

**Comparison of three methods for cell surface proteome extraction of *Listeria*  
*monocytogenes* biofilms**

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**Short title:** *Methods for bacterial surface proteome extraction*

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## **Abstract**

The cell surface proteome of the foodborne pathogen *Listeria monocytogenes*, the etiological agent of listeriosis, is critical for understanding the physiological processes associated with stress resistance and persistence in the environment. In this context, the most widespread mode of growth for bacterial cells in natural and industrial environments is in biofilms. Cell surface proteins are, however, challenging to characterize due to their low abundance and poor solubility. Moreover, cell surface protein extracts are usually contaminated with cytoplasmic proteins that constitute the main signal in proteomic analysis. This study aimed to compare the efficiency of three methods to extract and explore surface proteins of *L. monocytogenes* growing in a biofilm: trypsin shaving, biotinylation and cell fractionation. Peptides separation and identification were performed by shotgun proteomics using high-performance liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). The biotinylation method was the most effective in extracting surface proteins, with the lowest rate of contamination by cytoplasmic proteins. Although presenting a higher contamination rate in cytoplasmic proteins, the other two techniques allowed the identification of additional surface proteins. Seven proteins were commonly retrieved by the three methods. The extracted proteins belong to several functional classes, involved in virulence, transport or metabolic pathways. Finally, the three extraction methods appeared complementary and their combined use improved the exploration of the bacterial surface proteome. These new findings collectively inform future discovery and translational proteomics for clinical, environmental health and industrial applications.

**Keywords:** *Listeria monocytogenes*; biofilms; cell surface proteome; protein extraction; LC-MS/MS

## Introduction

The most widespread mode of growth for bacterial cells in natural and industrial environments is in biofilms. Cell surface proteins are difficult to characterize due to low abundance and poor solubility. Moreover, cell surface protein extracts are usually contaminated with cytoplasmic proteins that constitute the main signal in proteomic analysis.

In this context, bacterial cell surface proteome constitutes the so-called proteosurfaceome of a bacterium (Cullen et al., 2005; Desvaux et al., 2018; Desvaux et al., 2006a). While the totality of the molecules found at the bacterial cell surface corresponds to the surfaceome, the proteosurfaceome specifically refers to the protein subset of the surfaceome, which can be involved in diversified processes and contributes to the interface between the bacterium and its environment.

Among bacteria involved in biofilm formation, *Listeria monocytogenes* is an important foodborne pathogen associated with high mortality rates in large outbreaks (Allerberger and Wagner, 2010). Capable of growing at refrigeration temperatures, in a wide range of pH (4.3 to 9.6), in salt concentrations up to 10% NaCl and low water activity ( $A_w$  down to 0.90), it can contaminate and persist in food-processing environment (Renier et al., 2011). In the context of pathogenesis, surface proteins are key factors to understand invasion mechanisms of host cells and thriving capabilities in stress conditions including hostile niche.

Annotation of *L. monocytogenes* EGDe (Glaser et al., 2001) genome predicted 133 surface proteins, accurately classified according to their anchoring systems and potential structural domains (Cabanes et al., 2002). Essential roles for surface proteins include bacterial growth, transport, virulence, sensing of and resistance to environmental conditions, signalling and adhesion to substrates and intercellular interaction for biofilm formation. These surface proteins correspond to nearly 5% of all the predicted proteins from the complete genome, and interestingly, represent the major difference between *L. monocytogenes* and the non-

pathogenic *L. innocua*, highlighting their potential role in pathogenesis (Cabanes et al., 2002). Even though the involvement of surface proteins in virulence is well documented, there are only few investigations concerning the role of cell surface proteome in other mechanisms like adaptation, resistance or persistence in the environment. Examining the proteosurfaceome of *L. monocytogenes* in biofilms is important and timely because it is the predominant mode of growth in food workshops (Giaouris et al., 2015; Giaouris et al., 2014), therefore influencing the physiology and the cell envelope composition of sessile cells (Azeredo et al., 2017). This study aimed to compare the efficiency of three methods to extract and explore surface proteins of *L. monocytogenes* growing in a biofilm: trypsin shaving, biotinylation and cell fractionation.

## **Materials and methods**

### *Strain and culture conditions*

*L. monocytogenes* EGD-e strain, serogroup 1/2a, whose genome was sequenced in 2001 (Glaser et al., 2001), was used throughout this study. Routine pre-culturing and culturing were carried out in Tryptic Soy Broth (TSB, Difco, Fisher Scientific) at 25 °C and 150 rpm. Bacterial growth was monitored by measuring the absorbance at 600 nm (OD<sub>600</sub>). Precultured cells in stationary phase were used to inoculate cultures to obtain a final OD<sub>600</sub> of 0.005. After 6 h of growth, cells were harvested by centrifugation (7 500 × g, 15 min) and resuspended in TSB diluted by 1:5 with sterile water in a volume equal to that of the supernatant collected, reaching an OD<sub>600</sub> between 0.6 and 0.7. Seven milliliters of the bacterial suspension was poured on each stainless steel (SS) disk (38.5 cm<sup>2</sup>), corresponding to an inoculation of 10<sup>8</sup> to 10<sup>9</sup> UFC/cm<sup>2</sup>. The SS disks were then placed in a sterile petri dish (55-mm diameter) and incubated at 25°C. Bacterial cells were allowed to adhere onto the disk for 3 h in static mode, before removing the medium to eliminate planktonic cells and adding fresh medium. The

disks were then incubated for 21 h to reach biofilm formation. To harvest the biofilm, the medium was removed and adherent cells were detached in 10 ml of Tryptone-salt (tryptone 0.1%, NaCl 0.85%, pH 7.0) by scraping the SS disk with a sterile spoonbill. Cell adhesion and biofilm formation after 24 h incubation at 25°C were evaluated by cell enumeration. Serial dilutions were plated on Tryptic Soy Agar (TSA, Difco, Fisher Scientific) and incubated for 24 h at 37 °C.

#### *Enzymatic shaving of surface proteins*

The shaving method consists of treating intact cells with proteases in an isotonic solution to release exposed peptides. Biofilm cells were harvested by low-speed centrifugation (1 000 × g, 15 min, 4°C) to prevent cell lysis. The bacterial cell pellet was gently washed twice with 1 ml of ice-cold Tris Buffer Saline (TBS, 20 mM Tris-HCl pH 7.4, 150 mM NaCl). Pellet was resuspended in 1 ml of shaving buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM CaCl<sub>2</sub> 6H<sub>2</sub>O, 1 M L-arabinose) and bacteria were treated with 0.5 µg/ml sequencing grade trypsin (Promega, Charbonnières-les-bains, France) under gentle shaking at 37°C for 1 h. Bacterial cells were removed by centrifugation (1 000 × g, 15 min, 4°C) and the supernatant, containing trypsin-shaved peptides, was collected and filtered (0.22 µm). Digestion of peptides was completed overnight with 0.6 µg of trypsin at 37°C. Peptides purification and concentration were carried out using Sep-Pak C18 Plus Short cartridges (Waters), pre-equilibrated in two steps with 65% ACN/0.1% trifluoroacetic acid (TFA) and 2% ACN/0.1% TFA. Peptides were loaded onto the cartridges, washed with 2% ACN/0.1% TFA and eluted with 65% ACN/0.1% TFA. Purified peptides were dried with a speed-vacuum and resuspended in 20 µL of 2% ACN/0.1% TFA.

#### *Biotinylation of bacterial cell surface proteins*

In the biotinylation method, intact cells are treated with sulfo-NHS-SS-Biotin, to which the cell membrane is impermeable. This marker molecule reacts specifically with the ε-amino-

group of lysine residues of surface-exposed proteins. Subsequently, labelled proteins can be separated from non-labelled proteins by affinity chromatography with neutravidin and then analyzed by LC-MS/MS (Hempel et al., 2011; Hempel et al., 2010; Romero-Saavedra et al., 2014; Voss et al., 2014). Biofilm cells were suspended in 10 ml buffer A (PBS, 0.01 mM, pH 8 + 1 mM PMSF). The suspension was transferred into weighted tubes and centrifuged at 4 000 x g for 10 min at room temperature (RT). The bacterial pellet was washed twice, and the weight of wet cells was calculated. Each 100 mg of cells was resuspended in 300 µl buffer A supplemented with 1.5 mM EZ-Link Sulfo-NHS-SS (Thermo Scientific). Biotinylation was performed for 15 min at RT under gentle agitation. Free biotin was removed by centrifugation at 4 000 x g for 5 min at RT and pellet was washed three times with PBS (0.01 M, pH 8 + 500 mM glycine) to block non-reacted biotin. Cells were resuspended in 500 µl buffer A supplemented with 1% (v/v) Triton X100 and broken at 4°C by vigorous shaking in a Fastprep-24 cell breaker twice for 20 s. The cell extracts were centrifuged at 20 000 × g for 30 min at 4 °C to pellet the insoluble material. Labelled proteins were recovered by affinity chromatography in a Monomeric Neutravidin Resin (Thermo Scientific), with gravity flow, using phosphate-buffered saline (PBS, pH 8) + 1% Triton X-100 as equilibration and wash buffer. Proteins were eluted with an elution buffer (50 mM DTT, 2% SDS 5% β-mercaptoethanol, 20% glycerol in 62.5 mM Tris-HCl, pH 6.8). A control without biotin labelling was carried out in parallel with the same protocol.

### *Cell fractionation*

The third method tested in this study is the fairly well-established separation of membrane and cell wall components by cell fractionation. However, extractions from cell fractionation are usually heavily contaminated with cytoplasmic proteins and often give insufficient information regarding surface proteins (Rodriguez-Ortega et al., 2006). Biofilm cells were washed twice in Tris-EDTA (TE, 20 mM Tris, 5 mM EDTA, pH 7). Pellet was resuspended

in 1 ml TE, and bacterial cells were broken using a cell disrupter (One shot cell disrupter, 1-8 ml, 2.7 KBar max, constant Systems Ltd, Daventry, UK) by applying 2.5 kBar pressure. Insoluble materials containing cell walls were removed by centrifugation (13 000 x g, 15 min, 4°C) and the supernatant was ultracentrifuged (200 000 x g, 1 h, 4°C). The pellet, containing membranes was washed twice in 1 ml Tris 40 mM, pH 8.5. Membranes were suspended in denaturing buffer (1% SDS, 0.1 M DTT, 20 mM Tris-HCl, pH 7.5) before heat-treatment (5 min, 95°C). Membrane and cell wall protein extracts were suspended in 100 mM ammonium bicarbonate pH 7.5. Proteins were reduced with 2.5 mM DTT at 56°C for 30 min, alkylated with 25 mM iodoacetamide during 20 min in the dark, and after digested overnight at 37°C with 20 µg of trypsin (Promega) per sample. Extracted peptides were purified and concentrated with Sep-Pak C18 Plus Short cartridges as described above.

#### *LC-MS/MS and bioinformatic analyses*

Each of the three methods described above was applied to three independent biofilm cultures to obtain triplicate protein samples. Samples were loaded onto SDS-PAGE gels and subjected to a short electrophoresis to concentrate the soluble proteome in one single band in the first few millimeters of the resolution gel. Excised bands were washed in 25 mM ammonium bicarbonate with 5% acetonitrile (ACN) for 30 min and twice in 25 mM ammonium bicarbonate with 50% acetonitrile for 30 min. Reduction and alkylation reactions were performed with 10 mM DTT and 55 mM iodoacetamide solutions respectively, and all bands were finally dehydrated with 100% acetonitrile. The samples were hydrolyzed overnight with 600 ng trypsin and peptides extracted from the gel bands with 100% acetonitrile.

All peptide mixtures were analysed by nano-LC-MS/MS (Thermo Fisher Scientific) using an Ultimate 3000 system coupled to a LTQ Velos mass spectrometer (MS) with a nano ion source. For each sample, eight microliters of peptide mixture were first preconcentrated on a C18 pre-column (300 µm inner diameter × 5 cm length) equilibrated with Trifluoroacetic

Acid 0.05% in water at 30  $\mu\text{L}/\text{min}$ . After 6 min of desalting, the pre-column was switched online with the analytical C18 column (75  $\mu\text{m}$  inner diameter  $\times$  25 cm length; 2  $\mu\text{m}$ , Acclaim C18 Pepmap RSLC) equilibrated in 96 % solvent A (99.5 %  $\text{H}_2\text{O}$ , 0.5 % formic acid) and 4 % solvent B (80%ACN, 19.5%  $\text{H}_2\text{O}$ , 0.5 % formic acid). Peptides were eluted using 4 to 55% gradient of solvent B for 50 min at 400 nL/min flow rate. Eluates were electrosprayed in positive-ion mode at 2.7 kV through a nanoelectrospray ion source heated to 275°C. The LTQ Velos MS was used in CID top 10 mode (i.e. 1 full scan MS and the 10 major peaks in the full scan were selected for MS/MS). For raw data processing, Thermo Proteome Discoverer v1.2 was used with Mascot v2.3 for database search (<http://www.matrixscience.com>). The following parameters were considered for the searches: precursor mass tolerance of 1.5 Da and fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation and oxidation set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identification Mascot scores  $> 13$  ( $p < 0.05$ ). Ions score is  $-10 \log(P)$ , where  $P$  is the probability that the observed match is a random event. Individual ions scores  $> 13$  indicate identity or extensive homology. Interrogations were performed against a custom database containing distinct entries corresponding to raw protein sequences and the different predicted mature proteins of *L. monocytogenes* EGD-e (i.e. DB-Mature-LmoEGDe v2.0, 5838 sequences) based on putative cleavage sites of the signal peptide (SP) (Renier et al., 2015). The subcellular location of identified proteins was determined with the rational secretomics-based strategy for genomic and proteomic analyses developed by Renier et al. (2012) (Renier et al., 2012). Briefly, the flowchart and decision trees designed to predict the subcellular localization use available bioinformatic tools and mimic the biology of protein secretion taking into consideration successively the presence or not of a SP, the type of SP, the secretion system pathway, the export of proteins lacking a SP, the transmembrane domains and conserved cell wall domains.

## Results

### *Global results on proteins identified by the three methods*

The data included in our results concerned protein identified with at least two unique peptides to ensure MS/MS results and in at least two biological replicates to take into account the variability inside each extraction methods ([see the report protein analyses in the supplementary data table](#)). In this way, the total number of proteins identified were 39, 62 and 125 for trypsin shaving, biotinylation and cell fractionation, respectively (Fig. 1). They were first subdivided into three broad categories according to their predicted localization: intracellular proteins or cytoproteins, cell envelope proteins and exo-cellular proteins. Despite the precautions taken to avoid cell lysis and using surface-targeted extraction methods, a significant number of intracellular proteins was present in the three methods tested. Thus, 23, 30 and 100 cytoproteins were retrieved by trypsin shaving, biotinylation, and cell fractionation (considering both cell wall and membrane subfractions), corresponding to 59%, 48% and 77% of total identified proteins in each of the fractions, respectively (Fig. 2). It is noteworthy that only one cytoprotein was identified in the membrane subfraction. The majority of the remaining proteins in the different samples were classified as cell envelope proteins (Renier et al., 2012). They represented 28% (11 proteins), 44% (27 proteins), and 19% (25 proteins) of the proteins extracted by trypsin shaving, biotinylation, and cell fractionation, respectively. In these last samples, 17 proteins were identified in the cell wall subfractions and 8 in the membrane subfractions, in which they represented an uneven proportion of 14% and 89% of the total proteins identified, respectively. This notable difference is related to the higher contamination of the cell wall subfractions by cytoplasmic proteins, contrary to the membrane sub-fractions but that contained a limited number of

proteins. Finally, five proteins predicted to be extracellular were present in each of the samples from the three approaches, representing 4 to 13% of the total identified proteins.

#### *Surface proteins identified by the three methods*

To evaluate the efficiency and specificity of each methods, all cell envelope proteins identified were distributed according to a Venn-diagram (Fig. 3). Thanks to the biotinylation protocol, 16 proteins were specifically extracted, while the shaving and cell fractionation methods allow the specific extraction of 2 and 8 proteins, respectively. Seven proteins were extracted and identified by the three methods, which represents a shared core of 18% of all surface proteins identified. Considering the cell fractionation method, 3 proteins were present in the membrane subfraction and 4 in the cell wall subfraction. The analysis of these two subfractions showed that among the 14 other surface proteins identified, only the protein Lmo0392 was identified in both the cell wall and membrane samples (data not shown). On the contrary, Lmo0690, Lmo2335 and Lmo2638 were specifically recovered in the membrane subfraction while the lipoprotein of unknown function, Lmo0791, was found both in this subfraction and with the shaving method. The others proteins retrieved in the cell wall fraction were either specific (Lmo0592, Lmo1374, Lmo1529 and Lmo1538), common with the biotinylation (Lmo0220, Lmo0292, Lmo0723 and Lmo2089) or with the shaving (Lmo2558) methods (data not shown).

#### *Predictive location of identified surface proteins*

The accurate subcellular localization of the cell envelope proteins was defined using a secretomics-based prediction workflow compiling numerous bioinformatics tools (Renier et al., 2012) and taking into account the presence or absence of a SP, the secretion pathway, the binding motifs or domains to the cell wall or to the cytoplasmic membrane (Desvaux et al., 2006b; Desvaux M. and M., 2009). The biotinylation method enabled the extraction and identification of 2 exoproteins (Lmo0971 and Lmo1499), 12 lipoproteins, 9 cytoplasmic

membrane-associated proteins (CMAP) and 4 cell wall-associated proteins (CWAP), while the shaving technique resulted in the identification of 6 lipoproteins, 1 CMAP and 4 CWAP and the cell fractionation of 6 lipoproteins, 12 CMAP and 3 CWAP (Fig. 3). The *lmo0690* gene product, namely the flagellin, which is a cell surface protein forming the filament of the bacterial flagella, was identified in the cell wall subfraction.

## **Discussion**

The bacterial proteosurfaceome is relatively hydrophobic and can only be extracted from the cell membrane using detergents. Due to its variability and instability, its analysis presents many hurdles including extraction, solubilisation, purification, analysis with in-gel (one or two-dimensional gel electrophoresis) (Hebraud, 2014; Sun et al., 2011) or off-gel (shotgun proteomics) approaches (Cordwell, 2006). Despite the challenge of working with surface proteins, their study remains an important research area, especially due to their role in the control of fundamental biochemical processes and their importance as pharmaceutical targets. Because proteins from the cell surface proteome of microorganisms are difficult to extract and identify using the conventional "in-gel" proteomics approach, particularly from Gram-positive bacteria, the efficacy of three extraction methods was evaluated on the pathogen *L. monocytogenes*. Several methods, including the three evaluated in this study, have been previously assessed and compared, but on monoderm bacteria growing in planktonic mode (Annette et al., 2010; Annette et al., 2011b; Hempel et al., 2011; Jessica et al., 2004; Tiong et al., 2015), and few have been performed on biofilms (Tiong et al., 2016). This growth mode in biofilms is the most widespread in the environment as noted earlier. The exploration of the proteosurfaceome of sessile bacteria is an additional challenge because of cell aggregation and the presence of extracellular compounds that form a matrix around the cells. The three methods compared in this study are based on enzymatic shaving of the cell surface, biotin

labelling of cell envelope proteins and cell fractionation, then followed by an LC-MS/MS analysis of tryptic peptides.

#### *Presence of intracellular proteins in cell surface proteome samples*

Irrespective of the methodology used for cell surface proteome extraction and the measures used to avoid cell lysis, a significant number of intracellular proteins was present in the extracted samples, contaminating the proteosurfaceome.

In the case of enzymatic shaving, this contamination may be due to the lysis of some bacteria within the biofilm or the alteration of the cell envelope during the preparation of the proteosurfaceome samples. The frailty or robustness of the cell envelope to the surface extraction process may differ depending on whether addressing a monoderm or diderm (= Gram-negative), as well as on the genus and bacterial species. Consequently, some methodological parameters must be adapted to the microorganism studied, such as temperature, incubation time, buffer for enzymatic digestion (Annette et al., 2010), quantity of enzyme and centrifugation speed to recover cells. These parameters influence both the efficiency to extract surfaceome peptides and the degree of contamination by intracellular proteins. The surface proteins identified in this study on *L. monocytogenes* biofilms were consistent with those reported in the literature on planktonic cells (Harold et al., 2008; He and De Buck, 2010; Olaya-Abril et al., 2012), despite the presence of a significant proportion of cytoplasmic proteins.

For the biotinylation method, *L. monocytogenes* intact cells were treated with sulfo-NHS-SS-Biotin, a marker molecule which is supposed to be membrane-impermeable. In fact, this technique, initially implemented on eukaryotic cells, has been applied on diderm bacteria by using different biotinylation reagents whose sulfo-NHS-SS-Biotin appeared the most suitable, even if some periplasmic, cytoplasmic and inner membrane proteins were identified (Hempel et al., 2011; Monteiro et al., 2018).

With the cell fractionation methods, the washing steps to remove potential contaminating intracellular proteins were efficient for the membrane subfractions but to a lesser extent for the cell wall subfractions. Intracellular proteins appear to be trapped or to have an affinity for components of the cell envelope and thus to be carried along them in the subsequent steps of the extraction process.

In addition, among the proteins considered as contaminants, certain proteins may have a double localization with a different function than known at the cytoplasmic level. Some of these proteins can be moonlighting proteins, that is exhibiting an additional function when localized at the bacterial cell surface. Similarly, some secreted exoproteins may be in transit from the cytoplasm and be retrieved in the proteosurfaceome. We must also consider that the subcellular localization of proteins predicted as cytoplasmic maybe incorrect or incomplete because they potentially harbour unknown motifs for cell-envelope attachment or their secretory pathway could not be identified.

Despite these drawbacks, the optimization of each of these methods should not be at the expense of their efficiency in extracting proteins or peptides from the bacterial cell surface and the presence of cytosolic proteins is not critical as it does not interfere with their identification by mass spectrometry.

#### *Comparison of the three methods*

Among the various strategies for exploring the proteosurfaceome, the enzymatic shaving method has been applied in recent years, mainly on monoderm bacteria (Nestor and J., 2011), with contrasting results depending on the bacterial species. Shaving method has been previously used to explore the cell surface proteome of a wide variety of microorganisms (Olaya-Abril et al., 2014), among them Gram-positive pathogens such as *L. monocytogenes* (Tiong et al., 2015), *Streptococcus* spp. (Pribyl et al., 2014; Rodriguez-Ortega et al., 2006; Severin et al., 2007), *Staphylococcus aureus* (Annette et al., 2010; Annette et al., 2011a;

Annette et al., 2011b; Monteiro et al., 2015; Nestor et al., 2010; Pribyl et al., 2014; Tiong et al., 2015; Ventura et al., 2010) and other monoderm bacteria such as *Bacillus subtilis* (Harold et al., 2008) or the dairy starter *Lactococcus lactis* (Meyrand et al., 2013). This technique has been successfully used to explore *L. monocytogenes* proteosurfaceome with 19 surface proteins out of a total of 174 proteins identified by Zhang et al. (2013) (Zhang et al., 2013) or 18 proteins out of 155 proteins identified by Tiong et al. (2015) (Tiong et al., 2015). In this study, 11 surface proteins were identified from a total of 39 proteins, with 6 lipoproteins, one membrane and 4 cell wall proteins. In comparison, our samples had less contamination by intracellular proteins but fewer surface proteins were characterized. This can be a consequence of the biofilm mode of growth, reflecting a more difficult access of the trypsin to the cell surface due to the presence of extra polymeric substances. By using another protocol to extract proteins from the cell envelope, called UB-Ghost and implementing a hypotonic extraction buffer and then an 8 M urea buffer before trypsin digestion, Tiong et al. (2016) showed similar results. Indeed, they identified 14 and 109 surface proteins from sessile and planktonic cells, representing 2.3 % and 17.6 % of the total proteins identified, respectively (Tiong et al., 2016).

Concerning *Streptococcus* spp., shaving appeared more efficient than for *L. monocytogenes* since it allowed the identification of 68 and 58 surface-localized proteins in *Streptococcus* group A (Rodriguez-Ortega et al., 2006) and *S. pyogenes* (Severin et al., 2007), respectively. However, by using immobilized trypsin on agarose beads, Pribyl et al. (2014) identified only 6 surface proteins in *S. pneumoniae* (4 lipoproteins and 2 LPXTG-anchored proteins) (Pribyl et al., 2014). All these proteins were also identified by the biotinylation method; this approach was also more effective than shaving with 39 surface proteins identified (29 lipoproteins and 10 LPXTG-anchored proteins) (Pribyl et al., 2014). The biotin labelling method of bacterial cell envelope protein has been mainly performed on diderm bacteria and few attempts on

monoderm bacteria have been reported to date. The biotinylation approach was also applied on the vancomycin resistant *Enterococcus faecium* E155, allowing the extraction of 43 proteins, among which 27 proteins with solely extracytoplasmic localization (Romero-Saavedra et al., 2014). The shaving method on the same bacterium was more efficient with 39 extracytoplasmic proteins identified, although the original sample was severely contaminated by cytoplasmic proteins (390 proteins identified in total).

The cell surface proteome of the opportunistic pathogen *Staphylococcus aureus* was one of the first explored by a range of different approaches. For example, Solis et al. (2010) evaluated the cell shaving method by using trypsin and proteinase K (Nestor et al., 2010). The overlap between the two enzymes resulted in the identification of 10 surface-exposed proteins, while 12 and 16 were specific to trypsin and proteinase K, respectively, reflecting both their specific cleavage and their complementarity. In addition, the proteins were classified as lipoproteins and proteins with a LysM (noncovalent) or LPXTG (covalent) motifs bounding to peptidoglycans, demonstrating the efficacy of the enzymatic method. Even more conclusive results from trypsin shaving were reported with the identification of 72 (Annette et al., 2011b) and 101 surface-associated proteins (Hempel et al., 2011), about half of which were transmembrane proteins, the other half belonging to the other categories listed above. By comparison, the biotinylation implemented in each of these two studies allowed to identify 49 and 110 cell-surface proteins respectively. In the study of Hempel et al. (2011), about two-thirds of the total proteins identified, and predicted as surface associated, were common to both methods (*i.e.* 66/101 and 110 for shaving and biotinylation, respectively) (Hempel et al., 2011). Noticeably, by combining the two methods, almost all lipoproteins were identified by biotinylation (39 out of 40), while all the 15 sortase substrates, proteins covalently linked to the cell wall, were identified by trypsin shaving. We observed similar results for *L. monocytogenes*, 13 lipoproteins were identified in total using both methods with

12 found with biotinylation, 7 of which specific to this method. On the other hand, the two approaches did not reveal any specificity at the level of sortase substrates and/or cell wall proteins. It should also be noted that the conventional cell fractionation approach was the most effective in identifying membrane proteins with 11 of the 17 proteins obtained by the three methods we compared, including 7 specific proteins.

#### *Focus on cell surface proteins identified*

The cell envelope of Gram-positive bacteria (= monoderm) is primarily composed of a single biological membrane and a cell-wall of peptidoglycan (Shockman and Barrett, 1983). To ensure their functions, surface proteins are both targeted to the cytoplasmic membrane and the cell wall (Desvaux et al., 2006a). Membrane-associated proteins include membrane-integrated proteins, peripheral membrane proteins and lipoproteins. Anchoring to the cell wall is allowed by two primary mechanisms; (i) cell wall sorting and (ii) targeting (Navarre and Schneewind, 1999). Cell wall sorting is the covalent attachment of surface proteins to the peptidoglycan via a C-terminal sorting signal that contains a consensus LPXTG sequence. Cell wall targeting involves the noncovalent attachment of proteins to the cell surface via specific binding domains (Desvaux et al., 2018; Desvaux et al., 2006a). Six cell-wall associated proteins were recovered with the combination of the 3 extraction methods, with 2 containing a LysM motif (Buist et al., 2008). This motif is found in 6 *L. monocytogenes* proteins (Bierne and Cossart, 2007), including the invasion-associated protein Iap, (Lmo0582). This protein, also called CwhA, has been characterized as an autolysin with a relevant role in infection (Dussurget et al., 2004; Lenz et al., 2003). Two other proteins containing noncovalent cell wall binding domain were identified, the peptidase S41 (Lmo1851) with a PGBD1 domain and Lmo2558 with a GW module. A protein of unknown function containing an LPXTG motif for covalent binding to peptidoglycan (Lmo2714) was also retrieved. This family of LPXTG proteins, firmly anchored to the cell wall, comprises 41 proteins in *L. monocytogenes* (Cabanès et al.,

2002). Considering that only few proteins with LPXTG motif were recovered in our study, the three methods tested do not appear efficient to monitor them. To access these proteins strongly associated with peptidoglycans, a method based on boiling cell envelopes of *L. monocytogenes* in the presence of SDS was previously shown more effective and allowed to identify 13 LPXTG-containing proteins (Graciela et al., 2005).

Based on their characteristic signal sequences and conserved lipobox motif in the C-domain (Tjalsma et al., 2000), 68 of 133 surface proteins were classified as lipoproteins in *L. monocytogenes* EGD-e strain (Glaser et al., 2001), and 26 were later confirmed *in situ* (Baumgartner et al., 2007). Almost half of these lipoproteins are presumed to act as substrate-binding components of ABC transporter systems (Bierne and Cossart, 2007), performing the equivalent functions of periplasmic solute-binding proteins in Gram-negative bacteria (Tam and Saier, 1993). Despite their predominance as surface proteins, very few lipoproteins have been biochemically characterised like LpeA (Lmo1847), which belongs to the LraI family of manganese-importing ABC transporter components (Novak et al., 1998) and TscA (Lmo1388), a CD4+ T cell-stimulating antigen.

Concerning the cytoplasmic membrane-associated proteins (CMAP), 17 were identified, mainly by the biotinylation and cell fractionation methods. The CMAP are anchored within the lipid bilayer and systematically exhibit at least one  $\alpha$ -helical transmembrane domains (TMD) and sometimes multi-membrane spanning domain as it is the case for Lmo2335, Lmo2360 or Lmo2638 (Desvaux M. and M., 2009; Renier et al., 2012). Thus, the CMAP are classified according to the TMD topology (Desvaux M. and M., 2008) but irrespective of their topology, these hydrophobic TMD domains make their extraction challenging by classical proteomic approaches.

The cell fractionation allowed the identification of the flagellin in the cell wall fraction. This cell surface protein, exported via the flagella export apparatus (FEA), is the constitutive

subunit of the flagella filament. FlaA exhibits also a peptidoglycan-hydrolyzing activity that might play a role during flagella assembly (Popowska, 2004; Popowska and Markiewicz, 2004). Indeed, some flagellar components are assembled on the bacterial cell surface like for the filament proteins FlaA and FliD (Desvaux M. and M., 2008), which could explain its presence in the cell wall subfraction.

Overall, the three methods implemented in our experimental conditions allowed to extract and to identify 39 proteins out of the 133 *L. monocytogenes* surface predicted proteins (29%). A relatively low recovery rate (18%) was found between the different methods with only 7 surface proteins in common, highlighting the complementarity of these approaches.

## **Conclusion**

The cell surface proteome comprises proteins usually difficult to extract and is subject to several factors of variation such as mode (planktonic or sessile), phase and conditions (*e.g.* medium, temperature and pH) of growth, as well as differences between monoderm and diderm bacteria. In this study, we used three different extracting methods for exploring the proteosurfaceome of *L. monocytogenes*. The three methods were applied on bacterial cells growing in a biofilm, which is the most widespread growth mode in natural and agri-food environments of this pathogen. The proteosurfaceome, due to its importance for adhesion, biofilm formation and bacterial persistence in natural and in food processing environments, requires a particular attention. Despite the lack of abundant extra polymeric substances in *L. monocytogenes* biofilms, accessing the cell surface proteome of these sessile and aggregated bacteria for their proteomic analysis is extremely challenging and presents more difficulties than for planktonic cells.

Our results demonstrated that a multi-approach strategy, taking advantage of the complementary of several methods, is needed to fully explore the cell surface proteome of *L.*

*monocytogenes* in biofilm. Other additional approaches have been shown to be effective on planktonic bacteria and could be used to further expand the analysis of the biofilm proteosurfaceome. Among them, the Ghost urea method has been already used on adhered cells (Tiong et al., 2016). Moreover, the method using cell wall extracts boiled with SDS before their trypsin digestion (Graciela et al., 2005) could allow to target more specifically the LPXTG proteins.

In conclusion, these different methods for deciphering the proteosurfaceome often require modifications and improvements according to the bacterial species, and their selection depends on whether a profiling or a targeted analysis is required. These new findings collectively inform future discovery and translational proteomics for clinical, environmental health and industrial applications.

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### **Author Disclosure Statement**

The authors declare that no competing financial interests exist.

### **Alphabetical list of abbreviations used**

ABC	ATP-binding cassette
ACN	Acetonitrile
CFU	Colony-forming unit
CID	Collision-induced dissociation
CMAP	Cytoplasmic associated proteins
CWAP	Cell wall associated proteins
DTT	Dithiotreitol
EDTA	Ethylenediamine tetraacetic acid
FEA	Flagella export apparatus
LC-MS/MS	Liquid chromatography– tandem mass spectrometry
LPXTG	Leu-Pro-any-Thr-Gly motif
LTQ	Linear Trap Quadropole
LysM	Lysin motif
MS	Mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
RT	Room temperature
SDS	Sodium dodecyl sulfate
SP	Signal peptide
SS	Stainless steel
TBS	Tris-buffered saline
TE	Tris-EDTA
TFA	Trifluoroacetic acid
TMD	Transmembrane domain
TSA	Tryptic soy agar
TSB	Tryptic soy broth

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