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Tiago Santos. Role of proteome in biofilm development and adaptation of *Listeria monocytogenes* to controlled environments. Bacteriology. Université Clermont Auvergne [2017-2020], 2019. English. NNT : 2019CLFAC027 . tel-02426255

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UNIVERSITÉ CLERMONT-AUVERGNE
CLERMONT-FERRAND
N° D.U.

Ecole Doctorale
des Sciences de la Vie, Santé, Agronomie et Environnement
N° d'ordre :



Thèse

Présentée à l'Université Clermont-Auvergne pour l'obtention du grade de

DOCTEUR D'UNIVERSITÉ

Spécialité

Génétique, Physiologie, Pathologie, Nutrition, Microbiologie Santé et Innovation

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Role of proteome in biofilm development and adaptation of *Listeria monocytogenes* to controlled environments

Soutenue publiquement le **12 juin 2019** devant le jury composé de :

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Directeur de thèse	Michel Hébraud, Directeur de Recherche – INRA de Theix

À minha família por estar sempre lá para mim.

"Do not go gentle into that good night", Dylan Thomas

Rôle du protéome dans le développement de biofilms et l'adaptation de *Listeria monocytogenes* à des environnements contrôlés

Résumé

Listeria monocytogenes est une bactérie pathogène et ubiquitaire capable de s'adapter et de survivre dans plusieurs environnements. C'est l'une des principales causes de décès liés à la contamination des aliments dans l'Union européenne, qui coûte des millions d'euros par an en soins médicaux. Ce projet de doctorat tente d'établir un lien entre le rôle des protéines exprimées lors de la formation de biofilms et de leur adaptation à des environnements contrôlés (ex : faible humidité relative de l'air). Des approches protéomiques innovantes ont été utilisées telles que (i) l'imagerie par spectrométrie de masse MALDI (IMS) développée sur des biofilms soumis à un stress de déshumidification et (ii) la chromatographie liquide haute performance couplée à la spectrométrie de masse en tandem (LC-MS/MS) semi-quantitative et sans marquage (« shotgun proteomics ») sur des sous-protéomes extraits par différentes méthodes complémentaires (fractionnement cellulaire, biotinylation, « shaving » enzymatique) de cellules bactériennes planctoniques ou en biofilms cultivées à trois températures (10°C, 25°C et 37°C).

Cette thèse peut être divisée en 3 chapitres principaux. Le premier chapitre comprend l'analyse de l'adaptation du biofilm de *Listeria monocytogenes* à la déshumidification par IMS. Le deuxième chapitre est consacré à l'extraction des sous-protéomes de cellules de *Listeria monocytogenes* en biofilm, après croissance à trois températures différentes jusqu'à un stade précoce ou mature, en biofilm ou planctonique, puis à l'analyse par « shotgun proteomics ». Le troisième et dernier chapitre est constitué de la même approche que le deuxième chapitre; toutefois, des extractions ont été réalisées dans des cultures planctoniques suivies d'une comparaison *in silico* avec les données obtenues dans le deuxième chapitre.

Premier chapitre - Analyse de l'adaptation de biofilm de *Listeria monocytogenes* à la déshumidification par IMS

Contexte

Dans le mode de croissance en biofilm, *L. monocytogenes* peut résister à de nombreuses conditions environnementales difficiles, telles qu'une basse température et un faible taux d'humidité relative de l'air. Il est donc d'un intérêt majeur d'étudier les lipides, les peptides et les protéines présents dans la matrice du biofilm et/ou à la surface cellulaire qui jouent un rôle dans l'adaptation et la survie de *Listeria* à ces conditions. La spectrométrie de masse par imagerie MALDI constitue un bon outil pour analyser la présence, l'abondance et la distribution de ces molécules dans un biofilm. Cette approche est basée sur la technique de spectrométrie de masse MALDI-TOF (Matrix Assisted Laser Desorption Ionisation – Time of Flight) ; les principales différences se situant au niveau de la nature de l'échantillon et de l'analyse finale des données de masse recueillies. Une lame de verre électriquement conductrice, revêtue d'oxyde d'étain (ITO), est nécessaire pour disposer le tissu analysé, qui dans ces cas est un biofilm mature de *L. monocytogenes*. Ensuite, la lame est insérée dans un appareil (« sprayer ») qui pulvérise une molécule de matrice. La pulvérisation dure 2 à 3 heures avec plusieurs cycles de pulvérisation/séchage/cristallisation afin d'obtenir finalement une couche uniforme de matrice sur l'ensemble du biofilm. Ensuite, la lame est placée dans un support en acier inoxydable et introduite dans le spectromètre de masse pour effectuer l'analyse.

L'étape critique de cette technique est le dépôt de la matrice sur le biofilm bactérien. Celle-ci doit cristalliser avec les analytes en surface du biofilm sans les délocaliser. Chaque fois que le laser du spectromètre de masse frappe l'échantillon, des molécules (protéines) sont ionisées et désorbées pour ensuite, sous l'effet d'un champ électrique, parcourir un tube dans lequel règne un vide très poussé. Le temps de vol de chaque ion pour atteindre le détecteur situé à l'extrémité du tube est fonction de la valeur du rapport masse sur charge (m/z). Ainsi, le spectromètre de masse mesure la masse précise des molécules désorbées en surface de l'échantillon. Un logiciel de traitement et d'analyse des données d'imagerie est couplé à l'appareil. Celui-ci permet de visualiser chaque groupe d'espèces d'ions avec une couleur différente, reflétant ainsi l'abondance et la distribution des analytes le long du biofilm. C'est une technique développée depuis une vingtaine d'années, initialement dans des domaines tels que

la recherche de marqueurs du cancer sur de biopsies de tissus tumoraux. Cependant, l'utilisation d'une telle technologie pour étudier des bactéries, et plus précisément des biofilms bactériens, est très récente et présente de nombreux écueils qu'il faut résoudre. Tout d'abord, une cellule bactérienne, telle que *L. monocytogenes*, a une taille moyenne de 1 à 2 μm et la résolution spatiale maximale d'un MALDI est d'environ 20 μm . Dans cette étude, la résolution utilisée sera d'environ 100 μm . Ainsi, chaque tir de laser n'ionise pas seulement une cellule mais il atteint un ensemble de cellules constituant le biofilm, ce dernier étant dans ce cas assimilé à un tissu.

Le contrôle de *L. monocytogenes* est difficile à réaliser dans les industries agro-alimentaires (IAA) en raison de ses capacités de survie face à des conditions difficiles et de son aptitude à former des biofilms. Les bactéries sont notamment soumises à des variations quotidiennes de l'humidité relative de l'air suite aux procédures de nettoyage-désinfection. Malgré cela, une partie de la population est capable de survivre et de persister, mais les mécanismes moléculaires par lesquels les biofilms s'adaptent à la déshumidification ne sont pas bien connus. L'objectif était de développer une approche IMS pour explorer l'expression des protéines et leur distribution *in situ* au sein de biofilms de *L. monocytogenes* exposés à la déshumidification.

Matériel et méthodes

Les différentes étapes de la méthodologie mises au point et optimisées pour obtenir un biofilm bactérien et des spectres de masse de qualité sont décrites dans la Figure 1.

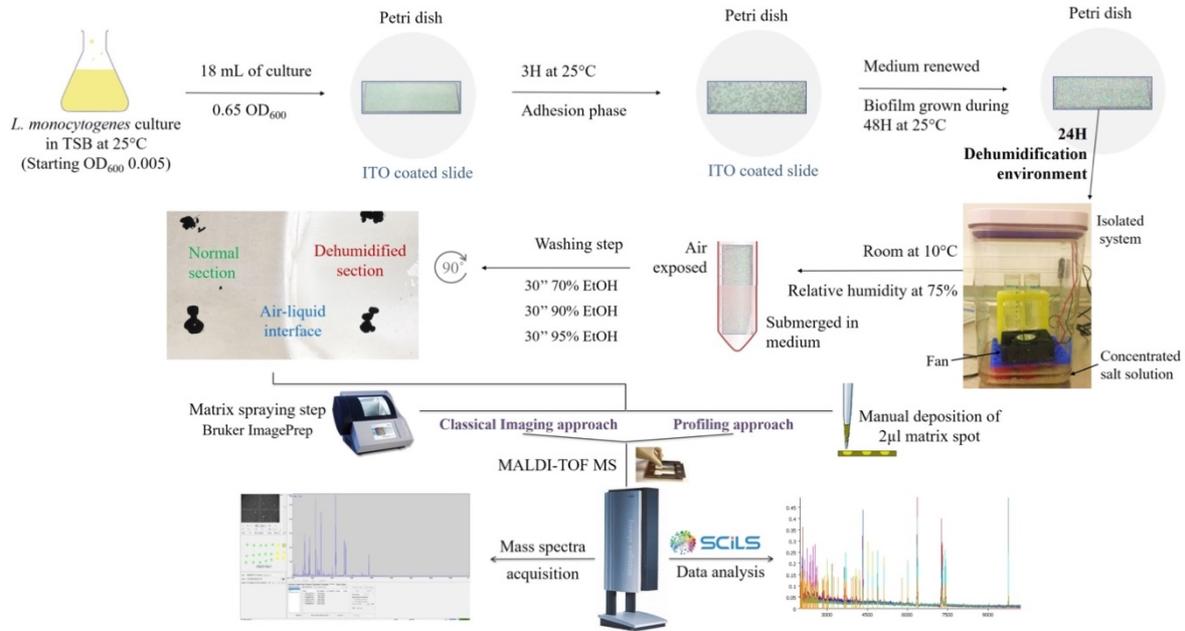


Figure 1 - Méthodologie utilisée pour étudier les biofilms de *L. monocytogenes* par imagerie MALDI.

Des cellules pré-cultivées jusqu'en phase stationnaire sont utilisées pour inoculer des cultures afin d'obtenir une DO_{600} finale de 0,005. La souche a ensuite été cultivée pendant 6 h. Les cellules sont récoltées par centrifugation ($7500 \times g$, 15 min) et remises en suspension dans du TSB dilué au 1/5 avec de l'eau stérile dans un volume égal à celui du surnageant recueilli atteignant une DO_{600} comprise entre 0,6 et 0,7. Dix-huit ml de la suspension bactérienne sont versés sur chaque lame de verre revêtue d'oxyde d'indium-étain (ITO) placée dans une boîte de Pétri stérile (diamètre de 55 mm) et incubée à 25°C . Les cellules bactériennes sont laissées adhérer pendant 3 h avant de retirer le milieu, ce qui permet d'éliminer les cellules planctoniques. Du milieu de culture frais est ajouté et les cellules sont incubées pendant 48 h supplémentaires à 25°C . Les lames sont placées verticalement dans un flacon, avec un milieu liquide jusqu'à mi-hauteur, favorisant l'exposition aux conditions atmosphériques de la moitié supérieure de la lame. Le flacon a ensuite été placé dans un récipient fermé hermétiquement dans lequel l'humidité relative est contrôlée à 75% ; le tout est incubé pendant 24 h dans une pièce à 10°C . Les lames de verre sont ensuite lavées pour éliminer les sels interférents et les lipides lors de lavages successifs de 30 secondes avec de l'éthanol de qualité HPLC à 70, 90 et 95%. Au final, cette méthode permet d'obtenir des biofilms de *L. monocytogenes* avec 3 sections, l'une qui est restée immergée dans le milieu de culture, une autre soumise

à une humidité relative de l'air de 75% (stress de déshumidification) et une interface entre elles (Figure 1). Celles-ci sont analysées sur un spectromètre de masse MALDI-TOF/TOF Autoflex Speed avec un laser Smartbeam à l'aide des logiciels FlexControl 3.4 et FlexImaging 3.0 (Bruker Daltonics).

Au préalable, deux approches ont été mises en œuvre pour appliquer la matrice sur le biofilm. La première, qui correspond à une méthodologie d'imagerie classique, consiste à pulvériser la matrice sur toute la lame à l'aide de la station de préparation d'échantillons ImagePrep (Bruker Daltonics). Cette matrice est constituée de 7 mg/ml d'acide α -cyano-4-hydroxycinnamique (CHCA) dans un mélange eau / acétonitrile 50:50 (v/v) avec 0,2% d'acide trifluoroacétique. Pour cette approche classique, la résolution latérale est fixée à 100 μm . La seconde approche utilise des paramètres de « profilage », avec une résolution latérale inférieure à 2000 μm et des dépôts manuels de gouttes de matrice de 2 μl , permettant d'augmenter la résolution spectrale et de réaliser l'acquisition spectrale dans une zone plus étendue.

Pour l'imagerie protéique, 500 tirs laser sont accumulés par pixel à puissance laser constante et les ions sont détectés en mode linéaire positif dans une plage de masse allant de 2000 à 20000 m/z. La déflection est fixée à 1500 m/z et la focalisation laser à « moyen ». Les analyses sont effectuées en utilisant un gain de détecteur de 2,783 V, une tension de source ionique 1 à 19,5 kV, une tension de source ionique 2 à 18,15 kV et une tension de lentille à 7 kV. Un étalon protéique a été utilisé pour l'étalonnage des spectres, effectué de manière externe sur la même cible avant chaque mesure.

Pour identifier les m/z observées par IMS, les protéines ont été extraites directement des différentes sections du biofilm (Figure 2). L'échantillon est recouvert de 2 μL d'acétonitrile à 7,5% dans de l'acide trifluoroacétique à 0,2% et incubé pendant 1 min. Le liquide contenant l'extrait protéique est recueilli et cette extraction est répétée une fois. La même zone est ensuite recouverte de 1 μL d'acétonitrile à 60% dans 0,2% d'acide trifluoroacétique et le liquide contenant l'extrait protéique est immédiatement recueilli et combiné avec les extraits précédents (5 μL au total).

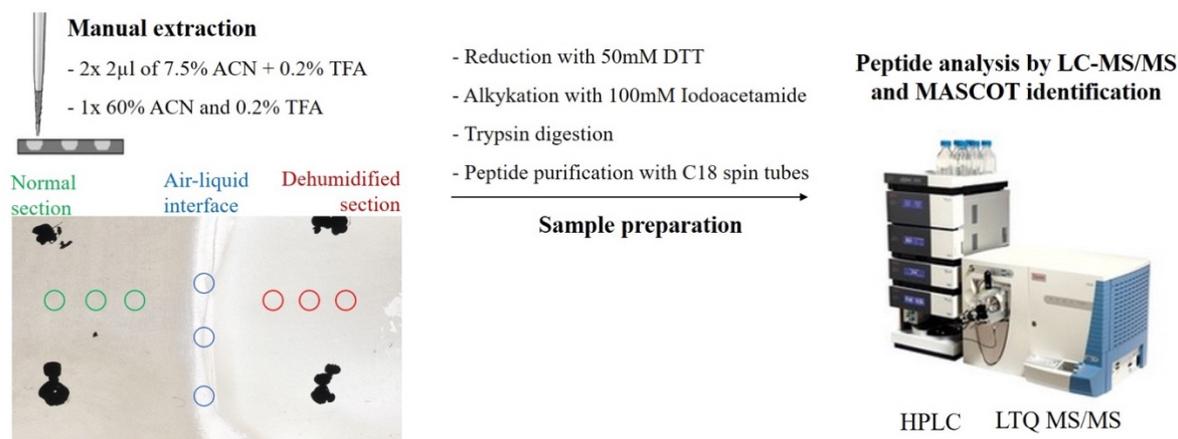


Figure 2 - Extraction manuelle de protéines suivie d'une identification par LC-MS/MS.

Les extraits protéiques sont réduits avec 20 µL de dithiothréitol 100 mM dans du bicarbonate d'ammonium 50 mM (pH 8) pendant 15 min à 55°C. Après refroidissement, l'alkylation est réalisée en ajoutant 20 µL d'iodoacétamide 100 mM dans du bicarbonate d'ammonium 50 mM, pendant 15 min à 20°C et à l'obscurité. Après neutralisation, la digestion des protéines est obtenue en ajoutant 25 µL de solution de trypsine (20 ng/µL) dans du bicarbonate d'ammonium à 50 mM et par une incubation pendant une nuit à 37°C. La digestion à la trypsine est arrêtée avec de l'acide trifluoroacétique à 2% et après 5 minutes de centrifugation à 3000 x g, le surnageant est recueilli. Le culot est lavé avec une solution d'acétonitrile 2% et d'acide trifluoroacétique 0,05% dans de l'eau, puis soumis à une sonication pendant 5 min, centrifugé 5 min à 3000 g pour récupérer le surnageant et le combiner avec le précédent, donnant des échantillons de 100 µL. Les mélanges de peptides sont ensuite analysés par nano-LC-ESI-MS/MS. Les données d'imagerie sont traitées et analysées avec le logiciel SCiLS et l'identification des protéines est réalisé par LC-Progenesis.

Résultats et discussion

Des spectres de bonne qualité ont été obtenus à partir des deux approches, l'imagerie classique et le « profilage » (Figure 3).

Profiling approach

Classical imaging approach

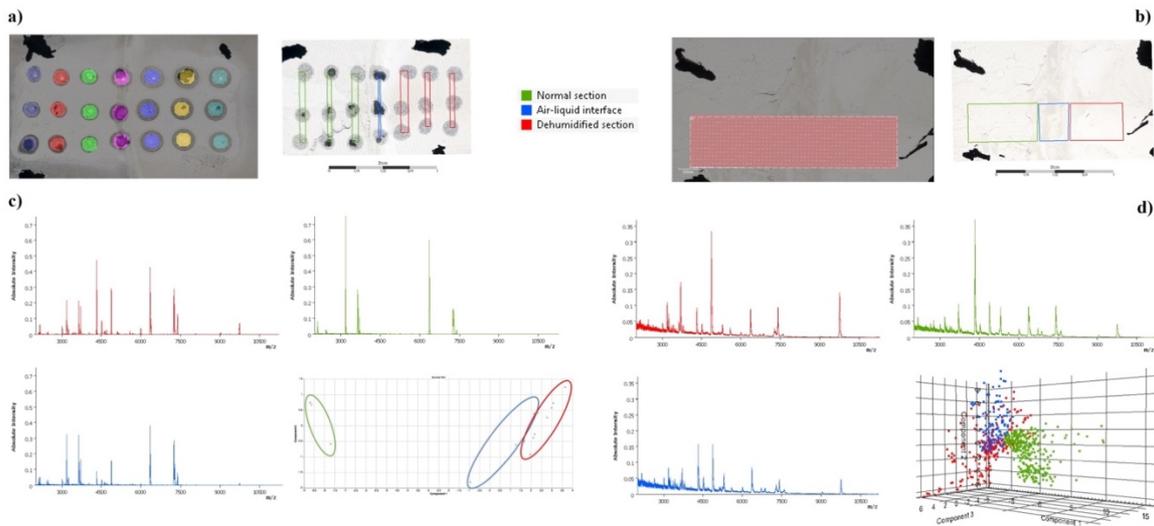


Figure 3 - Résultats obtenus par les deux approches de préparation d'échantillon et d'analyse : imagerie classique (à gauche) et « profilage » (à droite).

Le profilage a montré un niveau de bruit de fond et une intensité de pics plus faibles que l'approche classique. Cela pourrait être dû à la présence d'un rapport échantillon/matrice plus élevé dans le profilage et à l'accumulation supérieure de signal obtenue par point d'analyse. Pour les deux approches, un test ANOVA a été réalisé et les intervalles significatifs m/z résultants ont été utilisés pour l'ACP, ce qui a permis de séparer les spectres des 3 régions. Cependant, il n'est pas possible d'affirmer que les 3 régions montrent de nettes différences puisque ce résultat pourrait être lié aux contraintes techniques. Le taux et la qualité de la cristallisation matrice/analyte sont affectés par l'humidité résiduelle dans la région qui était restée immergée. Cette section normale et l'interface sont en effet immergées dans le liquide tout au long de la méthodologie, contrairement à la section déshumidifiée, cette dernière ayant un niveau d'humidité plus bas (75%) et donnant des spectres avec des ions d'intensité plus élevée. La majorité des protéines détectées par les méthodes MALDI à cellules intactes sont de petites protéines ribosomales, idéales pour l'identification bactérienne robuste, mais pas idéales pour dépister les mécanismes d'adaptation au stress. En effet, certaines protéines <15 kDa sont abondantes (protéines ribosomales) et peuvent perturber l'ionisation d'autres protéines d'intérêt beaucoup moins abondantes. Néanmoins, on peut supposer la présence possible de protéines de choc froid dans ces spectres, comme celles dans la famille des CspL ($\pm 7\ 200$ Da).

La combinaison de MALDI MSI avec des méthodes telles que la chromatographie en phase liquide et la spectrométrie de masse en tandem (LC-MS/MS) peut fournir des informations supplémentaires sur la nature de protéines obtenues par imagerie, permettant ainsi leur identification. La stratégie utilisée pour l'identification des protéines a été découplée de l'analyse MSI ; elle met en œuvre une analyse protéomique « bottom up », à partir de laquelle un nombre reproductible de protéines a été identifié à partir de 3 réplicats biologiques (en moyenne, 423 protéines identifiées sur les 3 lames de biofilm). Un total de 513 protéines différentes a été détecté à partir des extraits protéiques des trois réplicats biologiques. Les diagrammes de Venn de la figure 4 montrent que dans tous les cas, la majorité des protéines uniques a été détectée dans la section déshumidifiée. Cela pourrait être lié à un pourcentage plus élevé de cellules mortes dans cette section et, par conséquent, à une extraction plus efficace des protéines, mais également à une humidité résiduelle plus élevée dans la section restée immergée (non stressée) qui aurait pu diluer les protéines extraites. En MSI, un m/z peut être le résultat de plusieurs séquences protéiques comprises dans l'intervalle de masse de l'analyse. En conséquence, cela représente un véritable défi d'identification des protéines. Cela explique pourquoi dans la majorité des publications de MALDI MSI, seules quelques-unes font état d'identifications protéiques et rapportent la nature de protéines présentes dans les spectres obtenus.

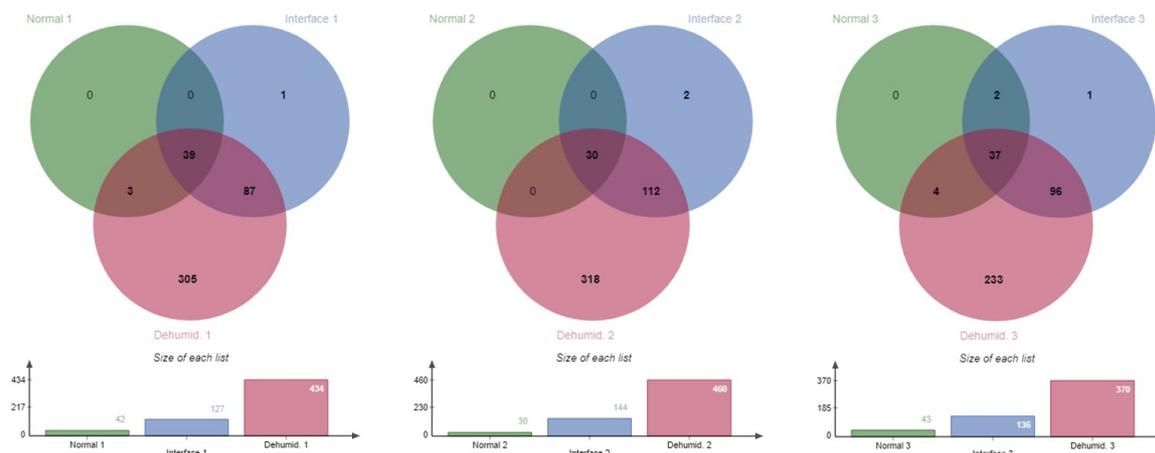


Figure 4 - Diagrammes de Venn pour toutes les protéines identifiées provenant de chacune des répétitions biologiques (Répliquat 1 : 435 protéines; répliquat 2 : 462 protéines; répliquat 3 : 373 protéines).

La figure 5 est une représentation visuelle de la connexion entre les spectres de masse moyens des approches d'imagerie classique vs profilage et les protéines

identifiées obtenues. Nous avons tout d'abord évalué les listes de protéines obtenues par LC-MS/MS, en reprenant l'analyse des protéines identifiées dans toutes les répliques biologiques de chacune des trois sections (section normale - Fig. 5a; interface air-liquide - Fig. 5b; section déshumidifiée - Fig. 5c). Dans un deuxième temps, nous avons pris en compte l'intervalle de masse entre 2000 et 15000 m/z que nous avons utilisé pour la MSI et avons établi les listes de protéines identifiées en deçà de cette valeur seuil de 15 kDa. En conséquence, sept protéines dans la section immergée, 8 dans l'interface air-liquide et 19 dans la section déshumidifiée correspondaient à ces critères. Ces protéines sont représentées dans la figure 5 par un réseau d'interaction protéine-protéine et sont également listées avec leur description détaillée.

La « back correlation » entre le m/z significatif et l'identification *in situ* a été établie avec une tolérance de masse de 0,5%, ce qui a conduit à l'assignation de 5 protéines différentes pour la section déshumidifiée. Dans les sections « normale ou immergée » et « interface air-liquide », deux protéines ont été assignées aux spectres moyens de la MSI, la protéine de choc froid CspLA (CspL) et la protéine ribosomique L29 50S (RmpC). La famille des petites protéines de choc froid (Csp), avec un domaine de choc froid, est généralement associée à la résistance au stress, mais plus récemment, elles sont aussi apparues comme impliquées dans la virulence, l'agrégation cellulaire et la motilité extracellulaire flagellaire chez *L. monocytogenes*.

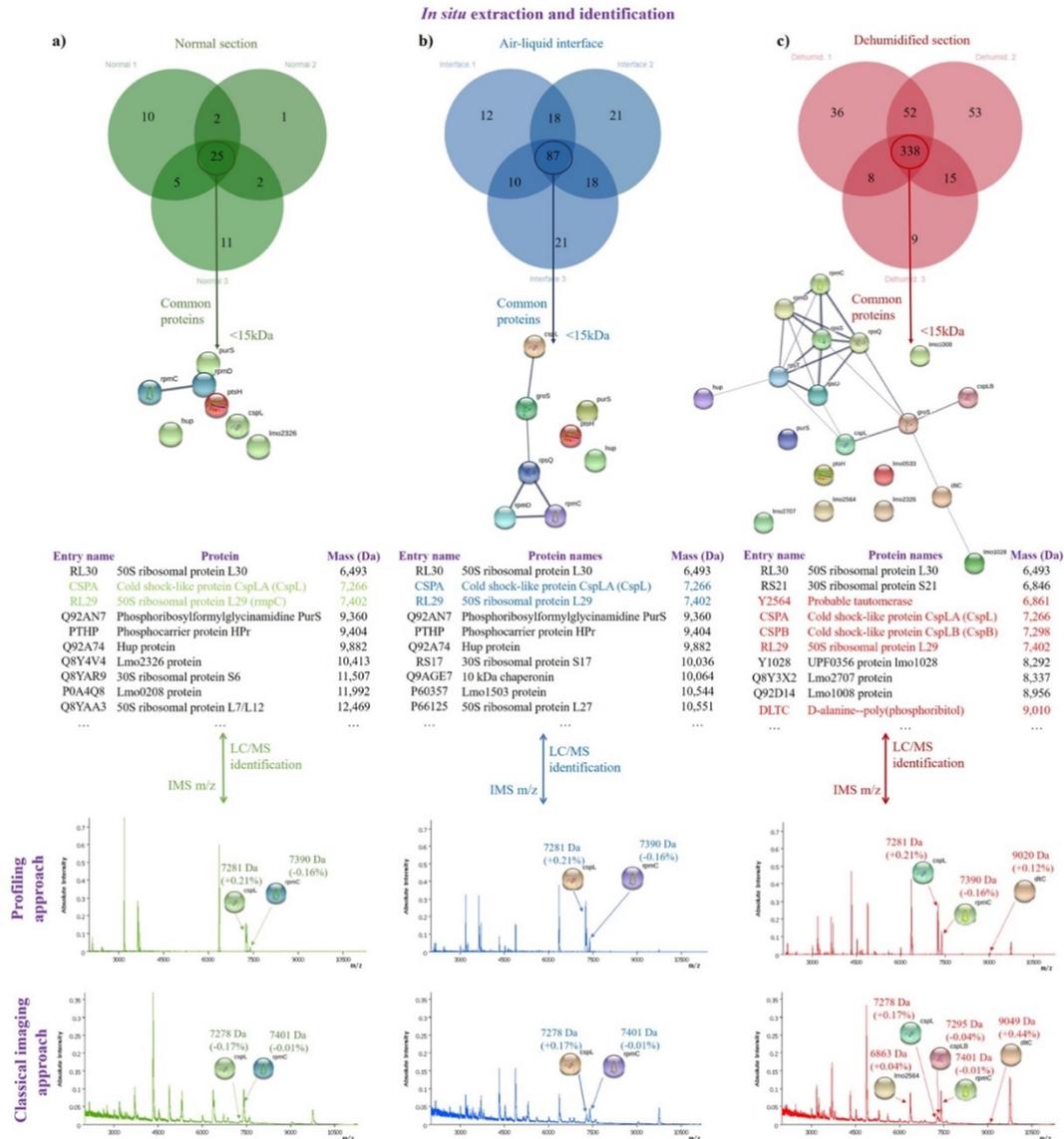


Figure 5 - Schéma de la « back correlation » entre les espèces d'ions obtenues par MALDI (en bas) et l'identification des protéines par LC-MS/MS (en haut). a) Identification des protéines assignées à la section « normale ou immergée ». b) Identification des protéines assignées à la section « interface air-liquide ». c) Identification des protéines assignées à la section « déshumidifiée ». De haut en bas : diagrammes de Venn pour toutes les protéines identifiées dans les trois sections (section normale: 56 protéines; interface: 187 protéines; section déshumidifiée: 511 protéines); Interactions protéine-protéine établies avec le logiciel STRING pour les protéines identifiées de masse inférieure à 15 kDa dans chacune des trois sections ; Description de la liste des protéines présentes dans les interactions protéine-protéine (focalisé sur celles corrélées par leur m / z en IMS et en « profilage », avec une tolérance de masse de 0,5%); Spectres de masse moyens pour chacune des 3 sections des deux approches et identification des protéines assignées avec succès.

CspA a été caractérisée comme la plus pertinente dans l'adaptation au froid. Par conséquent, il n'est pas surprenant que cette protéine ait été identifiée dans la section « normale ». Cette section du biofilm, comme l'ensemble de la lame, a été exposée à une basse température de 10°C pendant 24 h, à l'origine d'une telle réponse. Les deux protéines citées plus haut ont également été assignées dans la section déshumidifiée avec en plus une seconde protéine de choc froid (CspLB), également impliquée dans l'adaptation à des conditions atypiques et également une protéine de type tautomérase et une D-alanine-poly (phosphoribitol) ligase. Cette dernière est impliquée dans la voie de biosynthèse de l'acide lipotéichoïque. La perte de D-alanylation des acides lipotéichoïques modifie la charge de la surface cellulaire et entraîne une réduction de l'attachement à une surface biotique et de la production de biofilm. Cette protéine assignée dans la section déshumidifiée est un élément important dans le lien entre la formation de biofilm et la résistance à des conditions environnementales difficiles. Les acides lipotéichoïques sont essentiels à l'adhésion en surface des cellules de *Listeria* et dans notre cas, c'est dans les conditions de stress que cette protéine a été identifiée, ce qui suggère que des mécanismes similaires à ceux qui se produisent lors de la formation de biofilm sont induits pour résister à des conditions de diminution de l'humidité relative.

Conclusions

Le premier objectif de ce travail était de développer et d'optimiser une méthodologie permettant l'utilisation de l'imagerie MALDI sur des biofilms de bactéries à Gram positif, puis de l'appliquer pour étudier l'impact, au niveau moléculaire, de contraintes environnementales sur les cellules sessiles. Ces contraintes consistaient à imiter les conditions d'atelier alimentaire dans lesquelles, après chaque procédure de nettoyage-désinfection des surfaces et des équipements, l'air est déshumidifié pour éliminer rapidement l'eau résiduelle, assainir l'atmosphère ambiante et limiter la propagation des contaminations.

Le protocole d'imagerie MALDI développé nous a permis de comparer les sections de biofilms immergés et déshumidifiés, révélant la localisation spatiale de 47 protéines différentes au sein d'une seule analyse. Une approche de micro-protéomique nous a ensuite conduit à identifier plusieurs protéines et à établir enfin une « back correlation » avec les données d'imagerie, permettant l'identification de 5 protéines parmi les spectres obtenus. Il serait particulièrement intéressant d'étudier les

variations d'expression de ces protéines identifiées dans d'autres conditions environnementales et d'appliquer éventuellement cette méthodologie à d'autres bactéries productrices de biofilms. Ces approches d'IMS et d'identification de protéines innovantes ont contribué à disséquer le protéome spatial d'un biofilm bactérien intact, donnant un aperçu de l'expression des protéines liées à l'adaptation des biofilms à la déshumidification. D'un point de vue plus général, cette approche enrichit de manière prometteuse les différentes techniques et méthodologies déjà utilisées pour explorer et comprendre la physiologie des microorganismes dans les biofilms. Le travail rapporté dans ce chapitre a été accepté en septembre 2018 comme article de recherche original dans « Journal of Proteomics », volume 187, pages 152-160. (<https://www.sciencedirect.com/science/article/pii/S1874391918302902>).

Deuxième chapitre: Adaptation de biofilms de *Listeria monocytogenes* à différentes températures vue par une approche de « shotgun proteomics »

Contexte

Listeria monocytogenes est un agent pathogène d'origine alimentaire pouvant causer une maladie humaine invasive grave, la listériose, chez les patients sensibles. La plupart des cas de listériose humaine semblent être dus à la consommation d'aliments prêts-à-manger réfrigérés. Bien que les niveaux de contamination initiale dans les aliments soient généralement faibles, la capacité de ces bactéries à survivre et à se multiplier à basse température leur permet d'atteindre des niveaux suffisamment élevés pour provoquer une maladie. *L. monocytogenes* peut se développer dans un large intervalle de températures, entre 1 et 45°C, et peut modifier la composition de son enveloppe cellulaire et son métabolisme afin de maintenir l'homéostasie cellulaire, en fonction des conditions thermiques. Ce chapitre explore l'ensemble des protéines pouvant être associées à l'adaptation de *L. monocytogenes* à différentes températures et les différences qui peuvent potentiellement constituer un avantage à un mode de croissance en biofilm *versus* planctonique.

Matériel et méthodes

La souche EGD-e de *L. monocytogenes*, de séro groupe 1/2a, a été utilisée pour cette étude. Une pré-culture est réalisée dans le milieu TSB à 25°C et sous agitation à 150

tr/min. La croissance est contrôlée en mesurant l'absorbance à 600 nm (DO_{600}). Des cellules précultivées jusqu'en phase stationnaire sont utilisées pour inoculer des cultures afin d'obtenir une DO_{600} finale de 0,005. Pour les cultures sessiles, la souche est cultivée pendant 6 h. Les cellules sont récoltées par centrifugation ($7500 \times g$, 15 min) et remises en suspension dans du TSB dilué au 1/5 avec de l'eau stérile dans un volume égal à celui du surnageant recueilli, atteignant une DO_{600} comprise entre 0,6 et 0,7. Sept millilitres de la suspension bactérienne sont versés sur chaque disque en acier inoxydable (SS) ($38,5 \text{ cm}^2$) ; ces derniers sont ensuite placés dans une boîte de Pétri stérile (diamètre de 55 mm) et incubés à 25°C . Les bactéries sont laissées à adhérer pendant 3 h avant de retirer le milieu, ce qui permet d'éliminer les cellules planctoniques. Les disques sont ensuite incubés à l'une des 3 différentes températures (10, 20 et 37°C). Après incubation, le milieu est retiré et les cellules adhérentes sont détachées dans 10 ml de TS en grattant le disque SS avec une spatule stérile. Pour une biomasse suffisante, les cellules adhérentes de 10 disques sont combinées. L'extraction des protéines est réalisée par trois méthodes : le shaving enzymatique avec de la trypsine, le marquage à la biotine et le fractionnement cellulaire (sous-fractions membranaire, pariétale et intracellulaire). Chacune des trois méthodes est appliquée à trois cultures indépendantes en biofilm et trois cultures indépendantes planctoniques après deux temps d'incubation correspondant à la phase exponentielle de croissance et à la phase stationnaire pour chacun des modes de croissance. Au final, nous avons trois réplicats biologiques pour chaque condition, ce qui correspond à 180 échantillons protéiques.

La méthode de shaving consiste à traiter des cellules intactes avec une protéase (trypsine le plus souvent) dans une solution isotonique afin de libérer les peptides des régions protéiques hydrophiles exposées à la surface. Pour la méthode de biotinylation, les cellules intactes sont traitées avec de la sulfo-NHS-SS-Biotine, à laquelle la membrane cellulaire est imperméable. Cette molécule marqueur réagit spécifiquement avec le groupe ϵ -amino des résidus de lysine de protéines exposées en surface. Par la suite, les protéines marquées peuvent être séparées par chromatographie d'affinité avec la neutravidine à partir de lysats de cellules entières. La troisième méthode consiste en des séparations assez bien établies des fractions intracellulaire, membranaire et pariétale par fractionnement, c'est-à-dire le cassage des cellules bactériennes suivi par des centrifugations différentielles. Cependant, les sous-fractions membranaires et pariétales issues du fractionnement sont

généralement fortement contaminées par des protéines cytoplasmiques et fournissent souvent des informations insuffisantes sur les protéines de surface.

Pour le procédé de shaving enzymatique, les cellules sont récoltées par centrifugation à basse vitesse (1 000 x g, 15 min, 4°C) pour éviter la lyse cellulaire. Le culot de cellules bactériennes est lavé doucement deux fois avec 1 ml d'une solution saline tamponnée au Tris (TBS : Tris-HCl 20 mM, pH 7,4, NaCl 150 mM). Le culot est remis en suspension dans 1 ml de tampon (TBS + CaCl₂ 6H₂O 10 mM, L-arabinose 1 M) et les bactéries sont traitées avec 0,5 µg/ml de trypsine (Promega, Madison WI, USA) sous agitation douce à 37°C pendant 1 h. Les cellules bactériennes sont ensuite éliminées par centrifugation pendant 15 min à 1000 tr/min et à 4°C et le surnageant contenant les peptides coupés par la trypsine sont recueillis et filtrés (0,22 µm).

Pour la biotinylation des protéines de surface des cellules bactériennes, nous avons utilisé une sulfo-NHS-SS-Biotine clivable, imperméable aux cellules, pour marquer les amines primaires des protéines exposées à la surface des cellules intactes. Les cellules en biofilm sont mises en suspension dans 10 ml de tampon A (PBS, 0,01 mM, pH 8 + 1 mM PMSF). La suspension est transférée dans des tubes et centrifugée à 4 000 x g pendant 10 min à température ambiante (RT). Le culot bactérien est lavé deux fois et le poids des cellules humides est calculé. Cent mg de cellules sont remisés en suspension dans 300 µl de tampon A additionné de 1,5 mM de sulfo-NHS-SS EZ-Link. La biotinylation est effectuée pendant 15 min à température ambiante sous agitation douce. La biotine libre éliminée par centrifugation à 4 000 x g pendant 5 min à température ambiante et le culot est lavé trois fois avec du PBS (0,01 M, pH 8 + 500 mM de glycine) pour bloquer la biotine n'ayant pas réagi. Les cellules sont remises en suspension dans 500 µl de tampon A additionné de triton X100 à 1% (v/v) et cassées à 4°C par agitation vigoureuse dans un Fastprep-24 pendant deux fois 20 s. Les extraits cellulaires sont centrifugés à 20 000 x g pendant 30 min à 4°C pour sédimenter les parois cellulaires insolubles. Les protéines marquées sont récupérées par chromatographie d'affinité dans une résine monomère de neutravidine avec un écoulement gravitaire, en utilisant une solution saline tamponnée au phosphate (PBS, pH 8) + 1% de Triton X-100 en tant que tampon d'équilibrage et de lavage. Les protéines sont éluées avec un tampon d'éluion (DTT 50 mM, DTT 2%, β-mercaptoéthanol 5%, glycérol 20% dans du Tris-HCl à 62,5 mM, pH 6,8).

Dans le procédé de fractionnement cellulaire, les cellules sont lavées deux fois dans du Tris-EDTA (TE, Tris 20 mM, EDTA 5 mM, pH 7). Le culot est remis en suspension

dans 1 ml de TE et les cellules bactériennes sont cassées à l'aide d'un disrupteur de cellules en appliquant une pression de 2,5 kBar. Les matériaux insolubles contenant les parois cellulaires ont été éliminés par centrifugation (13 000 x *g*, 15 min à 4°C) et le surnageant est ultracentrifugé (200 000 x *g*, 1 h à 4°C). Les membranes contenant le culot sont lavées et ultracentrifugées (200 000 x *g*, 1 h, 4°C) deux fois dans 1 ml de Tris 40 mM, pH 8,5. Les membranes sont mises en suspension dans un tampon de dénaturation (SDS 1%, DTT 0,1 M, Tris-HCl 20 mM, pH 7,5) avant le traitement thermique (5 min, 95°C). Les extraits protéiques de membrane et de paroi cellulaire sont mis en suspension dans 100 mM de bicarbonate d'ammonium à pH 7,5.

L'analyse par LC-MS/MS et l'identification des protéines sont effectuées sur tous les extraits de la fraction intracellulaire. Afin de réaliser les analyses, tous les échantillons ont été chargés sur un gel de SDS-PAGE afin de se concentrer les protéines sur une seule bande dans le premier millimètre du gel de résolution. Les bandes excisées sont lavées dans du bicarbonate d'ammonium 25 mM avec 5% d'acétonitrile (ACN) pendant 30 min et deux fois dans du bicarbonate d'ammonium 25 mM avec 50% d'acétonitrile pendant 30 min. Les réactions de réduction et d'alkylation sont réalisées avec des solutions de DTT 10 mM et d'iodoacétamide 55 mM respectivement et toutes les bandes sont finalement déshydratées avec 100% d'acétonitrile. Les échantillons sont hydrolysés pendant une nuit avec 600 ng de trypsine et les peptides sont extraits des bandes de gel avec 100% d'acétonitrile. Tous les mélanges de peptides sont analysés par nano-LC-MS/MS en utilisant un système Ultimate 3000 (Dionex) couplé à un spectromètre de masse (MS) Impact II avec une source d'ions CaptiveSpray. L'analyse au MS est réalisée avec un piège à ions FTMS à une résolution de 60 000 (tolérance 10 ppm) et les spectres sont acquis dans une fenêtre de *m/z* 400 - 1400. Pour l'obtention des spectres de fragmentation MS/MS, les paramètres suivants sont utilisés : largeur d'isolement des ions précurseurs fixée à 1 *m/z* avec le rejet des espèces monochargées, énergie de collision normalisée à 37% pour la fragmentation et un temps d'activation de 10 ms. Pour le traitement des données brutes, une recherche MS/MS a été effectuée avec Mascot v2.5 dans la base de données (<http://www.matrixscience.com>). Les paramètres suivants ont été pris en compte pour les recherches : tolérance de masse du précurseur de 10 ppm et tolérance de la masse des fragments de 0,5 Da, un maximum de deux sites de clivage de la trypsine manqués, carbamidométhylation (C) et oxydation (M) définies comme modifications variables. L'identification des protéines est validée lorsqu'au moins deux peptides

provenant d'une protéine présentent une identité statistiquement significative > 13 pour les scores de Mascot avec un taux de faux positifs de 1% (seuil de signification ajusté $p < 0,05$). Le score en ions est égal à $-10 \log(P)$, où P est la probabilité que la correspondance observée soit un événement aléatoire. Des scores d'ions individuels > 13 indiquent une identité ou une homologie étendue. Les interrogations sont effectuées sur une base de données personnalisée contenant des entrées distinctes correspondant à des séquences de protéines brutes et aux différentes protéines matures prédites de *L. monocytogenes* EGD-e, basées sur les sites de clivage putatifs du peptide signal. La localisation subcellulaire des protéines identifiées est déterminée avec la stratégie d'analyses génomiques et protéomiques basée sur la sécrétomique développée par Renier et al. (2012). Pour l'analyse de la quantification des protéines, LC-Progenesis a été utilisé avec Mascot v2.3 et les mêmes paramètres d'identification décrits ci-dessus. Les diagrammes de Venn ont été réalisés par l'outil en ligne Jvenn. La catégorie fonctionnelle des protéines est basée sur les clusters de groupes orthologues (COG) via eggNOG en ligne. Les réseaux d'association protéine-protéine sont réalisés avec la base de données String. Une « heat map » présentant les abondances des protéines normalisées est obtenue via Xlstat (2018.5). Les « volcano plots » sont réalisés par le logiciel R avec l'intégration des logiciels graphiques ggplot2 et ggrepel pour l'analyse des données.

Résultats et discussion

Le plus grand nombre de protéines uniques identifiées par MS, contenant au moins deux peptides uniques, a été obtenu par la méthode de fractionnement cellulaire (910, comprenant à la fois les sous-fractions de pariétale et membranaire), suivies des méthodes de biotinylation et de « shaving », 141 et 98 protéines identifiées, respectivement (Figure 6A). La même évaluation peut être faite à la figure 6B avec tous les résultats obtenus en intégrant les trois sous-fractions de la méthode de fractionnement. Il existe un recouvrement notable des protéines identifiées entre les trois méthodes et le nombre le plus élevé de protéines identifiées uniques est de nouveau obtenu avec le fractionnement cellulaire, en particulier dans la fraction intracellulaire.

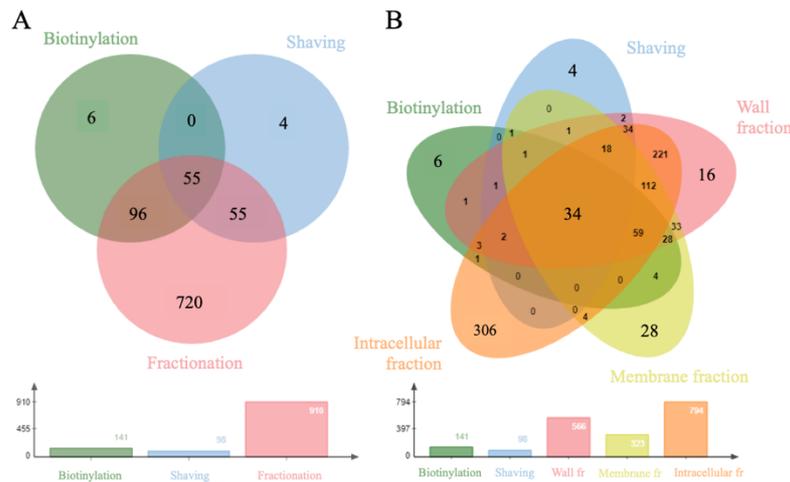


Figure 6-A : diagrammes de Venn représentant la distribution des 920 protéines uniques identifiées par les différents procédés d'extraction de protéines; B: Même représentation mais en intégrant les différentes sous-fractions de la méthode de fractionnement.

Les protéines identifiées ont d'abord été annotées et séparées en fonction de leur localisation sub-cellulaire: intracellulaires, associée à la membrane, associée à la paroi cellulaire et extracellulaires. L'un des objectifs de cette étude était d'identifier le plus grand nombre possible de protéines associées à la surface cellulaire, d'où l'utilisation de trois méthodes d'extraction complémentaires. Malgré les précautions prises pour éviter la lyse cellulaire, en particulier dans les méthodes d'extraction par rasage enzymatique et par biotinylation, un nombre important de protéines cytoplasmiques était présent dans toutes les méthodes et dans toutes les fractions (Figure 7). Le pourcentage le plus élevé de protéines intracellulaires dans les sous-fractions de protéosurfaceome a été trouvé dans le décapage à la trypsine (82%), suivi par la paroi, la fraction membranaire et la biotinylation, correspondant à 75%, 53% et 41% du total des protéines identifiées dans chacune des fractions, respectivement.

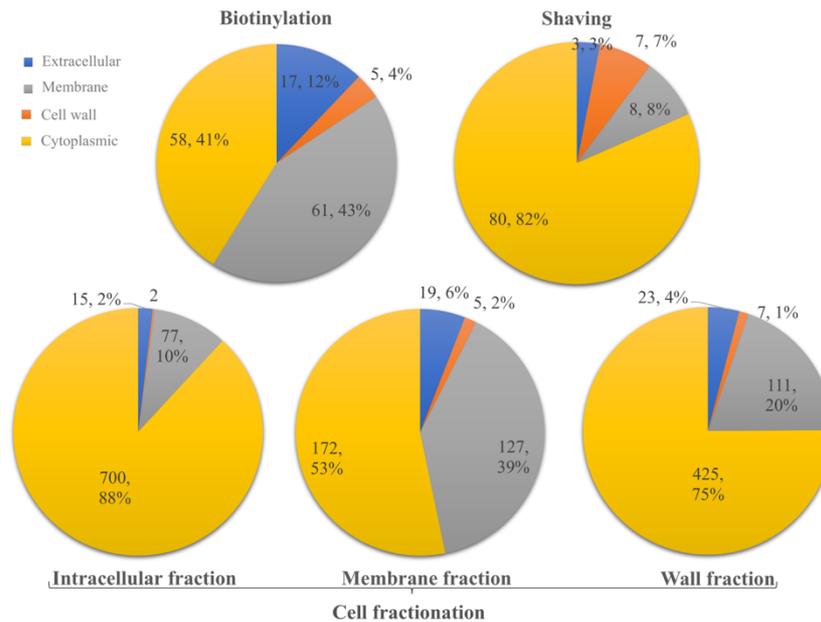


Figure 7 - Diagrammes illustrant le nombre de protéines identifiées et l'efficacité de chaque méthode d'extraction en tenant compte de la localisation subcellulaire prédite de ces protéines.

Pour mieux comprendre les modifications du protéome de *L. monocytogenes* dans les différentes conditions, il est important d'examiner les protéines d'intérêt qui pourraient constituer des biomarqueurs potentiels pour l'adaptation à des conditions environnementales et d'industries agroalimentaires. Pour effectuer ces analyses, des « volcano plots » ont été utilisés avec des seuils de coupure d'une valeur de $p = 0,05$ ($\log_{10} (p\text{-value}) = 1,30103$) et un ratio (FC) = 2 ($\log_2 (\text{Fold Change}) = 1$). Les comparaisons quantitatives entre les différentes températures sont représentées par des « volcano plots » différents, 10°C vs 37°C (Figure 8A), 10°C vs 25°C (Figure 8B) et 25°C contre 37°C (Figure 8C). Au total, 139, 68 et 58 protéines sont comprises dans ces critères de seuils de coupure, respectivement. Les 50 protéines présentant les plus fortes variations d'expression sont marquées par une couleur correspondant à la température à laquelle elles présentaient une condition moyenne plus élevée. Dans la représentation 10°C vs 37°C, les protéines associées à 10°C qui présentaient le ratio le plus élevé sont associées au métabolisme et au transport des acides aminés. Les protéines mises en évidence comprennent des protéines associées à l'adaptation au stress, comme OpuCA (Q7AP65_LISMO) et CspLA (CSPA_LISMO), et à la motilité cellulaire (Q8Y954_LISMO, Lmo0680). Il y a également des protéines associées à la virulence telles que LmaA (Q7AP93_LISMO), LmaB (Q7AP94_LISMO), CspLB (CSPB_LISMO) et à l'adaptation à la chaleur (Lmo1580, Q8Y6V1_LISMO et CcpA,

Q8Y6T3_LISMO). En ce qui concerne l'évaluation entre la basse température (10°C) et la condition de contrôle (25°C), le transport était la principale fonction moléculaire trouvée dans les protéines à 10°C avec des « fold change » importants, principalement le transport des acides aminés, avec en particulier les multiples « ATP-binding cassette (ABC) » transporteurs d'acides aminés (OpuCA, Q7AP65_LISMO; Lmo0538, Q8Y9J0_LISMO; SERC_LISMO, SerC) et en relation avec les mécanismes de défense (Lmo2371, Q8Y4R3_LISMO; Lmo2372, Q8Y4R2_LMO06, L8H05). À la température de contrôle, la majorité des protéines avaient un lien avec la machinerie de traduction et de production d'énergie, incluant cependant des protéines chaperonnes telles que GroEL (CH60_LISMO) et PrsA2 (PRSA2_LISMO). Concernant la comparaison de 25°C vs 37°C, la majorité d'entre elles avaient des fonctions dans la traduction ou l'utilisation de la source de carbone.

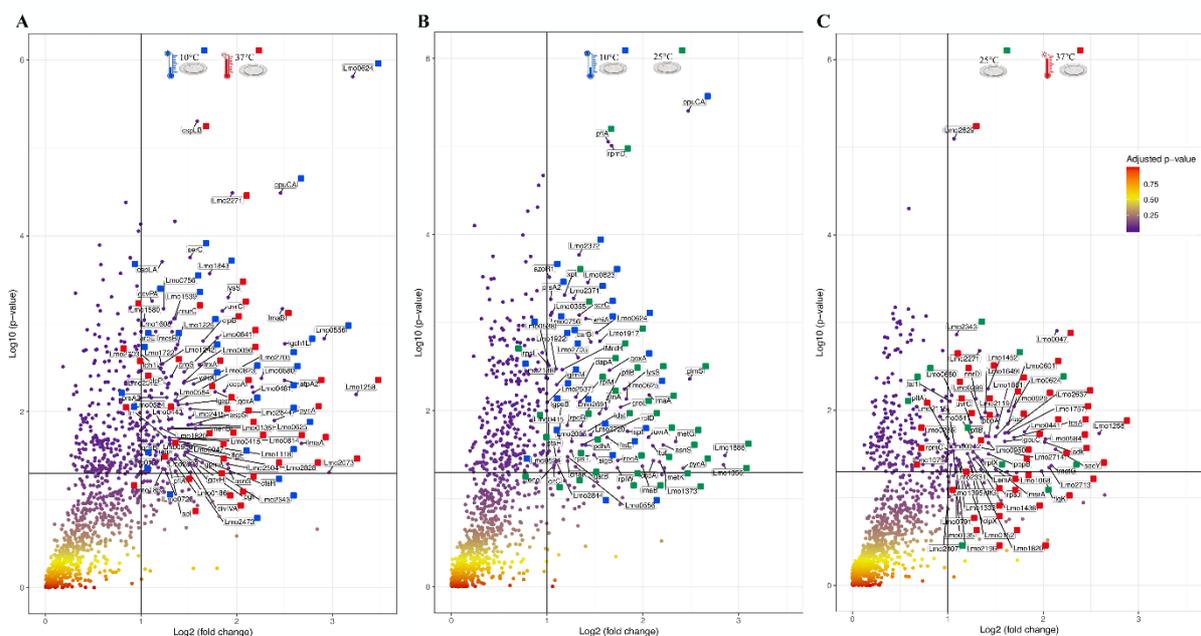


Figure 8 - Volcano plots représentant la distribution des protéines identifiées en tenant compte de leur ratio d'expression et de leur p-value. Les seuils sont représentés par des lignes noires à un ratio de 2 ($\text{Log}_2(\text{ratio}) = 1$) et une p-value de 0,05 ($\text{Log}_{10}(\text{p-value}) = 1,30103$). Les 50 protéines avec les plus forts ratios sont marquées de la couleur correspondant à la température à laquelle elles sont les plus abondantes. A: Volcano plot pour la comparaison entre 10°C et 37°C; B: Volcano plot pour la comparaison entre 10°C et 25°C; C: Volcano plot pour la comparaison entre 25°C et 37°C.

Conclusions

Listeria monocytogenes résiste à divers types de stress, notamment au stress froid associé aux basses températures dans les environnements de production et de

stockage de produits alimentaires. Les mécanismes d'adaptation au stress froid sont donc une compétence essentielle de *Listeria*, lui permettant de survivre et de proliférer pour atteindre des niveaux infectieux minimaux sur les aliments réfrigérés. Dans ce fait, les conditions froides dans les usines de produits alimentaires peuvent sélectionner des sous-types de *L. monocytogenes* dotés des capacités physiologiques d'adaptation appropriées permettant leur survie efficace et leur propagation lors de la manipulation et du stockage des aliments. Des approches analogues à l'avenir aideront à mettre en évidence d'autres gènes cibles potentiels de la résistance au stress froid chez *Listeria monocytogenes*. Il sera particulièrement intéressant de faire une comparaison entre ces données protéomiques sur biofilm et des approches similaires sur des cellules planctoniques. Ce travail a été accepté en juin 2019 comme article de recherche original dans *Frontiers in Nutrition*, 6 : 89 (doi: 10.3389/fnut.2019.00089).

Troisième chapitre: Croissance planctonique vs sessiles de *Listeria monocytogenes* : apport de la protéomique pour détecter les avantages?

Contexte

Listeria monocytogenes est un agent pathogène d'origine alimentaire et l'agent responsable de la listériose, une infection dont le taux de mortalité atteint 20%. Il s'agit avant tout d'une bactérie psychrotrophe capable de former un biofilm, de s'adapter, de résister et de croître dans des conditions difficiles, notamment celles rencontrées dans les industries alimentaires, telles que les basses températures et la faible disponibilité en eau. La capacité de *Listeria* à survivre à des températures aussi basses que zéro degré est directement associée à la majorité des cas de listériose causés par des aliments contaminés par cette bactérie. De multiples études de génomiques, transcriptomiques et protéomiques tentent de comprendre cette adaptation aux changements physico-chimiques et biologiques. Dans tous les cas, l'adaptation à la température est liée d'une manière ou d'une autre à la surexpression des mécanismes de stabilité par la modulation des structures d'acides nucléiques (ADN et ARN), au maintien de l'intégrité structurale des membranes cellulaires, à l'absorption de solutés compatibles, à des mécanismes de réponse au stress non spécifiques et à la production de diverses protéines de stress froid. Ici, trois méthodes

d'extraction de protéines, à savoir la biotinylation, le shaving et le fractionnement, ont été utilisées conjointement pour explorer le protéome de cellules de *L. monocytogenes* planctoniques en croissance à trois températures différentes (10°C, 25°C et 37°C). De plus, une comparaison *in silico* a été faite avec les données protéomiques obtenues précédemment à partir de cultures en biofilms à ces trois mêmes températures.

Matériel et méthodes

La même méthodologie d'analyse du protéome que celle utilisée dans le deuxième chapitre a également été appliquée ici, mais pour des cultures planctoniques de *Listeria monocytogenes*.

Résultats et discussion

Sur l'ensemble des méthodes d'extraction et des trois températures, 931 protéines uniques ont été identifiées, représentant 32,6% du protéome de *Listeria monocytogenes* (Glaser et al., 2001). Le nombre de protéines identifiées dans cette étude était significativement plus élevé que celui rapporté dans la publication où ces trois méthodes d'extraction de protéines ont été mises au point et comparées pour étudier les sous-protéomes de *L. monocytogenes* (Esbelin et al., 2018). Le plus grand nombre de protéines identifiées par MS contenant au moins deux peptides uniques a été obtenu par la méthode de fractionnement cellulaire (668, incluant les sous-fractions de la paroi cellulaire et de la membrane), suivies des méthodes de biotinylation et de « shaving », 169 et 94 protéines identifiées respectivement (Figure 9A). Il existe un chevauchement notable des protéines identifiées entre les trois méthodes et le nombre le plus élevé de protéines identifiées est obtenu avec le fractionnement cellulaire, en particulier avec la fraction intracellulaire. Les protéines identifiées ont d'abord été annotées et séparées en fonction de leur localisation sub-cellulaire : protéines intracellulaires, protéines associées à la membrane, protéines associées à la paroi cellulaire et protéines extracellulaires. La grande majorité des protéines identifiées ont été caractérisées en tant que cytoprotéines, suivies des protéines associées à la membrane, des protéines extracellulaires et des protéines associées à la paroi cellulaire. Le pourcentage le plus élevé de protéines identifiées était cytoplasmique (79%), quelle que soit la méthodologie utilisée pour l'extraction du protéome à la surface des cellules et les mesures prises pour éviter la lyse cellulaire.

Ce résultat était attendu, si l'on prend en compte le fait que la majorité du protéome cellulaire est composé de protéines cytoplasmiques, en particulier de protéines ribosomales qui représentent plus de 20% des protéines cellulaires totales (Ryzhov et Fenselau, 2001).

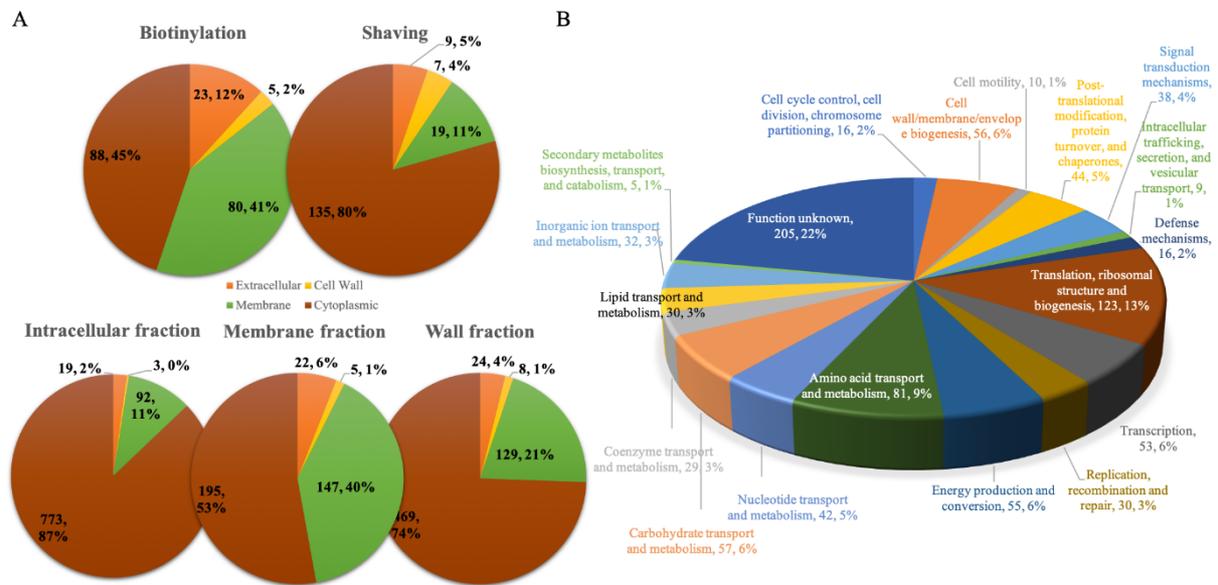


Figure 9-A: Diagrammes à secteurs illustrant le nombre de protéines identifiées et l'efficacité de chaque méthode d'extraction de protéines en tenant compte de la localisation subcellulaire prédite de ces protéines; B: Diagramme à secteurs illustrant la distribution des protéines uniques identifiées par leur fonction moléculaire prévue de COG.

De plus, les conditions techniques ajoutent également à la grande quantité de cytoprotéines identifiées. Les méthodes d'extraction peuvent provoquer la lyse d'une partie des cellules, en particulier avec la méthode de « shaving », ce qui entraîne la présence d'un nombre important de protéines intracellulaires identifiées. La catégorie fonctionnelle de cet ensemble de protéines était basée sur les clusters de groupes orthologues (COG) (Figure 10B). Étant donné que la majorité des protéines identifiées proviennent ont une localisation cytoplasmique, il n'est pas surprenant que la catégorie fonctionnelle présentant le pourcentage le plus élevé de protéines identifiées est «Traduction, structure ribosomale et biogenèse» (13%), suivie de «Transcription». et 'Biogenèse paroi cellulaire / membrane / enveloppe', 6% chacun.

Listeria monocytogenes est capable de se développer aux températures de réfrigération. L'adaptation est largement basée sur les modifications de la synthèse et du repliement des protéines et sur l'absorption d'osmolytes. L'analyse quantitative de

l'abondance des protéines réalisée par LC-Progenesis permet de faire des comparaisons deux à deux aux trois températures utilisées, 10°C vs 37°C (Figure 10), 10°C vs 25°C et 37°C. Dans tous les cas, des abondances normalisées des protéines identifiées statistiquement significatives ($p < 0,05$) ont été utilisées pour chaque comparaison. La carte d'interaction protéine-protéine obtenue par le logiciel String (Figure 11) révèle que chacune des 434 protéines statistiquement significatives était plus abondante lorsque l'on compare la croissance à 10°C et à 37°C. Cette évaluation met en contraste une condition de température imitant celle trouvée dans l'industrie alimentaire (10°C) et la température de l'hôte humain (37°C), dans laquelle *L. monocytogenes* exprime un niveau de virulence plus élevé. Un nombre proche de protéines plus abondantes a été détecté aux deux températures, à savoir 237 à 10°C et 198 à 37°C. L'adaptation de *L. monocytogenes* à la croissance au froid s'effectue par le biais de fluctuations d'un grand nombre de composants cellulaires. De plus, les mécanismes de protection contre la basse température peuvent être partagés ou conférer une protection croisée à d'autres conditions difficiles. Le métabolisme général et les voies biochimiques sont ceux qui ont finalement montré un plus grand changement dans l'abondance des protéines, confirmant qu'il s'agissait des processus principalement affectés par une exposition au froid.

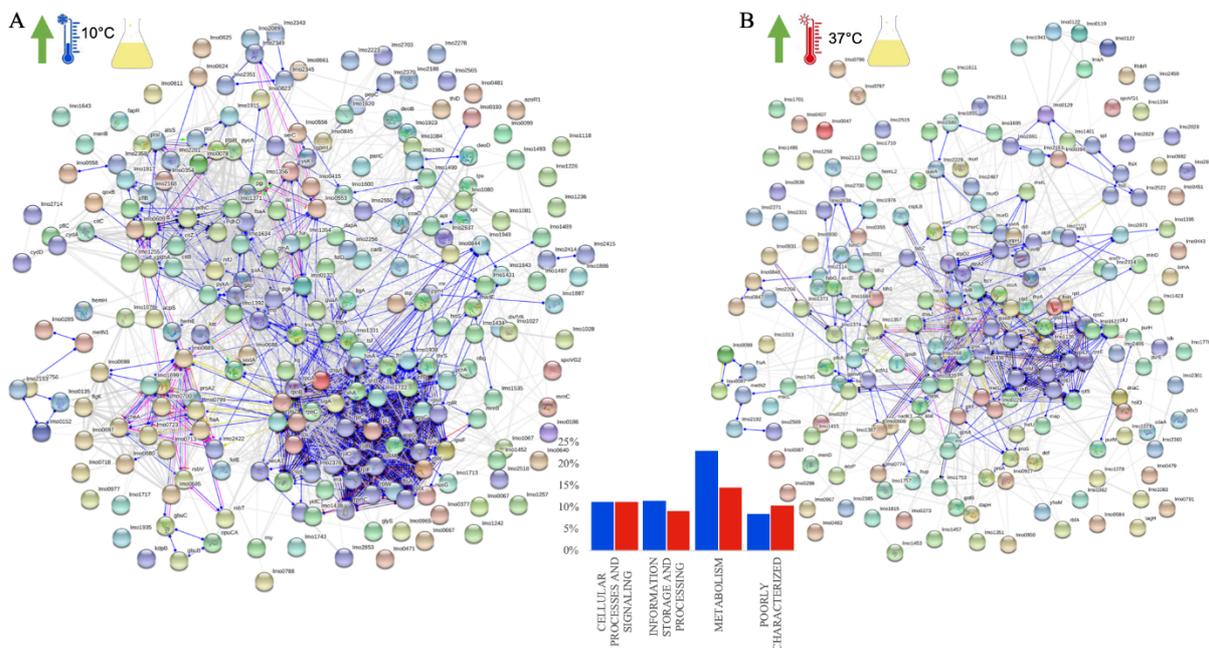


Figure 10 - ANOVA LC-Progenesis comparant *Listeria monocytogenes* à 10°C et à 37°C. A: A: carte d'interactions protéine-protéine des protéines plus abondantes à 10°C; B: carte d'interactions protéine-protéine des protéines plus abondantes à 37°C; Diagramme à barres vertical montrant le pourcentage

de protéines ayant une abondance plus élevée dans chaque température en fonction de leur fonction moléculaire prédite de COG.

La résistance physiologique de *Listeria monocytogenes* est accompagnée par sa capacité à se lier rapidement aux surfaces et à former un biofilm qui est le facteur principal de la persistance de ce microorganisme dans l'environnement alimentaire. Les données protéomiques obtenues ici à partir de cellules se développant en mode planctonique ont permis de visualiser les modifications du protéome à différentes températures de croissance. Notre équipe a déjà appliqué ces mêmes méthodes d'extraction des protéines et la même approche de protéomique aux cellules sessiles de *L. monocytogenes* adhérees sur une surface en acier inoxydable (données et article accepté dans *Frontiers of Nutrition*). Cela nous a permis d'effectuer une comparaison en profondeur de l'abondance en protéines entre les deux modes de croissance et les trois températures. L'abondance des protéines ne conduit pas automatiquement à différents phénotypes. Néanmoins, les cellules bactériennes planctoniques et les cellules de biofilm fonctionnent de manière distincte, ce qui permet une évaluation des données protéomiques sur la base de leur mode de croissance et de leur adaptation à différents contextes. Les protéines associées aux glucides étaient plus abondantes dans les cellules planctoniques aux trois températures. En ce qui concerne les protéines associées au métabolisme du transport des acides aminés et des ions inorganiques, un plus grand nombre d'entre elles était plus abondantes dans le biofilm pour les trois températures. Par contre, un plus grand nombre de protéines associées à la transcription et à la traduction étaient plus abondantes dans les cellules planctoniques vs les cellules sessiles, sauf à 37°C où l'inverse. Les protéines des petites sous-unités ribosomales, telles que RpsD, RpsE, RpsF, RpsM, RpsL, RpsP, RpsS et RpsT, ont toutes montré une abondance plus élevée dans les cellules planctoniques ; pour certaines, elles apparaissent dans les figures de « Volcano plots » avec un fort ratio (Figure 11). Ces résultats suggèrent que les cellules sessiles ont potentiellement une activité traductionnelle plus faible.

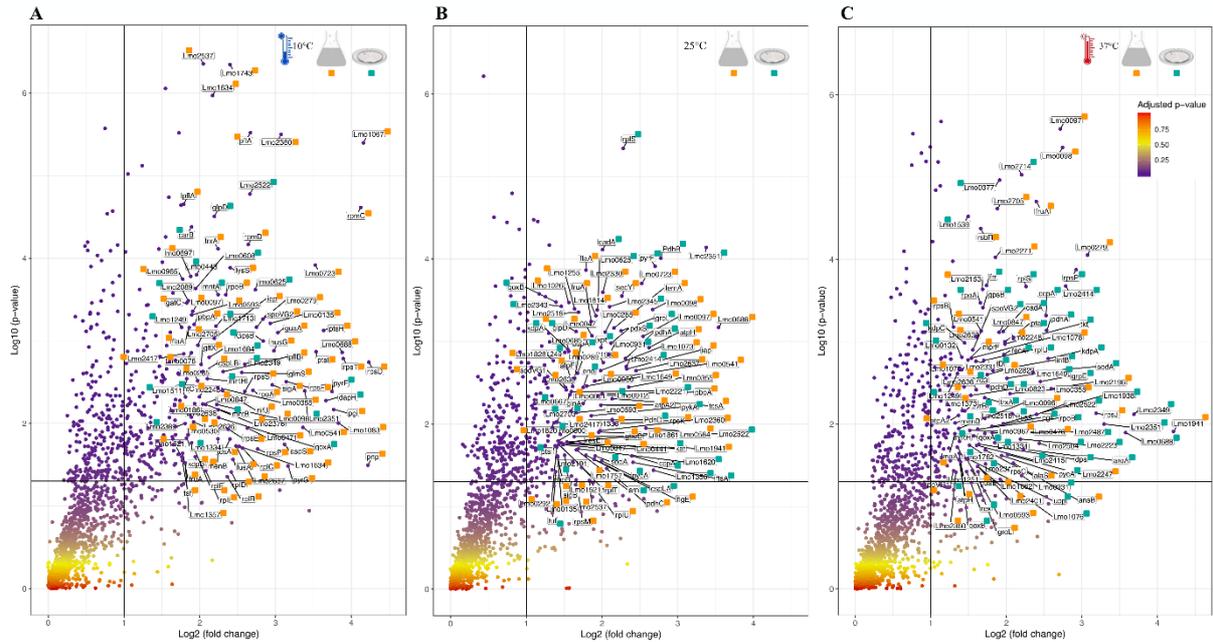


Figure 11 - « Volcano plots » représentant la distribution des protéines identifiées en tenant compte de leur ratio et de leur p-value. Les seuils sont représentés par des lignes noires pour un ratio de 2 (Log_2 (changement de repli) = 1) et une p-value de 0,05 (Log_{10} (valeur p) = 1,30103). Les 100 principales protéines avec le plus fort ratio sont marquées d'une couleur représentant la température où elles étaient le plus abondante. A : « Volcano plots » pour la comparaison entre croissance planctonique et en biofilm à 10°C ; B : « Volcano plots » pour la comparaison entre croissance planctonique et en biofilm à 25°C ; C : « Volcano plots » pour la comparaison entre croissance planctonique et en biofilm à 37°C.

L'abondance réduite de la protéine de structure des flagelles (FlaA) est révélatrice du développement d'un biofilm, car la synthèse de cette protéine est inhibée à ce stade. Cette caractéristique est l'une des plus importantes pour différencier les biofilms phénotypiquement. L'abondance réduite des protéines associées à la motilité cellulaire dans le mode de croissance en biofilm suggère que les cellules sessiles sont moins flagellées que les cellules planctoniques. Nous avons observé dans les cellules sessiles une plus faible abondance de FlaA ainsi que de la protéine crochet des flagelles (FlgE), ce qui suggère que les cellules sessiles sont globalement moins flagellées ou perdent leurs flagelles. Les données précédemment obtenues indiquent que le rôle principal des flagelles chez *L. monocytogenes* est de promouvoir le mouvement, ce qui signifie que pendant le développement sessile, la motilité et la production de matrice extracellulaire sont inversement régulées ; lorsque les cellules planctoniques entrent en contact avec une surface, elles se mettent principalement à

produire de la matrice. Les protéines senseur du chimiotaxisme (CheA et CheY) présentent également des niveaux plus élevés dans les cellules planctoniques à basse température, ce qui indique que les cellules sessiles sont moins performantes à ce niveau-là. Un certain nombre de protéines liées à l'adhésion étaient plus abondantes dans le biofilm que dans les cellules planctoniques. On a observé que la protéine DltD était détectée à des niveaux plus élevés dans le biofilm à 25°C, celle-ci est impliquée dans l'estérification de la D-alanine de l'acide lipotéichoïque et de l'acide teichoïque de la paroi (TA) et favorise l'adhésion en augmentant la D-alanylation de TA. De plus, *L. monocytogenes* dépourvu de gène DltD présente une réduction significative de la capacité d'adhésion. L'ADN translocase FtsK, responsable de l'ADN extracellulaire (ADNe) apparaît plus abondant dans le biofilm à 10°C. L'ADNe de poids moléculaire élevé joue un rôle dans la phase d'adhésion précoce de *Listeria*. Comme indiqué dans le chapitre précédent, le stress oxydatif est l'un des effets de la croissance à basse température. Le régulon *Fur* qui contrôle l'apport en fer de la cellule était plus abondant dans les cellules sessiles. Cela indique que les cellules en biofilm présentent une réponse au stress oxydatif particulièrement élevée. De la même façon, la superoxyde dismutase (Sod) et la protéine de protection de l'ADN pendant la déplétion en nutriments (Dps) étaient présentes à des niveaux plus élevés dans le biofilm. La SOD est produite de manière constitutive en réponse à de multiples facteurs environnementaux et peut également jouer un rôle dans le mode de croissance. Ces protéines impliquées dans le transport des ions inorganiques et le métabolisme favorisent la protection contre les dommages oxydatifs et jouent un rôle important dans les biofilms de *Listeria*. Les gènes régulateurs qui ont une influence sur la formation de biofilm chez *Listeria*, comme le gène *luxS* du système Agr régulant le quorum sensing, ont également montré une surexpression en biofilm. La présence de LuxS s'avère essentielle chez d'autres bactéries formant des biofilms, telles que *Enterococcus faecalis* et *Streptococcus pneumoniae*. Le manganèse est un micronutriment essentiel lors de l'infection et pour la survie en situation de stress oxydatif. Les bactéries possèdent un système de transport à haute affinité pour la consommation de manganèse et son maintien au niveau intracellulaire. Comme montré avec un mutant délété du gène *MntH*, l'absence de système de transport MntABC pour le manganèse entraîne une réduction de la formation de biofilms chez *Neisseria gonorrhoeae* et *Enterococcus faecalis*. De plus, le manganèse est également essentiel au développement des biofilms de *Bacillus subtilis*. Nous avons

observé des niveaux plus élevés du système de transport du manganèse MntABC et de la protéine MntH dans le mode de croissance en biofilm, et à 10°C ces protéines présentaient un facteur de surexpression supérieur à 2, ce qui suggère son importance non seulement pour le développement du biofilm, mais également pour son adaptation à basse température. La protéine PrfA, régulateur notamment de l'îlot de pathogénicité LIPI-1 était présente à un niveau plus élevé dans les cellules sessiles. La perte de PrfA réduit considérablement la capacité de développement sessile. Il est à noter que parmi les protéines plus abondantes en mode de croissance sessile, nous avons trouvé des enzymes impliquées dans la recombinaison et la réparation de l'ADN, telles que l'exonucléase UvrB et RecA. L'endopeptidase ClpC était également plus abondante en mode de croissance sessile, ce qui suggère que, dans le métabolisme des cellules en biofilm, le cycle du citrate est plus actif. Dans une étude antérieure comparant les exoprotéomes de *L. monocytogenes* issus de cellules en biofilms et cellules planctoniques, le rôle de la protéine Lmo2504 dans la formation de biofilms, putativement liée à la paroi cellulaire, a été confirmé par un mutant de délétion codant cette protéine. Cette protéine appartient à un groupe d'enzymes ayant de multiples fonctions dans la croissance et la division cellulaire, y compris des rôles dans le développement de biofilms. Nous avons observé Lmo2504 à des niveaux plus élevés dans le biofilm à 37°C et avec un fort taux de surexpression (x 5,42), confirmant ainsi la pertinence de cette protéine dans le développement sessile. Les protéines chaperon, telle que la protéine de choc thermique chaud DnaK, ont déjà été décrites comme étant essentielles au développement des biofilms de *L. monocytogenes*. Nous avons enregistré des niveaux plus élevés de cette protéine à l'état sessile, laissant supposer un rôle du système de réponse au stress dans la formation de biofilm. Outre les protéines associées à la réparation de l'ADN, à la réponse au stress et à la motilité, d'autres protéines précédemment décrites comme jouant un rôle dans le biofilm étaient également plus abondantes ici dans les données des cellules sessiles, telles que SrtA et RelA. Cependant, il faut tenir compte du fait que la modification de l'abondance de ces protéines pourrait être due à la croissance normale dans différents contextes, dans ce cas la température. Néanmoins, il est raisonnable de supposer que la variabilité du protéome observé ici entre les cellules libres et les cellules sessiles peut éventuellement être liée à la capacité de *L. monocytogenes* à s'adapter à de multiples environnements, que ce soit cliniques ou d'ateliers agro-alimentaires.

Conclusions

Listeria monocytogenes survit et prolifère dans de multiples environnements hostiles grâce à divers mécanismes lui permettant de survivre. En raison de sa capacité à persister dans les environnements alimentaires, un effort continu a été fait pour comprendre les mécanismes moléculaires impliqués afin de trouver les moyens de mieux contrôler et réduire la contamination des produits alimentaires. Nos résultats ont révélé dans les cellules planctoniques de *L. monocytogenes* cultivées à 10°C un taux d'expression plus important que dans les cellules sessiles au niveau de protéines impliquée dans le transport d'osmolytes, dans la glycolyse et le métabolisme des glucides, dans l'absorption des nutriments et dans la réponse à plusieurs conditions de stress, comme le stress oxydatif. Inversement, nous avons observé des protéines significativement surexprimées dans le protéome de *L. monocytogenes* en biofilm par rapport au mode de croissance planctonique. Ces protéines interviennent à divers niveaux de la physiologie des cellules. Cela indique que le phénotype sessile est à l'origine de structures complexes de régulation des gènes. Compte tenu de la pertinence de la formation de biofilms pour la persistance de *Listeria* sur les surfaces dans les ateliers alimentaires, il est essentiel de comprendre le rôle de ces protéines dans le développement sessile et les mécanismes de résistance de cette bactérie dans ces environnements normalement peu favorables à sa croissance.

Les résultats obtenus dans ce troisième chapitre feront l'objet d'un article de recherche original qui sera soumis prochainement pour publication dans *Frontiers in Microbiology*.

Acknowledgments

Science progresses in collaboration and with the help of thousands of researchers. The same stands for my PhD, I want to acknowledge some amazing people that in one way or another helped me accomplish this important career and life mark. During these three years I have a lot of people to thank and in at least three languages so bear with me.

I would like to start by thanking my thesis reviewers and jury members, Jean Armengaud, Graziella Bourdin, Jean Guzzo, Pascal Piveteau and Pascale Gueirard for their willingness to judge my work and participate in my PhD thesis defence.

First and foremost, I want to address my sincere gratitude to my thesis director, Michel Hébraud, I am truly grateful to have had him as a supervisor. He was a great academic and scientific support throughout these three years. Moreover, he was always available to help in any situation and to share some truly remarkable moments. Michel, more than a supervisor I now consider you my friend.

A special thanks to everyone in the Marie-Skłodowska Curie ITN List_MAPS project. I now realise how fortunate I was to be a part of this project and to have shared with you enriching workshops, summer schools and congresses. In my memory and photo recordings I will save these three years as one of the best periods of my life. I particularly would like to express my gratitude to some people of the project. To Pascal Piveteau for the coordination of this colossal project and to have received me, together with Laurant Gal, with open arms in my secondment in Dijon. To Cécile for all the help in the huge task that was managing this project. To Hanne Ignmer, Marianne Larsen and Gitte Peterson to have been so welcoming and helpful in my secondment in Copenhagen. To Patrícia, Ibrahim, Ignasi and Miguel, friends that I made in the project that I am certain will be part of the next brilliant generation of researchers.

A big thanks to everyone in the INRA Medis unit, either through scientific discussions or conviviality in countless coffee breaks, you have helped me grow during these three years. I would like to highlight the guidance and smile of Julia, the kindness of Nelly, the contagious happiness of Caty, Stephanie as the best secretary ever and the always open hand to help attitude of Carine and Ingrid.

Un remerciement spécial à ma maman française. Sylvie, tu es une personne extraordinaire, gentille et belle. Tu étais là pour moi dans les moments heureux mais spécialement dans les moments sombres. Patrice et toi êtes comme une famille pour moi.

A special thanks to Didier, Christophe and Laëtitia, from the Proteomics Unit of INRA. Thank you for all your technical and critical help in all things related with mass spectrometry and also for the long talks and great moments we shared in congresses.

Thanks Aline, I only had one master student during this PhD but how lucky was I? Not only you are a great researcher, you were also an amazing company in the lab and in the office. I bet you will have a successful scientific career and I wish all the best in your future.

Thank you Raphaële, one of the best things that happened to me during these three years is to have had the chance to meet you. You become a trusty friend, an adviser and an always available shoulder to cry on. You have a beautiful heart and wherever life takes us you have a friend in me.

I do not even have the words to thank these next three people, Geoffrey, Bertrand and Audrey. Geoffrey the time that I shared the office with you made me realize how brilliant you are, particularly dancing and singing Despacito. Now seriously, you become a brother to me, you have a kind and beautiful soul. It is hard to not say the same things about Bertrand, an always there friend for the good and bad moments. I treasure in my heart every hiking adventure and beer shared with a smile and a friendly advice. Thank you Audrey for every laugh that we shared during these three years, we lived untimely memories that I will save as a gem. I am already anxious for the great moments in the near future that I will have with these three new members of my family.

A special thanks to my Mexican sunshine by the name of Maricarmen. I saved all the 4.30 pm apple breaks and your smile in my heart. You were always there with a kind word and a new adventure to conquer. Your brilliant mind will take you all over the world, but wherever you are, know that I will be always a phone away from you my friend.

Thank you Marc and Thomas, it is true that I met you guys by your lovely wife and future wife to be, respectively. But I am so glad I did. You guys are great people and in happy and some dark moments you were there for me.

Um obrigado a todos os amigos do laboratório de Vila Real, Professor Gilberto, o meu mestre Alexandre, Susana, Júlio, Eduardo, Luís, Hajer, Rui e Darinka foi convosco que comecei a minha carreira científica e aprendi a ser um bom companheiro de laboratório. Um especial obrigado ao Ricardo, um amigo do peito com quem tive o prazer de partilhar a primeira parte do doutoramento em Clermont.

A toda a família Moreira Marinho, muito obrigado por todos os bons momentos que vivemos durante os últimos três anos. Um especial abraço a uma tia e um irmão emprestado, Elisa e Renato. Ah, e uma lambidela ao Kinder que por duas vezes durante este doutoramento nos tentou fugir.

Obrigado aos amigos de Touguinhó, Magalhães, Mário, Célia, Renato, Ana, Stéphane, André, Pedro e Plácido, mesmo longe conversamos e partilhamos momentos hilariantes e por mais distantes que estejamos uns dos outros haveremos sempre de nos reencontrar (Azul e Branco é o coração).

Um obrigado aos meus amigos Maiatos, Patrícia, Teixeira, Aninhas, Tiago, Luís e prima Carla. Mesmo longe estiveram sempre disponíveis para uma palavra amiga e de apoio.

Um obrigado sem limites à minha família. Obrigado Mãe Arminda, obrigado Pai Francisco, obrigado mana Daniela, obrigado cunhado João, obrigado sobrinha Leonor e obrigado afilhada Julieta (para te ver nascer até escrevi a tese mais rápido). Obrigado por estarem sempre do meu lado, obrigado pelo vosso amor e por me acolherem de braços abertos em todos os momentos.

Last but not least, the person to whom I dedicate this thesis. A person that has helped me in more ways than she can ever imagine. A person that moment after moment gave me the strengths to keep going and bring down all barriers. The most correct, kind and generous person I have ever met. Ilham I will cherish your friendship until my last breath.

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List of abbreviations

2-DE	Two-dimensional gel electrophoresis	FPE	Fimbrillin-protein exporter
ActA	Sugar phosphate permease	GAD	Glutamate Decarboxylase
ADI	Arginine Deaminase	GC	Guanine-cytosine
AhpCF	Alkyl hydroperoxide	GHP	Good hygiene practice
ATR	Adaptive acid tolerance response	GSR	General stress response
AUC	Area under the curve	HACCP	Hazard analysis and critical control points
a_w	Water activity	HGF	Hepatocyte growth factor
BAP	Biofilm-associated proteins	IF2	Initiation translation factor
CAP	cAMP receptor protein	Kat	Catalase
CFU	Colony-forming unit	LC	Liquid chromatography
CHCA	Cyano-4-hydroxycinnamic acid matrix	LDI	Laser desorption/ionization
Csp	Cold shock protein	LLO	Listeriolysin
DESI	Desorption electrospray ionization	LPXTG	Leu-Pro-any-Thr-Gly
DHB	2,5-Dihydroxybenzoic acid matrix	LRR	Leucine-rich repeats
eDNA	Extracellular DNA	LTA	Lipoteichoic acid
EFSA	European Food Safety Authority	m/z	Mass to charge ratio
EPS	Extracellular polymeric substances	MALDI	Matrix assisted laser desorption/ ionization
ESI	Electrospray ionization	Mpl	Protease
EU	Europe	MS	Mass spectrometry
FDR	False discovery rate	MS/MS	Tandem mass spectrometry
FEA	Flagellar export apparatus	PlcA	Lecithinase A
Flp	Ferritin-like protein	PlcB	Lecithinase B

PrfA	Virulence transcriptional factor
PSM	Peptide-spectrum match
PTM	Post-translation modification
QAC	Quaternary ammonium compound
RH	Relative humidity
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RP	Reversed phase
RTE	Ready-to-eat
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labelling with amino acids in culture
SIMS	Secondary ion mass spectrometry
Spases	Signal peptidases
Sod	Superoxide dismutase
TA	Teichoic acid
Tat	Twin-arginine translocation
TOF	Time-of-flight
UPEC	UroPathogenic <i>Escherichia coli</i>
US	United States
WHO	Worldwide Health Organization
WSS	WXG100 secretion system
σ^B	Sigma factor sigma B

Introduction

In the world of bacterial pathogens, there is a double-faced microbe named *Listeria monocytogenes*, that it is considered to be on the forefront when it comes to pathogenicity. *L. monocytogenes* is a biofilm-forming bacterium that has a set of skills that make it a considerable risk for human health. In its natural realm, the soil, *Listeria* evolved to use various sources of carbon and to adapt to multiple extreme conditions, such as low temperatures, pH, osmotic stress and desiccation. In this environment, it is relatively harmless, however the problem resides in its saprotrophic and ubiquitous nature. This meaning that there are multiple pathways of transmission for it to cross from soil to livestock, food products until reaching humans. In sufficient numbers, *Listeria* can lead to a hyper threatening infection named listeriosis, that is particularly lethal in immunocompromised patients, elderly and new-borns. In fact, *Listeria* is in Europe (EU) the leading cause of mortality and food recalls due to a foodborne pathogen, costing EU millions of euros per year in medical care and associated costs in the food sector. Just in 2015, the European Food Safety Authority reported more than twenty-two hundred food-borne related human invasive listeriosis cases that lead to 270 deaths.

Taking into account this panorama, in 2015, a European project with ten PhD students, entitled List_MAPS, funded by the Research and Innovation programme of the European Union Horizon 2020 under the Marie-Skłodowska Curie actions (ITN-ETN), was set in motion in order to tackle food safety through the combination of high throughput Epigenetics, Deep sequencing of transcripts, Proteomics, Bioinformatics, Mathematics and Microbiology to decipher the transcriptional regulatory circuitry that drives adaptation and virulence of *L. monocytogenes* from farm to fork. Integrated in this European network, my PhD project had as an overall goal to gather more information surrounding the proteome shifts in *L. monocytogenes* biofilms and adaptation to controlled environments.

In the first section of this manuscript, I make a non-exhaustive review of the main topics that surround my PhD project. In the first chapter, I describe all the crucial up-to-date knowledge about the pathogen *L. monocytogenes*, including an epidemiologic overview of listeriosis, *Listeria*'s virulence mechanisms and its biofilm-forming capabilities. With a greater bearing to the scope of this thesis, *Listeria*'s strategies to overcome environmental stresses are outlined in the following chapter. I also lay the background of some of the stress settings that this microbe is subjected

in nature and in the food industry realm, particularly the two stress conditions tested in this thesis work, dehumidification and adaptation to different temperatures. Finally, in the third chapter, I describe the state of art of proteomics and mass spectrometry, particularly the two main mass spectrometry workflows applied in this dissertation, MALDI-TOF Imaging Mass Spectrometry and label-free shotgun proteomics by LC-MS/MS. Within this bibliographic review there are also parts of two manuscripts published within the extent of this PhD project, a review duelling on the topic of desiccation and a manuscript detailing three methods to explore the surfaceome of *L. monocytogenes*.

The results' section is divided in three manuscripts reflecting my PhD research from the last three years. The first paper describes the optimization and use of an Imaging Mass Spectrometry approach to explore the proteome of an intact *L. monocytogenes* biofilm. Moreover, the development of an *in situ* microproteomics extraction to try to bridge the gap between the imaging data and the identification of proteins through a bottom-up approach. This work has been published in Journal of Proteomics on September 2018. As for the second manuscript, it has been submitted in Frontiers in Nutrition on November 2018 and it is a shotgun proteomic analysis into *Listeria's* biofilm adaptation to three different temperatures (10°C, 25°C and 37°C). The third paper implemented the same physical stress to *Listeria* cells, but this time in liquid culture. Furthermore, it explores by an *in silico* comparison, the protein abundances seen in the biofilm and in the planktonic mode of growth. Finally, I present a general discussion and conclusions of my PhD project, expanding the research to future directions.

Bibliographical review

Chapter 1 - *Listeria monocytogenes* and biofilm

1.1 Historical context

Listeria monocytogenes is a Gram-positive bacterium first described in England by Murray et al. in 1926. In this first report, *Listeria* was named *Bacterium monocytogenes* after showing a marked increase in the number of monocytes in the bloodstream of infected rabbits (Murray et al., 1926). One year after, Pirie isolated the same organism from the liver of gerbils in South Africa. In this instance, it was proposed the genus name *Listerella*, honouring the surgeon J. Lister. Murray and Pirie eventually realized they were in the presence of the same bacterium and combined the two names to form *Listerella monocytogenes*, later one due to taxonomic reasons this name was rectified to *Listeria monocytogenes* (Pirie, 1940). In regards to *Listeria*'s natural environment, it was proposed in the 70's that soil was the predominant realm (Welshimer and Donker-Voet, 1971). However, soil contamination with this bacterium may derive from other sources like animal manure, sewage and decomposing plant vegetation (Fenlon et al., 1996).

L. monocytogenes can give rise to a threatening human infection named listeriosis. Prior to 1926, there were already mentions in disease reports that were probably due to listeriosis, as was the case of the "diphtheroid" isolated from a soldier in 1919 that was later identified as *L. monocytogenes* (Cotoni, 1942). For almost four decades, listeriosis was classified as a zoonotic infection, meaning a disease affecting mainly animals, that could be transmitted to humans (Stavru et al., 2011). However, during this period, multiple cases were reported of human infections caused by *Listeria*. For example, *L. monocytogenes* was isolated from blood culture of Danish patients with mononucleosis in 1929 (Nyfeldt, 1929) and in 1936, Burn made the connection between this bacterium and cases of sepsis in newborns (Gray and Killinger, 1966). In the 1960s, this bacterium started to gain some traction as an important human pathogen following Mackaness's studies that described the role of cellular immunity in the control of intracellular infection using *L. monocytogenes* as an intracellular lifestyle model (Mackaness, 1962).

L. monocytogenes was official characterized as a human foodborne pathogen after the first epidemics in the 80s (Stavru et al., 2011), and the rise in the total numbers of

human listeriosis linked with intake of contaminated coleslaw, milk and soft cheese (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988). Since then, it is well established that *L. monocytogenes* leads to a severe and life-threatening disease connected with the ingestion with contaminated foods with resultant high economic burden to health services and the food industry. This prompted the intense study in the molecular basis of *L. monocytogenes* virulence that established this bacterium as a model system to explore the molecular mechanisms involved in host-pathogen interactions (Stavru et al., 2011).

1.2 Genus *Listeria*

The genus *Listeria* belongs to the phylum Firmicutes, the latter is comprised of Gram-positive bacteria with low GC-content (36–42%) and also contains the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and *Staphylococcus* (Collins et al., 1991). *Listeria* is composed of 17 non-sporeforming, rod-shaped bacterial species (Korsak and Szuplewska, 2016). This genus was well separated in four clades through phylogenetic analysis: (I) the clade comprising the six *Listeria sensu stricto*, *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*; (II) *L. fleischmannii*, *L. aquatica* and *L. floridensis*, (III) *L. borriae*, *L. cornellensis*, *L. grandensis*, *L. newyorkensis*, *L. riparia*, *L. rocourtiae*, *L. weihenstephanensis* and (IV) *L. grayi* (den Bakker et al., 2014).

Listeria spp. cell wall surface consists mainly of peptidoglycan, teichoic acids and lipoteichoic acids. This composition will be further discussed in this scope of the secretome (Section 1.6.1). The bacterial species of this genus are nutritionally undemanding to reach optimal growth and can be isolated from a wide range of sources, including soil (Falardeau et al., 2018), sewage (Watkins and Sleath, 1981), plant (Brandl, 2006), animal feed (Skovgaard and Morgen, 1988), farms (Castro et al., 2018), households and hospitals (Jacks et al., 2015) and food processing industries (Korsak and Szuplewska, 2016). The former will be further reviewed in Chapter two. *Listeriae*, apart from *L. grayi*, are naturally susceptible to diverse antimicrobial agents, including glycopeptides, tetracyclines, trimethoprim, penicillins, carbapenems, rifampicin, macrolides, lincosamides, chloramphenicol and naturally resistant to most cephalosporins (Troxler et al., 2000). However, antibiotic resistant *L. monocytogenes* strains have been uncovered with various levels of multiresistance depending mostly

on the proximity to reservoirs of antimicrobial resistance (Lotfollahi et al., 2017). *Listeria* spp. are notorious for its capacity to thrive under harsh environmental conditions such as a wide range of temperature and pH, high salt concentrations and other stresses that are further tackled in Chapter 2 (Cole et al., 1990).

Only the *sensu stricto* strains have been isolated in the gut of humans and animals and only two of those have been registered as leading to disease, *L. monocytogenes* and *L. ivanovii*. They are facultative intracellular pathogens and the etiological agents of listeriosis. This notorious capacity from clade I species to survive and even grow in the mammalian gastrointestinal tract is given in part to more than 150 gene products only present in *sensu stricto* strains (Schardt et al., 2017). The other fifteen species are commensal environmental saprophytes. However, there are reported cases of illness involving some of these species, but mainly in severely immunocompromised patients (Liu, 2013).

In regards to the association between *Listeria* and human illness, *L. monocytogenes* is the predominant *Listeria* species. Table 1 describes the twelve different serotypes of *L. monocytogenes* that have been identified and can be divided in four serotype lineages (Rawool et al., 2016). Lineage I is comprised of strains with serotype 4b, 1/2b, and 3b, while lineage II strains are of serotype 1/2a, 1/2c, 3a, and 3c. Lineage III contains strains of serotype 4a and 4c, as well as certain strains of serotype 4b. Lineage IV includes serotype 4a (Ward et al., 2008). Strains from lineage I and II are more often found than strains from lineage III or IV. This can be connected to the fact that 97% of human clinical cases are caused by serotypes 1/2a, 1/2b and 4b (Swaminathan and Gerner-Smidt, 2007).

Table 1 - Genetic lineages of *Listeria monocytogenes*. From (Donaldson et al., 2015).

Lineage	Serotypes	Distribution	Genetic characteristics
I	1/2b, 3b, 3c, 4b	Mainly human isolates	Low diversity among lineages
II	1/2a, 1/2c, 3a	Mainly food products and natural environments	Most diverse
III	4a, 4b, 4c	Majority ruminant isolates	Very diverse
IV	4a, 4b, 4c	Majority ruminant isolates	Few isolates

1.3 Listeriosis

The genus *Listeria* become renowned in public health and in the food industry due to its capacity to lead to a life-threatening disease denominated listeriosis (Schlech et al., 1983). In the case of other foodborne pathogens, such as *Salmonella* and *E. coli* O157:H7, a low amount of bacterial cells can lead to an infection, as low as a food product contaminated with 10 of this *E. coli* strain can cause disease in an individual (Swaminathan and Gerner-Smidt, 2007). As for *L. monocytogenes*, food products with a high level of contamination (e.g. 10^9 bacteria) can result in mild to severe gastroenteritis (Radoshevich and Cossart, 2018). In the United States (US), it is estimated that, per year, a healthy person ingests at least a meal contaminated with 10^6 to 10^9 *L. monocytogenes* cells without any consequent harmful reaction (Chan and Wiedmann, 2009). Reason why, listeriosis incidence is more dependent on the health condition of the individual than any other factor. On the same note, the number of infections per year is relatively low when compared to other foodborne pathogens. In 2010, the worldwide report on listeriosis estimated the number of events to be just shy above the twenty-three thousand that resulted in more than 5463 fatalities (de Noordhout et al., 2014). However, listeriosis potential severity makes it one of the most notorious foodborne pathogens in Europe and worldwide (Antonia Ricci et al., 2018). Particularly due to its unusually high mortality rates, between 20 to 30% of clinical cases end up in deceased. The groups most at risk are newborns, pregnant woman, elderly and individuals with weakened immunity system (Swaminathan and Gerner-Smidt, 2007).

The European Food Safety Authority (EFSA) upheld a quantitative model regarding invasive listeriosis that concluded that more than 90% of cases are due to the consumption of ready-to-eat foods (RTE) (Antonia Ricci et al., 2018). RTE foods are sold directly to the consumer without any prior heat-treatment and have been connected with multiple human listeriosis outbreaks in different points around the globe, among them are included the US (Angelo et al., 2017), Canada (Thomas et al., 2015), Chile (Cordano and Rocourt, 2001), Japan (Shimojima et al., 2016), and throughout Europe (Lomonaco et al., 2015). As a ubiquitous pathogen, *Listeria* can be isolated in food processing pipelines and other multitude of environments (Gandhi and Chikindas, 2007), increasing the chances for it to result in infection in animals and humans.

1.3.1 Pathology in animals

Listeriosis in animals are caused in the majority of instances by *Listeria monocytogenes* and it is believed that the main route of transmission is through contaminated feed. *L. monocytogenes* can survive in animal feed and in liquid manure for months (Nightingale et al., 2004). This contaminated faecal matter is a pathway of pathogen transmission if used as an agricultural fertilizer (Ferreira et al., 2014). However, in some animals, the main cause of septicemia is due to *L. ivanovii* (Wagner M. and McLauchlin J., 2008). In sheep as in goats, listeriosis causes encephalitis, septicaemia and abortion.

The presence of this pathogen in livestock farms increases the possibility of transmission to humans, and subtypes connected with listeriosis in humans have been isolated in cattle. A report of such occurrence was a listeriosis outbreak with 42 human patients, which was traced to the ingestion of cabbage products that were fertilized with sheep manure from a farm that had previous reports of *Listeria* contamination (Swaminathan and Gerner-Smidt, 2007). In the farm environment cattle shows the highest capacity in the spread of *Listeriae*. There are reports of healthy cattle being a reservoir of *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. grayi* (Hutchison et al., 2004).

Bovine farm environments have frequent high prevalence of *L. monocytogenes*, and also with human listeriosis subtypes being isolated (Nightingale et al., 2004). A study in bovine from a dairy farm isolated *L. monocytogenes* 1/2a in faecal samples of asymptomatic cows, hinting that farm animals can also be reservoirs of this pathogen (Borucki et al., 2005). *Listeria* spp. is frequently isolated from other animals sources, such as lambs (Wardrope and MacLeod, 1983), horses (Gudmundsdottir et al., 2004) and chickens (Kurazono et al., 2003).

1.3.2 Pathogenicity and epidemiology

Upon consumption of contaminated food products by an individual, *Listeria monocytogenes* faces the intestinal epithelium and has to cross the intestinal epithelial barrier to disseminate via the lymph nodes and blood to reach its preferred target organs, the liver and spleen (Radoshevich and Cossart, 2018). Figure 1 illustrates the pathways of *L. monocytogenes* spreading in the human host. This invasive intracellular bacterium responsible for listeriosis is described by several clinical

features, such as meningitis, meningo-encephalitis, fetus infections, abortions, perinatal infections and febrile gastroenteritis (Cossart, 2002). These clinical complications are attributed to *Listeria*'s remarkable abilities to cross the intestinal barrier, the feto-placental barriers and the blood brain barrier (Cossart and Toledo-Arana, 2008).

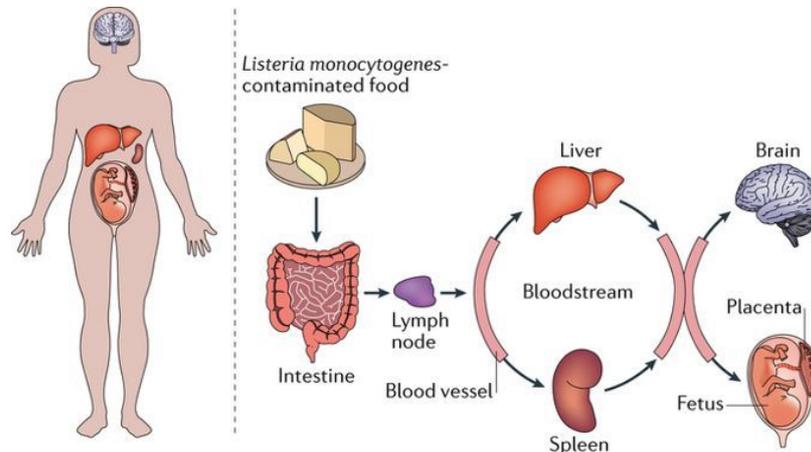


Figure 1 - Diagram of the *L. monocytogenes* infection of an individual and its path of infection. Infection occurs with ingestion of contaminated food products. If *L. monocytogenes* manages to cross the intestinal barrier then it can disseminate to its preferential organs (liver and spleen), but also to the brain and placenta. From (Radoshevich and Cossart, 2018).

Listeria has the capacity to escape the humoral immune defences by invading a variety of mammalian cells, to survive and replicate inside them reaching sufficient numbers to spread to nearby cells (Stavru et al., 2011).

The diagram in Figure 2 illustrates the multiple pathways of *Listeria* transmission between soil, livestock, food products and humans. Being widespread in the environment, *Listeria* can harbour for long periods of time in sewage, vegetation, soil, farm environments and food processing plants (Allerberger and Wagner, 2010).

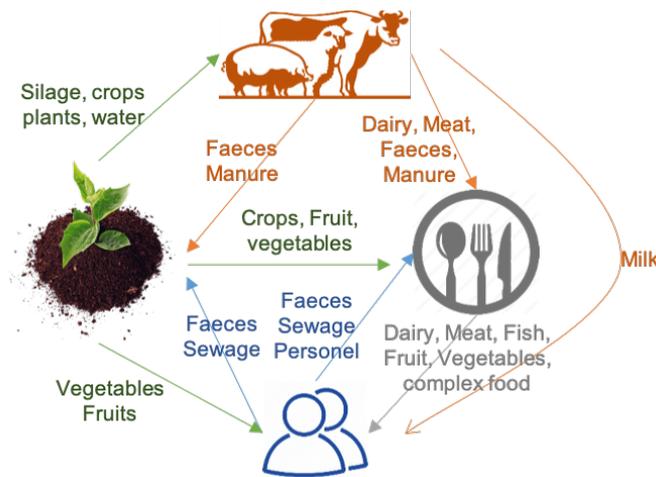


Figure 2 - Potential transmission pathways for *L. monocytogenes* that allow it to reach humans. Adapted from (Walland J. et al., 2015).

The risk groups more affected by listeriosis are well defined, such as pregnant women, newborns, elderly, immunocompromised patients and patients with transplants and diabetes (Hernandez-Milian and Payeras-Cifre, 2014). However, healthy individuals can also suffer from this invasive illness, in this case the major factor is the ingestion dose of contaminated food (Hernandez-Milian and Payeras-Cifre, 2014). In general, the number of listeriosis cases are higher in people over 65 years old and in newborns. The most common clinical form of invasive *L. monocytogenes* is bloodstream infection, followed by meningitis (Antonia Ricci et al., 2018). The incubation time is very dependent on the strain and patient but it can range from 1 to 70 days (Swaminathan and Gerner-Smidt, 2007). The longer incubation periods were found to be in pregnancy-related cases and the shortest ones in bacteraemia and central nervous system cases.

The annual incidence of listeriosis is estimated in between 0.1 and 1 case per 100 thousand people but an increase amount of sporadic cases and outbreaks has been registered over the years (Hernandez-Milian and Payeras-Cifre, 2014). In Europe, period reports since 2003 have registered this same progressive increase in *Listeria* infections (Allerberger and Wagner, 2010; ECDC, 2015; Antonia Ricci et al., 2018). This invasive illness happens mostly due to contaminated foods related outbreaks or sporadic cases. The number of outbreaks per year is low, around five worldwide, however some of the sporadic listeriosis registered cases might be in fact connected to outbreaks to which casualty could not be confirmed (Ferreira et al., 2014). Figure 3 shows the number of human invasive listeriosis cases reported annually in Europe

connected with food outbreaks, as well as the daunting number of deaths resulted from these cases.

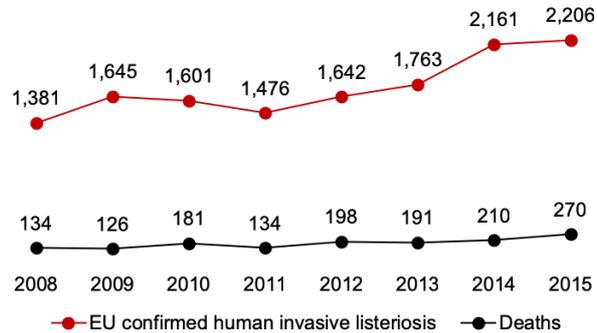


Figure 3 - Graphic representing the number cases and connected fatalities of confirmed human invasive listeriosis reported in Europe resulted from outbreaks of food contaminated with *L. monocytogenes* in the period from 2008 to 2015. Data from (Antonia Ricci et al., 2018).

A decade long danish report overviewing this illness in Denmark reported a case-fatality rate of 21% and the majority of patients presented a bloodstream infection (Gerner-Smidt et al., 2005). As in other regions of the world, in the US listeriosis is also considered rare. Though, it is the third cause of death by a foodborne pathogen. A two year clinical report identified 1651 cases of listeriosis with a 17.6% mortality rate (Hernandez-Milian and Payeras-Cifre, 2014). Invasive listeriosis leads to admission in intensive-care units, which makes *Listeria* the third most costly foodborne pathogen in the US, with a projected annual cost between 2 and 22 billion dollars (de Noordhout et al., 2014).

1.3.3 Human listeriosis and food products

Since the large majority of human listeriosis cases are linked to the consumption of foods contaminated with this pathogen (Schlech et al., 1983), throughout the years diverse food sources have been linked with listeriosis outbreaks. A particular type of foods that causes great concern are the ready-to-eat (RTE) foods, which do not go through any heat-treatment and are sold directly to the consumer, particularly delicatessen meats have one of the highest per serving risk of listeriosis. In 1992, a large listeriosis outbreak with 279 registered cases was reported in France due to cross-contamination of RTE food products (Ferreira et al., 2014). In the US, delicatessen turkey meat contaminated with *L. monocytogenes* resulted in an outbreak with 29 patients and 4 fatalities (Olsen et al., 2005). The relevance of this outbreak is the food facility source of contamination, which had been the harbour of a

different outbreak 12 years before. This *Listeria* persistence in food plants is a public health risk and has a heavy financial costs, in the previous mentioned outbreak it lead to the recall of more than seven thousand tons of processed turkey and chicken meat (Wenger et al., 1990). Case in point, it is estimated that a single recall of a food product contaminated with *Listeria* results in losses up to a quarter of a million euros (Ivanek et al., 2004). Table 2 lists some of the food related outbreaks of listeriosis reported around the globe. The clear public health burden and economic cost of listeriosis has prompt researchers to explore the virulence mechanisms and adaptation strategies to different conditions that makes *L. monocytogenes* an all-around pathogen.

Table 2 - List of some listeriosis outbreaks with source in foods contaminated with *L. monocytogenes*.

Country	Year	Food source	N.° of cases	Death toll (%)	Reference
Switzerland	1983 - 1987	Soft cheese	112	31 (27.7)	(Nocera et al., 1990)
Finland	1998 - 1999	Dairy butter	25	6 (24)	(Mikkola et al., 2000)
Switzerland	2005	Tomme cheese	10	3 (30)	(Bille et al., 2006)
Germany	2006 – 2007	Cheese	187	26 (13.9)	(Koch et al., 2010)
Canada	2008	Deli meat	57	24 (42.1)	(Andrea et al., 2015)
Denmark	2009	Beef meat	7	2 (28.6)	(Smith et al., 2011)
Austria - Germany	2009	Cheese	14	4 (28,6)	(Fretz et al., 2010)
Portugal	2009 - 2012	Cheese	30	11 (36.7)	(Magalhães et al., 2015)
US	2010	Diced celery	10	5 (50)	(Kingsley et al., 2012)
Switzerland	2011	Cooked ham	9	-	(Hächler et al., 2013)
US	2011	Cantaloupe	147	33 (22.4)	(McCollum et al., 2013)
US	2012	Cheese	22	4 (18.2)	(Heiman et al., 2015)
Denmark	2013 - 2015	Smoked fish	20	7 (35)	(Gillesberg Lassen et al., 2016)
Denmark	2014	Delicatessen meat	41	17 (41.5)	(Mølbak et al., 2016)
Denmark	2015	Smoked salmon	6	1 (16.7)	(Schjørring et al., 2017)
US	2015	Ice cream	4	-	(Pouillot et al., 2016)

1.4 *Listeria monocytogenes*, a unique model in infection biology

During the last three decades, the study around the facultative intracellular pathogen *L. monocytogenes* lead to paramount knowledge regarding infection, immunity and host cell biology (Schlech et al., 1983; Dramsi et al., 1993; Cossart, 2002; Johansson et al., 2002; Greene and Freitag, 2003; Desvaux and Hébraud, 2006; Cossart and Toledo-Arana, 2008; Carvalho et al., 2014).

Current infectious process knowledge was obtained through research in humans and various animal models, mainly the mouse model (Cossart and Toledo-Arana, 2008). These reports showed that during infection *L. monocytogenes* main site of entry in the organism is the intestine and that the induction of the T-cell response does not have an impact in the recovery from the infection or defences against a second moment of infection (Dramsi et al., 1996). Mackaness showed that *Listeria* is capable of surviving and replicating in murine macrophages (Mackaness, 1962), but other relevant factors for this bacterial infection have been found since then. In particular, *Listeria's* capacity to survive in the cytosol avoiding both innate and adaptive immune responses makes it a proficient invasive pathogen (Cossart, 2002).

The infection into mammalian cell can be envisioned in three stages: internalization, where internalins (InIA and InIB) help the entry process; evading the vacuole and surviving the cytosol through the pore-forming toxin listeriolysin (LLO) and the lecithinase (PlcA); and cell to cell spreading through the actin-based motility and lysis of the second vacuole, ensured by a second lecithinase (PlcB) and also by LLO (Cossart and Toledo-Arana, 2008).

1.4.1 *PrfA*, the virulence gene locus

The transcription factor *prfA* is the major regulator of virulence factors (Figure 4), comprising some of the agents in the virulence mechanism agents such as InIA and InLB, LLO, phospholipases PlcA and PlcB, Mpl protease, sugar phosphate permease, ActA, and InIC (Cossart, 2002), which will be further discussed. This virulence pleiotropic regulator is encoded on a 10 kb pathogenicity island and it is a protein similar to protein CAP, the cAMP receptor protein of *Escherichia coli* (Ireton, 2007). The transcriptional factor *prfA* has a role in overall regulation of virulence genes for the transition from extracellular to intracellular pathogen and also in extracellular biofilm development (Lemon et al., 2010).

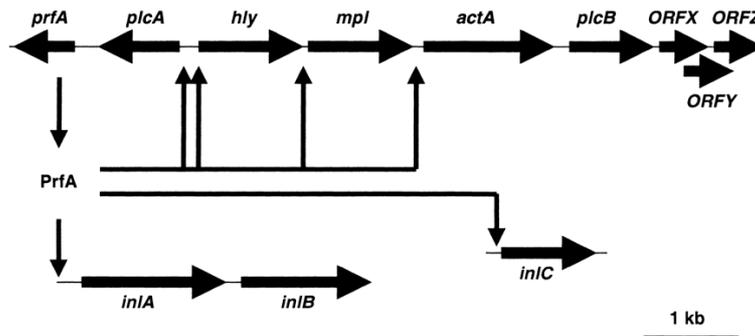


Figure 4 - The virulence gene locus. From (Cossart, 2002).

1.4.2 Internalization

L. monocytogenes is able to invade and proliferate within macrophages and epithelial nonphagocytic cells. When in contact with the host target tissues, internalization results from the tight apposition of the plasma membrane that surrounds the incoming microbe (Figure 5).

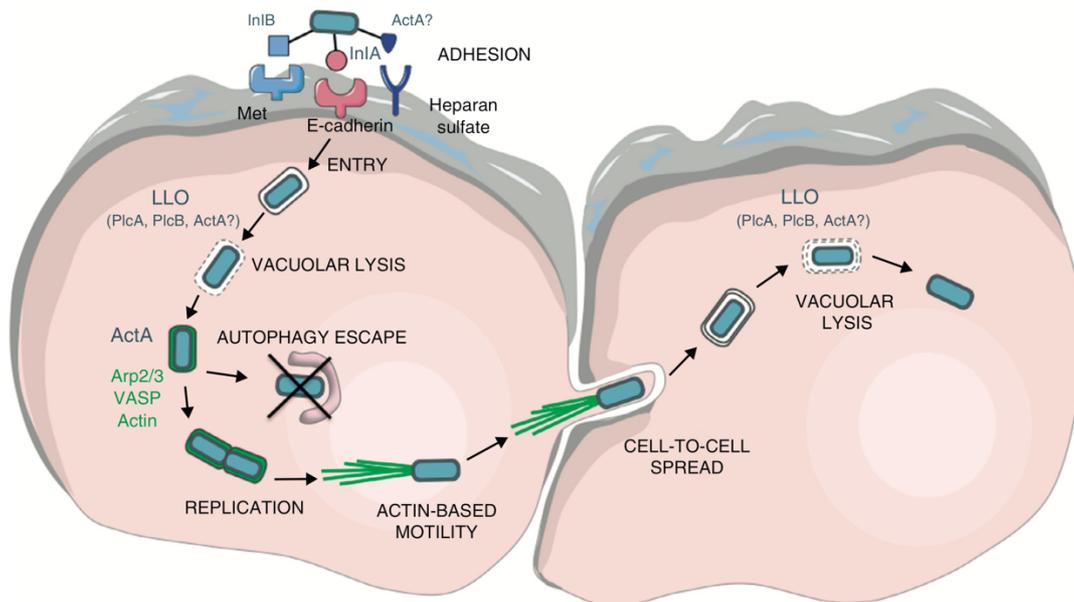


Figure 5 - *In vitro* cell cycle of *L. monocytogenes*. It binds to epithelial host cells through InlA–E-cadherin, InlB– Met and ActA–heparan sulfate interactions and it promotes its entry into the cell. It escapes the vacuole by LLO, together with PlcA and PlcB and possibly ActA. Production of ActA triggers the recruitment of Arp2/3, VASP and actin and allows autophagy escape and *Listeria* replication. ActA induces actin polymerization that will propel the bacteria within the cytosol and within membrane protrusions into neighbouring cells. The lysis of the second vacuole finish the cell-to-cell spread and it starts the new infection cycle. From (Travier and Lecuit, 2014).

This mechanism is referenced as the “zipper mechanism”, since it requires a progressive interaction between the bacterial surface ligands and their counterpart cellular receptors (Cossart and Toledo-Arana, 2008). This *L. monocytogenes* infection stage has been extensively characterized and it is mainly based on the activity of two internalins. InIA and InIB are two surface proteins members of a family of 25 internalins that bind to eukaryotic host cell ligands. InIA has an N-terminal leucine-rich repeat (LRR) that binds with the human host E-cadherin. As for InIB, it cooperates with InIA and allows the entry into non-polarized epithelial cells by binding to Met, the receptor of the hepatocyte growth factor (HGF). These surface proteins are the major mediators of several virulence related functions and are either anchored to the cell wall, associated with the cell wall or secreted (Cossart and Helenius, 2014; Bigot et al., 2005; Bierne et al., 2007).

1.4.3 Vacuolar stage and life in the cytosol

L. monocytogenes interaction with the host cell receptors results in the engulfment and internalization of the bacteria in a membrane-bound vacuole (Figure 5). In around 30 minutes the entrapped bacteria manages to escape the vacuole (Cossart and Toledo-Arana, 2008). This escape is mediated by the cholesterol-dependent pore-forming toxin LLO, in conjunction with two phospholipases (PlcA and PlcB). LLO toxin induces haemolysis and it is encoded by the *hly* gene. PlcA and PlcB also have a role in the evasion of *Listeria* from primary vacuoles and from secondary vacuoles during bacteria cell-to-cell spread (Pizarro-Cerda and Cossart, 2018).

Free bacterial cells in the cytosol replicate with a division time close to that in rich broth medium and it also starts inducing the recruitment and polymerisation of cellular actin. This mechanism produces enough energy to propel *Listeria* in the cytoplasm at a speed around 10 mm per minute. In this way, *Listeria* escapes intracellular autophagy and extracellular humoral immune responses (Cossart, 2002; Kortebi et al., 2017).

1.4.4 Bacteria cell-to-cell spread

When free in the plasma membrane, *Listeria* pushes the membrane inducing the formation of protrusions which allow the invasion to nearby cells. These protrusions lead to a two-membrane vacuole that the bacteria will eventually lyse to replicate in the cytosol of the second cell (Figure 5). As mention before, this vacuole rupture and

escape is mediated by LLO, PlcA and PlcB. All these crucial steps in *L. monocytogenes* pathogenesis allow it to disseminate in various infected tissues while escaping from host defences (Cossart, 2002; Cossart and Toledo-Arana, 2008; Radoshevich and Cossart, 2018).

1.5 Biofilms

Biofilms are the predominant growth mode and survival mechanism undertaken by microorganisms in most man-made and natural environments (Khemiri et al., 2016). The sessile mode of growth is defined as communities of microorganisms attached to a surface which is surrounded by an extracellular matrix that embeds these microorganisms. This is a natural and frequent phenomenon that happens whenever there is a close proximity between microorganisms and suitable surfaces (Kostaki et al., 2012).

Even if biofilms can be formed by a single bacterial species, particularly in most laboratory conditions (Khemiri et al., 2016), monospecies biofilms are uncommon. Most of the times, biofilms are found in different environments as complex multi-species communities (Steinberger and Holden, 2005; Bogino et al., 2013; Korsak and Szuplewska, 2016). In this complex fabric of different bacterial species and matrix, the metabolic products of one species can be shared with others and the attachment to surfaces is reinforced. However, on the negative side there is bacterial competition for space and nutrients, and over accumulation of toxic products (da Silva Fernandes et al., 2015). Nevertheless, multiple advantages arise for bacteria living within a biofilm. The main one is the increased protection against harsh conditions, such as antimicrobial treatments and fluctuations in physical conditions (e.g. temperature and pH). Within a biofilm, bacteria cells have a better chance to survive the host defence responses and acquire advantageous genetic traits by horizontal gene transfer, such as antibiotic resistance genes (Costerton et al., 1999).

1.5.1 Biofilm stages

Bacterial biofilm development can be divided in four main stages: (i) attachment to surface, (ii) sessile growth, (iii) colonization, and (iv) dispersal (Figure 6). The formation of a biofilm starts with the attachment of planktonic cells to a surface through low energy interactions (electrostatic and Van der Waals forces, steric and

hydrophobic interactions). In this initial step, non-specific bacterial-surface binding occurs just after seconds the first contact with the surface (Takhistov and George, 2004). This first cell adhesions are scarce, but overtime evolves to the formation of micro colonies.

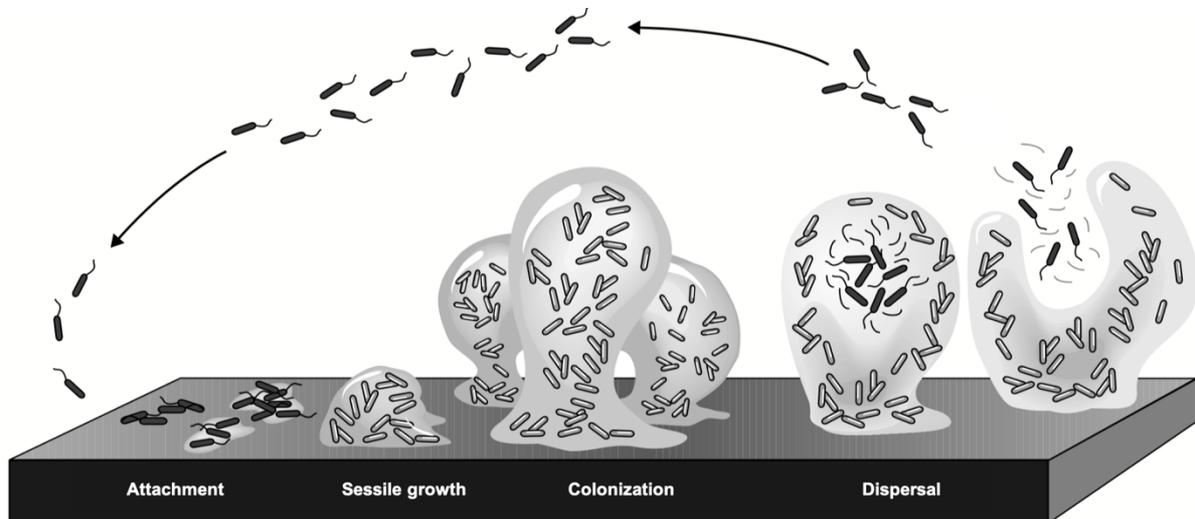


Figure 6 - Stages of biofilm formation. Initial surface attachment, followed by production of extracellular polymeric substance and adhesins leading to irreversible surface attachment. Then, formation of bacterial microcolonies and colonization of empty areas during biofilm maturation. Finally, bacterial dispersion from the biofilm. Adapted from (Sauer, 2003).

This first stage is composed by both reversible and irreversible bound cells (Costerton et al., 1999). In this stage, bacteria can aggregate loosely or dissociate and go back to planktonic state. The presence of adhesins and bacterial appendages (pili, fimbriae, flagella) help bacteria to sustain the electrostatic interactions that happen with the surface (Park et al., 2011). The second stage, sessile growth is marked by the formation and growth of micro colonies from the irreversibly attached cells. These initial cells and aggregates start producing extracellular polymeric substances (EPS) that will form the matrix. In conjunction with the colonization stage it marks the development of the biofilm. In the following step, microcolonies start growing towards areas with higher nutrient availability and fewer cells. The resulting bacterial web is composed of a higher number and bigger size of cells with intercolony bridges (Hanna and Wang, 2006). However, with time and depletion of nutrients, the inner cells enter in static growth and the outer layers keep a stable growth. This results in the decrease of EPS that maintains the cohesiveness of the aggregates. In the beginning of the dispersal stage, it becomes easier for cells to detach and disseminate as a single or

clustered cells. Bacterial cells in the inner core of the biofilm lyse prior to the dispersal of the outer layer cells. This provides nutrients for the bacteria in dispersion. In this orchestrated mechanism, the cells in dispersion also change their metabolism shutting down genes encoding for exopolysaccharides and fimbriae, and upregulating chemotactic proteins and flagella that will be required to enter in the planktonic mode (Kumar et al., 2017).

1.5.2 Planktonic vs sessile mode of growth

One of the aims of this work thesis is to make a protein abundance comparison between planktonic and sessile cells. Case in point, significant changes in the cellular physiology between planktonic and biofilm cells have been reported in different microorganisms (Figure 7).

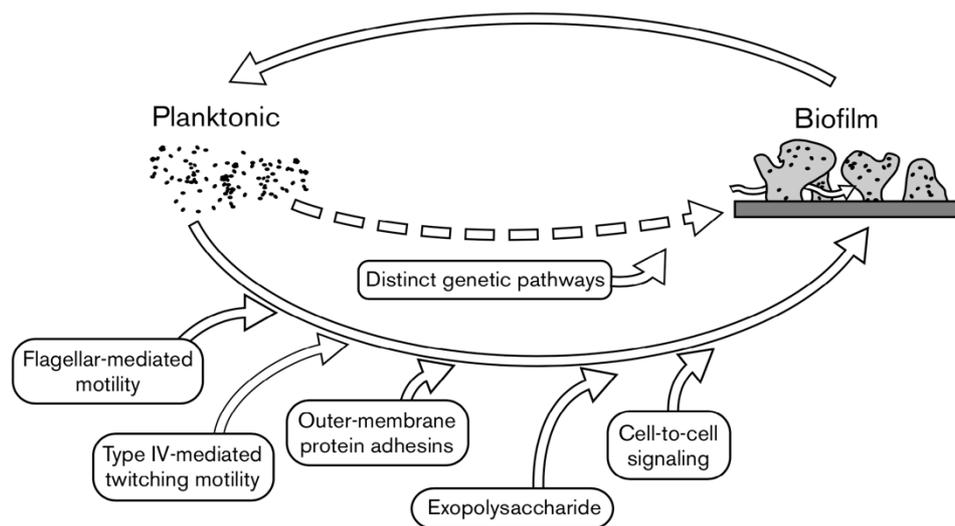


Figure 7 - Diagram of genetic components relevant for the transition between planktonic and biofilm mode of growth. From (Pratt and Kolter, 1999).

The transition between the two growth modes results from a shift of cell genes and abundance of proteins, such as initiation factors and other cascade proteins (Qayyum et al., 2016). Genes linked with quorum sensing, iron acquisition and motility were upregulated in *Acinetobacter baumannii* sessile cells (Rumbo-Feal et al., 2013). Increased expression of genes connected with production of extracellular matrix were found in *Pseudomonas aeruginosa* biofilms (Dotsch et al., 2012). Compared to planktonic growth mode, cells in a biofilm have an increased production of

recombinant proteins and other variations in gene expression were also observed in relation to different stages of biofilm development (Lim et al., 2017). Through the multiple reports comparing planktonic *versus* biofilm state, there is still a research gap reflecting all the shifts that happen between these two growth modes, particularly big data sets of differentially expressed proteins (Rani and Babu, 2018).

1.5.3 Biofilm matrix and flagella

The biofilm extracellular matrix that surrounds bacteria is mainly composed of water and extracellular polymeric substances (EPS: exopolysaccharides, proteins and extracellular DNA). All these molecules play a structure-stabilizing and protective role to bacterial cells in a biofilm (Sutherland, 2001a). EPS effects biofilm shape and size, determines resistance to shear forces and resistance against stress conditions and antimicrobial treatments (Kumar et al., 2017). The overall abundance and composition of the matrix can vary significantly depending on the bacterial species. In the majority of biofilm-forming bacteria, exopolysaccharides are the most abundant and important components of the biofilm (Limoli et al., 2015). Extracellular DNA (eDNA), besides the shared structural role with the other EPS components, it serves as an energy and nutrition source (Colagiorgi et al., 2017). In regards to the biofilm-associated proteins (Bap), they are surface proteins of high molecular weight that support biofilm formation and as referenced before, they also have a role in infectious processes (Lasa and Penadés, 2006). *L. monocytogenes* EPS are mainly constituted by proteins while the major polysaccharides are teichoic acids (Brauge et al., 2015), the latter are essential in biofilm development and structural stability (Combrouse et al., 2013).

Flagella are crucial for biofilm formation in multiple bacterial species. The flagellum in *L. monocytogenes* is comprised of thousands of flagellin monomers encoded by the *flaA* gene. This gene is regulated by temperature and *Listeria* has up to six peritrichous flagella per cell (Griffin and Robbins, 1944). The role of flagella in *Listeria* biofilm is controversial, particularly due to the different growth experiments (static or flow cell mode of growth) and use of different temperatures. Some reports observed that its presence is relevant in early stages of *L. monocytogenes* adherence, particularly surface attachment and subsequent biofilm formation (Lemon et al., 2007). Other reports found that motility does not reveal a positive correlation with biofilm formation

(Di Bonaventura et al., 2008), or that motility is not critical for biofilm development on flat surfaces but might be required on “hanging” surfaces (Piercey et al., 2016).

1.5.4 Biofilms in the food processing environment

Biofilms in the food industry are a major source of concern in regards to food safety. Bacteria that are able to attach to surfaces in food processing plants and form biofilms can lead to food spoilage and the spread of bacterial pathogens (Ferreira et al., 2014). A strain that efficiently colonizes a surface in a sessile mode of growth is up to one thousand times more tolerant to disinfection procedures than their free-living cells counterparts (Dubois-Brissonnet et al., 2016). Furthermore, some of these strains can be repeatedly isolated from the same processing plant over a period up to years, also known as persistent strains. *L. monocytogenes* biofilm formation is considered one of the main reasons for such persistence (Kathariou, 2002). Bacterial biofilms have a heavy cost in public health but also in the world economy that affects industries, the health sector, agriculture and water quality (Khemiri et al., 2016).

1.5.5 *Listeria monocytogenes* biofilm

L. monocytogenes biofilms are composed of two homogeneous layers of cells or microcolonies. Figure 8A shows the overall described shape of this honeycomb biofilm instead of a more frequent mushroom shape architecture as in other biofilm-forming bacteria (e.g. *Pseudomonas aeruginosa*). *Listeria's* biofilm upper and lower layers contain more than 10^5 cells/cm² with low amount of EPS (Hanna and Wang, 2006; da Silva Fernandes et al., 2015). Confocal laser scanning microscopy images showed that this honeycomb structure of scattered colonies is composed of hollow voids of 5 to 50 μ m (Guilbaud et al., 2015) (Figure 8B).

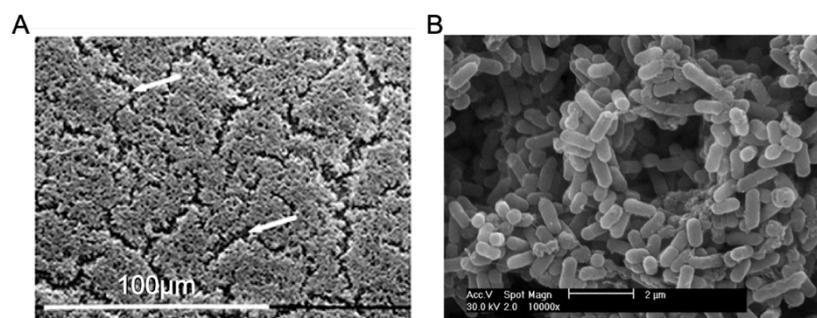


Figure 8 - A: Scanning electron microscopy observations of *L. monocytogenes* biofilm formation after 7 days. From (Renier et al., 2011). **B:** The “honeycomb” biofilm structure by *L. monocytogenes*. From (Hanna and Wang, 2006).

This biofilm architecture is shared with other species, such as *Staphylococcus aureus* (Bridier et al., 2010).

In terms of biofilm forming proficiency and lineages, some studies found no putative correlation between the ability to form biofilms and lineage (Renier et al., 2011). Other reports found that strains of lineage I have a tendency to produce less biofilm than lineage II strains (Combrouse et al., 2013). The common ground in *L. monocytogenes* biofilm formation is that it protects *Listeria* from harsh conditions and increases its resistance to disinfectants and antimicrobials (Chavant et al., 2004). Temperature wise, *Listeria* can develop biofilms at low temperatures, but as expected at 37 °C it produces a significantly denser biofilm (Chavant et al., 2002). At low temperatures, there are fewer cells and low EPS production (Di Bonaventura et al., 2008). *L. monocytogenes* capacity to produce biofilms in cold settings used during food processing environment increases the probability of food product contamination which will be further reviewed in the following Chapter 2.

1.6 *Listeria monocytogenes* proteome

The circular genome of *L. monocytogenes* EGDe strain (serogroup 1/2a) is 2,944,528 base pairs long and predicted to have a total of 2853 open reading frames, including 133 surface proteins (Glaser et al., 2001; Cabanes et al., 2002). This genome has a high level of synteny with *Bacillus subtilis* genome and it has a large portion connected to transcription regulation pathways, with 209 transcriptional regulators (Glaser et al., 2001). Taking into account *Listeria*'s pathogenicity and adaptation capacity, there have been multiple studies exploring the proteome of this genus.

Particularly, proteomic studies focusing on the comparison of different strains, aiming to determine the proteomic basis of the more virulent strains. Donaldson et al. compared the proteome of the EGDe strain (serogroup 1/2a) with the outbreak strain F2365 (serotype 4b) and observed that 413 proteins had significant difference in abundance, including proteins with roles in cell wall maintenance, flagellar biosynthesis, DNA repair, and stress responses (Donaldson et al., 2009). On the other hand, Dumas et al., while comparing 12 strains of *L. monocytogenes* (serotypes 1/2a, 1/2b and 4b), shown that no correlation could be established between protein abundances patterns and the levels of virulence. However, a correlation was found between the protein expression patterns and the serotypes (Dumas et al., 2008), and

later on revealed a connection between the subproteomic data and the origin of serotype 4b (Dumas et al., 2009). Still on the matter of virulence, a different proteomic study identified 43 proteins only present in *L. monocytogenes* EGDe and not in the non-pathogenic *L. innocua*. Furthermore, 16 proteins in *L. monocytogenes* had no orthologues in *L. innocua*, including nine virulence factors (PlcA, LLO, Mpl, ActA, PlcB, InlA, InlB, InlC, and InlH) (Trost et al., 2005). This reveals that surface proteins have a major role in the particular virulence of *L. monocytogenes*, since most of the proteins mentioned before are cell surface or cell wall metabolism associated proteins with a crucial role in the different stages of mammalian cell invasion.

1.6.1 Cell wall and membrane

L. monocytogenes membrane is constituted by 55 to 60% of proteins, 30 to 35% of lipids, and 1.3 to 2.3% of carbohydrates (Ghosh and Carroll, 1968). Lipid composition is mainly of phospholipids. The membrane fatty acid is composed by branched-chain fatty acids which can vary depending on the temperature to maintain optimal membrane fluidity (Phadtare et al., 1999; Cacace et al., 2010) as it will be further discussed in Chapter 2. The teichoic acids (TAs) covalently bound to the peptidoglycan, and the lipoteichoic acids (LTAs) form the *L. monocytogenes* cell wall. These components have a crucial cellular importance since they are involved in the anchoring of surface proteins and transport of molecules (Bierne and Cossart, 2007).

1.6.2 Secretome

The secretome is a concept that contains the secretion machineries and their secreted proteins. The extracellular proteome (exoproteome) is a subset of proteins present in the extracellular milieu that are also part of the secretome (Bierne and Cossart, 2007). The first point of contact between the environment and the bacterial cell is through its surface. Such communication is predominantly achieved through proteins that are exposed to some extent in the outside of the microorganism. These include secreted proteins and surface-attached proteins (Olaya-Abril et al., 2014). However, it is relevant to take into account that first, the cell wall is not an impermeable barrier and so proteins in the cell envelope can cooperate with the outside environment without having a domain external to the cell wall. Secondly, the putative protein localization in the cell envelope as a cell surface exposed *stricto sensu* can sometimes be falsely

attributed due to other overlying components (Desvaux and Hébraud, 2008). A secreted protein can be in the outside of the cell as a soluble protein, a surface-associated protein or a subunit part of a surface appendage. In the extracellular milieu, it is also possible to find proteins expelled from lysed cells which cannot be considered as secreted proteins. Therefore, the exoproteome comprises all the proteins found in its culture supernatant (Cabrita et al., 2014).

1.6.2.1 Secretion systems

Figure 9 illustrates the six secretion systems, annotated in *L. monocytogenes*, that prompt proteins to pass through the membrane (Desvaux and Hébraud, 2006). Secretion is primarily dependent on the sec pathway, which is predicted to secret 96% of the proteins (Bierne and Cossart, 2007). The putative proteins secreted by this system are associated with cell wall metabolism, adhesion and biofilm formation (Renier et al., 2013). The other secretion systems are: Tat (twin-arginine translocation), FEA (flagellar export apparatus), FPE (fimbriillin-protein exporter), Holins, and Wss (WXG100 secretion system) (Desvaux and Hébraud, 2006). For secreted proteins to be placed in the cell surface they need to translocate the membrane through one of these secretion systems and then connect with a cell surface component. Protein displacement on the cell surface is dependent on specific domains or motifs that meddle secretion and attachment to the cell envelope. Sec, Tat and FPE pathways of secretion require a specific N-terminal secretion signal peptide (Bierne and Cossart, 2007). Whereas, proteins lacking a signal peptide are translocated via FEA, Holins and WSS secretion systems. Cytoplasmic membrane translocators target signal peptides which are generally cleaved by signal peptidases during the course of translocation. In the major Sec pathway two groups of signal peptidases (Spases) have been described: three Spases of type I (SipX, SipY and SipZ) and two of type II (LspA and LspB), the latter favour the cleavage of lipoproteins (Desvaux and Hébraud, 2006).

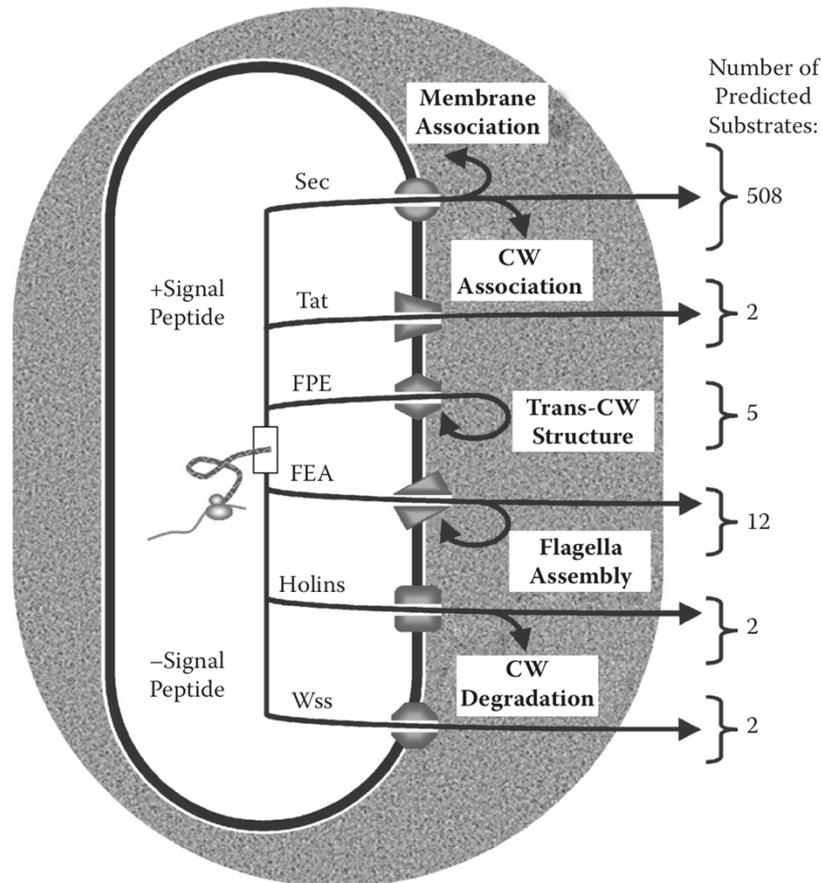


Figure 9 - Schema of the protein secretion pathways in *L. monocytogenes* EGDe. Proteins exported via the Sec pathway either stay membrane associated or cell wall associated, or are released into the extracellular milieu, or would even be injected into a eukaryotic host cell. Proteins secreted via Tat would most certainly be cell surface or released into the extracellular milieu. FPE would be involved in the formation of trans cell wall structures. FEA is involved in flagella assembly. Proteins translocated by holins seem secreted into the extracellular milieu or involved in cell wall degradation. WSS proteins would be secreted into the extracellular milieu. From (Desvaux and Hebraud, 2008).

1.6.2.2 Surfaceome and extracellular proteins

The surfaceome comprises surface proteins involved in multiple biological processes, such as host invasion, responses to different harsh conditions and bacterial growth (Bierne and Cossart, 2007). Therefore, surface proteins have a wide range of molecular functions: some can have a role as transporters of nutrients or ions; be receptors for molecules; be binding factors to surfaces or other cells; be degradation mechanisms for molecules and have enzymatic activities (e.g. sortase mediated anchoring of proteins to the surface of the cell) (Olaya-Abril et al., 2014). In pathogenic bacteria, as described before, surface proteins also have a role in adhesion, invasion and general virulence. Since they are exposed to the exterior of the cell, surface

proteins are as well the first interacting molecules with the host immune response, thus being recognized as antigens. Meaning that surface proteins are good drug targets for vaccine development (Grandi, 2006).

L. monocytogenes EGDe has a total of 133 surface proteins, including 41 LPXTG (Leu-Pro-any-Thr-Gly) proteins, nine GW proteins with a signal peptide, 11 hydrophobic tail proteins, four P60-like proteins and 68 lipoproteins (Figure 10).

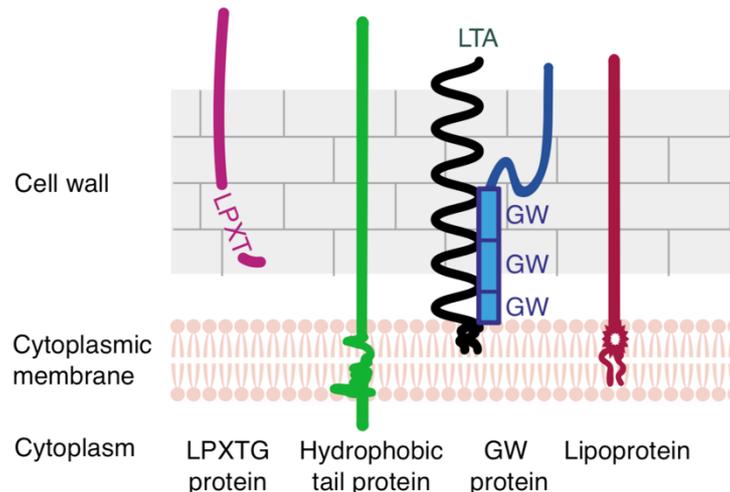


Figure 10 - The major types of surface proteins found in *L. monocytogenes*: proteins that possess an LPXTG carboxy-terminal sorting signal; proteins anchored by a hydrophobic tail motif; proteins containing GW modules; and lipoproteins. From (Cabanés et al., 2002).

LPXTG motif proteins are the most explored class of surface proteins and they have been identified in more than one hundred bacterial species (Davies et al., 2009). In particular, *Listeria* has the highest number of this class of proteins in comparison to the other bacterial genomes. LPXTG motifs have a short C-terminal sorting signal that attaches to the peptidoglycan. The signal is comprised by conserved LPXTG sequence motif (where X equals any amino acid), which is succeeded by a hydrophobic domain of around 20 amino acids and a tail of positively charged amino acids (Cabanés et al., 2002). The polypeptide is retained by the sorting signal in the cell membrane. Via membrane sortases, sortase A and B are described in *L. monocytogenes*, the LPXTG motif is cleaved between the threonine and glycine residues and direct linkage of the carboxyl group of the threonine residue to the meso-diaminopimelic acid of the cell wall peptide precursors (Pucciarelli et al., 2005).

Some examples of *L. monocytogenes* surface proteins are: the major invasive internalins, the LPXTG protein InlA and the GW protein InlB, that promote the entry into mammalian cells (Bierne et al., 2007); the virulence factor actA for intracellular movement (Travier and Lecuit, 2014); and multiple transporter systems such as OppA, which mediates the transport of oligopeptides with a role in growth at low temperature and intracellular survival in macrophages (Angelidis and Smith, 2003). The abundance and diversity of *Listeria* surface proteins are also connected to its proficient capacity to adapt to diverse harsh environmental conditions.

Chapter 2 – *Listeria* in the food industry and its adaptation to different environments

2.1 Adaptation and virulence of *L. monocytogenes* from farm to fork

L. monocytogenes as a ubiquitous bacterium it is widely spread in the environment. The primary realms are soil and water but farm and livestock are also considered significant reservoirs that contaminate raw material (Linke et al., 2014). But it is the persistence of this invasive foodborne pathogen in the food processing plants that is the main factor of a multitude of human listeriosis food-related outbreaks (Ferreira et al., 2014). Figure 11 illustrates all the food sources contaminated with *L. monocytogenes* that lead to listeriosis outbreaks registered in Europe from 2008 to 2015 (Antonia Ricci et al., 2018).



Figure 11 - Reported human invasive listeriosis food-related outbreaks in EU from 2008 to 2015. Data from (Antonia Ricci et al., 2018).

RTE foods are a particular risk case taking into account its lack of processing and elevated levels of contamination during handling. In Europe, food industries have to show that *L. monocytogenes* levels do not go above 100 CFU/g during the course of the shelf life (Antonia Ricci et al., 2018). As for the US, it is required a total non-existence of *Listeria* from the RTE foods. *Listeria* persistence in the food processing environments is particularly due to cross-contamination events. This is connected with badly designed food processing areas where residues can be accumulated and be a sustainable source for the development of a biofilm such as joints, parts of conveyor belts and cutting machines or slicers (Gandhi and Chikindas, 2007). To note that contamination can also occur in the household environment due to storage over at

long periods of time or at inappropriate refrigeration temperatures. The temperature range in the European food plant environment is from 2.7 to 5.6 °C and storage temperature is considered to be the most important factor influencing the risk of *Listeria* presence in the main food products known to harbour it, such as deli meats, fish products, dairy product and leafy vegetables (Antonia Ricci et al., 2018). *Listeria*'s ability to resist and sustain growth at these temperatures (Bayles et al., 1996), and to adapt to various stress conditions make it a true nightmare for public health and food industry.

A stress condition is an environmental variation which leads to a decrease in growth rate or that can impeach cell survival. The bacterium response to a stress implies a high energy cost in order to maintain homeostasis, to produce macromolecules and to repair or replace damaged components. *Listeria* stress response mechanisms allow it to persist in the natural and the food processing realms, withstanding acidic environments, low water activity, desiccation and low temperature (NicAogain and O'Byrne, 2016).

2.2 Methods for controlling *L. monocytogenes* in the food industry

L. monocytogenes is resilient to the majority of physical treatments employed by the food processing practices. As food products are a nutrient-rich hold for bacteria, its survival going through the processing environment increases the risk of causing infection after human consumption of the contaminated product (Colagiorgi et al., 2017). The survival of the microorganism is also dependent on the type of food and applied processing. As described before, RTE do not suffer a step of heat processing and are therefore the number one risk in potentially causing listeriosis. Reason why strict rules, different disinfection procedures and security guidelines were set in motion in order to reduce at maximum the presence of *Listeria* in the food industry pipeline. The success of the control and monitoring procedures is based in appropriate agricultural practices at the farm step and the hazard analysis and critical control points (HACCP) programme and good hygiene practices (GHP) at the processing and retail steps (Antonia Ricci et al., 2018).

Food preservation approaches include mainly the control of physical conditions that inhibit somewhat microbial development, such as reduced water activity, acidic pH and low temperature environments (NicAogain and O'Byrne, 2016). However, there

are other disinfection approaches already established and other innovative ones that seem promising. The well proven use of sanitizers such as quaternary ammonium compounds (QACs), hydrogen peroxide, peracetic acid and sodium hypochlorite are effective in eliminating *L. monocytogenes* cells from food plants (Kastbjerg and Gram, 2012). Likewise, the use of light and antibacterial peptides, such as nisin, have been proven effective in preventing the growth of *L. monocytogenes* (Ruiz et al., 2009; Endarko et al., 2012). Multiple innovative biocontrol methods to limit the growth of *L. monocytogenes* in food surfaces are also under development. It is worth mentioning the use of endolysins, hydrolytic enzymes produced by bacteriophages that can break the cell wall of Gram-positive bacteria leading to cell lysis. Other approaches such the use of bacteriocin proteins and natural essential oils are being developed for biopreservation and extending the food product shelf life (Gray et al., 2018). It is reasonable to contemplate in the future a target approach that would encumber the *Listeria* regulators for stress tolerance (σ^B) and virulence (PrfA) (NicAogain and O'Byrne, 2016).

2.3 The stressosome, σ^B and GSR

L. monocytogenes response to the ever-changing environmental factors and stress conditions is linked to the activation of the alternative sigma factor sigma B (σ^B) that controls the general stress response (GSR). A cascade of reactions that starts with the sensing of the stress until transcription of vital stress resistance genes leads to the maintenance of cellular homeostasis (NicAogain and O'Byrne, 2016). In *L. monocytogenes* EGDe, 7.6% of the genome is under the regulation of σ^B (Hain et al., 2008). Functions of genes regulated by this transcriptional factor include a diversity of metabolic pathways, transport associated proteins, stress proteins and other transcriptional factors (Cossart, 2002). σ^B also seems to have a partial role in virulence by regulation of the virulence transcriptional factor PrfA, controlling the transcription of internalin genes and supporting the *Listeria* survival in the harsh conditions present in the gastrointestinal tract (NicAogain and O'Byrne, 2016). However, σ^B is not a virulence factor, in fact it is a transcriptional factor shared with other non-pathogenic species of the *Listeria* genus (Raengpradub et al., 2008).

Figure 12 represents the *L. monocytogenes* stressosome, a large protein complex with 40 RsbR proteins and 20 copies each of RsbS and RsbT (Marles-Wright and Lewis,

2010). The centre of this complex is formed by RsbS and RsbT, where the RsbR C-terminal region is rooted and its N-terminal region protruded out into the extracellular milieu (Dorey et al., 2019). In a study to identify translation initiation sites by N-terminomics, it was found that the mini protein Prli42 which is located in the membrane, anchors the stressosome by interacting with the N-terminal domain of RsbR (Impens et al., 2017). The activation of σ^B occurs through a complex chain reaction with several players in action. The connection of anti-sigma factor RsbW with σ^B precludes the transcriptional function of the latter. The main factor of this chain reaction that leads to the expression of σ^B is the phosphorylation state of the anti-anti-sigma factor RsbV. Unphosphorylated RsbV binds to RsbW, releasing σ^B . Upon sensing a stress condition, the cell increases the activities of two phosphatases, RsbU and RsbP, dephosphorylate RsbW leads to the release and induction of the σ^B regulon activity (Dorey et al., 2019).

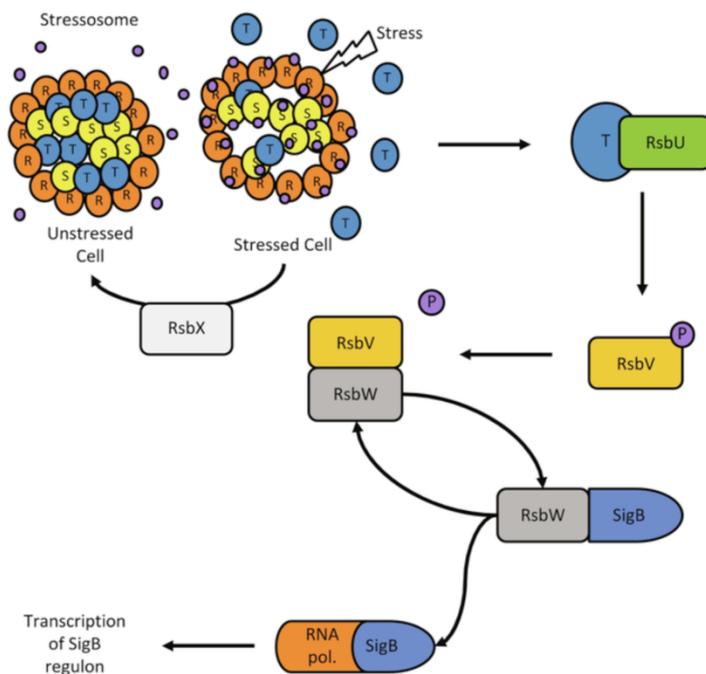


Figure 12 - Model for the *L. monocytogenes* σ^B regulatory mechanism. From (Dorey et al., 2019).

2.4 Adaptation to different conditions: from natural environment to food industry

2.4.1 Desiccation

Water is essential for all living organisms, for animals as well as for plants and microorganisms. For the latter, the presence of water or a humid environment with a high air relative humidity (RH) is necessary for their survival and growth. Thus, variations in the availability of water or in the air relative humidity constitute widespread environmental stresses which challenge microorganisms, and especially bacteria. Indeed, in their environment, bacteria are often faced with conditions that remove cell-bound water through air-drying of the atmosphere. Bacterial cells are subject to daily or seasonal environmental variations, sometimes going through periods of severe desiccation. This is also the case in the food industry, where air dehumidification treatments are applied after the daily cleaning-disinfection procedures. In the scope of this thesis, we published a review duelling with all the aspects of desiccation conditions in microbes, case in point this was the induced stress condition in the first paper of this dissertation (Esbelin et al., 2018a).

2.4.1.1 Context

In terrestrial habitats, bacteria habitually live as multicellular aggregates adhering to biotic or abiotic surfaces and/or to each other (sessile cells) rather than in free suspension in liquid medium (planktonic cells). In this lifestyle, a.k.a. biofilm, cells are often involved in highly complex communities that enable efficient resource use in hostile environments (Bogino et al., 2013; Vogeleeer et al., 2014). Hence, about 99% of the world's bacterial population is seen in the form of biofilms at various stages of growth (Costerton et al., 1987). Water availability is of particular importance in all environmental habitats since bacteria are submitted to daily (including nycthemeral rhythms) and seasonal variations of air relative humidity (RH) due to rain, drought periods or to drainage. Variations in air RH also occur in food processing plants due to cleaning and disinfection procedures followed by air dehumidification.

Microorganisms are settled in a wide range of environments, their genetic and physiological adaptability enables them to withstand numerous harsh and sometimes combined environmental factors (Moissl-Eichinger et al., 2016). This ability to adapt

and persist in harsh environments lies in how cells are able to sense and respond to environmental changes. The switch between planktonic and sessile mode of growth, as well as the adaptation to physicochemical variations surrounding the cells, requires profound physiological changes which occur through the regulation of gene expression in response to various signals (Renier et al., 2011). Furthermore, it has been reported that bacterial cells growing in biofilm have significantly higher survival rate to stress conditions (Giaouris et al., 2014). The biofilms present in food industry plants may be of major concern when containing pathogen and/or spoilage bacteria, since they can produce significant public health and economic consequences (Carpentier and Cerf, 1993). Moreover, bacterial cells in biofilms are embedded in extracellular polymeric substances (EPS) that are able to give protection against stresses (Lequette et al., 2010). In spite of the relevance of biofilm, the physiological and molecular responses of bacteria to desiccation stress remains poorly known. Furthermore, most of the desiccation tolerance data comes from planktonic cell reports, as evidenced by the data summarized in Table 3.

Table 3 - Examples of different bacteria and their responses to fight against desiccation.

Bacteria	Cell state	Mechanism of resistance to desiccation	Reference
Cyanobacteria	Planktonic	Trehalose and sucrose accumulation	(HersHKovitz et al., 1991)
<i>Pseudomonas</i> spp.	Planktonic	Hygroscopic secreted polysaccharides and fatty acid <i>trans</i> -configuration to maintain membrane fluidity	(Roberson and Firestone, 1992)
<i>Escherichia coli</i>	Planktonic	Trehalose synthesis	(Welsh and Herbert, 1999)
<i>Salmonella enteritidis</i> and <i>S. Typhimurium</i>	Planktonic	Filament formations	(Mattick et al., 2000)
<i>Listeria monocytogenes</i>	Planktonic	Osmolytes uptake	(Bayles and Wilkinson, 2000)
Enterobacteriaceae	Planktonic	Production of extracellular cellulose, EPS, fimbriae, changes to membrane permeability	(Ramos et al., 2001)
<i>Chroococcidiopsis</i> spp.	Planktonic	Development of thick multilayered envelopes rich in polysaccharides, lipids and proteins	(Billi and Potts, 2002)
<i>Shewanella baltica</i>	Planktonic	Express of a proteinaceous osmotic shock response	(Leblanc et al., 2003)

Bacteria	Cell state	Mechanism of resistance to desiccation	Reference
<i>Salmonella</i> spp.	Planktonic	Fimbriae and cellulose protection	(Gibson et al., 2006)
<i>Listeria monocytogenes</i>	Biofilm	Increase in extrapolymeric substances (EPS)	(Chae et al., 2006)
<i>Staphylococcus aureus</i>	Planktonic	SigB activity via oxidative stress	(Chaibenjawong and Foster, 2011)
<i>Pseudomonas</i> spp.	Planktonic	Upregulation of alginate synthesis and flagellar genes	(Gulez et al., 2012)
<i>Deinococcus radiodurans</i>	Planktonic	Increased levels of Mn(II)	(Anderson et al., 2015)

2.4.1.2 Water relevance for bacteria

Before analysing the effects of desiccation stress on bacterial cells, it is important to shed light on the different phenomena related to water. Thus, it is first indispensable to outline some definitions.

Water activity (a_w) is the ratio between water vapour pressure in a material (p) and the vapour pressure of pure water (p_0) at the same temperature. A_w is suitable to predict the growth of microorganisms because they can only use "available" water. The a_w value for each bacterium is generally the minimum a_w which can lead to growth. Growth is minimal at the minimum a_w , increasing as a_w increases. At a_w values below the minimum required for growth, bacteria do not necessarily die. Some bacteria, such as *Deinococcus radiodurans* (Blasius et al., 2008) and *Mycobacterium* (Harland et al., 2008), are extremely resistant to prolonged desiccation times while others, such as *Neisseria gonorrhoeae* (Tzeng et al., 2014), can survive only short periods of desiccation.

The relative humidity (RH) of air is the ratio between vapour pressure of air and its vapour pressure saturation. When vapour and temperature levels are stable, the a_w of the sample matches the RH of the air surrounding the sample in a sealed measurement chamber. The percentage of the relative humidity equilibrium (ERH) can be obtained by multiplying a_w by 100.

$$a_w = p/p_0 = \text{ERH} (\%) / 100$$

As described by the above equation, a_w is the ratio between vapour pressures and therefore it has no units. It ranges from 0.0 a_w (bone dry) to 1.0 a_w (pure water).

Microorganisms that are able to grow in low a_w conditions are qualified as xerophilous (Madigan et al., 2006). A xerophile is an organism that can grow and multiply in an

environment with an extremely low water availability. They can often survive in situations with a_w below 0.8, as is the case with arid desert soil environments. Among this class of organisms is *Chloroflexus aurantiacus*, a well-known isolated from hot springs.

Desiccation leads to the exit of water from a body and this phenomenon can be natural or forced. Several mechanisms can be used to dry an atmosphere or a specific biological entity, such as a bacterial cell. Removing air from water by the use of physical means is a way to reach desiccation; drying, usually by exposure to dry air, is a special case of desiccation.

Desiccation tolerance is the ability to undergo nearly absolute dehydration through air drying without being killed (Billi and Potts, 2002). A desiccated cell is characterized by its singular lack of water, with contents as low as 0.02 g of H₂O (dry weight)⁻¹. Low water potential is considered the biggest life threatening abiotic stress and it negatively affects all biological functions (Krisko et al., 2010). Drying is often associated with osmotic stress but in fact, they are two different stresses. In drying air, dried cells are surrounded by an atmosphere, while under osmotic stress they are immersed in an aqueous solution. This review focuses in the main aspects of desiccation and its impact in bacterial communities.

Water is essential for life and it is the most abundant molecule in cells, where it normally comprises $\geq 80\%$ of the total cell mass (Bratbak and Dundas, 1984). The early steps in the evolution of living organisms on Earth occurred in water and its presence is being actively explored on other planets, in order to find extra-terrestrial forms of life. Water is a source of protons, hydrogen and oxygen in photosynthesis and the solvent of biomolecules. It is essential in biochemical reactions, for the structure of macromolecules and molecular mobility (Ball, 2008). Water confers structural order to cells, stabilizes proteins, lipids and nucleic acids and maintains a cellular microenvironment in which vital metabolic systems and chemical reactions occur (Potts, 1994). Microorganisms need water for their growth, but depending on the species, the amount of water to reach multiplication differs. With the decrease of a_w , the microbial metabolism is progressively inhibited until conditions become unsuitable for growth. All the microbial multiplication is stopped when a_w is below 0.60 (Beuchat et al., 2013). Nonetheless, a few organisms can resist nearly absolute dehydration, in a phenomenon known as anhydrobiosis. This ability to withstand extreme dehydration

is found in a number of bacteria, terrestrial microalgae, lichens and yeasts. Anhydrobiosis is not a very common phenomenon and it remains rare in nature, (e.g., no vertebrate exhibits desiccation tolerance) (Dupont et al., 2014).

Water is also an important factor that has a role in the microbial deterioration of foods and in the persistence of microorganisms in food plants. Nutrients and suitable a_w , pH, redox potential, and temperature are required for a growing bacterial community. During the production of dried foods, the control of moisture, and consequently a_w , is key to control microbial growth. Low- a_w foods and food ingredients are either naturally low in moisture or they are produced from high- a_w foods that are purposely dried. Whether in their natural environment or in industrial processes, water environmental disturbances constantly impose significant physical and physiological constraints for microorganisms.

2.4.1.3 Foodborne bacteria in desiccated environments

Each microorganism has a minimum, an optimum, and a maximum a_w level suitable for growth. Yeasts and moulds can grow at a lower a_w than most bacteria, but 0.85 and 0.90 is considered the lowest level for eukaryotic and prokaryotic human pathogen growth, respectively (Houtsma et al., 1993). A a_w of 0.85 is based on the minimum a_w required for *S. aureus* toxin production (Ding et al., 2016). However, the a_w values of fresh foods are generally between 0.95 and 0.99 allowing the growth of all microorganisms, whether it is technological, spoilage or pathogen bacteria (Grant, 2004). Consequently, some foods require careful control of a_w and the two primary ways for that consist on adding salt or sugar to bind the water molecules or dehydrating by freeze-drying or evaporation (Grant, 2004). The addition of a solute, either salt or sugar, is common in soy sauce, jams, fruit syrups, cured ham and salted fish. Additionally, drying is one of the oldest methods of food preservation allowing producers to significantly increase the shelf life of food products such as cereals, chocolate, dried fruits and vegetables, fermented dry sausages, seeds, herbs, spices or powder from different origins (egg, milk, vegetable or meat) (Beuchat et al., 2013). Nevertheless, although these low a_w -foods can prevent the growth of foodborne microorganisms, some bacterial cells including foodborne pathogens are able to survive for long periods in foods and food ingredients with $a_w < 0.85$, as well as in dry processing environments (Takahashi et al., 2011). So, it is important to consider that

few persistent cells of foodborne pathogens can be enough to cause disease (Beuchat et al., 2013). Sometimes, these low a_w -foods may be a vehicle of pathogenic bacteria and cause outbreaks of foodborne illnesses (Koyama et al., 2016). As example, the occurrence of *E. coli* O157:H7 infections was connected with the consumption of reduced- a_w meat products (Hwang et al., 2009), and numerous outbreaks of salmonellosis after consumption of contaminated chocolate (Tamminga et al., 1976). There were reported at least 7,315 cases of bacterial infection and 63 deaths due to consumption of contaminated low a_w -foods worldwide between 2007 and 2012 (Santillana Farakos and Frank, 2014).

However, many foods that cause infections (e.g. listeriosis) are related with RTE food products, and the primary suspected source of contamination is the food-processing environments. If surfaces are not properly cleaned, they remain soiled by food components, which can contribute to bacterial adhesion, survival and growth (Kuda et al., 2015). The Canadian *L. monocytogenes* outbreak in 2008 was traced to slicing equipment contamination from a processing plant. This contamination led to 22 deaths out of 57 reported cases of listeriosis. Even exposed to a multitude of severe environmental conditions, like sanitizers, preservatives, heat, cold or freezing temperatures, drying, and osmotic pressure changes, an enhanced tolerance to any of these stress factors could contribute to persistence of microbes in food processing facilities (Vogel et al., 2010). In combination with other strategies, hyperosmolarity and desiccation are frequently used in food processing industry as a means to prevent bacterial proliferation, and particularly product contamination with foodborne pathogens (Burgess et al., 2016). In fact, air-drying which has been applied in the pharmaceutical industry for a long time is now increasingly applied in food processing plants following the cleaning and disinfection procedures. This operation is part of an overall hygienic setup which aims to dry surfaces, eliminate cold spots and avoid water condensation which are source of microbial growth. The control of drying kinetics and final relative air humidity value reached after the dehumidification process are crucial for improving the hygiene of the surfaces (Zoz et al., 2016; Lang et al., 2017). The most commonly used drying process in these industry consists of air RH control by mechanical cold techniques or water sorption through a desiccant wheel. In food processing workshops, where air dehumidification operations systematically follow

after cleaning disinfection procedures, microorganisms are daily subjected to drastic RH variations.

These RH variations in household environments are also a major concern for food safety. It has been found that Shiga toxin-producing *Escherichia coli* and *Salmonella* may die after 1 to 2 months upon storage at room temperature or higher, however may survive for up to 2 years in a refrigerated setting, pointing to a bigger food safety hazard when dry foods contaminated with bacteria are stored in a refrigerator (Hiramatsu et al., 2005).

The rising number of food-borne bacterial outbreaks over the past decade is also linked to fresh fruits and vegetables, raises concerns over the role of plants in pathogen transmission. Fresh fruits and vegetables are an essential part of a healthy diet. Nevertheless, they have also been documented as a potential vehicle of foodborne pathogens (Poimenidou et al., 2016). In agricultural environment, microorganisms are subjected to harsh conditions that vary widely and rapidly over short periods of time (Hirano and Upper, 2000). The accumulation and production of xeroprotectants (e.g. trehalose) by some microbes and plants enables them to endure in extreme abiotic stresses. Furthermore, Vilchez et al. (2016) confirmed for the first time a link between the desiccation resistance of a microorganism and the protection they can confer to plants against drought.

In all the diverse environments subjected to harsh physico-chemical stresses or conditions, it is established that the sessile mode of growth is an efficient strategy for microorganisms to increase their survival (Giaouris et al., 2014; Azeredo et al., 2016). Over the past few decades, microbial biofilms have been reported in several industrial, domestic and environmental realms in which they constitute the most widespread lifestyle. They form communities, with complex intra- and inter-species interactions, offering better protection against various stresses than planktonic growing cells (Giaouris et al., 2015). Indeed, they are frequently exposed to strong insolation, extreme temperatures, concurrent hyper salinity and periodic desiccation in natural environments, to low temperature, daily cleaning-disinfection procedures and relative humidity variations in food plants. Among these hard environmental conditions, it turns out that desiccation is a widespread and common stress. The effects of water level limitation on bacterial biofilms have been studied in samples from the rhizosphere (Bogino et al., 2013), groundwater aquifers (Weaver et al., 2015) and soil (LeBlanc et

al., 2008). In all these examples, biofilms were able to withstand periods of desiccation and adapt to their surroundings. Thus, harsh environmental conditions contribute to the selection of resistant and biofilm-forming strains within bacterial communities (Selasi et al., 2016). However, despite the predominance of biofilm lifestyle and its particular relevance in food industry regarding the health risks posed by foodborne pathogens, most studies aiming to understand the mechanisms implemented by bacteria to survive and withstand unfavourable and/or stresses conditions concern planktonic cells and/or natural environments as underlined above. The importance and frequency of desiccation stress deserves particular attention in order to understand how bacteria can tolerate and survive it. The following section provides an overview of the molecular mechanisms described in the literature that bacteria are capable of implementing to adapt to desiccation.

2.4.1.4 Cellular mechanisms towards desiccation tolerance

Microbial response of the non-spore forming bacteria involves a regulated molecular response to various conditions (for example osmotic, heat, or cold) that includes a cascade of sigma transcription factors, heat and cold shock proteins, and RNA chaperones (Stone et al., 2016). Bacteria can develop distinct strategies to tolerate desiccation stress; nevertheless, several of them share cellular pathways to survive. Increasing desiccation tolerance of bacteria is also linked to the protective effect of the biofilm matrix, in the case of aggregate or biofilm formation, modifications in cell envelope composition and fluidity and/or shift in metabolism. Nevertheless, the main mechanisms of resistance to desiccation to date concern bacteria in planktonic rather than sessile mode of growth.

1) Import and synthesis of osmolytes

Desiccation stress leads to the exit of water from microorganisms, which ultimately culminates in loss of turgor pressure and an increase of molecular crowding (Minton, 2001). The water replacement hypothesis is a well-known theory for the underlying mechanism to battle cell lysis by importing and synthesizing osmolytes. In this hypothesis, the nonreducing disaccharides sucrose and trehalose are considered to preserve protein structures and thus the membrane function by replacing bacterial membranous water in desiccated conditions (Hiramatsu et al., 2005). Trehalose and

sucrose hydrogen bond to membrane phospholipids replacing the water shell around macromolecules, thus preventing the transition of membranes to gel phase (Potts, 1994; Billi and Potts, 2002).

Aquaporin- and/or aquaglyceroporin like channels such as AqpZ and GlpF, respectively, are part of the first response against desiccation stress, together with the efflux of osmoprotectants to prevent cell lysis (Potts, 2001). The entrance of biomolecules balances the lack of water and the sugar molecules protect the cells through the formation of a vitreous cytoplasmatic matrix (Wolkers et al., 2002). Upon exposure to osmotic stress, bacteria imports potassium ions through Kup, Trk, and Kdp transport systems (Csonka, 1989). Despite its toxicity, potassium is essential for cell turgor pressure recovery (Epstein, 2003). The uptake or synthesis of sugars, free amino acids, polyols, quaternary amines, sulphate esters, inositol phosphates or manosylglyceramides are a common bacterial response to tolerate desiccation (Anderson et al., 2015).

II) Role of proteins

Bacterial membrane proteins are responsible for detecting environmental signals, promoting downstream regulatory events, and numerous metabolic functions. Protein damage induced by osmotic stress can be linked to desiccation resistance and intracellular Mn/Fe concentration ratios (Fredrickson et al., 2008). Protein aggregation, enzymatic inactivation, and changes in tertiary architecture are some of the protein damage induced by desiccation (Allison et al., 1999; Tarek and Tobias, 2002). The desiccation stress response is also associated with a rapid synthesis of stress proteins. Heat shock proteins (HSP) are able to counterbalance the loss of water through the formation of hydrogen bonds to other molecules (Franca et al., 2007; Boumahdi et al., 2001).

The desiccation stress response by bacteria is commonly connected to DNA damage repair. For any DNA damage, the efficiency of DNA repair and replication systems determines whether a bacterial cell lives or dies (Minton, 1996). Protection of proteins against reactive oxygen species (ROS) damage is a point at least as important and critical for preserving bacteria in a viable state as DNA repair. This theory means that bacteria with robust systems for protecting proteins against ROS induced damage will exhibit better long-term cell survival, as is the case for *D. radiodurans* (Fredrickson et

al., 2008). It has also been proposed that in desiccation microbial cell uses DNA to stabilize proteins, resembling the trehalose role. Therefore, DNA could also be a molecular shield against drying stress (García-Fontana et al., 2016).

III) Biofilms and matrix protection

Generally speaking, all bacterial species have increased resistance to desiccation after attached cells have grown to form a biofilm. This may be due to the protective effect of extracellular polymeric substances, which form a water-rich gel around bacterial cells (Schnider-Keel et al., 2001; Sutherland, 2001b). In a biofilm mode of growth, bacterial cells are embedded in a matrix of extracellular polymeric substances that contain polysaccharides, proteins, lipids and nucleic acids. Moreover, some cells and particularly those of the upper layers, form a physical barrier between cells in the lower layers and the surrounding milieu. These features, combined with a low metabolic activity, confer bacterial cells in biofilms a higher resistance to environmental stresses than planktonic cells (Steinberger and Holden, 2005; Johnson, 2008; Hansen and Vogel, 2011; Limoli et al., 2015). EPS are a relevant part of this matrix and are produced by a large range of bacterial species (Bazaka et al., 2011). Due to their high concentration of water (about 95%), EPS are highly hydrated. Thus, this ability of water retention creates hydrated microenvironments providing bacterial cells in biofilms the means to survive desiccation (Roberson and Firestone, 1992). Moreover, this property confers nutritional advantages over their planktonic counterparts, by acting as a force that binds and concentrates organic molecules and ions close to the cells (Kawaguchi and Decho, 2000). Hansen and Vogel (2011) showed that *L. monocytogenes* in a biofilm survived up to 49 days against a desiccated environment (43% RH and 15°C). One can reasonably assume that, due to their high hydrophilic properties, the exopolymeric substances produced during biofilm formation, including exopolysaccharides but also eDNA and peptidoglycans (Harmsen et al., 2010), were the main contributor to the *L. monocytogenes* desiccation survival (Hingston et al., 2013). In the case of *Pseudomonas* spp., the biofilm matrix plays an essential protection role during osmotic stress. The upregulation of alginate synthesis genes as well as flagella genes are associated with this tolerance in *P. putida* (Gulez et al., 2012). Desiccation tolerance in this bacteria was also associated

with nutrient or solute acquisition, energy generation and cell envelope structure (van de Mortel and Halverson, 2004).

2.4.1.5 Reported mechanisms from different bacteria

Gram-positive bacteria, due to their thicker peptidoglycans, are more resistant to dryness (Bale et al., 1993). It has been reported that *S. aureus* survived on dry hospital mops for several weeks (Oie and Kamiya, 1996). As is shown in the following results, there is not a single response to desiccation stress among the studied bacteria; the more cited responses comprise the regulation and accumulation of osmoprotectants, cross protection in response to other stresses and the contribution of EPS (Burgess et al., 2016).

Some enterobacteriaceae members have high tolerance to dry environments (Hiramatsu et al., 2005). Desiccation tolerance strategies for this family include the production of extracellular cellulose, EPS and fimbriae, changes in membrane permeability and synthesis and/or uptake of compatible solutes such as trehalose (Garmiri et al., 2008). In the case of *E. coli*, the induction of trehalose by osmotic shock supports its desiccation resistance, while intracellular glycine and betaine have no influence against this stress (Billi and Potts, 2002). As for *L. monocytogenes*, initially the accumulation of osmolytes was considered restricted to betaine, carnitine, and proline, but the list of compatible solutes promoting osmotic tolerance in *Listeria* has been extended to include also acetylcarnitine, gamma-butyrobetaine, and 3-dimethylsulfoniopropionate (Bayles and Wilkinson, 2000; Sleator et al., 2003).

Salmonella has the capacity to survive under osmotic stress for extended periods of time and it is considered one of the main biological hazards by dry-food manufacturers (Podolak et al., 2010). The filamentation properties of *S. enterica* contribute to its desiccation stress survival; nonetheless, the efficiency of this response may vary depending on temperature and the presence of other stresses (Stackhouse et al., 2012). The outer membrane antigen of lipopolysaccharide and cell surface components, like fimbriae and cellulose, are crucial for *Salmonella* desiccation tolerance (Vanderlinde et al., 2010). Cross-protection to low a_w and acid stress was also demonstrated through the increased expression of *rpoS* and *otsB*, involved in response to multiple stresses and in the production of trehalose, respectively (Aviles et al., 2013).

Cyanobacteria species are known for having significant resistance to several stresses due to the presence of multiple genome copies, which ultimately leads to better interchromosomal recombination and repair of DNA-induced damage (Billi and Potts, 2002). The same is reported to happen in *D. radiodurans* that also acquired, by horizontal gene transfer, several putative plant desiccation resistance-associated genes, which may contribute to its extreme resistance phenotype (Makarova et al., 2001). Increased intracellular levels of Mn(II) and upregulation of genes have been detected in *D. radiodurans* upon radiation and desiccation tolerance (Anderson et al., 2015).

These examples provide evidence that desiccation stress affects and promotes reactions in different cellular processes. Therefore, no single gene or protein could offer a complete protection from this stress and bacterial cells must employ a vast number of synergistic mechanisms to survive desiccation. In addition, desiccation leads to other stresses such as oxidative stress, resulting in several overlapping response pathways, making it very difficult to deconstruct them and to understand which ones are potentially unique and/or specific to desiccation and which ones are common to several stresses. Nevertheless, these overlaps are common features which allows bacteria to adapt to harsh environmental conditions, since one stress response pathway can protect against another stress (Begley et al., 2002).

2.4.2 Low temperature adaptation

L. monocytogenes adaptation to low temperatures is one of its crucial attributes that supports *Listeria* persistence and dissemination in refrigerated products. This adaptation to cold temperatures renders the use of such physical settings insufficient for the control of *L. monocytogenes* presence in long-term storage under refrigeration products (Tasara and Stephan, 2006). It is therefore relevant to explore *Listeria*'s adaptation mechanisms in low temperatures. Case in point, the second and third paper of this thesis have as one of the aims to explore the protein abundance shifts of *L. monocytogenes* under adaptation to a cold setting.

L. monocytogenes is able to growth at temperatures as low as -0.4 °C but also survive in freezing temperature such as -18 °C (Walker et al., 1990; Nowak et al., 2015). Moreover, this pathogen adheres and develops a biofilm regardless of the exposed temperature (Di Bonaventura et al., 2008). In short, upon exposure to low

temperatures, bacterial membranes become more rigid and the metabolic rate decreases. To overcome the hurdles imposed by a cold stress, bacteria have to increase the expression of genes involved in cell membrane function, production of cold shock proteins and multiple other molecular strategies to maintain homeostasis (NicAogain and O'Byrne, 2016).

2.4.2.1 Effects of low temperature

At low temperatures *L. monocytogenes* doubling time increases to up to 50 hours (Angelidis and Smith, 2003). This is caused by a general slowdown of the majority of the metabolic processes. In a cold condition, the bacterial membrane structural stability is compromised due to an increased rigidity in the lipid bilayer. It is observed a reduced protein and enzyme activity, a speed decrease in transport and nutrient uptake processes, increased superhelical coiling of DNA, stalled gene expression processes, secondary structures in RNA affecting translation, and increased destabilization of ribosomes and protein damage (Soni et al., 2011). Oxidative and amino acid starvation stresses have also been observed in *Listeria* cell exposed to cold (Liu et al., 2002).

2.4.2.2 Molecular mechanisms to adapt to cold

Bacterial adaptation to low temperatures can be subdivide in 3 stages, initial cold shock, acclimation and cold adapted status (Hebraud and Potier, 1999; 2000). Upon sensing initial cold shock, bacterial synthesis of proteins non-related to cold adaptation is reduced to residual values and synthesis of cold shock associated proteins increases significantly in the acclimation period. If bacterial cells reach the cold adapted status, synthesis of normal metabolic associated proteins increases to regain molecular stability and growth rate, while cold shock protein production decreases (Chan and Wiedmann, 2009). Figure 13 illustrates some of the major response systems implemented by *Listeria* to adapt to a cold condition. One of the major adaptative strategies is the induction of osmolyte and peptide transporters which will increase the amount of this molecules in the cytosol and maintain turgor pressure (Miladi et al., 2017). A combination with other mechanisms of cellular homeostasis maintenance, such as: control of membrane fluidity, gene expression events, protein folding and degradation, assimilation of carbon sources, oxidative stress response and

production of specific amino acids and lipids culminate in *Listeria's* successful persistence in the cold temperatures of the food processing environment (Soni et al., 2011).

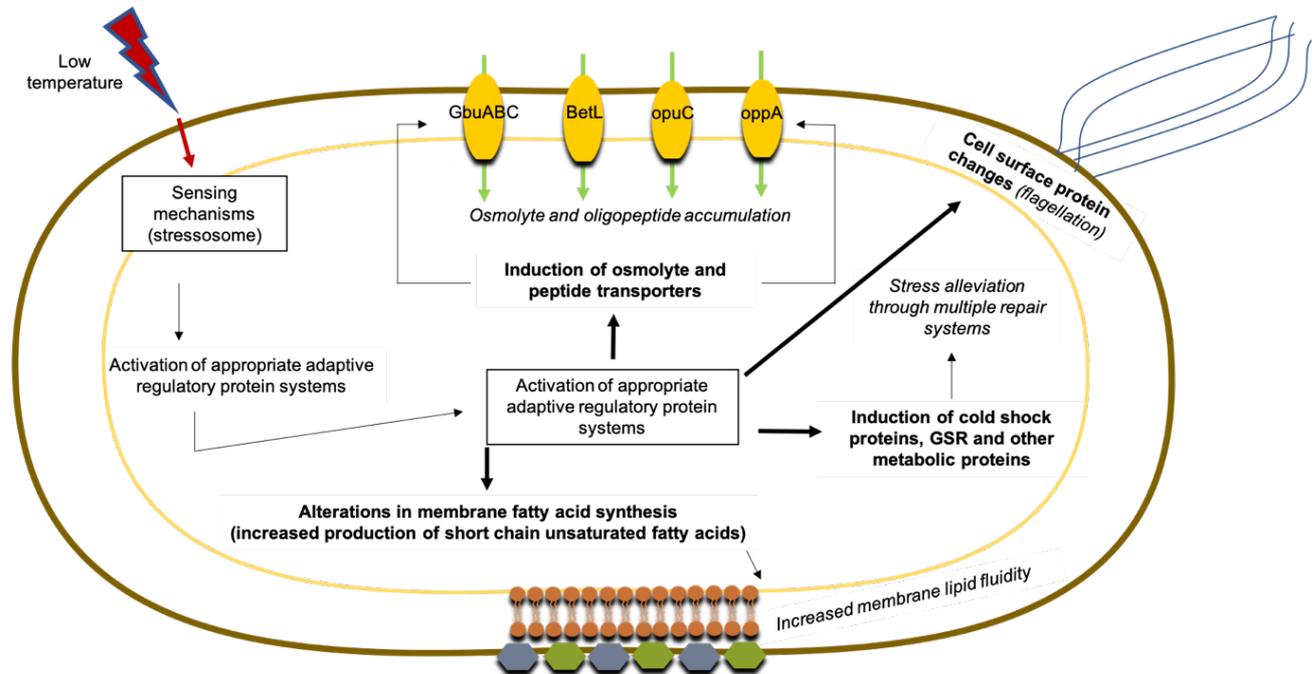


Figure 13 - Diagram outlining some of the response mechanisms present in *L. monocytogenes* to adapt to low temperatures. Adapted from (Tasara and Stephan, 2006).

1) Uptake of osmoprotectant molecules

The critical uptake of osmoprotectant molecules for *Listeria's* adaptation to low temperature is made through auto transporters for compatible osmolytes and short oligopeptides (Wemekamp-Kamphuis et al., 2004). Osmolytes like glycine betaine, carnitine, γ -butyrobetaine, proline betaine, and 3-dimethylsulphoniopropionate help survival at low temperatures through stabilization of enzymatic functions and the cell-membrane lipid bilayer. These low-molecular osmolytes can be reserved in the cytosol at high concentrations, however *Listeria* does not synthesize these cryoprotective molecules. Therefore it is through transporters that it manages to obtain them, particularly from food products (Angelidis and Smith, 2003; Wemekamp-Kamphuis et al., 2004; Miladi et al., 2017). The main carnitine transporter, OpuC, encoded by the opuCABCD operon, was observed in high abundance in *Listeria* cells exposed to low temperatures. As for betaine cryotolerance role, its uptake is mainly controlled by Gbu,

but also via BetL and OpuC. To note that, carnitine has a described higher role in the tolerance to cold stress than betaine (Cetin et al., 2004; Miladi et al., 2017).

In *L. monocytogenes*, the intake of short oligopeptides is partially explained by the surface lipoprotein OppA. This ABC autotransporter showed high levels in stationary-phase cells grown at 4 °C and it is considered essential for *Listeria's* cryotolerance (Borezee et al., 2000). The short peptides are used as a nutrient source, to recycle cell wall peptides, and also to sense extracellular changes in physicochemical conditions (Cabrita et al., 2014).

II) Cold shock and chaperone proteins

In cold environments, bacteria also deal with protein damage, particularly protein misfolding and aggregation. To counteract this damage, cells have at their disposal a network of molecular chaperones that assist in maintaining proteins in their native states. A key role of chaperones is preventing protein aggregation. Furthermore, chaperone proteins have other functions in quality control, for example pursuing terminally misfolded proteins for proteolytic degradation (Kim et al., 2013).

Cold shock proteins (Csps) are molecular chaperones that help stabilize replication, transcription and translation in a condition of stress, including exposure to low temperatures (Phadtare et al., 1999). In its sequence, they have a conserved domain that has high level of homology with the “cold shock domain” of eukaryotic Y-box proteins. This family of small proteins (around 70 amino acids long) bind to single-stranded nucleic acids stabilizing its conformation and preventing degradation (Bucur et al., 2018). The bulk of the cold-adaptation research has been made in bacterial cells exposed to sudden decrease of temperature, and few had a look into the acclimation and adaptation stages of the cold stress. Temperature settings in these studies normally range between 4 to 10 °C with the detection of at least 10 Csps (Bayles et al., 1996). The main Csp is an 18-kDa polypeptide ferritin-like protein (Flp) which is highly expressed in response to cold shock (Hebraud and Guzzo, 2000). *L. monocytogenes* harbours three proteins from the CspA family (CspLA, CspLB and CspD) and genome-wide expression studies showed a significant increase in expression of CspL at low temperatures in stationary phase (Chan and Wiedmann, 2009). In *E. coli*, CspA is the main cold shock protein which has been observed in high abundance during cold shock as well in *B. subtilis* where cold shock protein also

showed relevance in adaptation to other harsh conditions (Goldstein et al., 1990; Graumann et al., 1997). Under cold adaptation *L. monocytogenes* induces the production of other molecular chaperones, such as GroEL, DnaK and heat-shock protein GrpE (Cacace et al., 2010).

III) Alterations in the cell membrane

Under cold stress, bacteria promote alterations in the membrane fatty acid synthesis by changing the fatty acid chain lengths, altering the degree of fatty acid unsaturation and changing the type of branching at the methyl end of the fatty acids (Tasara and Stephan, 2006). These modifications in the membrane composition will keep its fluidity during low temperature exposure and prevent the creation of a gel-like state that could lead to leakage of cytosolic content (Zhang and Rock, 2008). One other change in the bacterial cell surface during the cold stress is the induction of flagellation. The hypothesis is that under stress, bacteria promote motility with the goal of biofilm development and consequent better protection against the cold, or simply to support the bacteria translocation to available locations with higher nutritional or osmolyte sources (Mattila et al., 2011).

IV) Role of ribosomes and σ^B

Ribosomes are the basis of protein synthesis and are also key components of the bacterial adaptation to low temperatures. In the immediate aftermath of a cold shock, there is a decrease in protein synthesis caused by loss of ribosome function. In *E. coli* ribosomes are used as early sensors of cold shock and the lack of some ribosomal structures unable the activation of the GSR (VanBogelen and Neidhardt, 1990; Zhang et al., 2001). At low temperatures, it has been observed an increase in the level of translation initiation factor (IF2), responsible for the formation of 30S complex, pointing to the relevance of this structure during this stress. The role of 50S ribosomal protein L25 (general stress protein Ctc) has been observed in *Listeria's* adaptation to osmotic stress (Duche et al., 2002). Even if Ctc role in adaptation to cold setting has not yet been observed, it is reasonable to assume a connection taking into account that as in osmotic stress, low temperature adaptation much relies on uptake of osmoprotectant molecules. Furthermore, Ctc is regulated by σ^B and efficient intake of solutes betaine and carnitine in a cold setting is dependent on this transcriptional regulator (Bayles et

al., 1996). Particularly the osmolyte carnitine, which as mentioned before is transported via OpuC. The expression of this autotransporter is also regulated by σ^B . Other genes associated with cold stress resistance, *ltrC* (Chan et al., 2007a) and *fri* (Hebraud and Guzzo, 2000), are also controlled by σ^B regulon. In conclusion, even if a direct link between cryotolerance and σ^B has not yet been obtained, there are clear signs that this transcriptional factor has a role in *Listeria's* adaptation to low temperature (NicAogain and O'Byrne, 2016).

2.4.3 Other stress conditions

Besides desiccation and low temperature, *L. monocytogenes* is able to resist other harsh conditions found in the environment and in the food processing pipeline. Some of them also have cross-protective molecular changes with the two previously described stresses.

2.4.3.1 Heat stress

The exposure to high temperatures leads to the stalling of bacterial cell growth and progressive loss of viability. *L. monocytogenes* has to endure heat conditions in the preservation treatments employed by the food industry but also upon host infection (Abee and Wouters, 1999; Hanawa et al., 2002). At the molecular level, the response to this stress triggers mainly an overexpression of class I (*grpE*, *dnaK*, *dnaJ*, *groEL*, and *groES*) and class III heat-shock genes (*clpC*, *clpP*, and *clpE*), and genes of the σ^B dependent class II stress response (Gahan et al., 2001). Heat-shock proteins (Hsps) act as intracellular chaperones and are also identified at low levels in normal conditions, but they are overexpressed upon exposure to elevated temperatures. In the host, they also act by helping to resist host defences or regulating virulence genes (Bucur et al., 2018).

2.4.3.2 Acidic condition

Similar with heat stress challenges, *L. monocytogenes* has to face acid conditions in the food processing environment (food preservatives and decontaminates) and in the gastrointestinal tract of the host (inorganic acids) (Soni et al., 2011). Keeping the cytoplasmic pH at a value close to neutrality despite fluctuations in external pH is crucial to its survival and required for infection. Bacterial survival in low pH conditions

is based in three main mechanisms, namely the adaptive acid tolerance response (ATR), the Glutamate Decarboxylase (GAD) system and the Arginine Deaminase (ADI) system (Davis et al., 1996).

2.4.3.3 Osmotic shock

Salt is a fundamental preservative in food products, therefore for *L. monocytogenes* to survive in such condition it has to implement strategies to tolerate osmotic stress. *Listeria* can resist in salt concentrations up to 3 M NaCl (Csonka, 1989). The primary osmotic stress response is based in the influx of potassium and glutamate into the cytosol, while the secondary response involves the uptake of small osmoprotectants, as reviewed in the cold shock section (Pittman et al., 2014). These strategies allow the bacteria to maintain turgor pressure, cell volume and protein stabilization upon an osmotic shock (Durack et al., 2013).

2.4.3.4 Oxidative stress

L. monocytogenes in the food industry can develop oxidative stress due to atmospheric modification and chemical reagents applied present in disinfectants. Oxidative stress can lead to a partial shutdown of molecular functions, as well as protein, membrane, and nucleic acid damage (Soni et al., 2011). To cope with the stress, *Listeria* activates ROS detoxification systems like superoxide dismutase (Sod), catalase (Kat) and alkyl hydroperoxide (AhpCF) enzyme systems (Franca et al., 2007). The ferritin protein has also been shown to have a role in the tolerance to oxidative stress (Dussurget et al., 2005).

2.4.3.4 Light

In the clinical environment and in the food industry, light has been employed in order to decontaminate surfaces (NicAogain and O'Byrne, 2016). In particular, blue light is a stress condition for *L. monocytogenes* that leads to the activation of the GSR. However, together with σ^B activation also internalin virulence genes are overexpressed upon exposure to light (Ondrusch and Kreft, 2011).

2.4.3.5 Quaternary ammonium compounds

Quaternary ammonium compounds (QAC) are antibacterial agents frequently used in the health, agriculture and food sector. The most regularly used QAC is the benzalkonium chloride (Tezel and Pavlostathis, 2015). However, strains of *L. monocytogenes* have been found to be resistant to this agent by the three small multidrug resistance protein transporters (QacH, BcrB, and BcrC) (Møretrø et al., 2017).

2.4.3.6 Nisin

The use of antimicrobial compounds is one of the food industry strategies to contain the presence of *L. monocytogenes*. Nisin, produced by *Lactococcus lactis*, is the most common and effective bacteriocin used against *Listeria* (Delves-Broughton et al., 1996). This molecule disrupts the cell membrane by the formation of pores that interfere with the cell wall biosynthesis. However, upon exposure to nisin, *Listeria* activates a two-component regulatory system and the GSR that promotes changes in the cell wall composition hampering the access of the bacteriocin to the cell (Kaur et al., 2013).

Chapter 3 - Proteomics and Mass Spectrometry

The ever-changing dynamics and complexity of bacterial proteomes means that not a single analytical method will give the full picture of the proteome. In this section, I revise briefly the state of art in proteomics and describe the mass spectrometry tools and approaches taken in order to explore the proteome of *L. monocytogenes*.

3.1 Putting Proteomics into context

After uncovering the double helical structure of DNA, James Watson and Francis Crick stated the following: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.” (Watson and Crick, 1953). This marked the start of molecular biology and less than 50 years later, the success of the Human Genome project lead to the start of the genomics era (Lander et al., 2001; Venter et al., 2015). The evolution of sequencers in delivering high-throughput results at progressively lower prices resulted in genome sequencing becoming ordinary (Yates et al., 2009). As for transcriptomics, it studies the complete set of transcripts in a cell, and their quantity, for a particular developmental stage or physiological condition. Recently, the advances in RNA sequencing (RNA-Seq) lead to the collection of vast data sets of eukaryotic and prokaryotic transcriptomes (Wang et al., 2009). Nonetheless, proteins are the basis of the cells main function. They are the cellular building blocks that control gene function via enzymatic catalysis, molecular signalling and physical interactions. Contrary to the genome, the proteome is an always changing entity due to intracellular or extracellular environmental factors. Moreover, events such as splicing, post-translational modifications and protein degradation intensify even more the magnitude of variance in proteomes (Gilmore and Washburn, 2010). Proteomics is considered the third downstream omics, that explores the identification, modification, quantification and localization of proteins (Yates et al., 2009). Proteomics was first devised by Mark Wilkins in 1996 to define the study of the total set of proteins encoded by a genome (Wilkins et al., 1996). Though it sounds a fare mission, it is in fact a daunting task in of itself that requires the use of many different advanced instrumental tools and complex methods (Santos et al., 2010). In the beginning of the 20th century, the physicist JJ Thomson envisioned the now considered the first mass spectrometer

(MS) (Marchetti-Deschmann and Allmaier, 2011). The second half of the century was marked by constant and crucial developments in mass spectrometers accuracy, resolution and overall capacity in analysing proteins, making its way into chemistry, biochemistry and also structural biology (Marchetti-Deschmann and Allmaier, 2011). The high point of this evolution in apparatus occurred at the end of the 20th century when Fenn and Tanaka postulated two mass spectrometry techniques that are still nowadays the benchmark in high throughput proteomics, namely the matrix-assisted laser desorption ionization (MALDI) and the electrospray ionization (ESI) (Tanaka et al., 1988; Fenn et al., 1989). These two mass spectrometry tools not only gave these two researchers the Nobel Prize in Chemistry but were also the driving forces behind the development of the world of “Omics” (Marchetti-Deschmann and Allmaier, 2011). In separation sciences, there is a pursuit for high resolution separations to conquer the analyses of complex samples (Rabilloud and Lelong, 2011). Proteomics first aim is the profiling of proteins and peptides, this is high a demanding analytical problem, due to high chemical variability, solubility differences between analytes and strong tendency for precipitation (Resing and Ahn, 2005). Two main strategies are recognized as the branches of proteomic approaches, the top-down and the bottom-up approach.

3.2 Top-down proteomics approach

The top-down approach studies intact proteins, as for the bottom-up approach it analyses peptide mixtures from enzymatic digestion of proteins. The first well established technical approach in top-down proteomics was the two-dimensional gel electrophoresis (2-DE), which allows to see changes in protein abundance and covalent modifications from protein staining intensity and electrophoretic mobility (Rabilloud, 2009). The streamlined workflow includes the excision of gel pieces, in-gel digestion and analysis by a mass spectrometry (Resing and Ahn, 2005). Though, the term “the déjà-vu in proteomics” was used in order to reflect one of the main issues with 2-DE (Petрак et al., 2008). This method only shows a limited pool of highly abundant and soluble proteins. These proteins comprehend mainly central metabolism, protein production, protein conformational control and degradation, and oxidative stress response (Rabilloud and Lelong, 2011). That is why nowadays most of the top-down proteomics are done by injection of intact protein ions into the gas

phase by electrospray ionization (ESI) followed by fragmentation by a mass spectrometer. This approach enables to explore the primary structure of the protein and its modification without prior enzymatic digestion and possible loss of information regarding their post translational modifications (Chait, 2006). The top-down approach can provide a full description of the protein primary structure but its use is not always feasible due to the difficulty to produce extensive gas-phase fragmentation of intact protein ions, particularly from large proteins (Jiang et al., 2000). Proteins undergo PTMs that modulate their structure and modify their function. It is estimated that a single protein on average carries at least three PTMs, but it is clear that there is still a gap between what is known and what is left to be detected (Ahrne et al., 2010). For sake of synthesis and since this thesis workflows duels in bottom-up approaches I will not go further in detail about the top-down proteomics. However, even if the bottom-up approach has been the main tool of choice by scientists in the proteomics field, the study of the intact protein through top-down is showing an increased demand, due to its capacity to explore in full detail all possible physicochemical variations of a protein (Penque et al., 2011). Further information about top-down proteomics can be available to the reader by multiple extensive reviews on the subject (Chait, 2006; Wynne et al., 2009; Huber and Huber, 2010; Calligaris et al., 2011).

3.3 Bottom-up proteomics approach

The most frequent proteomic approach, and also the one used throughout this work, is the bottom-up proteomics, also named “shotgun” proteomics. It was paramount in the progress and spread of proteomic analysis in multiple sciences (Huber and Huber, 2010). However, as the term indicates, the shot of pieces of proteins gives a level of uncertainty about the actual analysis. Opposite to the top-down method, here proteins are digested with an enzyme, frequently the enzyme of choice is trypsin, and the obtained peptide mixture is then analysed by mass spectrometry. The main advantage of this approach is its high level of protein identification output, since tryptic peptides are easily solubilized and separated in comparison to intact proteins. However, on the negative side only a fraction of these peptides is detected, meaning that this approach is normally insufficient for the analysis of post translational modifications (Chait, 2006; Calligaris et al., 2011). Since it explores a mixture of peptides, information associated

with the intact protein, such as its function, location, interaction and general relevance in biological processes, is lost in the way (Huber and Huber, 2010).

3.3.1 Workflow and protein extraction

The key steps of a shotgun proteomics workflow are: (a) sample preparation (e.g. enzymatic digestion of protein with trypsin), (b) peptide separation (e.g. high-performance liquid chromatography, HPLC); (c) mass detection; and (d) data analysis. All steps are essential to achieve a reproducible experiment, but the robustness of the preparation method is a vital step to attain a high throughput result (Bereman et al., 2011). Different laboratories tend to optimize in their way the sample preparation procedure, however typically they all follow common general stages including: (a) solubilisation and denaturation of proteins, (b) reduction of disulphide bonds, (c) alkylation of reduced cysteines, (d) digestion, and (e) sample clean-up (Resing and Ahn, 2005).

Even though these are the key steps to obtain a robust and reproducible bottom-up proteomic analysis, the protein extraction methods that precedes these tasks is no less important. In relation to that and integrated in the work of this thesis a paper was published standardizing three subproteomic extraction methods from *L. monocytogenes* bacterial cells, with particular focus on surface protein identification (Esbelin et al., 2018b). As reviewed in section one, bacterial cell surface proteome constitutes the so-called proteosurfaceome of a bacterium, which can be involved in diversified processes, and contributes to the interface between the bacterium and its environment (Cullen et al., 2005; Desvaux et al., 2006; Desvaux et al., 2018). The bacterial proteosurfaceome is relatively hydrophobic and can only be extracted from the cell membrane using detergents. Because of its variability and instability, its analysis presents many hurdles including extraction, solubilisation, purification, and analysis with in-gel (one- or two-dimensional gel electrophoresis) (Sun et al., 2011; Hebraud, 2014) or off-gel (shotgun proteomics) approaches (Cordwell, 2006). Several methods have been previously assessed and compared, but on monoderm bacteria growing in planktonic mode (Schaumburg et al., 2004; Dreisbach et al., 2010; Hempel et al., 2010; Dreisbach et al., 2011), 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 were enzymatic shaving of the cell surface, biotin labelling of cell envelope proteins and cell fractionation. The shaving method consists of treating intact cells with proteases in an isotonic solution to release exposed peptides. For the biotinylation method, bacterial cells are treated with Sulfo-NHS-SS-Biotin, a marker molecule that 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 seemed the most suitable, even if some periplasmic, cytoplasmic, and inner membrane proteins were identified (Hempel et al., 2011; Monteiro et al., 2018). The third method is the fairly well-established separation of membrane and cell wall components by cell fractionation. Among the various strategies for exploring the proteosurfaceome, the enzymatic shaving method has been applied in recent years, mainly on monoderm bacteria (Cordwell, 2006), 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. (Rodriguez-Ortega et al., 2006; Severin et al., 2007; Pribyl et al., 2014), *Staphylococcus aureus* (Dreisbach et al., 2010; Solis et al., 2010; Ventura et al., 2010; Dreisbach et al., 2011; Pribyl et al., 2014; Monteiro et al., 2015; Tiong et al., 2015), and other monoderm bacteria such as *B. subtilis* (Tjalsma et al., 2000) or the dairy starter *Lactococcus lactis* (Meyrand et al., 2013). In short, the results gathered in this study 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 of using cell wall extracts boiled with SDS before their trypsin digestion (Pucciarelli et al., 2005) could allow to target more specifically the LPXTG proteins.

3.3.2 Data analysis and quality assessments in shotgun proteomics

In proteomics, sample deepness has a huge role in achieving the coverage of a proteome. Shotgun proteomics approach works with proteolytic digests and to achieve a high percentage coverage of a proteome there is a need to collect as many MS/MS spectra of the peptides in mixture as possible, to ultimately achieve the relative abundance of a set of proteins. Though, the high number of sample replicates and accumulated runs is contra balanced with the cost, time and acquisition rate of the mass spectrometer (Hackett, 2008).

Shotgun proteomics is a powerful tool to identify peptides and proteins from the most diverse biological sources (Resing and Ahn, 2005). After enzymatic digestion of the protein extraction and separation by liquid chromatography, the peptide mixture is screened by MS/MS.

The match of MS/MS spectra is achievable because peptide molecular ions break preferentially at certain points along the sequence (Fernandez-de-Cossio et al., 1998). Through a search engine, the obtained fragmentation spectra are then compared and matched to theoretical spectra from a database of peptides of the studied organism. Search engines like SEQUEST, X! Tandem, OMSSA, Crux and MASCOT, the latter the one used in this thesis, give peptide-spectrum matches (PSMs) that are the result from the comparison between observed and theoretical spectra (Granholm and Kall, 2011). To note that, the search engines assign correct peptides to 10–50% of the spectra since only fraction of this PSMs have eventually an acceptable score for the identification (Ahrne et al., 2010).

The amount of spectral information that can be gathered in a single shotgun analysis is enormous. Taking into consideration the theoretically possible protein sequences the task becomes even more challenging. Plus, the different amino acids have very similar masses (mass difference of lysine and glutamine is as low as 0.04 Da), a very high amount of amino acid combination is feasible (Wohlbrand et al., 2013). That is why it is crucial to sort the obtain PSMs, not only by a method of threshold score, to define a cut-off for significant PSMs, but also through other statistical scores as p-value and false discovery rate (FDR) (Granholm and Kall, 2011). The p-value is the probability that an incorrect top-scoring PSM would have a score equal to or higher than a given threshold just by chance (Granholm and Kall, 2011). As for the FDR, it describes the expected fraction of incorrect PSMs above the current score threshold,

in this way it shows the quality of the PSMs that were considered significant and so takes into account the effects of multiple testing (Storey and Tibshirani, 2003).

3.3.3 Quantitative proteomics: Isotopic vs label-free workflows

To bridge the gap between genotype and phenotype, it is crucial to gather information regarding the shifts in abundances of proteins. Large-scale proteomics allows to quantify these abundances, resulting in a snapshot of protein concentrations connected to different states, e.g. adaption to stress conditions (Yates et al., 2009). There are two branches of quantitative approaches in mass spectrometry, either absolute quantitative proteomics or relative quantitative proteomics. Focusing on the latter, proteins can be analysed regarding its abundance through stable isotope-labelling or label-free methods.

In quantitative proteomics, the gold standard is the use of heavy-labelled peptides that are assimilated by the sample in precise concentrations in order to quantify a protein or a set of proteins of interest (Malmstrom et al., 2011). Target proteins of interest are marked with peptides synthesized with a heavier form of the C-terminal arginine or lysine residue. The biological sample, composed of isotopically lighter proteins, and the synthesized peptide are mixed. The relative intensity between the heavy and light version of the peptide can be used to find the absolute amount of the lighter peptides present in the mix, the biological sample. Taking into account the known amount of the heavy labelled reference peptide it is possible to calculate the amount of the endogenous peptide through the ratios of the specific ion currents (Gerber et al., 2003). Currently protein quantification by mass spectrometry can be achieved with several different strategies: ^{18}O -based labelling, stable isotope labelling with amino acids in culture (SILAC) (Ong and Mann, 2005); isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004); internal standard-based approaches such as QconCAT or the use of AQUA peptides (Capelo et al., 2010).

Besides isotopic labelling quantification workflows, label-free quantification approaches have become frequently used in proteomics. Even if it has lower accuracy when compared with isotope labelling, the label-free approach is however reliable, particularly in the comparison of similar samples (Otto et al., 2014). Case in point, in the workflow throughout the two last thesis papers, the label-free comparison was continuously made between *L. monocytogenes* cells. Two main models are in the

basis of the label-free quantitation. Quantitation made through spectral counting and quantitation based on the determination of the area under the curve (AUC) on the levels of the first mass spectrum level (Neilson et al., 2011). Spectral counting is found on the notion that the more abundant peptides will be selected for fragmentation and will result in higher abundance of MS/MS spectra, and so proportional to protein amount (Liu et al., 2004). AUC approaches have a similarity with protein abundance determination to a 2-DE gel. Though, instead of densitometry of stained spots, the ion chromatograms AUC are used to compare LC-MS runs (Wohlbrand et al., 2013). Spectral counting is the most frequently used method in label-free approaches, particularly because AUC requires a higher complexity in data acquisition and processing software (Neilson et al., 2011).

The shortcoming of either labelled or label-free quantitative approaches is the MS/MS protein identification method and the presence of shared peptides. Shared peptides meaning peptide sequences that are eventually matched to more than one protein (Neilson et al., 2011). From a single gene, hundreds of different proteins can be produced, due to splicing events, PTMs and protein isoforms, potentially causing erroneous protein matches (Black, 2000). This ambiguity of different proteins being identified due to a shared peptide is a limitation of quantification in proteomics which has been dealt with either ignoring the shared peptides, focusing only on unique peptides or spread the peptides across the homologous proteins. It is important to take into account that whatever option taken there is always a limitation and inference that will affect quantitation (Podwojski et al., 2010). Still, even with its caveats, label-free quantitation has a higher level of accuracy in determining protein abundance than the gel-based approach. Plus, it is more suitable to explore the protein abundance over a larger dynamic range than isotopic labelling (Neilson et al., 2011).

3.3.4 Proteomics in microbiology

Proteomics in microbiology has been considered more simplistic when compared to organisms with higher level of complexity, taking into account the smaller genomes, reduced proteins species, and fewer occurring PTM's in microbes compared to higher organisms (Otto et al., 2014). Plus, it is now possible to achieve microbial proteome coverages of up to 80%. Results hardly achievable in eukaryotic studies (Becher et al., 2009; Nagaraj et al., 2012). The natural next step in microbial proteomics was the

quantification of the identified proteins. However, this is no easy task, mass spectrometry is not inherently quantitative (Otto et al., 2014). Significant differences in the mass spectrometer detection can be retrieved due to differences in physicochemical properties for peptide species and variation in signal intensities. This is the reason why most quantitative studies use isotopic labelling, this enables the separation between peptide species, and, consequently, a better determination of quantitative shifts in protein abundances in the sample (Van Oudenhove and Devreese, 2013).

The majority of microbial proteomic studies have had the goal to identify and quantify active pathways and cellular adaptation to various intracellular and outside stimuli. The analysis of expressed proteins under different growth condition allows the identification of proteins and pathways crucial to a specific process, as is the adaptation to environmental conditions (Chao and Hansmeier, 2012).

One constant target in cellular proteomics is the study of the surfaceome, particularly the membrane proteins. Surface proteins are a key element in the interaction with the outside environment, including the transport of essential nutrients, first stress signals for cascade responses and even virulence mechanisms (Olaya-Abril et al., 2014).

The characterization of bacterial surface proteins of pathogenic bacteria such as *Mycobacterium tuberculosis* lead to the identification of novel proteins that are connected in host cell adhesion and invasion (Malen et al., 2007).

A trendy and promising approach in global proteomics is the single shot proteomics (Penque et al., 2011). With this approach, around 4000 proteins were identified per run in a yeast lysate, equivalent to its entire expressed proteome. The relevance here is that this result was obtained in a single run of HPLC, decreasing the levels of variability and also potentially enabling a higher quality in the quantification through isotope labelling (Nagaraj et al., 2012).

3.4 Mass spectrometry approaches used in this work

As mentioned before, proteins are a very challenging molecule to be analysed. Taking into consideration an average size protein of 450 amino acids, there can be more than ten thousand different primary sequences (Wohlbrand et al., 2013). Furthermore, proteome complexity is increased due to the microbes' capacity to adapt to a changing environment causing changes up to 100-fold in some adaptation associated proteins

(Malmstrom et al., 2011). The ever-changing dynamics and complexity of bacterial proteomes mean that not a single analytical method will give the full picture. In this section, I describe the mass spectrometry tools and approaches taken in order to explore the proteome of *L. monocytogenes*.

The recent developments in protein extraction methods, apparatus and data analysis approaches have resulted in an exponential use of mass spectrometry in different sciences. The polar, non-volatile, and thermally unstable nature of proteins and peptides are the barriers that hinder its ionization. Reason why, one of the most relevant advances in mass analysers was the use of soft ionization methods such as MALDI and ESI (Yates et al., 2009).

3.4.1 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The soft ionization technology MALDI is based on the co-crystallization event between sample and matrix, and its capacity to absorb the laser energy and transfer it to the acidified analyte. Ionization is achieved by short, intense pulses of a UV laser beam that will result in desorption of matrix and analyte ions into the gas phase (Ryzhov and Fenselau, 2001). The gas-phase ions formed by MALDI are given a fixed value of kinetic energy by acceleration in an electric field that is created by high voltage. These predominantly single charged ions then enter a vacuum tube where its time of flight until reaching the detector is measured (Figure 14) (Liao and Allison, 1995). The ions will travel at a velocity inversely proportional to their mass to charge ratio (m/z). Therefore, ions with smaller mass are faster than the ones with bigger mass and will reach the detector earlier. The m/z is equal to the ion velocity of the ion, which is retracted from the time required that the ion took to travel the field-free region (Dingle and Butler-Wu, 2013). The final result is given in the form of mass spectrum presenting the m/z to intensity of the ions. The MALDI-TOF MS is the most commonly used technical basis for Imaging Mass Spectrometry approach (IMS).

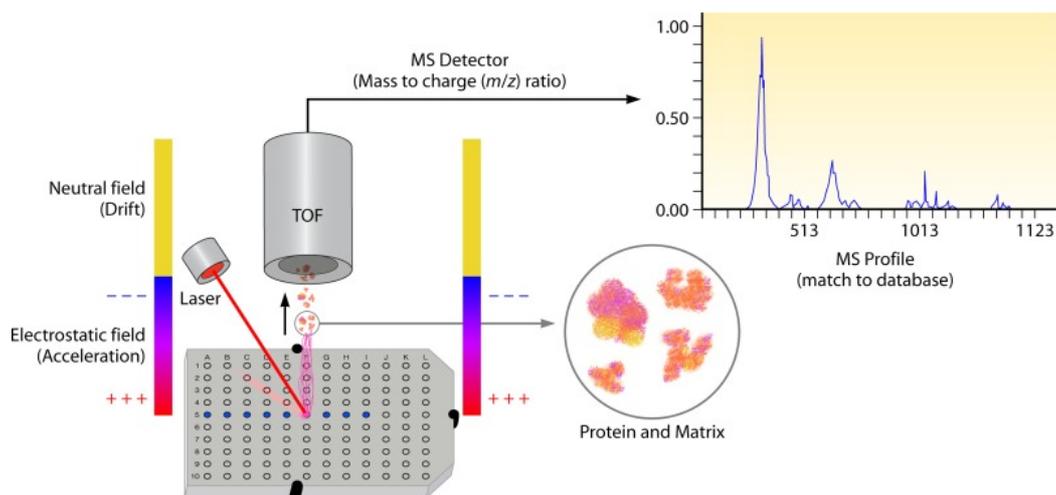


Figure 14 - Scheme of the MALDI-TOF MS pipeline. Analyte co-crystallized with matrix is bombarded by a UV laser which results in the desorption ionization of the sample. These ions are then separated in a vacuum tube by their mass-to-charge ratio. From (Clark et al., 2013).

3.4.1.1 MALDI-TOF Imaging Mass spectrometry (MALDI-TOF IMS)

The full characterization of molecular pathways requires not only the capacity to identify its constituents and dynamics but also the abundance and spatial localization of these molecules in different states (Sandrin and Demirev, 2017). The development of the imaging method enhanced MS detection with the spatial information of the detected ions. In a single experiment, it is possible to have the distribution and relative abundance of hundreds of different molecules (metabolites, peptides and proteins) within a biological sample (Baker et al., 2017). IMS was first introduced in Caprioli's lab just twenty years ago (Caprioli et al., 1997), but since then it has established itself as a go-to technique to obtain the spatial analysis of multiple molecules from numerous biological samples (Maier et al., 2013). Since 1997, more than seven thousand PubMed article entries addressed the use of IMS.

This tool is able to access complex molecular mixtures with high chemical specificity. The images obtained from IMS result from direct molecular analysis and are not based on target-specific reagents such as antibodies (Schwartz and Caprioli, 2010). Figure 15 makes a schematic representation of the technical steps to analyse a biological sample, from applying the matrix until data visualization.

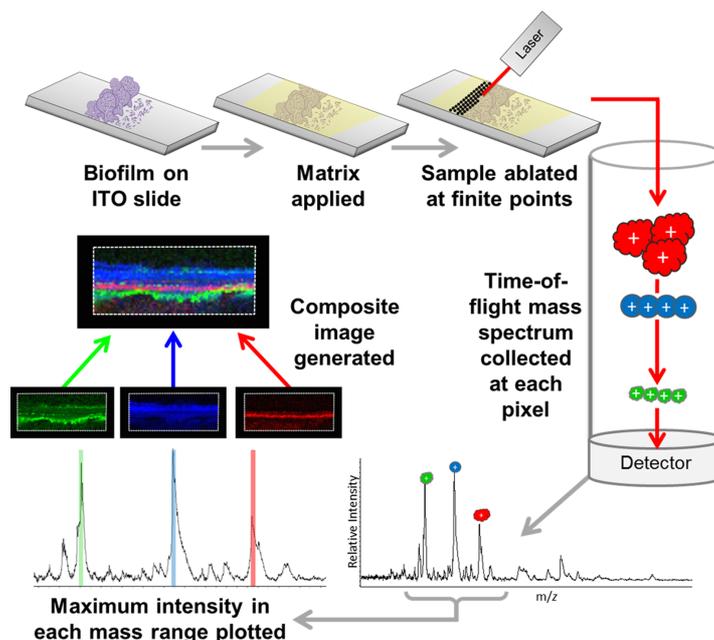


Figure 15 - Description of the MALDI-TOF IMS pipeline. In this case, a bacterial biofilm grown on top of Indium tin oxide coated (ITO) glass slide was covered with UV-absorbing matrix and analyzed by MALDI IMS. Each ion species can then be picked from the mass spectrum and its intensity and abundance across the biofilm can be checked via a heat map. From (Floyd et al., 2015).

Applications of these technologies were initially focused in clinical research, particularly cancer research (Yanagisawa et al., 2003; Schwartz et al., 2005; Cornett et al., 2006; Lemaire et al., 2007). With the progressive evolution of the technique and protocols, IMS was implemented with success not only in molecular histology of tissue (Chaurand et al., 2002; Groseclose et al., 2007; Djidja et al., 2010; Stauber et al., 2010; Martin-Lorenzo et al., 2016; Theron et al., 2016; Centeno et al., 2017), but also plant research (Anderson et al., 2009; Kaspar et al., 2011; Baker et al., 2017) and microbiology (Esquenazi et al., 2008; Blaze et al., 2012; Gonzalez et al., 2012; Debois et al., 2014; Floyd et al., 2015; Boya et al., 2017; Santos et al., 2018).

IMS studies can be achieved with different MS ionization techniques, such as secondary ion mass spectrometry (SIMS), laser desorption/ionization (LDI), desorption electrospray ionization (DESI) and MALDI (Caprioli et al., 1997). The latter being the most frequently used approach due to its versatility and accuracy in the analysis of peptides and small proteins. MALDI workflows rely on a suitable application of matrix, but prior to that sample preparation is imperative. Due to suppression effects, it is necessary to remove salts that impair a correct co-crystallization between matrix and sample. Furthermore, it is also advised to remove lipids and phospholipids,

in case that proteins are the target molecule of study, in order to reduce the complexity of the sample and the overabundance of ions species (Enthaler et al., 2013). The washing procedure usually involves the immersion of the glass slide in a washing solution. It is relevant to take into account, that this step might also lead to movement of cells in the sample and even reduce the amount of proteins available for ionization. As described in the previous section, the MALDI IMS workflow starts by the application of an uniform coating of energy absorbing matrix on top of the biological sample (Cornett et al., 2007). There are multiple matrices available in the market dependent on the ionization target molecule, such as alpha-cyano-4-hydroxycinnamic acid matrix (CHCA) suited for ionizing peptides and small proteins or 2,5-dihydroxybenzoic acid matrix (DHB) for the ionization of proteins (Dingle and Butler-Wu, 2013). Whatever the chosen matrix, there are two main approaches for its application on the biological sample surface, manual deposition of very small droplets or automated overspraying of matrix. The deposition of matrix is a crucial determinant of spatial resolution and it will influence the number of ions species detected (Cornett et al., 2007). The first paper of this thesis also compares these two strategies for matrix deposition and its obtained results.

IMS adds to the MALDI-TOF workflow through the gathering of individual mass spectra using continuous scanning motion over the sample area. From these spectra are generated intensity coloured pixels, which all combined form an image that allows the visualization of the abundance and spatial distribution of each ion species (Watrous et al., 2011).

Continuous developments and new IMS approaches are in motion, such a case is the three-dimension (3-D) IMS. Normal IMS analysis provides information about intensity and localization of ion species across the sample surface. Multiple sections can be computationally superimposed to provide three-dimensional analysis of the sample (Seeley and Caprioli, 2012).

3.4.1.2 MALDI-TOF IMS coupled with LC-MS/MS

As overviewed above, MALDI-IMS can provide a variety of insights into different biological samples without prior extraction of molecules. This innovative mass spectrometry approach provides a measurement of molecular weight of a specific ion, its distribution and also abundance across space, but not the identification of the

protein present (Groseclose et al., 2007). Coupling IMS with bottom-up approaches such as liquid chromatography (LC) and tandem MS fragmentation can add crucial information about the ion species detected in IMS, including its identification (Stasulli and Shank, 2016). The most common workflow for protein identification of the ion species detected in IMS is based on extraction, enzymatic digestion followed by mass spectrometry analysis (Theron et al., 2016). Even if this approach is effective, it is also tedious and it breaks the link between the imaging and the identification of the ions species, resulting in a loss of spatial information. However, this procedure remains an efficient way of identification of an unknown peak in an imaging spectrum (Groseclose et al., 2007). It was the identification approach implemented in the first paper of this thesis and other publications have used the same work pipeline to explore the metabolites of different microbes (Phelan et al., 2014; Floyd et al., 2015; Phelan et al., 2015; Frydenlund Michelsen et al., 2016; Santos et al., 2018).

3.4.1.3 IMS in Microbiology

Over three decades ago, a mass spectrometer was for the first time used in order to differentiate bacterial species (Anhalt and Fenselau, 1975). Since then, MALDI-TOF mass spectrometry has led to the identification of over 1000 different species. This is made by the comparison of sample mass spectrum against spectral libraries with reference bacterial mass spectra (Randall et al., 2014). This approach has reached a sufficient level of accuracy that it is used in diagnosis laboratories in order to faster identify the bacterial agents' causative of disease from a particular patient (Risch et al., 2010). The reason for the success of this approach is due to a heavy abundance of small ribosomal proteins in the detected ions by MALDI intact cell analysis (mass range until 15 kDa). Which due to their uniqueness within each genus and species are well suited for the reliable identification of microbes (Santos et al., 2015). This molecular stability in this range could be seen as an obstacle for the discovery of less abundant biomarkers in imaging approaches, however IMS has been able to detect chemical signals in a spatially resolved manner in order to unveil bacterial inter- and intraspecies interactions (Watrous et al., 2010).

The most usual method in IMS approach to study microbes is the growth of microbial colonies on top of thin slices of agar that are after covered with matrix. It has been successful in exploring the spatial distribution of secondary metabolites produced by

marine cyanobacteria filaments (Gonzalez et al., 2012) and to identify m/z intervals that have a putative relation with biofilm development in *Bacillus cereus* (Stasulli and Shank, 2016). A similar approach managed to determine metabolic differences in *Lysobacter* strains culture with *Rhizoctonia solani* fungus (de Bruijn et al., 2015). A different study that also implemented a co-culture method explored the interaction between *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (Moree et al., 2012). However, this agar based approach is encumbered by the presence of high concentrations of salts that can lead to ion suppression or uneven matrix crystallization (Hoffmann and Dorrestein, 2015).

The study of bacterial pathogens with implication in human health has also been a target of IMS studies. Antibiotic moxifloxacin action was monitored through imaging in rabbit lungs infected with *Mycobacterium tuberculosis* (Prideaux et al., 2011). In a similar approach to the one undertaken in this thesis, Floyd et al. detected protein species within UroPathogenic *Escherichia coli* (UPEC) biofilm surface. In this approach, the biofilm was grown on top of the glass slide and the study resulted in the determination of the spatial localization of two adhesive fibers critical for UPEC biofilm formation and virulence (Floyd et al., 2015). With a similar method but this time in *Enterococcus faecalis* biofilms it was possible to explore and localize multiple peptides and proteins within this intact biofilm (Blaze et al., 2012).

3.4.1.4 Data analysis in IMS

A single Imaging Mass Spectrometry study can originate a vast data set of spectra (up to 50 thousand spectra), with each raw spectrum detailing the intensities detected for hundreds of different ions species (m/z values) representing multiple molecules (Alexandrov, 2012). This level of complexity requires automated high throughput software in order to extract useful information, one example is the Scils lab software for imaging data set analysis. For each given m/z value, a heat-map can be obtained of its intensity across the sample region analysed. Meaning that the signal intensity at this m/z through all gathered spectra can be pictured as a coloured image where each pixel is rendered according to its intensity (Alexandrov et al., 2010). The ultimate goal of IMS studies is to find ions of interest from the collected m/z values. These ions and their differential abundance or spatial localization can add relevant information for the discrimination of tumour regions when compared with a control section (Djidja et al.,

2010) or bacterial biofilm adaptation to a stress condition when compared to an ambient one (Santos et al., 2018). However, one of the limitations of the MALDI intact cell analysis is that the more abundant proteins will result in a higher accumulation of ions species of these proteins, shadowing less abundant but potentially interesting biomarkers (Stauber et al., 2010). A second major challenge is spot-to-spot variability within the same IMS analysis due to shifts in desorption/ionization efficiency (Blaze et al., 2012). These differences in ionization are due to ion suppression, non-uniform concentration of matrix throughout the sample and detector noise (Watrous and Dorrestein, 2011; Vertes et al., 2012). Approaches to overcome these obstacles have been put in motion, as for example the use of isotope labelled internal standards and comparison with LC-MS data (Koeniger et al., 2011; Li et al., 2017). Other advanced data processing tools are in development to battle this variability in IMS works (Watrous et al., 2011).

3.4.2 Bottom up shotgun proteomics by label-free quantitative LC-MS/MS

The proteome high level of complexity requires efficient separation technologies to simplify its analysis. As discussed before, the “soft-ionization” method that enable the transfer of peptides and proteins as whole molecules into the gas phase was crucial to reduce this complexity (Fenn et al., 1989). A mass spectrometer determines a m/z from gas-phase ions and eventually deduces their molecular mass.

First, the peptides are ionized in the ion source, then dependent on the mass these ions will be differentially separated and finally detected. The end result is a mass spectrum plotting the ion abundance versus m/z (Wohlbrand et al., 2013). Ion velocities and oscillation frequencies in electromagnetic fields are intensely dependent on the ions m/z . This dependency is the basis of a correct mass determination by MS (Marchetti-Deschmann and Allmaier, 2011). The reliable protein identification relies on high mass accuracy and sensitivity, and these factors are related to the ability of the MS pipeline to resolve peptide ions with similar m/z (Chait, 2006).

3.4.2.1 Reversed phase HPLC separation

Proteins are identified through the detected mass-to-charge ration of their peptides and fragments, thus sufficient separation is needed for un ambiguous identification (Yates et al., 2009). Reason why an optimal HPLC separation is crucial prior to mass

analyses. Reversed phase (RP) HPLC separation is based on the molecule hydrophobicity within an acidic aqueous mobile phase with a gradient of increased hydrophobicity. The mixture of peptides runs in a non-polar stationary phase C18 chains bound to base silica composition (Wohlbrand et al., 2013). In the mobile phase peptides are separated in low organic solvent. (Nirenberg, 1994). The hydrophobic nature of the peptides is based on its amino acid composition which in turn will influence the retained peptides elution in order of increasing strength of hydrophobic interaction with the stationary phase (Qiu et al., 2011). RP-HPLC presents as an optimal separation method to be coupled with ESI (Wohlbrand et al., 2013).

3.4.2.2 Electrospray ionization (ESI)

Electrospray ionization (ESI) through high voltage (2-6kV) produces ions from a solution. This voltage is applied between the capillary end after LC separation and the inlet of the mass spectrometer. Figure 16 is a macro photograph of the electrospray mechanism establishing a continuous electrically charged spray, followed by formation and desolvation of analyte-solvent droplets (Taylor Geoffrey, 1964). In this thesis workflow, a technique named nano-ESI was used in order to lower the flow rates to a nanoliter-per-minute. This improves sensitivity, plus it is compatible with RP-HPLC columns (Griffin et al., 1991).



Figure 16 - Photograph of a stable cloud, also known as Taylor cone, created by electrospray ionisation. From (Smith et al., 1988).

3.4.2.3 Orbitrap MS/MS

Throughout this thesis, a particularly powerful and versatile mass analyser was used. The Orbitrap, that features high resolution (up to 240,000), high mass accuracy (2–5 ppm), a mass-to-charge range of 6000, and a dynamic range greater than 10^3 . The high mass accuracy of the Orbitrap enables a vaster data acquisition and analysis approaches compared with a low-resolution mass spectrometer (Yates et al., 2009). The Orbitrap system illustrated in Figure 17 has a central electrode with a constant electrostatic potential, analyte ions will be electrostatically attracted to the inner electrode creating orbital trajectories. Ions with different velocity will have different rotational frequencies. Ions will then spread into rings that oscillate along the inner electrode. Through a Fourier transformer it is possible to obtain the oscillation frequencies, resulting in an accurate mass spectrum (Makarov, 2000; Hu et al., 2005). In tandem MS (MS/MS), there are two consecutive steps of mass determination. In the first step, a precursor ion of interest is isolated through its m/z . Followed by a second step, where this precursor ion is fragmented and the masses of the fragments are examined (Wohlbrand et al., 2013). Orbitrap shotgun proteomic based approaches are time and cost efficient, enabling comparisons of a large number of proteins out of samples from multiple physiological conditions (Armengaud, 2013). Such facet is perfect for the challenge of exploring the proteome shifts induced by different temperatures in this thesis second and third papers.

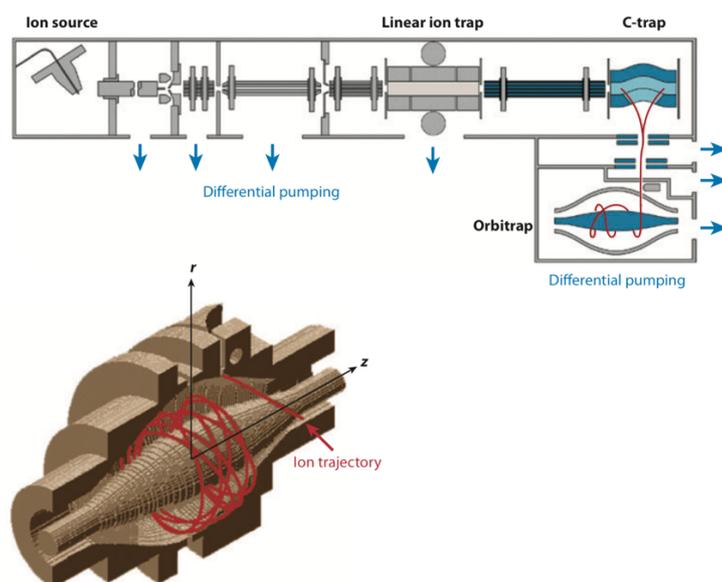


Figure 17 - Scheme of the ion trap-orbitrap hybrid instrument and cross section of the Orbitrap analyser. Ions point of entry is by marked by the red arrow. From (Hardman and Makarov, 2003; Hu et al., 2005; Yates et al., 2009).

Results

1. Summary and objectives

Almost a century has passed since *L. monocytogenes* was recognised as human pathogen. This ubiquitous Gram-positive facultative intracellular bacterial pathogen is the etiological agent causative of listeriosis. Although this infection is rare in occurrence, in comparison with infections caused by other common foodborne pathogens, listeriosis is associated with a high mortality rate of roughly 20–30%. *L. monocytogenes* has also the capacity to adapt and survive to a multitude of harsh conditions, such as desiccation, low temperature and pH, which makes it a long-time concern for food industries that rely on the control of these physical conditions and other decontamination procedures to which *Listeria* seems resilient. Case in point, the epidemiological research of the last decades confirmed with no reservation that listeriosis outbreaks are largely caused by consumption of contaminated food products. Furthermore, the number of listeriosis cases has been increasing over the years, partly due to changes in consumer lifestyles, with a decrease in time available for food preparation and increased consumption of RTE and takeaway foods. This pathogen ability to form biofilm and the difficulty of eliminating sessile cells is a concern for the food industry with recurrent contamination by persistent strains. Though biofilms are the prominent growth mode of bacteria in the environmental realm, the model of choice in microbiology research is still the planktonic mode of growth.

Proteomics is considered as a pertinent science that brings new relevant information from the active and continuous changes inside of a bacterial cell. Plus, due to the evolution of mass spectrometry apparatus and robustness in proteomic techniques, it is possible to explore through the complexity of a sessile agglomerate of cells. The putative proteins of *L. monocytogenes* biofilms that are differentially expressed in regards to different environmental conditions might further help us understand its unique resistance capacity. Although multiple genomic and transcriptomic research approaches have been developed around *Listeria*'s temperature and dehumidification adaptation, the same cannot be mentioned for proteomic approaches, particularly in the biofilm mode of growth.

On these bases, the general goal of my thesis work was to explore the proteome of *L. monocytogenes*, particularly in biofilm mode of growth, under different environmental and food plant conditions, specifically dehumidification and adaptation to three

temperatures. The first work task has three particular aims. First, to develop an Imaging Mass Spectrometry (IMS) approach that would allow to explore the proteome of intact cells in biofilms. Secondly, to subject this biofilm to a dehumidification condition and implement the imaging approach to sort any differences in the biofilm proteome. Lastly, to identify the proteins from the normal and stressed sections of the biofilm, exploring *L. monocytogenes* adaptation to a dehumidification condition, while developing an *in situ* extraction of proteins and bottom-up identification to bridge the gap with the imaging data. The second work task aims to explore, by a label-free shotgun proteomics approach, the proteome of *L. monocytogenes* grown in different temperatures. Preceded by a paramount comparison, made with LC-Progenesis, of the protein abundance shifts registered in planktonic and biofilm mode of growth. To note that the work of this thesis was seconded by the List_MAPS project, which received funding from the European Union's Horizon 2020 research and innovation programme under the Marie- Skłodowska Curie grant agreement n° 641984.

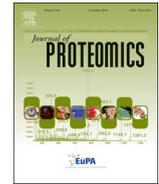
2. MALDI IMS to explore *Listeria monocytogenes* biofilms subjected to dehumidification

Until recently, the study of bacterial biofilms implied the use of traditional techniques to visualize protein distribution. Methods like fluorescent microscopy or use of specific antibodies to a protein of interest, which are limited to previously identified protein targets. MALDI TOF Imaging Mass spectrometry is a surface-sampling technology that can determine spatial information and relative abundance of molecules directly from a biological sample, without any prior protein extraction. In order to explore and compare the proteome of a normal *L. monocytogenes* biofilm to one subjected to a dehumidification condition, we developed an IMS approach to study intact biofilms. Further analysis by shotgun proteomics allowed the cross connection between imaging ion species and the identified proteins in 5 instances.

MALDI Mass Spectrometry Imaging and *in situ* Microproteomics of *Listeria monocytogenes* biofilms

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Published in Journal of Proteomics on September 2018, 187 (2018) 152–160.



MALDI mass spectrometry imaging and *in situ* microproteomics of *Listeria monocytogenes* biofilms



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ARTICLE INFO

Keywords:
MALDI MSI
LC-MS/MS
Listeria monocytogenes
Biofilm
Dehumidification stress

ABSTRACT

MALDI-TOF Mass spectrometry Imaging (MSI) is a surface-sampling technology that can determine spatial information and relative abundance of analytes directly from biological samples. Human listeriosis cases are due to the ingestion of contaminated foods with the pathogenic bacteria *Listeria monocytogenes*. The reduction of water availability in food workshops by decreasing the air relative humidity (RH) is one strategy to improve the control of bacterial contamination. This study aims to develop and implement an MSI approach on *L. monocytogenes* biofilms and proof of concept using a dehumidified stress condition. MSI allowed examining the distribution of low molecular weight proteins within the biofilms subjected to a dehumidification environment, mimicking the one present in a food workshop (10 °C, 75% RH). Furthermore, a LC-MS/MS approach was made to link the dots between MSI and protein identification. Five identified proteins were assigned to registered MSI *m/z*, including two cold-shock proteins and a ligase involved in cell wall biogenesis. These data demonstrate how imaging can be used to dissect the proteome of an intact bacterial biofilm giving new insights into protein expression relating to a dehumidification stress adaptation. Data are available via ProteomeXchange with identifier PXD010444. **Biological significance:** The ready-to-eat food processing industry has the daily challenge of controlling the contamination of surfaces and machines with spoilage and pathogenic microorganisms. In some cases, it is a lost cause due to these microorganisms' capacity to withstand the cleaning treatments, like desiccation procedures. Such a case is the ubiquitous Gram-positive Bacterium *Listeria monocytogenes*. Its surface proteins have particular importance for the interaction with its environment, being important factors contributing to adaptation to stress conditions. There are few reproducibly techniques to obtain the surface proteins of Gram-positive cells. Here, we developed a workflow that enables the use of MALDI imaging on Gram-positive bacterium biofilms to study the impact of dehumidification on sessile cells. It will be of the most interest to test this workflow with different environmental conditions and potentially apply it to other biofilm-forming bacteria.

1. Introduction

After the first MALDI imaging application by Caprioli and colleagues [1], imaging mass spectrometry has become a widespread method for the analysis of small molecules and biomarkers. Mass spectrometry imaging (MSI) enables the *in situ* analysis of molecular species in intact biological samples, overcoming the limitation of other techniques that require cell disaggregation for analyte extraction or the use of exogenous labels [2–4]. It allows visualizing the spatial distribution of metabolites and proteins from mammalian tissues [5–8], plants [9, 10] and several reports involving microbiology approaches [3, 4, 9, 11–16]. MALDI-TOF MS is by now, a frequently used tool for the bacterial

species identification. The resulting average mass spectra can be used as a bacterial biomarker, corresponding mostly to ribosomal proteins [17]. This composition represents a significant challenge for the detection of a fine and residual bacterial adaptation to an environmental condition [18]. Still, MSI goes beyond MALDI profiling with a reduced intra-sample variability, potentially shading a light into discretely concentrated ion species [4, 19].

The variety and number of proteomic approaches in microbiology studies are vast. Today, most proteomic studies are carried out using shotgun approaches, 2D-PAGE being considered useful only for a restricted number of applications [20]. This approach has been gradually replaced by the use of tandem mass spectrometry analysis of tryptic

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<https://doi.org/10.1016/j.jprot.2018.07.012>

Received 28 February 2018; Received in revised form 16 July 2018; Accepted 16 July 2018

Available online 30 July 2018

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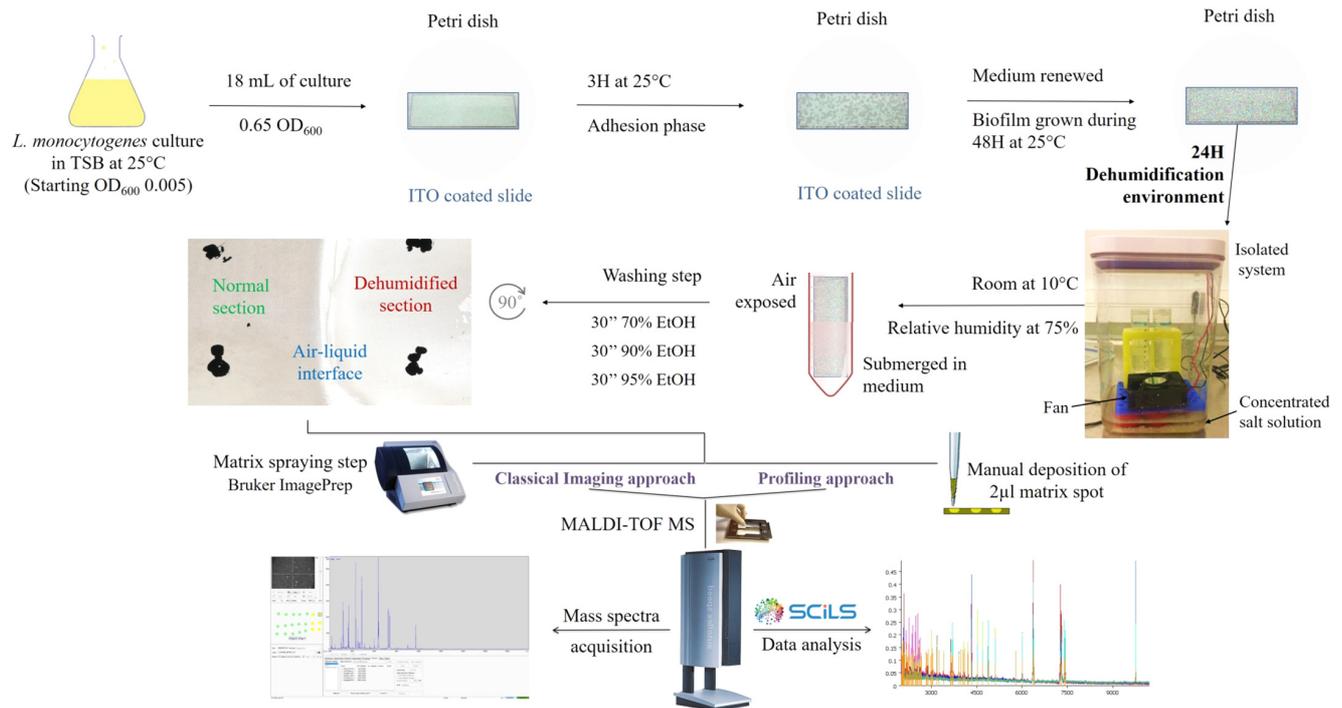


Fig. 1. Schematic workflow for the MALDI mass spectrometry imaging analysis of *Listeria monocytogenes* biofilms using two approaches: a classical imaging approach (matrix sprayed on top of the slide) and a profiling one (manual deposition of the matrix).

peptides derived directly from protein mixtures [21–23]. In both cases, there are numerous study examples of bacterial adaptation to different environments and stress conditions [24–30]. One particular interest focus on bacterial proteomics is the study of surface proteins. Surface proteins are “identity cards” of bacterial cells and have particular relevance in the bacterial interaction with its environment [31]. They are involved in signalling events, transport, adherent growth and therefore are the predominant factors contributing to adaptation to stress conditions [32]. However, there are few reproducible techniques to obtain the surfaceome of Gram-positive cells [33]. Moreover, no single method provides the all range of surface proteins, leading to a multitude of complementary and non-exhaustive methods to achieve this sub-proteome [34]. In this study, we intend to use first generation proteomics, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and a second-generation proteomic-based approach, liquid chromatography-tandem mass spectrometry (LC-MS/MS), to evaluate its potential as a faster tool to evaluate the surfaceome.

Listeria monocytogenes is a ubiquitous Gram-positive bacterium that can grow at refrigeration temperatures ($> 1^\circ\text{C}$) [35], a wide pH range (pH 4–9) [36] and form biofilms [37, 38]. *L. monocytogenes* is widespread, and it is of great concern in the food industry since this pathogen is the causative agent of listeriosis, by the ingestion of contaminated foodstuff [39]. It is listed to be one of the five major food-related microorganisms causing foodborne disease and being able to survive for several months on stainless steel surfaces [40], and in a biofilm mode of growth, it was reported the survival of *L. monocytogenes* up to 49 days against a desiccated environment [41]. Bacterial studies tend to be made with controlled laboratory condition isolates in planktonic cultures. However, in the natural and food environments, microbes are often found as populating complex, these surface-attached communities are exposed and respond to different environmental conditions [13]. One ubiquitous changing condition is water availability, and bacterial communities are subjected to daily and seasonal variations of air relative humidity (RH). These variations are also frequent in

food industries due to cleaning and disinfection procedures [42]. Bacterial cells growing in a biofilm have higher resilience against stress conditions, including increased resistance to desiccation after cell attachment [42, 43]. Here, a dehumidification condition was used as a proof of concept of the MALDI IMS approach.

Bacterial mediums are required for the formation of a bacterial mat; the latter can take a three-dimensional biofilm shape. This structure resembles a biological tissue, and thus it can be explored by MSI. However, the challenge associated with this biological material is the presence of salts in the culture medium which, even after the latter has been eliminated, impairs a correct co-crystallization between a matrix layer and the sample [3]. The highly lipid-rich extracellular composition is an added complication [3]. Thus, commonly used cleaning procedures in imaging tissue research may be applied [44]. Possibly due to this technical constraint, little work has been done with *in situ* imaging proteomics in intact bacterial biofilms, except an approach to the adhesion capacity of *Escherichia coli* biofilms and the interaction between *E. coli* and *Enterococcus faecalis* biofilms co-cultured on an agar surface [45, 46].

In this study, pre-grown *L. monocytogenes* biofilms were submitted to dehumidification, mimicking the food industry daily cleaning-disinfection events. Then, a combination of MSI, MALDI profiling and LC-MS/MS was used to explore the effect of dehumidification stress on *L. monocytogenes* biofilms. To our knowledge, this is the first report that applies an MSI approach to study an environmental stress effect on a Gram-positive bacterial biofilm.

2. Material and methods

2.1. Sample preparation

The *L. monocytogenes* EGD-e strain was grown in tryptic soy broth (TSB) medium at 25°C . A pre-culture in stationary phase was used to inoculate a culture at a final $\text{OD}_{600\text{nm}}$ of 0.005 and cells were allowed to grow during 3 h. Cells were then harvested by centrifugation ($7500 \times g$,

15 min) and resuspended in MCDB medium with sterile water in a volume equal to that of the supernatant collected reach an OD_{600nm} between 0.6 and 0.7. Eighteen mL of the bacterial suspension was poured on each indium tin oxide coated glass slide (ITO) placed in a sterile petri dish (55-mm diameter) and incubated at 25 °C. Bacterial cells were allowed to adhere during three hours before removing the medium, eliminating the planktonic cells. Fresh culture medium was added, and the cells were incubated for 48 h more at 25 °C. The slides were placed in a 50-mL tube, with liquid medium until half, promoting the exposure to the air conditions of half of the slide. The open tube was then arranged inside a container enclosing a saturated sodium chloride solution allowing to obtain and control the air RH at 75% (Fig. 1). This device was placed in a cold room at constant 10 °C. After 24 h of dehumidification exposure, the glass slides were washed to remove salts and deplete lipids in sequential 30-s washes of 70, 90, and 95% HPLC-grade ethanol [44, 45]. As a result, the workflow yield *L. monocytogenes* biofilms with 2 sections of analysis and an interface between them.

2.2. MALDI mass spectrometry imaging

For matrix application and mass spectra acquisition, two approaches have been put in motion (Fig. 1). One, using a standard imaging methodology (classical approach), the matrix was applied using the ImagePrep station (Bruker Daltonics), and a section of the biofilm was screened. The lateral resolution was set to 100 μ m, and a total of 500 laser shots were accumulated per pixel at constant laser power. The second approach implemented profiling parameters with a lower lateral resolution of 2000 μ m, a manual deposit of a 2 μ L droplet of the matrix, and a high accumulation of shots per spot (4000 hits). In both approaches, the matrix used was 7 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA) in water/acetonitrile 50:50 (v/v) with 0.2% trifluoroacetic acid. The α -cyano-4-hydroxy-cinnamic acid (CHCA, 99%), trifluoroacetic acid (TFA, 99%), acetonitrile (\geq 99.9%), and methanol (\geq 99.9%) HPLC grade were purchased from Sigma-Aldrich (Steinheim, Germany), conductive indium tin oxide (ITO)-coated glass slides and peptide calibration standard II from Bruker Daltonics (Bremen, Germany). The mass spectrometer used was an Autoflex Speed MALDI-TOF/TOF with a Smartbeam laser using FlexControl 3.4 and FlexImaging 3.0 software packages (Bruker Daltonics). Ions were detected in positive linear mode at a mass range of m/z 2000–15,000 with a sampling rate of 0.31 GS/s. Deflection was set at m/z of 2000, and laser focus on medium. The analysis was performed using a detector gain of 2.500 V, ion source voltage 1 at 19.56 kV, ion source voltage 2 at 18.11 kV and lens voltage at 7 kV. A protein standard (Protein Calibration Standard I, Bruker Daltonics) was employed for external calibration of spectra, which was done externally on the same target before each measurement. Spectral data were loaded into SCiLS Lab 2016 software (HTTP://scils.de/; Bremen, Germany). The following workflow was used for data treatment: baseline subtraction (TopHat algorithm), normalization (total ion current algorithm), manual peak picking, peak alignment and spatial denoising [47].

2.3. Microproteomics in situ protein extraction and peptide analysis by LC-MS/MS

Proteins extracts for shotgun proteomic were extracted from different biofilm slides grown in the same conditions as the ones used for MALDI Imaging and MALDI profiling approach, to identify m/z ion species observed in MSI (Fig. 2). Three spots per section were manually extracted and three biological replicates performed. The sample was covered with 2 μ L of 7.5% acetonitrile in 0.2% trifluoroacetic acid and incubated for 1 min. The liquid containing the protein extract was collected, and this extraction was repeated once. The same area was then covered with 1 μ L of 60% acetonitrile in 0.2% trifluoroacetic acid and the liquid containing the protein extract was immediately collected and combined with previous extracts (5 μ L total). Samples were purified

using C18 spin columns (Pierce, Thermo Scientific) and the supplier protocol. Proteins were reduced with 20 μ L of 100 mM dithiothreitol in 50 mM ammonium bicarbonate (pH = 8), during 15 min at 55 °C. After cooling, protein alkylation was performed by adding 20 μ L of 100 mM iodoacetamide in 50 mM ammonium bicarbonate, during 15 min at 20 °C in darkness. After neutralization, protein digestion was achieved by adding 25 μ L of trypsin solution (20 ng/ μ L) in 50 mM ammonium bicarbonate, and overnight incubation at 37 °C. Trypsin digestion was stopped with 2% trifluoroacetic acid, and after 5 min centrifugation at 3000 \times g, the supernatant was collected. The pellet was washed using 2% acetonitrile, 0.05% trifluoroacetic acid in water, sonicated for 5 min, centrifuged for 5 min at 3000 \times g, and the supernatant was collected and combined with the previous one, resulting in 100 μ L samples. The mixtures of peptides were then analyzed by nano-LC-ESI-MS/MS. For these analyses, 2 μ L of peptide mixture was run in duplicate by online nanoflow liquid chromatography using the Ultimate 3000 RSLC (Dionex, Voisins le Bretonneux, France) with a nanocolumn of 15 cm length \times 75 μ m I.D., 3 μ m, 100 Å (Acclaim PepMap100C18, Dionex, Thermo Fisher Scientific, Waltham, MA, USA). After concentration 6 min on a loading column (0.5 cm, 300 μ m, Thermo Fisher), the peptides were separated on nanocolumn, with a linear acetonitrile gradient from 4% to 50% acetonitrile in 0.5% formic acid at a flow rate of 400 nL/min for 50 min. The eluate was electrosprayed in positive-ion mode at 2.7 kV in a LTQ-VELOS mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source which was operated in a CID top 10 mode (i.e., one full scan MS and the 10 major peaks in the full scan were selected for MS/MS). Full-enhanced-scan MS spectra were acquired with one microscan (m/z 400–1400). Dynamic exclusion was used with one repeat counts and 120 s exclusion duration. For MS/MS, isolation width for ion precursor was fixed at 2 m/z , single and four and above-charged species were rejected; fragmentation used 37% normalized collision energy and a default activation of 0.250. After transformation of raw in .mgf data, Mascot Daemon (version 2.5.1) was used for database search. For protein identification, the *listeria_mEGDe* (2844 sequences, 2017/08) protein database was used. The following parameters were considered for the searches: peptide mass tolerance was set to 500 ppm, fragment mass tolerance was set to 0.6 Da, and a maximum of one missed cleavages was allowed. Variable modifications were methionine oxidation (M) and carbamidomethylation (C) of cysteine. Protein identification was considered valid if at least two peptide with a statistically significant Mascot score assigned it (False Discovery Rate (FDR) at 1%). Protein-protein interaction networks were determined using the STRING v.10 software (with the default setting). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [48] partner repository with the dataset identifier PXD010444 and <https://doi.org/10.6019/PXD010444>.

3. Results and discussion

3.1. *Listeria monocytogenes* biofilms and dehumidification stress induction

Here, we present an MSI approach and used it to explore the protein expression and their *in situ* distribution within intact *Listeria monocytogenes* biofilms exposed or not to a dehumidification condition. To achieve that, ITO coated slides were submerged in bacterial culture and grown during 48 h to form a bacterial mat on top of the slide (Fig. 1). However, the bacterial mat obtained was not entirely homogeneous, and it was composed of prominent cell aggregates and small gaps with low cell abundance [49]. This lack of homogeneity may result in low reproducibility or accumulation of a noisy signal [13]. After the slides were placed vertically into media, such that only half of the slide was submerged within the media, the other half was exposed to the controlled air dehumidification condition present in an airtight container. The workflow development yield *L. monocytogenes* biofilms with two sections, one immersed in the culture medium and one exposed to 75%

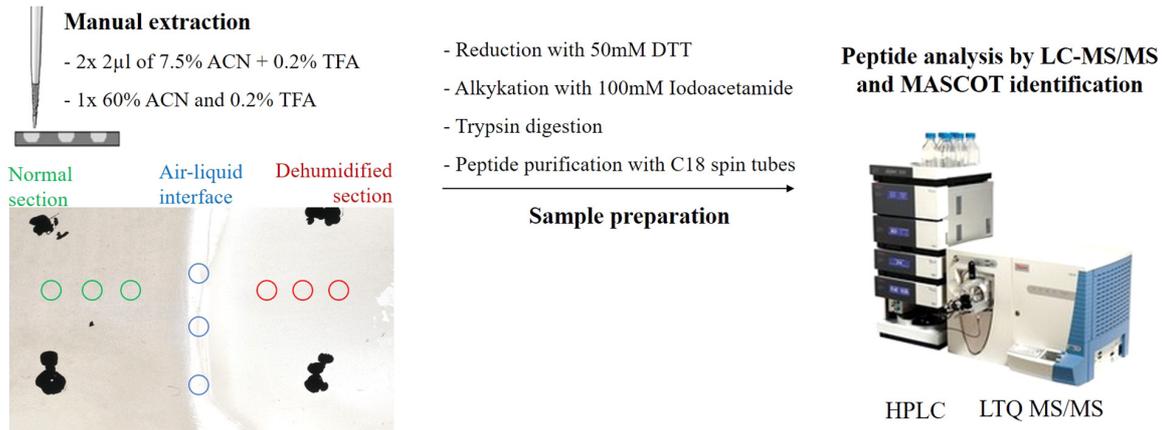


Fig. 2. Schematic workflow for the *in situ* protein extraction and LC-MS/MS peptide analysis from 3 spots per section of *L. monocytogenes* biofilms subjected or not to air dehumidification.

air RH, plus an interface between them. Two mass spectrometry approaches were implemented to compare their ability in dissecting the spatial proteome of *L. monocytogenes* biofilms.

3.2. MALDI MSI and MALDI profiling of intact biofilms

MALDI profiling or MALDI intact cell analysis is nowadays a regularly used tool in clinical microbiology, due its reproducibility and accuracy in the identification of bacterial species [50]. When compared with a classical imaging approach, the main differences are in the deposition of the matrix and the mass spectrometer parameters [19]. For MALDI profiling, deposition of the matrix was made manually with 2µl matrix spot and mass spectra was acquired with a low spatial resolution (2000µm), but a higher spectral resolution (Fig. 3a), meaning a mass spectrum with a lower level of background noise and well-defined ion peaks. As for MSI, matrix deposition was performed by a spraying

device (Bruker ImagePrep), resulting in an even layer of the matrix on top of the biofilm. In this approach schemed in Fig. 3b, a section of the biofilm was screened with a higher spatial resolution of 100µm and lower accumulation of shots per spot (500 hits). The MALDI approaches and matrix selected were optimized for lower molecular weight protein species. Thus, all analyses were carried out over an ion range of mass-to-charge ratio (*m/z*) 2000–15,000. Spectral and image datasets were analyzed using the software SCiLS Lab, and high-quality mass spectra were obtained in both approaches (Fig. 3c, d). The profiling presented a lower level of background noise and higher intensity of peaks than the classical approach. This result can be attributed to the higher ratio matrix-sample present in the profiling and the superior signal accumulation obtained in the one spot analysis. Still, after SCiLS processing, MSI data gave a higher amount of statistically significant (ANOVA *p*-value < .05) *m/z* than the profiling (47 vs 31 ion species detected, Supplementary Table 1). In these *m/z* lists, only 10 *m/z* (± 5 Da) where

Profiling approach

Classical imaging approach

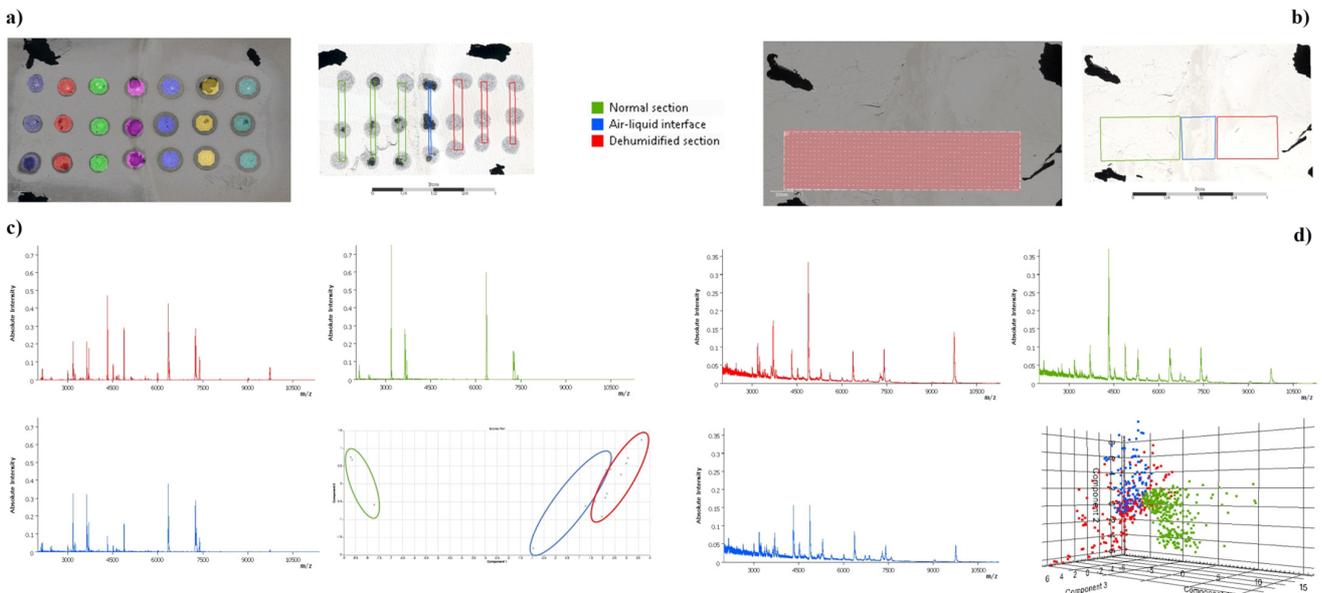


Fig. 3. Mass spectral data obtained from the two approaches. a) Spot to spot analysis of a biofilm implemented with an intact cell analysis parameter (Resolution: 2000 µm; 4000 shots per spot); b) Screening across a large section of biofilm by classical imaging approach (Resolution: 100 µm; 500 shots per spot). c) Profiling result displayed in the average mass spectrum per section of analysis and principal component analysis. d) Profiling result displayed in the average mass spectrum per section of analysis and principal component analysis.

commonly found in both approaches. The resulting table of mass-to-charge ratios (m/z) is provided in Supplementary Table 1. Via the manual matrix deposition, the dried droplet matrix suffers a nonuniform co-crystallization of matrix and sample, which causes sample and matrix segregation into separate regions within the dried droplet [51], together with a lower lateral resolution these could be the factors behind the fewer peaks present in the profiling approach. Within this mass range, we observed fewer m/z when compared with the analysis of *E. coli* biofilms [45]. However, in this study, we are dissecting a Gram-positive bacteria, the thick cell wall could be the obstacle avoiding the ionization of a higher number of ions species [50].

Several well-known limiting conditions can lead to weak mass signal and hamper a quality mass spectrometry result, i.e., spot-to-spot variability due to differences in desorption/ionization efficiency; a likely uneven sample concentration in biofilm due to heterogeneous matrix application; detector noise and limits of detection [4, 46]. However, here, the workflow showed a good average mass spectrum from each section of analysis through both methods (MSI and profiling). The resulting principal component analysis (Fig. 3c, 3d) managed to group the replicate's average mass spectra from each section and separate the different sections. The spatial and depth resolution should also be taking into account in microbiology studies, even more, when dealing with biofilms. The spatial resolution in most MALDI MSI studies is between 100 and 150 μm [46], meaning that each MALDI shot will induce the ionization of molecules from large biofilm aggregates. *L. monocytogenes* biofilms form honeycomb-like structures consisting of layers of cohesive cells intercalated with hollow voids with diameters ranging from 5 to 50 μm [52]. Thus, the MSI screening across the biofilm accumulates mass spectra from areas without sufficient biomass, and when ionization occurs, it will also lead to cell lysis and a strengthening of the primary limiting factor of the imaging technique, the observed ion species are typically those most abundant within the sample [45]. Despite these constraints, the results shown in this section demonstrate that even relatively low spatial resolution can provide useful information on bacterial biofilms, in particular, to obtain a set of average spectra that enable the separation between a normal and stressed biofilm section.

3.3. In situ microproteomics of intact *L. monocytogenes* biofilm

Combining MALDI MSI with methods such as liquid chromatography and tandem mass spectrometry (LC-MS/MS) can provide additional information about unknown protein species obtained from imaging, enabling its identification [16]. The workflow for protein identification implemented here was decoupled from the MSI analysis and resorted to a detailed bottom-up proteomic analysis, from which, a reproducible number of proteins were identified from 3 biological replicates (on average 423 identified proteins from the 3 biofilm slides). 513 different proteins were detected from the protein extracts of the three biological replicates. Venn diagrams in Fig. 4 show that in all cases, the majority of unique proteins was detected in the dehumidified section. This could be linked to a higher percentage of dead cells in this section, and therefore, more efficient extraction of proteins, but also the higher humidity in the normal section could have diluted the cell extraction from this section. An m/z from MSI can be a result of any protein sequence within the mass range of the analysis, so it represents a challenge for protein identification. This fact might figure the reason why from the majority of MALDI MSI publications, only a few have managed to reveal which were the proteins present in the obtained MALDI spectra [53].

Looking at all the identified proteins by section in the m/z range of this study, there was a noticeable high amount of ribosomal proteins identified: 35% in the normal section, 36% in the air-liquid interface and 38% in the dehumidified section (Suppl. Table 2). This result is consistent with the high abundance of these proteins in the bacterial cell, where they account for > 20% of total cell protein [54].

Nonetheless, some stress adaptation associated proteins were identified from the air-liquid interface and dehumidified section (beyond the 15 kDa mass limit, Supplementary Table 2). In the latter, CshA and CshB DEAD-box RNA helicases possibly involved in RNA degradation were identified. These proteins are essential for cold adaptation, and interact with cold shock proteins in *Bacillus subtilis* [55]. A similar annotation has been hypothesized in *Listeria* [56]. Moreover, four proteins from the stress-induced multi-chaperone system, involved in response to hyperosmotic stress, were identified in the dehumidified section. The ClpB, DnaK, DnaJ and GrpE act in the processing of protein aggregates and repair of stress-induced protein damage [57–59].

SpoVG protein-like family in *Listeria* has been associated with lysozyme resistance and hypervirulence [60]. It is relevant to point out the identification of two of these proteins in the dehumidified section, SpoVG1 and SpoVG2 suggests that they might be connected with stress resistance. The protein data from the dehumidified section also showed a new possible association to the hypothesized existence of a stress-sensing complex in *L. monocytogenes* [61]. Stress tolerance in *L. monocytogenes* can be explained partially by the presence of the general stress response (GSR), a transcriptional response under the control of the RNA polymerase alternative sigma B factor (σ^B). The stressosome which is composed of Rsb proteins has been well described in *B. subtilis*. Sensory signals lead to the signalling cascade downstream of the stressosome which ultimately leads to the activation of σ^B in response to the stress [62]. The ability of *L. monocytogenes* to resist many adverse environmental conditions has been attributed in part to activation of σ^B [63], which was not identified in this study. However, four proteins (RsbR, RsbT, RsbV and RsbW) part of the stressosome cascade that activates the general stress response system have been identified in the dehumidified section (RsbW and RsbV were also found in the air-liquid interface). The σ^B -dependent response to cold [64] and osmotic stress [65] was already reported, but here is the first potential association with a dehumidification condition.

As previously mentioned, MALDI MSI shows an interesting insight into the spatial proteome, but the identification of the ion species present in the imaging data is not always an easy task, mainly due to the MALDI's low mass resolution [16]. The use of microproteomics for extraction of proteins followed by shotgun proteomics was well suited for the confident identification of numerous proteins, and several associated with mechanisms of stress adaptation/resistance.

3.4. Connecting the dots between MSI and LC-MS/MS data

Over the years, it has progressively increased the number of MSI workflows that try to make the identification of the detected m/z through a back-correlation with the proteins identified by a shotgun proteomics approach [44]. It is in fact, a challenging task to connect the MSI peaks to a protein identification. The extraction and identification method for proteins applied in this study is one that has been already used in different studies [53]. Many researchers have decoupled the imaging experiment from the protein identification experiment and resorted to bottom-up proteomic analysis of a separate similar sample on which the MALDI imaging was made [66].

Fig. 5 is a visual representation of the dot connection between the average mass spectra from the classical imaging/profiling approach and the identified proteins obtained in Section 3.3. First, we assessed the obtained LC-MS/MS proteins lists, restricting the analysis to the identified proteins present in all biological replicates from each of the three sections (normal section – Fig. 5a; air-liquid interface – Fig. 5b; dehumidified section – Fig. 5c). Furthermore, since MSI was set at a mass range of m/z 2000–15,000, 15 kDa was established as the cutoff mass value used in the lists of identified proteins. As a result, seven proteins in the normal section, 8 in the air-liquid interface and 19 in the dehumidified section followed these criteria (Supplementary Table 1). These proteins are represented in Fig. 5, by a protein-protein interaction network and also listed with their detailed description.

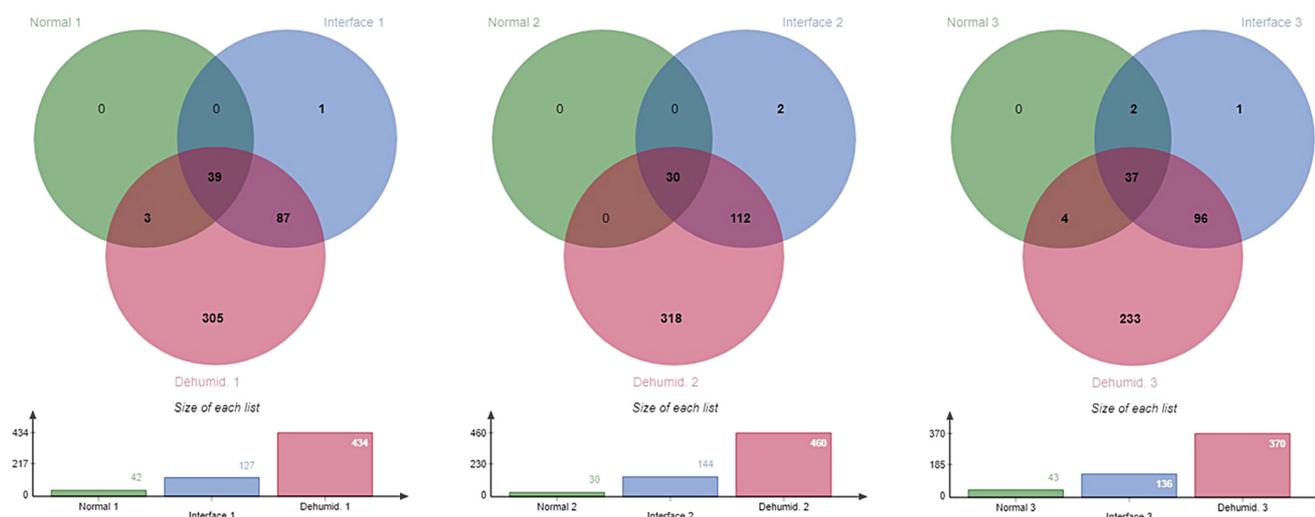


Fig. 4. Venn diagrams for all identified proteins from each of the biological replicates (slide 1: 435 proteins; slide 2: 462 proteins; slide 3: 373 proteins).

The back correlation between the significant m/z and the *in situ* identification was made with a mass tolerance of 0.5% [44], which lead to 5 different proteins being assigned to the mass spectra (dehumidified section). The assigned proteins are highlighted in Fig. 5 lists. From the normal section and air-liquid interface, two proteins were assigned to the average spectra of the MALDI imaging approach, the cold-shock protein CspLA (CspL) and the 50S ribosomal protein L29 (RmpC). The family of small cold shock proteins (Csps), with a cold-shock domain, is commonly associated with stress resistance, but more recently has been reported its involvement in virulence, cell aggregation and flagella-based extracellular motility in *L. monocytogenes* [67]. CspA was characterized as being the most relevant in cold adaptation [68]. Therefore, it is not surprising that this protein was identified in the normal section. Since, this section was also exposed to cold shock stress, thus triggering such a response [67]. These two proteins were also assigned in the dehumidified section together with a second cold shock protein (CspLB), also involved in the adaptation to atypical conditions [69]; plus a tautomerase-like protein and a D-alanine-poly(phosphoribitol) ligase. The latter is involved in the pathway lipoteichoic acid biosynthesis. Loss of D-alanylation of lipoteichoic acids alters the cell surface charge and results in reduced biotic attachment and biofilm production [70]. This protein assigned in the dehumidified section is a new dot in the connection between biofilm formation and resistance to harsh environmental conditions [43]. Lipoteichoic acids are essential for the surface adhesion of *Listeria* cells, and here, it was in the stress condition that this protein was allocated, which suggests that mechanisms similar to those occurring during biofilm formation are induced to withstand conditions of decreasing RH.

Lastly, a classical imaging result, using SCiLS software, shows the spatial distribution of the two cold shock proteins across the biofilm (Fig. 6a, b). In our view, there are some technical challenges with this tool for MSI data analysis: like pixel-to-pixel variability, data quality assurance, noise-tolerant statistical learning algorithms [19]. However, and in agreement with the previous results, the heat-map from the imaging approach data shows that CspLA is present in the three sections but with more intensity in the dehumidified section. As for CspLB, it was identified in the dehumidified section and the air-liquid interface. However, the connection to the MSI m/z was only made in the dehumidified section.

The MALDI profiling mass spectra was also analyzed through bloxspot, to check sample variability (Fig. 6c). The level ion species assigned being the lipoteichoic acid associated protein (dltC) confirmed a higher detection of this protein in the dehumidified section compared

to the other two regions. This fact corroborates the data obtained from the protein identification and back-correlation with the ions species observed in MSI.

Even though, the shotgun approach breaks the link between the identified proteins and the m/z peaks detected in MALDI MSI, the list of identified proteins serves as a base for cross connection. This is for know the best technical option. The direct mass spectrometric sequencing of the detected m/z in a MALDI mass spectrum is usually not possible due to the generally inadequate sensitivity, resolution, mass accuracy and mass range of these instruments in tandem MS mode [53]. In comparison with direct MALDI MS/MS, the shotgun approach has the gain of superior sensitivity and a higher amount of proteins identified. Moreover, here most of the proteins identified by shotgun proteomics were labelled as being ribosomal proteins (< 20 kDa), and the spectral data obtained are similar to the average spectrum obtained in MALDI intact cell analysis of *L. monocytogenes* cells [71, 72]. The MALDI intact cell approach, for biotyping bacterial species, is based on the close similarity of the ribosomal profile in this range, giving weight to some of the peak connections here achieved. This work presents a strategy to connect the dots between MSI data and LC-MS/MS in 5 cases, a similar level of back-correlation to that obtained by Floyd et al. [45], confirming that the approach implemented here was successful. In the foreseeable future, back-correlation levels can reach even higher numbers. For that, it will be crucial to overcome technical constraints involving the limitation to relatively high abundance proteins [46, 73] and the MALDI limitations into ionizing, in linear mode, high molecular weight proteins potentially essential to biofilm adaptation [45]. Additionally, the approach showed the identification of proteins with vital importance in the biofilm adaptation to dehumidification.

4. Conclusions

The first aim of this work was the development and optimization of a workflow that would enable the use of MALDI imaging on Gram-positive bacterium biofilms and then, applying it to study the impact, at the molecular level, of environmental stresses on sessile cells. These stresses consisted of mimicking food workshop conditions in which, after each cleaning-disinfection procedure of surfaces and equipment, the air is dehumidified to eliminate residual water quickly, sanitize the ambient atmosphere and limit contamination spreading.

The MALDI imaging protocol developed allowed us to compare immersed and dehumidified biofilm sections, revealing the spatial localization of up to 47 different protein species within a single analysis.

In situ extraction and identification

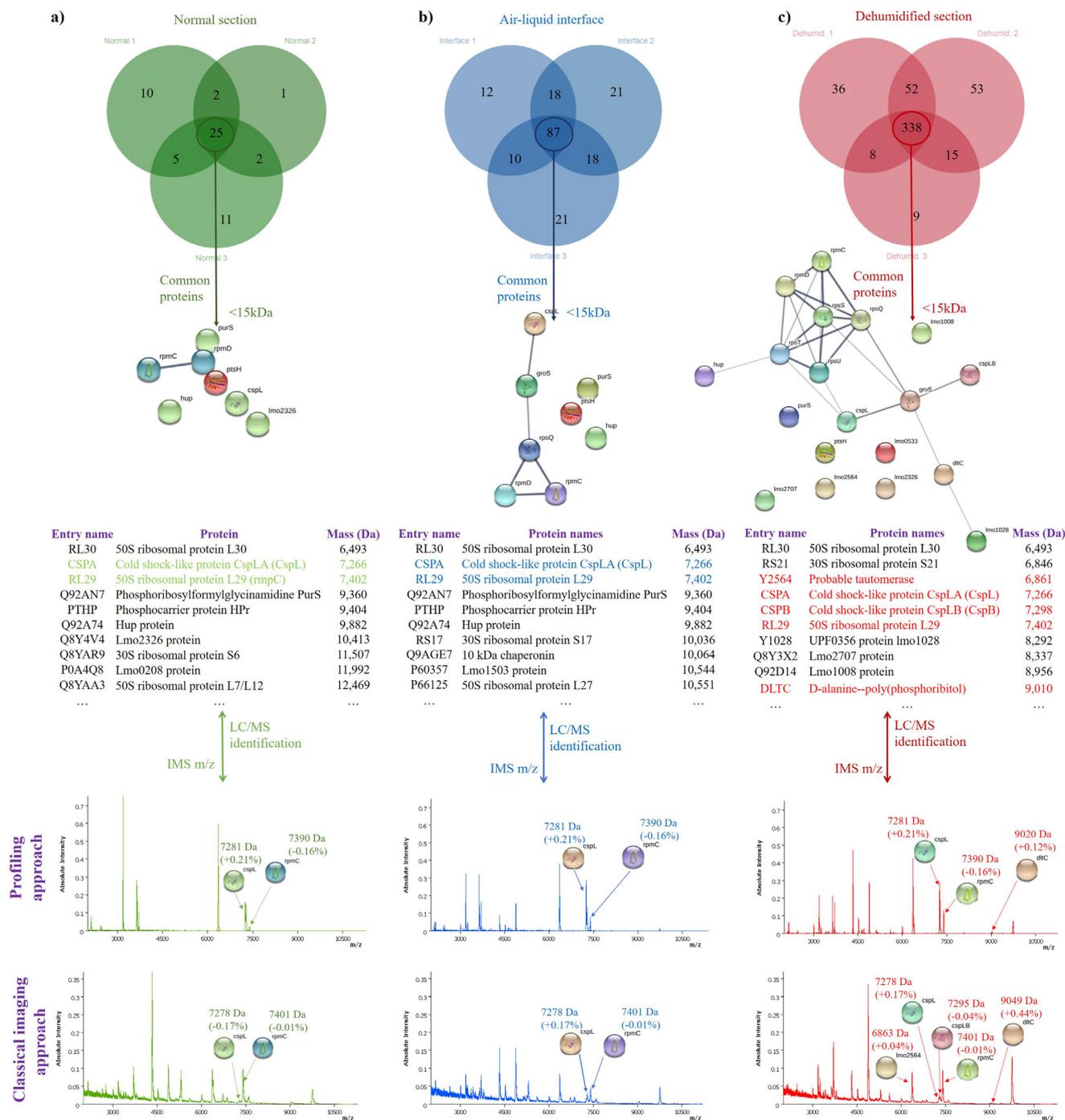


Fig. 5. The schema for the back-correlation between ions species obtained by MALDI (bottom) and protein identification by LC-MS/MS (top) (all mass lists present in Supplementary Table 1). a) Normal section assigns protein identification. b) Air-liquid interface assigns protein identification. c) Dehumidified section assigns protein identification. From top to bottom: Venn diagrams for all identified proteins in the three sections (normal section: 56 proteins; interface: 187 proteins; dehumidified section: 511 proteins); STRING protein-protein interaction with the shared proteins identified (with mass below 15 kDa) from each of the three sections; List description of the proteins present in protein-protein interaction (highlighted proteins correspond to the ones correlated with m/z from IMS and profiling, mass tolerance: 0.5%); Average mass spectra for each of the 3 sections from the two approaches and identification of the proteins successfully assigned.

A microproteomics approach then led us to identify several proteins and to lastly make a back correlation with imaging data the confident identification of 5 proteins in these spectra. It will be of the most interest to detect the variance of these identified proteins in other

environmental conditions and potentially apply this workflow to other biofilm-forming bacteria.

Resorted MALDI IMS and innovative protein identification approaches contributed to dissect the spatial proteome of an intact

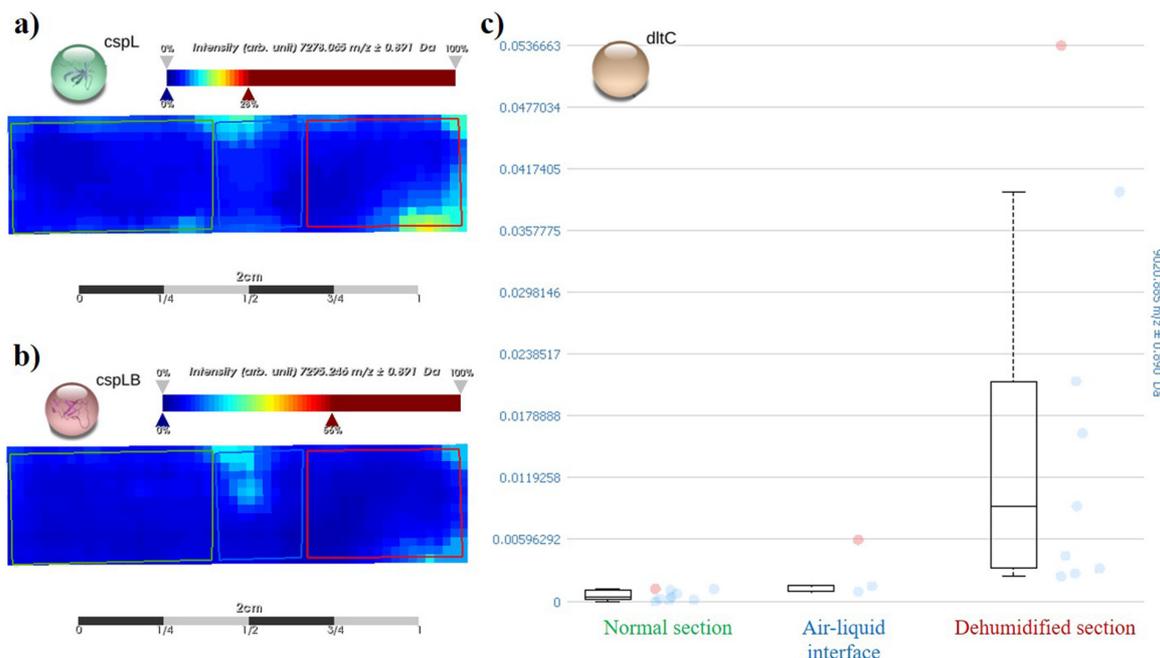


Fig. 6. SciLS output results regarding the abundance of some ion species. a) and b) Heat maps showing the abundance of two ion species across the biofilm, after that assigned as two cold shock proteins from data of the classical imaging approach. c) Boxplot is displaying the abundance of an ion species obtained by profiling, assigned as D-alanine-poly(phosphoribitol) ligase (DltC).

bacterial biofilm giving new insight into protein expression relating to biofilm adaptation to dehumidification. From a general point of view, this approach enriches promisingly the different techniques and methodologies already used to explore and understand the physiology of microorganisms in biofilms [74].

Acknowledgments

Tiago Santos was seconded by List_MAPS project, which received funding from the European Union's Horizon 2020 research and Innovation programme under the Marie-Sklodowska-Curie grant agreement no 641984.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2018.07.012>.

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3. *Listeria monocytogenes* Adaptation to Different Temperatures Seen Through Shotgun Proteomics

L. monocytogenes has the capacity of prevailing and even growing at temperatures close to zero degrees. This temperature adaptation strength combined with its capacity to attach and form a complex biofilm mark this bacterium as a problematic in the food industry. Since proteome changes occur in a very promptly fashion, particularly in a response to a stress condition, the two manuscripts in this section aim to explore and compare variations in protein abundance of *L. monocytogenes* grown in two modes of growth (planktonic vs biofilm) and three different temperatures (10°C, 25°C and 37°C).

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Published in Frontiers in Nutrition on June 2019, 6 (2019) 89.



Listeria monocytogenes Biofilm Adaptation to Different Temperatures Seen Through Shotgun Proteomics

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OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to
Food Microbiology,
a section of the journal
Frontiers in Nutrition

Received: 26 November 2018

Accepted: 24 May 2019

Published: 14 June 2019

Citation:

Santos T, Viala D, Chambon C,
Esbelin J and Hébraud M (2019)
Listeria monocytogenes Biofilm
Adaptation to Different Temperatures
Seen Through Shotgun Proteomics.
Front. Nutr. 6:89.
doi: 10.3389/fnut.2019.00089

Listeria monocytogenes is a foodborne pathogen that can cause invasive severe human illness (listeriosis) in susceptible patients. Most human listeriosis cases appear to be caused by consumption of refrigerated ready-to-eat foods. Although initial contamination levels in foods are usually low, the ability of these bacteria to survive and multiply at low temperatures allows it to reach levels high enough to cause disease. This study explores the set of proteins that might have an association with *L. monocytogenes* adaptation to different temperatures. Cultures were grown in biofilm, the most widespread mode of growth in natural and industrial realms. Protein extractions were performed from three different growth temperatures (10, 25, and 37°C) and two growth phases (early stage and mature biofilm). *L. monocytogenes* subproteomes were targeted using three extraction methods: trypsin-enzymatic shaving, biotin-labeling and cell fractionation. The different subproteomes obtained were separated and analyzed by shotgun proteomics using high-performance liquid chromatography combined with tandem mass spectrometry (LC-OrbiTrap LTQVelos, ThermoFisher Scientific). A total of 141 (biotinylation), 98 (shaving) and 910 (fractionation) proteins were identified. Throughout the 920 unique proteins identified, many are connected to basic cell functions, but some are linked with thermoregulation. We observed some noteworthy protein abundance shifts associated with the major adaptation to cold mechanisms present in *L. monocytogenes*, namely: the role of ribosomes and the stressosome with a higher abundance of the general stress protein Ctc (R125) and the general stress transcription factor sigma B (σ^B), changes in cell fluidity and motility seen by higher levels of foldase protein PrsA2 and flagellin (FlaA), the uptake of osmolytes with a higher abundance of glycine betaine (GbuB) and carnitine transporters (OpucA), and the relevance of the overexpression of chaperone proteins such as cold shock proteins (CspLA and Dps). As for 37°C, we observed a significantly higher percentage of proteins associated with transcriptional or translational activity present in higher abundance upon comparison with the colder settings. These contrasts of protein expression throughout several conditions will enrich databases and help to model the regulatory circuitry that drives adaptation of *L. monocytogenes* to environments.

Keywords: *Listeria monocytogenes*, temperature adaptation, biofilm, subproteomes, shotgun proteomics

INTRODUCTION

The ready-to-eat (RTE) food sector is in constant expansion, offering a wide variety and number of products to consumers. Unfortunately, with that comes an increased chance of microbial contamination, as is the case of *Listeria monocytogenes* (1), a Gram-positive foodborne pathogen bacterium and the causative agent of the illness listeriosis (2). The European Food Safety Authority (EFSA) reported, in Europe, from 2008 to 2015, 37 food-borne outbreaks caused by *L. monocytogenes* that lead to 37 deaths (3). Just from June of 2018, 47 cases have been reported, and nine patients have died due to or with the infection (4). Even if the annual number of listeriosis cases comes behind other major foodborne pathogens (~23,150 listeriosis cases were estimated worldwide in 2010), the mortality among infected individuals is very high, reaching levels up to 30% (5). This ubiquitous biofilm-forming bacterium is found throughout the environment, including soil, vegetation, and animals (6). The agroecosystems play a major role in the spread of such pathogens in the food chain through the production of contaminated raw products (7).

Bacterial cells are often found in complex communities, termed biofilms, that provide resources and protection to harsh environments (8). The trifactor, that includes pathogenic power, ability to form biofilm and ubiquity qualify *L. monocytogenes* as huge risk for human health. Remarkably, most stress-related reports were performed in planktonic cultures (9), even if there is a clear need to study the bacterial responses associated with stress tolerance and the features and benefits conferred by the sessile mode of growth to bacterial cells. One other *L. monocytogenes* attribute is its proteosurfaceome. Surface proteins are the link between bacteria and its environment, playing a significant role in communication, chemical sensing, stress resistance and balance of nutrients and toxins in the cell (10). *L. monocytogenes* genome sequence revealed 133 genes coding for surface proteins. Notably, the phylogenetically close but non-pathogenic *L. innocua* genome presented despair results regarding this protein family, unveiling for the first time its potential role in virulence (11). Even with the clear importance of proteomes and proteosurfaceome, protein studies have a higher level of complexity in comparison to DNA or RNA, mainly due to its variations in abundance, physicochemical features and subcellular localization (10). As for surface proteins, these are especially hard to work, since they require a carefully balanced hydrophilic and lipophilic environment (12). In regards to the role of surface proteins in virulence, the *L. monocytogenes* proteosurfaceome has been well explored (13), but the same cannot be said for its part in the adaptation and resistance to different environmental settings. Even scarcer are the studies in the sessile mode of growth, the predominant growth state in food workshops (14). To overcome the challenges associated with properly identifying surface-associated proteins, three different but complementary extraction methods were used in this study. First, the biotinylation method which is based on the treatment of intact cells 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, labeled proteins can be separated from non-labeled proteins by affinity chromatography with neutravidin and then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (15). Secondly, the shaving method which consists on treating intact bacterial cells with proteases in an isotonic solution to promote the release exposed peptides (16). The third and final extraction method, the fractionation which allows to explore the proteome in separated subcellular fractions.

L. monocytogenes is an opportunistic bacterial pathogen that has the capacity to survive under extreme environmental conditions encountered in nature and in the food chain, such as high salt concentrations (17), large range of pH (18), desiccation (19, 20), and low temperatures (21). Maintaining the cold chain is an essential parameter throughout the processing and distribution of food, protecting it from the growth of mesophilic microorganisms and thus extending its shelflife. However, the temperatures used for refrigerated storage do not prevent the growth of psychrotrophic germs such as *L. monocytogenes* (22). Even if cold environments lead to a decrease in the rate of bacterial growth, they do not inhibit it completely (21). Temperature variation also has its role in virulence. As for many other bacterial pathogens, *L. monocytogenes* activates the expression of virulence genes at host body temperature (23). Throughout the years, multiple studies have been published exploring *Listeria's* temperature adaptation, either through physicochemical tests (21, 24–29), genomic and transcriptomic methods (30–38), and also through metabolic and proteomic approaches (39–44). However, there is still a knowledge gap in the comparison of subproteomic changes to different temperatures, particularly in the biofilm mode of growth.

L. monocytogenes adaptation to low temperatures is one of its crucial attributes that supports *Listeria* persistence and dissemination in refrigerated products. This adaptation to cold temperatures renders the use of such physical setting insufficient for the control of *L. monocytogenes* presence in long-term storage under refrigeration products (45). *L. monocytogenes* is able to grow at temperatures as low as -0.4°C but also survive in freezing temperature such as -18°C (21, 46). In short, upon exposure to low temperatures, bacterial membranes become more rigid and the metabolic rate decreases. To overcome the hurdles imposed by a cold stress, bacteria have to increase the expression of genes involved in cell membrane function, production of cold shock proteins and multiple other molecular strategies to maintain homeostasis (47).

One of the major adaptative strategies is the induction of osmolyte and peptide transporters which will increase the amount of this molecules in the cytosol and maintain turgor pressure (48). The critical uptake of osmoprotectant molecules for *Listeria's* adaptation to low temperature is made through auto transporters for compatible osmolytes and short oligopeptides (49). The main carnitine transporter, OpuC, encoded by the opuCABCD operon, was observed in high abundance in *Listeria* cells exposed to low temperatures (48, 50).

In cold environments, bacteria also deal with protein damage, particularly protein misfolding and aggregation. To counteract this damage, cells have at their disposal a network of molecular

chaperones that assist in maintaining proteins in their native states. A key role of chaperones is preventing protein aggregation (51). *L. monocytogenes* harbors three proteins from the CspA family (CspLA, CspLB, and CspD) and genome-wide expression studies showed a significant increase in expression of CspL at low temperatures (52).

L. monocytogenes response to the ever-changing environmental factors and stress conditions is linked to the activation of the alternative sigma factor sigma B (σ^B) that controls the general stress response (GSR). Functions of genes regulated by this transcriptional factor include a diversity of metabolic pathways, transport associated proteins, stress proteins, and other transcriptional factors (53). Particularly the osmolyte carnitine, which as mentioned before is transported via OpuC. The expression of this autotransporter is also regulated by σ^B . Other genes associated with cold stress resistance, *ltrC* (54) and *fri* (55), are also controlled by σ^B regulon. In short, even if a direct link between cryotolerance and σ^B has not yet been obtained, there are clear signs that this transcriptional factor has a role in *Listeria's* adaptation to low temperature (47).

A combination with other mechanisms of cellular homeostasis maintenance, such as: control of membrane fluidity, gene expression events, protein folding and degradation, assimilation of carbon sources, oxidative stress response, and production of specific amino acids and lipids culminate in *Listeria's* successful persistence in the cold temperatures of the food processing environment (56).

An understanding of how *L. monocytogenes* proteome changes in the biofilm mode of growth at different temperatures can help to unveil how it establishes and survives in the processing environment. To this point, this study aimed to explore the *Listeria monocytogenes* biofilm subproteomes and surface proteins under the influence of three temperatures: 10°C mimicking a common setting found in the food industry environment, 25°C as a baseline temperature, and 37°C as the human host setting.

MATERIALS AND METHODS

Strain and Biofilm Settings for Protein Extraction

The sequenced *L. monocytogenes* EGD-e strain, serogroup 1/2a, was used throughout this study (57). 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 (OD₆₀₀). Precultured cells in stationary phase were used to inoculate cultures to obtain a final OD₆₀₀ 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 OD₆₀₀ between 0.6 and 0.7. Seven milliliters of the bacterial suspension was poured on each stainless steel (SS) disk (38.5 cm²), corresponding to an inoculation of 10⁸ to 10⁹ CFU/cm² (colony-forming unit/ square centimeter). 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 add fresh medium. The disks incubation settings were dependent on the desired temperature (10, 25, or 37°C) and growth stage (early stage or mature biofilm) (Supplementary Table 1). 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 spatula. Three biological replicates with 10 disks per each were used for every analyzed setting. Cell adhesion and population in biofilm 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.

Protein Extraction Methods

The three extraction methods used in this study were biotin labeling, trypsin-enzymatic shaving and cell fractionation. All methods used were based on the protocol optimized for the extraction of surface exposed proteins developed by (58). In the biotinylation method, bacterial cells are treated with Sulfo-NHS-SS-Biotin, a marker molecule that is supposed to be membrane impermeable and interacts with surface exposed proteins. The shaving method consists of treating intact cells with proteases in an isotonic solution to release exposed peptides. The third method is the fairly well-established separation of membrane and cell wall components by cell fractionation. The technical details and result output of these extraction methods can be found in the Supporting information document and Supplementary Figures 1, 2.

Nano-LC-MS/MS and Bioinformatic Analysis

In order to obtain triplicate protein extracts, three independent biofilm cultures were used for each of the three methods described above. All samples, except those from the shaving method, were loaded onto SDS-PAGE gels to concentrate 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% ACN 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% ACN. The samples were hydrolyzed overnight at 37°C using 48 μl of a 25 mM NH₄HCO₃/12.5 ng.μl⁻¹ trypsin solution (Promega) per band. Peptides were extracted from the gel bands in an ultrasonic field during 10 min with 38.4 μl of 100% acetonitrile representing 80% of digestion volume. Supernatants were transferred in eppendorf vials and dried using Speed Vac for 45 min and 40 μl of equilibration solution (H₂O/Trifluoroacetic Acid –99.95/0.05) was added.

All peptide mixtures were analyzed by nano-LC-MS/MS (Thermo Fisher Scientific) using an Ultimate 3000 system coupled to a LTQ Orbitrap Velos mass spectrometer (MS) with a nanoelectrospray ion source. For each sample, one microliter of peptide mixture was first preconcentrated on a C18 pre-column 5 cm length X 100 μm I.D. (Acclaim PepMap 100 C18, 5 μm, 100 A nanoViper), equilibrated with Trifluoroacetic Acid

0.05% in water at 30 $\mu\text{L}/\text{min}$. After 6 min of desalting and preconcentration, the pre-column was switched online with the analytical C18 column (75 μm inner diameter \times 15 cm length; 2 μm , Acclaim PepMap 100 C18 Pepmap RSLC) equilibrated with 96 % solvent A (99.5 % H_2O , 0.5 % formic acid) and 4 % solvent B (80%ACN, 19.5% H_2O , 0.5% formic acid). Peptides were eluted according to their hydrophobicity at 300 nL/min flow rate using, respectively, a 4 to 52% gradient of solvent B for 31 min for biotin labeled and trypsin-enzymatic shaved fractions and a 4 to 40% gradient of solvent B for 56 min for cell fractions. Eluates were electrosprayed in positive-ion mode at 1.6 kV through a nanoelectrospray ion source heated to 250°C. The LTQ Orbitrap Velos MS was used in CID top 15 mode (i.e., 1 full scan MS and the 15 major peaks in the full scan were selected for MS/MS). The full scan MS was realized in the FTMS ion trap at a resolution of 60,000 (tolerance 10 ppm) and spectra were acquired with 1 microscan (m/z 400–1,400). For MS/MS, the following parameters were used: dynamic exclusion with 1 repeat counts, 20 s repeat duration and 80 s exclusion duration, isolation width for ion precursor was fixed at 1 m/z , single charged species were rejected, fragmentation used 37% normalized collision energy as the default activation of 0.25 and 10 ms activation time. For raw data processing, MS/MS ion search was performed with Mascot v2.5 for database search (<http://www.matrixscience.com>). The following parameters were considered for the searches: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C) and oxidation (M) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores 13 with a False Discovery Rate of 1% (adjusted significance threshold $p < 0.05$). Ions score is $-\log(P)$, where P is the probability that the observed match is a random event. Individual ions score >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 SP (59). The subcellular location of identified proteins was determined with the rational secretomics based strategy for genomic and proteomic analyses developed by Renier et al. (60). For protein quantitation analysis, LC-Progenesis was used with Mascot v2.3 and the same identification parameters described above. Normalization was based on the LC-Progenesis process with Log10 ratio calculation and scalar estimation in log space. The statistical method used was the comparison in groups of two temperature settings in a “Between-subject design.” For each temperature setting three biological replicas with three technical replicas were used. Venn diagrams were performed by the jvenn online tool (61). Functional category of proteins was based on the clusters of orthologous groups (COGs) via the eggNOG online framework (62). For proteins of interest, the average abundance from the 3 biological replicates in each condition was represented in a bar chart with standard deviation and statistical significance was tested by one-way ANOVA. Protein-protein association networks were made by the String database

(63). Heat map displaying the normalized protein abundances were obtained via Xlstat (2018.5). Volcano plots were performed by the R software with the integration of the ggplot2 and ggrepel graphical packages for data analysis (64).

RESULTS

Overall Protein Identification Results

From all extraction methods and temperature settings, 920 unique proteins were identified (**Figure 1A**), representing a significant proportion of the *Listeria monocytogenes* proteome (32.2%) (57). The vast majority, 79% of the identified proteins, were characterized as cytoproteins, followed by membrane-associated proteins, extracellular and cell wall-associated proteins represent 17, 3, and 1%, respectively. Functional category of this set of proteins was based on the clusters of orthologous groups (COGs) (**Figure 1B**). Taking into consideration that the majority of identified proteins are from the cytoplasmic subcellular localization, it is not surprising that the functional category with the highest percentage of identified proteins is “Translation, ribosomal structure, and biogenesis” (13%), followed by “Transcription” and ‘Cell wall/membrane/envelope biogenesis,” 6% each. In addition, there are a multitude of normal molecular functions with a similar number of identified proteins. Although the genome sequence of *L. monocytogenes* was performed 18 years ago, a significant part of its proteome is still unannotated and lacks relevant information. This is reinforced by the large number of proteins whose function is not known here (192, 21%). In regards to the subproteomic extraction approach here implemented, we identified a high percentage of the *L. monocytogenes* intracellular proteome and also a significant amount of its surfaceome. In order to look for potential protein biomarkers of temperature adaptation, the protein identification data was structured by temperature and growth condition. The column chart in **Figure 4A** illustrates the stable and steady amount of identified proteins that were found in the different settings. The six branched Venn diagram shows that the vast majority of proteins were shared between all settings (531 proteins). Nevertheless, 122 proteins were identified only in one of the six settings in the study. Some of them with potential interest with temperature adaptation. In the case of early stage biofilm (Biof Es) grown at 10°C (**Figure 2A**), 40 uniquely identified proteins were registered, including the RNA polymerase sigma factor SigB, documented as regulating the expression of genes necessary for survival under environmental stress conditions (65). We have also identified three proteins of the flagellar motility system (Lmo0713, Lmo0714, and Lmo0697) and a DNA replication, recombination and repair associated protein (ssb2). Interestingly, OpuCA which is part of a documented carnitine solute auto transporter was identified only at the 10°C settings. Likewise, a unique heat shock protein involved in cell division and virulence (clpE) was identified in the 37°C condition at mature biofilm growth (66). The distribution of molecular functions in each setting follows the same trend as in the analysis of the whole protein identification data set seen previously (**Figure 2B**). A higher amount of proteins related to translational machinery was present and a similar amount of

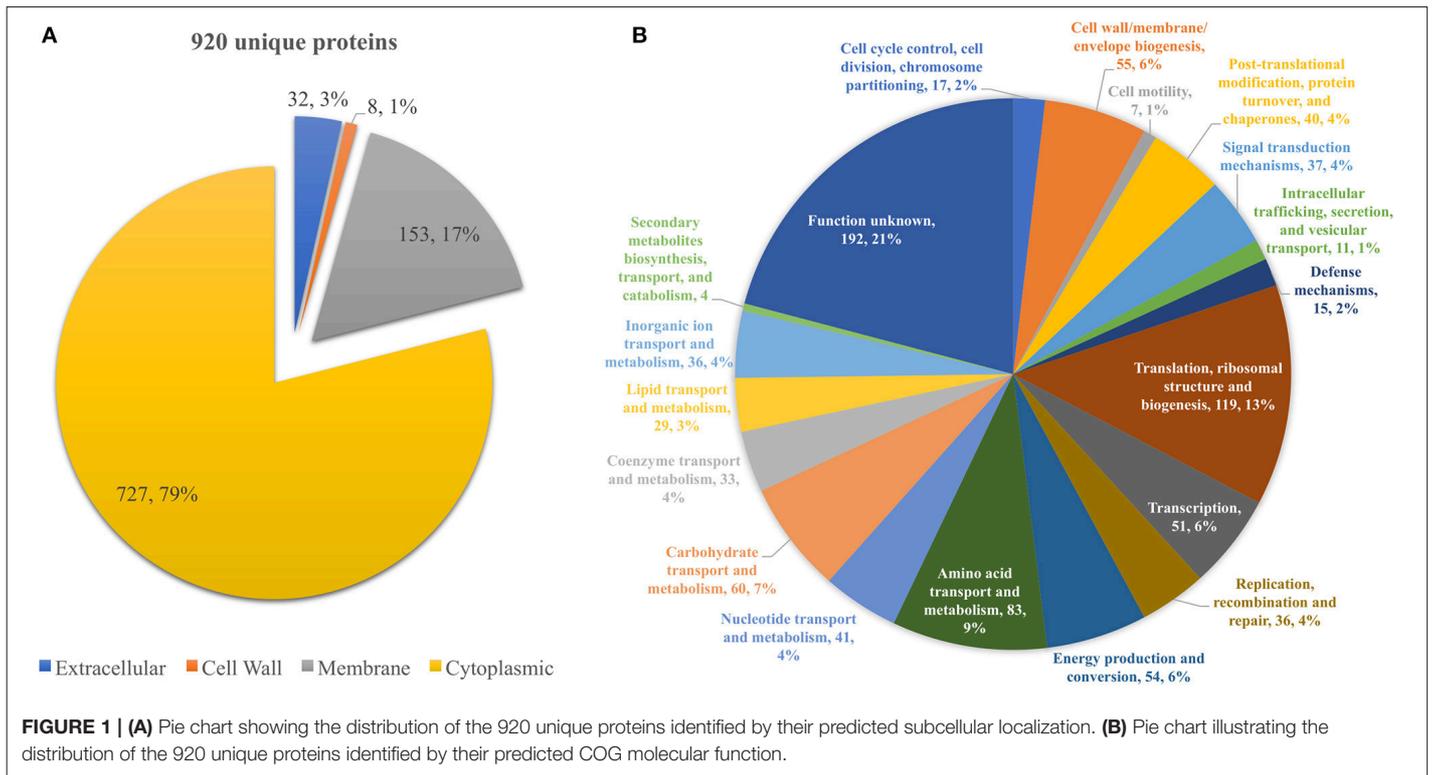


FIGURE 1 | (A) Pie chart showing the distribution of the 920 unique proteins identified by their predicted subcellular localization. **(B)** Pie chart illustrating the distribution of the 920 unique proteins identified by their predicted COG molecular function.

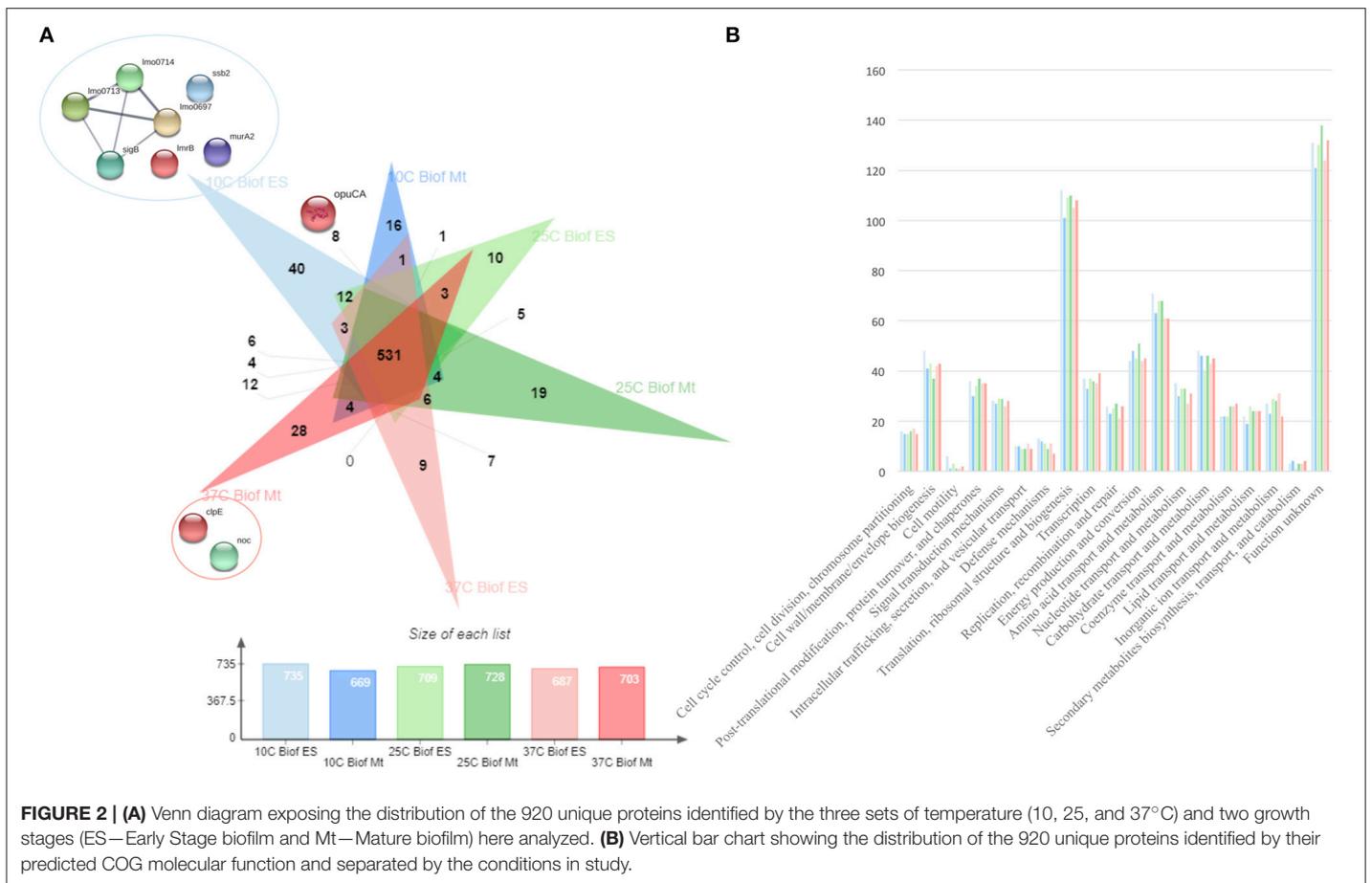


FIGURE 2 | (A) Venn diagram exposing the distribution of the 920 unique proteins identified by the three sets of temperature (10, 25, and 37°C) and two growth stages (ES—Early Stage biofilm and Mt—Mature biofilm) here analyzed. **(B)** Vertical bar chart showing the distribution of the 920 unique proteins identified by their predicted COG molecular function and separated by the conditions in study.

proteins identified for each molecular function across the six different settings. Though, a comparison of protein flows and changes that might have a relation to temperature adaptation can only be achieved by quantitation analysis.

Cross-Comparison of Temperature Adaptation Through Protein Quantitation

The quantitative analysis of protein abundance made by LC-Progenesis enabled the comparison of protein abundance through pairs of temperature settings, 10 vs. 37°C (**Figure 3; Supplementary Figure 4** and **Supplementary Table 2**), 10 vs. 25°C (**Figure 4; Supplementary Figure 5** and **Supplementary Table 3**), and 25 vs. 37°C (**Figure 5; Supplementary Figure 6** and **Supplementary Table 4**). In all cases, LC-Progenesis normalized abundances of the statistically significant identified proteins ($p < 0.05$) were used in order to compare the flows of proteins in each setting.

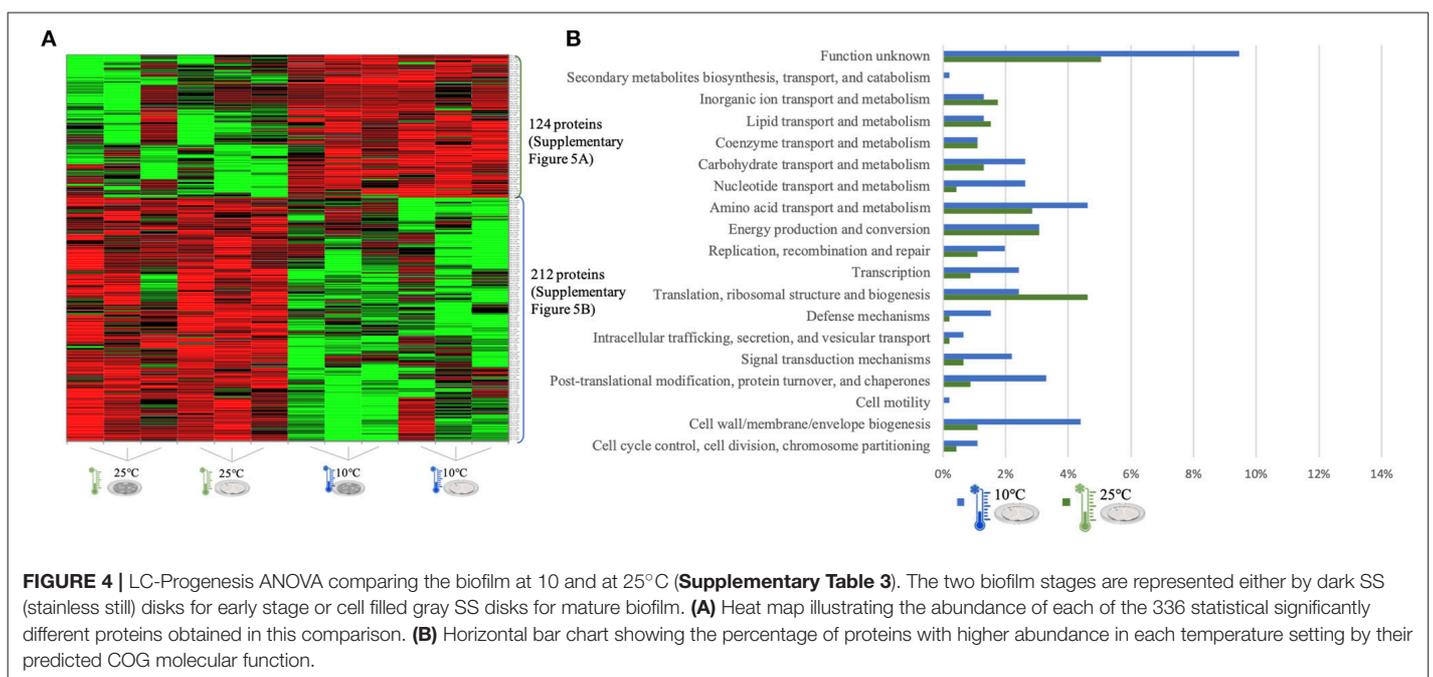
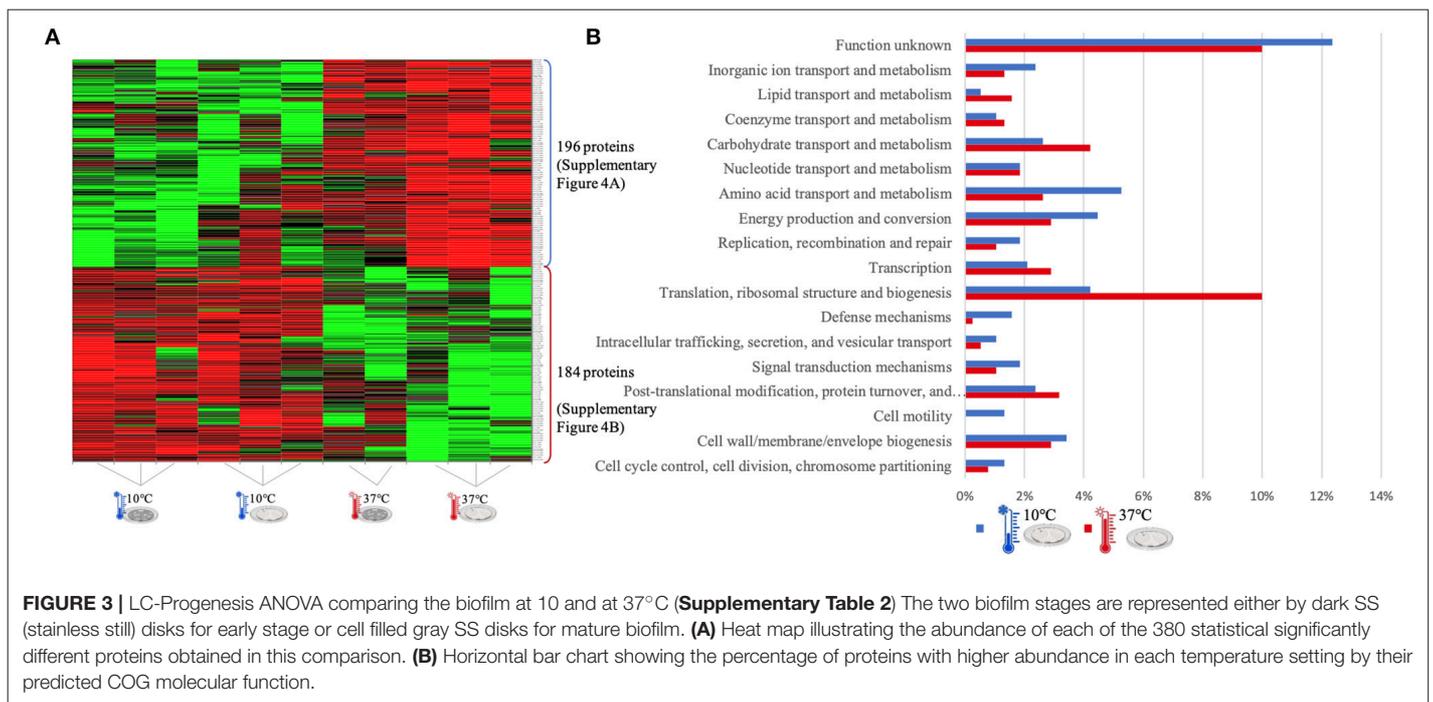
The Xlstat heat map in **Figure 3A** shows the relative abundance of each of the 380 statistically significant proteins retrieved when comparing the two growth phases at 10 and 37°C. This was the most contrasting comparison of growth temperatures, between a temperature mimicking the settings found in the food industry (10°C) and the temperature of the human host (37°C) to which *L. monocytogenes* expresses a higher level of virulence. A similar sum of proteins with higher abundance was detected in both temperatures, i.e., 196 at 10°C and 184 at 37°C. In some instances, there is a variance in one or more biological replicates regarding its abundance. The comparison of proteome changes can also be viewed by the shifts of protein abundance in regards to molecular function (**Figure 3B**). Regarding to transcriptional or translational activity, at 37°C, there are more proteins with higher abundance annotated with this molecular function. On the other hand, a higher percentage of proteins associated with a repair system mechanism were more abundant in the cold condition than at 37°C. The repair system molecular function is one of the categories COG (EggNOG orthologs). A temperature stress as 10°C induces DNA, RNA and protein damage in the cell (45). Some of the proteins that were more abundant at 10°C in this category have annotated involvement in solving protein aggregation and DNA damage (as for example DNAA chaperone protein, **Supplementary Table 2**). Similar result was seen for inorganic ion and amino acid transport associated proteins. A String map of protein interaction (**Supplementary Figure 4**) provides a graphical representation of the statistically significant proteins from this quantitation. Highlighted in blue are some of the proteins associated with the adaptation to harsh environmental conditions that were more abundant in the 10°C condition (**Supplementary Figure 4A**), as is the case of: the GTP-sensing transcriptional pleiotropic repressor CodY; the carnitine transporter OpuCA; the redox regulated molecular chaperone HslO, that protects both thermally unfolding and oxidatively damaged proteins from irreversible aggregation (67); the foldase protein PrsA2, which protects the cell by controlling the folding and stability of secreted proteins in stress conditions (68); the DNA protection during starvation Dps, that protects DNA from

oxidative damage by sequestering intracellular Fe²⁺ ion and storing it in the form of Fe³⁺ oxyhydroxide mineral (56); the ATP-dependent zinc metalloprotease FtsH, which plays a role in the quality control of integral membrane proteins (69); the DNA ligase LigA, essential for DNA replication and repair of damaged DNA under stress conditions (70); the superoxide dismutase SodA, responsible for destroying superoxide anion radicals which are produced within the cells (71); and the ATP-dependent RNA helicases CshA and CshB, which are involved in RNA degradation during cold tolerance, motility and alcohol tolerance (72).

The large sum of ribosomal proteins at 37°C validates the higher percentage of proteins previously associated with translational function (**Supplementary Figure 4B**). Likewise, highlighted in red are some of the chaperone proteins associated with heat adaptation and the general stress response system, that were more abundant at 37°C. They participate actively in the response to hyperosmotic and heat shock by the recognition and processing of DNA lesions, and preventing the aggregation of stress-denatured proteins and by disaggregating proteins (73), such proteins as: DnaK, DnaJ, GroES, GroEL, GrpE, UvrA, and ClpB. The virulence capacity of *L. monocytogenes* is also here underlined by the higher abundance of the endopeptidase p60 (Iap) which is a major extracellular protein involved in the invasion of phagocytic cells (11).

The quantitation appraisal of the cold condition to the control temperature setting in this study (25°C) resulted in 336 statistically significant proteins (**Figure 4A**). We observed 26% more proteins with a higher abundance at 10°C than at 25°C. In agreement with the previous comparison, sessile cells at 10°C showed a lower percentage of abundance of proteins associated with translational machinery and, once again, a higher percentage of proteins with higher abundance involved in amino acid transport. The mapping of this set of proteins reveals the same trend as previous results with stress response associated proteins being more abundant in the cold condition (e.g., ClspL and SigB) (**Supplementary Figure 5**). The higher number of ribosomal proteins at 25°C confirms the tendency toward fewer translation mechanisms at the cold condition. Furthermore, chaperone proteins (DnaK, GroEL) and some virulence associated proteins, like the internalin A (InlA) which mediates the entry of *L. monocytogenes* into host intestinal epithelial cells (74) and chemotaxis CheA involved in the transmission of sensory signals from the chemoreceptors to the flagellar motors in intracellular movement (75), were also present in lower levels at the colder setting.

The last quantitative comparison of protein abundance was between 25 and 37°C, which gave the lowest amount of statistically significant proteins among the three comparisons (233 proteins, **Figure 5A**). A similar number of proteins with higher abundance was found under both conditions, probably related to the proximity of this two temperature settings. However, changes in proteome abundance can be retrieved upon analysis of molecular functional categories (**Figure 5B**). As in the previous two analyses, the highest temperature showed a greater number of proteins associated with translation. At 37°C, there is a higher amount of proteins

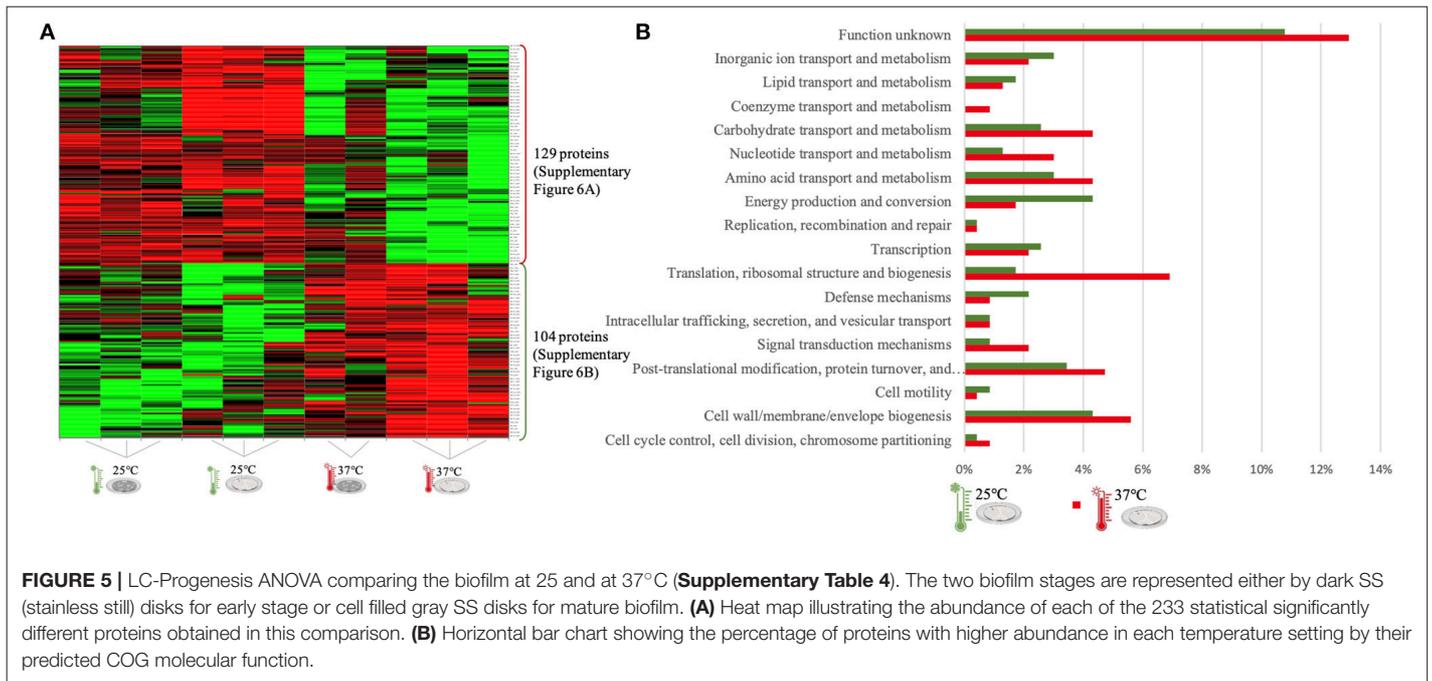


with higher abundance related to carbohydrate metabolism and also coenzyme transport. The flagella motor system was more abundant at 25°C (Supplementary Figure 6A). As in the first quantitative appraisal (10 vs. 37°C), the ribosomal, chaperone and virulence associated proteins were more abundant at 37°C (Supplementary Figure 4B).

Proteins of Interest for Temperature Adaptation

For an additional understanding of the *L. monocytogenes* proteome changes under different conditions, it was important

to have a look at sets of proteins of interest and also outliers that could be potential biomarkers for the adaptation to this environmental and food industry setting. Figures 6, 7 represent the average abundances of some of these proteins of interest between the two extreme poles of temperatures and across the two stages of biofilm growth (early stage, represented by dark SS disks, and mature biofilm, represented by cell filled SS disks). For each protein, average abundances were obtained from the three biological replicates in each condition. In the case of the DNA protection to starvation protein (Dps), a higher abundance was obtained at 10°C, particularly in the mature biofilm stage.



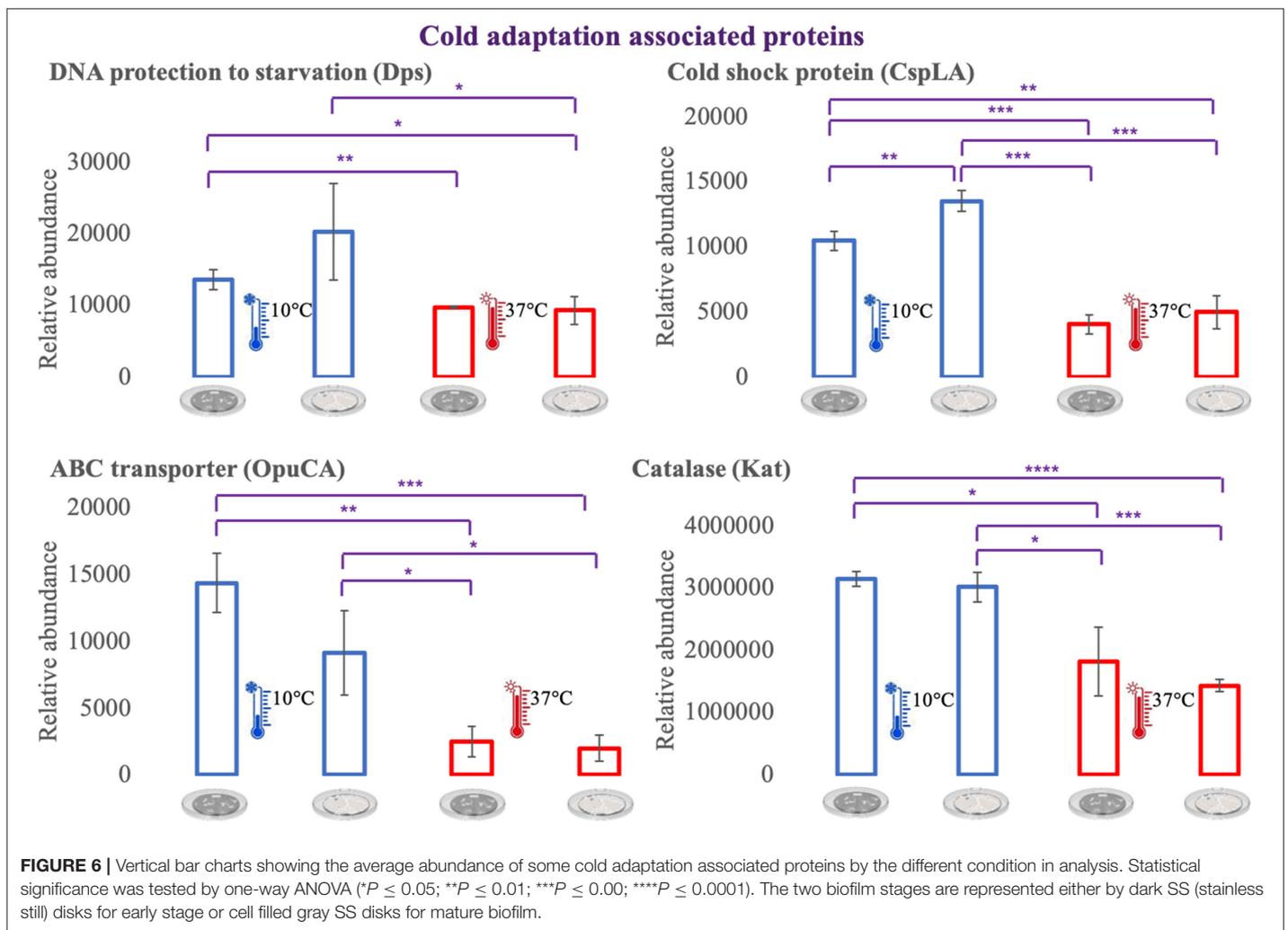
Dps is important for full resistance to heat and cold shocks and is essential for full virulence of this bacterium (56). The abundance of other three cold adaptation associated proteins was also greater at 10°C than at 37°C (Figure 6), namely: the cold shock protein (CspLA); ABC transporter OpuCA; and Kat, a catalase suggested to be one of the contributors to the ability of *L. monocytogenes* to grow at low temperatures (76). The cell motility associated proteins were more abundant at temperatures under 37°C, as it is the case of flagellin (FlaA) and flagellar hook (FlgE) with a higher abundance at 10°C, particularly in the early stage biofilm (Figure 7). A different tendency was seen for the cellular trafficking proteins, the abundance of these proteins were registered with higher levels at 37°C. Heat adaptation associated protein chaperones were detected, in most instances, with higher abundance at 37°C, particularly in the mature biofilm. To further retrieve protein drifts and outliers, volcano plots were generated from the three groups of temperature comparison, with cut-offs at p -value = 0.05 ($\log_{10}(p\text{-value}) = 1.30103$) and fold change (FC) = 2 ($\log_2(\text{fold change}) = 1$). Each of the quantitative comparisons was represented in a different volcano plot, 10 vs. 37°C (Figure 8A), 10 vs. 25°C (Figure 8B), and 25 vs. 37°C (Figure 8C). A total of 139, 68, and 58 proteins respected these cut-offs criteria, respectively. In all three plots, the top 50 proteins with higher fold change were marked with the color to which they presented higher mean condition (blue for 10°C, green for 25°C, and red for 37°C). In the 10 vs. 37°C plot, the proteins with the highest fold change at 10°C are associated with metabolism and amino acid transport. In the 10°C highlighted set of proteins are also included stress adaptation proteins, such as OpuCA (Q7AP65_LISMO) and CspLA (CSPA_LISMO), and cell motility associated proteins (Q8Y954_LISMO, Lmo0680). In the pool of proteins with high fold change at 37°C, there are proteins associated with virulence as LmaA (Q7AP93_LISMO), LmaB (Q7AP94_LISMO), CspLB (CSPB_LISMO) and proteins

involved in heat adaptation (Lmo1580, Q8Y6V1_LISMO, and CcpA, Q8Y6T3_LISMO). In regards to the evaluation between 10 and 25°C, transport associated proteins were observed with the highest fold change in the cold condition. This was particularly the case for amino acid transporter such as multiple ATP-binding cassette transporter (ABC) OpuCA, (Q7AP65_LISMO), Lmo0538 (Q8Y9J0_LISMO), and SerC (SERC_LISMO). At 25°C, most of proteins had a connection with energy production and translation machinery. At the level of the 25 vs. 37°C comparison, the majority of proteins were associated with translation or carbohydrate functions. To note the presence of two virulence associated proteins with FC>2 at 37°C, Iap (P60_LISMO) and UvrC (UVRC_LISMO).

DISCUSSION

Listeria monocytogenes is the causative agent of listeriosis, a highly fatal disease to fetuses, newborns, infants, pregnant women, elderly and immunocompromised individuals (77). Invasive listeriosis is considered the leading cause of death from foodborne infections in industrialized countries. Despite the application of the food safety criteria (FSC) for *L. monocytogenes* in RTE foods from 2006 onwards, a statistically significant increase in listeriosis cases has been reported in the European Union between 2009 and 2013 (3, 78).

The cleaning and disinfection of surfaces and materials in food industries is difficult to achieve due to *L. monocytogenes* capacity to survive under harsh condition, such as a wide pH range (79), desiccated environments (9, 20), and low temperatures (80). Since *L. monocytogenes* is a psychrotrophic microorganism able to multiply in food stored under refrigerated temperatures, a low contamination number present in RTE products can grow to a level which threatens consumer health (81). At low temperatures, *Listeria* faces multiple molecular constraints such as increased

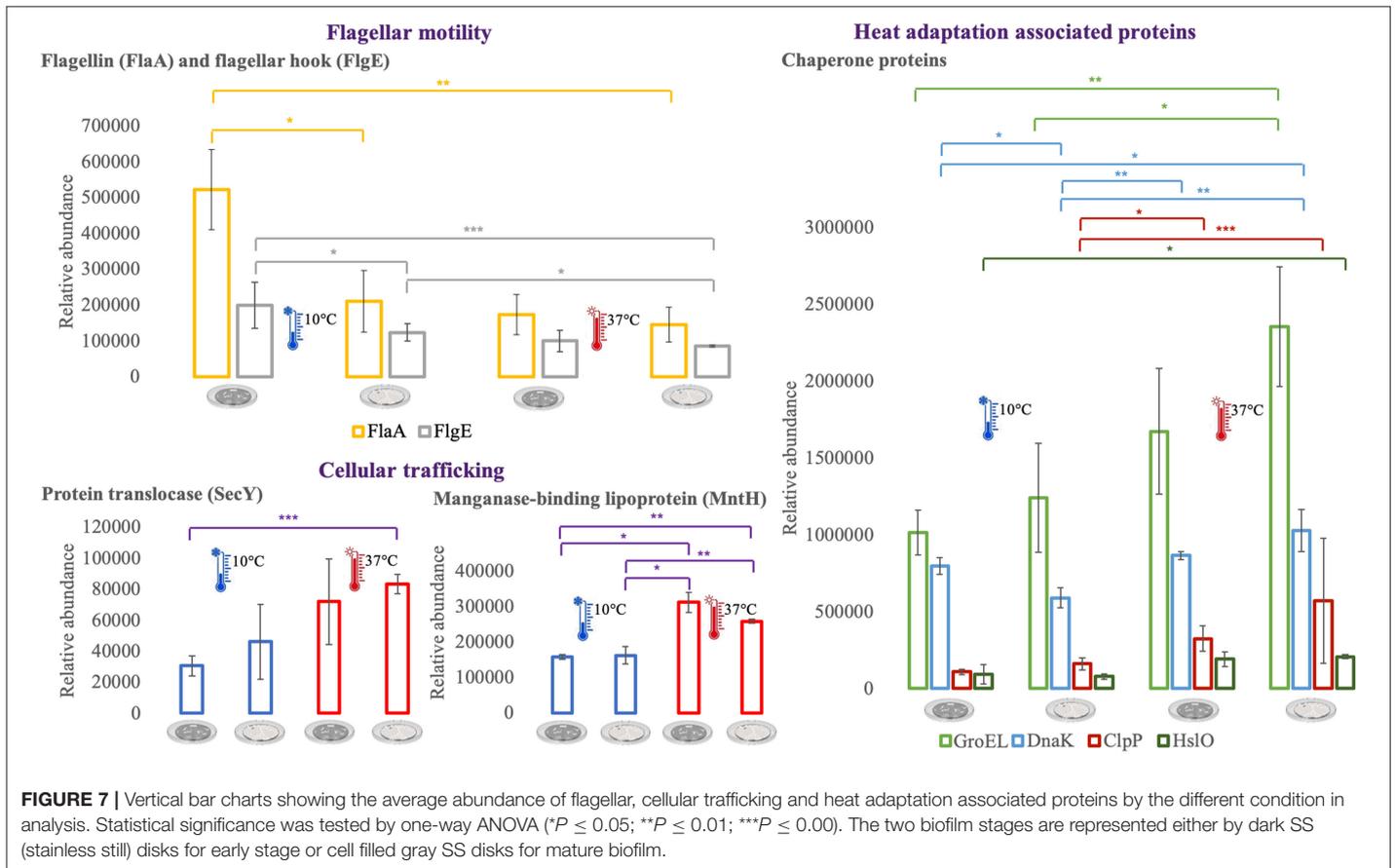


membrane rigidity, amino acid starvation, oxidative stress, aberrant protein synthesis, cell surface remodeling, reduced protein and enzyme activity, slow transport, and nutrient uptake processes (56, 82). *L. monocytogenes* response mechanisms to these hurdles has been extensively studied by transcriptomic approaches. Contrasting are the few studies to the changes at the proteome level that allow *Listeria* to survive at refrigeration temperatures (41, 43, 83), and none performed in a biofilm mode of growth. The present study sought to investigate the influence of three temperatures in the proteome changes of *L. monocytogenes* grown in the biofilm mode of growth. In the next subsections, we overview some noteworthy protein abundance shifts associated with the major adaptation to cold mechanisms present in *L. monocytogenes*, namely: the role of ribosomes and the stressosome, changes in cell fluidity and motility, the uptake of osmolytes, and the relevance of the overexpression of chaperone proteins such as cold shock proteins. Based on the proteomic data obtained in this study it is highly unlikely that a set of surface proteins play a unique role in the adaptation to the different temperatures. Hence, adaptation to low temperature growth is a complex response involving many aspects of the cell molecular biology and biochemistry (45). The typical molecular mechanisms of cold stress adaptation referenced in

bacteria include: overexpression of stability mechanisms by the modulation of nucleic acid structures, maintenance of structural integrity in cell membranes, uptake of compatible solutes, production of various cold stress proteins, including cold shock proteins (Csps), and nonspecific stress response mechanisms.

Cold Stress Adaptation and the Role of Ribosomes

One of the first side effects of exposure to low temperatures is the compromise of ribosomal structural stability and this deterioration causes a general reduction in the bacteria's protein synthesis capacity (45). In this study, the majority of ribosomal proteins were less abundant in the cold condition, and in some instances, ribosomal proteins had a high fold change in the warmer setting (Figure 8). The 50S ribosomal proteins (R1) are referenced as the first cold stress sensors in microbes, as is the case of R11. The former is essential for the activation of sigma B (σ^B) transcription factor in *Bacillus subtilis* (84), the general stress response transcriptional factor that controls the expression of dozens of stress adaptation related genes. We observed an increased level of R11 protein at 10°C, as well as the initiation factor IF2 which is implicated in the formation of 30S preinitiation complex, suggesting a role in ribosome assembling

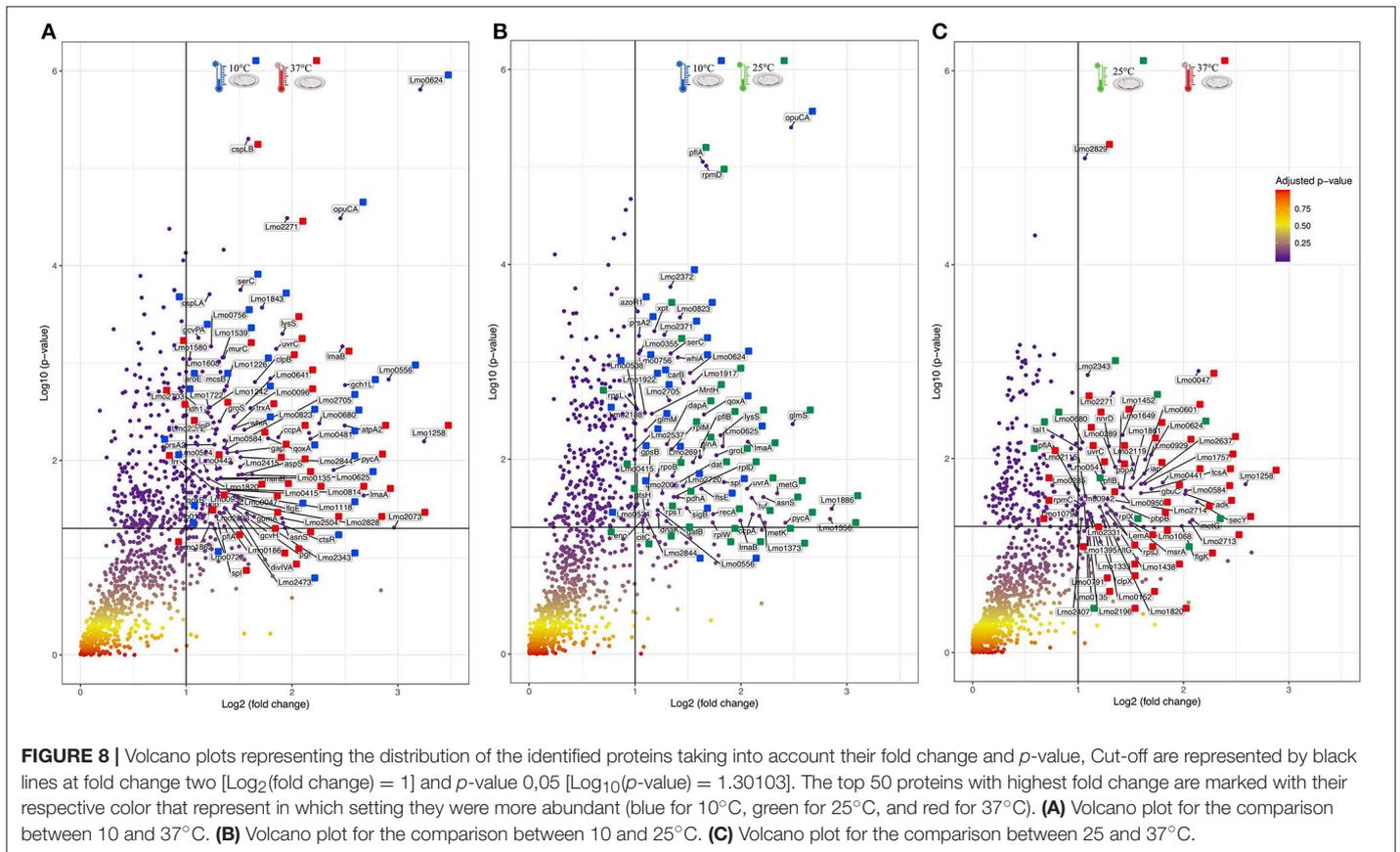


(41). The general stress protein Ctc (Rl25), was here more abundant at 10°C and the same was seen in *L. monocytogenes* upon exposure to a salt condition (85). Concerning σ^B , this general stress transcription factor was more abundant at 10°C than at the room temperature (Supplementary Table 3). The σ^B enables *Listeria* pathogenicity in two different ways. First, by controlling the expression of genes that enhance the survival during food industry pipeline settings. Second, σ^B plays a role upon infection by indirectly regulating PrfA, the main virulence regulator (86, 87). This transcriptional factor has also been associated with the efficient accumulation of betaine and carnitine as cryoprotectants (88), which will be further discussed in the next sections. On the contrary, it has been suggested that σ^B does not, in fact, play a pivotal role during cold adaptation in *L. monocytogenes*. So, it seems to contribute to the adaptation in a growth phase-dependent manner, particularly in the early stages of growth (89). This result was not seen here, since σ^B was more abundant in the mature biofilm than in the early stage one. The reasoning behind this can be linked with the different modes of growth applied in the two studies and the fact that σ^B also has a determinant role in biofilm formation (90).

Cell Membrane Fluidity, Chemotaxis, and Their Involvement in Cold Adaptation

Changes in the cell membrane fluidity and surface proteins are part of the bacterial adaptation to cold stress (30). For example, Lmo0624 has a role in lipid metabolism and higher

transcripts of this gene were reported at 4°C (91). In agreement with this result we also observed Lmo0624 (Q8Y9A8) more abundant in the cold condition with one of the highest fold changes (FC = 9.27) (Figure 8A). The foldase protein PrsA2, here found more abundant in the colder setting (FC = 2.41, Supplementary Table 2) is a member of a family of membrane-associated lipoproteins that play a role in the folding and stability of secreted proteins as they pass the bacterial membrane. PrsA2 contributes to the integrity of the *L. monocytogenes* cell wall as well as swimming motility and bacterial resistance to different stresses (92). Cell stability and bacterial attachment to surfaces are influenced not only by cell surface properties but also by the presence of surface appendages, such as flagella (93). All the cell motility associated proteins identified in this study, namely flagellin (FlaA), flagellar hooks FlgK and FlgE, Lmo0689 (Q8Y948), Flha (Lmo0680, Q8Y954), MotA (Lmo0685, Q7AP82) were more abundant at 10 and 25°C when compared with the *in vivo* setting (37°C). FlaA is the bacterial flagella main protein (94) and *L. monocytogenes* strains are motile and flagellated below 30°C (95), and typically not motile at 37°C (96). However, the role of the flagellum in biofilm formation is controversial. There are observations that give it an important role in biofilm formation (97), and in other reports strains with this gene deleted had improved sessile development (98). In regards to the putative role of the flagella, it was described to be required for the initial cell attachment phase by overcoming the van der Waals forces (93). We have observed several motility associated proteins with



significant abundance shifts that support the hypothesis that motile flagellum is needed for an optimal cold stress response in *L. monocytogenes*. Moreover, we have detected that all these cell motility proteins were more abundant in the early stage of biofilm. A flagellum-associated operon (consisting of Lmo0675 to Lmo0689) has been previously retrieved in high transcript levels from *L. monocytogenes* exposed to 4°C in log phase (91). Lmo0689 was also observed in this study to be more abundant at 10°C and in the early stage biofilm. Similarly, FlhA and MotA play a role in the cold tolerance of *L. monocytogenes* (99). This is consistent with our observation that MotA was one of the proteins with the highest fold change (FC = 4.78) in the 10 vs. 37°C comparison. Likewise, the ability to modulate membrane fatty acid composition and improve membrane fluidity is a crucial step for temperature adaptation (100). FabH, which was here more abundant at 10°C, is connected with the increased formation of anteiso branched-chain fatty acids during cold temperature adaptation, which ultimately is responsible for the increase in membrane fluidity (101).

The Crucial Role of Cryoprotective Solutes

The uptake of compatible solutes/osmolytes is one of the key steps for the survival of bacteria in stress conditions. Osmolytes are low-molecular weight organic compounds that are stored in high intracellular concentrations with minimal effects on the normal functioning of the cell. At low temperatures, cryoprotective solutes act through stabilization of enzymatic functions and the cell membrane lipid bilayer (102). Organic

compounds such as glycine betaine and carnitine help to relieve turgor pressure, in this manner they have an essential role in the survival of *Listeria* under elevated osmolarity and cold settings (25, 50). In the food industry, *L. monocytogenes* has access to these solutes from carnitine rich meat and dairy products, and glycine betaine from plants and shellfish (54). There are three solute import systems known to operate in *L. monocytogenes*: glycine betaine porter I (BetL), glycine betaine porter II (Gbu), and the carnitine transporter OpuC (25). Here, we have identified GbuB (Q7AP75) and OpuCA (Q7AP65). The non-identification of a BetL can be reasoned with the described more pronounced role of Gbu and OpuC as preferential cryoprotection systems in *L. monocytogenes* (49). Regarding the cryoprotective impact between Gbu and OpuC, a report comparing their role concluded that carnitine uptake at low temperatures is higher than betaine after cold temperature (48). Supporting this result, GbuB (Q7AP75) and OpuCA (Q7AP65) were in this study more abundant at 10°C. Interestingly, OpuCA had one of the highest fold changes (FC = 4.91, **Figure 8A**; FC = 5.56, **Figure 8B**), one more hint that cryoprotectants are essential for *Listeria* rapid response to the conditions typically found in food preservation.

Cold Shock Proteins and Other Proteins Potential Involved in Temperature Adaptation

A second key point into the adaptation of a microbe to a cold stress is the production of various stress-related proteins,

including cold shock proteins (Csps) (31). Our results showed a higher abundance of the cold shock protein CspLA (CSPA) at 10°C and with high fold change (FC = 2.33, **Figure 8A**). CspA deletion mutants have showed to abolish *L. monocytogenes* growth at refrigeration temperatures (31). However, there are also Csps downregulated at low temperature, pointing to their potential role under conditions other than cold growth (91). Similar result was here obtained with the cold shock protein CspLB (CSPB) more abundant in the warmer condition (FC = 3, **Figure 8A**). This infers that CspLB also have a function during normal growth (40).

Various other cold response associated proteins were identified in this study. The GTP-sensing transcriptional pleiotropic repressor CodY was more abundant at 10°C. CodY has a role in the regulation of relevant genes implicated in *Listeria* growth at low temperature (91). *L. monocytogenes* CodY also has a recognized role in virulence (65), in this matter, from the 16 proteins identified here encoded by CodY regulated genes there are cases of stress adaptation associated proteins (Dps and SerC—more abundant at 10°C) and virulence-associated proteins (LmaA, LmaB, GroES, GroEL, and ClpB—more abundant at 37°C) (**Supplementary Table 2**). DNA protection during starvation protein (Dps or Fri) is a 18 kDa *L. monocytogenes* major cold shock protein that is required for iron storage and protection against reactive oxygen species (55, 103). This major protein for low-temperature adaptation was here detected in high levels at 10°C. Similar result was obtained for enolase (Eno) (**Supplementary Table 2**), which has been found to be upregulated at low temperatures by others (43). Eno does not possess an N-terminal signal peptide, but it was found before to be exposed on the bacterial cell surface or extracellularly, thus meaning there should be an uncharacterized secretion pathway (94). Further proteins connected to cold adaptation mechanisms in *L. monocytogenes* were more abundant at 10°C, such as folding catalysts Lmo1583 and Lmo2376, that have been detected before in increased levels at 4°C (41). Proteins with a role in protection from reactive oxygen species (superoxide dismutase-Sod, catalase-Cat, Lmo0640, and Lmo1967) and in iron metabolism (Lmo2415-SufD and Lmo2411-SufB) were also more abundant in the cold condition. SufD and SufB are part of the Suf system, which in *Escherichia coli* is activated to enable the increase of *de novo* Fe-S assembly, maintaining Fe-S cluster biosynthesis under oxidative stress conditions (104). Two-component-system histidine kinases (TCSs) are among the major systems that aid bacteria in overcoming many of arduous stress factors encountered in nature and during food processing environment (28). LisK which is part of the TCSs was here more abundant at 10°C. As for RNA chaperones, like DEAD-box RNA helicases, they act by resolving secondary structures in mRNA that can be developed in a cell subjected to cold (105). The three putative DEAD-box RNA helicase genes (Lmo0866, Lmo1450, and Lmo1722) are required for cold tolerance and motility in *L. monocytogenes* (37, 106). We have observed the three helicases with higher abundances at 10°C. The overall protein abundance data obtained also showed some nonspecific stress response mechanisms or shared responses to other stress conditions. Such a case is the amino acid, lipid and carbohydrate

transport which have at least a double role in the protection against cold temperatures and salt exposure (83). In respect to the proteins associated with the amino acid transport and metabolism, the majority of them was more abundant in the cold condition (66.7% in 10 vs. 37°C, **Figure 3B**; 61.8% 10 vs. 25°C, **Figure 4B**). This suggests that the cells endure starvation in certain amino acids during a cold condition, counterbalancing this lack with the increase expression of such transportation systems. Moreover, some amino acid biosynthetic enzymes were also more abundant at 10°C, for example Cysteine tRNA ligase (CysS) and Shikimate dehydrogenase (AroE, Q8Y733), indicating that the stressed cell responds by increasing the production of enzymes needed to upturn the production of scarce amino acids (45, 82). Additionally, six proteins with amino acid transport presented a high fold change, including AroE with FC = 2.23.

Abundance Flows of Virulence-Associated Proteins

The comparison made between a food processing low temperature setting and the *in vivo* temperature enabled an outlook over the changes in heat shock proteins (HSP) and proteins typically associated with virulence. HSPs main function is to repair damage to proteins via chaperone activity, for example DnaK, GroEL, GroES, GrpE, ClpB, and HtpG (27). One might assume their importance in resolving protein damage from cold stress, however this class of stress response genes are usually less transcribed at lower temperatures (91). In this study, the majority of these chaperone proteins were more abundant in the warmer condition, particularly at 37°C. This is also connected to the fact that *L. monocytogenes* is a psychrotroph microorganism and growth in temperatures above 35°C may result in the induction of a stress response (107). Moreover, chaperone proteins are also required for *L. monocytogenes* maximum virulence potential (82). To approach the topic of virulence-associated proteins, it is pertinent to look into Lmo0443, a potential factor for low virulence. Lmo0443 is overexpressed in less virulent *L. monocytogenes* strains and underexpressed in more virulent ones (108). In this *L. monocytogenes* EGD-e strain, Lmo0443 had higher abundance at 37°C, consistent with its lower pathogenicity when compared with EGD or 10403S *Listeria* strains (109). In the same trend of results, internalins are essential for *Listeria* pathogenicity (110). Only one internalin was detected in this study. InlA which was observed with higher abundance at 25°C (10 vs. 25°C comparison, **Supplementary Table 3**). Internalins have a diversity of functions amongst the *Listeriae*, InlA in particular is documented as being crucial for *Listeria* capacity to invade human epithelial cell lines (111). The crucial topological factor (DivIVA) required for completion of cell division in *L. monocytogenes* and associated with host cell invasion was more abundant in the 37°C condition with high fold change (FC = 2.84) (13). Lastly, the *agr* locus of *L. monocytogenes* is recognized to be important in bacterial virulence. The open reading frame *lmo0047* that proceeds the *agrB* encodes a protein of unknown function (112), that was here more abundant at 37°C and with

high fold change (FC = 4.41), suggesting a potential role in a virulence mechanism.

Consistent with previous studies (30, 32, 35, 37, 38, 41, 43, 44, 56, 82, 83, 85, 91), we have observed a significant remodeling of protein abundance as a function of temperature, highlighting once again the predominant influence of this environmental parameter on protein expression and the need for the bacteria to adapt to it and maintain its homeostasis.

CONCLUSION

Listeria monocytogenes overcomes various kinds of stress, including the low temperatures present in food processing and storage. Cold stress adaptation mechanisms are therefore an essential skill of *Listeria*, enabling it to survive and proliferate to reach minimal infectious levels on refrigerated foods. In this aspect, the cold conditions in food plants may, in fact, be selecting for *L. monocytogenes* subtypes with the appropriate adaptive physiological attributes that lead to efficient survival and spread during food handling (45). Analogous approaches in the future will aid in highlighting additional potential target genes of cold stress resistance in *Listeria monocytogenes*. It will be of particular interest to make a direct comparison between

this biofilm proteomic data and similar planktonic approaches. Thus, enabling the study of possible molecular cold response and biofilm targets that currently await additional assessment.

AUTHOR CONTRIBUTIONS

TS and MH designed the study. TS wrote the manuscript. TS, CC, DV, and JE performed the experiments. TS, DV, and MH analyzed the data. All authors reviewed the manuscript.

FUNDING

TS was seconded by List_MAPS project, which received funding from the European Union's Horizon 2020 research and Innovation programme under the Marie-Slodowska Curie grant agreement n°641984. JE was seconded from the Agence Nationale de la Recherche (ANR) (France) as part of an EcoSec contract.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2019.00089/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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***Listeria monocytogenes* planktonic vs biofilm mode of growth: Are there any advantages detected by bottom-up proteomics?**

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Submitted in Frontiers in Microbiology on April 2019

***Listeria monocytogenes* planktonic vs biofilm mode of growth: Are there any advantages detected by bottom-up proteomics?**

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Abstract

Listeria monocytogenes is a foodborne pathogen and the causative agent of listeriosis, an infection with a mortality rate up to 20%. It is most of all a psychrotrophic biofilm forming bacterium capable of adapting, resisting and growing in harsh conditions, including those found in food industries as low temperatures and water availability. *Listeria* ability to survive refrigeration temperatures is directly associated with the majority of listeriosis cases being caused by food contaminated with this bacterium. Multiple genomic, transcriptomic and proteomic reports have duelled in understanding this adaptation at the physicochemical and biological changes. In all instances, temperature adaptation is in some way related to the overexpression or neosynthesis of a set of gene products aimed in particular at maintaining the fluidity and structural integrity of the cell membranes, some metabolic activities and the functioning of translational machinery. Here, three protein extractions methods, namely biotin-labelling, enzymatic shaving and cell fractionation were used in conjunction to explore the proteome of planktonic *L. monocytogenes* cells grown at three different temperatures (10°C, 25°C and 37°C). Plus, an in silico study was carried out using proteomic data previously obtained from biofilm cultures, in order to compare the two modes of growth at three different temperature settings. The combination of these data resulted in a *L. monocytogenes* proteome coverage of 32.6% for which we observed a significant remodelling of protein abundance as a function of temperature and mode of growth.

Keywords: *Listeria monocytogenes*, Temperature adaptation, Biofilm, Planktonic, Shotgun proteomics

Introduction

Listeria monocytogenes is a gram-positive bacterium that can be isolated from soil, vegetation, faecal matter, sewage, water, and animal feed (Moltz and Martin, 2005). Listeriosis, the disease caused by this human foodborne pathogen, is characterised by high mortality rates (reaching up to 30%) and affecting particularly immunocompromised patients, pregnant women, the elderly and newborns (Mead et al., 1999; Soni et al., 2011). *Listeria* is capable of surviving in harsh environments and stresses (high salt concentration, wide range of pH, low water availability), and it also thrives at temperatures ranging from – 0.4 to 45 °C (Cacace et al., 2010). Because of its ubiquitous and psychrotolerant nature, *L. monocytogenes* is very difficult to control along the entire food chain and it is frequently detected in refrigerated food products, mainly in meat and seafood (Tasara and Stephan, 2006; Bucur et al., 2018). In the food industry, *L. monocytogenes* also survives by the formation of biofilms on equipment such as drains, pipes and conveyor belts of preparation surfaces that ultimately contaminate food products (van der Veen and Abee, 2010). The contamination of the ready-to-eat (RTE) food industry and household environments by the foodborne pathogen *L. monocytogenes* is a considerable burden into public health and the economy. RTE shelf life is mostly dependent on refrigeration with low temperatures, to which some bacteria can easily endure, as is the case of *L. monocytogenes* (Cacace et al., 2010; Luque-Sastre et al., 2018). The first *Listeria* responses to cold settings are a decrease in metabolic rates, changes in membrane composition, production of cold shock proteins (Hebraud and Potier, 1999; 2000), and an increase uptake of cryoprotective osmolytes (Phadtare et al., 1999; Somers and Lee Wong, 2004; Neunlist et al., 2005). Furthermore, RTE were the source of half of the listeriosis outbreaks registered in Europe since 1985 (Luque-Sastre et al., 2018). In nature and frequently in food environments the majority of bacteria live attached to surfaces, as complex biofilm communities. Despite the use of disinfectants on food-contact surfaces, these treatments are less effective when applied on biofilms (Dubois-Brissonnet et al., 2016). Compared to the planktonic mode of growth, the compact microbial community within the biofilm is more tolerant to extreme settings and/ or biocides (Chavant et al., 2004; Folio et al., 2004; Dubois-Brissonnet et al., 2016). In sessile mode, *Listeria* can survive at 4 or 10°C for at least 5 days (Somers and Lee Wong, 2004) and most certainly much longer time. Within the biofilm there is

an increase opportunity for metabolic cooperation between bacteria and distinct environmental signalling that ultimately improves their capacity to resist to stress conditions (Davey and O'Toole G, 2000). Since the metabolic characteristics of bacteria within a biofilm are distinct from those of their free-living bacteria counterparts, also their proteomic responses to a cold stress differ. *Listeria* low-temperature growth capability is based on maintaining membrane fluidity and its structural integrity, controlling nutrient uptake, maintaining a functional translational machinery, dealing with inefficient or slow protein folding, reducing enzyme activities, decreasing ability in DNA replication and in RNA translation (Cacace et al., 2010). Proteomes are in constant change dependent on cellular stages (Folio et al., 2004) and particularly in adaptation to a stress condition. Protein expression is one of the bacteria main tools to interact with the environment (Caballero Gomez et al., 2013), therefore its study is of unique importance. Proteomic approaches have been used to study how *L. monocytogenes* adapts to cold environments (Bayles et al., 1996; Hebraud and Guzzo, 2000; Wemekamp-Kamphuis et al., 2002; Cacace et al., 2010; Singh et al., 2011; Cabrita et al., 2013; He et al., 2015). Additionally, proteomics has shown that adaptation to low temperatures improves protection to other stress conditions (Pittman et al., 2014).

Wide-ranging proteomics experiments and *in silico* proteome data analysis were here used to study the protein abundance changes in *L. monocytogenes* planktonic cultures grown under different temperatures and compare them to proteomic data previously obtained in biofilm mode of growth. In sum, the divergent protein abundances observed across the different settings showed temperature and growth mode-dependent differences, highlighting the relevance of growth condition in relation to environmental persistence.

Material and methods

Strain and culture settings for protein extraction

L. monocytogenes EGD-e strain, serogroup 1/2a, was used in the entirety of this study (Glaser et al., 2001). 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 (OD₆₀₀). Pre-cultured cells in stationary phase were used to inoculate cultures to obtain a final OD₆₀₀ of 0.005. The culture incubation settings were dependent on the desired temperature (10°C, 25°C or 37°C) and growth stage (mid-log or stationary phase) (Supplementary Table 1). Three biological replicates were used for each analysed setting.

Protein extraction methods

The protein extraction methods used in this study were biotin-labelling, trypsin-enzymatic shaving and cell fractionation. All three methods were based on the protocol optimization for the extraction of surface exposed proteins developed by Esbelin and colleagues (Esbelin et al., 2018b). The biotinylation method uses a cell-impermeable, cleavable Sulfo-NHS-SS-Biotin to label exposed primary amines of proteins on the surface of intact cells. Planktonic cultures 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. For the second method, enzymatic shaving of surface proteins, planktonic cells were harvested by low-speed centrifugation ($1,000 \times g$, 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 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 1 M L-arabinose) and bacteria were treated with 0.5 $\mu\text{g}/\text{ml}$ sequencing grade trypsin (Promega, Charbonnières-les-bains, France) under gentle shaking at 37°C for 1 h. Bacterial cells were removed by centrifugation ($1,000 \times g$, 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.

In the third and last method, cell fractionation, planktonic cultures 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 ($13,000 \times g$, 15 min, 4°C) and the supernatant was ultracentrifuged ($200,000 \times g$, 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). Membrane and cell wall protein extracts were suspended in 100 mM ammonium bicarbonate pH 7.5.

Nano-LC-MS/MS and bioinformatic analysis

All samples, except those from the shaving method, were loaded onto SDS-PAGE gels to concentrate 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 dithiothreitol and 55 mM iodoacetamide solutions respectively, and all bands were finally dehydrated with 100% ACN. The samples were hydrolysed overnight at 37°C using 48 µl of a 25mM NH₄HCO₃ - 12.5 ng.µl⁻¹ trypsin solution (Promega) per band. Extracted peptides were purified and concentrated with Sep-Pak C18 Plus Short cartridges. Peptides were extracted from the gel bands in an ultrasonic field during 10 mn with 38.4 µl of 100% acetonitrile representing 80% of digestion volume. Supernatants were transferred in Eppendorf vials and dried using Speed Vac for 45 minutes and 40 µl of equilibration solution (H₂O/ Trifluoroacetic Acid – 99.95/0.05) was added.

All peptide mixtures were analysed by nano-LC-MS/MS (Thermo Fisher Scientific) using an Ultimate 3000 system coupled to a LTQ Orbitrap Velos mass spectrometer (MS) with a nanoelectrospray ion source. For each sample, one microliter of peptide mixture was first pre-concentrated on a C18 pre-column (100 µm inner diameter X 5 cm length, Acclaim C18 PepMap nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water at 30 µL/min. After 6 min of desalting and pre-concentration, the pre-column was switched online with the analytical C18 column (75 µm inner diameter X 15 cm length; 2 µm, Acclaim C18 PepMap RSLC) equilibrated with 96% solvent A (99.5% H₂O, 0.5% formic acid) and 4% solvent B (80% ACN, 19.5% H₂O, 0.5% formic acid). Peptides were eluted according to their hydrophobicity at 300 nL/min flow rate using respectively a 4 to 52% gradient of solvent B for 31 min for biotin labelled and trypsin-enzymatic shaved fractions and a 4 to 40% gradient of solvent B for 56 min for cell fractions. Eluates were electrosprayed in positive-ion mode at 1.6 kV through a nanoelectrospray ion source heated to 250°C. The LTQ Orbitrap Velos MS was used in CID top 15 mode (i.e. 1 full scan MS and the 15 major peaks in the full scan were selected for MS/MS). The full scan MS was realized in the FTMS ion trap at a resolution of 60,000 (tolerance 10 ppm) and spectra were acquired with 1 microscan (m/z 400 – 1,400). For raw data processing, MS/MS ion search was performed with Mascot v2.5 for database search (<http://www.matrixscience.com>). The following parameters were considered for the searches: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C) and oxidation (M) set as variable modifications. Protein identification was validated when at least two peptides originating from one

protein showed statistically significant identity above Mascot scores > 13 with a False Discovery Rate of 1% (adjusted significance threshold $p < 0.05$). Ions score is $-10 \log(P)$, where P is the probability that the observed match is a random event. Individual ions score 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 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). For protein quantitation analysis, LC-Progenesis was used with Mascot v2.3 and the same identification parameters described above. Venn diagrams were performed by the jvenn online tool (Bardou et al., 2014). Functional category of proteins was based on the clusters of orthologous groups (COGs) via the eggNOG online framework (Huerta-Cepas et al., 2016). Protein-protein association networks were made by the String database (Szklarczyk et al., 2017). Volcano plots were performed by the R software with the integration of the ggplot2 and ggrepel graphical packages for data analysis (Wickham, 2009).

Results and discussion

Overall protein identification results

From all extraction methods and temperature settings, 931 unique proteins were identified, representing 32.6% of the *L. monocytogenes* proteome (Glaser et al., 2001). The highest number of identified proteins by MS containing at least two unique peptides was obtained by the cell fractionation method (668, including all fractions), followed by biotinylation and shaving methods, 169 and 94 identified proteins, respectively. Figure 1A shows the number and percentage of all proteins identified according to their predicted subcellular location from each subproteome studied and method used. There is a noticeable overlap of identified proteins between the three methods, and the highest number of unique identified proteins was once again obtained with the cell fractionation, particularly the intracellular fraction. It is noticeable a complementarity of the extraction methods and their relative efficiency to extract surface associated proteins targeted by the different methods, even if for some of these methods the identification number is low. The identified proteins were first annotated and separated accordingly to their subcellular localization: intracellular proteins, membrane-associated proteins, cell wall-associated proteins and extracellular proteins. The vast majority of the identified proteins were characterized as cytoproteins (79%), followed by membrane-associated proteins (16.5%), extracellular (3.7%) and cell wall-associated proteins (0.75%). The higher percentage of the identified proteins were cytoplasmic, regardless of the methodology used for cell surface proteome extraction and the measures used to avoid cell lysis. An expected result taking into account that the majority of the cell proteome is composed by cytoplasmic proteins, particularly ribosomal proteins which surpasses 20% of total cell protein (Ryzhov and Fenselau, 2001). However, there are drawbacks at the technical side that add to the large amount of identified cytoproteins. The extraction methods can cause lysis of part of the cells, particularly the shaving method, resulting in a significant number of intracellular proteins being identified (Quan et al., 2013). Functional category of this set of proteins was based on the clusters of orthologous groups (COGs) (Figure 1B). Taking into consideration that the majority of identified proteins are from the cytoplasmic subcellular localization, it is not surprising that the functional category with the highest percentage of identified proteins is 'Translation, ribosomal structure and biogenesis' (13%), followed by 'Transcription' and 'Cell wall/

membrane/ envelope biogenesis', 6% each. Plus, there is a range of normal molecular functions with even number of identified proteins.

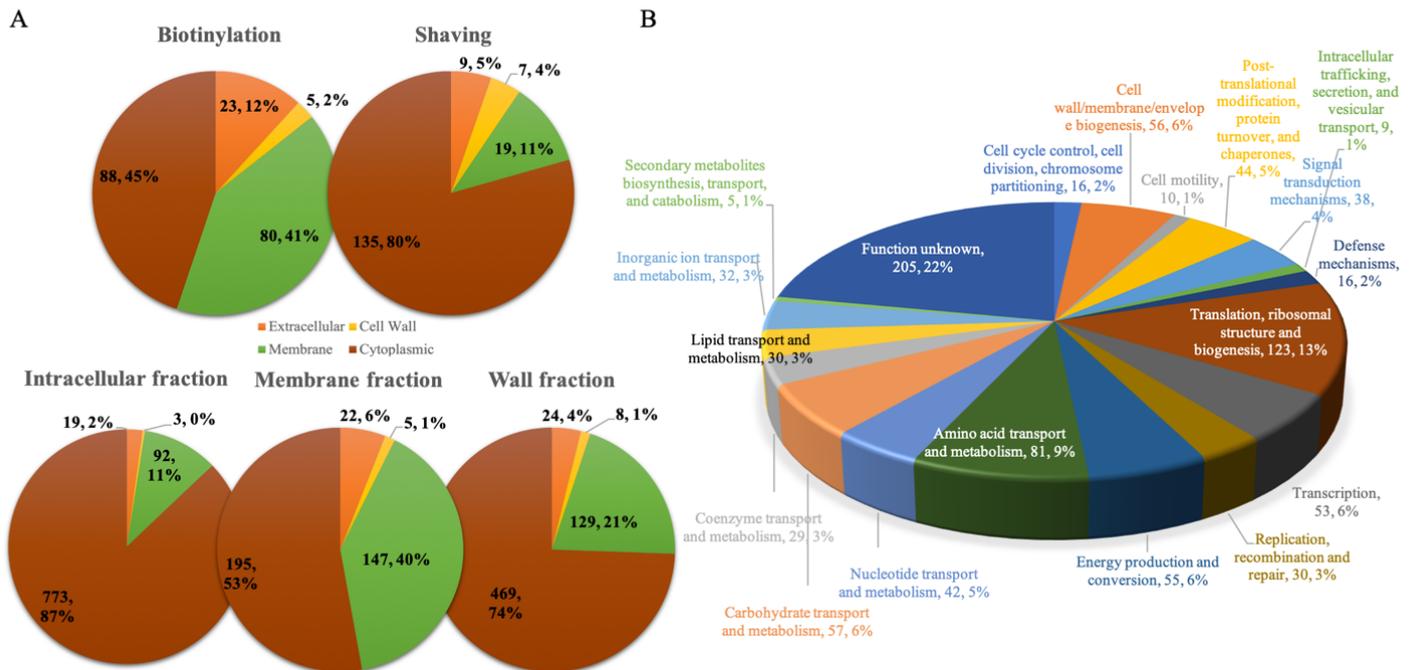


Figure 1 – A: Pie charts illustrating the number of identified proteins and the corresponding percentage for each protein extraction method taking into account the predicted subcellular localization of these proteins; B: Pie chart illustrating the distribution of the 931 unique proteins identified by their predicted COG molecular function (number and percentage by category).

Temperature adaptation of L. monocytogenes planktonic cells as seen through protein quantification

Listeria monocytogenes thrives even at refrigeration temperatures. The adaptation is largely based on changes in protein synthesis and folding, and osmolyte uptake (Cacace et al., 2010). The quantitative analysis of protein abundance made by LC-Progenesis enable the comparison of protein abundance through pairs of temperature settings, 10°C vs 37°C (Figure 2 and Supplementary Table 2), 10°C vs 25°C (Figure 3 and Supplementary Table 3) and 25°C vs 37°C (Figure 4 and Supplementary Table 4). In all cases, normalized abundances of the statistically significant identified proteins ($p < 0.05$) were used in order to compare the flows of proteins in each comparison. The String protein-protein interaction map in Figure 2 reveals the setting to each one of the 434 statistically significant proteins were more abundant when comparing the growth at 10°C and 37°C. This assessment puts into contrast a temperature condition mimicking the one found in the food industry (10°C), and the temperature of the human host (37°C), in which *L. monocytogenes* expresses its

virulence. A close number of proteins with higher abundance was detected at both temperatures, i.e. 237 at 10°C and 198 at 37°C (Supplementary Table 2). The comparison between the protein abundance retrieved from planktonic cells at 10°C and 25°C (an ambient temperature) resulted in the identification of 282 statistically significant proteins (Figure 3 and Supplementary Table 3). In this case a large portion of the identified proteins had higher levels at 10°C (215 proteins). The third and last evaluation, 25°C *versus* 37°C, was between the two best growth conditions for *L. monocytogenes* present in this study and resulted in 401 statistically significant proteins. In this case more proteins with higher levels were found at the 37°C (222 proteins; Figure 4 and Supplementary Table 4). Obviously, growth at 37°C leads to more adjustments in protein synthesis when compared to low or environmental temperature (more than 400 proteins differentially expressed) than the latter two conditions between them.

The adaptation of *L. monocytogenes* to cold growth is accomplished through fluctuations in a large number of cellular components (Cacace et al., 2010). Moreover, the cold protection mechanisms can be shared or confer cross-protection to other different harsh conditions (Soni et al., 2011). General metabolism and biochemical pathways are the ones that ultimately showed a larger shift in protein abundance (Figure 2 and 3), confirming that they are the processes mainly affected upon a cold exposure (Cabrita et al., 2013). Energy production associated proteins, such as glycolysis (Enolase - Eno, 10°C vs 37°C, Figure 2A) and Pta-AckA pathway (Acetate kinase - AckA, 10°C vs 37°C) were detected at higher levels in the cold setting. We detected other enzymes involved glycolysis and carbohydrate metabolism that were also more abundant at the low temperature. As is the case of the aldolase (FbaA) from the inositol phosphate metabolism, observed here more abundant at 10°C. All of these points contribute to the bacteria demand of energy to withstand low temperature growth (Cabrita et al., 2013).

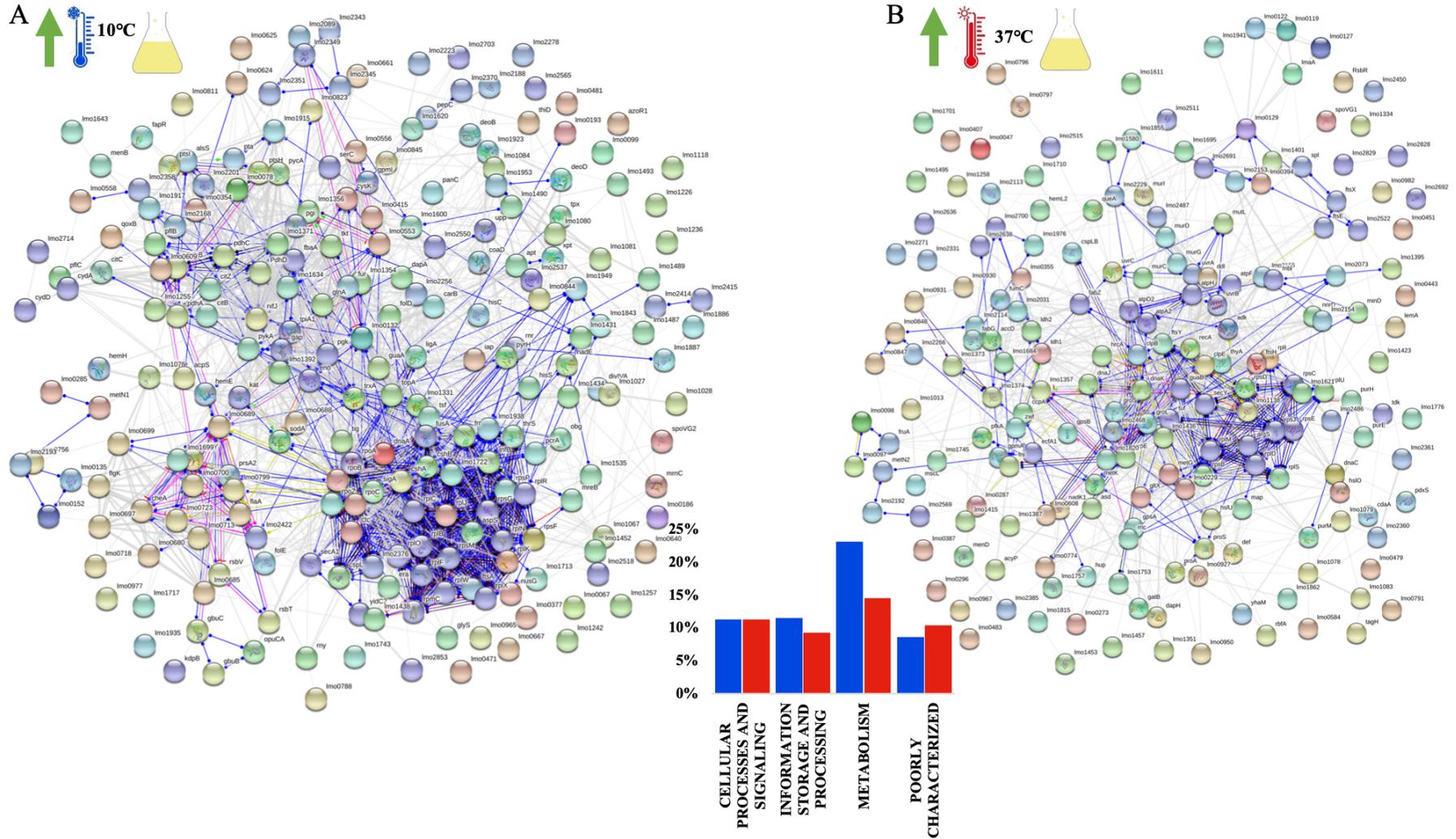


Figure 2 – LC-Progenesis ANOVA comparing planktonic growth of *L. monocytogenes* at 10°C and 37°C (Supplementary Table 2). A: Protein-protein map interaction of the 237 proteins that were more abundant at 10°C; B: Protein-protein map interaction of the 198 proteins that were more abundant at 37°C; Vertical bar chart showing the percentage of proteins with higher abundance in each temperature setting by their predicted COG molecular function.

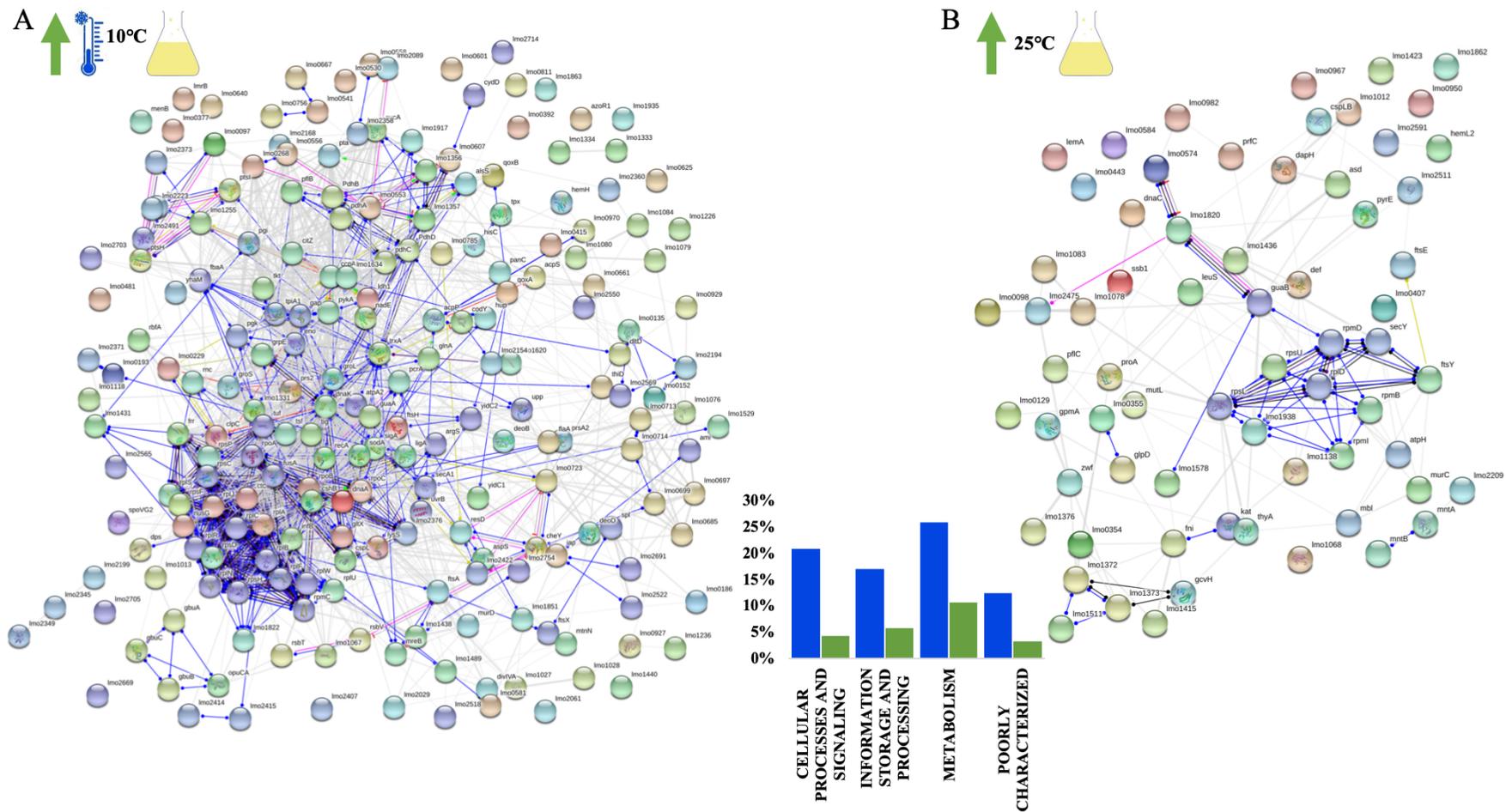


Figure 3 – LC-Progenisis ANOVA comparing planktonic growth of *L. monocytogenes* at 10°C and 25°C (Supplementary Table 3). A: Protein-protein map interaction of the 215 proteins that were more abundant at 10°C; B: Protein-protein map interaction of the 67 proteins that were more abundant at 25°C. Vertical bar chart showing the percentage of proteins with higher abundance in each temperature setting by their predicted COG molecular function.

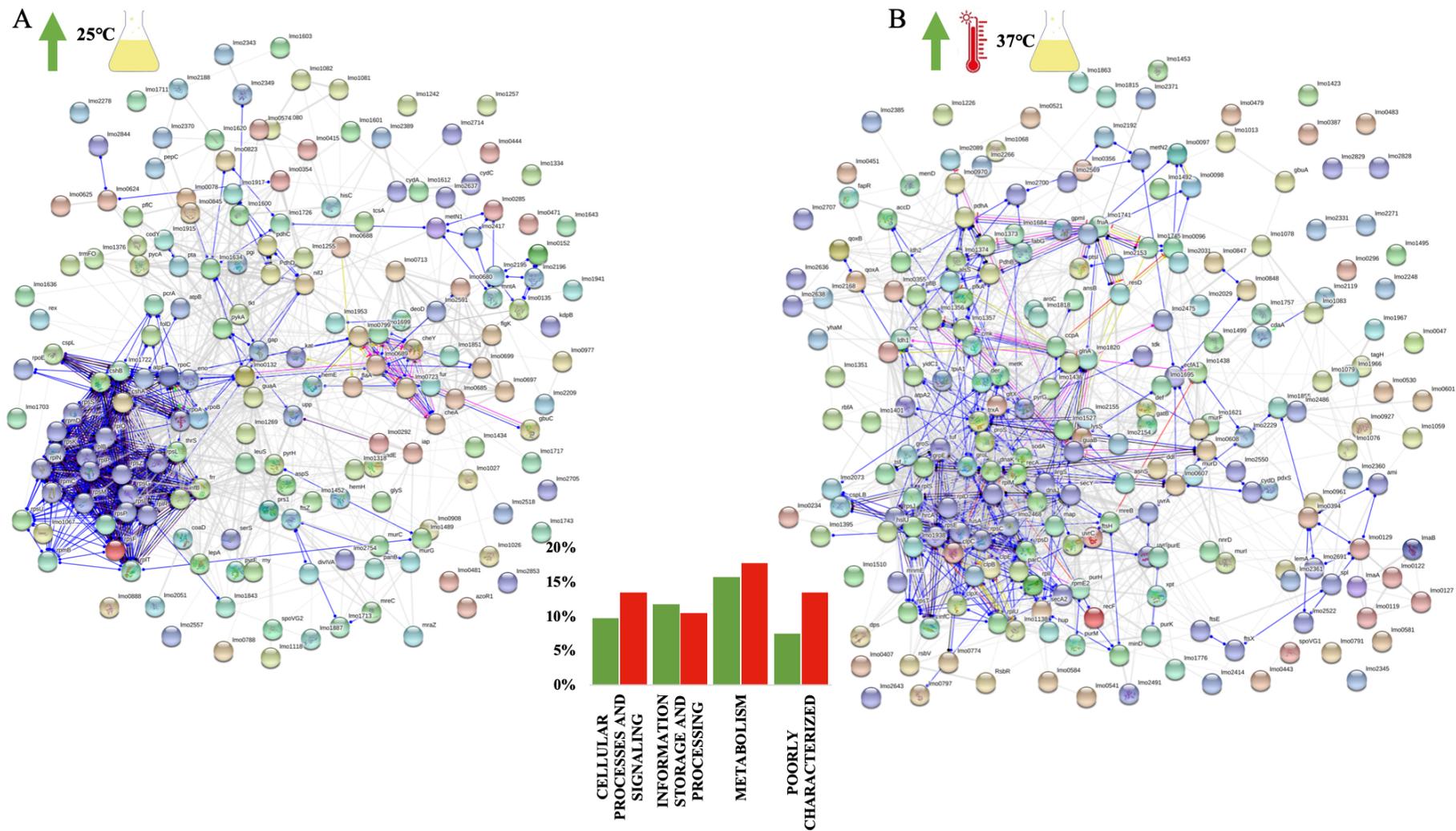


Figure 4 – LC-Progenesis ANOVA comparing planktonic growth of *L. monocytogenes* at 25°C and 37°C (Supplementary Table 4). A: Protein-protein map interaction of the 179 proteins that were more abundant at 25°C; B: Protein-protein map interaction of the 222 proteins that were more abundant at 37°C. Vertical bar chart showing the percentage of proteins with higher abundance in each temperature setting by their predicted COG molecular function.

One of the main response systems implemented by bacteria to face a cold condition is the intake and accumulation of cryoprotective osmolytes, such as glycine betaine and carnitine. This molecule entry is controlled by GbuABC and OpuC transport systems, respectively (Angelidis and Smith, 2003; Wemekamp-Kamphuis et al., 2004). We identified all three proteins from the Gbu system and in all cases, they were present with higher levels at the colder setting. Similar result was obtained for the carnitine intake system with the identification of OpuCA and its presence with a fold change superior to the threshold ($FC > 2$) in the 10°C setting. This is in accord with previous reports that Gbu-mediated glycine betaine uptake improves growth under cold stress and uptake of betaine via BetL and OpuC transport systems improves cryotolerance (Angelidis and Smith, 2003). Additionally, OpuC, the main carnitine transporter, showed higher relevance to cold resistance than the betaine transport system (Soni et al., 2011). In the same study, OpuC was shown to be the main carnitine transporter, which provided markedly higher resistance to cold stress than betaine uptake. Oligopeptide intake is also relevant for bacteria to endure a cold setting. The Lmo0135 is referenced as being part of an oligopeptide transport system highly expressed under low temperatures (Cabrita et al., 2013). This transmembrane protein was more abundant in the cold setting and with high fold change ($FC = 4.86$, 10°C vs 37°C; $FC = 4.76$, 10°C vs 25°C Figure 3A).

Ribosome function is seriously compromised upon growth at low temperatures (Tasara and Stephan, 2006). It is one of the main factors explaining the limit temperature of growth for a bacterial species, particularly when the 50S complex becomes unable to complete the initiation complex 30S, so the initiation of translation stops and there is no longer protein synthesis. For *Listeria*, this limit temperature of growth is below 4°C and at 10°C, growth is slowed down compared to an ambient temperature but it is still active.

Translation initiation factor IF2 is connected with the formation of 30S preinitiation complex, which was here more abundant at 10°C indicating its relevance in ribosome assembling during cold shock. RplY, 50S ribosomal protein L25, mentioned as the general stress protein Ctc (Duche et al., 2002), was observed with higher abundance at 10°C.

For the maintenance of membrane structural integrity under cold growth *L. monocytogenes* promotes changes in its lipid membrane and fatty acid chain

conformation (Cacace et al., 2010). Generally, fatty acids are shorter and unsaturated at low temperatures to maintain membrane fluidity, facilitate the transport and intake of nutrients and ions. Even if a direct link between proteomic data and lipid changes is hard to access, we observed higher levels of hypothetical protein Lmo2201 at 10°C, which catalyses the subsequent elongation steps of the fatty acid chain, indicating the trigger of the lipid biosynthetic pathway.

During growth at low temperatures bacteria also face oxidative stress due to the accumulation of reactive oxygen species (ROS) (Bowman et al., 2010). Oxidative stress can encumber multiple molecular pathways and cellular components. We observed three proteins associated with an adaptive response to oxidative stress with higher levels at 10°C, namely superoxide dismutase (SOD), catalase (Kat) and ferritin (DPS). *L. monocytogenes* SOD and Kat deletion mutants showed lower rates of growth and increased oxidative stress sensitivity (Archambaud et al., 2006; Azizoglu and Kathariou, 2010b). The DPS null *L. monocytogenes* cells displayed higher sensitivity to oxidative stress (Dussurget et al., 2005). Ferritin protein was also detected with high levels in response to a cold shock, indicating to its importance in response to a low temperature environment (Hebraud and Guzzo, 2000). Other proteins involved in iron metabolism, such as Lmo2414 and SufD were more abundant at 10°C. These electron transfer elements also have a role in gene regulation due to their sensitivity to cellular redox events (Lee et al., 2008)

A further relevant bacteria response to cold stress is the increase in levels of cold shock proteins and RNA helicases, which are crucial in RNA secondary structure resolution, contributing for the regain of regular transcription and translation process (Phadtare et al., 1999; Hunger et al., 2006). We observed a higher level of CspLA at 10°C with a fold change of 7.2 (10°C vs 37°C). It has been previously shown that the lack of this gene weakened *L. monocytogenes* growth ability under low temperatures (Schmid et al., 2009). Chan and colleagues described the existence of three putative DEAD box RNA helicases (Lmo0866, Lmo1722, and Lmo1450) that showed a role in the cold stress response (Chan et al., 2007b). All three helicases were here identified and with higher abundance at the cold setting. Moreover, *L. monocytogenes* lacking the Lmo0866 gene showed a decreased growth capacity under cold growth (Azizoglu and Kathariou, 2010a).

Motility in *L. monocytogenes* is described below 30°C, as it happens as well for the presence of flagella (Cabrita et al., 2013). In this study, all the cell motility associated proteins had higher levels at 10°C than at 37°C (Supplementary Table 2), particularly Flagellin (FlaA) was more abundant at the cold setting with a high fold change (FC=14).

As for the protein folding associated chaperones, such as DnaJ, Dnak, GrpE, HrcA, GroL and GroS, typically heat shock proteins that allow the refolding of heat denaturated proteins were present with higher levels at 37°C (10°C vs 37°C; 25°C vs 37°C, Figure 2B and 4B, Supplementary Table 2 and 4). Parallel findings were reported in a transcript level study on *L. monocytogenes* cultured at low temperatures (Chan et al., 2007b). The presence of these proteins at high temperatures indirectly protects cells by aiding in protein folding and assembling. Other heat stress adaptation mechanisms were also more abundant at the warmer condition in study. Signal transduction associated proteins, like HrcA, CtsR and MogR, presented higher levels at 37°C (Hu et al., 2007).

L. monocytogenes cells sense stress-associated molecular harms when exposed to a stress condition. This sense promotes the activation of stress protection processes that ultimately will end up in change of protein abundance and function. In the next section, we compare and discuss the protein changes detected in planktonic and biofilm mode of growth through the three tested temperatures.

In silico protein comparison between planktonic vs biofilm temperature adaptation

Listeria monocytogenes physiological sturdiness is accompanied by its capacity to rapidly attach to surfaces and form a biofilm which is the major factor to the persistence of this microorganism in the food environment (Schwab et al., 2005).

Proteomic data obtained here from *L. monocytogenes* grew in planktonic mode enabled a view over the proteome changes across different growth temperatures. The same extraction methods and overall workflow was previously applied by our group to attached sessile *L. monocytogenes* cells (data and article under review in *Frontiers of Nutrition*). This now allows a deep through protein abundance comparison between the two growth modes and the three temperatures. Protein abundance do not automatically result in different phenotypes (Mata et al., 2015). Nonetheless, free-living bacterial cells and biofilm cells perform in distinct ways that allow for a proteome

data assessment on the bases of their growth mode and the adaptation to different settings. Carbohydrate associated proteins were more abundant in planktonic cells in all the three temperatures (Figure 5). As for proteins associated with amino acid and inorganic ion transport metabolism, a higher amount of them were more abundant in biofilm across all settings. While a higher amount of proteins associated with Transcription and Translation were more abundant in planktonic cells, except at 37°C where we obtained a higher amount of these proteins in sessile cells. Small subunit ribosomal proteins, such as RpsD, RpsE, RpsF, RpsM, RpsL, RpsP, RpsS and RpsT, all showed higher abundance in planktonic cells, in some instances they can be observed in the volcano plots with a high fold change (Figure 6). These results suggest that sessile cells potentially have lower rates of protein translation (Mata et al., 2015).

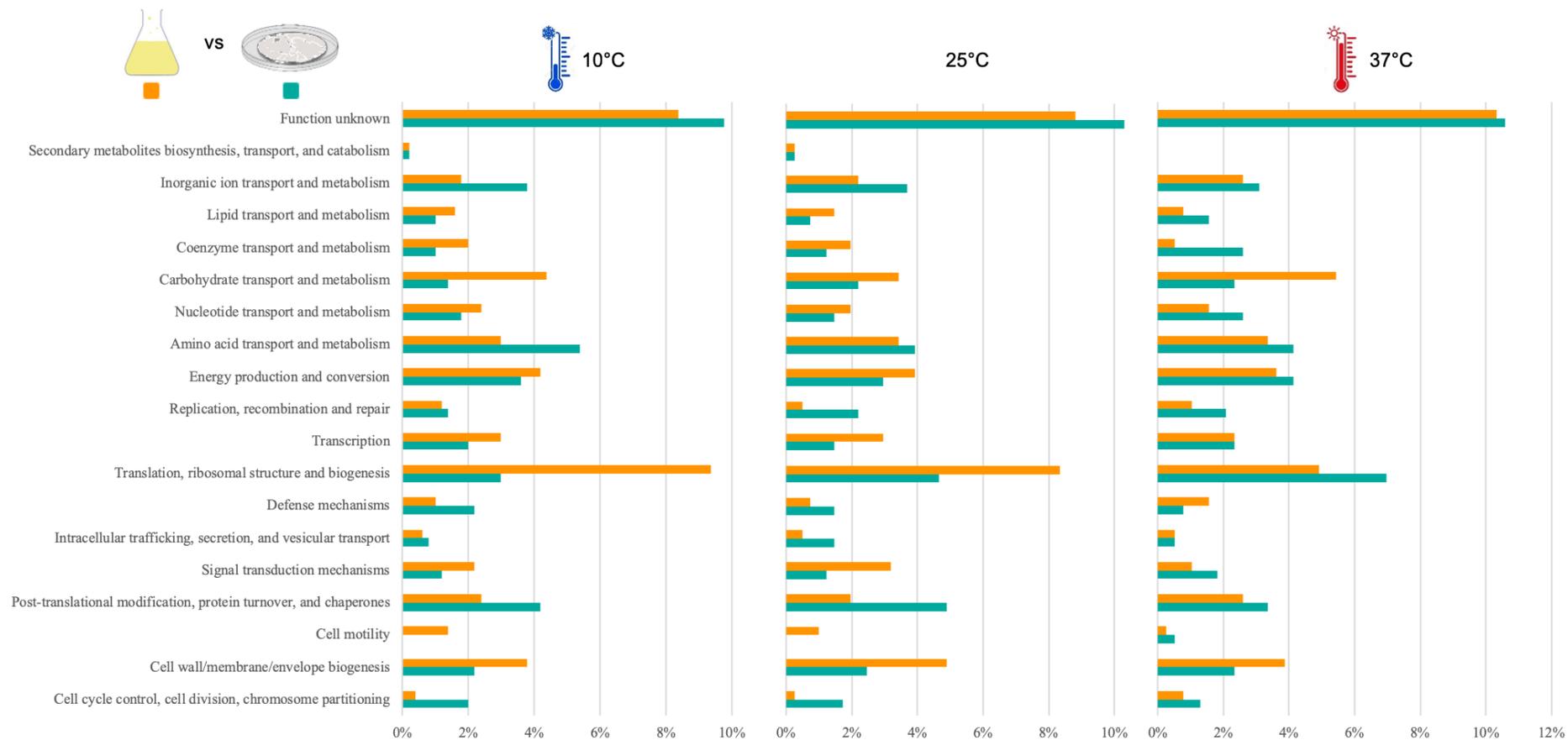


Figure 5 – Planktonic vs biofilm temperature adaptation mechanisms represented in vertical bar charts showing the percentage of proteins with higher abundance in each mode of growth and temperature setting by their predicted COG molecular function (Supplementary Table 5, 6 and 7).

The reduced abundance of flagella (FlaA) is indicative of a biofilm development since the synthesis of this protein is inhibited at this stage (Prigent-Combaret et al., 1999). This trait is one of the most important to differentiate phenotypically biofilms from planktonic cells (Tremoulet et al., 2002).

The overall reduced abundance of cell motility associated proteins in the biofilm mode of growth suggests that sessile cells are less peritrichously flagellated than planktonic cells. We observed in sessile cells a lower abundance of FlaA as well as the flagella hook protein (FlgE), suggesting that sessile cells have overall reduced flagellation (Mata et al., 2015). Data previously obtained indicates that the primary role of flagella in *L. monocytogenes* is to promote motion, meaning that during sessile development motility and extracellular matrix are inversely regulated, when planktonic cells come into contact with a surface their main focus shifts to matrix production (Kolter and Greenberg, 2006; Lemon et al., 2007). Chemotaxis sensor proteins (CheA and CheY) also presented higher levels in planktonic cells at the lower temperature pointing to that sessile cells have a reduced tactic performance.

A number of proteins linked with adherence were more abundant in biofilm compared to the planktonic mode of growth. DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid (TA) has been observed to promote adherence by increasing D-alanylation of TA and it was here detected in higher levels in biofilm at 25°C (Gross et al., 2001). Plus, *L. monocytogenes* deprived of DltD gene presented a significant reduction in adhesion capacity (Chang et al., 2012). The DNA translocase FtsK, involved in cytokinesis and chromosome segregation, is responsible for the presence of extracellular DNA (eDNA) outside of the cell (Rose and Bermudez, 2016). Lmo1386 encoding a putative highly homologous protein to the DNA translocase FtsK/SpoIIIE is more abundant in biofilm at 10°C. Furthermore, it has been shown that this protein plays a role in biofilm formation because the mutant is altered in this ability on abiotic surfaces. As DNA translocase functions as a DNA exporter, the authors hypothesized that Lmo1386 could act as a pump to transport intercellular DNA out of cells, producing eDNA which functions in the biofilm formation (Chang et al., 2013). As discussed in the previous section, oxidative stress is one of the effects of growth at low temperatures. The Fur regulon which controls iron intake by the cell was more abundant in sessile cells. This indicates that biofilm cells display a particular higher oxidative stress response (Ledala et al., 2010). In the same trend, superoxide

dismutase (Sod) and DNA protection during starvation protein (Dps) were present in higher levels in biofilm. SOD is constitutively produced in response to multiple environmental factors and may also play a role in the mode of growth (Tremoulet et al., 2002). These proteins involved in inorganic ion transport and metabolism promote protection against oxidative damage and have a significant role in *Listeria* biofilms (Mata et al., 2015). Regulatory genes with influence on biofilm formation, such as luxS gene of the quorum sensing regulatory Agr system, were also more abundant in biofilm (Chang et al., 2012). The presence of LuxS was shown to be essential in other biofilm forming bacteria such as *Enterococcus faecalis* and *Streptococcus pneumoniae* (Shao et al., 2012; Vidal et al., 2013). Manganese is a crucial micronutrient during infection and for survival under oxidative stress. Bacteria have a high-affinity transport system for manganese intake and its intracellular level maintenance is crucial (Colomer-Winter et al., 2018). The lack of MntABC transport system for manganese leads to reduced biofilm formation in *Neisseria gonorrhoeae* and an *Enterococcus faecalis* with a MntH gene deletion mutant presented less biofilm growth (Lim et al., 2008; Colomer-Winter et al., 2018). Furthermore, manganese is also essential for the development of *Bacillus subtilis* biofilms (Mhatre et al., 2016). We observed higher levels of the manganese transport system MntABC and MntH protein in the biofilm mode of growth, and at 10°C these proteins presented a FC>2 (Figure 6), suggesting its importance not only in biofilm development but also adaptation to temperature.

Listeriolysin regulatory protein PrfA has documented relevance in biofilm formation at room temperature and 37°C and it was here present in higher level in sessile cells (Taylor et al., 2002; Lemon et al., 2010). The loss of PrfA drastically leads to a reduced ability of sessile development (Greene and Freitag, 2003).

Of note, among the proteins more abundant at sessile mode of growth we found enzymes involved in DNA recombination and repair, such as excinuclease uvrB and recombination protein RecA (Greene and Freitag, 2003). Endopeptidase ClpC was also more abundant at biofilm mode of growth, suggesting that in sessile cells metabolism there is a higher activity in the citrate cycle (Resch et al., 2006).

In a previous study comparing *L. monocytogenes* exoproteomes from biofilm and planktonic state, the role of the putative cell wall binding protein Lmo2504 in biofilm formation was confirmed by a deletion mutant coding for this protein (Tremoulet et al., 2002). This protein belongs to a group of enzymes with multiple functions in cell growth

and division, including roles in biofilm development (Popowska, 2004). We observed Lmo2504 at higher levels in the biofilm at 37°C and with a high fold change (FC= 5.42, Figure 6C), supporting the relevance of this protein in sessile development.

Chaperone proteins such as DnaK, classed as a heat-shock protein, was previously described as essential in the development of *L. monocytogenes* biofilms (Korsak et al., 2010). We registered higher levels of this protein in the sessile state, proposing a role of the stress response system in biofilm formation (Cabrita et al., 2013). Besides DNA repair, stress response and motility associated proteins, other proteins previously described as having a role in biofilm were also more abundant here in the sessile data, such as SrtA and RelA (Karatan and Watnick, 2009). However, it should be taken into account that the altered abundance of these proteins might be due to the normal growth in different settings, in this case temperature (Lim et al., 2017). Nonetheless, it is reasonable to assume that the proteome variability seen here between planktonic and sessile cells can possibly be connected to the *L. monocytogenes* capacity to adapt to multiple food processing and clinical environments.

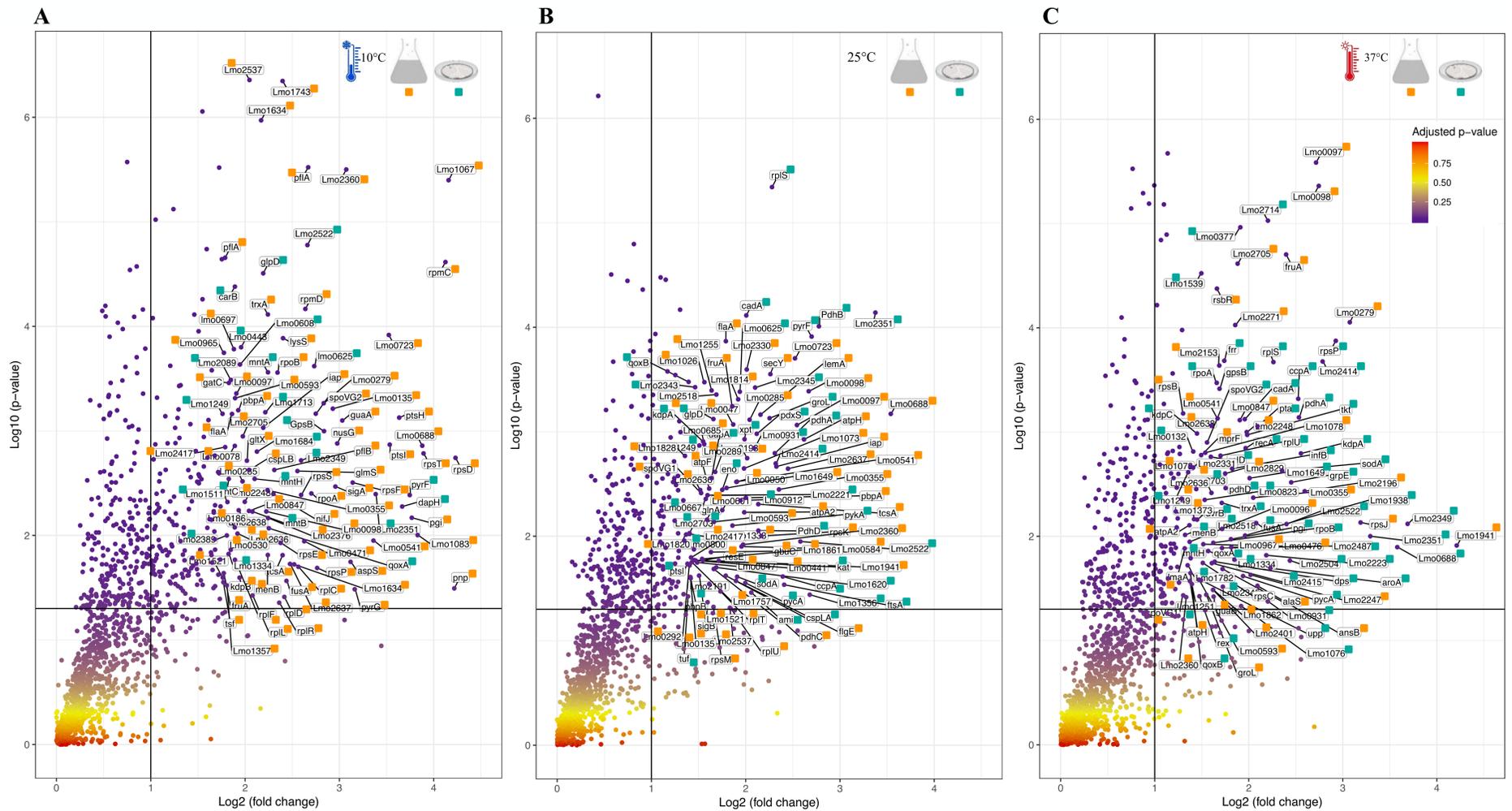


Figure 6 – Volcano plots of the comparison between planktonic and biofilm at 10°C (A), 25°C (B) and 37°C (C) (See also supplementary Table 5, 6 and 7, respectively). The plots represent the distribution of the identified proteins taking into account their fold change and p-value, cut-offs are represented by black lines at fold change two ($\text{Log}_2(\text{fold change}) = 1$) and p-value 0,05 ($\text{Log}_{10}(\text{p-value}) = 1.30103$). The top 100 proteins with highest fold change are marked with their respective colour that represent in which setting they were more abundant.

Conclusion

Listeria monocytogenes survives and proliferates in multiple harsh environments with the use of diverse molecular mechanisms. Due to its recurring recovery from food environments a continue effort has been made to understand these mechanisms and strategies to best control and reduce the contamination of food products with *L. monocytogenes* (Soni et al., 2011). Results here revealed in planktonic *L. monocytogenes* cells grown at 10°C an increased level of transporters for osmolytes, glycolysis and carbohydrate metabolism, nutrient uptake and proteins known to be involved in bacterial response to several stress conditions, as is the case of oxidative stress resistance associated proteins. We also observed a shift in the proteome of *L. monocytogenes* greatly influenced by the biofilm compared to planktonic mode of growth. The proteins that presented a significant change in abundance are in various levels of cellular physiology. This ultimately indicates that the sessile phenotype leads to complex patterns of gene regulation (Cabrita et al., 2013). Taking into account the relevance of biofilm formation for the persistence of *Listeria* on food surfaces, it is crucial to understand the role of specific proteins in the sessile development and the mechanisms behind the resistance of this bacteria in different environments.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

Tiago Santos was seconded by List_MAPS project, which received funding from the European Union's Horizon 2020 research and Innovation programme under the Marie-Sklódowska Curie grant agreement n° 641984. Julia Esbelin was seconded from the Agence Nationale de la Recherche (ANR) (France) as part of an ANR-12-ALID-0005 EcoSec contract.

Supplementary Data

Due to the sheer size of the Supplementary Tables 2, 3, 4, 5, 6 and 7, they could not be inserted in this manuscript. The raw data will soon be available in the PRIDE database upon acceptance of the paper, as it was made in the case of the first paper.

STable 1 - Times when the planktonic culture was harvested taking into account the temperature and stage of growth.

Planktonic			
Mid-log		Stationary	
Time of incubation	Temperature of incubation	Time of incubation	Temperature of incubation
<i>24H of growth</i>	10 °C	<i>48H of growth</i>	10 °C
<i>10H of growth</i>	25 °C	<i>20H of growth</i>	25 °C
<i>6H of growth</i>	37 °C	<i>16H of growth</i>	37 °C

General Discussion

Foodborne pathogens cause significant life-threatening illnesses, fatalities and economic losses worldwide. A study on the global burden of foodborne diseases by the World Health Organization (WHO) estimated that there are annually 2 billion infections resulting in over 1 million deaths caused by 22 foodborne pathogens (Kirk et al., 2015). In regards to food related outbreaks, EFSA reported more than 5 thousand occurrences in 2013 (ECDC, 2015). Per year, in the US, foodborne pathogens are the cause of 48 million cases which resulted in 3 thousand deaths. This colossal number of foodborne related infection results in hundreds of thousands of hospitalizations that together with food recalls lead to billions of euros in losses worldwide, ultimately having a huge impact in public health services and the food processing industry (Scharff, 2012).

As for listeriosis, the life-threatening infection caused by *Listeria monocytogenes*, the WHO study previously mentioned projected more than 14 thousand cases in 2010. This number of infections caused by *Listeria* comes behind other foodborne pathogens, such as *Salmonella enterica* with over 26 million cases estimated cases annually. The hazard and relevance of *L. monocytogenes* is the associated high case-fatality rate of listeriosis, which in this report was estimated to be of 22.4%. A much higher percentage than other foodborne pathogens such as *S. enterica* (0.9%) (Kirk et al., 2015). In Europe, the annual listeriosis incidence sums up to 2,300 cases and in the US to 1,600 (Lomonaco et al., 2015; Antonia Ricci et al., 2018). Those most susceptible to this infection are immunocompromised patients, elderly, infants, and pregnant women. Worldwide, listeriosis causes close to 300 stillbirths and, in 2010, a total of 5463 fatalities (de Noordhout et al., 2014).

There are three main factors that explain the number of listeriosis cases and their associated severity. Firstly, *L. monocytogenes* is a proficient invasive pathogen with the capacity to elicit both innate and adaptive immune responses and to cross the intestinal, brain and the feto-maternal barriers, resulting in meningitis, septicemia, abortion, and perinatal infections (Cossart, 2002; Allerberger and Wagner, 2010; Hernandez-Milian and Payeras-Cifre, 2014). Secondly, *Listeria* is an ubiquitous biofilm-forming bacterium that has been isolated from the most diverse environments, such as soil, water, sewage, hospitals, households and food industries (Schlech et al., 1983). Furthermore, the majority of the human listeriosis cases are linked to the consumption of contaminated RTE foods, some even predict that the underlying

source of all listeriosis is foodborne related (Kirk et al., 2015). Lastly, the third factor for *Listeria's* relevance in public health is connected to its persistence in the food industry causing numerous outbreaks. *L. monocytogenes* is able to endure and sustain in a multitude of harsh conditions and even resist to disinfectant procedures, prolonging its presence in food surfaces and increasing the chances of food contamination (NicAogain and O'Byrne, 2016).

Taking into account this dark outlook, multiple research groups and financial institutions have been intensively investing time and resources to unveil all the mechanisms that allow this foodborne pathogen to resist in the food industry. Case in point, in 2015 the European Union Horizon 2020 under the Marie-Skłodowska Curie actions (ITN-ETN) financed a 4-year project entitled List_MAPS with the goal to train ten PhD students and research in *L. monocytogenes* adaptation through proteomic and transcriptome deep sequencing analysis. Some of the specific aims were to: (i) understand how environmental conditions in soil, plants, biofilms and food matrices influence the capacity of *L. monocytogenes* to cause infection; (ii) Develop an integrated model of the regulatory circuitry of this pathogenic bacterium; (iii) Assess intraspecific diversity of virulence potential and biofilm in relation to environmental cues.

At this moment, the project already produced a substantial amount of data and several outcome papers. Lee and colleagues used crystal violet staining and the BioFilm Ring Test to explore the adhesion and biofilm formation of 22 listerial strains from different serogroups and origins under cold-stressed and cold-adapted conditions. The results showed that adhesion to stainless-steel and polystyrene was increased by cold stress, while cold-adapted cells remained mostly in planktonic form (Lee et al., 2017). Crespo Tapia and colleagues looked into glycerol metabolism and its relevance in *Listeria monocytogenes* biofilm formation, results indicated that the formation of biofilms at the air-liquid interface is dependent on glycerol-induced aerotaxis (Crespo Tapia et al., 2018). Dos Santos and colleagues explored a family of small regulatory RNAs (sRNAs) and their role in *Listeria's* response to heme toxicity. The outcome results made a connection between one of this sRNAs and the response to excess heme and cell envelope stress in *L. monocytogenes* (Dos Santos et al., 2018).

My PhD project is also part of this vast network and had as main aim to explore *L. monocytogenes* proteome shifts resulting from the adaptation to controlled environmental settings through different proteomic and mass spectrometry approaches. Deconstructing the title of this dissertation, which is "Role of proteome in biofilm development and adaptation of *Listeria monocytogenes* to controlled environments", gives us three main topics for discussion: (i) the proteomic approaches embarked in this work; (ii) *Listeria's* mechanisms of adaptation to controlled environments and (iii) the advantageous role of biofilm in *Listeria's* survival.

For the first topic I would like to overview the technical approaches undertaken during the course of this PhD work tasks, namely innovative (MALDI Imaging) and traditional (Shotgun proteomics) mass spectrometry workflows were ventured to study sessile and planktonic *L. monocytogenes* cells.

MALDI Imaging Mass spectrometry (IMS) is a well-established tool that enables to simultaneously identify metabolites and protein spatial distributions within cells/tissues without the need for chemical labels or antibodies (Penque et al., 2011). This powerful tool has been widely used in medical sciences, such as disease pathology and pharmaceutical research, particularly to analyse organs and tissues (Groseclose et al., 2007; Lemaire et al., 2007; Stauber et al., 2010; Maier et al., 2013; Theron et al., 2016; Centeno et al., 2017) and in the search of cancer biomarkers upon the comparison of a tumorous tissue to a healthy one (Djidja et al., 2010). Furthermore, IMS has also been employed in plant tissues (Anderson et al., 2009; Kaspar et al., 2011) and microbiology (Watrous and Dorrestein, 2011). In the latter, most approaches were developed in bacteria grown in thin slices of agar which are further screened in IMS (Hoffmann and Dorrestein, 2015). Further than that, some research groups have optioned instead to promote bacterial growth directly on top of the ITO coated slides (Blaze et al., 2012; Floyd et al., 2015).

An analogous approach was undertaken in the workflow of the first step of this thesis. Moreover, we implemented a comparison between the analysis using MALDI profiling parameters with a manually deposition of matrix and mass spectra acquired with a low spatial resolution of 2000 μm , and the traditional MALDI imaging settings with matrix deposition using a spraying device and a higher spatial resolution of 100 μm . The imaging of *L. monocytogenes* biofilms by MALDI was however not without its challenges.

First and foremost, *L. monocytogenes* biofilms are formed by a honeycomb structure with layers of cohesive cells that have scattered hollow voids with diameters of up to 50 μm (Guilbaud et al., 2015). When considering the MALDI imaging spatial resolution of 100 μm , it means that at some given points ionization will occur in areas with fewer or even deprived of sessile cells. This adds noise spectra to the final average mass spectrum, decreasing its quality and increasing background noise. Secondly, to achieve a suitable density and spread of sessile cells, biofilms had to be grown in suitable growth medium. In the latter, there are salts that can encumber a correct crystallization between matrix and sample, further impairing the ionization (Enthaler et al., 2013). Lastly, one of the big appealing points of MALDI imaging is the search of biomarkers for a specific condition and obtain its abundance and distribution in a tissue (Sandrin and Demirev, 2017). However, the MALDI IMS workflow for studying peptides and proteins has as basis the MALDI workflow for bacterial fingerprinting. MALDI-TOF MS fingerprinting approach enables the quick identification of genus, species and in some instances bacterial serotypes (Risch et al., 2010). This is possible due to the close similarity of ion profiles in this range (2 – 20 kDa) within the same species and the high number of abundant ribosomal proteins. These aspects are unflawed for the identification of a microorganism but negative for the discovery of less abundant potentially biomarker ion species. Nevertheless, we obtained 47 different ions species and its imaging profile across *L. monocytogenes* biofilm. This is a lower number of m/z than the one obtain by Floyd and colleagues upon studying *E. coli* biofilms (Floyd et al., 2015). Still, it should be noted that the thick cell walls of Gram-positive bacteria and its debris are a further obstacle in the ionization event (Santos et al., 2015).

To enhance the ion species spatial information obtained by MALDI IMS we opted for a shotgun approach and the analyses of peptide mixtures by LC-MS/MS. In truth, this breaks the link between the IMS data obtain by MALDI and the identification of proteins, however at this moment MALDI lacks in sensitivity and resolution to perform MS tandem identification from intact biofilms (Maier et al., 2013). A combination of IMS and bottom-up proteomics has been used before and in our study it resulted in a 5 cases being back correlated, a similar result reported by others (Floyd et al., 2015).

As for the second and third paper, a label-free semi-quantitative shotgun approach was used to study planktonic and sessile *L. monocytogenes*. As reviewed in Chapter 3, the bottom-up approach and the prior enzymatic digestion of proteins has its limitations, such as the loss of the intact protein's PTM information, essential for its function, location and general relevance in biological processes (Huber and Huber, 2010). Though, shotgun proteomics allows the efficient identification of large number of proteins from peptide mixtures (Chait, 2006), and it is particularly suited for the comparison of closed related sample extractions as was the case here. One particular target was the identification of as many cellular envelope (surfaceome) and intracellular proteins as possible. The surfaceome is particularly crucial in major biological processes, such as bacterial growth, biofilm formation, responses to environmental stress, host invasion, and interference with the immune system (Cabanes et al., 2002). For that reason, we implemented three complementary protein extraction methods: fractionation, enzymatic shaving and biotin labelling of surface exposed proteins (Eselin et al., 2018b).

The results on the overall protein identification were 920 identified proteins from sessile cell extraction and 931 unique proteins were identified from planktonic culture extraction, representing a significant proportion of the *L. monocytogenes* proteome (Glaser et al., 2001), 32.2% and 32.6%, respectively. The fractionation method was by far the one the lead to a higher number of protein identifications (79% from both growth modes), particularly from the intracellular fraction with cytoplasmic proteins. However, the combination of the three methods resulted in significant percentage of identified membrane, cell wall and extracellular associated proteins. In all extraction methods and fractions there was a significant percentage of cytosol proteins, even if a protocol is focused for the extraction of surface associated proteins, there are high chances of cell lysis, particularly in the shaving method (Quan et al., 2013).

A second major topic in this dissertation was focused on the *L. monocytogenes* ability to adapt to harsh conditions, particularly desiccation and low temperatures.

Desiccation leads to the exit of water from cells resulting in critical changes in cell turgor pressure and overall normal cellular functions. Water availability is an ever-changing condition in nature and also a controlled setting in the food processing environment. Drying allows to significantly increase the shelf life of food products and to reduce at minimum the moisture present in food surfaces after disinfection

procedures avoiding cross-contaminations (Potts, 1994). For microorganisms, desiccation tolerance is the ability to undergo nearly absolute dehydration through air drying without being lysed (Potts, 2001). *Listeria's* capacity to adapt in environments with low a_w has similarities with cellular mechanisms to survive other harsh conditions, such as the import and synthesis of protective osmolites to control turgor pressure and maintain membrane stability, the activation of aquaglyceroporin like channels and the overexpression of multiple chaperone proteins that help to counterbalance the loss of water through the formation of hydrogen bonds to other molecules (Burgess et al., 2016).

The *Listeria's* dehumidification adaptation was the focus on the first paper of this dissertation. Mature biofilms were subjected to 75% relative humidity in an isolated container placed in a room at 10°C, a model setting that can be found in the food industry. By the use of IMS and identification of proteins through LC-MS/MS the goal was to explore potential ion species that could be biomarkers for the adaptation to this condition and see its abundance and distribution across the normal section and the dehumidified section of the biofilm (slide divided at half). As previously mentioned, the mass comparison between the imaging m/z with the identified proteins by mass spectrometry resulted in five positive associations, including the cold-shock proteins CspLA and CspLB, the 50S ribosomal protein L29 (RmpC), a tautomerase-like protein (Lmo2564) and a D-alanine-poly(phosphoribitol) ligase (dltC). As chaperonins, small cold shock proteins are associated with protection against multiple stress conditions (Bayles et al., 1996; Hebraud and Guzzo, 2000; Wemekamp-Kamphuis et al., 2002) and have also a confirmed role in virulence, cell aggregation and flagella-based extracellular motility in *Listeria* (Lee et al., 2017).

The mass spectrometry approach employed in this paper was focused on the identification of proteins from the different biofilm sections and quantitation was not performed. Even if a definitive determination of abundance of the identified proteins cannot be performed, it is possible through the IMS data and representative heat maps to check the abundance and distribution of these identified ions species by shotgun. Both cold shock proteins were found more abundant in the dehumidified section of the biofilm, pointing to its involvement in protection against low a_w environments as they have also been associated with osmotic shock adaptation (Schmid et al., 2009).

Besides the proteins that were matched to the imaging ion species, there were also other relevant stress related proteins from the pool of 513 different proteins identified by LC-MS/MS. This included DEAD-box RNA helicases with a role in RNA degradation and previously annotated as taking part in *Listeria* and *Bacillus* adaptation to cold settings (Hunger et al., 2006; Markkula et al., 2012). Moreover, four stress-induced multi-chaperone systems (ClpB, DnaK, DnaJ and GrpE) were also identified in the dehumidified section of the biofilm. These chaperone proteins repair protein damage and have an observed role in the adaptation to hyperosmotic stress (Liberek et al., 1991; Hanawa et al., 2002; Haslberger et al., 2010).

The second environmental condition tested was temperature, particularly *Listeria*'s adaptation to a low temperature setting. To this point, we explored the subproteomes of *L. monocytogenes* biofilms and planktonic cells under the influence of three temperatures, that is 10°C, mimicking a common setting found in the food industry environment, 25°C as a baseline temperature and 37°C as the human host setting.

L. monocytogenes is able to growth at temperatures as low as -0.4°C (Walker et al., 1990). However, at low temperatures the doubling time of the bacterium can be up to 50 h or more (Angelidis and Smith, 2003). Furthermore, cold leads to a decreased membrane fluidity, an increased superhelical coiling of DNA, a formation of secondary structures in RNA, a reduced enzyme activity, and inappropriate protein folding (Walker et al., 1990; Bayles et al., 1996; Gandhi and Chikindas, 2007; Cabrita et al., 2013; Durack et al., 2013). Variations in temperature are a constant changing factor in nature to which ubiquitous bacteria such as *Listeria* have evolved to adapt. In the food industry environment, low temperatures are used throughout the processing pipeline in order to limit the growth of microorganisms and extend the product shelf life. *Listeria*'s ability to endure in such conditions is a huge threat for food safety (Kathariou, 2002; Colagiorgi et al., 2017).

L. monocytogenes adaptation to cold is mainly based on the following strategies: maintenance of membrane structural integrity, uptake of nutrients and osmoprotectant molecules, resumption of gene expression events, production of chaperone proteins, oxidative stress protection, energy production, and specific amino acid and lipid biosynthesis pathways (Phadtare et al., 1999; Cacace et al., 2010).

Moreover, the cold protection mechanisms are shared or provide cross-protection to other stress conditions (Soni et al., 2011).

Cell stability and cell adhesion at low temperatures is connected with surface appendages, such as flagella (Di Bonaventura et al., 2008). Surprisingly, all the cell motility associated proteins identified from sessile and planktonic cells were present in higher levels at 10°C.

As for ribosome function, it is seriously compromised upon growth at low temperatures (Tasara and Stephan, 2006). The 50S ribosomal proteins (RI) are referenced as the first cold stress sensors in microbes, RplY (RIL25) is the general stress protein Ctc (Duche et al., 2002) and we observed it more abundant at the cold setting in both growth modes.

A key point into the cold adaptation of a bacteria is the production of various stress-related proteins, including cold shock proteins (Csps) (Schmid et al., 2009). We observed a higher level of CspLA at 10°C with a high fold change from both modes of growth. DNA protection during starvation protein (Dps or Fri) is a major cold shock protein that is required for iron storage and protection against reactive oxygen species (Hebraud and Guzzo, 2000). This major protein for low-temperature adaptation was also detected in higher levels at 10°C.

Bacteria have a higher demand of energy to withstand low temperature growth (Cabrita et al., 2013). Energy production associated proteins, such as glycolysis (Enolase – Eno) and Pta-AckA pathway (Acetate kinase – AckA) were detected at higher levels in the low temperature condition.

One of the main response systems implemented by bacteria to face cold stress is the intake and accumulation of cryoprotective osmolytes. At low temperatures, cryoprotective solutes promote stabilization of enzymatic functions and the cell membrane lipid bilayer (Wemekamp-Kamphuis et al., 2004; Miladi et al., 2017). We identified all three proteins from the Gbu system and in all cases they were present with higher levels in the colder setting from both growth modes.

During growth at low temperatures bacteria also have to face oxidative stress due to the accumulation of reactive oxygen species (ROS) (Bowman et al., 2010). We observed three proteins associated with an adaptive response to oxidative with higher levels at 10°C, namely superoxide dismutase (SOD), catalase (Kat) and ferritin (DPS). DEAD-box RNA helicases act as RNA chaperones solving secondary structures in

mRNA that can be found inside the cell subjected to a cold condition (Markkula et al., 2012). The three putative DEAD-box RNA helicase genes are necessary for cold tolerance and motility in *L. monocytogenes* (Bareclev et al., 2014). The three helicases were identified here and presented higher abundance at 10°C.

We have also registered a considerable shift in protein abundance as function of a cold stress, underscoring the relevance of this environmental parameter on protein expression and the need for *Listeria* to adapt to it and maintain its homeostasis (Liu et al., 2002; Chan et al., 2007b; Arguedas-Villa et al., 2010; Durack et al., 2013; Pittman et al., 2014; He et al., 2015; Cordero et al., 2016).

The third and last topic of discussion is the value of biofilm mode of growth in the adaptation of *L. monocytogenes* to cold stress. To this point, in the third paper it was made an *in silico* comparison between the protein abundances from sessile and planktonic cells. Cells in a biofilm have a higher protection against harsh conditions and the sessile formation is the main cause for the persistence of some strains in food processing environments (Ortiz et al., 2010). Furthermore, regardless of temperature, *Listeria* is able to adhere to surfaces and develop a biofilm, pointing to a regulatory system empowering the organism to rapidly adapt to changing environmental conditions (Di Bonaventura et al., 2008). The transition from planktonic to sessile cells requires flagellar mediated motility, production of outer-membrane protein adhesins and exopolysaccharides, and cell-to-cell signalling (Pratt and Kolter, 1999).

Here, we observed protein abundance shifts that might have a relation to the two distinct growth modes. Small ribosomal proteins were more abundant in planktonic than in sessile cells, suggesting that within a biofilm cells possibly have lower rates of protein translation (Mata et al., 2015). We noticed an overall reduced abundance of cell motility associated proteins in sessile cells, confirming that cells in a biofilm are less peritrichously flagellated than planktonic cells (Prigent-Combaret et al., 1999). Several proteins connected with adhesion were more abundant in sessile cells than in planktonic cells. Some of these, like DltD, have been previously associated with the first stages of biofilm development (Gross et al., 2001). Oxidative stress is one of the side effects of growth at low temperatures. In biofilm, proteins associated with tolerance to this stress like the Fur regulon, SOD and Dps were more abundant than in planktonic cultures. This points to a higher capacity to tolerate oxidative stress displayed by biofilm cells (Tremoulet et al., 2002; Ledala et al., 2010).

Regulatory genes with influence on sessile development, such as the quorum sensing regulatory Agr system and the virulence locus *prfA* were also present at higher levels in sessile cells. Both systems have been proven to be associated with biofilm development (Greene and Freitag, 2003; Chang et al., 2012). Chaperone proteins, such as DnaK had higher levels in the sessile state, indicating a role of the stress response system in the biofilm formation (Cabrita et al., 2013).

The proteome shifts here obtained are one more clue to the capacity shown by *Listeria monocytogenes* to adapt to multiple food processing and clinical environments, particularly in biofilm mode of growth.

Conclusions

The foodborne pathogen *Listeria monocytogenes* is a highly adaptable microorganism that can persist in a wide range of environmental and food-related realms. The main source of human listeriosis cases and outbreaks is the proliferation of *L. monocytogenes* on RTE foods (Chan and Wiedmann, 2009).

MALDI MSI showed a noteworthy insight into the spatial proteome of *L. monocytogenes* biofilm, the use of microproteomics for extraction of proteins followed by shotgun proteomics was well suited for the confident identification of numerous proteins, including several related with mechanisms of stress tolerance.

Different harsh conditions such as high salt concentration, wide range of pH, low water availability and, particularly, low temperatures provoke pleiotropic effects on cell physiology. Research showed that the activation of common gene patterns occurs in different stress conditions that contribute to cross-protection occurrences, for example increase intake of osmolytes, folding chaperones and oxidative stress proteins (superoxide dismutase and catalase) which are highly expressed under low temperatures, desiccation and osmotic stress conditions (Cacace et al., 2010).

At 10°C, *L. monocytogenes* cells showed an increased level of osmolyte transporters, glycolysis and carbohydrate metabolism, nutrient uptake and proteins known to be involved in bacterial response to several stress conditions, as is the case of oxidative stress resistance associated proteins. Figure 1 summarizes the planktonic and biofilm protein dataset gathered here showed sets of proteins with higher abundance that have a registered connection with different temperature adaptation mechanisms. In the first moments upon exposure to a low temperature, *Listeria monocytogenes* senses this condition through multiple ribosomal proteins, as well as through the sigma factor sigma B (σ^B). The release of the alternative transcriptional factor eventually activates a series of cold adaptive regulatory protein systems, like: alterations in membrane fatty acid synthesis, induction of cold shock proteins, cell surface changes and induction of osmolyte and peptide transporters. Sets of proteins associated with these regulatory systems were here found more abundant in the cold setting.

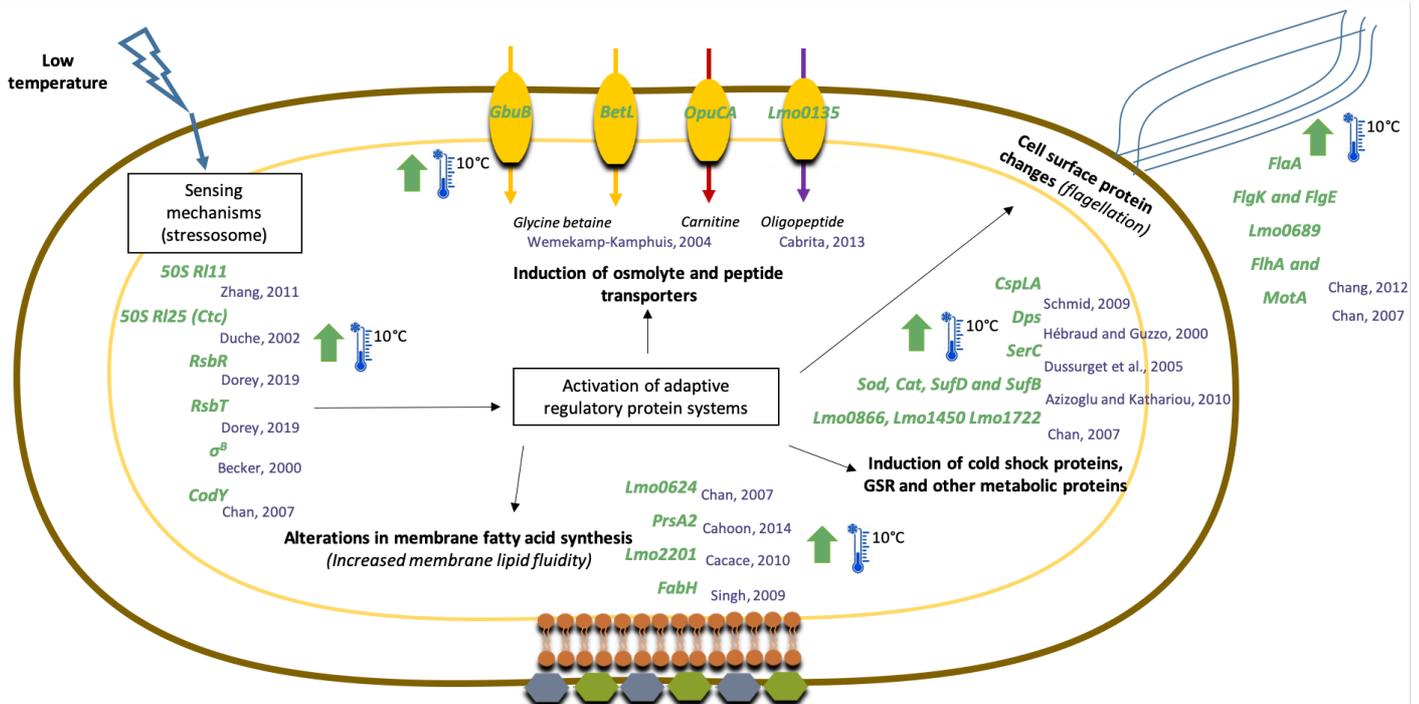


Figure 1 – A data outcome schema representing the different temperature adaptation mechanisms in *L. monocytogenes*.

On the basis of the proteomic data obtained in this dissertation it is unlikely that a set of surface proteins play a unique role in the adaptation to a single stress tolerance. Therefore, adaptation to low temperature growth is a complex response involving many aspects of the cell molecular biology and biochemistry (Tasara and Stephan, 2006). Analogous approaches in the future will aid in highlighting additional potential target genes of cold stress resistance in *L. monocytogenes*.

Bacterial stress adaptation research in the past has privileged the planktonic growth mode. These investigations are the basis of protein functional annotations. This means that potential sessile-specific proteins still have unknown or hypothetical functions (Khemiri et al., 2016). The comparison between planktonic and sessile cells displayed proteome shifts that might have a connection with the biofilm mode of growth. Ultimately meaning that the biofilm growth results in a complex pattern of gene regulation (Cabrita et al., 2013). Figure 2 summarizes the obtained data with connection with relevant changes in protein abundance between the biofilm and planktonic mode of growth. In brief, in one hand, sessile cells have lower rates of protein translation and flagellation. On the other hand, in biofilm mode of growth cells seem to have better adhesion capacity and protein repair systems. Moreover, proteins associated with amino acid and micronutrient intake, oxidative resistance and molecular regulation had higher abundance in this complex growth mode.

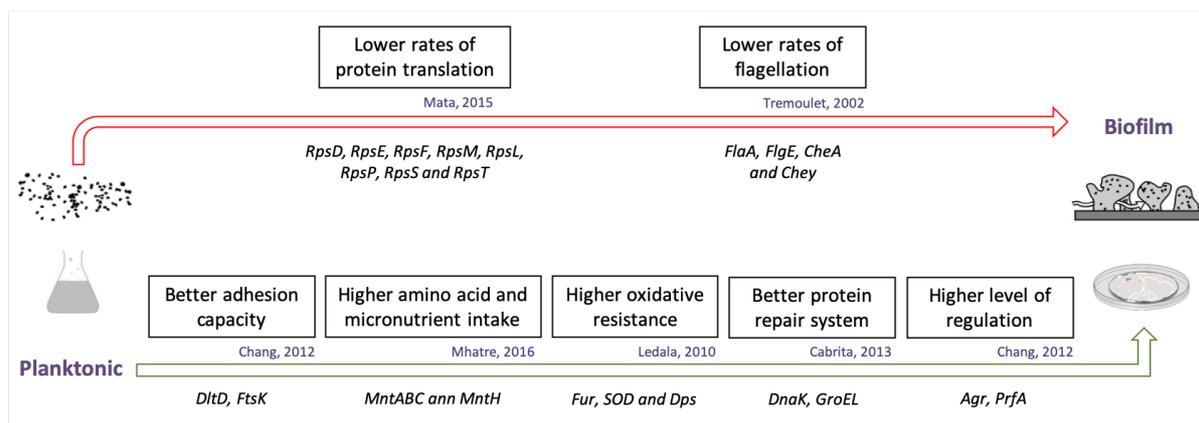


Figure 2 – A data outcome schema illustrating the changes between the planktonic and biofilm growth mode.

Taking into account the relevance of biofilm formation for the persistence of *Listeria* in food surfaces it is crucial to understand the role of specific proteins in the sessile development and the mechanisms behind the resistance of this bacteria in different environments.

The upcoming trend for healthier, minimally processed food products also puts into evidence the foodborne pathogen hazard (Bucur et al., 2018). Innovative treatment procedures like inactivation with visible light or using inhibitors aimed to target the regulatory machinery of this pathogen, such as novel ways to block the expression of transporter proteins that oversee cold/osmotic stress adaptation are promising strategies in the future of food biopreservation (Soni et al., 2011; NicAogain and O'Byrne, 2016). The proteomic approaches developed and used in this dissertation are relevant tools to study the effects on and resistance of *L. monocytogenes* to such combinations of disinfection approaches.

Through the development of an IMS approach it was possible to explore small proteins ionized from an intact *L. monocytogenes* biofilm subjected to a condition of dehumidification. On the basis of the results here obtained and as future application of this technique, it would be relevant to use the same approach to compare the effects of other stress conditions on sessile cells. Furthermore, optimization of an *in situ* trypsin protein digestion will allow to focus the study on peptides to further enrich the analysis. In the same trend, the protein extraction workflow and peptide analysis by label-free semi-quantitative shotgun proteomics here applied can be used to explore *L. monocytogenes* proteome changes resultant from other environmental conditions. Moreover, the huge proteomic dataset will be cross-linked with transcriptomic data

obtained by other partners in the List_MAPS project, to further decipher the transcriptional regulatory circuitry that drives the adaptation of this bacteria. At the personal level, this PhD project enriched substantially my knowledge in microbiology and proteomics, providing the needed tools to embrace a postdoctoral project in these fields or even embark in an industry or a regulatory food safety agency related job.

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Scientific Production

I. Publications

1. **Santos T.** and Hebraud M. 2019. Extraction and preparation of *Listeria monocytogenes* subproteomes for mass spectrometry analysis. In: Methods in Molecular Biology, Ed: Edward Fox, Hélène Bierne and Beatrix Stessl. Humana Press, Springer Protocols, New York, NY, USA. (In preparation).
2. **Santos T.**, Viala D., Chambon C., Esbelin J., and Hebraud M. 2019. *Listeria monocytogenes* planktonic vs biofilm realm: are there any advantages detected by bottom-up proteomics? (Submitted to *Frontiers in Microbiology*).
3. **Santos T.**, Viala D., Chambon C., Esbelin J., and Hebraud M. 2019. *Listeria monocytogenes* biofilms adaptation to different temperatures seen through shotgun proteomics. *Frontiers in Nutrition*, <https://doi.org/10.3389/fnut.2019.00089>
4. Esbelin J., **Santos T.**, Ribiere C., Desvaux M., Viala D., Chambon C., and Hebraud, M. 2018. Comparison of three methods for cell surface proteome extraction of *Listeria monocytogenes* biofilms. *OMICS: A Journal of Integrative Biology*, 22:779-787.
- 5.
6. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D., Esbelin J., and Hebraud M. 2018. MALDI mass spectrometry imaging and *in situ* microproteomics of *Listeria monocytogenes* biofilms exposed to air dehumidification. *Journal of Proteomics*, **187**:152-160.
7. Esbelin* J., **Santos* T.** and Hebraud M. 2018. Desiccation: a common environmental and food industry stress to which bacteria have to face. *Food Microbiology*, 69:82-88. *Review*. * shared first authorship

II. Communications in congresses, conferences, symposiums

II.1 Oral communications

8. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D., and Hebraud M. 2018. MALDI mass spectrometry imaging and *in situ* microproteomics of *Listeria*

monocytogenes biofilms. 35^{ème} SFEAP Congress, joint with the SEProt, Albi, France, October 09-12.

9. **Santos T.**, Chambon C., Viala D., Esbelin J., and Hebraud M. 2018. *Listeria monocytogenes* planktonic and sessile cells adaptation to different temperatures seen through shotgun proteomics. XII EuPA. Santiago de Compostela, Spain, June 16-20.
10. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D., and Hebraud M. 2018. Développement d'une approche d'imagerie par spectrométrie de masse pour explorer des biofilms de *Listeria monocytogenes* exposés à un stress de déshumidification. Journées de l'Ecole Doctorale SVSAE de l'Université Clermont Auvergne. Clermont-Ferrand, France, June 14-15.
11. **Santos T.**, Chambon C., Viala D., Esbelin J. and Hebraud M. 2018. *Listeria monocytogenes* planktonic and sessile cells adaptation to temperatures seen through shotgun proteomics. Foodborne pathogens; from farm to pharmacy! List-MAPS symposium, Kinsale, Ireland, April 26.

II.2 Posters

12. **Santos T.**, Viala D., Chambon, C., Esbelin J. and Hebraud M. 2019. Human Invasive Pathogen *Listeria monocytogenes* Proficient Temperature Adaptation Seen Through Shotgun Proteomics. ASM Microbe, San Francisco, USA, June 20-24.
13. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D. and Hebraud M. 2019. MALDI Mass Spectrometry Imaging and *in situ* Microproteomics of *Listeria monocytogenes* Biofilms. ASM Microbe, San Francisco, USA, June 20-24.
14. **Santos T.**, Chambon, C., Viala D., Esbelin J. and Hebraud M. 2018. *Listeria monocytogenes* planktonic and sessile cells adaptation to different temperatures seen through shotgun proteomics. 35^{ème} SFEAP Congress, joint with the SEProt, Albi, France, October 09-12.
15. **Santos T.**, Theron L., Centeno D., Viala D., Chambon C., and Hebraud M. 2018. A new insight into *Listeria monocytogenes* biofilm adaptation to air relative dehumidification through MALDI mass spectrometry imaging. Microbial stress:

from systems to molecules and back, European Federation of Biotechnology, Kinsale, Ireland, April 23-25.

16. **Santos T.**, Centeno D., Viala D., Chambon C., and Hebraud M. 2017. Development of a MALDI imaging mass spectrometry (IMS) approach to bacterial proteomics: first application to *Listeria monocytogenes* biofilms exposed to a desiccation. FEMS 2017, 7th Congress of European Microbiologists, Valencia, Spain, July 9-13.
17. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D., and Hebraud M. 2017. Développement d'une approche d'imagerie par spectrométrie de masse pour explorer des biofilms de *Listeria monocytogenes* exposés à un stress de déshumidification. 8^{ème} Colloque du Réseau National Biofilms. Clermont-Ferrand, France, December 5-6.
18. **Santos T.**, Chambon C., Viala D., Esbelin J., and Hebraud M. 2017. Approche protéomique pour explorer les mécanismes d'adaptation de *Listeria monocytogenes* à son environnement. 8^{ème} Colloque du Réseau National Biofilms. Clermont-Ferrand, France, December 5-6.
19. **Santos T.**, Theron, L., Chambon, C., Viala, D., Centeno, D., and Hebraud, M. 2017. MALDI imaging and profiling mass spectrometry approach for the analysis of *Listeria monocytogenes* biofilms exposed to a desiccation. SMMAP 2017, Spectrométrie de Masse, Métabolomique et Analyse Protéomique, Marne-la-Vallée-Chessy, France, October 3-5.
20. **Santos T.**, Chambon C., Viala D., Esbelin J., and Hebraud M. 2017. Adaptation of *Listeria monocytogenes* to temperature: exploration of intracellular subproteome through shotgun proteomics. SMMAP 2017, Spectrométrie de Masse, Métabolomique et Analyse Protéomique, Marne-la-Vallée-Chessy, France, October 3.

III. Public dissemination

21. **Santos T.**, Theron L., Chambon C., Viala D., Centeno D., Esbelin J., and Hebraud M. 2018. L'imagerie par spectrométrie de masse pour l'exploration moléculaire de biofilms. Faits marquants Dpt MICA.

IV. Workshops, Summer schools and Secondments

1. Workshop “Outreach”, 13- 14 October 2015, Dijon, France.
2. French course Level A1 (44h), 8 February – 6 June 2016, Clermont-Ferrand, France.
3. Summer school “Next generation sequencing”, 4 – 6 July 2016, Frankfurt, Germany.
4. Workshop “Introduction to statistical analysis of expression data with R”, 10 – 14 October 2016, Jouy-en-Josas, France.
5. Online joint syllabus on a business plan (52h), January to June 2017.
6. Summer school “Quantitative proteomics”, 3 – July 2017, Clermont-Ferrand, France.
7. Workshop “Use of model to optimize experimental design and extract biological knowledge”, 9 – 11 October 2017, Copenhagen, Denmark.
8. Secondment “Invasion capability assays with *Listeria monocytogenes* mutants in Caco-2 cells”, 21 November – 20 December 2017, Copenhagen, Denmark.
9. Workshop “Career and job opportunities”, 2 – 3 July 2018, Dijon, France.
10. Secondment “*Listeria monocytogenes* infection capability assays in larvae of *Galleria mellonella*”, 4 – 22 July 2018, Dijon, France.

ABSTRACT

Listeria monocytogenes is a Gram-positive bacterium implicated in serious food-borne infections. Most cases of human listeriosis are caused by the consumption of refrigerated ready-to-eat foods. The ability of these bacteria to survive and multiply in a wide range of harsh conditions make this pathogen a major concern in agro-food industries. These properties of *L. monocytogenes* are enhanced by its ability to form biofilms. The aim of this project was to explore the adaptation of this pathogen to dehumidification and low temperatures by two proteomic approaches. The first approach, based on the MALDI-TOF mass spectrometry imaging (IMS), allows the mapping of molecules from biological samples. This work aimed to develop this approach, considering a bacterial biofilm as a tissue, in order to access information on the distribution of proteins in *L. monocytogenes* biofilms subjected to a dehumidification stress. In addition, an LC-MS/MS approach was used to link spectral data of interest obtained by IMS and protein identification. The IMS allowed to examine the distribution of 47 low molecular weight proteins within the biofilms. Five identified proteins were assigned by LC-MS/MS using IMS m/z data, including two cold-shock proteins. The results demonstrate that imaging can be used to dissect the spatial proteome of a bacterial biofilm. The second proteomic approach consisted on a relative semi-quantitative label-free (shotgun proteomic) comparison of proteins expressed under different culture conditions. With the method, we explored protein expression according to the mode of growth (biofilm vs planktonic) and temperature (10°C, 25°C and 37°C). Throughout the 920 and 931 unique proteins identified, from sessile and planktonic cells, respectively, many are connected to basic cell functions, but some are linked with thermoregulation. A shift was observed in the proteome of *L. monocytogenes* biofilms compared to planktonic cells indicating different patterns of regulation according to the mode of growth. These comparisons of protein expression throughout several conditions (mode of growth and temperatures) will enrich databases and help to model regulatory circuitry that drives adaptation of *L. monocytogenes* to environments.

Keywords: *Listeria monocytogenes*, biofilms, adaptation, Proteomics, Imaging Mass Spectrometry

Rôle du protéome dans le développement de biofilms et adaptation de *Listeria monocytogenes* à des environnements contrôlés

RESUME

Listeria monocytogenes est une bactérie à Gram positif impliquée dans des infections graves d'origine alimentaire. La plupart des cas de listériose humaine sont causés par la consommation d'aliments réfrigérés prêts à consommer. La capacité de ces bactéries à survivre et à se multiplier dans une large gamme de conditions difficiles fait de ce pathogène une des préoccupations majeures dans les industries agro-alimentaires. Ces propriétés de *L. monocytogenes* sont renforcées par son aptitude à former des biofilms. Le but de ce projet était d'explorer l'adaptation de ce pathogène à la déshumidification et aux basses températures par deux approches de protéomique. La première approche, basée sur la technique d'imagerie par spectrométrie de masse (IMS) MALDI-TOF, permet de réaliser la cartographie de molécules à partir d'échantillons biologiques. Ce travail a consisté à développer cette approche, en considérant un biofilm bactérien comme un tissu, afin d'accéder à des informations sur la distribution de protéines dans des biofilms de *L. monocytogenes* soumis à un stress de déshumidification. En outre, une approche LC-MS/MS a été utilisée pour relier les données spectrales d'intérêt obtenues par l'IMS et l'identification des protéines. L'IMS a permis d'examiner la distribution de 47 protéines de bas poids moléculaire dans les biofilms. Cinq protéines ont été identifiées par LC-MS/MS grâce aux données m/z de l'IMS, y compris deux protéines de choc thermique. Les résultats démontrent que l'IMS peut être utilisée pour disséquer le protéome spatial d'un biofilm bactérien. La deuxième approche protéomique a consisté en une comparaison semi-quantitative relative et sans marquage (shotgun proteomic) des protéines exprimées dans différentes conditions de culture. Par cette méthode, nous avons exploré l'expression protéique en fonction du mode de croissance (biofilm vs planctonique) et de la température (10°C, 25°C et 37°C). Parmi les 920 et 931 protéines uniques identifiées, provenant respectivement de cellules sessiles et planctoniques, beaucoup sont liées à des fonctions cellulaires de base, mais certaines sont liées à la thermorégulation. Des changements ont été observés dans le protéome de *L. monocytogenes* en biofilm par rapport aux cellules planctoniques, ce qui indique des modes de régulation différents selon le mode de croissance. Ces comparaisons de l'expression des protéines dans plusieurs conditions (modes de croissance, températures) enrichiront les bases de données et aideront à modéliser les circuits de régulation qui conduisent à l'adaptation de *L. monocytogenes* aux environnements.

Mots clés: *Listeria monocytogenes*, Biofilm, Adaptation, Protéomiques, Imagerie par spectrométrie de masse
