo1128*

PTS mannose/fructose/sorbose transporter family subunit IID	1.09	CM	Transport	Transmembrane transporter activity	*lmo0098*
Efem/EfeO family lipoprotein	1.34	CM	Transport, respose to stress	N/A	*lmo0366*
Foldase protein PrsA 2	2.90	CM	Protein folding, cellular protein modification process, transport, cellular component assembly, homeostatic process, membrane organization	Isomerase activity, protein binding, unfolded protein binding	*prsA2*
Actin assembly-inducing protein	1.93	CM	Pathogenesis	N/A	*actA*
Signal recognition particle protein	1.51	CM	protein targeting, transport, catabolic process, cellular nitrogen compound metabolic process, nucleobase-containing compound catabolic process, small molecule metabolic process	RNA binding, GTPase activity, hydrolase activity, ion binding	*ffh*
Pyridine nucleotide-disulfide oxidoreductase family protein	1.27	CM	Generation of precursor metabolites and energy, homeostatic process	Oxidoreductase activity, ion binding	*lmo2638*
Listeriolysin O	1.36	EX	Cell death, pathogenesis	Lipid binding	*hly*
Translation initiation factor IF-1	3.44	EX	Translation, biosynthetic process	RNA binding, translation factor activity, nucleic acid binding	*infA*
50S ribosomal protein L31 type B	1.13	EX	Translation, biosynthetic process	Structural constituent of ribosome, structural molecule activity, RNA binding, rRNA binding, ion binding	*rpsM*
CamS family sex pheromone protein	1.09	EX	N/A	N/A	*lmo1757*
Flavocytochrome c	4.85	ND	Generation of precursor metabolites and energy, biosynthetic process, cellular nitrogen compound metabolic process, small molecule metabolic process, cofactor metabolic process, lipid metabolic process, catabolic process	Oxidoreductase activity, ion binding	*lmo0355*

Uncharacterized protein	1.70	ND	N/A	N/A	*lmo0791*
Hemin/hemoglobin-binding protein 2	3.67	CW	Transport	Ion binding, protein binding	*hbp2*
Internalin B	4.32	CW	Pathogenesis, locomotion, symbiosis, encompassing mutualism through parasitism, catabolic process, cell wall organization or biogenesis.	Hydrolase activity, hydrolase activity, acting on glycosyl bonds, hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	*inlB*
Peptide ABC transporter substrate-binding protein	1.34	CW	Transport, cell differentiation, anatomical structure formation involved in morphogenesis, anatomical structure development, locomotion	Transmembrane transporter activity, ion binding	*lmo2196*
ABC transporter substrate-binding protein	1.19	CW	Transport, cell differentiation, anatomical structure formation involved in morphogenesis, anatomical structure development, locomotion	Transmembrane transporter activity, ion binding, DNA binding	*lmo0135*

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3.4 Fourth Scientific Article

Lipidomic and proteomic analysis of Listeria monocytogenes reveals modulation of biofilm formation by exposure to antimicrobial peptides

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Lipidomic and proteomic analysis of Listeria monocytogenes reveals modulation of biofilm formation by exposure to antimicrobial peptides

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Abstract

The outset of *Listeria monocytogenes* resistance to the bacteriocin nisin, promote the study of alternative antimicrobial peptides (AMPs) to overcome this concern in the food sector. In this work, an omics approach was employed in order to investigate proteins and lipids directly involved in the possible resistance strategy of *L. monocytogenes* upon exposure to AMPs. The commercial bacteriocin nisin and the already characterized fengycin secreted by *Bacillus velezensis* P34 were compared through lipidomic and proteomic approaches. The differential regulation of biofilm formation was detected by the action of these AMPs, confirming the promotion of cell attachment and biofilm assembling after treatment with sub-lethal concentration of nisin, whereas an inhibition was observed in fengycin-treated samples. Supporting this hypothesis the, fatty acids quantification of branched chain fatty acids BCFAs were detected in higher percentages by the fengycin than nisin treated and untreated samples with respectively 46.6, 39.4 and 43.4%. As consequence an higher relative abundance of 30:0, 31:0 and 32:0 phosphatidylglycerol species were detected. Supporting the lipidomic data suggesting the inhibition of biofilm by the fengycin treated samples, the proteomics data revealed downregulation of important cell wall proteins involved on the building of the multibacterial organization such as the LTA backbone synthesis (Lmo0927) and the flagella-related (Lmo0718) proteins among the others. Moreover, we discussed a group of additional proteins that may collaborate on the bacterial cytoplasm maintaining to contrast the AMPs action.

Keywords: antimicrobial peptides; antimicrobial resistance; nisin; fatty acids; membrane proteins

1. Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis, a severe disease afflicting immunocompromised humans, pregnant women and children, causing septicaemia, meningitis and aborts (Radoshevich and Cossart, 2018). The main route of *L. monocytogenes* transmission in cases of food contamination occurs during the production process (Duze et al., 2021). Antimicrobial compounds secreted by bacteria might be utilized for extending shelf life in the food industry limiting the spread of foodborne pathogens, including *L. monocytogenes* (Zhao and Kuipers, 2016). Bacteria produce diverse types of antimicrobial peptides (AMPs), including those ribosomally synthesized also known as bacteriocins (Chikindas et al., 2018), and non-ribosomally synthesized AMPs, e.g., cyclic lipopeptides (CLPs) (Fira et al., 2018).

The lantibiotic nisin, a positively charged bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, has been considered one of the widely used strategies to control *L. monocytogenes* in foods (Cotter at al., 2005; Alvarez-Sieiro et al., 2016). However, the acquisition of nisin tolerance or resistance in *L. monocytogenes* has already been observed (Wu et al., 2018). Among the strategies employed by *L. monocytogenes* to evade nisin action, the upregulation of membrane proteins involved in biofilm secretion was reported in a previous study (Stincone et al., 2020a). The antimicrobial resistance of *L. monocytogenes* can be influenced by environmental conditions and genetic characteristics; considering the genetic characteristics some strains have been described to own or yield high natural nisin resistance compared with others, as consequence a reduction or increasing of the potency of this bacteriocin can be observed (Malekmohammadi et al., 2017; Wambui et al., 2020). The bacterial growth inhibition process caused by nisin is mediated by depolarisation of charged cell wall components, followed by the generation of pores in the cell membrane and interruption of the cell-wall biosynthesis through specific interaction with the lipid II,

recognized as a bacterial cell walls precursor component (Huang et al., 2002; Prince et al., 2016).

Bacillus strains dispose the ability to produce CLPs, collectively grouped into the surfactin, fengycin and iturin families. *Bacillus velezensis* P34, a stain isolated from the intestine of an Amazonian fish, produces CLPs, mainly fengycin (Stincone et al., 2020b). Although fengycin has been mostly reported as a potent antifungal lipopeptide (Vanittanakom and Loeffler, 1986), its antibacterial activity is also recognized (Medeot et al., 2020). Fengycin activity has been confirmed against Gram-positive bacteria, including the effect on reducing the presence of *Staphylococcus aureus* in human intestine by modulating quorum sensing (Piewngam et al., 2018).

The phospholipids phosphatidylglycerol (PG), along with phosphatidylethanolamine (PE), cardiolipin (CL) and lipoteichoic acid (LTA) are considered key factors of Gram-positive bacterial membranes playing a crucial role in membrane stability (Seltmann and Holst, 2013; Fishetti et al., 2019). Changes in lipid membrane have been included among the variety of resistance mechanisms to AMPs. Bacteria AMPs resistance were correlated with the induction of changes in surface charge by modifications of TAs, LPS and membrane lipids (Mishra and Bayer, 2013; Sahl et al., 2015; Omardien et al., 2016). The cytolytic activity of AMPs can be often explained by two different mechanisms (Sani and Separovic, 2016; Lee et al., 2016): the case in which the peptide penetrates the membrane by forming channels, where nisin is included (Sur et al., 2018); and a second case in which the peptide aggregates on the membrane surface and causes injuries, in this latter may include the fengycin (Horn et al., 2013).

Membrane proteins should not be considered as isolated entities but part of a complex structure surrounded by lipid molecules, playing a pivotal role in maintaining the full structural and functional integrity (Donnarumma et al., 2018). The investigation of the critical

lipid-protein interactions through instrumentation and bioinformatics tools together with biophysical and biochemical techniques have become fundamentally important in this field (Bolla et al., 2019).

Listeria monocytogenes biofilm is mainly composed of teichoic acids and can be developed in different surfaces and inaccessible locations of a food processing facility, representing an important concern. Knowing about the biofilm formation process permit to enable adopting best hygienic practices against this pathogen (Silva et al., 2008; Kocot and Olszewska, 2017).

In this work, the *L. monocytogenes* resistance strategies to AMPs was investigated comparing the application of sub-lethal concentrations of the bacteriocin nisin and the *B. velezensis* P34 fengycin, through a lipidomics and proteomics approach. For the first time the action of fengycin was investigated on the modulation of biofilm formation by *L. monocytogenes*, already considered as one of the resistance strategies of this foodborne pathogen.

2. Materials and methods

2.1 Chemicals

Reagents to perform the derivatization of lipid components into fatty acid methyl ester (FAMEs) derivatives, sodium methoxide (MeONa) and boron trifluoride-methanol solution (BF_3, 14% in methanol) were acquired from Merck Life Science (Merck KGaA, Darmstadt, Germany). A reference standard solution of C_4-C_{24} even carbon saturated FAMEs (1000 μg/mL) was acquired from Merck Life Science for determining experimental linear retention index (LRI) of fatty acid methyl esters (FAMEs).

2.2 Antimicrobials

Nisin (Chrisin®) was provided by Chr. Hansen A/S (Hørsholm, Denmark). In agreement with the manufacturer, the formulation contains 2.5% (w/w) pure nisin. Nisin stock solution (1 mg/mL) was prepared by diluting Chrisin® in 0.01 M HCl and stored at 4°C. The working concentrations were obtained by dilution of the nisin stock solution in 10 mM sodium phosphate buffer (pH 7.0). The sub-lethal nisin concentration of 0.1 µg/mL was previously determined through the inhibitory effect on microbial growth curves (Stincone et al., 2020a).

The production of fengycin peptides by *Bacillus velezensis* P34 was carried out as described previously (Stincone et al., 2020b). The obtained product showed 3,200 activity units (AU) per mL. Thus, dilutions in buffer phosphate pH: 7 were performed to achieve the sub-lethal concentration of 100 AU/mL on *L. monocytogenes*, as demonstrated by a previous work (Motta et al., 2008; Stincone et al., 2020c).

2.3 Listeria monocytogenes cultivation for omics analysis

Listeria monocytogenes ATCC 19116 was pre-cultured in Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) agar to prepare a bacterial suspension in saline solution (8.5 g/L NaCl) with an OD_{600} of 0.150, corresponding to a 0.5 McFarland turbidity standard. This suspension (3 mL) was inoculated in 300 mL BHI broth to obtain a concentration of 10^6 colony forming units per mL (CFU/mL) and incubated in a rotary shaker at 37°C and 150 rpm. After 6 h cultivation, the antimicrobials (fengycin or nisin) were added to the cultures and samples were incubated for additional 1 h before the protein and lipid extraction. Untreated bacteria were used as control samples.

2.4 Extraction of lipid components

The lipid fraction of *L. monocytogenes* was initially extracted by using Bligh and Dyer protocol as reported recently by Hines et al. (2020). Briefly, 4 mL of a methanol/chloroform solution (2:1, v:v) was added to each cellular pellet contained in 25 mL Falcon tube. The extraction mixture was homogenized by using vortex mixing (5 min). After, 1 mL of chloroform and 2 mL of sodium chloride (NaCl) saturated water solution were added to the mixture. The sample was vortexed and centrifuged for 10 min at 2.000 rpm to separate methanol and chloroform layers. The lower organic phase containing lipid compounds was collected by using a Pasteur pipette and transferred to a 2 mL vial. The chloroform solvent was removed under constant nitrogen flow.

2.5 Derivatization of lipid extract

The conversion of lipid compounds into FAME derivatives for gas chromatography (GC) analysis was performed as reported earlier (Kováčik et al., 2020). Briefly, the procedure involved the usage of two different derivatizing agents: methanolic solution of MeONa (0.5% v/w) and methanolic solution of BF_3 (14% v/w). 500 µL of the derivatizing agents were added to the lipid extract in subsequent manner, and the reaction temperatures were maintained at 95°C for 30 min. Subsequently, 300 µL of *n*-heptane and 200 µL of saturated NaCl solution were added to the mixture, The upper heptanic FAMEs layer was collected and injected directly into GC instrumentations for chromatographic separation.

2.6 GC-MS and GC-FID analysis

The separation and identification of FAME derivative compounds were carried out by using a GCMS-QP2010 Ultra (Shimadzu, Duisburg, Germany) instrument equipped with a split-splitless injector (280°C) and an AOC-20i autosampler. A medium-polarity ionic liquid (IL) column, named SLB-IL60 30m x 0.25mm *id* x 0.25µm d_f (Merck Life Science) was utilized

for the FAMEs separation. Quantitative analyses were performed through the use of a GC-2010 instrument (Shimadzu) equipped with a split-splitless injector (280°C), a flame ionization detector (FID) and an AOC-20i autosampler. Chromatographic conditions such as volume injections, programmed oven temperatures, gas carrier linear velocity, MS and FID parameters, data collection and peak assignment were same as those described in detail previously (Kováčik et al., 2020).

2.7 Lipidomics UHPLC-MS/MS analysis

The analyses were performed on a Shimadzu Ultra High-Performance Liquid Chromatograph-Nexera X-2 system (Shimadzu), including two LC-30 AD dual-plunger parallel-flow pumps, a DGU-20A5R degasser, a CTO-20AC column oven and a SIL-30AC autosampler. The UHPLC system was coupled with an LCMS-8060 triple quadrupole mass spectrometer equipped with ESI interface (Shimadzu). Mobile phases were: A) 20 mM $HCOONH_4$ in water and B) 2-propanol/ACN/water (60:36:4 v/v/v) with 0.1% formic acid. The gradient program was: 0-6 min, 80-100% B (held for 16 min). The flow rate was 0.4 mL/min; column oven was set at 40 °C and the injection volume was 5 µL. MS and MS/MS acquisitions were performed using ESI source operating both in positive (+) and negative ionization modes (-), with the following parameters: interface temperature, 450 °C; CDL temperature, 250 °C; heat block temperature, 200 °C; nebulizing gas flow (N_2), 3 L/min; drying gas flow (N_2), 5 L/min; acquisition range, 350-1250 *m/z* (+) and 150-1250 *m/z* (-). Additional MS/MS experiments were optimized through the injection of single phospholipid (PL) standards, ~~and the selected events are reported in Table 2~~ (Rigano et al, 2021). Data acquisition and processing was handled by the LabSolution ver. 5.95 software (Shimadzu Europa, Duisburg, Germany). The LIPID MAPS Structure Database (LMSD) was employed

for compound identification (available at https://www.lipidmaps.org/data/structure/) (Sud et al. 2007).

2.8 Total protein extraction, digestion and desalinization

The cellular pellets collected by a previous centrifugation at 10 000 g for 15 min at 4°C were washed with PBS buffer pH 7.4 and subjected to protein extraction according to the methodology previously reported (Ritter et al., 2018) with some modifications. Bacterial cells were suspended in Tris–HCl buffer pH 7.5 containing 10 μL of Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and sonicated for 5 cycles of 30s with intervals of 1 min in an ultrasonic cell disrupter in ice bath to maintain low temperatures. The cell lysed solutions were centrifuged at 10000 g for 20 min and the supernatants were lyophilized before the total protein quantification with the Bradford method.

A concentration of 10 μg/mL protein of each sample was digested carrying out the protocol of (Villèn and Gygi, 2008). Digestion of proteins were performed diluting urea with 50 mM ammonium bicarbonate to a final concentration of 1.6 M, lastly 1 mM of calcium chloride was added to the samples and incubating overnight at 37 °C (2 μg of trypsin), 2% (v/v) of trifluoroacetic acid was employed to stop the enzymatic reaction. Sample desalinations were performed employing C18 stage tips. Specifically, water and 0.1% formic acid solution were used to wash the peptides from the salt residues, and peptides elution was performed using a 60% (v/v) acetonitrile and 0.1% (v/v) formic acid (Rappsilber et al., 2007). The samples were dried in a vacuum concentrator and stored at -20 °C for successive analysis by LC-MS/MS.

2.9 Proteomics mass spectrometry analysis

The dried samples were reconstituted in 10 μL formic acid (0.1% v/v). Then, an aliquot of 3 μL was analyzed using LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to the EASY-nLC system (Proxeon Biosystem, West Palm Beach, FL, USA) through a Proxeon nanoelectrospray ion source. The Peptide separation was carried out employing an acetonitrile gradient (2–90% v/v) containing 0.1% (v/v) formic acid, and using a PicoFrit Column (20 cm x ID75 μm, 5 μm particle size, New Objective) at a flow rate of 300 μL/min over 65 min. The instrument methods were set up in the data dependent acquisition mode. The complete setup of the mass spectrometer used in this work, was the same reported by Stincone et al. (2020). The data was processed using MaxQuant v1.3.0.3 software (Cox and Mann, 2008) and MS/MS results were investigated in the UniProt database against *L. monocytogenes*, using the Andromeda search tool (Cox et al., 2011).

2.10 Biofilm assay

The biofilm quantification was carried out to check the possible inhibition of the biofilm by the fengycin treated *L. monocytogenes* samples. The assay employed was the crystal violet method detailed by Stepanović et al. (2007). Briefly, a fresh liquid culture in BHI was prepared at an initial concentration of 10^6 CFU/mL and incubated for 6 h at 37 °C in aerobic condition. Aliquots were used to create respectively: (i) untreated samples, cultures treated with (ii) 0.1 μg/mL of nisin and cultures treated with (iii) 100AU/mL of fengycin lipopeptides. 10 wells of 96-well microplate for each of these three cultures were filled and incubated at 37 °C for 24 h. After incubation, the formed biofilm into the wells was washed three times and fixed with methanol and the biofilm was dryed overnight at room temperature. 2% (w/v) violet crystal was used for the staining of the fixed biofilm before to wash the wells for residual dye elimination. Before the optical density reading, 95% ethanol was added to the wells for extraction of the dye and quantify adherent bacteria biofilm

included. The microplate reader (SpectraMax M2^e) at 570 nm was employed for the optical density detection of the ethanol solution.Optical density comparison was made for interpreting the results coming from the wells containing *L. monocytogenes* respectively: untreated (OD_{Cont}), treated with nisin (OD_{Nis}) and treated with fengycin (OD_{Fen}). The effect of the antimicrobials nisin and fengycin on biofilm formation was considered as inductive when the OD_{Nis} or OD_{Fen} >OD_{Cont}, inhibitor when OD_{Nis} or OD_{Fen} <OD_{Cont}, and without effect when OD_{Nis} or OD_{Fen} =OD_{Cont}

2.11 Statistical analysis

MetaboAnalyst 3.068 on line software was employed for the statistical analysis of lipidomics and proteomics data. Principal component analysis (PCA) and Partial least squares discriminate analysis (PLS-DA) were carried out to determine the variation grade between the control and the samples treated with nisin and fengycin. The important features were selected with a threshold of VIP (Variable Importance in Projection) ≥1.0, and this data was used for the hierarchical clustering heatmaps analysis. Moreover, all the proteins with VIP ≥1.0 were submitted to String v11 Database for constructing protein-protein interactions (Szklarczyk et al., 2019), and the information were used to detect proteins involved on the structural lipidic membrane.

3. Results

3.1 Differential fatty acids profile

A total of 14 fatty acids were detected by the GC analysis, including saturated fatty acids (SFA), branched-chain fatty acids (BCFA) and a mono-unsaturated fatty acid (MUFA). FAMEs GC profile reported can be seen in Fig. 1. Lipid quantification permitted to detect a higher percentage of SFA content (35.9%) in *L. monocytogenes* treated with nisin, in

comparison with both control and lipopeptides treated samples (30.2% and 27.8%, respectively). Contrarily, BCFA were detected in higher percentages in both control and fengycin-treated samples (Supplementary Table S1). Statistical analysis including both PCA and PLS-DA revealed higher statistically differences by the fengycin-treated samples compared with the control and nisin-treated samples (Fig. 2). Three fatty acids presenting VIP>1.0 were detected (Fig. 3). The BCFA *anteiso*-C17:0 showed the highest VIP value and higher concentration in samples treated with fengycin, while detected as downregulated in the nisin treatment. The *iso*-C15:0 appeared downregulated by the treatment with fengycin, whereas the C16:0 was upregulated in the nisin-treated samples and downregulated by the treatment with fengycin.

3.2 MS/MS lipidomic analysis

The intact polar lipid profile detected in *L. monocytogenes* comprises mostly of phosphatidylglycerols (PGs), diglycosyl-diacylglycerols (DGDGs) and diacylglycerols (DGs), as described in Supplementary Table S2. Detected ions, class, partition number (PN), carbon number (CN), double bond number (DB) and retention time (RT) values are reported for each lipid species. Sodium adducts were observed for DGDGs, while DG were present as dehydrated protonated ions. On the other hand, LPGs and PGs were detected in negative ion mode, by precursor ion scan experiments, through selective monitoring of 153 *m/z* fragment (data not shown). Elution of the lipid species occurs according to increasing PN, derived from the equation PN = CN - 2DB. The mass identification was carried out following the information reported by Tatituri et al. (2015). Relative abundance percentage of all the PG compounds (27:0, 29:0, 30:0, 31:0, 32:0, 31:1, 33:0, and 34:0) permitted to detect differences among the treatments as reported in Fig. 4.

3.3 Proteomics analysis

A total of 951 LFQ (label free quantified) protein intensities, among the control and treated samples, were detected by OrbiTrap mass spectrometry analysis. After database normalization, 797 LFQ protein intensities were selected for proteomics data analysis. Statistical analysis included the PCA, which showed discrimination between control group and both treated samples, and between these latter a sight discrimination was observed (Fig. 5A). PLS-DA analysis instead showed a clear discrimination among the three groups of samples (Fig. 5B). This latter analysis permitted to calculate the protein VIP scores and only 142 protein resulted with VIP $\geq$1.0 (Supplementary Table S3).

3.4 Differential biofilm organization

The fengycin treatment showed specific protein regulation towards the inhibition of biofilm formation, compared with the control and the nisin treatment (Table 1). Negative action on *Listeria* biofilm organization was indicated from the downregulation of LTA backbone synthesis (Lmo0927), supported by downregulation of the precursor *lmo2120* gene coding for a di-adenylate cyclase involved on the bacteria response against the host cell defences. In this context, additional proteins with high VIP score including putative pyruvate phosphate di-kinase regulatory protein, Lmo1875 protein, Lmo0718 protein, and Lmo1819 protein were observed by proteomic analysis (Table 1). All these proteins work on the biofilm promotion and were detected downregulated in fengycin-treated samples. Experimental biofilm quantification assay permitted to detect significant differences among samples (Fig. 6). Biofilm promotion was induced by the treatment with the lantibiotic nisin ($OD_{Nis} > OD_{Cont}$), while a strong inhibition of this multicellular structure was observed by the action of the lipopeptide fengycin ($OD_{Fen} < OD_{Cont}$).

3.5 Resistance membrane proteins

The proteomic analysis also revealed a group of proteins associated with the membrane resistance responses to the AMPs (Table 2), including glutathione synthase, Lmo0927 protein, di-adenylate cyclase, Lmo2555 protein and Lmo1539 protein.

The Lmo0927 protein acts in LTA backbone synthesis (LtaS), work also structurally on the bacterial cell membrane extending the glycerol phosphate backbone chain. Beside to this latter protein acting on the LTA synthesis two required distinct glycosyltransferases in *Listeria monocytogenes*, LafA and LafB coded by *lmo2555* and *lmo2554* (not differentially observed in this work) genes , play a pivotal role to produce monoglucosyldiacylglycerol and diglycosyl-diacylglycerols, respectively (Webb et al., 2009). The last protein differentially detected and included in this group of proteins related with the membrane stress resistance homeostasis was the Lmo1539 protein highly similar with the glycerol uptake facilitator protein (GlpF-2).

4. Discussion

The exposure of *L. monocytogenes* to sub-lethal concentrations of two antimicrobial peptides resulted in different bacterial responses based on lipidomics and proteomics approaches. Under the applied conditions of antimicrobial stress, it could be highlighted the propensity to inhibit the biofilm formation in the fengycin-treated samples, and the differentially expressed proteins group collaborating with membrane resistance to these stress agents.

The exanimate fatty acids profile of *L. monocytogenes* in general permitted to detect membranes highly enriched in BCFAs, mainly *anteiso*-C15:0 and *anteiso*-C17:0, as previously reported by Mastronicolis et al., (1996 a,b), similar to others gram positive bacteria such as: *Staphylococcus* and *Bacillus* species (Whittake et al., 2005), conferring to these bacterial groups major fluidity compared with others. Modification in membrane lipid

composition were observed in nisin/fengycin treatments (which was effectively measured), suggesting an alteration of membrane fluidity probably due to different antimicrobial peptides way of action and consequence bacteria response. The characterization of membrane lipids in *L. monocytogenes* after exposure to some bacteriocins has been reported. These studies describe the modulation of the saturated-unsaturated membrane fatty acids ratio, including either reduction of the membrane fluidity coming from an improvement of the SFAs concentrations (Martinez and Rodriguez, 2005; Naghmouchi et al., 2008; Ming and Daeschel 1993) or improving of the membrane fluidity through the increase of the BCFAs concentrations (Mendoza et al., 1999; Vadyvaloo et al., 2002 and 2004). A previous work showed the BCFAs modulation ratio by antimicrobials, specifically the sub-inhibitory concentration action of the major cinnamon essential oil component *trans*-cinnamaldehyde for example, act on the whole-cell fatty acid profile with an almost complete loss of *anteiso* BCFAs, compensated by elevated concentration of SFA and *iso*-BCFAs with a consequence reduced membrane fluidity (Rogiers et al., 2017).

The differences in the polar lipid fingerprints of *L. monocytogenes* treated with nisin and fengycin were additional results of this work. The examination of the obtained MS/MS data permitted to detect the PG components as the main polar lipid class, with a relative higher abundance for the species 30:0, 31:0 and 32:0 in fengycin-treated samples, probably correlated toe the higher quantification of anteiso BCFAs forms **(Table S1)**. The nisin-treated samples showed increased amounts of long acyl chain saturated fatty acids comparing it with the fengycin lipopeptides treatment, indicating an increasing thermodynamically stable interactions between acyl-chains and so increasing of the bilayer rigidity (Denich et al., 2003) . Some studies showed as gram-positive bacteria increase the percentage of saturated fatty acids in bacterial cells forming biofilm, with a concomitant decrease of branched-chain fatty acids (Giannotti et al., 2008; Dubois-Brissonnet et al., 2016). One of the

L. monocytogenes responses to the antimicrobial exposure is the switch from planktonic to sessile organization, building biofilms to tolerate the environmental stress. In agreement, the treatment with sub-lethal nisin concentration induces the upregulation of proteins associated with biofilm formation in this species (Stincone et al., 2020). Contrarily, the treatment of *L. monocytogenes* with sub-lethal fengycin concentration showed an improvement of the BCFAs species with possible preference for a planktonic state, which can be related to the effective reduction in biofilm formation. Furthermore, the *anteiso*-BCFAs were observed as a *L. monocytogenes* resistance mechanism against the host stress coming from the antimicrobial defence mechanisms including both antimicrobial peptides and peptidoglycan hydrolases into the phagosome. In the meantime, this fatty acid modulation has showed to compromise the key virulence factor suggesting influence on the virulence regulation (Sun et al., 2012).

In accordance with the fatty acid and biofilm formation data, a group of proteins was differentially regulated in fengycin-treated *L. monocytogenes*. Like other compounds acting against biofilm formation through inhibition of LTA synthesis (Naclerio et al., 2020), fengycin treatment strongly downregulated the Lmo0927 protein (LtaS). Being typical of gram-positive bacteria, the LTA biosynthesis machinery provides an attractive target for the development of novel antimicrobial interventions (Rahman et al., 2009). The LTA deficiency in *S. aureus* altered the bacterial surface hydrophobicity and resulted in reduction of biofilm formation (Fedtke et al., 2007; Percy and Grundling, 2014). The strong reduction of Lmo0927 could be related with the possible action of fengycin on the multi-drug resistance (MDR) transporters required for full production of LTA, possibly via c-di-AMP efflux (Tadmor et al., 2014). In support to this hypothesis, the proteomic results showed a downregulation of the related dacA protein (*lmo2120* gene), a di-adenylate cyclase with c-di-AMP synthesis activity involved in triggering the type I interferon response within the host

cell (Woodward et al., 2010). In fact, the typical gram-positive c-di-AMP showed a role on the osmotic homeostasis on the adaptation to its environment (Devaux et al., 2018) including acid and oxidative stress factors (Rallu et al., 2000; Thibessard et al., 2004), resistance to extreme cell wall stress in *S. aureus* (Corrigan et al., 2011) and biofilm formation associated antibiotic tolerance (Römling and Balsalobre, 2012). In this context, c-di-AMP was also considered to play a role on the negative regulation of fatty acid synthesis in *Mycobacterium smegmatis* (Zhang et al., 2013).

Moreover, proteins supporting the hypothesis of biofilm inhibition were detected, including the downregulation of both putative pyruvate phosphate dikinase regulatory protein and Lmo1875 protein, previously reported among the upregulated proteins in sessile *L. monocytogenes* cells inside biofilms, when treated with lactocin AL705 in a comparative study with planktonic cells (Melian et al., 2021). As widely known, the flagellum contributes to the biofilm organization and its biosynthesis requires dozens of genes coming from a large operon working on the motility including the protein coded by the gene *lmo0718*, detected as downregulated in fengycin-treated samples. Thus, inhibition of flagella-related proteins may represent a possible loss on the biofilm building capacity (Toledo-Arana and Lasa, 2020; Bidnenko et al., 2017). Another deregulated protein in the fengycin-treated samples was the Lmo1819 protein with unknown function, although a deficient mutant effectively reduced the propensity of *L. monocytogenes* to form biofilms (Chang et al., 2012).

The biofilm and membrane modification represent two of the several mechanisms involved in bacterial resistance. The presence of proteins into membranes was recognised as highly functional in this direction. Molecular dynamics simulations studies offer a powerful complementary approach to gain atomic-level structural and energetic information on lipid-protein interactions, to give a new light the challenges coming from the lipid-protein interactions (Muller et al., 2019). The amounts of membrane branched fatty acids were

increased in *L. monocytogenes* treated with fengycin, in this direction studies including phospholipids reconstitution models or *in silico* molecular dynamics simulations confirmed that branching of acyl chains reduce the water permeability within the hydrophobic core (Shinoda et al., 2004; Tristram-Nagle et al., 2010). Moreover, considering the effect of differentially regulated proteins on the membrane structure, those related with LTA metabolism should be emphasised. LTAs play a pivotal role in the ability of gram-positive bacteria to grow, replicate and form biofilm, modulating the membrane structural stability and integrity under different physiological conditions (Bharatiya et al., 2021). The LTAs, indeed, play a critical role in processes such as osmotic stability, antimicrobial resistance, cell division (Hesser et al., 2020) and membrane lipid metabolism (Koch et al., 1984). Moreover, the bacterial resistance strategy includes modification of teichoic acids with D-alanine (Abachin et al., 2002) and modification of negatively charged phosphatidylglycerol with positively charged lysine (Thedieck et al., 2006), to avoid interactions with cationic AMPs. All these characteristics can be associated with the differential responses of *L. monocytogenes* to nisin and fengycin.

In many Gram-positive bacteria the abundant lipid species like monoglucosyl-diacylglycerols (MGDG) and diglycosyl-diacylglycerols (DGDG) act as membrane anchors for the LTA constituents of the cell wall (Neuhaus and Baddiley, 2003). The upregulation of the Lmo2555 protein, acting as MGDG synthase, observed by both the treatments with AMPs may work maintaining the membrane composition, as shown by the construction of deletion mutant, obviating the complete loss of glycolipids with the consequent phenotypic changes (Theilacker et al., 2011). The presence of MGDG in the *L. monocytogenes* membrane guaranteed by Lmo2555 protein support a protein-mediated osmotic stress adaptation against osmotic stressors like salts and sucrose (Wikström et al., 2004), and on the maintaining the anionic lipid surface charge density as observed in *Acholeplasma laidlawii* (Edman et al.,

2003). Specifically, a simulation with bacterial MGDG synthetase demonstrated local enrichment of cardiolipin (CL) and phosphatidylglycerol (PG) in the immediate area of this protein through interaction with their positive residues (Ge et al., 2014).

Another possibly discriminated protein related to the bacterial reaction to the membrane perturbation determined by the antimicrobial actions was the glutathione (GSH) biosynthesis protein gshAB. Although the knowledge about the physiological function of GSH in Gram-positive bacteria is very limited, it was showed plays important roles in cellular antioxidant mechanisms. Previous studies have demonstrated that bacterial cells containing GSH were capable to protect the membrane structure when exposed to various environmental stresses, such as osmotic pressure, oxidative, and acid stress (Zhang et al., 2010; Zou et al., 2014; Wang et al., 2015). In our study, gshAB protein was upregulated by the nisin treated, whereas fengycin action downregulated this protein in *L. monocytogenes*.

Then the protein GlpF was upregulated in both treatments compared with the control. Activities related with this latter include the modulation of the membrane lipid composition changes. In this context, the *sn*-glycerol-1-phosphate is an intermediate compound used for the synthesis of membrane lipid components while the GlpF membrane channel acts as the glycerol uptake facilitator (Blotz & Stulke, 2017). However, supposing a possible introduction of glycerol to be used on the membrane components synthesis, the glycerol kinase encoded by *glpK* gene representing another important enzyme to form *sn*-glycerol-1-phosphate had to be upregulated, but no significance difference was observed in our proteomics data. Contrarily, Cao et al. (2020) have related both Glpf and Glpk were upregulated when the antimicrobial activity of essential oils acted on destroying the phospholipids of cell membrane. Thus, an alternative motivation to explain our contradictory results may come from dynamic simulations studies. In fact, molecular dynamic simulation on the acyl-chain order in lipids around *Escherichia coli* aquaporins AqpZ and GlpF shows

an increase in the acyl-chain order parameter. Still the bilayer thickness decreases towards the protein and meets effectively the hydrophobic matching condition. It was possible to observe that the lipid bilayer adapts to the channel by a hydrophobic matching condition reflecting the propensity of the lipid molecules for forming curved structures, and also the transport function of the channel was modulated (Jensen and Mouritsen, 2004).

5. Conclusion

This study provided evidence that there is a link between the use of a sub-lethal dose of the cyclic lipopeptide fengycin and inhibition of biofilm formation in *L. monocytogenes*. Contrarily, it was confirmed that the bacterial physiology was organized to promote the biofilm when a sub-lethal concentration of nisin was employed. Moreover, it could be hypothesized that proteins may collaborate on the cell membrane stability through protein-lipid interactions. For the first time, biofilm inhibition by cyclic lipopetides was observed in *L. monocytogenes*, representing an important application for these AMPs to confront the serious hazard represented by bacterial pathogens. In addition, considering that the lipopeptide fengycin has been understudied for antibacterial activity, this study support the idea that it should be more investigated for inhibition of gram-positive bacteria including multi-resistant strains.

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Conflicts of interest

Authors declare no conflicts of interest.

Authors contribution statement

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Table 1. Detected proteins by proteomic analysis involved on *Listeria monocytogenes* biofilm process inhibition in fengycin treated samples, followed by the respective VIP score and gene name.

Protein Name	VIP Score	Regulation	Treatment	Gene Name
Putative Pyruvate Phosphate Dikinase regulatory protein	4.54	Down	Fengycin	*lmo0234*
Lmo1875 protein	2.71	Down	Fengycin	*lmo1875*
Lmo0718 protein	2.92	Down	Fengycin	*lmo0718*
Lmo 1819 protein	2.90	Down	Fengycin	*Lmo1819*
Lmo0927 protein*	2.76	Down	Fengycin	*lmo0927*

* protein shared with the membrane adaptation responds group

Table 2. Detected proteins by proteomic analysis involved on *Listeria monocytogenes* involved on membrane adaptation responds to AMPs treatments nisin and fengycin, with the respective VIP score, activity and gene name.

Protein Name	VIP Score	Regulation	Treatment	Gene Name
Glutathione biosynthesis bifunctional protein GshAB	4.81	Down	Fengycin	*gshAB*
Lmo0927 protein*	2.76	Down	Fengycin	*lmo0927*
Diadenylate cyclase	1.40	Down	Fengycin	*dacA /lmo2120*
Lmo2555 protein	2.88	Up	Fengycin and Nisin	*lmo2555*
Lmo1539 protein	4.65	Up	Fengycin and Nisin	*lmo1539*

* protein shared with the biofilm inhibition group of proteins

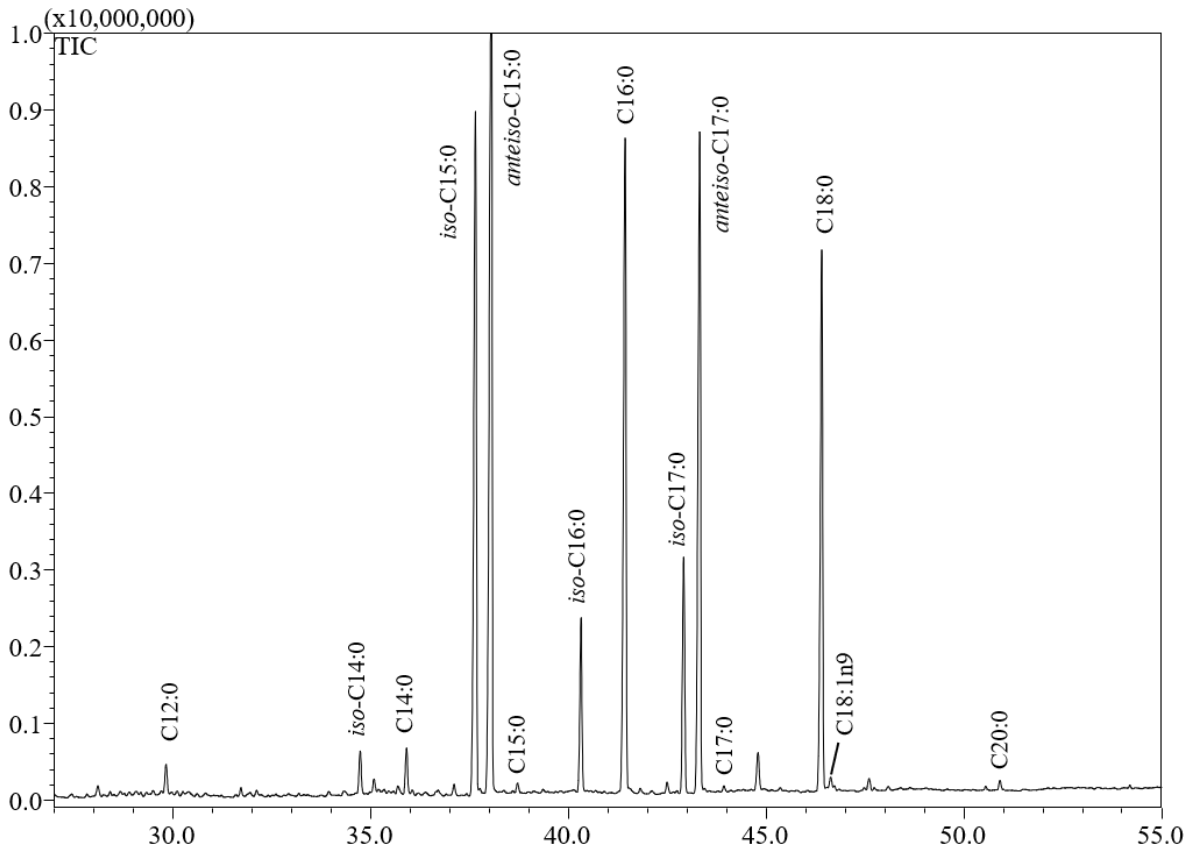


Figure 1. FAMEs GC-MS chromatogram in *Listeria monocytogenes* Gram-positive bacterium.

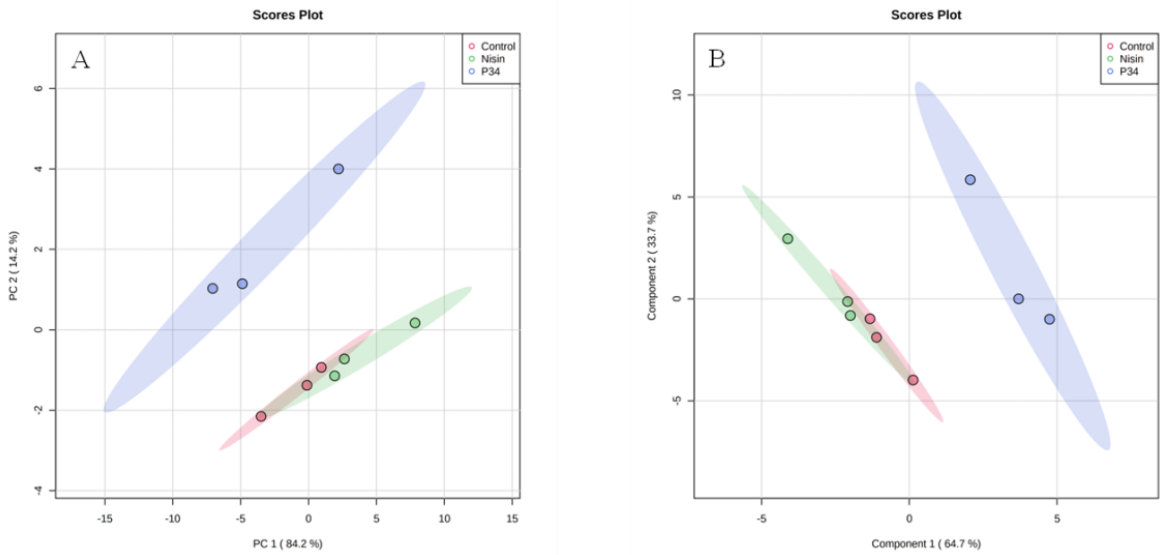


Figure 2. (A) Principal Component Analysis (PCA) and (B) Partial Least Squares - Discriminant Analysis (PLS-DA) in fatty acids samples of *L. monocytogenes* treated with sub-lethal doses of Fengycin (blue), Nisin (green) and control (red).

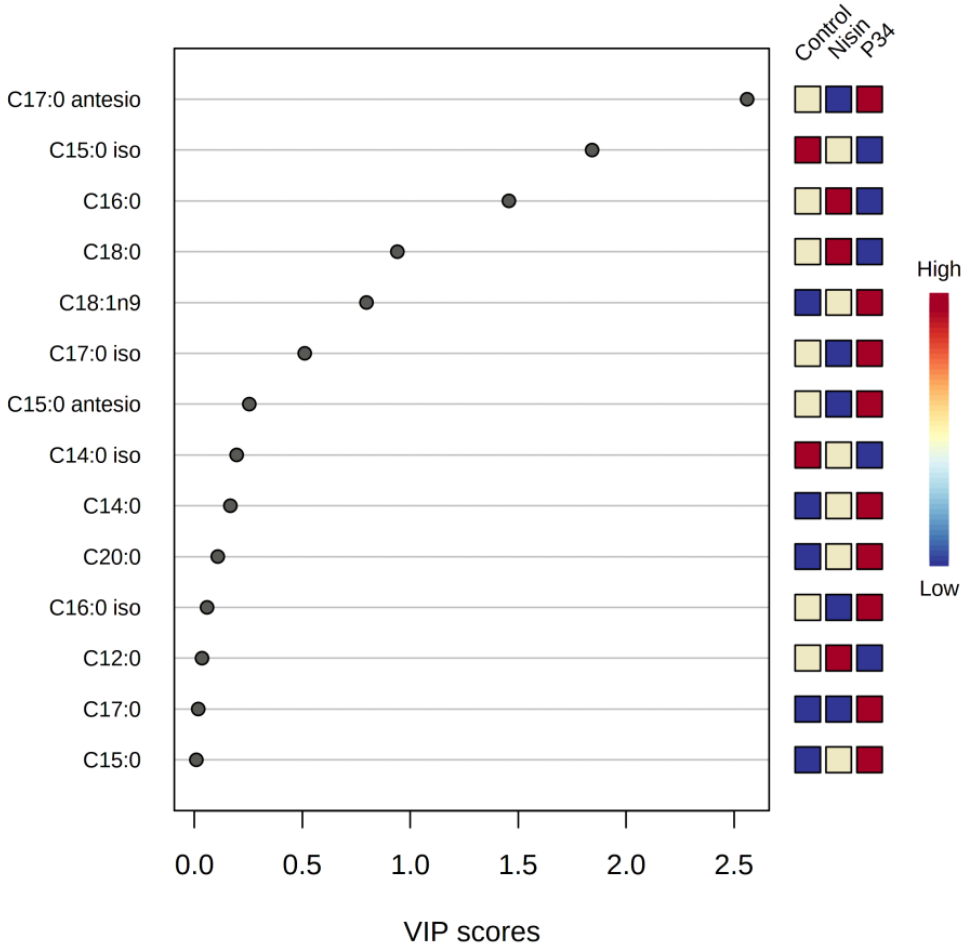


Figure 3. Partial Least Squares Discriminant Analysis (PLS-DA) with the variable importance peojection (VIP) score and the coulored boxes indicating realtive concentration of the corresponding fatty acids in *Listeria monocytogenes* cultures for each specific group including the control and the treated with sub-lethal concentration of nisn and fengycin.

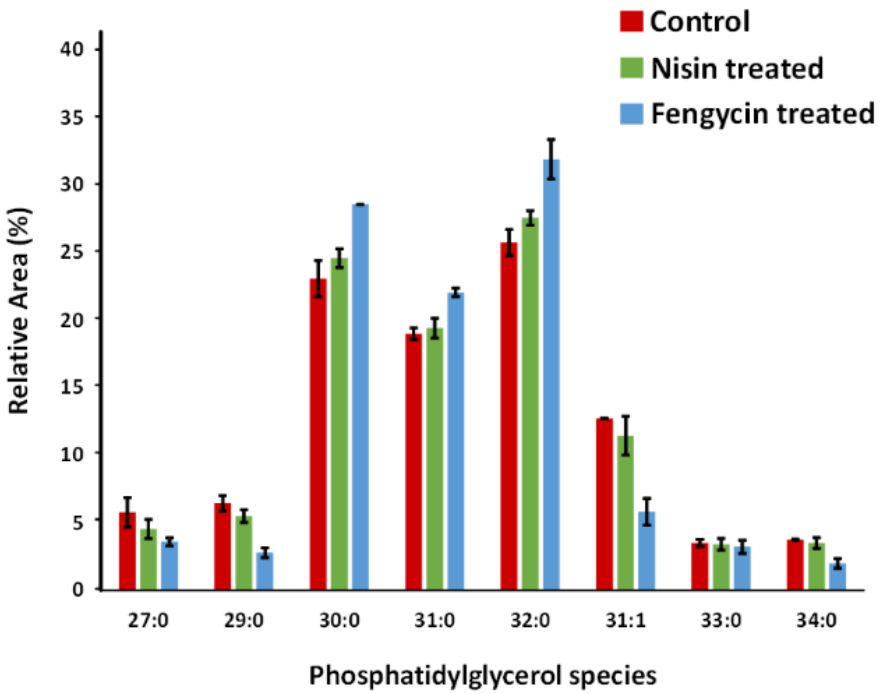


Figure 4. Comparison of relative quantification of individual PG molecular species in total lipid extracts ($n = 3$ biological replicate sets of treated bacteria) employing MS/MS using electrospray ionization ESI in negative ionization modes (-), by precursor ion scan experiments, through selective monitoring of 153 *m/z* fragment.

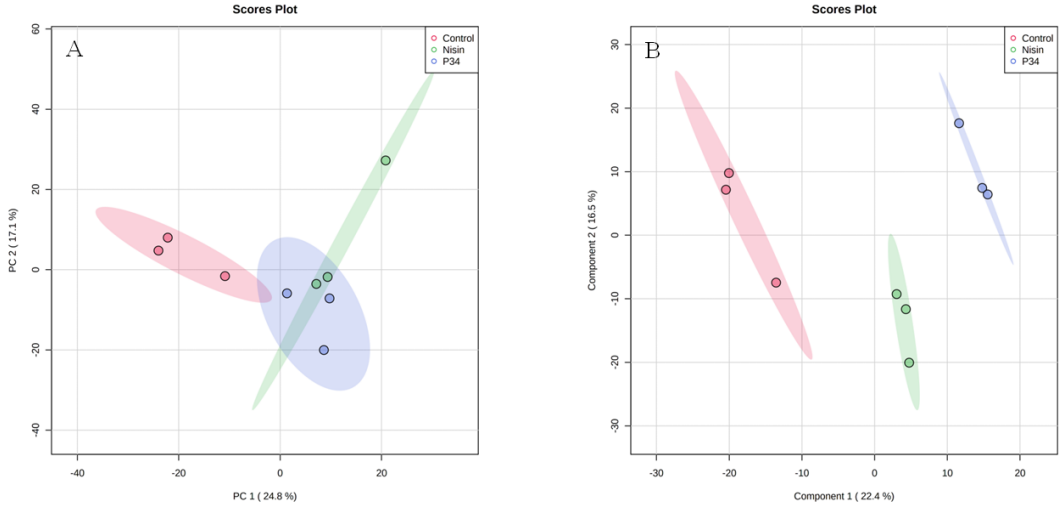


Figure 5. (A) Principal Component Analysis (PCA) and (B) Partial Least Squares - Discriminant Analysis (PLS-DA) in proteome samples of *L. monocytogenes* treated with Fengycin (blue), Nisin (green) and control (red).

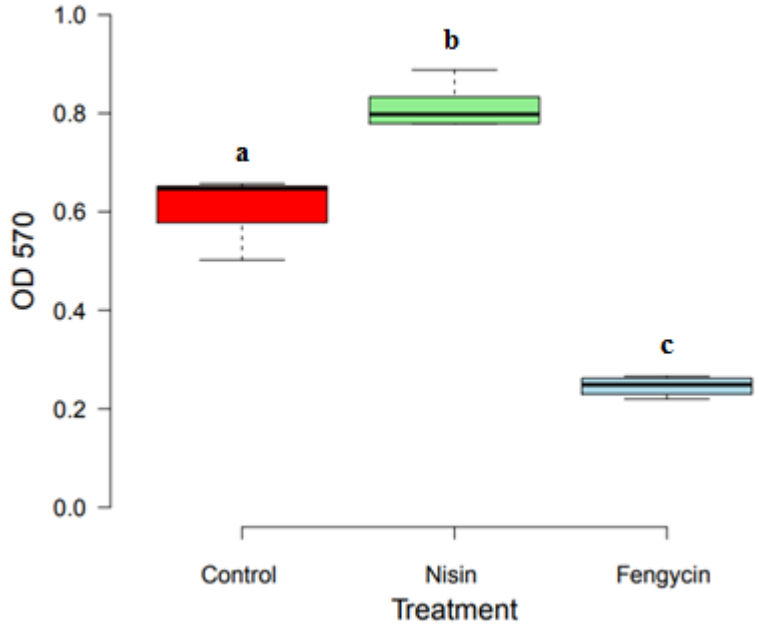
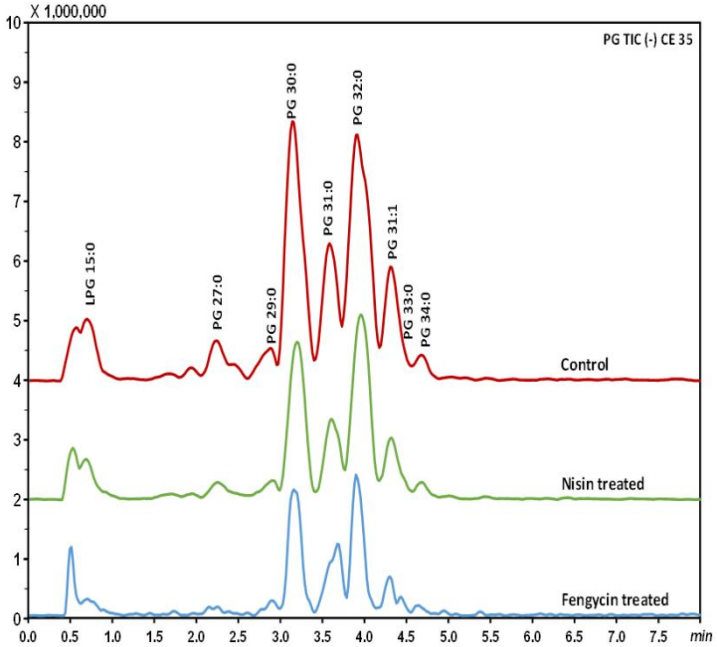


Figure 6. Boxplot of spectrophotometry data at 570 nm showing the optical density (OD) of the *Listeria monocytogenes* biofilm after 24 h growth in BHI broth under different treatments: negative control, nisin (0.1 μg/mL) and fengycin (100 UA/mL) treatments, which are represented by the red, green and blue color, respectively. Different lower case letter over the plot of each treatment indicate significant differences ($P < 0.05$).

Supplementary material

Lipidomic and proteomic comparative study of *Listeria monocytogenes* treated with AMPs revealed biofilm inhibition by Fengycin lipopeptides

Paolo Stincone, Flávio F. Veras, Giuseppe Micalizzi, Danilo Donnaruma, Gaetano Celano, Maria de Angelis, Luigi Mondello, Adriano Brandelli



Supplementary figure 1. Comparison of the Lysophosphatidylglycerols/Phosphatidylglycerols (LPG/PG) specific chromatograms in negative precursor ion scan mode, obtained by samples of *Listeria monocytogenes* treated with nisin, control and treated with cyclic lipopeptides, mainly fengycin, secreted by *Bacillus velezensis* P34. .

Supplementary Table 1. List of FAME derivatives identified in *Listeria monocytogenes* treated with nisin, fengycin and untreated samples. Abbreviations: MS Sim: database spectral similarity; LRI_{ref} : reference LRI; LRI_{exp} : experimental LRI;. FAMEs profile is expressed as mean percentage (%) of total fatty acids ± standard deviation (SD). The FAMEs are also grouped in saturated, branched and unsaturated compounds.

Compounds	**MS $_{Sim}$**	**LRI $_{ref}$**	**LRI $_{exp}$**	**Control**		**Nisin**		**Fengycin**	
				mean	**st. dev.**	**mean**	**st. dev.**	**mean**	**st. dev.**
C12:0	96	1200	1200	0.81	0.15	1.04	0.05	0.77	0.15
***iso*-C14:0**	93	1361	1361	0.82	0.03	0.73	0.07	0.60	0.10
C14:0	96	1400	1400	0.98	0.07	1.12	0.02	1.17	0.09
***iso*-C15:0**	93	1462	1461	17.24	1.04	15.76	0.98	15.14	2.16
***anteiso*-C15:0**	94	1476	1475	27.88	0.89	25.01	1.94	28.17	2.49
C15:0	90	1500	1500	0.19	0.02	0.19	0.01	0.20	0.01
***iso*-C16:0**	93	1560	1560	3.21	0.13	3.11	0.10	3.27	0.35
C16:0	96	1600	1600	16.13	1.53	18.83	1.87	14.46	2.11
***iso*-C17:0**	94	1660	1660	4.70	0.27	4.43	0.14	5.28	0.33
***anteiso*-C17:0**	96	1676	1676	15.53	0.43	14.38	0.54	18.45	1.06
C17:0	90	1700	1700	0.10	0.01	0.10	0.01	0.12	0.02
C18:0	96	1800	1800	11.87	1.27	14.53	1.69	10.79	3.09
C18:1n9	96	1811	1810	0.40	0.12	0.61	0.10	1.31	0.71
C20:0	90	2000	2000	0.14	0.02	0.18	0.02	0.27	0.28
TOTAL				100.00		100.00		100.00	
Saturated				30.21	3.07	35.99	3.65	27.78	5.75
Branched				69.38	2.78	63.41	3.76	70.91	6.48
iso				25.97	1.46	24.03	1.28	24.29	2.93
anteiso				43.41	1.32	39.39	2.47	46.62	3.55
Unsaturated				0.40	0.12	0.61	0.10	1.31	0.71

Supplementary Table 2. List of intact lipids identified by *UHPLC-MS/MS* analysis in *Listeria monocytogenes*. Abbreviations: LPC, lysophosphatidylcholine; LPG, lysophosphatidylglycerol; LPS; lysophosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerols; PS; phosphatidylserine; DG, diacylglycerols; DGDG; Digalactosyl-diacylglycerols; PN, partition number; RT, retention time

***m/z* product ions**					**Lipid Type**		**PN**	**RT**
$[M+H]^+$	$[M+H-H_2O]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$				
		485.3			LPC	14:0	14	0.48
		487.3			LPS	14:0	14	0.49
				469.1	LPG	15:0	15	0.7
				651.3	PG	27:0	27	2.27
				679.1	PG	29:0	29	2.88
	523.4				DG	30:0	30	3.18
				693.7	PG	30:0	30	3.21
	537.2				DG	31:0	31	3.59
			873.2		DGDG	29:0	29	3.65
				707.2	PG	31:0	31	3.62
				721.4	PG	32:0	32	3.95
	551.4				DG	32:0	32	3.97
			887.4		DGDG	30:0	30	3.97
				705.3	PG	31:1	29	4.32
	565.6				DG	33:0	33	4.46
			901.3		DGDG	31:0	31	4.46
				735.3	PG	33:0	33	4.48
				749.1	PG	34:0	34	4.67
	579.5				DG	34:0	34	4.72
			915.3		DGDG	32:0	32	4.72
			929.4		DGDG	33:0	33	5.09
680.3					PS	28:0	28	5.22
	607.2				DG	36:0	36	5.22
692.1					PE	32:0	32	5.23
			943.6		DGDG	34:0	34	5.42

Supplementary Table S3. PLS-DA values used to identify differentially expressed proteins related to the exposure of *L. monocytogenes* sub-lethal doses of nisin, and fengycin, with the VIP threshold > 1.0 in the first component of PLS-DA.

Protein name	**Gene name**	**VIP Score**		
		Comp. 1	Comp. 2	Comp. 3
Internalin B	*inlB*	5.03	4.87	4.87
Quinol oxidase subunit 2	*qoxA*	4.92	4.85	4.84
Lmo0066 protein	*lmo0066*	4.88	4.71	4.71
Glutathione biosynthesis bifunctional protein GshAB	*gshAB*	4.81	4.75	4.74
Lmo1539 protein	*lmo1539*	4.65	4.50	4.49
Uncharacterized PIN and TRAM-domain containing protein Lmo0234	*lmo0234*	4.54	4.51	4.51
Putative pyruvate, phosphate dikinase regulatory protein 1	*lmo1457*	4.53	4.40	4.39
Probable transaldolase 1	*tal1*	4.44	4.29	4.28
Lmo2074 protein	*lmo2074*	4.29	4.15	4.15
Lmo1030 protein	*lmo1030*	4.28	4.14	4.14
Lmo1976 protein	*lmo1976*	4.06	3.92	3.92
Lmo0847 protein	*lmo0847*	3.54	3.42	3.42
Peptide methionine sulfoxide reductase MsrB	*msrB*	3.31	3.21	3.21
Lmo0541 protein	*lmo0541*	3.31	3.28	3.28
Lmo0335 protein	*lmo0335*	3.30	3.20	3.20
Arginine repressor	*argR*	3.26	3.21	3.21
Lmo2469 protein	*lmo2469*	3.17	3.10	3.10
Putative zinc metalloprotease Lmo1318	*lmo1318*	3.15	3.05	3.05
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	*murA2*	3.12	3.08	3.07
Regulatory protein Spx	*spxA*	3.10	3.00	3.00
AA3-600 quinol oxidase subunit III	*qoxC*	3.09	3.09	3.08
Lmo0665 protein	*lmo0665*	3.09	3.00	3.00
Lmo0027 protein	*lmo0027*	3.06	3.00	2.99
ATP synthase subunit delta	*atpH*	3.04	3.02	3.01
Calcium-transporting ATPase lmo0841	*lmo0841*	3.03	2.99	2.99
Lmo2164 protein	*lmo2164*	3.00	2.96	2.96
Cell division protein FtsX	*ftsX*	3.00	3.00	2.99
Lmo2167 protein	*lmo2167*	3.00	2.91	2.91
Lmo1421 protein	*lmo1421*	2.98	2.91	2.91
Lmo0936 protein	*lmo0936*	2.96	2.87	2.87
Lmo0966 protein	*lmo0966*	2.96	2.86	2.85
Lmo0718 protein	*lmo0718*	2.92	2.85	2.84
Lmo1726 protein	*lmo1726*	2.91	2.83	2.82
Lmo1918 protein	*lmo1918*	2.90	2.83	2.83
Lmo0121 protein	*lmo0121*	2.88	2.81	2.81

Lmo2555 protein	*lmo2555*	2.88	2.78	2.78
Lmo1616 protein	*lmo1616*	2.86	2.78	2.78
Spermidine/putrescine import ATP-binding protein PotA	*potA*	2.82	2.79	2.78
Lmo2429 protein	*lmo2429*	2.79	2.76	2.75
Lmo1132 protein	*lmo1132*	2.76	2.67	2.68
Lmo0927 protein	*lmo0927*	2.76	2.77	2.76
Lmo0257 protein	*lmo0257*	2.76	2.67	2.66
Lmo1642 protein	*lmo1642*	2.74	2.67	2.67
Lmo1875 protein	*lmo1875*	2.71	2.64	2.64
Lmo0099 protein	*lmo0099*	2.66	2.61	2.61
Lmo2222 protein	*lmo2222*	2.56	2.56	2.56
Homoserine dehydrogenase	*hom*	2.09	2.11	2.11
Macro domain-containing protein lmo2759	*lmo2759*	1.92	1.90	1.90
Pyridoxal 5-phosphate synthase subunit PdxT	*pdxT*	1.92	1.89	1.89
Lmo0900 protein	*lmo0900*	1.90	1.84	1.84
Serine acetyltransferase	*cysE*	1.90	1.87	1.87
Lmo1255 protein	*lmo1255*	1.89	1.85	1.85
Two-component response regulator	*lisR*	1.87	1.82	1.82
Lmo0906 protein	*lmo0906*	1.86	1.82	1.82
Thymidylate synthase	*thyA*	1.86	1.80	1.80
Putative tRNA (cytidine(34)-2-O)-methyltransferase	*lmo0935*	1.82	1.76	1.76
Serine/threonine phosphatase stp	*stp*	1.77	1.73	1.73
Lmo2452 protein	*lmo2452*	1.75	1.82	1.81
Lmo0279 protein	*lmo0279*	1.73	1.69	1.69
Lmo1763 protein	*lmo1763*	1.72	1.93	1.92
Putative pyruvate, phosphate dikinase regulatory protein 2	*lmo1866*	1.71	1.70	1.72
Endopeptidase La	*lmo2051*	1.70	1.67	1.67
Peptide methionine sulfoxide reductase MsrA	*msrA*	1.68	1.63	1.63
Lmo1521 protein	*lmo1521*	1.67	1.64	1.64
Uncharacterized RNA methyltransferase lmo1751	*lmo1751*	1.67	1.62	1.62
Lmo0536 protein	*lmo0536*	1.66	1.64	1.65
UPF0176 protein lmo1384	*lmo1384*	1.66	1.61	1.61
Listeria epitope LemA	*lemA*	1.66	1.65	1.65
UPF0109 protein lmo1796	*lmo1796*	1.64	1.65	1.66
Probable endopeptidase p60	*iap*	1.64	1.59	1.59
Lipoprotein	*lmo2417*	1.63	1.61	1.61
Lmo2491 protein	*lmo2491*	1.63	1.57	1.57
Lmo0097 protein	*lmo0097*	1.61	1.64	1.64
Protease HtpX homolog	*htpX*	1.61	1.59	1.59
D-alanine--D-alanyl carrier protein ligase	*dltA*	1.61	1.57	1.57
Lmo0288 protein	*lmo0288*	1.61	1.64	1.64
Lmo2515 protein	*lmo2515*	1.61	1.56	1.57
3-hydroxy-3-methylglutaryl coenzyme A	*lmo0825*	1.61	1.56	1.57

reductase				
Copper homeostasis protein CutC	*cutC*	1.60	1.63	1.63
Lmo2195 protein	*lmo2195*	1.59	1.91	1.91
Uncharacterized RNA methyltransferase lmo1703	*lmo1703*	1.58	1.57	1.58
Lmo0415 protein	*lmo0415*	1.58	1.56	1.55
Lmo1022 protein	*lmo1022*	1.57	1.52	1.53
PbpA protein	*pbpA*	1.56	1.54	1.54
Lmo2462 protein	*lmo2462*	1.56	1.51	1.51
Lmo1643 protein	*lmo1643*	1.54	1.58	1.58
Lmo2683 protein	*lmo2683*	1.54	1.74	1.74
DNA mismatch repair protein MutL	*mutL*	1.54	1.49	1.49
Cytidylate kinase	*cmk*	1.54	1.54	1.54
Lmo1932 protein	*lmo1932*	1.54	1.50	1.50
Lmo2460 protein	*lmo2460*	1.54	1.51	1.50
Lmo2077 protein	*lmo2077*	1.53	1.66	1.67
Lmo1231 protein	*lmo1231*	1.53	1.50	1.50
Probable dual-specificity RNA methyltransferase RlmN	*rlmN*	1.53	1.75	1.75
Pyrroline-5-carboxylate reductase	*proC*	1.53	1.48	1.48
Phosphoribosylaminoimidazole-succinocarboxamide synthase	*purC*	1.52	1.49	1.49
Lmo2798 protein	*lmo2798*	1.51	1.50	1.50
DNA primase	*dnaG*	1.50	1.47	1.47
Lmo1223 protein	*lmo1223*	1.49	1.52	1.52
Primosomal protein N	*priA*	1.49	1.45	1.45
Lmo0673 protein	*lmo0673*	1.49	1.44	1.44
Amidophosphoribosyltransferase	*purF*	1.48	1.53	1.53
Lmo0640 protein	*lmo0640*	1.47	1.43	1.43
Lmo1324 protein	*lmo1324*	1.46	1.43	1.43
Lmo1744 protein	*lmo1744*	1.46	1.43	1.43
Lmo1862 protein	*lmo1862*	1.45	1.42	1.42
Lmo2705 protein	*lmo2705*	1.45	1.45	1.45
Lmo2427 protein	*lmo2427*	1.45	1.42	1.42
Lmo1467 protein	*lmo1467*	1.45	1.41	1.40
Lmo2184 protein	*lmo2184*	1.44	1.42	1.42
Lmo1395 protein	*lmo1395*	1.44	1.40	1.41
Lmo2831 protein	*lmo2831*	1.44	1.50	1.50
Lmo1090 protein	*lmo1090*	1.44	1.39	1.39
Aminotransferase	*lmo2252*	1.43	1.66	1.66
Lmo1941 protein	*lmo1941*	1.43	1.49	1.49
Lmo2518 protein	*lmo2518*	1.42	1.39	1.39
Lmo1745 protein	*lmo1745*	1.42	1.46	1.46
Lmo2710 protein	*lmo2710*	1.42	1.37	1.37
Lmo1353 protein	*lmo1353*	1.41	1.51	1.52
Lmo0773 protein	*lmo0773*	1.41	1.57	1.57
Diadenylate cyclase	*dacA*	1.40	1.73	1.73

Lmo1829 protein	*lmo1829*	1.40	1.36	1.36
Phosphoserine aminotransferase	*serC*	1.37	1.36	1.37
Pyridoxal phosphate homeostasis protein	*lmo2031*	1.37	1.49	1.49
Endoribonuclease YbeY	*ybeY*	1.36	1.54	1.54
Lmo1091 protein	*lmo1091*	1.36	1.34	1.35
DNA repair protein RecN	*recN*	1.35	1.31	1.33
Lmo1525 protein	*lmo1525*	1.34	1.71	1.72
Lmo1285 protein	*lmo1285*	1.34	1.31	1.31
RsbU protein	*rsbU*	1.34	1.30	1.30
dITP/XTP pyrophosphatase	*lmo1239*	1.34	1.32	1.32
Prephenate dehydratase	*pheA*	1.33	1.31	1.30
Uncharacterized protein Lmo0216	*lmo0216*	1.32	1.29	1.29
Citrate synthase	*citZ*	1.32	1.30	1.30
S-adenosylmethionine:tRNA ribosyltransferase-isomerase	*queA*	1.31	1.49	1.49
Lmo1511 protein	*lmo1511*	1.28	1.34	1.34
Ribosome biogenesis GTPase A	*lmo1272*	1.28	1.24	1.26
Lmo0955 protein	*lmo0955*	1.25	1.46	1.46
Isopentenyl-diphosphate delta-isomerase	*fni*	1.24	1.63	1.63
FolK protein	*folK*	1.16	1.15	1.15
Lmo2752 protein	*lmo2752*	1.11	1.41	1.40

Supplementary Table 4. MS/MS events optimized for each lipid class detected in Listeria monocytogenes when *exposed* to sub-lethal doses of nisin and fengycin.

Class	MS/MS event	Polarity	Diagnostic fragment	Energy (V)
LPC/PC/SM	Precursor ion	+	184 *m/z*	-25
LPE/PE	Neutral loss	+	141 Da	-30
LPG/PG	Neutral loss	-	153 Da	+35
LPI/PI	Precursor ion	-	241 *m/z*	+50
LPS/PS	Neutral loss	+	185 Da	-28

Abbreviation: LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophophatidylinositol; LPG, lysophosphatidylglycerol; LPS; lysophosphatidylserine; PC, phosphatodylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerols; PS; lysophosphatidylserine; SM, sphyngomyelin.

4 GENERAL DISCUSSION

The four papers reported in this thesis have given information about the possible use of cyclic lipopeptides (CLPs) in the food industry to face the contamination of *L. monocytogenes*. These CLPs were secreted by a bacterium included into the *Bacillus* genus isolated from a freshwater fish of Brazilian Amazon basin.

To begin with, the phylogenetic identification through MLSA as *Bacillus velezensis* P34 of the bacterium chosen in this work as producer of CLPs. Full genome sequencing permitted the annotation and identification of the main bioactive gene cluster compounds synthesized by this group of bacteria. Beside that, the characterization through mass spectrometry analysis of the cyclic lipopeptides compounds secreted by the bacterium were included in this thesis. In addition, the physiological reaction of the foodborne bacteria *L. monocytogenes* to AMPs were investigated using proteomics and lipidomics approaches, a comparison of the sublethal concentrations of CLPs and nisin and their action on the menthinate pathogen were carried out; specifically, the synthesized compounds by the *Bacillus velezensis* P34 and the commercial nisin secreted by *Lactococcus lactis* were employed.

Thus, investigating the results, coming from the use of -omics approaches, two main processes were observed deeply involved by the action of these AMPs, specifically the *L. monocytogenes* differential biofilm regulation and virulence processes blockage.

The discovery and/or development of new antibacterial drugs was considered a crucial step to overcome the great challenge posed by the emergence of antibiotic resistance (DA CUNHA *et al.*, 2017). The characterization of the antimicrobial compounds synthesized by *Bacillus velezensis* P34 and released into the supernatant cultures medium included the concentration of CLP compounds through n-butanol extraction. These compounds responsible for the

antimicrobial activity were detected as CLPs components by the group previously (MOTTA *et al.,* 2007),

During the last decades, mass spectrometry analysis has become the dominant analytical technology for the bioactive peptide discovery (MOYER *et al.,* 2021). In this work, Maldi-tof and Qtof UPLC Ms-Ms were employed for the compounds' characterization. Metabolomics is one of the main omics techniques aiming to qualitatively and quantitatively describe a microbial metabolome. Two of the most popular analytical strategies prevailing in the metabolomics field were considered the mass spectrometry (MS) and nuclear magnetic resonance (NMR) (GAO AND XU, 2015). For the identification of the CLPs employed in this work, the liquid chromatography coupled with mass spectrometry was chosen. In general, LC-MS include targeted and non-targeted approaches, respectively: (i) non-targeted LC–MS metabolomics need to be capable of analysing complex biological samples and should ideally operate at high scan rates with high mass accuracy and resolution; (ii) whereas the targeted LC–MS is the technique of choice for the reliable quantization of known, pre-selected metabolites often previous discovered by non-targeted metabolomics; (iii) an additional approach is typically associated with a specific sample extraction protocol for the metabolites of interest and was named “semi-targeted” LC-MS (VIANT AND SOMMER, 2013). In this work, a “semi-targeted” LC-MS method was employed considering a previous specific n-butanol extraction method for CLPs, this latter extraction method was considered as one of the more efficient methods for the cyclic lipopeptides extraction by liquid medium (JIAO *et al.,* 2021). In order to obtain the exact characterization of the AMPs secreted by the bacterium beside these analytical investigations, the genetic information coming from the genome analysis was also considered. The bacterial AMP targets that did not appear directly act on the membrane integrity but up to specific conserved components including lipopolysaccharides (LPS), lipoteichoic acids (LTA) and the peptidoglycan precursor Lipid

II. The molecular interaction became essential for the AMPs antibacterial activity action, in this context the genetic bases of AMPs variants revealed the shape of new functions and functional divergence (SCHMITT *et al.,* 2016). Antismash annotations of *Bacillus*' whole genome represent a pivotal tool for the detection of the gene clusters related with bioactivities, among others. This online software permitted the identification of the biosynthetic clusters of surfactin, iturin, and fengycin; these latter compound family groups are the main components of the CLPs (XU *et al.,* 2018). Thus, the genomics guided discovery of the CPLs component was realized in this work employing the Antismash annotation that provided the sequence genetic clusters, permitting the alignment of the core DNA sequences for the iturin synthesis cluster confirming the analytical detection of the family isoforms bacillomycin L as a component produced by *Bacillus velezensis* P34. The other two components quantified as a major fraction of the n-butanol extract were the Fengycin A and B, which differ by the nature of the amino acid at position 6 being Ala or Val, respectively in fengycin A and B (TAO *et al.,* 2011, YANG *et al.,* 2015). Fengycin like plipastatin consists of a decapeptide chain (L-Glu – D-Orn – D- (L)-Tyr – D-Thr – L-Glu – D-Ala – L-Pro –L-Gln – L-(D)-Tyr – L-Ile), eight of these amino acids form a peptide ring and remaining two are linked to a 3-hydroxy fatty acid with 14–19 carbon atoms that may be saturated or unsaturated (WU *et al.,* 2007; GAO *et al.,* 2018). However fundamental become the differentiation between plipastatin and fengycin which are almost identical, the changes involved the tyrosine amino acids in position 6 and 9 showed enantiomers form inverted respectively L- and D-form in plipastatin and D- and L-form in fengycin (RAAIJMAKERS *et al.,* 2010; INES AND DHOUHA, 2015; HUSSEIN, 2019). In a recent study correlating the biosynthetic gene clusters and phylogeny it was possible to get information about the distribution of these CLPs inside the Bacillus genus, specifically fengycin and plipastatin were observed respecting a clade distribution, with fengycin found in *B. velezensis* and *B.*

amyloliquefaciens, whereas plipastatin was detected in *B. subtilis* and *B. atrophaeus* (STEINKE *et al.,* 2021). In addition, after the characterization of the antimicrobial compounds synthesized by *Bacillus velezensis* P34, in this thesis work, sublethal concentrations of AMPs were administered in *L. monocytogenes* liquid cultures to stress the bacteria and detect the possible strategies put in place to resist these antimicrobials. In this context besides the fengycin lipopeptides secreted by mentioned *Bacillus* strain, the commercial bacteriocin named nisin was employed as a comparison treatment trying to design the possible employment of the fengycin lipopeptides in food industry AMPs treatment. The carried out high-throughput technologies permitted to detect a biofilm promotion as a resistance strategy of *L. monocytogenes* to contrast the sublethal nisin administration.

The *L. monocytogenes'* ability to form biofilm on several surfaces used in the food industry has been largely documented and represents a serious concern for food safety being a possible source of contamination (COLAGIORGI *et al.,* 2017). Scientific studies have so far focused on *L. monocytogenes* persistence in the food industry and the harborage sites represent for the bacteria the places favoring the growth (CARPENTIER AND CERF, 2011) and in this way also cooked food can be contaminated by *L.monocytogenes* as the result of post-process contamination. Moreover, the promotion of the biofilm by this bacteria seems to be influenced by environmental factors including among those extensively studied temperature, salts, nutrition, and pH (SILVA *et al.,* 2020). Besides these abiotic factors, the biofilm organization permits the community to protect themselves against antimicrobial agents including those synthesized from the bacterial community (MAH AND O'TOOLE, 2001).

Food-grade sanitizers play an essential role in the *L. monocytogenes* control of its presence, considering also the ability to build a biofilm structure organization that reduces the bacteria

susceptibility to sanitizers in comparison to the planktonic organization (ANDRADE *et al.*, 2020). The difficulty to eradicate and control *L. monocytogenes* in biofilm has become harder than suspended bacterial cells, although many attempts have been made to develop control strategies to be applied, in the food industry seems to be no clear direction on how to manage the risk coming from this kind of pathogen (MAZAHERI *et al*., 2021).

The search for strategies acting on the *L. monocytogenes* biofilm inhibition represents an increasing trend in the food industry researches, including green technology approaches to prevent or control the formation of this multicellular organization, a serious concern being a possible source of contamination (OLOKETUYI AND KHAN, 2017). Novel biocontrol methods were reported for their action in biofilms organization present in food industries, representing effective means to help improve control of *L. monocytogenes* and decrease cross-contamination of food (GRAY *et al.*, 2018). Essential oils have been studied for their possible action against the biofilm organization in *L. monocytogenes* (ZHANG *et al.*,2021). However, such compounds that have been reported for disadvantages are usually correlated with the high dosage or long contact period, low solubility in water, or strong smell that might lead to products deterioration (FORAUER *et al.*, 2021). Recently, other strategies have been explored as possible alternatives capable of eliminating the mature *L. monocytogenes* biofilm, including the recent enzymatic treatments (MAZAHERI *et al.*, 2020; ZHANG *et al.*, 2021); the bacteriophages treatment with their action on the cell wall of Gram-positive bacteria even if many factor act on its effectiveness (PARASION *et al.*, 2014); non-pathogenic microorganisms, which can assist sanitation approaches in controlling biofilms preventing or eradicating unwanted species like foodborne pathogens. In this context, the lactic acid bacteria (LAB) showed important results against *L. monocytogenes* biofilm (ZHAO *et al.*, 2013).

In fact, studies investigating the Listeria biofilm production and the inhibitory effect of bacteriocins synthesized by LAB, considered as "friendly" antimicrobial agents, have been carried out with encouraging results in biofilm reduction in various food industries surfaces, suggesting the possible pre-treatment with LAB or by their bacteriocins (WINKELSTRÖTER *et al.*, 2015; PÉREZ-IBARRECHE *et al*., 2016; CAMARGO *et al*., 2018).

However, in this study contradictory results were revealed. The application of sublethal concentrations of the commercial bacteriocin nisin, produced by *Lactococcus lactis subsp. lactis*, a LAB bacterium, promoted the biofilm organization involving a set of proteins located between the *L. monocytogenes* cytoplasmatic membrane and the cell wall named peripheral cell component (PCC), including moonlight proteins. In this context, quantities employed seem to be the main factor acting on the biofilm promotion. However, considering these results and the fact that similar quantities of nisin were allowed to be used in some milk prepared products, pivotal became the evaluation of the risks on food industries. Moreover, nisin resistance is slowly being reported into the last decades, thus a great deal of research has gone into identifying more strategies active against *L. monocytogenes* planktonic cells and biofilms.

Following the idea developed through this thesis, the possibility to use cyclic lipopeptides against the foodborne *L. monocytogenes* was proposed. The obtained results, indeed, have shown the biofilm inhibition already in a sublethal concentration of the fengycin lipopeptides secreted by *Bacillus velezensis* P34. For the first time, a possible application of these compounds to contrast *L. monocytogenes* biofilm was shown, a huge concern for the food industries. Proteomic and lipidomic analysis on Listeria suggested the action on the biofilm building inhibition activity, the data were supported by biofilm quantification analysis. One of the main proteins downregulated by the fengycin lipopeptides treatment was the wall

teichoic acids synthetase, whose absence impacts antigenicity, sensitivity to antimicrobials, and virulence and for this reason, emerged as promising drug targets (MEIRELES *et al.*, 2020).

The use of antimicrobials needs to overcome some concerns before being applied in food industries and the use of nanostructures may work in this direction. Nanotechnology in food science represents an interesting alternative to protect and deliver antimicrobials including AMPs in food, providing the controlled release and the structural stability maintaining of these actives compounds (LOPES AND BRANDELLI, 2018). Evaluation of the fengycin lipopeptides encapsulation into nanoliposomes was also considered in this work and the action of sublethal concentration of CLPs in *L. monocytogenes* was detected through proteomics analysis. Previous work showed the nanoencapsulated cyclic lipopeptides produced by the *Bacillus velezensis* P34 mode of action; specifically, it was demonstrated how nanoparticles contact but not fusion with the bacterial cell wall of *L. monocytogenes*, and probably for a controlled release of the AMPs the bacteria inhibition was retarded compared with the free cyclic lipopeptides application (DA SILVA MALHEIROS *et al.*, 2011). The nanoliposome encapsulating bioactive peptides may offer the possibility of application in food science and technology areas to increase the shelf life of food products reducing the action of foodborne pathogens, considering the ability to maintain the nanostructured physicochemical parameters for 30 days (CORRÊA *et al.*, 2019). The proteomic analysis revealed a group of proteins differentially regulated in *L. monocytogenes* cultures treated with nano encapsulated fengycin lipopeptides P34. Specifically, these results indicate a correlation with the decreased virulence, registered also by the action of free fengycin.

The virulence process, indeed, was another detected as a fundamental target when the pathogen considered in this work was treated with the fengycin lipopeptides in both cases free and encapsulated in nanoliposome.

A strong reduction of a set of proteins related with the stress tolerance and under the transcription of the σB and VirR regulons were reported together with a possible negative regulation of the main key virulence factors Prfa involved in the pathogenicity of *L. monocytogenes*. The results showed an AMPs stressful condition for the bacteria compromising the ability to contrast environmental stress and the possible pathogenic action into the human cells.

The regulatory protein PrfA integrates various environmental signals that coordinate the transition between two contrasting bacterium lifestyles and activate a set of virulence factors during host infection (DE LAS HERAS *et al.,* 2011). Moreover in the *L. monocytogenes* cycle of life, the σB regulator also coordinates with PrfA the transcriptional activity to ensure the shift between the environmental stress conditions and the host conditions during the infection (GABALLA *et al.,* 2019). The observed deregulation of the important VirR regulon for *L. monocytogenes* virulence also represents another important result considering the revealed role played by this on bacteria resistance to commercially applied antimicrobial compounds that are used in food, including nisin, lauric arginate, and chitosan (KANG *et al.,* 2015).

Furthermore, the key virulence factor PrfA coordinates the transcription of the well-characterized bacterial virulence factors required for entry, phagosomal escape, and cell-to-cell spread into the human host cells. Some of these pivotal proteins for the pathogenicity were detected downregulated among the PCC by the sublethal treated samples with nisin. Also in this context, as on the biofilm promotion process by the nisin treated samples, the moonlight proteins were considered full involved in the process. The moonlight proteins were defined as single polypeptides proteins showing more than one function. Often, these proteins were involved in metabolic regulation or the cell stress response and additional biological

actions including bacterial virulence (HENDERSON AND MARTIN, 2011). Thus, these multitasking proteins represent an important strategy developed by a pathogen, being proteins highly conserved between pathogen and also by the host counterparts share of some epitopes, and, for this reason, the host immune system will not elicit protective antibodies against these pathogen proteins to avoid autoimmune diseases (FRANCO-SERRANO *et al.,* 2018).

Another important process detected differentially downregulated by the fengycin lipopeptides action was related with the membrane proteins involving the metal ions transport, essential in *L. monocytogenes* for the host invasion process. Specifically, the strong downregulation of the manganese transport proteins, important lipoprotein also on the *S. aureus* virulence (RADIN *et al.*,2018) were already reported as possible antibiotic target methicillin-resistant *S. aureus* MRSA (DIEP *et al.,* 2014). Thus, future studies about the action of the fengycin lipopeptides on the metal ions transport are strongly encouraged following the preliminary results obtained by this work. In addition, the results coming from this Ph.D. work permit to suggest future studies for the application of fengycin CLPs metabolites in the food industry as a natural sanitizer or food additive, to contrast the concern represented by the bacterial pathogen biofilm multicellular organization. In this context, future feasibility studies may be planed including the fengycin activity evaluation when added to other chemical sanitizers commonly used in food industries or the possible application of this CLPs component directly in food where *L. monocytogenes* biofilm has been detected with the related possible dangerousness.

5 CONCLUSION

In conclusion, considering all the results of this work through the analysis of the genome and the mass spectrometry characterization of the CLPs, as well as the others -omic approaches employed to detected the *L. monocytogenes* responses to these AMPs , it can be inferred that

the fengycin detected as the major CLPs components synthesized by *B. velezensis* P34 showed the potential to be used in food industries among the others possible application. The application of fengycin on the foodborne pathogen *L. monocytogenes* has provided interesting results with the involvement of fundamental processes, including bacterial pathogenicity and resistance. For the first time, the carried out proteomic study showed the association of fengycin with the reduction of proteins related with the σB and VirR regulons acting against the environmental stress and the PrfA key virulence factor during the host intracellular invasion. Proteomic and lipidomic comparison analysis between nisin and fengycin action on *L. monocytogenes* permitted to detect the fengycin action on the biofilm inhibition. This latter represents one of the *Listeria* resistance process to contrast the stress coming from the AMPs action, as demonstrated by the biofilm promotion when the commercial bacteriocin nisin was administered in sublethal concentration, with the active involvement of the "proteosurfaceome" including moonlight proteins.

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7 APPENDICES

7.1 Manuscript published on the journal Microbiological Research

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Diversity of cyclic antimicrobial lipopeptides from *Bacillus* P34 revealed by functional annotation and comparative genome analysis

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ARTICLE INFO

Keywords:
Antimicrobial peptides
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ABSTRACT

Cyclic lipopeptides (CLPs) from *Bacillus* strains have demonstrated a wide range of bioactivities making them interesting candidates for different applications in the pharmaceutical, food and biotechnological industries. Genome sequencing, together with phylogenetic analysis of the *Bacillus* sp. P34, isolated from a freshwater fish gut, showed that the bacterial strain belongs to the *Bacillus velezensis* group. *In silico* investigation of metabolic gene clusters of nonribosomal peptide synthetases (NRPS) revealed the genetic elements associated with the synthesis of surfactin, fengycin and iturin family component bacillomycin. Further, an assay was conducted to investigate the production of CLPs in the presence of heat inactivated bacterial cultures or fungal spores. Maximum fengycin concentration was observed at 24 h (2300 – 2700 mg/mL), while maximum iturin amounts were detected at 48 h (250 mg/mL) in the presence of heat-inactivated spores of *Aspergillus niger*. Heat-inactivated cells of *Listeria monocytogenes* caused a reduction of both fengycin and iturin amounts. The production of fengycins A and B and the iturin family component bacillomycin L was confirmed by mass spectrometry analyses. This study reinforces the potential of *B. velezensis* P34 as a valuable strain for biotechnological production of CLPs recognized as important antimicrobial substances.

7.2 Manuscript published on the journal Molecular Omics

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Proteomic analysis of *Listeria monocytogenes* exposed to free and nanostructured antimicrobial lipopeptides†

Paolo Stincone, Carolina Baldisserotto Comerlato and Adriano Brandelli *

In this work, the effect of antimicrobial lipopeptide P34 on *Listeria monocytogenes* was evaluated for the first time through a proteomics approach. Bacteria were treated with sub-lethal doses of peptide P34 (F-P34) and P34 encapsulated into nanoliposomes (N-P34), while empty nanoliposomes (NE) and fresh buffer were used as controls. The proteomic analysis allowed the detection of one group of proteins commonly differentially represented in response to free and encapsulated P34 exposure. A second group of proteins was found to be exclusively differentially represented after exposure with encapsulated P34 only. The antimicrobial peptide P34 caused a significant downregulation of proteins associated with the transport of manganese and the over-representation of proteins related with iron transport in *L. monocytogenes*. In addition, reduction of stress tolerance proteins related to the σB and VirR regulons, together with the modulation of phosphoenolpyruvate phosphotransferase systems (PTS) for sugar transport were observed. The sugar and oligopeptide transporters regulated by antimicrobial action may influence the key virulence factor PrfA, reducing the pathogenicity of this microorganism.

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Nisin influence on the expression of *Listeria monocytogenes* surface proteins

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Keywords:
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ABSTRACT

In this work, a comparative analysis of the peripheral cell component (PCC) proteins of *Listeria monocytogenes* was carried out. The study was conducted on two set of samples consisting of bacteria treated with sub-lethal concentration of nisin and untreated bacteria as control. PCC proteins were extracted by Tris-Urea-EDTA treatment and then subjected to trypsin digestion and mass spectrometry analysis. The whole cell proteome was analyzed through label-free quantitative proteomics approach. Proteomic analysis was carried out using OrbiTrap Mass Spectrometer coupled to nanoflow liquid chromatography. The treatment with sub-lethal nisin concentration resulted in 62 up regulated and 97 down regulated proteins compared to untreated samples. Using PSORTb 3.0, 19 and 18 surface proteins were detected among the up regulated and down regulated proteins, respectively. Proteins related with increased biofilm formation by *L. monocytogenes*, such as moonlight proteins of the pyruvate dehydrogenase complex and flagellin-related proteins, were identified as up regulated surface proteins. Proteins associated with virulence of *L. monocytogenes*, including listeriolysin O, internalin B and actin assembly-inducing protein, were detected among the down regulated proteins. To confirm proteomics data, increased production of biofilm was experimentally confirmed in nisin-treated cells through crystal violet method.

Biological significance: Proteosurfaceomics can be defined as the "omics" science applied to the proteins of the peripheral cell component (PCC). The surface proteins of *Listeria monocytogenes*, an important foodborne pathogen were investigated after treatment with nisin, a bacteriocin approved as a natural food preservative by regulatory agencies. Recent cases of nisin tolerance by *Listeria* spp. were documented, and deeper studies on the molecular process behind the bacterial survival may help in both understanding the development of tolerance process and comparing nisin effect with other antimicrobial compounds.

7.4 Manuscript published on the journal Critical Reviews in Biotechnology

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CRITICAL REVIEWS IN BIOTECHNOLOGY
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REVIEW ARTICLE

Marine bacteria as source of antimicrobial compounds

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ABSTRACT

The marine environment encompasses a huge biological diversity and can be considered as an underexplored location for prospecting bioactive molecules. In this review, the current state of art about antimicrobial molecules from marine bacteria has been summarized considering the main phylum and sources evolved in a marine environment. Considering the last two decades, we have found as most studied group of bacteria producers of substances with antimicrobial activity is the Firmicutes phylum, in particular strains of the *Bacillus* genus. The reason for that can be attributed to the difficult cultivation of typical Actinobacteria from a marine sediment, whose members are the major producers of antimicrobial substances in land environments. However, a reversed trend has been observed in recent years with an increasing number of reports settling on Actinobacteria. Great diversity of chemical structures have been identified, such as fijimicyns and lynamicyns from Actinomycetes and macrolactins produced by *Bacillus*.

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Antibiotic; antimicrobial; bioactive compounds; fijimycins; macrolactins; marine bacteria; marine biology

7.5 Manuscript submitted on the journal Cellular and Molecular Life Sciences

"Lipidomic and proteomic analysis of Listeria monocytogenes reveals modulation of biofilm formation by exposure to antimicrobial peptides"
Full author list: Paolo Stincone; Flávio Fonseca Veras; Giuseppe Micalizzi; Danilo Donnaruma; Gaetano Vitale Celano; Maria de Angelis; Luigi Mondello; Adriano Brandelli

Dear Dr. Stincone,

We have received the submission entitled: "Lipidomic and proteomic analysis of Listeria monocytogenes reveals modulation of biofilm formation by exposure to antimicrobial peptides" for possible publication in Cellular and Molecular Life Sciences, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Prof. Adriano Brandelli who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Cellular and Molecular Life Sciences

8 CURRICULUM VITAE

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2) EDUCATION

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3) INTERNSHIP

Voluntary internship (September – November 2020, Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Italy, Prof. Dr. Maria De Angelis)
Curricular Internship (March- June, 2014 Spanish National Research Council -IPNA, Spain, Dr. Mikel Becerro)
School of Advanced Science on Mass Spectrometry-based Proteomics (August 2017, Brazilian Biosciences National Laboratory (LNBio), Campinas, Brazil)

4)PROFESSIONAL EXPERIENCE

Research collaborator (2013 – 2015**,** Italian National Research Council, Italy, Dr. Angela Cuttitta)
Collaborator on research vessel (July-August 2015, 2013 – 2015, Italian National Research Council, Italy, Dr. Angela Cuttitta and Dr. Bennardo Patti)

5) PUBLISHED ARTICES

STINCONE, P; COMERLATO, C; & BRANDELLI, A. Proteomic analysis of Listeria monocytogenes exposed to free and nanostructured antimicrobial lipopeptides. *Molecular Omics*, 2021.

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