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ORIGINAL ARTICLE

Listeria monocytogenes exposed to antimicrobial peptides displays differential regulation of lipids and proteins associated to stress response

Paolo Stincone[1] · Flávio Fonseca Veras[1] · Giuseppe Micalizzi[2] · Danilo Donnarumma[2] · Gaetano Vitale Celano[4] · Daniel Petras[5] · Maria de Angelis[6] · Luigi Mondello[2,3] · Adriano Brandelli[1]

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Abstract

With the onset of *Listeria monocytogenes* resistance to the bacteriocin nisin, the search for alternative antimicrobial treatments is of fundamental importance. In this work, we set out to investigate proteins and lipids involved in the resistance mechanisms of *L. monocytogenes* against the antimicrobial peptides (AMPs) nisin and fengycin. The effect of sub-lethal concentrations of nisin and lipopeptide fengycin secreted by *Bacillus velezensis* P34 on *L. monocytogenes* was investigated by mass spectrometry-based lipidomics and proteomics. Both AMPs caused a differential regulation of biofilm formation, confirming the promotion of cell attachment and biofilm assembling after treatment with nisin, whereas growth inhibition was observed after fengycin treatment. Anteiso branched-chain fatty acids were detected in higher amounts in fengycin-treated samples (46.6%) as compared to nisin-treated and control samples (39.4% and 43.4%, respectively). In addition, a higher relative abundance of 30:0, 31:0 and 32:0 phosphatidylglycerol species was detected in fengycin-treated samples. The lipidomics data suggest the inhibition of biofilm formation by the fengycin treatment, while the proteomics data revealed downregulation of important cell wall proteins involved in the building of biofilms, such as the lipoteichoic acid backbone synthesis (Lmo0927) and the flagella-related (Lmo0718) proteins among others. Together, these results provide new insights into the modification of lipid and protein profiles and biofilm formation in *L. monocytogenes* upon exposure to antimicrobial peptides.

Keywords Antimicrobial peptides · Antimicrobial resistance · Nisin · Fengycin · Fatty acids · Membrane proteins

Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis, a severe disease affecting primarily immunocompromised people, pregnant women and children, causing septicemia, meningitis and abortions [1]. The main route of *L. monocytogenes* transmission in cases of food contamination occurs during the production process [2]. The food industry uses antimicrobial compounds for extending shelf life and limiting the spread of foodborne pathogens, including *L. monocytogenes* [3]. Bacteria produce diverse types of antimicrobial peptides (AMPs), including those ribosomally synthesized, such as bacteriocins [4], and non-ribosomally synthesized AMPs, e.g., cyclic lipopeptides (CLPs) [5].

The lantibiotic nisin, a positively charged bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, is widely used to control pathogens like *L. monocytogenes* in foods [6, 7]. Nisin inhibits bacterial growth through a dual mode of action, combining the generation of pores in the cell membrane and interruption of the cell wall biosynthesis mediated by specific interaction with the lipid II, which is recognized as a precursor component of bacterial cell wall [8, 9]. However, the acquisition of nisin tolerance or resistance in *L. monocytogenes* has already been observed. The antimicrobial resistance of *L. monocytogenes* to nisin can be influenced by environmental conditions and genetic characteristics [10, 11]. Some strains have high natural nisin resistance compared with others. As a consequence, the efficacy of this bacteriocin might be reduced [12, 13]. The upregulation of membrane proteins involved in biofilm

✉ Adriano Brandelli
abrand@ufrgs.br

Extended author information available on the last page of the article

formation was previously reported among the strategies employed by *L. monocytogenes* to evade nisin action [14].

Bacillus strains host multiple biosynthetic gene clusters that confer the ability to produce CLPs, collectively grouped into the surfactin, fengycin and iturin families [15]. *Bacillus velezensis* P34, a strain isolated from the intestine of an Amazonian fish, produces CLPs, mainly fengycin [16]. Although fengycin has been mostly reported as a potent antifungal lipopeptide [17], its antibacterial activity is also recognized [18]. 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 [19].

The phospholipid phosphatidylglycerol (PG), along with cardiolipin, lipoteichoic acid (LTA) and phosphatidylethanolamine (PE) has been described to play a crucial role in membrane stability in *Listeria* [20, 21]. Surface charge modifications induced by changes in teichoic acids and membrane lipids have been included among the variety of resistance mechanisms to AMPs [22, 23]. Such modifications influence the interaction of AMPs with bacterial cells and consequently their bacteriolytic activity, often explained by two different mechanisms [24, 25]: (a) after binding to cell surface components, the peptide penetrates the membrane by forming channels, as in the case of nisin, or (b) the peptide aggregates on the membrane surface causing damage, a mechanism that may be exerted by fengycin [26, 27].

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 of biological membranes [28]. The composition of bacterial cell surface also influences the biofilm formation capacity, already considered as one of the resistance strategies of *L. monocytogenes*. Biofilms formed by this foodborne pathogen contain teichoic acids as a major matrix component [29] and can be developed on different surfaces and inaccessible locations of food processing facilities, which is an important problem for the management of *L. monocytogenes* contaminations. Taking into account the biofilm formation process allows proposing better hygienic practices against this pathogen [30, 31].

The hypothesis we set out to test in this study is that *L. monocytogenes* is able to form biofilm as resistance strategy to sub-lethal concentration of nisin, and that the use of AMPs such as the CLP fengycin might overcome this concern. To test this hypothesis, *L. monocytogenes* was exposed to sub-lethal doses of nisin and fengycin and the molecular responses behind the bacterial resistance and biofilm modulation were investigated via mass spectrometry-based lipidomics and proteomics approaches.

Materials and methods

Chemicals

Reagents to perform the derivatization of lipid components into fatty acid methyl ester (FAME) 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).

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 a 10 mM sodium phosphate buffer (pH 7.0). The sub-lethal nisin concentration of 0.1 µg/mL was previously determined through the study of inhibitory effects on microbial growth curves [14].

The production of fengycin peptides by *B. velezensis* P34 was carried out as described previously [16]. 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 elsewhere [32].

Listeria monocytogenes cultivation

Listeria monocytogenes ATCC 7644 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 defined quantities of antimicrobials (fengycin or nisin) dissolved in 10 mM phosphate buffer pH 7.0 were added to the cultures and samples were incubated for additional 1 h. Control samples of untreated bacteria were prepared using phosphate buffer only. The cellular pellets were collected by centrifugation at 10,000g for 15 min at 4 °C, washed with PBS buffer pH 7.4 before

protein and lipid extraction. Additional bacterial cultures were performed to determine *L. monocytogenes* growth curves for each treatment. All tests were performed using three biological replicates.

Extraction of lipid components

The lipid fraction of *L. monocytogenes* was initially extracted using Bligh and Dyer protocol as recently reported [33]. Briefly, 4 mL of a methanol/chloroform solution (2:1 v:v) was added to each cellular pellet contained in a 25 mL Falcon tube. The extraction mixture was homogenized using vortex mixing (5 min). After, 1 mL of chloroform and 2 mL of aqueous sodium chloride (NaCl) saturated solution were added to the mixture. The sample was vortexed and centrifuged for 10 min at 2000 g to separate methanol and chloroform layers. The lower organic phase containing lipid compounds was collected using a Pasteur pipette and transferred to a 2 mL vial. The chloroform solvent was removed under constant nitrogen flow.

Derivatization of lipid extract

The derivatization of lipid compounds into FAMEs for gas chromatography (GC) analysis was performed as reported earlier [34]. Briefly, the procedure involved the use of two different derivatizing agents: methanolic solution of MeONa (0.5% w/v) and methanolic solution of BF_3 (14% w/v). An aliquot (500 µL) of the derivatizing agents was added to the lipid extract, 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 directly injected into GC instrumentation for chromatographic separation in triplicate.

GC–MS and GC–FID analysis

The separation and identification of FAME derivatives were carried out using a GC/MS QP2010 Ultra (Shimadzu, Duisburg, Germany) instrument equipped with a split–splitless injector (280 °C) and an AOC-20i auto-sampler. A medium-polarity ionic liquid (IL) column, named SLB-IL60 30 m × 0.25 mm *id* × 0.25 µm d_f (Merck Life Science) was used for the separation of FAMEs. Quantitative analyses were performed with a GC-2010 instrument (Shimadzu) equipped with a split–split-less injector (280 °C), a flame ionization detector (FID) and AOC-20i auto-sampler. Chromatographic conditions included the volume injections of 3.0 µL, the following temperature program 50 °C–280 °C at 3.0 °C/min, the helium used as carrier gas at 30 cm/s linear velocity and a pressure of 26.6 kPa. MS parameters were as follows: mass range 40–550 amu, ion source temperature 220 °C, interface temperature 250 °C. The FID parameters include detector temperature settled at 300 °C (sampling rate 40 ms) and gas flows were 40 mL/min for hydrogen, 40 mL/min for make up (nitrogen) and 400 mL/min for air, respectively. Carrier gas was helium, at a constant linear velocity of 30.0 cm/s and a pressure of 99.4 kPa. Data collection and peak assignment were the same as those described in detail previously [34].

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 auto-sampler. The UHPLC system was coupled to a LCMS-8060 triple quadrupole mass spectrometer equipped with ESI interface (Shimadzu). Mobile phases were: (A) 20 mM ammonium formate and (B) 2-propanol/acetonitrile/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 used was Ascentis Express C18, 100 × 2.1 mm, 2.7 µm dp (Merck Life Science, Darmstadt, Germany), the oven was set at 40 °C and the injection volume was 5 µL. MS and MS/MS acquisitions were performed using ESI source switching between 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 elsewhere [35].

Lipidomics data analysis

Data acquisition and processing were performed with the LabSolution ver. 5.95 software (Shimadzu Europa, Duisburg, Germany). The LIPID MAPS Structure Database (LMSD; https://www.lipidmaps.org/data/structure/) was employed for compound identification [36] by searching for the ion types reported in the Table S1, using a mass tolerance of ± 0.2 m/*z*. Additionally, raw data were converted to mzML format using MSconvert [37] and XIC was manually inspected via the GNPS Dashboard [38]. The urls for XIC visualizations through the GNPS dashboard are provided in the supplementary information.

Total protein extraction and processing

The preparation of protein samples and proteomic data acquisition were performed as reported previously [39,

40]. Briefly, bacterial cells were suspended in Tris–HCl buffer pH 7.5 containing 10 µL Halt™ protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and sonicated for 5 cycles of 30 s with intervals of 1 min in an ultrasonic cell disruptor in ice bath to maintain low temperatures. The lysed cell solutions were centrifuged at 10,000 g for 20 min and the supernatants were lyophilized before protein quantification by the Bradford method. Protein samples at a concentration of 10 µg/mL were digested following standard protocols [41], using 2 µg MS grade trypsin (Promega, Madison, WI, USA). Sample desalination was performed employing C18 stage tips, using 60% (v/v) acetonitrile and 0.1% (v/v) formic acid for peptides elution [42]. The samples were dried in a vacuum concentrator and stored at −20 °C for further analysis by nanoLC–MS/MS.

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. Nano-electrospray voltage was set to 2.2 kV, and the source temperature was set to 275 °C. The DDA setting included a resolution $r = 60{,}000$ and the 20 peptides with higher intense peptide ions (top 20) with charge states ≥ 2 were sequentially isolated to a target value of 5000 and fragmented in the high-pressure linear ion trap by CID (collision-induced dissociation) with a normalized collision energy of 35%. Dynamic exclusion was enabled with an exclusion size list of 500 peptides, an exclusion duration of 60 s and a repetition count of 1. The data were processed using MaxQuant v1.3.0.3 software [43] and MS/MS where matched against UniProt database against *L. monocytogenes* (total of 2844 protein sequences), using the Andromeda search tool [44]. MaxQuant parameters were set as follows: trypsin was used as a protease, with maximum 2 missed cleavages and minimum peptide length of 7. Carbamidomethylation (C) was set as a fixed modification. Oxidation (M) and acetylation (Protein N-term) were set as variable modifications. Mass tolerance was set to 20 ppm, peptide and protein false discovery rate (FDR) cut-off was set to 0.01.

Biofilm assay

The influence of antimicrobials on biofilm formation by *L. monocytogenes* was evaluated by the crystal violet method [45]. Briefly, a fresh culture in BHI broth was prepared with an initial concentration of 10^6 CFU/mL *L. monocytogenes* and incubated for 6 h at 37 °C under aerobic condition. Aliquots were used to create respectively: (i) untreated samples, and cultures treated with (ii) 0.1 µg/mL nisin or (iii) 100 AU/mL fengycin. Ten wells of a 96-well microplate were prepared for each of these treatments and incubated at 37 °C for 24 h. After incubation, the wells were washed three times with sterile saline, fixed with methanol and the biofilm was dried overnight at room temperature. Crystal violet (2% w/v) was used for staining the fixed biofilm before washing the wells for residual dye elimination. Then, 95% (v/v) ethanol was added to the wells for dye extraction and the optical density of ethanol solutions was measured at 570 nm using a microplate reader (SpectraMax M2e). Optical density comparison was made for interpreting the results from wells containing different treatments [45].

Statistical analysis

The MetaboAnalyst 3.068 online software infrastructure was used for the statistical analysis of both the lipidomics and proteomics data. Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) were carried out to determine the variation between the control and the samples treated with nisin and fengycin. The cutoff value VIP (Variable Importance in Projection) ≥ 1 was used to prioritize important features from PLA-SDA statistical analysis, as established by Chong and Jun [46]. Proteins with VIP ≥ 1.0 were submitted to the String v11 Database for constructing protein–protein interactions [47], and the networks were visualized with Cytoscape (www.cytoscape.org/). One-way ANOVA followed by Student's *t* test (95% confidence level) was used to evaluate the differences among treatments (control, nisin and fengycin) on lipids composition.

Results and discussion

Differential fatty acids profile

To investigate the changes in lipid profiles of *L. monocytogenes* after the treatments with nisin and fengycin, we performed a GC–MS analysis of fatty acids methyl esters (FAMEs) derivatives. In our analysis, we observed a total of 14 fatty acids, including the three most abundant FAs classes, namely straight FAs, iso-FAs and anteiso-FAs, and a mono-unsaturated fatty acid (MUFA). The GC profile of reported FAMEs can be seen in Fig. S1. According to the

statistical analysis, the only class of FAs that showed significant differences ($P < 0.05$) was the anteiso-FA, whose values were significantly increased in the samples treated with fengycin (46.62 ± 3.55%) in comparison to those from nisin-treated samples (39.39 ± 2.47%) (Table 1). Accordingly, the additional multivariate statistical analysis including both PCA and PLS-DA, considering each fatty acid separately, revealed statistically significant differences ($P < 0.05$) for the fengycin-treated samples in comparison to the control and nisin-treated samples (Fig. 1A). Three fatty acids with variable importance in the projection (VIP) scores > 1.0 were detected (Fig. 1B). The FA *anteiso*-C17:0 showed the highest VIP score and higher concentration in samples treated with fengycin, and 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.

In previous studies, the characterization of membrane lipids in *L. monocytogenes* after exposure to some bacteriocins, such as nisin A, divergicin M35 and leucocin A, 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 straight FAs concentrations [48, 49] or improving of the membrane fluidity through the increase of the branched FAs concentrations [50]. In this study, the nisin-treated samples showed increased amounts of long acyl chain straight saturated fatty acids (C16:0), in comparison with the control, suggesting increased thermodynamically stable interactions between acyl chains and thus increasing the membrane bilayer rigidity [51]. On contrary, the upregulation of branched fatty acid (*anteiso*-C17:0) improved the membrane fluidity on the fengycin-treated samples. Some studies showed that Gram-positive bacteria, such as *Staphylococcus aureus* and *L. monocytogenes*, increase the percentage of saturated fatty acids in bacterial cells forming biofilm, with a simultaneous decrease of iso-FAs and anteiso-FAs [52, 53].

LC–MS/MS-based lipidomic analysis

The intact polar lipid profiles detected in *L. monocytogenes* comprises mostly phosphatidylglycerols (PGs), diglycosyl-diacylglycerols (DGDGs) and diacylglycerols (DGs). Detected masses, class, partition number (PN) employed to simplify the process of identification, carbon number (CN), double bond number (DB) and retention time (RT) values are shown for each lipid species in Table S1. Sodium adducts were observed for DGDGs, while DGs 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 Da fragments (Table S2). Elution of the lipid species occurred according to increasing PN (Fig. S2), derived from the equation PN = CN–2DB.

Table 1 List of FAME derivatives identified in *Listeria monocytogenes* treated with nisin, fengycin and untreated samples. The FAMEs were also grouped as saturated, branched and unsaturated compounds

Compounds	MS_{Sim}	LRI_{ref}	LRI_{exp}	Control	Nisin	Fengycin
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
Straight-FA				30.21 ± 3.07	35.99 ± 3.65	27.78 ± 5.75
Iso-FA				25.97 ± 1.46	24.03 ± 1.28	24.29 ± 2.93
Anteiso-FA				43.41 ± 1.32	39.39 ± 2.47	46.62 ± 3.55
Unsaturated-FA				0.40 ± 0.12	0.61 ± 0.10	1.31 ± 0.71

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)

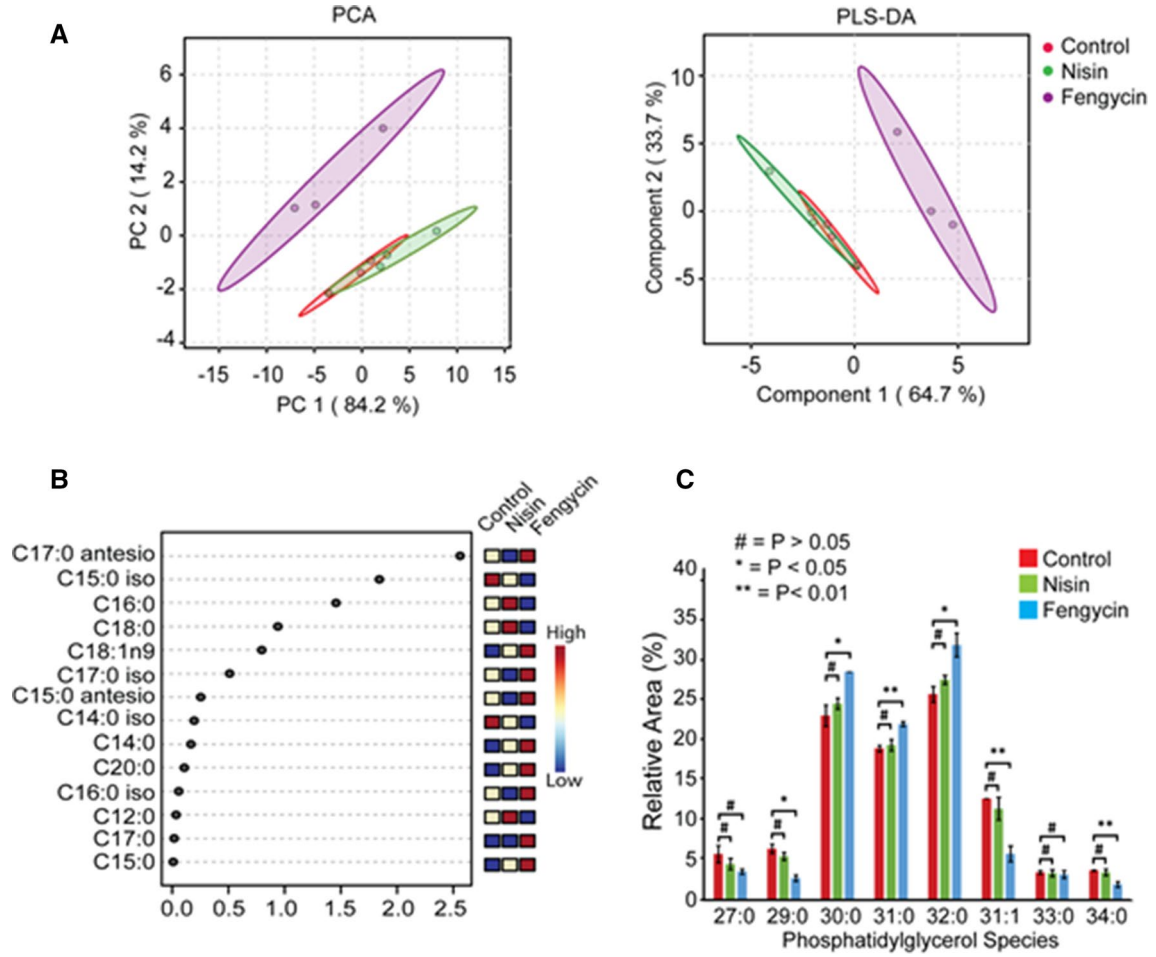

Fig. 1 Lipidomic analysis of *Listeria monocytogenes* exposed to nisin and fengycin. **A** Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) in fatty acids samples of *L. monocytogenes* treated with sub-lethal doses of fengycin (blue), nisin (green) and control (red). **B** Partial Least Squares-Discriminant Analysis (PLS-DA) with the variable importance projection (VIP) score. The boxes indicate relative concentration of the corresponding fatty acids in *L. monocytogenes* cultures for each specific group including the control and the treated with sub-lethal concentration of nisin and fengycin. **C** Comparison of relative quantification of individual PG molecular species in total lipid extracts ($n=3$ biological replicates of treated bacteria) employing MS/MS using electrospray ionization in negative ionization modes (-), by precursor ion scan experiments, through selective monitoring of 153 Da fragment. The statistical analysis was performed using the two-tailed Student's *t* test ($^{\#}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$)

The mass identification was carried out by comparing the obtained data with the information reported by Tatituri et al. [54] and further confirmed using the LIPID MAPS Structure Database [37]. Relative abundance percentages of all the reported PG compounds allowed us to detect significant differences among the treatments as shown in Fig. 1C. In particular, the most abundant PG species 30:0, 31:0 and 32:0 were found in higher amounts in fengycin-treated samples. MS/MS analysis confirmed the fatty acids 15:0/15:0, 15:0/16:0 and 15:0/17:0 as component of the PG species (Figure S3). The examination of the obtained MS data allowed us to correlate the relative abundance for some of the PG species in fengycin-treated samples to the higher quantification of *anteiso* branched FAs forms [54].

Several factors can influence the lipid profile of biological samples, including the culture medium and conditions, isolation and handling of lipid fraction, and even the activity of endogenous lipases ex vivo [55]. In this study, cardiolipin could not be identified due to a limitation of the analysis, while PE was detected in low amounts. Although these compounds are recognized as major lipids in *Listeria*, their abundances halved during elongation phase in *Listeria innocua* [56].

Proteomics analysis

The changes of protein expression after the treatment with nisin and fengycin were investigated through a proteomics

approach. A total of 951 LFQ (label-free quantified) proteins were detected, among the control and treated samples. After eliminating features that were not present in at least half of the samples, the database was reduced to 797 LFQ protein intensities. PCA showed discrimination between the control group and both treated sample groups, although no discrimination between nisin and fengycin groups was observed. Supervised PLS-DA analysis showed better discrimination among the three groups of samples (Fig. 2A) with 142 proteins being considered as drivers for the discrimination

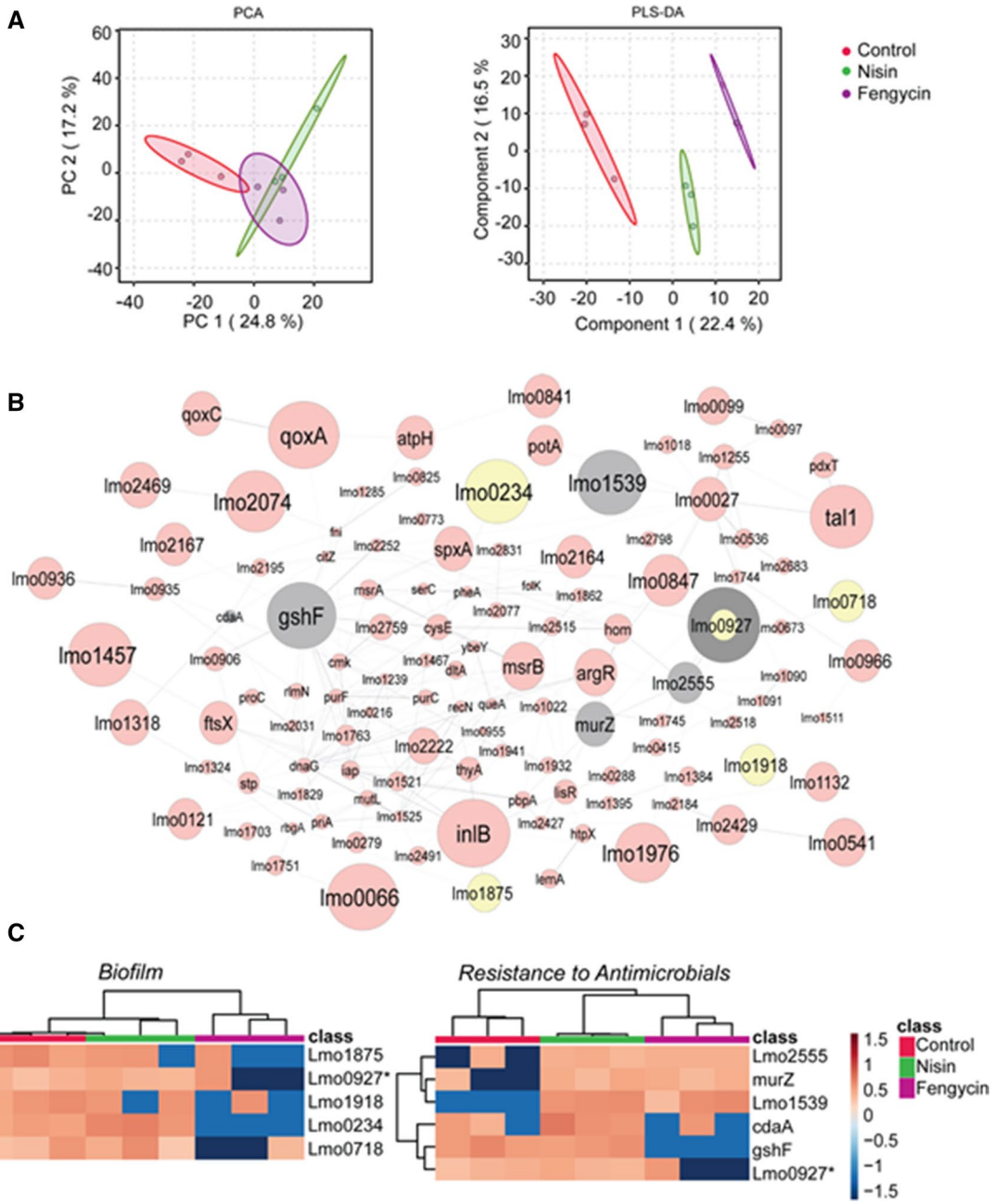

Fig. 2 Proteomic analysis of *Listeria monocytogenes* exposed to nisin and fengycin. **A** Principal Component Analysis (PCA) and **B** Partial Least Squares-Discriminant Analysis (PLS-DA) in proteome samples of *Listeria monocytogenes* treated with fengycin (blue), nisin (green) and control (red). **B** Protein–protein interaction networks generated on STRING v11 database based on the identified proteins, all the proteins with VIP > 1 where reported in the figure, the node size represents the score obtained for each protein. The proteins involved in the biofilm process are colored in gray while those involved in the resistance process in yellow. **C** Heatmap of the proteins identified by PLS-DA statistical analysis. Samples are represented in columns, as the triplicate control samples (red), triplicate samples treated with sub-lethal nisin concentration (green) and triplicate sub-lethal fengycin concentration. Each colored cell on the map corresponds to the relative abundance of the proteins

(VIP ≥ 1.0) (Table S3). This protein group was used to create a protein interaction network using functional enrichment analysis with STRING (LIT).With this network and according to their VIP scores, we prioritized protein families involved in biofilm regulation and the bacterial resistance process (Fig. 2B). Heatmaps with the proteins considered for their role on both resistance to antimicrobials and biofilm formation are shown in (Fig. 2C). The implications of these results are detailed in the next sections.

Proteins associated with membrane stress resistance

The proteomic analysis also revealed a group of proteins associated with membrane resistance responses to AMPs (Table 2), including the LTA backbone synthase Lmo0927 protein, di-adenylate cyclase protein and glutathione synthase GshF protein that were downregulated exclusively in the fengycin-treated samples, while Lmo2555 protein, MurZ and Lmo1539 protein were upregulated in both treatments considered in this work.

The Lmo0927 protein is involved in LTA backbone synthesis, working also structurally on the bacterial cell membrane by extending the glycerol phosphate backbone chain. Besides Lmo0927, two glycosyltransferases, coded by *lmo2555* and *lmo2554* genes (the latter not differentially observed in this work), play a pivotal role to produce monoglucosyl-diacylglycerol and diglycosyl-diacylglycerols, respectively [57].

In general, the resistance strategy in Gram-positive bacteria includes modification of teichoic acids with D-alanine [58] and modification of negatively charged phosphatidylglycerol with positively charged lysine [59], to avoid interactions with cationic AMPs. In many Gram-positive bacteria, lipid species like monoglucosyl-diacylglycerols (MGDG) and diglycosyl-diacylglycerols (DGDG) act as membrane anchors for the LTA constituents of the cell wall [60]. The upregulation of Lmo2555 acting as MGDG synthase, observed in both nisin and fengycin treatments may act toward maintaining the membrane composition, as shown by the construction of deletion mutant, counteracting the complete loss of glycolipids with the consequent phenotypic changes [61]. The presence of MGDG in *L. monocytogenes* membrane guaranteed by Lmo2555 protein supports a protein-mediated adaptation against osmotic stressors like salts and sucrose [62], and maintaining the anionic lipid surface charge density as observed in *Acholeplasma laidlawii* [63]. Specifically, a simulation with bacterial MGDG synthetase demonstrated local enrichment of cardiolipin and phosphatidylglycerol in the immediate area of this protein through interaction with their positively charged residues [64]. In addition, the strong reduction of Lmo0927 could be related with the possible action of fengycin on the multi-drug resistance (MDR) transporters needed for full production of LTA, possibly via c-di-AMP efflux [65]. In support of 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 type I interferon response within the host cell [66]. C-di-AMP showed a role on the osmotic homeostasis during environmental adaptation of Gram-positive bacteria [67], including resistance to acid and oxidative stress [68], resistance to extreme cell wall stress in *S. aureus* [69] and antibiotic tolerance associated with biofilm formation [70]. In this context, c-di-AMP has also been shown to play a role in *Mycobacterium smegmatis* for the negative regulation of fatty acid synthesis [71].

Another differentially expressed protein possibly associated with bacterial response to the membrane perturbation caused by antimicrobials was the glutathione (GSH) biosynthesis protein gshAB. In this study, gshAB was upregulated in nisin-treated samples, whereas the exposure to fengycin was related with the downregulation this protein in *L. monocytogenes* (Table 2). Although the knowledge about the physiological function of GSH in Gram-positive bacteria is limited, it has been shown to play important roles in cellular antioxidant mechanisms. Previous studies have demonstrated that bacterial cells containing GSH were capable of protecting the membrane structure when exposed to various environmental stresses, such as osmotic pressure, oxidative, and acid stress [72, 73]. Moreover, glutathione has emerged

Table 2 Detected proteins involved in membrane stress response in *Listeria monocytogenes*, with the respective VIP score and gene name

Protein name	VIP score	Regulation	Treatment	Gene name
Glutathione biosynthesis protein GshAB/(GshF)	4.81	Down	Fengycin	*gshAB*
Lmo0927 protein[a]	2.76	Down	Fengycin	*lmo0927*
Diadenylate cyclase	1.40	Down	Fengycin	*dacA/lmo2120*
Lmo2555 protein	2.88	Up	Fengycin/nisin	*lmo2555*
UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2 (MurZ)	3.12	Up	Fengycin/nisin	*lmo2552*
Lmo1539 protein	4.65	Up	Fengycin/nisin	*lmo1539*

[a]Protein shared with the group of proteins associated with biofilm formation

as a posttranslational regulator of protein function under conditions of oxidative stress, by the direct modification of proteins via glutathionylation.

Finally, both AMPs caused the upregulation of both UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (MurZ, *lmo2552* gene) and putative glycerol uptake facilitator protein (GlpF-2, *lmo1539* gene), which are associated to membrane stress resistance under the regulation of SigB. Lmo2552 protein has been associated with vancomycin tolerance in *L. monocytogenes* [74], whereas the Lmo1539 protein can be associated with the fact that a number of genes controlled by the key virulence factor PrfA are upregulated when *L. monocytogenes* growth in the presence of glycerol [75]. However, an alternative explanation for these results may come from dynamic simulations studies. Molecular dynamic simulation of the acyl-chain order in lipids around *Escherichia coli* aquaporins AqpZ and GlpF showed an increase in the acyl-chain order parameter. Yet the bilayer thickness decreases toward the protein and meets effectively the hydrophobic matching condition. The simulation also indicate that the lipid bilayer adapts to the channel by a hydrophobic matching condition reflecting the tendency of the lipid molecules for creating curved structures, and the transport function of the channel was modulated [76].

Differential biofilm organization after fengycin treatment

The fengycin treatment showed specific protein regulation toward the inhibition of biofilm formation, compared with the control and the nisin treatment. The proteomic analysis revealed a group of proteins suggesting the negative action on *Listeria* biofilm organization. Like other compounds acting against biofilm formation through inhibition of LTA synthesis [77], fengycin treatment strongly downregulated the Lmo0927 protein (LtaS). The LTA biosynthesis machinery represents an attractive target for the development of novel antimicrobial drugs against Gram-positive bacteria. The LTA deficiency in *S. aureus* altered the bacterial surface hydrophobicity and resulted in reduction of biofilm formation [78, 79]. Additional proteins with high VIP scores including putative pyruvate phosphate di-kinase regulatory protein, Lmo1875 protein, Lmo0718 protein, and Lmo1918 protein were observed (Table 3). All these proteins work on the biofilm promotion and were detected as downregulated in fengycin-treated samples.

The putative pyruvate phosphate dikinase regulatory protein and Lmo1875 protein, have been previously reported to be among the upregulated proteins in sessile *L. monocytogenes* cells inside biofilms, when treated with lactocin AL705 in a comparative study with planktonic cells [80]. The flagellum contributes to the biofilm organization and its biosynthesis require dozens of genes from a large operon working on the bacterial motility. The operon also includes the protein Lmo0718, which was detected as downregulated in fengycin-treated samples. Thus, inhibition of flagella-related proteins may represent a possible loss on the biofilm building capacity [81]. Another downregulated protein in the fengycin-treated samples was the Lmo1918 protein with unknown function, although a deficient mutant effectively reduced the propensity of *L. monocytogenes* to form biofilms [82].

The growth curves of *L. monocytogenes* treated with AMPs showed a rapid reduction of viable cell numbers after the addition of nisin and fengycin (Fig. 3A). At 7 h cultivation, when the bacterial cells were collected for the omics analysis, a similar number of viable cells was revealed for both treatments. However, cell counts of *L. monocy*togenes treated with nisin were higher than fengycin treatment at 24 h. Moreover, experimental biofilm quantification assay permitted to detect significant differences among samples (Fig. 3B). Biofilm promotion was induced by the treatment with sub-lethal concentration of nisin ($OD_{Nis} > OD_{Cont}$), while a strong inhibition of biofilm was observed by the action of fengycin ($OD_{Fen} < OD_{Cont}$).

Concluding remarks

The exposure of *L. monocytogenes* to sub-lethal concentrations of nisin and fengycin resulted in different molecular responses. The proteomics analysis enabled us to illuminate possible mechanisms involved with membrane resistance in

Table 3 Detected proteins involved in biofilm formation by *Listeria monocytogenes*, with 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*
Lmo1918 protein	2.90	Down	Fengycin	*lmo1918*
Lmo0927 protein[a]	2.76	Down	Fengycin	*lmo0927*

[a]Protein shared with the group of proteins associated with the membrane stress response

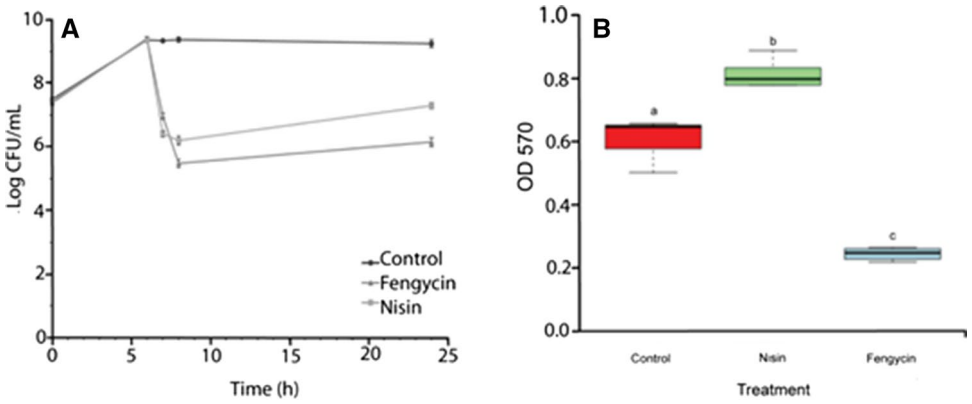

Fig. 3 **A** Growth curves of *Listeria monocytogenes* in BHI broth during 24 h at 37 °C. The bacterial cultures were treated with antimicrobial peptides (nisin and fengycin) after 6 h cultivation. The data represent the mean of three experiments and the bars indicate standard deviations. Significant differences ($P < 0.05$) by Tukey's test were observed among the microbial growth curves only after adding the antimicrobials. **B** Boxplot of spectrophotometry data showing the optical density (OD_{570}) of *L. monocytogenes* biofilm after 24 h growth in BHI broth under different treatments: negative control, nisin (0.1 µg/mL) and fengycin (100 AU/mL), respectively. Different lower case letter over the plot of each treatment indicates significant differences ($P < 0.05$)

both AMP treatments, as well as the propensity of biofilm inhibition by the fengycin-treated samples.

Lipidomics analysis of the *L. monocytogenes* fatty acids profiles showed that membranes are enriched in branched FAs, as reported previously [83, 84]. Similarly, higher amounts of branched FAs have been reported in other Gram-positive bacteria, such as *Staphylococcus* and *Bacillus* species [85], conferring increased membrane fluidity as compared with other bacterial groups. Alterations in membrane lipid composition were observed after both nisin and fengycin treatments, suggesting an alteration of membrane fluidity as bacterial response to these AMPs. The variation of relative abundance of PG species in the fengycin-treated samples might be associated to the downregulation of Lmo0927 protein (LTA synthase) and with the related variation of available LTA [86].

Bacteria can randomly differentiate into persister cells during regular growth, but this phenotypic change can be also induced by environmental signals indicating potential threats for the bacteria. In bacterial populations, this phenomenon is largely controlled by stress signaling pathways, such as the general stress response or the SOS response. Thus, formation of persister cells is stimulated under conditions that favor the activation of these signaling pathways, such as biofilm formation, hostile environments, and response to injury caused by sub-lethal doses of antimicrobials [87]. Some studies indicate that fengycin auto-aggregates by inserting their acyl chains into model membrane bilayers, which is related to membrane perturbation and damage [26, 27]. Fengycin binding and aggregation disturb phospholipid bilayers, appearing to affect bacterial membranes in a dose-dependent manner [88], playing a key role in cell damage. Thus, we hypothesize that the exposure to sub-lethal concentration of fengycin induces a stress–response mechanism that results in altered membrane fluidity, allowing the surviving cells to face the potential damage caused by surface-active lipopeptides like fengycin. This mechanism could involve the regulation of proteins related to the synthesis of surface lipids, as suggested above, and mediated by general stress response regulons, such as σB, VirB or PrfA, which have been largely associated with the resistance of *L. monocytogenes* to environmental stresses [89].

One of the *L. monocytogenes* responses to the nisin exposure was 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 [14]. 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 stress produced by host defense mechanisms including both AMPs and peptidoglycan hydrolases inside the phagosome. This fatty acid modulation has also shown to compromise the production of the key virulence factor listeriolysin O, suggesting an influence on the virulence regulation [90].

The biofilm formation and membrane modification represent two of the several mechanisms involved in bacterial resistance. Considering the effect of differentially regulated proteins on the membrane structure, the proteins related with LTA metabolism should be emphasized. 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 [91, 92].

This study provides evidence that there is a link between the use of a sub-lethal dose of fengycin and inhibition of biofilm formation in *L. monocytogenes*. It was also confirmed that the bacterial physiology was reorganized to promote the biofilm formation when a sub-lethal concentration of nisin was employed. We hypothesize that several proteins contribute to increase the cell membrane stability through protein–lipid interactions. Together, our results provide new insights into the molecular mechanisms of biofilm inhibition in *L. monocytogenes*, which could be leveraged as possible new strategies to confront *L. monocytogenes* and other Gram-positive bacteria, including multi-resistant strains.

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Author contributions PS, AB, LM, and MdA contributed to the study conception and design. Material preparation, data collection, data analysis, and statistical analysis were performed by PS, FFV, GM, DD, DP and GVC. The manuscript was written by PS and critically revised by AB. All the authors read and approved the final manuscript.

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Availability of data and material The lipidomics and proteomics mass spectrometry data are publicly available through the MassIVE repository under the following accession numbers: MSV000088672 and MSV000088738.

Declarations

Competing interests Authors declare no conflicts of interest.

Ethical approval This research did not involve human participants.

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Authors and Affiliations

Paolo Stincone[1] · Flávio Fonseca Veras[1] · Giuseppe Micalizzi[2] · Danilo Donnarumma[2] · Gaetano Vitale Celano[4] · Daniel Petras[5] · Maria de Angelis[6] · Luigi Mondello[2,3] · Adriano Brandelli[1]

[1] Laboratório de Bioquímica e Microbiologia Aplicada, ICTA-UFRGS, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre 91501-970, Brazil

[2] Chromaleont S.R.L., c/o Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, viale Annunziata, 98168 Polo AnnunziataMessina, Italy

[3] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Polo Annunziata, viale Annunziata, 98168 Messina, Italy

[4] Department of Veterinary Medicine, University of Bari Aldo Moro, Prov. le Casamassima, km 3, Valenzano, 70010 Bari, Italy

[5] CMFI Cluster of Excellence, Interfaculty Institute of Microbiology and Medicine, University of Tübingen, 72076 Tübingen, Germany

[6] Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, 70126 Bari, Italy