Identification of Virulence Biomarkers in a *Listeria monocytogenes* ST7 Strain Through Immunoproteomic and Transcriptomic Analysis

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The main objective of this PhD project was to identify specific virulence biomarkers of a *Listeria monocytogenes* 1/2a strain, cultivated under different stress conditions, and to gain insights into the proteins implicated in its pathogenesis mechanisms.

Identificazione di biomarkers di virulenza in un ceppo di *Listeria monocytogenes* ST7 mediante analisi di immunoproteomica e trascrittomica

Il principale obiettivo del presente progetto di dottorato è stato quello di identificare specifici biomarker di virulenza in un ceppo di *L. monocytogenes* 1/2a, coltivato in diverse condizioni di stress e ottenere informazioni sulle proteine chiave coinvolte nei meccanismi di patogenesi.

**Key words**: *Listeria monocytogenes*; immunoproteomics; biomarkers; transcriptional profiling.

# **1. Introduction**

The present PhD project aimed to identify specific virulence biomarkers of a *L. monocytogenes* ST7 strain grown at different stress environmental conditions. *L. monocytogenes* causes listeriosis, a severe foodborne infection in humans. The investigation of key proteins involving in virulence mechanisms of the pathogen is crucial to develop effective control strategies of public health surveillance. In order to achieve this objective, a comprehensive analysis of a *L. monocytogenes* 1/2a strainimmunoproteome was conducted by mean of nLC-MS/MS technique combined with an immunoinformatic approach. Furthermore, a correlation between the immunoproteome and transcriptome was studied by the development of a bioinformatics pipeline, integrating data from both immunoproteomic and transcriptional profiling analyses. By combining different omics approaches, this project aimed to improve our understanding of *L. monocytogenes* pathogenicity and identify specific biomarkers which can help in early detection, risk assessment, and development of targeted interventions.

# **2. Materials and Methods**

*L. monocytogenes* ST7 strain was cultivated at C1 (control): T 37°C, pH 7.0, NaCl 0.5%; C2: T 37°C, pH 5.5, NaCl 7%; C3: T 12°C, pH 7.0, NaCl 0.5%; C4: T 12°C, pH 5.5, NaCl 7% (D’Onofrio *et al*., 2021). Biological and technical triplicates were carried out for each experimental condition. The proteins were extracted, purified, and quantified. Protein extracts were resolved by SDS PAGE and Western blotting (WB) was carried out (D’Onofrio *et al*., 2022). The immunogenic proteins highlighted by WB were digested in-gel. The peptide extracts were analyzed by nLC-ESI-MS/MS, identified by Proteome Discoverer v1.4.1.14 against the database “uniprot\_listeria\_monocytogenes”, and then analyzed by immunoinformatic pipeline (Paci *et al*., 2021). The proteins encoded only in C1 and C4 conditions were correlated with their respective transcripts, obtained by RNAseq analysis, using Cufflinks version 2.2.1 for transcript assembly and Tophat version 2.1.1 for transcripts abundance estimation, and differential expression analysis.

# **3. Results**

In the present project an immunoproteomics approach combined with bioinformatics was applied to identify proteins showing differential expression in *L. monocytogenes* ST7 strain under different stress conditions and therefore relevant for the presence of the strain in meat products. By detecting antigen-antibody immunocomplexes (Ag-Ab ICs), potential immunogenic proteins specific to each stress condition have been predicted by different bioinformatic tools.

Through a comprehensive analysis using nLC-ESI-MS/MS-based proteome identification, a total of 226 proteins were identified. Among them, 58 proteins (28.3%) were classified as potential antigens, of which 6 membrane proteins were detected as unique in the 3 experimental stress conditions (C2, C3 and C4).

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, membrane proteins were involved in the functional categories as listed below.

A1) General stress response: Q8Y3Z9 (*lmo2679*), O32823 (*trxB*), Q8YAJ3 (*lmo0132*).

A2) Cell morphology and motility: Q8Y942 (*lmo0699*), Q8Y919 (*lmo0723*).

A3) Carbohydrate transport and metabolism and energy production: Q8Y864 (PdhB).

## **3.1 Immunoblotting**

## Immunoblotting analysis was conducted using a *L. monocytogenes* positive ovine serum (Fig. 1). Specific Ag-Ab ICs were detected at 50 kDa and 40 kDa under all growth conditions. Furthermore, Ag-Ab ICs were observed at 110 and 75 kDa in C2 and C3, respectively. Then, specific Ag-Ab ICs were identified at 80 kDa in C3 and C4, and at 60 kDa in C1 and C4.

## **3.2 Bioinformatic**

A total of 226 proteins were identified using nLC-ESI-MS/MS. To predict the subcellular localization of selected proteins, different tools were utilized, including LipoP 1.0 Server, TMHMM Server version 2.0, SignalP 4.1 Server, PSORTb version 3.0.2, and CELLO version 2.5. Among the identified proteins, 76 were non-cytosolic. These proteins were further analyzed using NetSurfP version 1.1 and BepiPred version 1.0 Server in order to assess their localization and predict epitopes exposed to solvent. Fifty-eight proteins were classified as potential antigens using Virulent Pred, Vaxign tool, and VaxiJen Server. Among them, 30 proteins were exclusively present in C1, while 6 proteins were identified in C2, C3, and C4 (Fig. 2).

A specific histidine kinase with a molecular weight (MW) of 100 kDa was found exclusively in C2. Only in C3, the flagellar motor switch protein (FliM) with a MW of 38 kDa was identified, together with lmo0723 (MW 66 kDa) encoded by the gene *lmo0723*.

In C4, the proteins identified were PdhB (MW 35 kDa), which bears similarity to pyruvate dehydrogenase (E1 b-subunit), thioredoxin reductase (MW 34 kDa), and lmo0132 (MW 55 kDa) involving in DNA repair and maintenance (Table 1) (Wang *et al*., 2016). Moreover, in C4 the expressed proteins were associated with a higher degree of stress, which could lead to cell damage and cell death. One of the identified proteins was the pyruvate dehydrogenase E1 beta-subunit (PdhB), encoded by the *lmo1053* gene. PdhB is part of the pyruvate dehydrogenase enzyme complex (PDH), which played a role in carbohydrate metabolism. Another expressed protein in C4 was thioredoxin reductase, encoded by *trxB*. This protein is considered part of the oxidative stress response and is regulated by the peroxide operon PerR. Q8YAJ3, encoded by *lmo0132* was identified in C4 condition.

## **3.3 Transcriptomic**

The integrated analysis of immunoproteomics and transcriptomics data provided valuable insights into the activation of stress response genes in C4. Among them, gene *lmo0613* encoding for lmo0613 proteins-oxidoreductase, exhibited significant upregulation. The oxidoreductases played a crucial role in increasing resistance to oxidative and antibiotic stress in bacteria (Pleitner *et al*., 2014). The activation of lmo0613 suggested that *L. monocytogenes* employed this pathway to cope with the challenging environmental conditions in C4. Another gene, *lmo1525*, encoding for the recombination protein RecS, also displayed pronounced upregulation in C4. Recombination proteins, such as RecS, are known to contribute to various cellular processes, including cold tolerance and DNA repair and recombination. The upregulation of lmo1525 indicated that *L. monocytogenes* activates DNA repair mechanisms and recombination pathways in response to the combined stressors in C4.



**Figure 1** *Immunoblotting of L. monocytogenes ST7 strain, cultivated at four different environmental conditions (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

**Figure 2** *Identification of immunogenic proteins of L. monocytogenes ST7 cultivated at four different combinations of T, pH and NaCl %: bioinformatic workflow (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

**Table 1** *List of identified L. monocytogenes ST7 immunogenic proteins (St: Standard; C1: 37°C, pH 7.0, NaCl 0.5%; C2: 37°C, pH 5.5, NaCl 7%; C3: 12°C, pH 7.0, NaCl 0.5%; C4: 12°C, pH 5.5, NaCl 7%).*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Gene** | **Protein** |  **Function** | **Localization** |  **MW (kDa)** | **Condition** |
| *lmo2679* | **Q8Y3Z9** | Histidine kinase | Adaptation to high-osmolarity conditions by regulating the expression of a high-afﬁnity potassium uptake system encoded by the *kdpABC* genes | Membrane | 100 | C2 |
| *lmo0699* | **Q8Y942** | Flagellar motor switch protein - FliM | Part of the switch complex that is involved in switching the direction of the flagella rotation | Membrane | 38 | C3 |
| *lmo0723* | **Q8Y919** | lmo0723 | Methyl-accepting chemotaxis protein | Membrane | 66 | C3 |
| *lmo1053* | **Q8Y864** | PdhB protein | Highly like pyruvate dehydrogenase (E1 beta-subunit) | Membrane | 35 | C4 |
| *trxB* | **O32823** | Thioredoxin reductase | Protection against oxidative stress by regulating the expression of thioredoxins | Membrane | 34 | C4 |
| *lmo0132* | **Q8YAJ3** | lmo0132 | DNA repair and mantainance | Membrane | 55 | C4 |

# **4. Discussion**

Listeriosis is recognized as the fifth most frequently reported foodborne disease among humans, and its notification is mandatory in the European Union (EFSA and ECDC, 2021). Consequently, the prevention and control of *L. monocytogenes* continue to be of greatest importance to ensure food safety worldwide. In recent times, different omics approaches, including proteomics, genomics, and transcriptomics, have emerged as valuable tools for studying the biodiversity of *L. monocytogenes* strains. In particular, immunoproteomics and transcriptomics provide appreciated insights into the behavior of the pathogen under stress conditions.

In response to stressors, *L. monocytogenes* can undergo changes in cell morphology, motility, and metabolism to enhance their growth and survival. The present study revealed the presence of histidine kinase (Q8Y3Z9) encoding by the *lmo2679* gene when the microorganism is exposed under mild acid and salt stress conditions at 37°C (C2). Histidine kinases are transmembrane proteins that play crucial roles in two-component regulatory signaling systems that induce the transcription of genes encoding membrane desaturases, thereby enhancing membrane fluidity. The expression of the histidine kinase encoded by *lmo2679* could be a response to the combined stresses of acidity and salt. In C3, where the only stressor was low temperature, only 2 expressed proteins involved in cell motility were identified.

Previous studies have demonstrated that *L. monocytogenes* can modulate flagellar motility in response to temperature, salt stress, and pH (Horlbog *et al*., 2019). The genes associated with cell motility in *L. monocytogenes* were downregulated under stress conditions. This regulation is crucial because flagella consume energy and could trigger the immune response. In *L. monocytogenes*, the flagellar motor-switch protein FliM, encoded by *lmo0699*, is essential for motility and involved in the secretion of other flagellar-related proteins. Most strains exhibit motility below 25°C, so it is not surprising to find the expression of FliM at 12°C. However, in the presence of acid and salt stress (C4) at the same temperature, FliM (lmo0699) was not detected. This finding suggests the impact of combined stress factors on the proteome. Interestingly, another protein Q8Y919, encoded by *lmo0723*, was identified in C3. Q8Y919 is potentially involved in chemotaxis and motility. In C4, the expressed proteins were associated with a higher degree of stress, which could lead to cell damage and cell death. PdhB, encoded by the *lmo1053*, plays a role in carbohydrate metabolism. TrxB, encoded by *trxB*, is considered part of the oxidative stress response, and is regulated by the peroxide operon PerR. TrxB is upregulated in response to harsh environmental conditions, such as after 48 h of desiccation on stainless steel surfaces and under acid shock at pH 3.0 with HCl in highly acid-tolerant field strains (Horlbog *et al*., 2019). This protein is an enzyme involved in the purine biosynthesis pathway called inosine 5-monophosphate dehydrogenase. It catalyzes the synthesis of xanthosine monophosphate through the NAD+-dependent oxidation of inosine monophosphate. The upregulation of genes, involved in severe stress response and DNA repair, suggested that the cultivation conditions used in C4 induced a response to extreme environmental conditions.

The correlation between immunoproteomics and transcriptomics demonstrated the activation of numerous stress response genes under the most severe stress conditions, such as *lmo0613* and *lmo1525*, involved in cold tolerance and antibiotic resistance, respectively. This observation highlighted a robust and coordinated cellular response by *L. monocytogenes* to the combined stressors present in C4.

# **7. Conclusions and Future Perspectives**

# In conclusion, the present study focused on understanding the proteome’ changes of *L. monocytogenes* when cultivated under sub-optimal temperature along with mild acid and salt stress conditions commonly found in meat products. Despite extensive research into the stress response of *L. monocytogenes*, this pathogen continues to pose a significant threat in food production environments, raising concerns for consumer safety. By studying the functions of different expressed proteins and relating them to their transcripts, this research has contributed to understanding their potential roles in the emergence or reemergence of listeriosis outbreaks. The identification of effector proteins secreted under real-time conditions has provided valuable information that can help in the development of new strategies for the treatment and prevention of listeriosis.

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