International Journal of Medical Microbiology 309 (2019) 199–212

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Comprehensive proteomic analysis and pathogenic role of membrane vesicles of *Listeria monocytogenes* serotype 4b reveals proteins associated with virulence and their possible interaction with host

Raman Karthikeyan[a], Pratapa Gayathri[b], Paramasamy Gunasekaran[c], Medicharla V. Jagannadham[b,*], Jeyaprakash Rajendhran[a,*]

[a] Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai, 625021, Tamil Nadu, India
[b] CSIR - Centre for Cellular and Molecular Biology, Tarnaka, Hyderabad, 500007, India
[c] VIT University, Bhopal, Madhya Pradesh, India

ARTICLE INFO

Keywords:
Listeria monocytogenes
Membrane vesicles
Caco-2 cells
Cytotoxicity
Endocytosis
Host-pathogen interactions

ABSTRACT

Membrane vesicles (MVs) are produced by various Gram positive and Gram negative pathogenic bacteria and play an important role in virulence. In this study, the membrane vesicles (MVs) of *L. monocytogenes* were isolated from the culture supernatant. High-resolution electron microscopy and dynamic light scattering analysis revealed that *L. monocytogenes* MVs are spherical with a diameter of 200 to 300 nm in size. Further, comprehensive proteomic analyses of MVs and whole cells of *L. monocytogenes* were performed using LC/MS/MS. A total of 1355 and 312 proteins were identified in the *L. monocytogenes* cells and MVs, respectively. We identified that 296 proteins are found in both whole cells, and MV proteome and 16 proteins were identified only in the MVs. Also, we have identified the virulence factors such as listeriolysin O (LLO), internalin B (InlB), autolysin, p60, NLP/P60 family protein, UPF0356 protein, and PLC-A in MVs. Computational prediction of host-MV interactions revealed a total of 1841 possible interactions with the host involving 99 MV proteins and 1513 host proteins. We elucidated the possible pathway that mediates internalization of *L. monocytogenes* MV to host cells and the subsequent pathogenesis mechanisms. The in vitro infection assays showed that the purified MVs could induce cytotoxicity in Caco-2 cells. Using endocytosis inhibitors, we demonstrated that MVs are internalized via actin-mediated endocytosis. These results suggest that *L. monocytogenes* MVs can interact with host cell and contribute to the pathogenesis of *L. monocytogenes* during infection.

1. Introduction

Listeria monocytogenes is a Gram-positive intracellular foodborne pathogen that causes listeriosis in human as well as in animals. The major symptoms of human listeriosis include meningitis, septicemia, miscarriage and fetal death. The fatal rate among the high-risk group is approximately 20–30%, which is relatively high among food-borne diseases (Hernandez-Milian and Payeras-Cifre, 2014). *L. monocytogenes* infection is acquired by ingestion of contaminated food and subsequent invasion of cells in the host duodenum. *L. monocytogenes* can also enter the bloodstream and spread systemically to other organs, including the placenta and the central nervous system (Lecuit, 2005). *L. monocytogenes* is widespread in nature, and it can survive in a wide range of temperature, pH and osmolarity (Farber and Peterkin, 1991; Ribeiro and Destro, 2014). *L. monocytogenes* is classified into 13 serotypes, such as 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 based on the surface antigenic determinants (Seeliger and Höhne, 1979; Orsi et al., 2011., Shen et al., 2013., Karthikeyan et al., 2015; Wang et al., 2017). Of these, most of the clinical isolates from the sporadic and outbreak cases in humans were reported to belong the serotype 4b, followed by serotypes 1/2a and 1/2b (Jacquet et al., 1995; Pan et al., 2009; Burall et al., 2017; Maurella et al., 2018)

L. monocytogenes expresses multiple virulence factors for the entry and intracellular survival in the host cells (Ireton, 2007; Mostowy and Cossart, 2012). The internalin A encoded by *inlA* helps to cross the intestinal epithelial cell barrier (Ireton, 2007). The internalin B encoded by *inlB* is responsible for the entry of *L. monocytogenes* into intestinal cells (Pentecost et al., 2010). To establish the intracellular survival, *L. monocytogenes* must escape from the hostile phagosomal environment. *L. monocytogenes* secrets a cholesterol-dependent cytolysin, listeriolysin

* Corresponding authors.
E-mail addresses: jagan@ccmb.res.in (M.V. Jagannadham), jrajendhran@gmail.com (J. Rajendhran).

https://doi.org/10.1016/j.ijmm.2019.03.008
Received 17 November 2018; Received in revised form 19 March 2019; Accepted 25 March 2019

O (LLO) that mediates the destruction of the phagosome (Schnupf and Portnoy, 2007), and two phospholipases encoded by LIPI-1 (*Listeria* Pathogenicity Island 1) that mediate *L. monocytogenes* escape from the endocytic vesicle into the cytoplasm. Subsequently, *L. monocytogenes* divides and undergo F-actin based motility to spread from cell to cell (Camejo et al., 2009).

The majority of the virulence proteins are located on the cell surface or secreted to the extracellular milieu. *L. monocytogenes* contains highly specialized secretion systems to deliver the proteins and to reach specific targets. Six secretion systems were identified in *L. monocytogenes* (Desvaux and Hébraud, 2006). Components of the Sec and flagella export apparatus (FEA) systems were reported to be required for virulence (Carvalho et al., 2014). Also, *L. monocytogenes* can interact with the host cells through MVs (Vdovikova et al., 2017). However, very little information is available on MVs of *L. monocytogenes*.

MVs are the bilayered structure of 50 to 500 nm in size containing various macromolecules, such as phospholipids, proteins, lipopolysaccharide (LPS), and nucleic acids. The synthesis and release of MVs are common phenomena in growing bacterial populations. MVs are produced by the outward bulging of the outer membrane, followed by constriction and subsequent release from the bacterial cell, a process referred as vesiculation (Schwechheimer et al., 2014). A wide variety of Gram-negative bacterial species release MVs. Recently, Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus spp*. *Clostridium perfringens*, *Streptococcus spp*, *Lactobacillus* and *L. monocytogenes* were reported to produce MVs (Gurung et al., 2011., Lee et al., 2013; Brown et al., 2014; Olaya-Abril et al., 2014; Grande et al., 2017). MVs play a multifunctional role such as, transferring DNA and toxic protein to other cells, promoting biofilm formation, providing resistance to antimicrobial compounds and phages. MVs also have roles in interaction with other bacteria and cell to cell communication and protecting the bacteria from various stresses (Tashiro et al., 2017).

Recently, it has been shown that *L. monocytogenes* secretes active MVs and play a role in the inhibition of autophagy in host cell (Vdovikova et al., 2017). We here report the comprehensive proteome analysis of MVs of *L. monocytogenes* 4b. We have identified protein profiles of MVs secreted by *L. monocytogenes* 4b strain, which could be relevant to its virulence. Also, we have validated the proteomics data by acetylation of the tryptic peptide followed by mass spectrometry, and the activity of MV-associated proteases using zymogram analysis. We determined and compared the proteomes of whole cells and MVs. We found that large numbers of proteins in MVs. Also, we found that *L. monocytogenes* MVs are internalized by epithelial cells via actin-dependent endocytosis. The in vitro infection assays showed that purified MVs induced cytotoxicity in Caco-2 cells, suggesting a role of MVs in pathogenesis. Therefore, MVs might be an important vehicle for delivering virulence factors to host cells during *L. monocytogenes* infection. Also, we have constructed a *L. monocytogenes* MV and the host protein-protein interaction (PPI) network based on bioinformatics prediction. The predicted network analysis indicated that the internalin B, listeriolysin O, ClpB, PepT, GroL proteins of MVs interact with human proteins involved in endocytosis, autophagy, immune response and mitochondrial-mediated apoptotic pathways. These findings may serve as the basis for understanding the interactions between *L. monocytogenes* MVs and human host cells at the molecular level.

2. Materials and methods

2.1. Bacterial strains and culture growth

L. monocytogenes MTCC 1143 (serotype 4b) (Microbial Type Culture Collection, IMTECH, Chandigarh, India) was routinely grown on brain heart infusion agar (BHI) (Himedia; Mumbai India) at 37 °C.

2.2. Cell culture and growth conditions

The Caco-2 human colon adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC; Virginia USA). Cells were routinely maintained in Dulbeccos Modified Eagle Medium (DMEM) F12 Ham supplemented with L-glutamine, sodium pyruvate, antibiotics and 20% of heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified chamber with 5% CO_2 supply. The cryopreserved cells were thawed rapidly by placing the vial at 37 °C. Cells were transferred to a T_{25} flask containing 5 ml of pre-warmed complete culture media (DMEM with 20%FBS). The cells were seeded at a density of 4.2×10^6 cells/ml and grown at 37 °C in a 95% air-5% CO_2 for 10–15 days till they form a monolayer with 70% confluency. At 70–80% confluence, cells were trypsinized (1X) for 3–5 min. Complete medium was added to flask and mixed with the trypsinized cells to inactivate trypsin. Cells were centrifuged at 1000 rpm for 2 min at room temperature in a 15-ml centrifuge tube. The supernatant was removed, and the pellet was resuspended in 5 ml of culture medium.

2.3. Extraction of L. monocytogenes whole cell proteome

Colonies from freshly streaked plates of *L. monocytogenes* 1143 was inoculated into BHI broth and grown overnight at 37 °C on a rotary shaker. After overnight growth, cultures were diluted in the ratio of 1:100 in 20 ml of BHI broth and grown again with shaking at 37 °C until the optical density at 600 nm (OD 600) reached approximately 1.0. Cells were harvested by centrifugation (8000 x *g* for 10 min at 4 °C) and resuspended in 4 ml of lysis solution (2% Triton X-100, 0.1 M Tris (pH 8.0), 8 mM phenylmethanesulfonyl fluoride). Subsequently, lysozyme (2 mg/ml) was added and incubated for 2 h at 37 °C. Cells were lysed by sonication with four 30-s pulses (Sonics Vibra Cell, Model VCX 750, USA) on ice with 1 min interval between pulses. Samples were treated with DNase I and RNase A for 30 min at 37 °C, and cell debris was pelleted by centrifugation (18,000 x *g* for 5 min at 4 °C). The supernatant containing the cellular proteins was stored at -20 °C until further use.

2.4. Isolation and purification of MVs

L. monocytogenes was inoculated into fresh BHI broth and incubated overnight at 37 °C. A 10 ml of overnight culture was transferred to 1 l of medium and was allowed to grown until the optical density at 600 nm (OD 600) reached 1.0 at 37 °C with shaking. Cells were pelleted using centrifugation at 6000 ×g for 20 min at 4 °C twice. The supernatant was collected and filtered through 0.4 µm membrane filter (Millipore, Billerica, USA). The filtrate thus obtained was subjected to ultracentrifugation at 142,032 x g, for 2 h at 4 °C in Type 45 Ti in Beckman Ultracentrifuge. The MVs settled as pellet were then resuspended in 100 µl of PBS. The MVs were further purified by sucrose density gradient centrifugation as described earlier (Kulkarni et al., 2014). Briefly, layers of equal volumes of 70%, 60%, and 20% sucrose were added in polyallomer tubes from bottom to top, and the MV suspension was added on the top of the layers. The tubes were ultracentrifuged in Beckman SW 60 Ti at 164,609 x g at 4 °C for 6 h. Different fractions were collected and diluted in 50 mM phosphate buffer (pH 7.4), and the presence of MVs was detected by dynamic light scattering (Nano particle analyser, Horiba scientific, Z-100 obtained from Japan) . The fractions containing MVs were pooled together and ultracentrifuged again in Beckman Type 60 Ti for 90 min at 250,000 x g at 4 °C. The pellet thus obtained was reconstituted in 50 mM phosphate buffer (pH 7.4) and stored at −80 °C until further use.

2.5. Particle size characterization or dynamic light scattering (DLS)

Purified MVs were diluted with PBS to a final protein concentration of 0.06 µg/ml. The size distribution analysis was performed and

recorded by at 90°angle with a laser of wavelength 632 nm. The data was analyzed by Horiba software and the average hydrodynamic radius was obtained. The measurements were conducted at 25 °C with 40 to 50 runs for each sample, and the average intensity weighted diameter was calculated. The average diameter was obtained for MVs isolated from three independent batches.

2.6. TEM analysis of isolated MVs

Three microlitres of purified MVs were negatively stained with freshly prepared 3% uranyl acetate for 1 min on 300 mesh-size formvar carbon-coated copper grids (Electron Microscopy Sciences, USA). Excess of stain was blotted, and the grid was washed once with distilled water and dried. Micrographs were obtained by screening around 30 fields of each grid from three independent batches of MVs with a high-resolution transmission electron microscope (FEI Tecnai F20 S/TEM**)** at 120 kV and JEOL, Japan).

2.7. SDS PAGE and in-gel digestion

The cellular and MV proteins of *L. monocytogenes* were precipitated and resolved in 12% SDS-PAGE. The protein samples (150 µg) were dissolved in SDS-PAGE loading buffer, and heated at 95 °C for 5 min and finally loaded in different wells of 1 mm thick gel. Once the protein separation is completed, the gel lanes were cut into 5 pieces and subjected to trypsin in-gel digestion. In brief, the gel pieces were cut into 1 mm x 1 mm fragments washed using 50 mM ammonium bicarbonate solution for 15 min, later with 50% acetonitrile and treated with 50 mM IAA for 1 h at 60 °C. The samples were then subjected to in-gel trypsin digestion for 16 h. Finally, obtained peptides were dried in vacuum and were dissolved in 50% acetonitrile with 5% trifluro acetic acid and subjected to MALDI-TOF MS (Applied Biosystems, Foster City, CA, USA) and Q-Exactive LC/MS/MS (Thermo Scientific, San Jose, CA, USA).

2.8. MALDI-TOF/TOF mass spectrometric analysis

Tryptic digested peptides were extracted from gel plugs by adding 50 µl of 5% (v/v) trifluoroacetic acetic acid, and 50% acetonitrile and vortexed for 15 min. Then, samples were desalted and concentrated using Zip-Tip columns (Millipore) and loaded onto the MALDI plate using 5 mg/ml of CHCA in 50% acetonitrile as the matrix. The samples were analyzed on 4800 MALDI TOF/TOF (Applied Biosystems, Foster City, CA, USA) equipped with Nd: YAG laser (355 nm). The mass spectra were recorded in positive ion reflector mode. The CID mass spectra were recorded for different peaks manually selected from MS spectra. The collision gas used was air. The most abundant peptide ions were then subjected to fragmentation analysis (MS/MS). The MS/MS spectra were analyzed/observed using Data explorer software provided by the manufacturer. The MALDI spectra were recorded to evaluate trypsin digestion and also to identify the sequence of some peptides randomly and compare the results with ESI-MS/MS.

2.9. LC/MS/MS analysis

The tryptic peptides of cellular proteins and MVs of *L. monocytogenes* 4b were subjected using an LC − ESI − MS/MS Orbitrap velos (Thermo Scientific, San Jose, CA, USA) and Q-Exactive HF LC/MS/MS (Thermo Scientific, San Jose, CA, USA) respectively. The collected mass spectrometry (MS/MS) spectra of the peptides were analyzed using the SEQUEST and PEAKS algorithm (Matrixscience) against databases of *L. monocytogenes* serotype 4b str. LL195 (For *L. monocytogenes* MTCC1143 4b). The search parameters were (i) enzyme specificity, trypsin; (ii) maximum allowed missed cleavages of 2; (iii) carbamidomethylation at cysteine residues (C) as a fixed modification; (iv) oxidation at methionine residues as variable modification; (v) MS tolerance of 10 ppm and (vi) MS/MS tolerance of 0.6 Da. The LC − ESI MS/MS spectra were analyzed using the Proteome Discoverer Version 1.4.0.288 (Thermo Scientific, San Jose, CA, USA). All the MS/MS spectra were analyzed using SEQUEST (Thermo Fisher Scientific) and PEAKS (Thermo Scientific, San Jose, CA, USA) selecting the enzyme trypsin, the precursor tolerance of 10 ppm and a fragment tolerance of 0.6 Da. The increase in mass due to the oxidation of methionine (15.99 Da) and carbamidomethylation of cysteine (57.02 Da) was set as a variable and fixed modification, respectively. Only peptides identified with high confidence were considered and all the proteins were identified by at least two unique peptides. Proteins were also identified by PEAKS 6 software, using the same search parameters. Peaks algorithm determines the *de novo* sequencing of the peptides from the MS/MS spectra, and matched against the database for identification of the proteins. The average local confidence (ALC) score of 30 and above were considered for *de novo* sequences. Since the total local confidence depends upon the length of an individual peptide cutoff values were not fixed. The −10 log P score was set at 50 for accepting the match from the database search. The probability of a wrong hit at this score is ≤0.001%. The SEQUEST and PEAKS result files were exported and filtered for a false-discovery rate (FDR) of less than 5% at the peptide level.

2.10. Validation of MV proteomics data

The tryptic peptides obtained from proteins of *L. monocytogenes* MVs were acetylated as described earlier with slight modification (Kuchibhotla et al., 2017). The acetylation mixture of 12% acetic anhydride and 3% triethylamine and were prepared in methanol. Five microlitres of the acetylation mixture was added to the tryptic digests of proteins and incubated for 20 min. After incubation, the samples were completely dried and re-dissolved in 10 µl of 5% ACN containing 0.1% formic acid and analysed by Q-Exactive HF LC/MS/MS. The collected mass spectrometry (MS/MS) spectra of the peptides were analyzed using the SEQUEST. A mass difference of 42 Da was observed in each of the peptides after acetylation, indicating that the reaction leads to the formation of acetylated derivatives.

Proteolytic activity of MVs was determined by zymogram analysis as described earlier (Elhenawy et al., 2014; Frankowski et al., 2012). *L. monocytogenes* MVs were solubilized in urea buffer and total protein content was quantified by Lowry et al., 1951 method. SDS acrylamide (12%) was co-polymerized with 0.5% (w/v) porcine skin gelatin (Gelatin, Sigma Aldrich, St. Louis, MO) as a protein substrate. MV proteins (20 µg) was used loaded into appropriated wells and electrophoresis was performed at 4 °C for until the dye front reaches the bottom of the gel. Following separation under denaturing conditions, the proteins were renatured to allow substrate cleavage. Colloidal Coomassie brilliant blue G-250 staining of gel was used to visualize areas where the substrate (gelatin) was digested by proteases.

2.11. Bioinformatic analysis of proteomic data

The subcellular localization of a protein and the pathway analysis was predicted by PSORTb (version PSORTb v3.0) (Yu et al., 2010) and CELLO2GO (Yu et al., 2014) respectively. Virulence proteins associated with MVs were predicted by VirulentPred (Garg and Gupta, 2008). Cellular and MV associated lipoproteins were predicted using LipoP (Juncker et al., 2003), DOLOP (Babu et al., 2006), and PredLipo (Bagos et al., 2008). Prediction of a signal peptide for the general secretory (Sec) or the twin-arginine translocation (Tat) pathway of the vesicular proteins was performed by PRED-TAT (Bagos et al., 2010), and the predicted Tat motif was obtained from TATFIND 1.4 server (Rose et al., 2002). Additional prediction of the signal peptide for vesicular proteins was made using the SignalP 4.1 (Petersen et al., 2011). Also, non-classical secretary proteins were identified by SecretomeP (Bendtsen et al., 2005).

2.12. Prediction of MV-host protein interaction network

We retrieved the proteome of *Homo sapiens* (human) (Proteome id: UP000005640; 71, 599) from UniProt. Protein-protein interactions (PPI) of MV proteome and the host proteome were predicted using the Host-Pathogen Interaction Database (HPIDB) (Kumar and Nanduri, 2010) with percentage identity and query coverage of MV proteins as 30% and the host percentage identity and query coverage as 50%, respectively. The HPIDB predicts PPI based on the homology approach from the plentiful template eukaryotic–prokaryote inter-species PPIs available among 68 hosts and 602 pathogens. The PPI network analysis and its visualization were performed using Cytoscape (Shannon et al., 2003). The cellular localization of host proteins that interact with MVs was predicated using EuLoc (Chang et al., 2013) and CELLO2GO (Yu et al., 2014).

2.13. Fluorescent labeling of MVs

Purified MVs were fluorescently labeled by incubating with fluorescein isothiocyanate (FITC) reagent (Sigma-Aldrich) (1 μg FITC/μg vesicle protein in 50 mM Na_2CO_3, pH 9.2), for 2 h at 25 °C with mixing. Free FITC was removed by washing three times in HEPES (100,000 × *g*, 30 min). Labelled vesicles were resuspended in PBS.

2.14. MVs association with Caco-2 cells

To monitor MVs entry into Caco-2 cells, a total of 1×10^5 Caco-2 cells were seeded and grown in coverslip till reaching 70% confluence. The Caco-2 cells were stained with Hoechst (1 μg/ml), incubated for 30 min at 37 °C in a 5% CO_2. Cells were then washed once with PBS. The medium was replaced with FITC-labelled MVs (10 μg/well) suspended in DMEM without fetal bovine serum and antibiotics. Cells were incubated for various time points (30 min, 2 h, 4 h, 6 h, and 12 h) and fixed to glass slide using methanol and glycerol. The fixed cells were observed, and images were captured using high content screening system (Operetta, PerkinElmer, USA).

2.15. Kinetics of MVs uptake by Caco-2 cells

MVs were isolated from *L. monocytogenes* were labelled with FITC. Cells were grown to confluence in 75 cm2 culture flasks, trypsinized and resuspended in cell culture medium. To monitor the kinetics of MV uptake, 10 μg of FITC labelled MVs were added to a suspension of Caco-2 cells and incubated for various time points 30 min, 2 h, 4 h, 6 h and 12 h at (37 °C, 5% CO_2). Samples were taken at each time points and fixed to glass slide using methanol and glycerol. The fixed cells were observed, and images were captured using high content screening system (Operetta, PerkinElmer, USA). To determine the proportion of internalized MVs, extracellular FITC-MV fluorescence was quenched with trypan blue (final concentration of 0.2% (w/v)) and thus allows to detect only internalized MVs. Fluorescence intensities were measured before (total amount of cell-associated MVs) and after (internalized MVs) adding trypan blue using a Enspire multimode plate reader.

To determine the mechanism involved in the endocytosis process, inhibition assay was performed as described earlier (Kunsmann et al., 2015). Briefly, monolayers of Caco-2 cells grown in 96-well plates were pre-treated with the endocytosis inhibitors: β-Methyl cyclodextrin (β-MCD), chlorpromazine 15 μg/ml, Cytochalsin B 10 μg/ml, and nystatin 10 μg/ml (Sigma-Aldrich) for 1 h at 37 °C prior to the addition of labeled-MVs. Control cells were not treated with the inhibitors. FITC labelled MVs (10 μg protein/well) were added to the monolayer and incubated at 37 °C for 4 h. After washing with PBS twice, and cell permeabilization with 1% Triton-X-100 (3 min), fluorescence was measured by Enspire multimode plate reader and normalized to FITC labeled MVs incubated without cells. MV uptake in the presence of each inhibitor was expressed as the percentage of MV uptake by inhibitor-untreated cells. Cells were observed, and images were captured using high content screening system-Operetta.

2.16. Cell viability assay

Cell viability was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, based on the mitochondrial reduction of tetrazolium to formazan. Cells were treated with different concentration of MVs and incubated for 24 h. The medium was then removed, and 1 ml of solubilization reagent (99% dimethyl sulfoxide) was added (Himedia, Mumbai). Cell viability was measured at 570 nm in enspire multimode plate reader (PerkinElmer). The results were expressed as a percentage of cell survival relative to untreated cells.

2.17. Statistical analysis

All experiments were performed in triplicates. Values were expressed as mean ± standard deviation of the mean. GraphPad statistical software was used. Data were analysed using the paired Student's *t*-test and $p < 0.05$ was considered as significant. Variables were compared using two way analysis of variance.

3. Results

3.1. Isolation and characterization of MVs of L. monocytogenes

The isolated MVs were fixed and examined by high-resolution transmission electron microscopy (Fig. 1A). The secreted MVs were observed on the surface of the bacterial cells and the surrounding milieu (Fig. 1B). The electron micrographs revealed that the MVs of *L. monocytogenes* are spherical and 200 nm–300 nm in size (Fig. 1C). The mean hydrodynamic radius of vesicles was 263.9 nm as determined by DLS (Fig. 1D). All further experiments were performed using these purified MVs.

3.2. Comprehensive proteome analysis of L. monocytogenes cells and MVs

L. monocytogenes cells and density gradient fractions were resolved by SDS-PAGE to visualise the MV associated proteins (Fig. S1). The proteome of the *L. monocytogenes* cells and purified MVs were analyzed by LC–MS/MS. Based on SEQUEST and PEAKS analysis, 12,124 and 1437 high confident peptides were identified in *L. monocytogenes* cells and MVs respectively. The complete list of the identified peptides and proteins are provided in Table S1 and S2, respectively. The total number of 1355 and 312 MV proteins were identified in *L. monocytogenes* cells and MV respectively. Of these, 60 vesicular proteins were predicted to be localized via the sec pathway while 46 proteins were identified to use the nonclassical secreted pathway (Fig. S2). The oligopeptide transport system permease protein (oppB) was predicted to use the Tat apparatus. Among the proteins identified, few MV proteins posses LPXTG motif, known to mediate the covalent attachment of the protein to the cell wall and a few carry LysM motif that allows non-covalent attachment of the protein to peptidoglycan. The PSORTb 3.0 was used to predict the subcellular localisation. Among the 312 proteins of MVs, 187 (60%) were predicted as cytoplasmic, 66 (21%) as a cytoplasmic membrane, 10 (3%) as cell wall-associated protein, 13 (4%) as extracellular. Localization of 36 proteins (11%) could not be predicted using Psortb. (Fig. S3).

We compared the MV and cellular proteomes of *L. monocytogenes* using Markov Clustering (MCL) of OrthoMCL software. As shown in venn diagram, we determined that 296 common proteins were found in both whole cell and MV proteome (Fig. 2). A total of 16 unique proteins were identified only in MVs, including PI-PLC, autolysin, uncharacterized protein *yabE,* competence protein ComEC/Rec2, flagellar proteins and other uncharacterized proteins (Table 1). These proteins

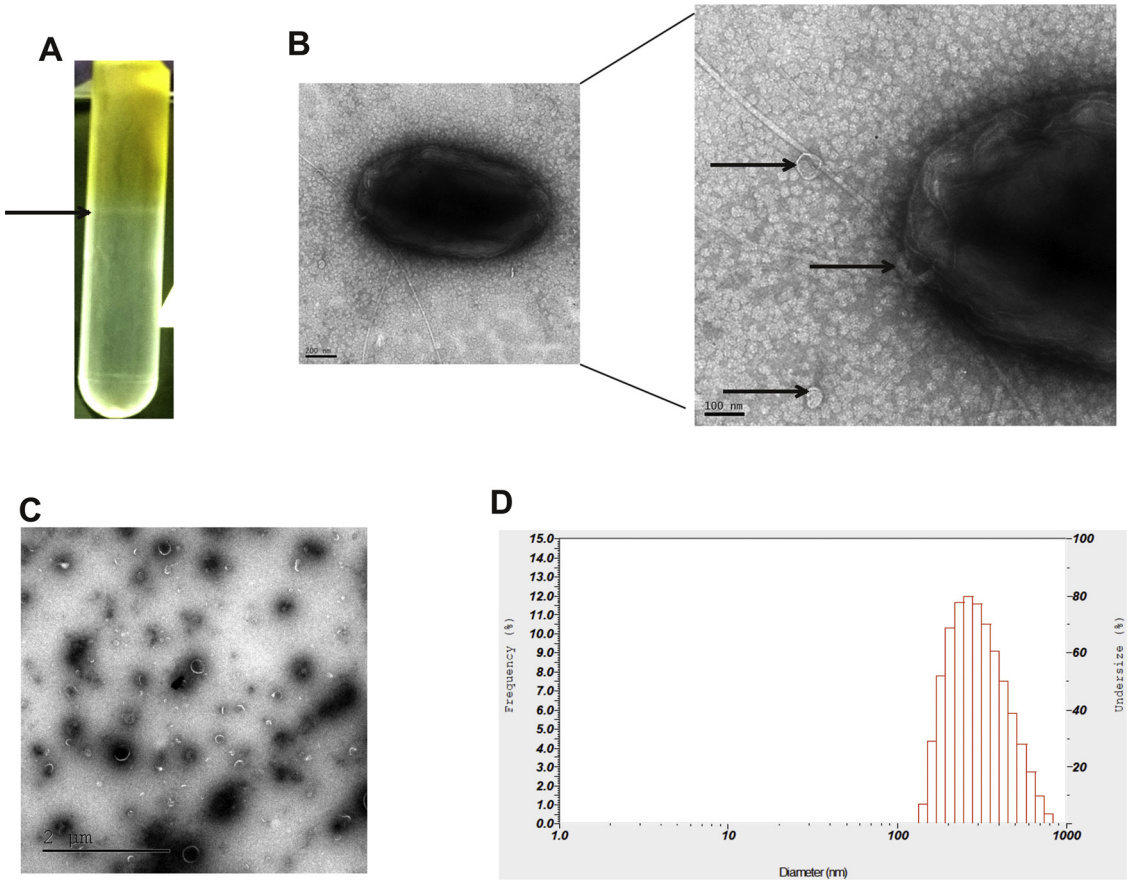

Fig. 1. Characterization of MVs produced by *L. monocytogenes*. (A) Purification of *L. monocytogenes* MVs using sucrose density gradient centrifugation. Black arrow indicates the clear white band observed in 60% sucrose fraction composed of MVs. (B) HR-TEM image of the negatively stained *L. monocytogenes* cells in mid-log phase. Black arrows indicate the MVs released in surrounding milieu and middle arrow indicates MVs being released or blebbing out from *L. monocytogenes* cell. (C) Bilayer and spherical structures of purified vesicles of *L. monocytogenes*. (D) Mean size distribution of vesicles released from *L. monocytogenes* cells, determined by dynamic light scattering method.

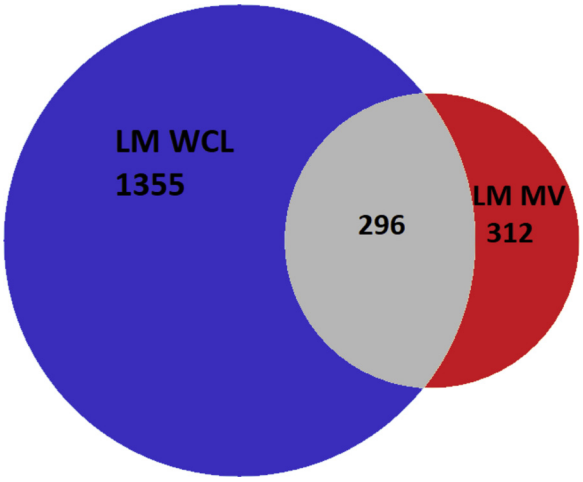

Fig. 2. Proteomic profiling of *L. monocytogenes*. The venn diagram describes individual differences in the total number of proteins identified exclusively (the proteins were not identified in cells proteome) MVs. LM WCL- *L. monocytogenes* whole cell lysate, LMMV- *L. monocytogenes* membrane vesicles.

have roles in virulence, adaptation, metabolism, and regulation. Flagellar hook protein (*FlgE*), flagellin, cell wall binding protein (*YocH*), M48 family peptidase, listeriolysin O, internalin B, a serine protease (*yyxA*), probable endopeptidase p60, flagellin, Lmo0955 were also identified in MVs. These proteins, which are low abundant in the bacteria could have enriched in the MVs. Also, we have identified several transcriptional regulator proteins in MVs, included Rex regulatory protein, TR/Xre regulatory proteins, lacI family regulator, padR negative transcription regulator and redox sensing transcriptional regulatory protein. The vesicular proteins were categorized by the subcellular localizations and biological functions (Fig. 3A-B).

3.3. Virulence factors associated with MVs

Known virulence factors of *L. monocytogenes* such as, listeriolysin O, internalin B, autolysin, p60, PLC-A, prsA, oligopeptide-binding protein OppA, murA X- prolyl aminopeptidase, SecDF, SecA2, superoxide dismutase, FlaA, were found in the purified MVs (Table 2). The antigenic lipoprotein TcsA, peptidase T were also identified in MVs. Using the Virulentpred tool, several other MV proteins were predicted to be associated with virulence (Table S4).

3.4. Validation of MV proteomics data

In bottom up approaches of proteomics, determining the sequence of the peptide plays a crucial role in the identification and validation of proteins. We found that acetylation at the N-terminal end of the tryptic peptides resulted in an increase in the relative intensity of b ions or detection of b1 ion or greater number of b ions in the MS/MS spectra as compared to unacetylated peptide. In some peptides all the three features were observed. The MS/MS spectra of a peptide generated from listeriolysin O and its acetylated peptides are shown in Fig. 4A which confirms the sequence of the peptides. The proteins of the MVs were

Table 1
List of proteins identified only in MVs of *L. monocytogenes*.

Gene	Protein	*L. monocytogenes* EGD-e locus	Subcellular localization
plcA	1-phosphatidylinositol phosphodiesterase	lmo0201	Extracellular
parC	DNA topoisomerase 4 subunit A	lmo1287	Cytoplasmic
yabE	Uncharacterized protein	lmo0186	Extracellular
BN389_02980	Competence protein ComEC/Rec2-related protein	lmo0275	Membrane
pcrA	ATP-dependent DNA helicase	lmo1759	Cytoplasmic
BN389_26590	Autolysin	lmo2691	Extracellular
gyrA	DNA gyrase subunit A	lmo0007	Cytoplasmic
Smc	Chromosome partition protein	lmo1804	Cytoplasmic
parE	DNA topoisomerase 4 subunit B	lmo1286	Cytoplasmic
gyrB	DNA gyrase subunit B	lmo0006	Cytoplasmic
ispG	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)	lmo1441	Cytoplasmic
lytG	Exo-glucosaminidase	lmo1216	Extracellular
yqgS	Lipoteichoic acid synthase-like	lmo0644	Membrane
BN389_17790	Uncharacterized protein	lmo1752	Membrane
flgK	Flagellar hook-associated protein 1	lmo0705	Extracellular
BN389_26820	Uncharacterized protein	lmo2713	Extracellular

also identified after acetylating the tryptic peptides. Using these criteria, 210 proteins from the MVs of the *L. monocytogenes* were also identified/validated. The MS/MS spectra of some representative peptides were shown the supplementary Fig. S4. As shown in the venn diagram (Fig. 4B and C), 210 proteins and 512 peptides were common in normal and acetylated MV proteins. The complete list of the identified acetylated peptides and proteins are provided in Table S3.

We identified a few proteins in MVs that were annotated as proteases. Therefore, we tested if they display activity using a zymogram analysis. MVs were harvested from *L. monocytogenes* and run on an SDS PAGE gel containing gelatin as the substrate for protease. Following renaturation and incubation at 37 °C, the gel was stained with coomassie blue to detect proteolytic activity. As shown in Fig. 4D, several clear bands were visualized after staining the gel, indicating that gelatin was digested at these sites. Thus, we can conclude that at least some of the proteases detected in the vesicles are active.

3.5. Prediction of MVs-host protein-protein interaction (PPI) network and pathogenicity mechanisms

We predicted the interactions of *L. monocytogenes* MV proteins with the human proteome (*Homo sapiens*). We have predicted a total of 1841 possible interactions with the host involving 90 MV proteins and 1513 host proteins. The targeted proteins are predicted to be localized in the cellular compartments relevant to the pathogenesis mechanisms. The predicted host proteins that could interact with MV proteins were localized extracellularly or associated with the respective organelles such as, lysosome, mitochondria, Golgi complex, endoplasmic reticulum (ER), nucleus, plasma membrane and the cytoplasm (Fig. S5). *L. monocytogenes* MVs may interact with endocytosis pathway at multiple stages and internalize via an actin-dependent endocytic pathway. The roles of these predicted target proteins in the host endocytosis-signaling pathway and other possible host interactions are schematically represented in Fig. 5. We have predicted that InlB, ClpB from MV could interact with the host proteins dynamin-binding protein, signal transducing adapter molecule 2, respectively (Fig. S6). The MetK and AlsS of MVs predicted to be interact with cathepsin L1 and WD repeat domain phosphoinositide-interacting protein 2 (WIPI-2) of the host protein (Fig. S7). These predicted proteins are essential for the autophagy during *L. monocytogenes* infection. The PepT, GroL, PpaC, BN389_16270, GlyA, DeoB, Pyc, DnaK, HemL1, and UvrA_2 were predicted to interact with host immune system related proteins. The highest connected node in this sub-network include predicted MV proteins such as InlB, Tkt, OppD, UrA and ClpP of MVs and these proteins may interact with NF-κB signaling pathway (Fig. 5 and Fig. S8). The predicted host proteins involved in this pathway include the inhibitor of nuclear factor kappa-B kinase subunit alpha, nuclear factor NF-kappa B p105 subunit, rab GTPase-activating protein, histone acetyltransferase type B catalytic subunit. These proteins are essential for degrading IκBα and activating NF-κB. The MV proteins Hly, ClpP, Gap, DeoB, Dak2, DnaK, MetK, Heml1, and AckA2 are predicted to be associated with host mitochondrial proteins such as thioredoxin-interacting protein, BCL2, SAFB-like transcription modulator, serine protease HTRA2, thioredoxin-dependent peroxide reductase (Fig. 5 and Fig. S8).

3.6. MVs-host interaction and cytotoxicity

We evaluated the role of MVs in pathogenicity using Caco-2 cell line as a model. Fluorescently labeled MVs (FITC-MVs) from late log-phase cultures were incubated with Caco-2 cells, and examined under high content screening imaging facility, and the kinetics of MVs uptake were monitored over time using a multimode plate reader. We found that the FITC-MVs bound to the cells, and internalized in to the Caco-2 cells in a time-dependant and dose dependant manner (Fig. 6). After 30 min, MVs were observed on the cell membranes (Fig. 6A, 30 min). After 2 and 4 h, MVs were internalized and accumulated in perinuclear regions.

3.7. Uptake of MVs by Caco-2 cells

MVs were labelled with FITC and fluorescence was quenched with trypan blue. Uptake of FITC MVs by Caco-2 cells showed a time-dependent increase in fluorescence, which was consistent with MVs internalization (Fig. 6B and C, $p < 0.05$). The fluorescence level of control samples containing only Caco-2 cells did not increase above the background levels. To distinguish internalized from cell surface-bound MVs, extracellular MV fluorescence was quenched with trypan blue. Results presented in Fig. 6A shows that FITC-labelled MVs were bound and internalized in Caco-2 cells in a time dependent manner.

To understand the mechanism of the *L. monocytogenes* MVs uptake, we studied the effect of endocytosis inhibitors on the internalization of MVs. Cytochalasin B, an inhibitor of actin formation, inhibited the MV uptake to a level of more than 75% when compared to the untreated control cells ($P < 0.001$) (Fig. 7A and B). In contrast, β-methyl cyclodextrins which disrupt lipid rafts and caveolae and chlorpromazine an inhibitor of clathrin-mediated endocytosis had no significant effects on MV uptake by the Caco-2 cells (Fig. 7A and B). These experiments indicated that *L. monocytogenes* MVs are internalized via actin-dependent endocytosis.

To determine the MVs induced cytotoxicity, Caco-2 cells were treated with various concentrations of MVs for 24 h and a cytological change was observed. The morphology of Caco-2 cells treated with 50 μg/ml of MVs showed cellular shrinkage, nuclear condensation suggesting that *L. monocytogenes* MVs induce host cell cytotoxicity. Cell viability was analyzed using the MTT assay. MVs induced Caco-2 cell

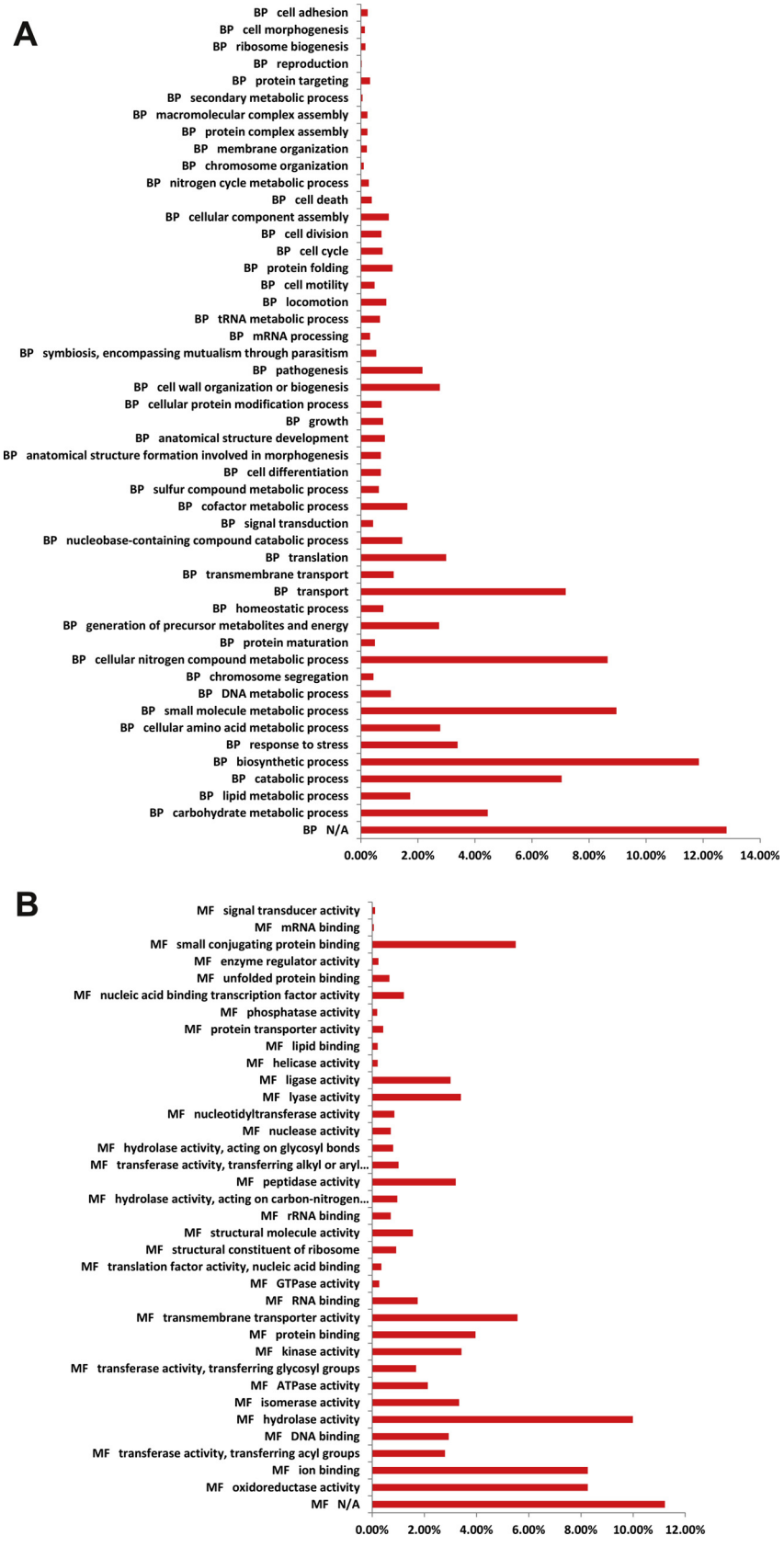

Fig. 3. Classification of MVs of *L. monocytogenes.* (A) MV associated proteins were grouped based on their GO (A) Biological processes (BP) (B) Molecular functions (MF).

Table 2
List of virulence proteins in MVs of *L. monocytogenes*.

Gene	Protein	*L. monocytogenes* EGD-e Locus	Subcellular localization	function	Reference
BN389_26590	Autolysin	lmo2713	Extracellular	Response to stress, pathogenesis	Cabanes et al., 2004
hly	Thiol-activated cytolysin	lmo0202	Extracellular	Pathogenesis,cell death	Gedde et al., 2000; Carrero et al., 2008; Seveau, 2014; Hamon et al., 2012
iap	Probable endopeptidase p60	lmo0582	Extracellular	Pathogenesis	Sashinami et al., 2010; Chandrabos et al., 2015
inlB_2	Internalin B	lmo1786	Extracellular	Pathogenesis, locomotion	Mengaud et al., 1996; Mansell et al., 2000
flaA	Flagellin OS	lmo0690	Extracellular	Locomotion and pathogenesis	Way et al., 2004; Wu et al., 2010
BN389_04110	NLP/P60 family protein	lmo0394	Extracellular	catabolic process	Schmidt et al., 2011
plcA	1-phosphatidylinositol phosphodiesterase OS	lmo0201	Extracellular	Pathogenesis	Camargo et al., 2016; Camilli *et al.*, 1993
BN389_09840	Lmo0955 protein	lmo0955	Cytoplasmic	Stress response	Laursen et al., 2015

cytotoxicity at a concentration of $\geq$ 20 μg/ml and was dose-dependent (Fig. 7C).

4. Discussion

Extracellular MVs are playing major roles in pathogenicity of several pathogens. MVs of Gram negative bacteria and their roles in pathogenesis are well characterised (Mondal et al., 2016; Jung et al., 2016; Bielaszewska et al., 2017; Bauman and Kuehn, 2009; Finethy et al., 2017). Recent data suggest that MVs of Gram positive bacteria also shown to involve in pathogenesis. MVs of pathogenic bacteria deliver toxins and other virulence factors in to the host cells (Jin et al., 2011; Bielaszewska et al., 2017). The synthesis and release of MVs into extracellular milieu appears to be similar in both Gram negative and Gram-positive bacteria (Deatheragea and Cooksona, 2012). However, very little information is available on the MVs of *L. monocytogenes*.

Lee et al. (2013) have reported that the stress transcription factor σB play an essential role in the MV production by *L. monocytogenes*. Vdovikova et al. (2017) have demonstrated that *L. monocytogenes* secretes active MVs inhibiting the autophagy in the host cells. Protective role of MVs in the inhibition of LLO-mediated pore formation, autophagy and the cell lysis, which helps *L. monocytogenes* to survive inside the eukaryotic cells. Recently, Lee et al., (2018) have reported that salt stress affects the MV production by *L. monocytogenes*. Proteomic analyses showed that the number of MV proteins expressed in the wild-type strain was similar to that in Δ*sigB* mutant under the salt stress. Earlier studies have reported the presence of approximately 130–150 proteins in the MVs of *L. monocytogenes* (Lee et al., 2013, 2018), whereas, we have identified 312 proteins in the MVs. Majority of the previously reported proteins were identified in this study too. However, we could detect a large number of proteins in MVs when compared to the earlier studies. The variation in the number of MV proteins could be due to the use of advanced Q-Exactive HF LC/MS/MS, which could detect lower levels of proteins. Presence of the major virulence proteins such as Listeriolysin O (LLO), internalin B (InlB), and several transporters including osmotic stress (OpuCA, OpuCB, OpuCC, and OpuCD) was reported previously. Consistent with the earlier studies, we have also identified these proteins in the *L. monocytogenes* MVs. In addition, several other virulence proteins could be detected in this study, which includes PI-PLCA, PrsA, oligopeptide-binding protein (OppA), murA X-prolyl aminopeptidase, secDF, secA2, flaA, mnt (manganese bind lipoprotein), Yidc (membrane protein insertase), NLP/P60 family protein, etc. The analysis of the MV proteome unveiled the vesicular protein pool providing insight into the role of MVs in bacterial physiology and pathology. From the infection point of view, the compositions of the listerial MV proteomes are important as they can interact with host cells.

In this study, 312 proteins were identified in *L. monocytogenes* MVs. The present study focused on the comprehensive proteome analysis of MV proteins from the pathogenic serotype 4b of *L. monocytogenes* MTCC 1143. As far as we are aware, this is the first comprehensive MV protein catalog of *L. monocytogenes* serotype 4b. The majority of them were predicted to be cytoplasmic proteins. Recent studies on Gram-positive bacteria are suggesting that MVs naturally incorporate a large number of cytoplasmic proteins into MVs (Gurung et al., 2011; Haas and Grenier, 2015; Resch et al., 2016). In contrast, few recent studies are reported that cytoplasmic proteins in MVs are depleted in Gram-negative OMVs (Choi et al., 2011; Yun et al., 2017; Veith et al., 2014). The mechanism by which these cytoplasmic proteins are differentially enriched is uncertain. Many of the identified cytoplasmic proteins are considered to be moonlighting proteins at the cell surface of *L. monocytogenes*. This group includes many conserved proteins involved in central metabolic pathways, cellular responses to stress, and/or virulence. Also, *L. monocytogenes* MVs are enriched with extracellular and surface-associated virulence proteins which could play critical roles in host-pathogen interactions. Several of these proteins have been

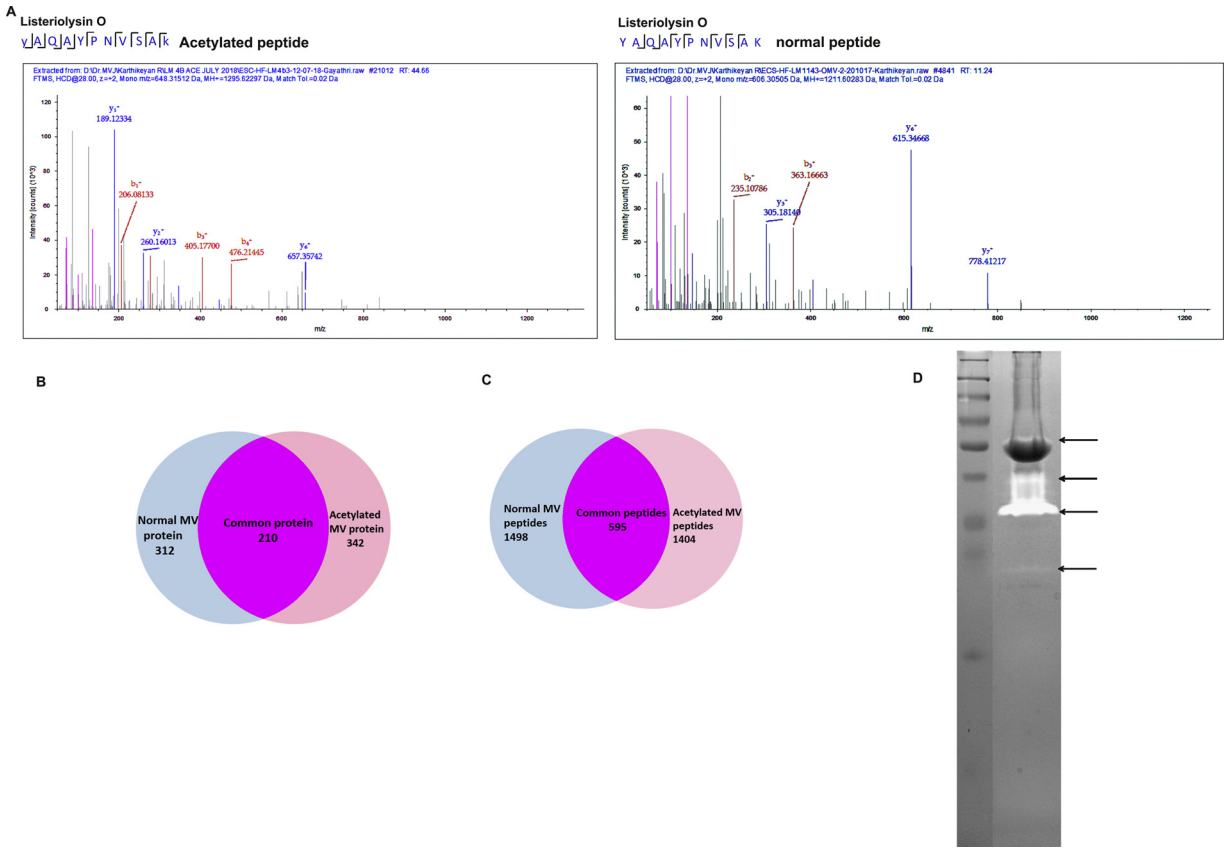

Fig. 4. Validation of MVs proteins. (A) Validation of Listeriolysin O in MV by actylation. Left panel shows acetylated peptide and Right panel shows normal peptide. (B) (C) Venn diagram represents the number of acetylated and normal proteins (Left panel) and peptides (Right panel). (D) MV proteins showed proteolytic activity by Zymogram. Black arrow indicates clear proteolytic activity.

reported to be immunogenic and have clear implications in virulence (Camejo et al., 2011).

MVs act as corgo for delivering various virulence factors, especially toxin delivery, such as cytolysin A in *Escherichia coli* (Wai et al., 2003), the heat-labile toxin in enterotoxigenic *E. coli* (Horstman and Kuehn, 2000), hemolytic phospholipase C in *Pseudomonas aeruginosa* (Bomberger et al., 2009), leukotoxin in *Actinobacillus actinomycetemcomitans* (Kato et al., 2002) and listeriolysin O in *L. monocytogenes* (Vdovikova et al., 2017). We identified several virulence factors in the *L. monocytogenes* MVs, including listeriolysin O, internalin proteins, PI-PLC, serine proteases, autolysin and pheromone lipoproteins. Flagellin is another important virulence factor associated with the host-pathogen interaction during infection (Dons et al., 2004). Also, we have identified several lipoproteins in MVs. In Gram-positive bacteria, lipoproteins are involved in nutrient transport, Toll-like receptor 2 activations, and pathogenicity (Shahmirzadi et al., 2016). A recent study has demonstrated that lipoproteins are essential for the virulence of *L. monocytogenes* (Machata et al., 2008).

Interestingly, we have found few transcriptional regulatory proteins in MVs. These regulatory proteins are located in the cytoplasm. The presence of transcriptional regulators, such as AbrB and TR/Xre in MVs is curious because regulator-mediated transcriptional activity occurs in the cytoplasm harboring nucleic acids and other transcription machinery and their role in MVs are unknown.

The comprehensive proteomic profiling of MVs performed in this study provides a foundation for understanding the virulence regulation and the general physiological attributes of MVs and the MV-mediated virulence mechanisms in *L. monocytogenes*. The MVs from Gram-negative bacteria could interact with host cells via either a receptor-mediated endocytic pathway or fusion with the host cell plasma membrane (Cañas et al., 2016). Bomberger et al. (2009) showed that MVs secreted by *Pseudomonas aeruginosa* delivered multiple virulence factors directly into the cytoplasm of host cells by fusion of OMV with lipid rafts in the plasma membrane. A recent study has also shown that uptake of *Helicobacter pylori* vesicles via clathrin-dependent and independent pathway (Olofsson et al., 2014). We found that *L. monocytogenes* MVs were internalized into host cells through the interactions with actin-mediated machinery.

Also, we have validated our proteomics data by the acetylation of tryptic peptides generated from the proteins as described in earlier method (Kulkarni et al., 2010). The chemical modification of the tryptic peptides followed by mass spectrometry support the validation of the peptide sequence and also improved the efficiency of *de novo* sequencing. It was observed that acetylation influenced the fragmentation of synthetic peptides and also tryptic peptides (Jagannadham et al., 2018). Acetylation facilitated the formation of more fragments in the CID mass spectra (mostly b ions, or y ions) that allows the confirmation of the sequence of the peptides. The MS/MS spectra of the peptide from Listeriolysin O and its acetylated derivative corresponding to the sequence YAQAYPNVSAK are shown in Fig. 4A. The increase in b-ion intensities in acetylated peptides were clearly validated the sequence obtained by database search.

Further, we assessed the proteolytic activity of MVs by zymogram analysis. We have identified few proteolytic activities in MVs as mentioned earlier. Previous reports suggested the presence of active proteases in OMVs from other bacteria (Ramírez Rico et al., 2017; Elhenawy et al., 2014; Surve et al., 2016). Although this study was conducted in vitro, these proteases could participate in the pathogenesis process.

L. monocytogenes express arsenal of virulence determinants, which allow the bacterium to enter the host cells and to establish its intracellular niche. The virulence loci are controlled by the master

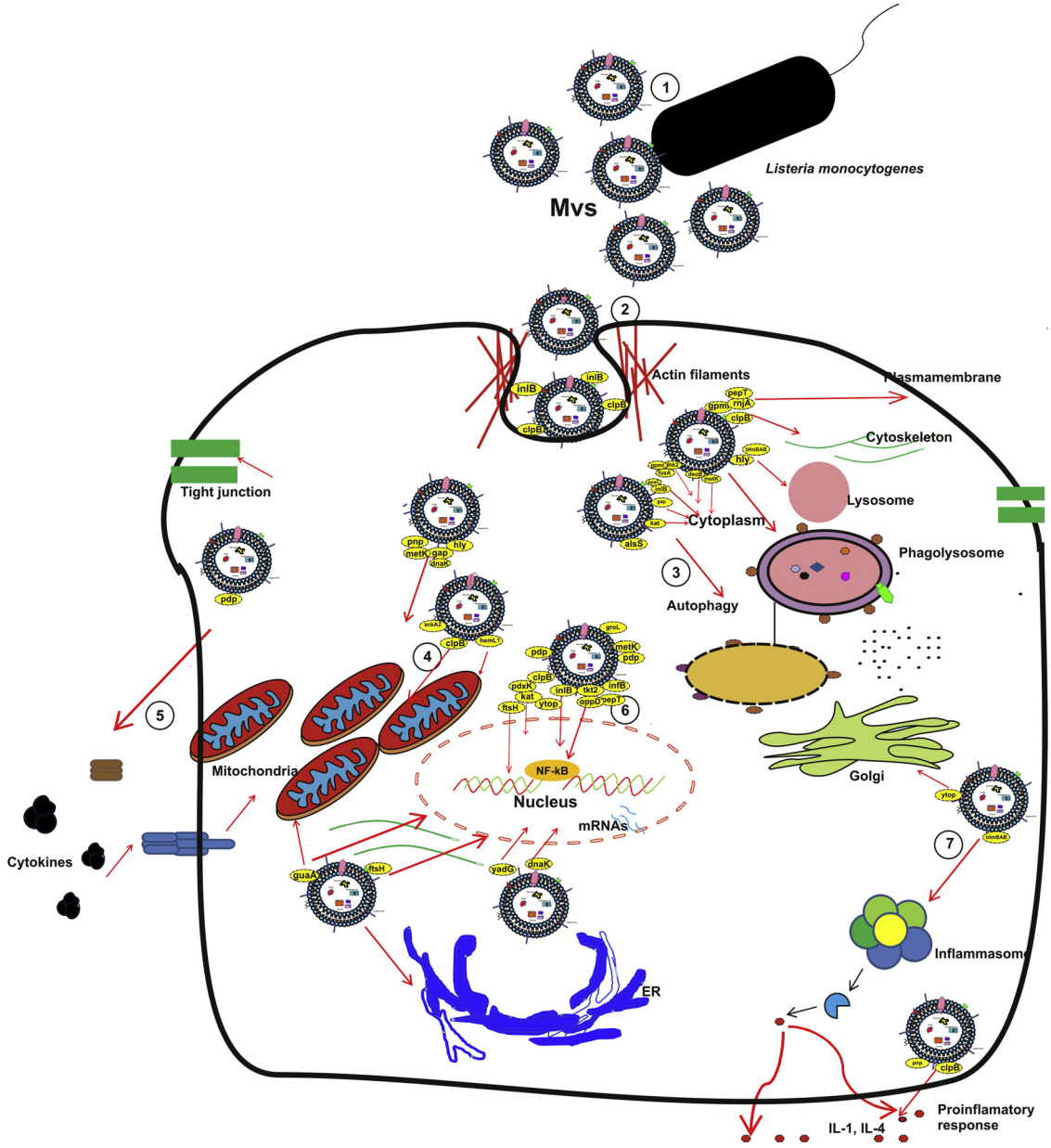

Fig. 5. Schematic representation of predicted *L. monocytogenes* MV-host protein-protein interactions network. (1) *L. monocytogenes* secrete biologically active MVs; (2) MVs are internalized into the host cell via actin-dependent endocytosis pathway; (3) MV carry a cocktail of proteins and enter to the endosomal compartment of the host cell and interact with proteins related to autophagy (4), mitochodria (5), cytokines (6) MV proteins interact with host nuclear proteins involved in NF-kB signaling pathways, MAPK signaling pathway, apoptosis (7) MV proteins interact with inflammasome and activate pro-inflammatory response.

regulator PrfA, which would enable *L. monocytogenes* to enter into the non-phagocytic cells, to escape from the internalization vacuole, to replicate intracellular, and to spread from cell to cell (Lecuit, 2005). The essential and well-characterized loci of this regulon include the listeriolysin (LLO) (Schnupf and Portnoy, 2007), actin polymerization (ActA), the Mpl metalloprotease, two phospholipases encoded by PlcA and PlcB, major internalins, InlA and InlB, the secreted protein InlC.

Two surface virulence proteins promote internalization of *L. monocytogenes*, InlA and InlB, which respectively target E-cadherin (Mengaud et al., 1996) and the hepatocyte growth factor receptor Met, which are host plasma membrane proteins. InlB-mediated entry requires localized polymerization of the host actin cytoskeleton. InlB activated each of these mTOR-containing complexes on the host receptor tyrosine kinase Met and the activity of PI3K (Bhalla et al., 2017). Besides, human SNARE proteins are required for the delivery of the human GTPase Dynamin 2, which promotes InlB-mediated entry (Van Ngo et al., 2017). We also found that the InlB as a major virulence proteins in MVs and predicted to interact with in several host proteins involved in the modulation of host cell death pathways. Previous studies have shown that InlB can activate NF-κB signaling to modulate host death (Mansell et al., 2000). Uptake of *Listeria* also requires clathrin, Dynamin 2, and several other host proteins that generally participate in endocytosis (Veiga and Cossart, 2005).

Another essential virulence factor LLO is found in MVs (encoded by the hly gene) which primary determinants of *L. monocytogenes* pathogenesis. Upon entry to the host cell, LLO mediates the escape from host cell phagosome before lysosomal fusion. Also, it activates/modulates various host signaling pathways such as IκB kinase complex-NF-κB signaling to stimulate immune activity, and modulates host cell epigenetics through histone modifications and chromatin remodeling (Kayal et al., 2002; Hamon et al., 2012). LLO induces apoptosis, and in high concentration, it can cause rapid lysis of the host cells (Carrero et al.,

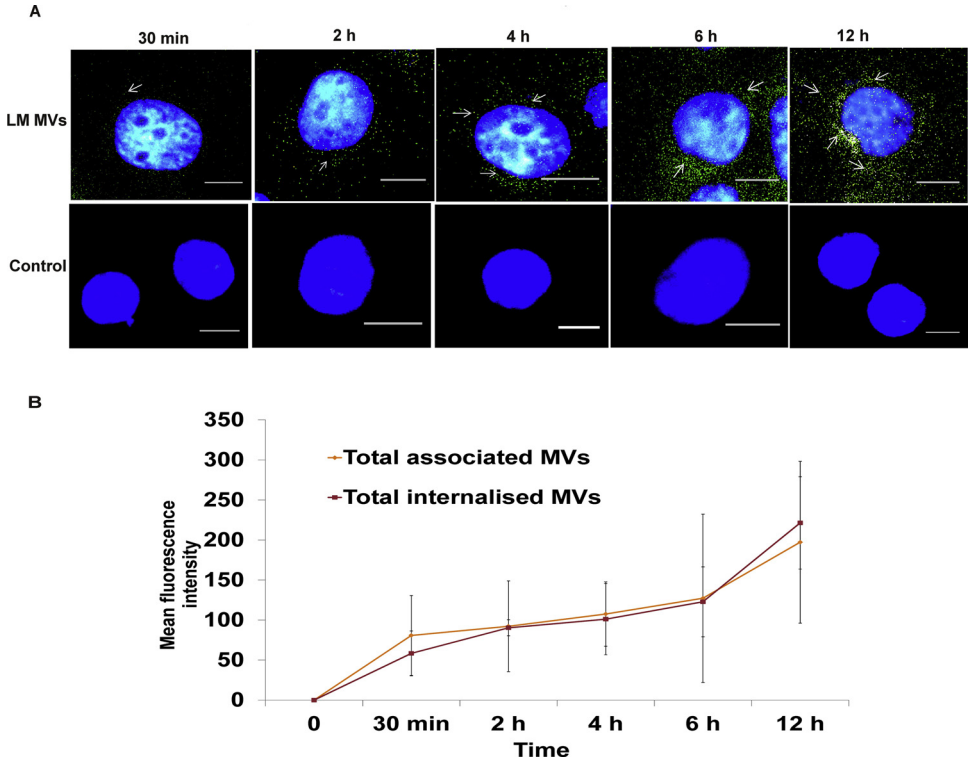

Fig. 6. Uptake of *L. monocytogenes* MVs by Caco-2 cells. (A) Microscopy images of cell associated and internalised MVs. Caco-2 cells were incubated with FITC labelled MVs for 30 min, 2 h, 4 h, 6 h, 12 h. The cells were stained with Hoechst. Internalized FITC labelled MVs are visualized in green. White arrows indicates the accumulation of FITC labelled MVs. Scale bars are set at 0.5 µm. (B) Caco-2 cells were incubated at 37 °C with FITC labelled MVs for 30 min, 2 h, 4 h, 6 h, 12 h. Fluorescence was measured in each time points. Caco-2 cells and MVs alone were used as controls of background fluorescence. Values are means ± standard error from four independent experiments ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2008; Seveau, 2014). LLO is also involved in posttranslational modifications such as deSUMOylation in the host cell and mitochondrial fragmentation (Ribet and Cossart, 2010; Stavru et al., 2013). A previous study on MVs of *L. monocytogenes* reported that the LLO of the MVs is essential for the host autophagy (Vdovikova et al., 2017). In this study, the predicted host-PPI network also suggests that the MV proteins can interact with the host proteins involved in autophagy machinery. The PPI network associated with these proteins indicates that *L. monocytogenes* MV proteins can target multiple host regulators involved in the autophagy. Another vital virulence factor is PlcA, which affect the lysis of the primary vacuole (Camargo et al., 2016; Camilli et al., 1993). In this study, PlcA was found in MVs, and thus it could play an essential role in MV-mediated pathogenesis. Besides, the secreted lipoproteins of *L. monocytogenes* interact with the host and induce inflammatory cytokines (tumor necrosis factor α [TNF-α] and interleukin 6) in a Toll-like receptor 2 (TLR2)–pathway during the infection (Machata et al., 2008).

Overall, the predicted host proteins in the PPI with *L. monocytogenes* MV proteins are involved in NF-κB signaling pathways, MAPK signaling pathway, apoptosis, endocytosis, autophagy, Wnt signaling pathway, T-

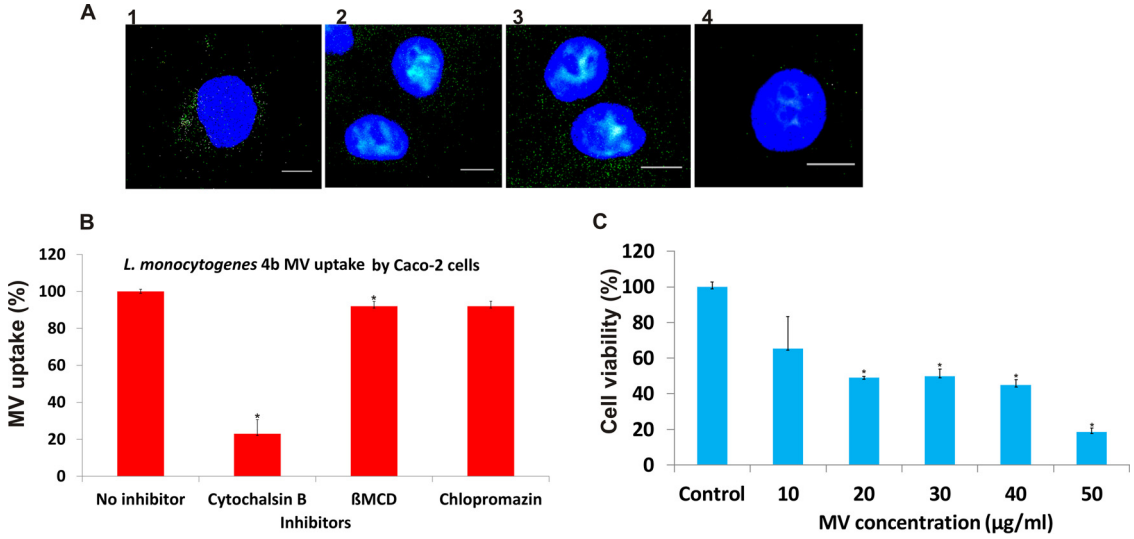

Fig. 7. Uptake mechanism of *L. monocytogenes* MVs by Caco-2 cells. (A) Effect of inhibitors on MV uptake. 1. Cells were treated without inhibitors (Control). Vesicles were internalized in to Caco-2 cells, 2. Cells were treated with β-MCD. 3. Cells were treated with chlopromazine and 4. Cells were treated with cytochalsin B and reduced uptake of MVs is observed. (B) Caco-2 cell fluorescence was measured and MV uptake in the presence of each inhibitor was expressed as the percentage of MV uptake by control, inhibitor-untreated cells (100%). Data are means ± standard deviations from three independent experiments. Scale bars are set at 0.5 µm (C) Host cell death induced by MVs of *L. monocytogenes*. Values are means ± standard error from four independent experiments ($P < 0.01$, versus untreated control cells).

cell and B-cell signaling pathways (Fig. 5). The NF-κB is one of the master regulators of the innate immune response, and it can activate the host inflammatory signaling pathway and cell survival pathway. Pathogens use a variety of mechanisms to manipulate the NF-κB–regulated survival pathway to modulate the host cell death response and thus, promote intracellular replication and pathogenicity. Similarly, previous studies have shown that listerial glycolipids and lipoproteins can affect the host immune system and either promote an effective immune response or inhibit the response (Yu et al., 2007; Zenewicz and Shen, 2007).

L. monocytogenes MVs serve as a transport system for virulence-associated components, as the host cell death is induced by MVs. In conclusion, our study demonstrates that *L. monocytogenes* MVs provide an important vehicle for the delivery of bacterial effector molecules to host cells. We have predicted several important virulence factors in MVs have the interaction with host cells. To the best of our knowledge, this work represents the first study to demonstrate the entry mechanism, predication of MV-host interaction and cytotoxicity of *L. monocytogenes* MVs in host cells. Our findings have significant implications for the study of Gram-positive bacteria pathogenesis and the development of a new therapeutic target against *L. monocytogenes* infections.

Competing interests

The authors declare no competing interests.

Acknowledgments

The authors gratefully acknowledge University Grants Commission, New Delhi, India for providing financial support (UGC-MRP 41-1143/2012(SR)). RK thanks UGC-BSR for awarding UGC-Meritorious Fellowship. The authors like to thank Mr. B. Raman from proteomic facility of CCMB for technical help in LC/MS/MS. We acknowledge the Proteomics Facility at CCMB, Hyderabad. We also acknowledge the UGC-CAS, NRCBS, DBT-IPLS, and DST-PURSE Programs of the School of Biological Sciences, Madurai Kamaraj University.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.03.008.

References

Babu, M.M., Priya, M.L., Selvan, A.T., Madera, M., Gough, J., Aravind, L., Sankaran, K., 2006. A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. J. Bacteriol. 188, 2761–2773. https://doi.org/10.1128/JB.188.8.2761-2773.2006.

Bagos, P.G., Tsirigos, K.D., Liakopoulos, T.D., Hamodrakas, S.J., 2008. Prediction of lipoprotein signal peptides in Gram-positive bacteria with a Hidden Markov Model. J. Proteome Res. 7, 5082–5093. https://doi.org/10.1021/pr800162c.

Bagos, P.G., Nikolaou, E.P., Liakopoulos, T.D., Tsirigos, K.D., 2010. Combined prediction of Tat and Sec signal peptides with hidden Markov models. Bioinformatics 26, 2811–2817. https://doi.org/10.1093/bioinformatics/btq530.

Bauman, S.J., Kuehn, M.J., 2009. *Pseudomonas aeruginosa* vesicles associate with and are internalized by human lung epithelial cells. BMC Microbiol. 9. https://doi.org/10.1186/1471-2180-9-26.

Bendtsen, J.D., Kiemer, L., Fausbøll, A., Brunak, S., 2005. Non-classical protein secretion in bacteria. BMC Microbiol. 5. https://doi.org/10.1186/1471-2180-5-58.

Bhalla, M., Law, D., Dowd, G.C., Ireton, K., 2017. Host serine/threonine kinases mTOR and protein kinase C-α promote InlB-mediated entry of Listeria monocytogenes. Infect. Immun. https://doi.org/10.1128/IAI.00087-17.

Bielaszewska, M., Rüter, C., Bauwens, A., Greune, L., Jarosch, K.A., Steil, D., Zhang, W., He, X., Lloubes, R., Fruth, A., Kim, K.S., Schmidt, M.A., Dobrindt, U., Mellmann, A., Karch, H., 2017. Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: intracellular delivery, trafficking and mechanisms of cell injury. PLoS Pathog. 13. https://doi.org/10.1371/journal.ppat.1006159.

Bomberger, J.M., MacEachran, D.P., Coutermarsh, B.A., Ye, S., O'Toole, G.A., Stanton, B.A., 2009. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. PLoS Pathog. 5. https://doi.org/10.1371/journal.ppat.1000382.

Brown, L., Kessler, A., Cabezas-Sanchez, P., Luque-Garcia, J.L., Casadevall, A., 2014. Extracellular vesicles produced by the Gram-positive bacterium *Bacillus subtilis* are disrupted by the lipopeptide surfactin. Mol. Microbiol. 93, 183–198. https://doi.org/10.1111/mmi.12650.

Burall, L.S., Grim, C.J., Datta, A.R., 2017. A clade of *Listeria m*onocytogenes serotype 4b variant strains linked to recent listeriosis outbreaks associated with produce from a defined geographic region in the US. PLoS One. https://doi.org/10.1371/journal.pone.0176912.

Cabanes, D., Dussurget, O., Dehoux, P., Cossart, P., 2004. Auto, a surface associated autolysin of *Listeria monocytogenes* required for entry into eukaryotic cells and virulence. Mol. Microbiol. https://doi.org/10.1111/j.1365-2958.2003.03945.x.

Camargo, A.C., Woodward, J.J., Nero, L.A., 2016. The continuous challenge of characterizing the foodborne pathogen Listeria monocytogenes. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2015.2115.

Camejo, A., Buchrieser, C., Couvé, E., Carvalho, F., Reis, O., Ferreira, P., Sousa, S., Cossart, P., Cabanes, D., 2009. In vivo transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. PLoS Pathog. 5. https://doi.org/10.1371/journal.ppat.1000449.

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S., Cabanes, D., 2011. The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence. https://doi.org/10.4161/viru.2.5.17703.

Camilli, A., Tilney, L.G., Portnoy, D.A., 1993. Dual roles of plcA in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. https://doi.org/10.1111/j.1365-2958.1993.tb01211.x.

Cañas, M.A., Giménez, R., Fábrega, M.J., Toloza, L., Baldomà, L., Badia, J., 2016. Outer membrane vesicles from the probiotic *Escherichia coli* Nissle 1917 and the commensal ECOR12 enter intestinal epithelial cells via clathrin-dependent endocytosis and elicit differential effects on DNA damage. PLoS One 11. https://doi.org/10.1371/journal.pone.0160374.

Carrero, J.A., Vivanco-Cid, H., Unanue, E.R., 2008. Granzymes drive a rapid listeriolysin O-induced T cell apoptosis. J. Immunol https://doi.org/181/2/1365 [pii].

Carvalho, F., Sousa, S., Cabanes, D., 2014. How *Listeria monocytogenes* organizes its surface for virulence. Front. Cell. Infect. Microbiol. 4. https://doi.org/10.3389/fcimb.2014.00048.

Chandrabos, C., M'Homa Soudja, S., Weinrick, B., Gros, M., Frangaj, A., Rahmoun, M., Jacobs, W.R., Lauvau, G., 2015. The p60 and NamA autolysins from *Listeria monocytogenes* contribute to host colonization and induction of protective memory. Cell. Microbiol. https://doi.org/10.1111/cmi.12362.

Chang, T.H., Wu, L.C., Lee, T.Y., Chen, S.P., Da Huang, H., Horng, J.T., 2013. EuLoc: a web-server for accurately predict protein subcellular localization in eukaryotes by incorporating various features of sequence segments into the general form of Chou's PseAAC. J. Comput. Aided Mol. Des. 27, 91–103. https://doi.org/10.1007/s10822-012-9628-0.

Choi, D.S., Kim, D.K., Choi, S.J., Lee, J., Choi, J.P., Rho, S., Park, S.H., Kim, Y.K., Hwang, D., Gho, Y.S., 2011. Proteomic analysis of outer membrane vesicles derived from Pseudomonas aeruginosa. Proteomics 11, 3424–3429. https://doi.org/10.1002/pmic.201000212.

Deatheragea, B.L., Cooksona, B.T., 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect. Immun. https://doi.org/10.1128/IAI.06014-11.

Desvaux, M., Hébraud, M., 2006. The protein secretion systems in *Listeria*: inside out bacterial virulence. FEMS Microbiol. Rev. https://doi.org/10.1111/j.1574-6976.2006.00035.x.

Dons, L., Eriksson, E., Jin, Y., Rottenberg, M.E., Kristensson, K., Larsen, C.N., Bresciani, J., Olsen, J.E., 2004. Role of flagellin and the two-component cheA/cheY system of *Listeria monocytogenes* in host cell invasion and virulence. Infect. Immun. 72, 3237–3244. https://doi.org/10.1128/IAI.72.6.3237-3244.2004.

Elhenawy, W., Debelyy, M.O., Feldman, M.F., 2014. Preferential packing of acidic glycosidases and proteases into Bacteroides outer membrane vesicles. MBio. https://doi.org/10.1128/mBio.00909-14.

Farber, J., Peterkin, P., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511. https://doi.org/10.1104/pp.110.161547.

Finethy, R., Luoma, S., Orench-Rivera, N., Feeley, E.M., Haldar, A.K., Yamamoto, M., Kanneganti, T.D., Kuehn, M.J., Coers, J., 2017. Inflammasome activation by bacterial outer membrane vesicles requires guanylate binding proteins. MBio 8. https://doi.org/10.1128/mBio.01188-17.

Frankowski, H., Gu, Y.H., Heo, J.H., Milner, R., Del Zoppo, G.J., 2012. Use of gel zymography to examine matrix metalloproteinase (Gelatinase): expression in brain tissue or in primary glial Cultures. Methods Mol. Biol. https://doi.org/10.1007/978-1-61779-452-0_15.

Garg, A., Gupta, D., 2008. VirulentPred: a SVM based prediction method for virulent proteins in bacterial pathogens. BMC Bioinformatics 9. https://doi.org/10.1186/1471-2105-9-62.

Gedde, M.M., Higgins, D.E., Tilney, L.G., Portnoy, D.A., 2000. Role of listeriolysin O in cell-to-cell spread of Listeria monocytogenes. Infect. Immun. https://doi.org/10.1128/IAI.68.2.999-1003.2000.

Grande, R., Celia, C., Mincione, G., Stringaro, A., Di Marzio, L., Colone, M., Di Marcantonio, M.C., Savino, L., Puca, V., Santoliquido, R., Locatelli, M., Muraro, R., Hall-Stoodley, L., Stoodley, P., 2017. Detection and physicochemical characterization of membrane vesicles (MVs) of *Lactobacillus reuteri* DSM 17938. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01040.

Gurung, M., Moon, D.C., Choi, C.W., Lee, J.H., Bae, Y.C., Kim, J., Lee, Y.C., Seol, S.Y., Cho, D.T., Kim, S.I., Lee, J.C., 2011. *Staphylococcus aureus* produces membrane-derived vesicles that induce host cell death. PLoS One 6. https://doi.org/10.1371/journal.pone.0027958.

Haas, B., Grenier, D., 2015. Isolation, characterization and biological properties of

membrane vesicles produced by the swine pathogen Streptococcus suis. PLoS One. https://doi.org/10.1371/journal.pone.0130528.
Hamon, M.A., Ribet, D., Stavru, F., Cossart, P., 2012. Listeriolysin O: the swiss army knife of Listeria. Trends Microbiol. https://doi.org/10.1016/j.tim.2012.04.006.
Hernandez-Milian, A., Payeras-Cifre, A., 2014. What is new in Listeriosis? Biomed Res. Int. https://doi.org/10.1155/2014/358051.
Horstman, A.L., Kuehn, M.J., 2000. Enterotoxigenic *Escherichia coli* secrete active heat-labile enterotoxin via outer membrane vesicles. J. Biol. Chem. 275, 12489–12496. https://doi.org/10.1074/JBC.275.17.12489.
Ireton, K., 2007. Entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells. Cell. Microbiol. https://doi.org/10.1111/j.1462-5822.2007.00933.x.
Jacquet, C., Catimel, B., Brosch, R., Buchrieser, C., Dehaumont, P., Goulet, V., Lepoutre, A., Veit, P., Rocourt, J., 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. Appl. Environ. Microbiol. 61, 2242–2246.
Jagannadham, M.V., Kameshwari, D.B., Gayathri, P., Nagaraj, R., 2018. Detection of peptides with intact phosphate groups using MALDI TOF/TOF and comparison with the ESI-MS/MS. Eur. J. Mass Spectrom. Chichester (Chichester). https://doi.org/10.1177/1469066717748115.
Jin, J.S., Kwon, S.-O., Moon, D.C., Gurung, M., Lee, J.H., Kim, S.I., Lee, J.C., 2011. *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via Outer membrane vesicles. PLoS One 6, e17027. https://doi.org/10.1371/journal.pone.0017027.
Juncker, A.S., Willenbrock, H., von Heijne, G., Brunak, S., Nielsen, H., Krogh, A., 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. 12, 1652–1662. https://doi.org/10.1110/ps.0303703.
Jung, A.L., Stoiber, C., Herkt, C.E., Schulz, C., Bertrams, W., Schmeck, B., 2016. *Legionella pneumophila*-derived outer membrane vesicles promote bacterial replication in macrophages. PLoS Pathog. 12. https://doi.org/10.1371/journal.ppat.1005592.
Karthikeyan, R., Gunasekaran, P., Rajendhran, J., 2015. Molecular serotyping and pathogenic potential of *Listeria monocytogenes* isolated from milk and milk products in Tamil Nadu, India. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2014.1872.
Kato, S., Kowashi, Y., Demuth, D.R., 2002. Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. Microb. Pathog. 32, 1–13. https://doi.org/10.1006/mpat.2001.0474.
Kayal, S., Lilienbaum, A., Join-Lambert, O., Li, X., Israël, A., Berche, P., 2002. Listeriolysin O secreted by *Listeria monocytogenes* induces NF-κB signalling by activating the IκB kinase complex. Mol. Microbiol. https://doi.org/10.1046/j.1365-2958.2002.02973.x.
Kuchibhotla, B., Kola, S.R., Medicherla, J.V., Cherukuvada, S.V., Dhople, V.M., Nalam, M.R., 2017. Combinatorial labeling method for improving peptide fragmentation in mass spectrometry. J. Am. Soc. Mass Spectrom. https://doi.org/10.1007/s13361-017-1606-2.
Kulkarni, H.M., Ramesh, V., Srinivas, R., Jagannadham, M.V., 2010. Acetylating tryptic peptides enhances b ion intensity in MALDI TOF/TOF: implications in peptide sequencing and identification of proteins in an Antarctic bacterium Pseudomonas syringae. Proteomics Insights. https://doi.org/10.4137/PRI.S3676.
Kulkarni, H.M., Swamy, C.V.B., Jagannadham, M.V., 2014. Molecular characterization and functional analysis of outer membrane vesicles from the Antarctic bacterium *Pseudomonas syringae* suggest a possible response to environmental conditions. J. Proteome Res. 13, 1345–1358. https://doi.org/10.1021/pr4009223.
Kumar, R., Nanduri, B., 2010. HPIDB - a unified resource for host-pathogen interactions. BMC Bioinformatics 11. https://doi.org/10.1186/1471-2105-11-16.
Kunsmann, L., Rüter, C., Bauwens, A., Greune, L., Glüder, M., Kemper, B., Fruth, A., Wai, S.N., He, X., Lloubes, R., Schmidt, M.A., Dobrindt, U., Mellmann, A., Karch, H., Bielaszewska, M., 2015. Virulence from vesicles: novel mechanisms of host cell injury by *Escherichia coli* O104:H4 outbreak strain. Sci. Rep. 5, 13252. https://doi.org/10.1038/srep13252.
Laursen, M.F., Bahl, M.I., Licht, T.R., Gram, L., Knudsen, G.M., 2015. A single exposure to a sublethal pediocin concentration initiates a resistance-associated temporal cell envelope and general stress response in Listeria monocytogenes. Environ. Microbiol. https://doi.org/10.1111/1462-2920.12534.
Lecuit, M., 2005. Understanding how *Listeria monocytogenes* targets and crosses host barriers. Clin. Microbiol. Infect. https://doi.org/10.1111/j.1469-0691.2005.01146.x.
Lee, J.H., Choi, C.-W., Lee, T., Kim, S.I., Lee, J.-C., Shin, J.-H., 2013. Transcription factor σB plays an important role in the production of extracellular membrane-derived vesicles in Listeria monocytogenes. PLoS One 8, e73196. https://doi.org/10.1371/journal.pone.0073196.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275. https://doi.org/10.1016/0304-3894(92)87011-4.
Machata, S., Tchatalbachev, S., Mohamed, W., Jansch, L., Hain, T., Chakraborty, T., 2008. Lipoproteins of *Listeria monocytogenes* are critical for virulence and TLR2-mediated immune activation. J. Immunol. 181, 2028–2035. https://doi.org/10.4049/jimmunol.181.3.2028.
Mansell, A., Braun, L., Cossart, P., O'neill, La.J., 2000. A novel function of InlB from *Listeria monocytogenes*: activation of NF-κB in J774 macrophages. Cell. Microbiol. 2, 127–136. https://doi.org/10.1046/j.1462-5822.2000.00038.x.
Maurella, C., Gallina, S., Ru, G., Adriano, D., Bellio, A., Bianchi, D.M., Chiavacci, L., Crescio, M.I., Croce, M., D'Errico, V., Dupont, M.F., Marra, A., Natangelo, U., Pomilio, F., Romano, A., Stanzione, S., Zaccaria, T., Zuccon, F., Caramelli, M., Decastelli, L., 2018. Outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* 1/2A in sliced cold beef ham, Italy, may 2016. Eurosurveillance. https://doi.org/10.2807/1560-7917.ES.2018.23.10.17-00155.
Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., Cossart, P., 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *Listeria monocytogenes* into epithelial cells. Cell. https://doi.org/10.1016/S0092-8674(00)81070-3.
Mondal, A., Tapader, R., Chatterjee, N.S., Ghosh, A., Sinha, R., Koley, H., Saha, D.R., Chakrabarti, M.K., Wai, S.N., Pal, A., 2016. Cytotoxic and inflammatory responses induced by outer membrane vesicle-associated biologically active proteases from Vibrio cholerae. Infect. Immun. 84, 1478–1490. https://doi.org/10.1128/IAI.01365-15.
Mostowy, S., Cossart, P., 2012. Virulence factors that modulate the cell biology of *Listeria* infection and the host response. Adv. Immunol. 113, 19–32. https://doi.org/10.1016/B978-0-12-394590-7.00007-5.
Olaya-Abril, A., Prados-Rosales, R., McConnell, M.J., Martín-Peña, R., González-Reyes, J.A., Jiménez-Munguía, I., Gómez-Gascón, L., Fernández, J., Luque-García, J.L., García-Lidón, C., Estévez, H., Pachón, J., Obando, I., Casadevall, A., Pirofski, Lanne, Rodríguez-Ortega, M.J., 2014. Characterization of protective extracellular membrane-derived vesicles produced by Streptococcus pneumoniae. J. Proteomics 106, 46–60. https://doi.org/10.1016/j.jprot.2014.04.023.
Olofsson, A., Skalman, L.N., Obi, I., Lundmark, R., Arnqvist, A., 2014. Uptake of *Helicobacter pylori* vesicles is facilitated by clathrin- dependent and clathrin-independent endocytic pathways. MBio 5. https://doi.org/10.1128/mBio.00979-14.
Orsi, R.H., de Bakker, H.C., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. https://doi.org/10.1016/j.ijmm.2010.05.002.
Pan, Y., Breidt, F., Kathariou, S., 2009. Competition of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed-culture biofilms. Appl. Environ. Microbiol. 75 (18), 5846–5852.
Pentecost, M., Kumaran, J., Ghosh, P., Amieva, M.R., 2010. *Listeria monocytogenes* internalin B activates junctional endocytosis to accelerate intestinal invasion. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1000900.
Petersen, T.N., Brunak, S., Von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods. https://doi.org/10.1038/nmeth.1701.
Ramírez Rico, G., Martínez-Castillo, M., González-Ruíz, C., Luna-Castro, S., de la Garza, M., 2017. Mannheimia haemolytica A2 secretes different proteases into the culture medium and in outer membrane vesicles. Microb. Pathog. https://doi.org/10.1016/j.micpath.2017.10.027.
Resch, U., Tsatsaronis, J.A., Le Rhun, A., Stübiger, G., Rohde, M., Kasvandik, S., Holzmeister, S., Tinnefeld, P., Nyunt Wai, S., Charpentier, E., 2016. A two-component regulatory system impacts extracellular membrane-derived vesicle production in Group A Streptococcus. MBio. https://doi.org/10.1128/mBio.00207-16.
Ribeiro, V.B., Destro, M.T., 2014. *Listeria monocytogenes* serotype 1/2b and 4b isolates from human clinical cases and foods show differences in tolerance to refrigeration and salt stress. J. Food Prot. 77 (9), 1519–1526. https://doi.org/10.4315/0362-028X.
Ribet, D., Cossart, P., 2010. Post-translational modifications in host cells during bacterial infection. FEBS Lett. https://doi.org/10.1016/j.febslet.2010.05.012.
Rose, R.W., Brüser, T., Kissinger, J.C., Pohlschröder, M., 2002. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol. Microbiol. https://doi.org/10.1046/j.1365-2958.2002.03090.x.
Sashinami, H., Hu, D.L., Li, S.J., Mitsui, T., Hakamada, K.I., Ishiguro, Y., Fukuda, S., Nakane, A., 2010. Virulence factor p60 of *Listeria monocytogenes* modulates innate immunity by inducing tumor necrosis factor α. FEMS Immunol. Med. Microbiol. https://doi.org/10.1111/j.1574-695X.2010.00666.x.
Schmidt, R.L., Filak, H.C., Lemon, J.D., Potter, T.A., Lenz, L.L., 2011. A LysM and SH3-domain containing region of the *Listeria monocytogenes* p60 protein stimulates accessory cells to promote activation of host NK cells. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1002368.
Schnupf, P., Portnoy, D.A., 2007. Listeriolysin O: a phagosome-specific lysin. Microbes Infect. https://doi.org/10.1016/j.micinf.2007.05.005.
Schwechheimer, C., Kulp, A., Kuehn, M.J., 2014. Modulation of bacterial outer membrane vesicle production by envelope structure and content. BMC Microbiol. 14. https://doi.org/10.1186/s12866-014-0324-1.
Seeliger, H.P.R., Höhne, K., 1979. Serotyping of *Listeria monocytogenes* and related species. Methods Microbiol. 13, 31–49. https://doi.org/10.1016/S0580-9517(08)70372-6.
Seveau, S., 2014. Multifaceted activity of listeriolysin O, the cholesterol-dependent cytolysin of Listeria monocytogenes. Subcell. Biochem. https://doi.org/10.1007/978-94-017-8881-6_9.
Shahmirzadi, S.V., Nguyen, M.T., Götz, F., 2016. Evaluation of *Staphylococcus aureus* lipoproteins: role in nutritional acquisition and pathogenicity. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01404.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software Environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.
Shen, J., Rump, L., Zhang, Y., Chen, Y., Wang, X., Meng, J., 2013. Molecular subtyping and virulence gene analysis of *Listeria monocytogenes* isolates from food. Food Microbiol. https://doi.org/10.1016/j.fm.2013.02.014.
Stavru, F., Palmer, A.E., Wang, C., Youle, R.J., Cossart, P., 2013. Atypical mitochondrial fission upon bacterial infection. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.1315784110.
Surve, M.V., Anil, A., Kamath, K.G., Bhutda, S., Sthanam, L.K., Pradhan, A., Srivastava, R., Basu, B., Dutta, S., Sen, S., Modi, D., Banerjee, A., 2016. Membrane vesicles of Group B Streptococcus disrupt feto-maternal barrier leading to preterm birth. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1005816.
Tashiro, Y., Hasegawa, Y., Shintani, M., Takaki, K., Ohkuma, M., Kimbara, K., Futamata, H., 2017. Interaction of bacterial membrane vesicles with specific species and their potential for delivery to target cells. Front. Microbiol. 8. https://doi.org/10.3389/

fmicb.2017.00571.
Van Ngo, H., Bhalla, M., Chen, D.Y., Ireton, K., 2017. A role for host cell exocytosis in InlB-mediated internalisation of Listeria monocytogenes. Cell. Microbiol. https://doi.org/10.1111/cmi.12768.
Vdovikova, S., Luhr, M., Szalai, P., Nygård Skalman, L., Francis, M.K., Lundmark, R., Engedal, N., Johansson, J., Wai, S.N., 2017. A Novel Role of *Listeria monocytogenes* membrane vesicles in Inhibition of autophagy and cell death. Front. Cell. Infect. Microbiol. 7. https://doi.org/10.3389/fcimb.2017.00154.
Veiga, E., Cossart, P., 2005. Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. Nat. Cell Biol. https://doi.org/10.1038/ncb1292.
Veith, P.D., Chen, Y.Y., Gorasia, D.G., Chen, D., Glew, M.D., O'Brien-Simpson, N.M., Cecil, J.D., Holden, J.A., Reynolds, E.C., 2014. *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. J. Proteome Res. https://doi.org/10.1021/pr401227e.
Wai, S.N., Lindmark, B., Söderblom, T., Takade, A., Westermark, M., Oscarsson, J., Jass, J., Richter-Dahlfors, A., Mizunoe, Y., Uhlin, B.E., 2003. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell 115, 25–35. https://doi.org/10.1016/S0092-8674(03)00754-2.
Wang, W., Zhou, X., Suo, Y., Deng, X., Cheng, M., Shi, C., Shi, X., 2017. Prevalence, serotype diversity, biofilm-forming ability and eradication of *Listeria monocytogenes* isolated from diverse foods in Shanghai, China. Food Control. https://doi.org/10.1016/j.foodcont.2016.10.025.
Way, S.S., Thompson, L.J., Lopes, J.E., Hajjar, A.M., Kollmann, T.R., Freitag, N.E., Wilson, C.B., 2004. Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. Cell. Microbiol. https://doi.org/10.1046/j.1462-5822.2004.00360.x.
Wu, J., Fernandes-Alnemri, T., Alnemri, E.S., 2010. Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. J. Clin. Immunol. https://doi.org/10.1007/s10875-010-9425-2.
Yu, W.L., Dan, H., Lin, M., 2007. Novel protein targets of the humoral immune response to *Listeria monocytogenes* infection in rabbits. J. Med. Microbiol. 56, 888–895. https://doi.org/10.1099/jmm.0.46977-0.
Yu, N.Y., Wagner, J.R., Laird, M.R., Melli, G., Rey, S., Lo, R., Dao, P., Cenk Sahinalp, S., Ester, M., Foster, L.J., Brinkman, F.S.L., 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26, 1608–1615. https://doi.org/10.1093/bioinformatics/btq249.
Yu, C.-S., Cheng, C.-W., Su, W.-C., Chang, K.-C., Huang, S.-W., Hwang, J.-K., Lu, C.-H., 2014. CELLO2GO: a web server for protein subCELlular LOcalization prediction with functional Gene Ontology annotation. PLoS One 9, e99368. https://doi.org/10.1371/journal.pone.0099368.
Yun, S.H., Lee, S.Y., Choi, C.W., Lee, H., Ro, H.J., Jun, S., Kwon, Y.M., Kwon, K.K., Kim, S.J., Kim, G.H., Kim, S.I., 2017. Proteomic characterization of the outer membrane vesicle of the halophilic marine bacterium *Novosphingobium pentaromativorans* US6-1. J. Microbiol. https://doi.org/10.1007/s12275-017-6581-6.
Zenewicz, La., Shen, H., 2007. Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview. Microbes Infect. 9, 1208–1215. https://doi.org/10.1016/j.micinf.2007.05.008.