„DUNĂREA DE JOS" UNIVERSITY OF GALAȚI

Doctoral School of Mechanical and Industrial Engineering

PhD THESIS

SUMMARY

Construction of two *Listeria monocytogenes* mutant strains in order to reveal the bacterium's resistance mechanism to high pressure and their characterization

PhD student,

Eng. Florentina Ionela BUCUR

Scientific coordinator,

Prof. dr. eng. Anca Ioana NICOLAU

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Keywords: *Listeria monocytogenes*, high pressure processing, food safety, food industry, stress resistance, site-directed mutagenesis, machanosensitive ion channels, penicillin binding proteins, nisin, hurdle technology, predictive modelling.

Justification of the research and its scientific objectives

Food is an essential aspect of life, and its safety is, consequently, a fundamental human right. Unfortunately, the consumption of food contaminated with pathogens continues to cause severe diseases that affect millions of people annually, of whom hundreds of thousands die. Ninety percent of food poisoning cases are caused by pathogenic bacteria belonging to the genera *Staphylococcus*, *Salmonella*, *Campylobacter*, *Listeria* and *Clostridium*. Although the infection with pathogenic species of *Listeria* (listeriosis), mainly *L. monocytogenes*, is rarer, the high mortality rate associated with it is a significant public concern. In 2018, the European Food Safety Authority (EFSA) showed that the incidence of listeriosis is following an increasing trend in Europe, with the highest mortality rate among diseases caused by foodborne pathogenic bacteria (13.8%).

L. monocytogenes is a ubiquitous bacterium, being present in soil, water, plant materials and animal farms, so that its transmission, through raw materials, to the food processing environment is almost inevitable. With a remarkable ability to adapt and survive, the bacterium can persist for a long time in food processing facilities, despite regular sanitation and disinfection procedures. Persistent *L. monocytogenes* strains are a continuous risk of food contamination, which can occur, mostly, during the post-processing handling steps. Unlike many other pathogenic bacteria, *L. monocytogenes* has the ability to proliferate in foods with relatively low humidity, acidic pH, high salt content and those stored at refrigeration temperatures, its control by traditional processing methods being, therefore, difficult. Thus, researchers and food industry specialists have recently paid a special attention to this pathogenic bacterium, trying to understand the genetic factors and molecular mechanisms underlying its ability to overcome the hurdles imposed by the conservation strategies applied on the industrial level.

Consumers' new habits and their increasing preference for minimally processed foods with superior sensorial and nutritional properties, have led to the need of alternative processing methods' implementation in food industry, which include high pressure processing (HPP). HPP is an alternative processing method to heat treatments, capable of inactivating most of the spoilage and pathogenic bacteria, while its impact on most food categories is low. However, the success of the processing method seems to be limited, since some bacteria, such as *L. monocytogenes*, manage to resist or recover after the treatment. There are many studies reporting the recovery of *L. monocytogenes* cells and their proliferation in HP treated foods stored under refrigeration conditions, even though the products were treated at 600 MPa, the industrial working regime. Advanced imaging techniques have allowed the scientists to study the effects of HPP on listerial cells, highlighting the injuries on the cell envelope level as the main consequence of the treatment. At the same time, transcriptome analyzes on high-pressure treated *L. monocytogenes* cells reported the increased expression of genes associated with cell wall biosynthesis and cell membrane functions.

By correlating all these aspects, the main objective of the Phd thesis was to reveal some of the molecular mechanisms involved in the resistance of *L. monocytogenes* to high pressure treatment. The present research aimed to eliminate two genes from the genome of *L. monocytogenes* EGDe (model strain), *lmo1013* and *lmo2999*, with possible importance for the bacterium's ability to survive after exposure to high pressure. The *lmo1013* gene encodes for the small mechanosensitive ion channels found on the cell membrane level, responsible, to a large

extent, with the membrane integrity maintenance under the effect of an extreme tension, being assumed a potential role of them in the *L. monoctogenes* response to HPP. Given that HPP exerts a mechanical stress on the cell wall, damaging it, and that several studies have reported the expression of the genes involved in the process of peptidoglycan biosynthesis after high pressure treatment, it was decided the deletion of the *lmo2229* gene, which encodes for the penicillin binding protein A2. The enzyme has both transglycosylase and transpeptidase activity, catalyzing the formation of the glycan chains and the cross-links between them, to ensure cell wall strength. Therefore, the function of this protein may be of major importance in the ability of *L. monocytogenes* cells to resist and repair the cell wall damage as result of a physical stress, such as HPP.

Starting from the main objective of the thesis, the following scientific key objectives were derived:

- Construction of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, by deleting from the bacterium's genome the previously mentioned target genes, *lmo1013* and *lmo2229*, respectively;
- Phenotypic characterization of the genetically modified strains, using as reference the wild-type strain, to understand their behavior;
- Evaluation of the resistance of the newly obtained strains, by comparison with the wild-type strain, to the treatment at high pressure in order to determine the role the proteins encoded by the deleted genes in the resistance of the bacterium to high pressure;
- Evaluation of *L. monocytogenes* mutant strains' susceptibility to other stress factors encountered by the bacterium in the food matrix;
- Selection of a *L. monocytogenes* strain with increased barotolerance, by testing the resistance to high pressure of some isolates from either foods or food processing environment;
- Proposing an alternative to the high pressure treatment applied by industry (600 MPa, 3 min) by using hurdle technology, in order to obtain a meat product safe for consumption and to reduce the operational costs associated with the technological process;
- Development of a predictive model, able to estimate the probability of *L. monocytogenes* recovery after high pressure treatments, with or without nisin, during product's storage under refrigeration conditions.

PhD thesis summary

The present PhD thesis contains six chapters and the final conclusions regarding the research results. The thesis includes 38 figures and 32 tables.

The first part of the Chapter I, entitled "The implications of the pathogenic bacterium *Listeria monocytogenes* regarding food safety", presents a summary of the information provided

by the recent literature on the pathogenic bacterium *L. monocytogenes*. Issues related to taxonomy, history, main phenotypic characteristics, serotypes associated with listeriosis, pathogenicity and virulence of the bacterium, problems caused by the presence of *L. monocytogenes* in the food processing environment, recent listeriosis epidemics in Europe and their consequences on consumers and food processors, as well as policies adopted by various countries to reduce the incidence of *L. monocytogenes* in foods are discussed.

The second part documents the mechanisms used by the pathogenic bacterium *L. monocytogenes* to survive and proliferate in foods, despite the hurdles imposed by industry through their preservation. The mechanisms applied by the bacterium to resist stressors associated with conventional preservation methods, such as high and low temperatures, acidity, osmotic stress and the presence of bacteriocins are discussed. Also, the information provided by the specialty literature regarding the resistance of *L. monocytogenes* to modern food production and preservation technologies, such as high pressure processing, UV light processing, and processing by pulsed electric fields, which generate new stress factors, as well as stress conditions encountered in the food processing environment (oxidative stress) is synthesized. The study contributes, through its complexity, to a deep understanding of the *L. monocytogenes* resistance, aiming to help food industry specialists in designing efficient processing methods to combat this pathogen and to ensure a better protection of the consumers.

Chapter II, entitled “Materials and equipment”, presents the detailed description of the materials and equipment used in the experimental studies. Information on bacterial strains, plasmid vectors, bacterial culture media, laboratory reagents (antibiotics, molecular biology analysis kits, buffers, enzymes, oligonucleotides, and chemicals), softwear, accessed databases, laboratory equipment and apparatus are presented in detail.

Chapters III-VI are organized according to the scientific articles, containing the following sections: introduction, materials and methods, results and their discussions by comparison to the literature, and conclusions.

Chapter III, entitled “Construction of the mutant strains, *Listeria monocytogenes* EGDe Δlmo*1013* and *Listeria monocytogenes* EGDe Δlmo*2229*”, presents the construction of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*. The site-targeted deletion method of the target genes, *lmo1013* and *lm02229*, was based on the lactococcal system formed of the plasmid vectors *p*ORI280 and *p*VE6007. The applied strategy, described step by step, can be a guide for the community of researchers studying the resistance of *L. monocytogenes* to different stressors.

Chapter IV, entitled “Phenotypic characterization of the constructed mutant strains, *Listeria monocytogenes* EGDe Δ*lmo1013* and *Listeria monocytogenes* EGDe Δ*lmo2229*”, performs the phenotypic characterization of the constructed *L. monocytogenes* mutant strains, by comparison to the wild-type strain, aiming to the determine their capacity of growth by discontinuous batch cultivation, the morphological characteristics of the mutant cells, the motility of the mutants and their capacity to form biofilm on hydrophobic surfaces (polystyrene). The induced mutations altered the behavior of the bacterium in terms of growth dynamics, morphology and motility. This research may constitute a reference point for studying the role of the homologous genes in other pathogenic bacteria.

Chapter V, entitled "Resistance of the mutant strains, *Listeria monocytogenes* EGDe Δ*lmo1013* and *Listeria monocytogenes* EGDe Δ*lmo2229*, to high pressure and other stress factors associated with food products", highlights, for the first time, the role of the proteins eliminated by deletion of the target genes, *lmo1013* and *lmo2229*, in the resistance of *L. monocytogenes* to high pressure. The mutant strain *L. monocytogenes* EGDe Δ*lmo1013*, deficient in the low-conductance mechanosensitive ion channels, has been shown to behave similarly to the wild-type strain at high pressure treatments, suggesting that the coding gene does not play a major role in the bacteria resistance to this stress. On the other hand, the mutant strain *L. monocytogenes* EGDe Δ*lmo2229*, deficient in the penicillin binding protein A2, proved to be significantly more sensitive to the action of high pressure treatments compared to the wild-type strain, highlighting, thus, a molecular mechanism that contributes to the recovery of the damaged *L. monocytogenes* cells. The chapter presents also the role of the studied proteins in the resistance of *L. monocytogenes* to other types of stress associated with the food matrix, such as osmotic stress (hyper- and hypoosmotic stress) and the presence of nisin, a bacteriocin approved as bio-preservative. The research has shown that penicillin binding protein A2 contributes to the bacterium's resistance to high concentrations of salt (NaCl) and its resistance to nisin.

Chapter VI, entitled "Assessment of the combined effect of high pressure processing and nisin on *Listeria monocytogenes* survival capacity in ready-to-eat ham", simulates the contamination of a ready-to-eat meat product (Prague ham) with a barotolerant *L. monocytogenes* strain (RO15), selected on the basis of its resistance to HPP, from a collection of strains isolated from either foods or food processing environment. After application of different treatment variants, the study proposes the combination of HPP, at a lower intensity (500 MPa, 8°C, 3 min), with the addition of nisin (25 mg/kg), as an alternative to the treatment of meat products practiced in industry (600 MPa, 8°C, 3 min), in order to reduce the operational costs associated with the technological process, and to obtain a food product safe for consumption. A predictive model based on logistic regression was also developed to estimate the likelihood of *L. monocytogenes* recovery after HPP of Prague ham, with or without nisin.

The final conclusions provide an overview of the results of the experimental research carried out in the present doctoral thesis. The original contributions of the author to the development of the knowledge in the approached field are indicated, as well as perspectives for the continuation of the research. Finally, the dissemination of the results obtained during the doctoral studies is presented.

The experimental studies within the doctoral thesis were carried out in the laboratories:

- The Laboratory of Physico-Chemical and Microbiological Analysis for Food (LAFCMA), belonging to the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galaţi
- The Genetics Laboratory, belonging to the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galaţi

The research was financially supported by the following projects:

- SafeFood Project - Development of a novel industrial process for safe, sustainable and higher quality foods, using biotechnology and cybernetic approach (ERA-IB-16- 014)
- European Social Fund through the Sectoral Operational Programme Human Capital 2014-2020 (in Romanian: Fondul Social European prin Programul Operațional Capital Uman 2014-2020) through the Financial Agreement „Burse pentru educaţia antreprenorială în rândul doctoranzilor şi cercetătorilor postdoctorat (Be Antreprenor!)", Contract No. 51680/09.07.2019- SMIS code: 124539

CHAPTER I

Implications of the pathogenic bacterium *Listeria monocytogenes* regarding food safety

Resistance of *L. monocytogenes* to high pressure

High pressure processing (HPP) is a technology used in food preservation as an alternative to thermal treatments, aiming to destroy food spoilage microorganisms and food-borne pathogens (Huang *et al*., 2014). Depending on the food and spoilage organisms, pressures applied for sterilization are usually between 250 and 700 MPa. Bacterial cells subjected to HPP treatments display morphological and physiological changes that may be reversible depending on pressure and holding time. Primary effects of HPP are an increase in the permeability of the cell membrane, the disruption of the protein structure and function, and, as a consequence, inhibition of the metabolism, replication, and transcription (Huang *et al*., 2014; **Figure 1.1**).

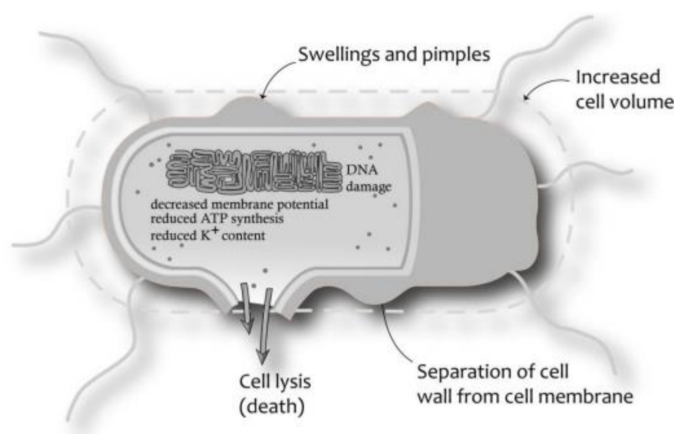

Figure 1.1. Effect of high pressure on listerial cell (Ferreira *et al*., 2016)

The effect of HPP on *L. monocytogenes* was investigated on the global transcriptomic level by microarray analysis with subsequent RT-PCR on some target genes (Bowman *et al*., 2008). This indicated that mRNA levels were reduced globally with increasing intensity and duration of the treatment. Nevertheless, HPP induced expression of genes associated with DNA repair, transcription, translation, cell division, protein secretion, motility, chemotaxis, and membrane and cell wall biosynthesis. On the other hand, reduced expression was observed for genes involved in carbohydrates' uptake, energy metabolism and virulence. Surprisingly, HPP seemed to reduce expression of the general stress sigma factor SigB and part of the SigB regulon. One of the genes showing highest induction by HPP was cspL encoding a cold-shock protein. This suggests that HPP also induces cross-resistance to other stresses. For example, HPP resistance in semi-skimmed milk was higher than in buffer and the resistant isolate was also more resistant to heat, acid, and oxidative stress (Karatzas and Bennik, 2002).

Mutations in CtsR, a class III stress genes repressor (Nair *et al*., 2000), have been linked to spontaneous resistance of *L. monocytogenes* cells to HPP. Mutants with a stable resistance showed point mutations, insertions or deletions in the ctsR gene that negatively affected its activity. This loss in ctsR function in HPP resistant variants of *L. monocytogenes* was

accompanied by increased expression of *clpB*, *clpC*, *clpE*, and *clpP* (Karatzas *et al*., 2003; Van Boeijen *et al*., 2010). Clp proteases have a clear role in degradation of misfolded or damaged proteins preventing their potentially harmful accumulation in bacterial cells (Krüger *et al*., 2000; Tomoyasu *et al*., 2001). Since protein denaturation is one of the consequences of HPP treatment (Moreirinha *et al*., 2016) increased Clp protease activity is in line with increased HPP tolerance in *L. monocytogenes*. However, isolation of resistant mutants that do not display these changes indicates that there may be other unknown mechanisms conferring resistance to HPP (Karatzas *et al*., 2005). Moreover, Chen *et al*. (2009) reported that different levels of HPP resistance among *L. monocytogenes* strains are not based on *ctsR* gene mutations.

L. mononcytogenes ScottA and a spontaneous HPP resistant isolate of this strain were shown to be more resistant to HPP in stationary compared to exponential growth phase (Karatzas and Bennik, 2002). Moreover, it seems that cells in stationary phase of growth do not exhibit the highest resistance to HPP treatment. *L. monocytogenes* cells found in long-term-survival phase showed even higher HPP tolerance, as transition back to log and stationary phases resulted in less survivors after pressurization. This phenomenon has been attributed to a change in cell morphology from rods to cocci that results in cytoplasmic condensation and, implicitly, reduction of intracellular water activity (Wen *et al*., 2009).

CHAPTER II

Materials and equipment

The materials and equipment used in the present PhD thesis were provided to the author by "Dunărea de Jos" University of Galaţi and Institute of Microbiology and Biotechnology, Ulm University, Germany.

Bacterial strains and plasmidial vectors

The bacterial strains and plasmidial vectors used in the experimental studies are presented in **Table 2.1** and **Table 2.2**, respectively.

Table 2.1. *Bacterial strains used in the experimental studies*

Bacterial strains	Description	Source
***Escherichia coli* strains**		
EC10B	Derivative of *E. coli* DH10B cloning strain, with *repA* and kanamycin resistance gene (Kan^r) integrated in the *glgB* gene	Monk *et al*., (2008)
EC10B/*p*ORI280(AD)Δ*lmo1013*	*E. coli* EC10B transformed with the *p*ORI280(AD)Δ*lmo1013* recombinant vector	This study
EC10B/*p*ORI280(AD)Δ*lmo2229*	*E. coli* EC10B transformed with the *p*ORI280(AD)Δ*lmo2229* recombinant vector	This study
Tulpini de *Listeria monocytogenes*		
EGDe	Wild type strain, serotype 1/2a	Glaser *et al*., (2001)
EGDe Δ*lmo1013*	*L. monocytogenes* EGDe genetically modified by *lmo1013* gene deletion	This study

Bacterial strains	Description	Source
EGDe Δ*lmo2229*	*L. monocytogenes* EGDe genetically modified by *lmo2229* gene deletion	This study
AB100	Strain isolated from food, serotype 1/2a (3a)	-
AB120	Strain isolated from food processing environment, serotype 1/2a (3a)	-
AB199	Strain isolated from food processing environment, serotype 1/2a (3a)	-
AB204	Strain isolated from food processing environment, serotype 1/2a (3a)	-
AB24	Strain isolated from food processing environment, serotype 1/2a (3a)	-
AB80	Strain isolated from food, serotype 1/2a (3a)	-
ABS43	Strain isolated from food, serotype 1/2c (3c)	-
ABS45	Strain isolated from food processing environment, serotype 1/2c (3c)	-
RO15	Strain isolated from food, serotype 4b (4d, 4e)	Ciolacu *et al.* (2015)
RO4	Strain isolated from food, serotype 1/2a (3a)	Ciolacu *et al.* (2015)

Table 2.2. *Plasmidial vectors used in the experimental studies*

Plasmidial vectors	Description	Source
*p*ORI280	RepA⁻ integrative vector, constitutive *lacZ*; Ori (replication origin of lactococcal vector *p*WV01); Em^r (erythromycin resistance gene)	Leenhouts *et al.*, (1996)
*p*ORI280(AD)Δ*lmo1013*	*p*ORI280 vector containing the DNA flanking fragments, AB (left fragment including the ATG start codon) and CD (right fragment including the TAA stop codon), of the *lmo1013* gene	This study
*p*ORI280(AD)Δ*lmo2229*	*p*ORI280 vector containing the DNA flanking fragments, AB (left fragment including the ATG start codon) and CD (right fragment including the TAA stop codon), of the *lmo2229* gene	This study
*p*VE6007	Helper thermosensitive vector (< 30˚C), RepA$^+$, Cm^r (chloramphenicol resistance gene)	Maguin *et al.*, (1992)

CHAPTER III

Construction of the mutant strains, *Listeria monocytogenes* EGDe Δ*lmo1013* and *Listeria monocytogenes* EGDe Δ*lmo2229*

The aim of this chapter was represented by the construction of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* Δ*lmo2229*, in order to reveal the mechanisms possibly used by the bacterium to resist high pressure processing. Deletion of the target genes, *lmo1013* and *lmo2229*, from the genome of *L. monocytogenes* EGDe, took place through a two-stage homologous recombination process based on the lactococcal system formed of *p*ORI280 (RepA$^-$) integrative vector, which allows the cloning of DNA fragments of

interest, and *p*VE6007 helper vector (RepA+; thermosensitive), which ensures the replication of the integrative vector in the host organism. *Lmo1013* gene encodes for the small mechanosensitive ion channels, responsible, to an important extent, with the membrane integrity maintenance under the effect of an extreme tension. *Lmo2229* gene encodes for the penicillin binding protein A2, involved in the peptidoglycan biosynthesis process.

Results and discussions

The deletion strategy of the target genes, *lmo1013* and *lmo2229*, consisted of DNA flanking fragments generation, AB (left fragment including the start codon, ATG) and CD (right fragment including the stop codon, TAA), their ligation into *p*ORI280 integrative vector, and transformation of listerial cells with the recombinant vector, in the presence of the Rep A protein provided in *trans* by a helper vector, to induce the homologous recombination on the host chromosome level.

The expected length of the AB and CD flanking sequences, amplified from the genomic DNA extracted from *L. monocytogenes* EGDe cells, was 543 bp. Fragment amplification was performed by using Gibson primers. The amplicons presented overlapping ends complementary to the ends of the *p*ORI280 vector cut with the restriction enzyme *Pst*I (20 bp), the other ends (20 bp) being created for their hybridization, with the formation of the AD fragment (**Figure 3.1. A**). As can be seen in **Figure 3.1. B**, the length of the amplified fragments, AB and CD, corresponded to the expected one, 543 bp.

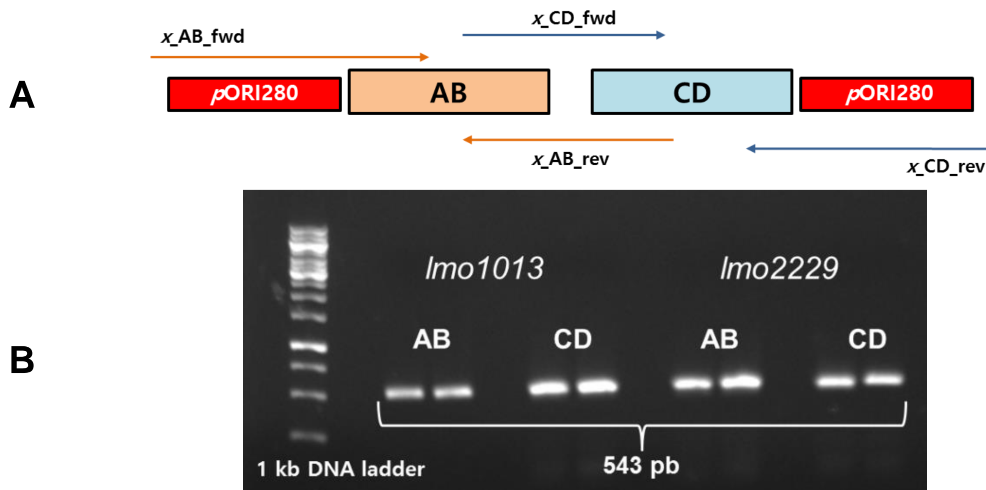

Figure 3.1. A. Schematic illustration of the target genes' DNA flanking fragments amplification, by using Gibson primers **B.** Separation by 1% agarose gel electrophoresis of the flanking DNA fragments, AB and CD (543 bp), located downstream and upstream, respectively, of the genes of interest, *lmo1013* and *lmo2229*

The ligation of the flanking DNA fragments into *p*ORI280 integrative vector, on the level of *Pst*I restriction site, was performed by Gibson assembly, resulting the recombinant vectors called *p*ORI280(AD) [*p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229*].

After *E. coli* EC10B cells transformation, in order to identify the clones carrying the recombinant vectors, it was amplified the AD fragment (1,046 pb) by employing the Colony PCR method. For the detection of each recombinant vector, an average of 50 transformants were tested, being identified 7 clones carrying the *p*ORI280(AD)Δ*lmo1013* recombinant vector and 6 clones carrying the *p*ORI280(AD)Δ*lmo2229* recombinant vector (**Figure 3.2**).

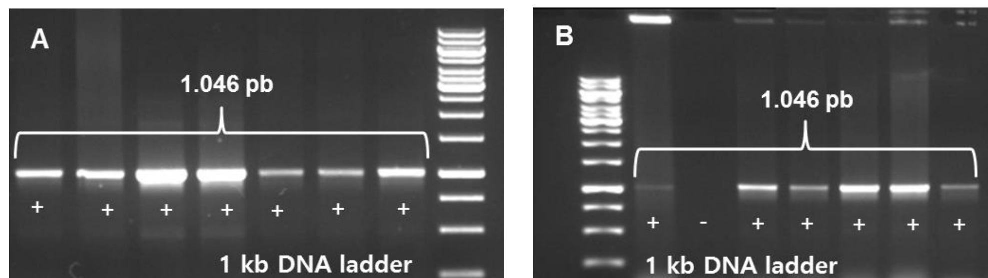

Figure 3.2. Separation by 1% agarose gel electrophoresis of the AD fragments (1,046 bp), amplified by Colony PCR from the *E. coli* EC10B transformants, in order to identify the positive clones. **A.** Identification of the clones carrying the *p*ORI280(AD)Δ*lmo1013* recombinant vector. **B.** Identification of the clones carrying the *p*ORI280(AD)Δ*lmo2229* recombinant vector

To confirm the correct insertion of the AD fragment, the isolated *p*ORI280(AD) recombinant vectors were digested with the restriction endonucleases *Bgl*II and *Not*I. The restriction sites of these enzymes are located upstream and downstream of the *Pst*I restriction site, at a distance of 53 and 38 pb, respectively (**Figure 3.3. A**). Therefore, the two DNA fragments resulting from the digestion of *p*ORI280(AD) recombinant vectors were expected to have a length of 5,145 and 1,097 bp, respectively, which was confirmed by electrophoretic migration of the digestion products and their visualization on the UV-transilluminator (**Figure 3.3 B**).

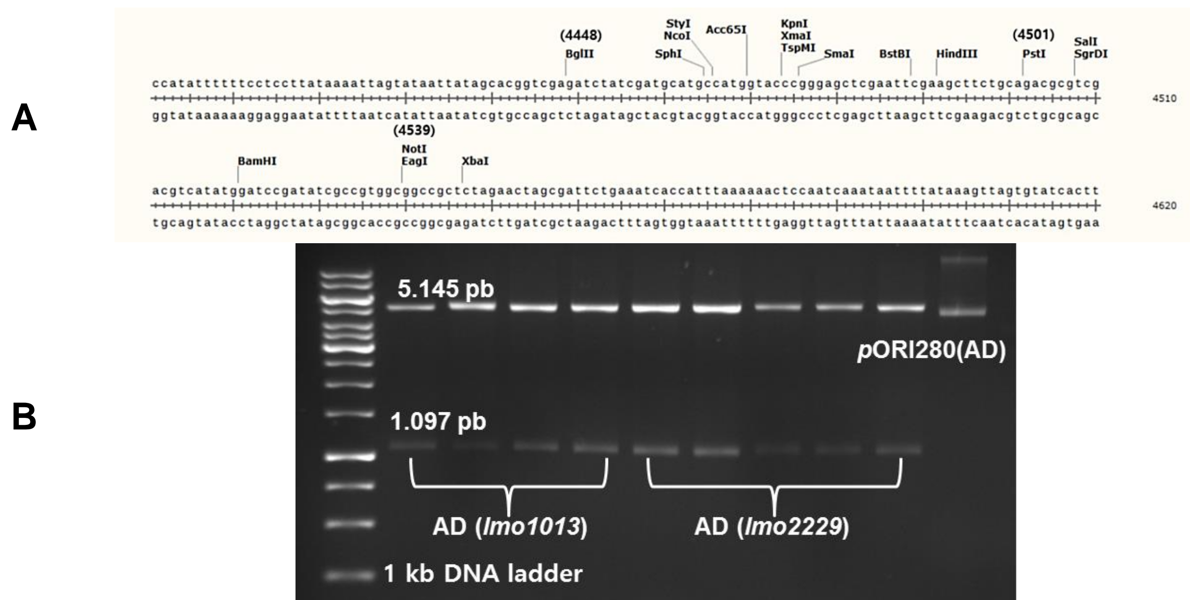

Figure 3.3. A. Multiple cloning site of the *p*ORI280 vector, the indicated restriction sites being unique. **B.** Separation by 1% agarose gel electrophoresis of the DNA fragments resulted from the digestion of

*p*ORI280(AD) recombinant vectors, isolated from the *E. coli* EC10B positive clones, with *Bgl*II and *Not*I restriction endonucleases; undigested *p*ORI280(AD) represents the negative control

L. monocytogene EGDe cells were transformed by electroporation with the *p*ORI280(AD) recombinant vector [*p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229,* respectively], and the *p*VE6007 helper vector, which ensures the replication of the latter at a permissive temperature (< 30°C). By incubating the transformants at 37°C, the inactivation of the thermosensitive protein RepA was induced, resulting in the loss of the *p*VE6007 vector and the integration of the recombinant vector in the host cell chromosome, through one of the flanking fragments of the target genes, AB or CD (Landete, 2016; **Figure 3.9**). The loss of the *p*VE6007 helper vector was confirmed by cultivation of the transformants on culture medium supplemented with chloramphenicol, since the vector includes the gene that gives bacteria resistance to this antibiotic. Chloramphenicol sensitive clones were selected and cultured statically at 37°C, in the absence of erythromycin. The cultures were passaged at 24 hour intervals and the cells were inoculated on culture medium supplemented with X-Gal, in order to performe the white – blue selection.

After 10 consecutive passages, following cells cultivation on agar medium supplemented with X-Gal, the white colonies developed at a frequency of approximately 5% (**Figure 3.4. A**), being indicated the second recombination step. At this stage, the *p*ORI280 vector is excised from the bacterial chromosome, *lacZ* gene, erythromycin resistance gene, and Ori replication origin being removed accordingly. Therfore, the colonies produced by the recombinant cells turn white when cultured on agar medium with X-Gal, in the absence of β-galactosidase which catalyzes its hydrolysis. The white recombinant colonies were streaked on a *master-plate* (**Figure 3.4. B**), tested on ALOA selective culture medium in order to determine their purity (**Figure 3.4. C**), and tested for the resistance to erythromycin. To identify the mutants, pure clones, which indicated sensitivity to erythromycin (**Figure 3.4. D**), were tested by Colony PCR in comparison to the wild type strain, *L. monocytogenes* EGDe. The strategy was to amplify the genomic region which includes both flanking fragments and gene of interest (**Figure 3.5**).

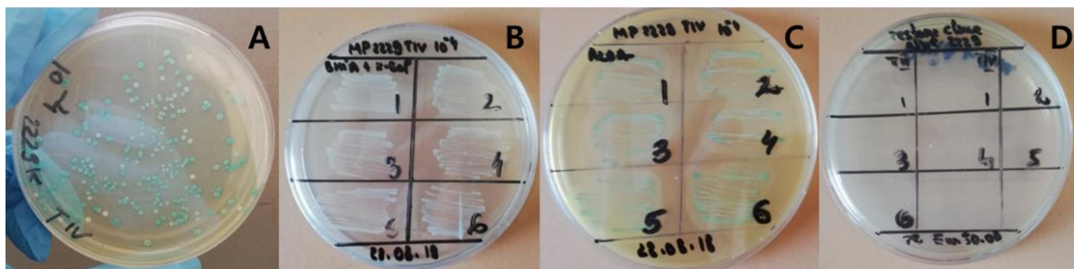

Figure 3.4. *L. monocytogenes* EGDe recombinant clones. **A.** Growth of white *L. monocytogenes* EGDe recombinant colonies on agar medium supplemented with X-Gal. **B.** Master-plate containing white *L. monocytogenes* EGDe recombinant colonies. **C.** Testing of white *L. monocytogenes* EGDe recombinant colonies on ALOA selective medium to determine their purity. **D.** Testing of *L. monocytogenes* EGDe recombinant colonies' susceptibility to erythromycin

Differentiation of the mutant clones from the wild type ones was performed by analyzing the length of the DNA fragments amplified by Colony PCR. The target genes' sequences, *lmo1013* and *lmo2229*, contain 846 and 2,145 nt, respectively. Therefore, amplicons with the

length of 1,046 bp correspond to the mutant clones, while amplicons with the length of 1,886 and 3,185 bp, respectively, correspond to the wild type clones.

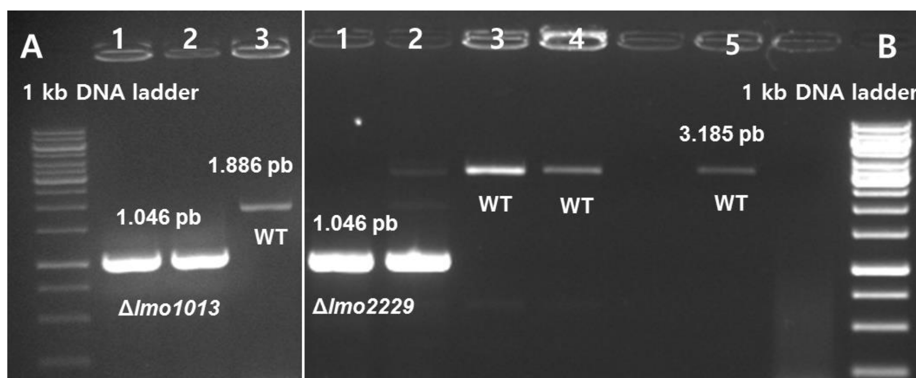

Figure 3.5. Separation by 1% agarose gel electrophoresis of the AD fragments amplified by Colony PCR from the *L. monocytogenes* EGDe recombinant clones. **A.** 1, 2 – amplicons with the length of 1,046 pb, indicating the Δ*lmo1013* mutant clones; 3 – amplicon with the length of 1,886 pb indicating the wild type strain (*L. monocytogenes* EGDe). **B.** 1, 2 - amplicons with the length of 1,046 pb, indicating the Δ*lmo2229* mutant clones; 3, 4, 5 – amplicons with the length of 3,185 pb, where 3 and 4 indicate the wild type clones and 5 indicates the wild type strain (*L. monocytogenes* EGDe)

To confirm the deletion of the target genes, *lmo1013* and *lmo2229*, respectively, from the genome of *L. monocytogenes* EGDe, the genomic DNA isolated from both genetically modified strains and wild type strain was sent for sequencing at the Institute of Biotechnology, University of Helsinki. The genomic DNA sequencing technology was represented by the next generation sequencing (NGS; **Figures 3.6** and **3.7**).

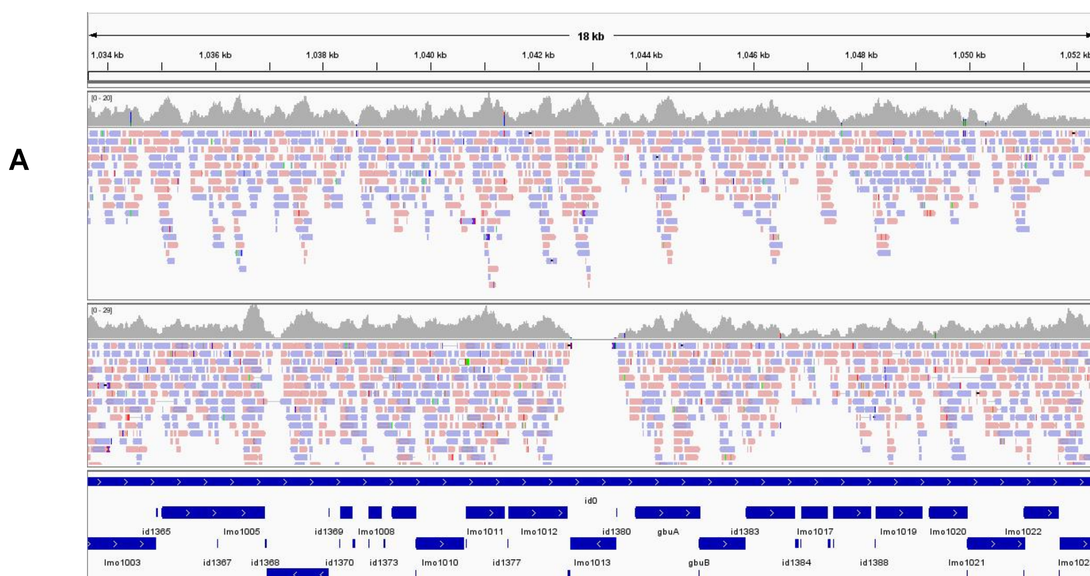

Figure 3.6. Confirmation, by NGS, of the *lmo1013* gene deletion from the genome of *L. monocytogenes* EGDe (visualized with the Integrative Genomics Viewer program). **A.** Framing of the *lmo1013* gene in the ~ 1,042 - 1,043 kpb region of the *L. monocytogenes* EGDe genome **B.** Highlighting the deletion of the *lmo1013* gene from the genome of *L. monocytogenes* EGDe

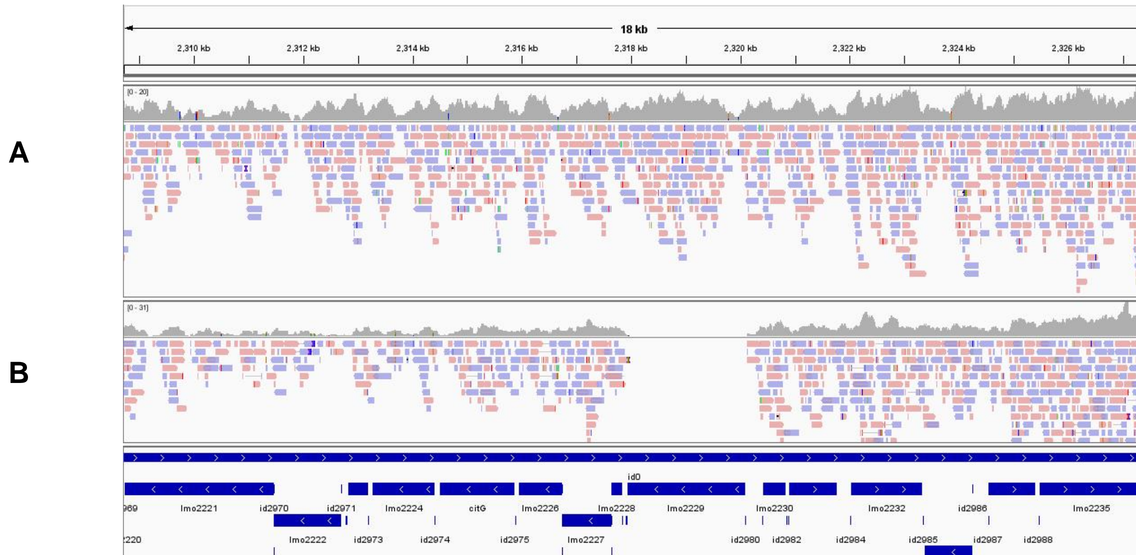

Figure 3.7. Confirmation, by NGS, of the *lmo2229* gene deletion from the genome of *L. monocytogenes* EGDe (visualized with the Integrative Genomics Viewer program). **A.** Framing of the *lmo2229* gene in the ~ 2.318 – 2.320 kpb region of the *L. monocytogenes* EGDe genome **B.** Emphasizing the deletion of the *lmo2229* gene from the genome of *L. monocytogenes* EGDe

Conclusions

This chapter presents the construction of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*. The deletion method, based on the lactococcal system formed of the *p*ORI280 and *p*VE6007 plasmidial vectors, consisted of seven key steps, which were successfully completed:

- Amplification of the target genes' flanking fragments, AB and CD, with primers configured for their assembly with the *p*ORI280 vector linearized with the *Pst*I restriction enzyme, by Gibson method;
- Obtaining of the *p*ORI280(AD) recombinant vectors [*p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229*] by Gibson ligation of the AB and CD fragments, specifique to each target gene, into *p*ORI280 integrative vector;
- Cloning of the recombinant vectors, *p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229*, in *E. coli* EC10B cells;
- Verification of the construction of the recombinant vectors, *p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229*, by their digestion with the restriction enzymes *Bgl*II/*Not*I;
- Transformation of the electrocompetent *L. monocytogenes* EGDe cells with the *p*ORI280(AD) recombinant vectors [*p*ORI280(AD)Δ*lmo1013* and *p*ORI280(AD)Δ*lmo2229*, respectively] and the *p*VE6007 RepA$^+$ helper thermosensitive vector;
- Induction of the homologous recombination on the *L. monocytogenes* EGDe chromosome level in order to eliminate the target genes, *lmo1013* and *lmo2229*, respectively, highlighted by Colony PCR;

- Confirmation by NGS of the *lmo1013* and *lmo2229* genes deletion from the genome of *L. monocytogenes* EGDe.

CHAPTER IV

Phenotypic characterization of the constructed mutant strains, *Listeria monocytogenes* EGDe Δ*lmo1013* and *Listeria monocytogenes* EGDe Δ*lmo2229*

This chapter presents the phenotypical characterization of the constructed mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, in comparison to the wild type strain, *L. monocytogenes* EGDe. The aspects followed by this study consisted of the growth capacity, in laboratory conditions, of the mutant cells, their morphological characteristics, motility and capacity to form biofilm on polystyrene surface.

Results and discussions

Growth dynamics determination, at 37°C, of *L. monocytogenes* mutant strains

The growth dynamics of the mutant cells, Δ*lmo1013* and Δ*lmo2229*, compared to that of the wild type cells, was determined by submerged batch cultivation. For this, the *L. monocytognes* cells were inoculated in a limited volume of TSB-YE culture medium (50 mL) and grown at the temperature of 37°C, for 24 hours. During the first 12 hours of incubation, at 2 hour intervals, samples were collected and analyzed to determine the OD_{600} and cell number (CFU/ mL). Final cell samples were collected after 24 hours of incubation and analyzed in a similar manner.

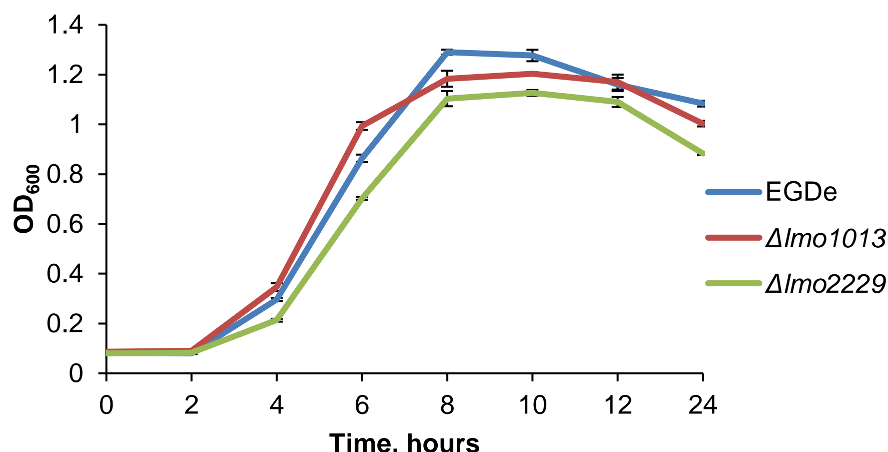

Figure 4.1. Growth dynamics of mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, in comparison to the wild type strain, *L. monocytogenes* EGDe, at the temperature of 37°C

The growth curves of the *L. monocytogenes* cells at 37°C, for 24 hours, monitored by determining the optical density (OD_{600}) of the cultures, are represented graphically in **Figure 4.1**. Δ*lmo1013* mutant cells behaved similarly to wild type ones, except for the earlier installation of

the stationary phase of growth. On the other hand, Δ*lmo2229* mutant cells indicated a slower growth rate. The generation time of the *L. monocytogenes* populations is presented in **Table 4.1**. In the case of the wild type strain, *L. monocytogenes* EGDe, the generation time was 1.16 hours. According to the previous observations, *L. monocytogenes* EGDe Δ*lmo1013* mutant indicated a generation time (1.15 hours) comparable to that of the wild type strain, while that of the *L. monocytogenes* EGDe Δ*lmo2229* mutant was greater (1.29 hours).

Table 4.1. *Generation time of L. monocytogenes cells, wild type strain and mutants, at the temperature of 37°C*

Strain	Generation time, hours
L. monocytogenes EGDe	1.16
L. monocytogenes EGDe Δ*lmo1013*	1.15
L. monocytogenes EGDe Δ*lmo2229*	1.29

Although PBP A2 is dispensable for cell viability, the mutant cells grow more slowly compared to the wild type ones. The study conducted by Zawadzka-Skomial *et al*., (2006) confirms that, by inactivating the gene that encodes for PBP A2, the growth capacity of the mutant cells is affected.

By analyzing the samples collected after 24 hours of incubation at 37°C, the death phase was noticed. The number of viable cells was reduced by approximately 1 log CFU/mL, for *L. monocytogenes* EGDe and *L. monocytogenes* EGDe Δ*lmo2229*, and by more than 2 log CFU/mL, for *L. monocytogenes* EGDe Δ*lmo1013*, compared to the cells number determined after 12 hours of incubation (**Table 4.2**).

Table 4.2. *Number of viable L. monocytogenes cells (log CFU/mL) determined at each time of analysis of the incubated cultures at 37°C*

	Number of cells, log CFU/mL		
Time, hours	*L. monocytogenes* EGDe	*L. monocytogenes* EGDe Δ*lmo1013*	*L. monocytogenes* EGDe Δ*lmo2229*
0	7.07	7.25	7.23
2	7.25	7.41	7.04
4	8.11	8.23	8.14
6	8.69	8.44	8.49
8	8.74	8.69	8.8
10	8.84	8.49	8.55
12	8.77	8.5	7.86
24	7.84	6.38	7.65

The more pronounced death phase observed in the case of the Δ*lmo1013* mutant cells may be a consequence of the small mechanosensitive ion channels elimination, which ensure the structural integrity of bacteria during the stationary phase of growth.

Determination of the morphological characteristics of *L. monocytogenes* mutant cells

To evaluate the effect of the mutations on cell morphology, both mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, and the wild type

one, *L. monocytogenes* EGDe, were analyzed by scanning electron microscopy. The morphology of *L. monocytogenes* cells was studied in both exponential and stationary phase of growth.

In the exponential phase of growth, Δ*lmo1013* mutant cells showed partially abnormal shape, compared to wild type cells (**Figure 4.3. B**). In the case of Δ*lmo2229* mutant cells, the transverse division septum determined the formation of unequal cells in length (**Figure 4.3. C**). Moreover, compared to wild type cells, Δ*lmo2229* mutant cells tended to form filaments, a behavior that could be attributed to a cell division defect (**Figure 4.4**). A study aimed at elucidating the contribution of PBPs in *L. monocytogenes* also showed that, after inactivation of the *lmo2229* gene by insertional mutagenesis, the mutant cells have a different morphology compared to the wild type ones, forming chains of 3 - 4 cells (Guinane *et al*., 2006). Moreover, inactivation of the PBP A2 coding gene in *Streptococcus gordonii* resulted in the growth of the mutants in extremely long chains, containing, on average, 40 cells (Haenni *et al*., 2006).

For a more precise analysis of the effect of the induced mutations (deletion of the *lmo1013* and *lmo2229* genes, respectively) on the morphology of *L. monocytogenes* EGDe cells, 40 cells were measured using the ImageJ program, in order to determine their length and diameter. According to the SEM images obtained for *L. monocytogenes* cells in the stationary phase of growth, in terms of mean length (**Figure 4.5**), no significant difference (p = 0.708) was found between Δ*lmo1013* mutant cells (1.248 ± 0.169 µm) and the wild type ones (1.221 ± 0.159 µm). In contrast, the mean diameter of Δ*lmo1013* mutant cells (0.568 ± 0.039 µm) was significantly larger (p < 0.001) than that of the wild type cells (0.496 ± 0.04 µm) (**Figure 4.6**). Bachin *et al.* (2015) also observed that, following the inactivation of the *mscL* gene (the coding gene of the large mechanosensitive ion channels) in *Synechocystis* sp. PCC 6803 (cyanobacteria), mutant cells have a larger diameter compared to the wild type ones.

Δ*lmo2229* mutant cells had a greater mean length (1.364 ± 0.17 µm) (p < 0.001) compared to the wild type cells. It should be noticed that, in this case, the determined lengths were heterogeneous, reaching values of up to 25 µm. Moreover, the mean diameter of Δ*lmo2229* mutant cells (0.449 ± 0.038 µm) was smaller (p < 0.001) than that of the wild type cells. The study of the role of PBPs in bacterial morphology conducted by Popham and Young (2003) concluded that the changes in the shape of class A PBPs deficient mutants may be due to the disruption of the cell wall biosynthesis as result of the lack of coordination between elongation and septation of the cells.

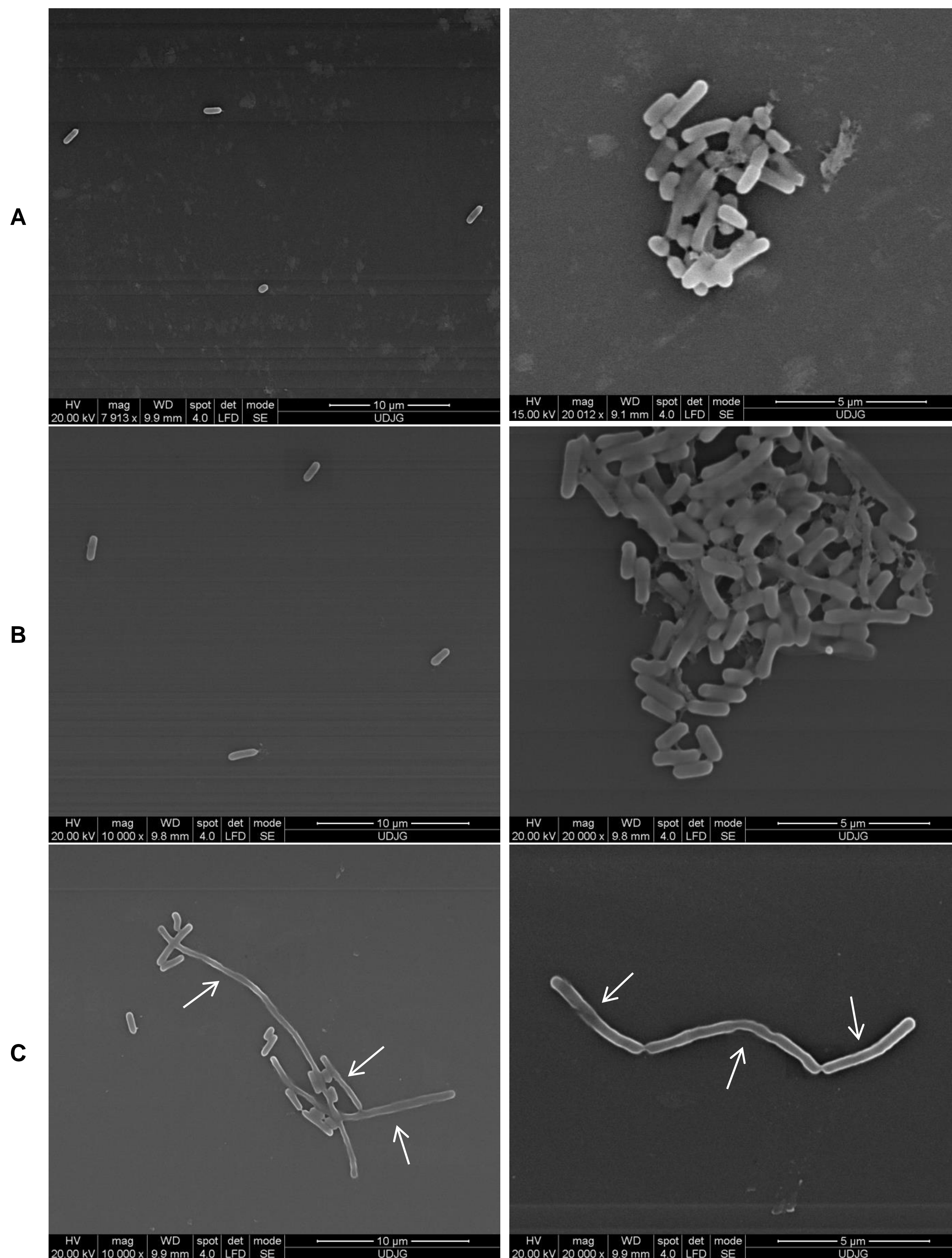

Figure 4.3. Morphology of *L. monocytogenes* cells in stationary phase of growth: **A** - *L. monocytogenes* EGDe; **B** - *L. monocytogenes* EGDe Δ*lmo1013*; **C** - *L. monocytogenes* EGDe Δ*lmo2229* (SEM analysis). Arrows indicate cells with abnormal shape

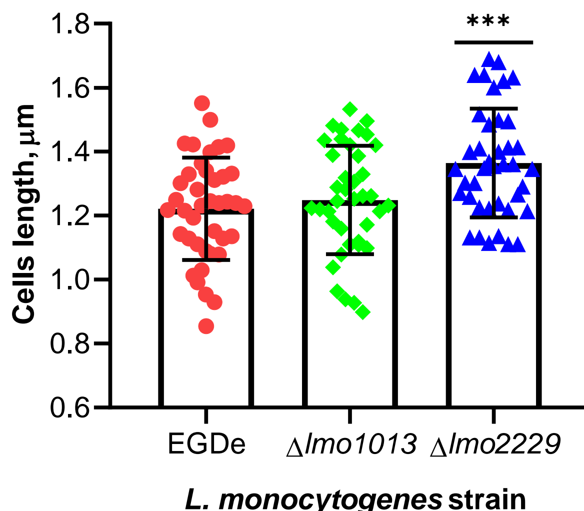

Figure 4.4. Length of *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* cells in stationary phase of growth (n = 40). Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test, where the length of *L. monocytogenes* EGDe cells represented the control (*** $p < 0.001$)

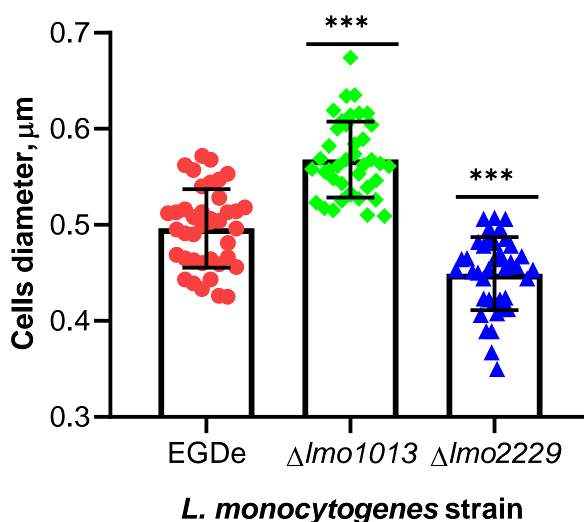

Figure 4.5. Diameter of *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* cells in stationary phase of growth (n = 40). Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test, where the diameter of *L. monocytogenes* EGDe cells represented the control (*** $p < 0.001$)

Motility of *L. monocytogenes* mutant strains

This stage of the phenotypic study aimed to evaluate the motility of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, compared to that of

the wild type strain, *L. monocytogenes* EGDe. Cells were cultured at 25°C in order to develop peritrichous flagella, as studies have shown that the expression of genes involved in *L. monocytogenes* motility is negatively regulated by the MogR repressor at temperatures above 30°C (Shen and Higgins, 2006), and then inoculated, by spotting technique, on semi-solid culture medium (0.3% agar) for the analysis of their migration capacity.

After 24 and 48 hours of incubation at 25°C (**Figure 4.6**), by determining the diameter of the growth zone related to the migration of bacteria, it was noticed that there are no significant differences ($p > 0.05$) between the mutant strains and the wild type one, in terms of motility. In contrast, after 120 hours of incubation of the colonies at 25°C, a lower motility of the mutant strains ($p < 0.001$), *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* was noticed, compared to that of the wild type strain, *L. monocytogenes* EGDe, with ~ 17 and ~ 24%, respectively.

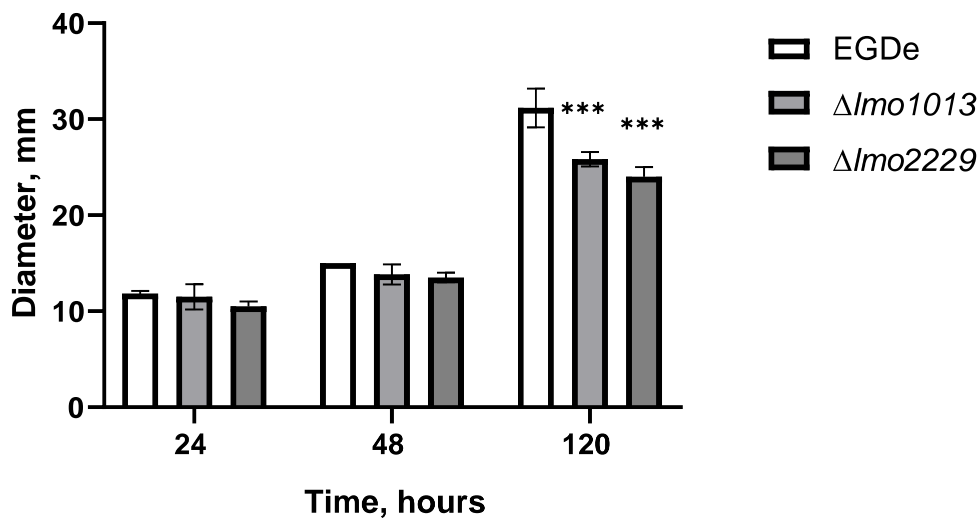

Figure 4.6. Quantification of the growth zone diameter around the spot inoculation of *L. monocytogenes* strains on 0.3% TSB-YE agar after incubation at 25°C for 24, 48 and 120 hours. The values represent the mean ± standard deviation of three independent experiments. Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test, where the wild type strain, *L. monocytogenes* EGDe, represented the control (***$p < 0.001$)

The rough appearance of the colony formed by *L. monocytogenes* EGDe Δ*lmo2229* mutant strain (**Figure 4.7**) and its reduced motility in relation to the wild type strain can be associated with the cell chaining phenotype, observed by SEM analysis. Similar observations were made by Durack *et al.* (2014), in the case of the *L. monocytogenes* 10403S Δ*secA2* mutant strain with a defect on the level of autolytic enzymes secretory system, SecA2.

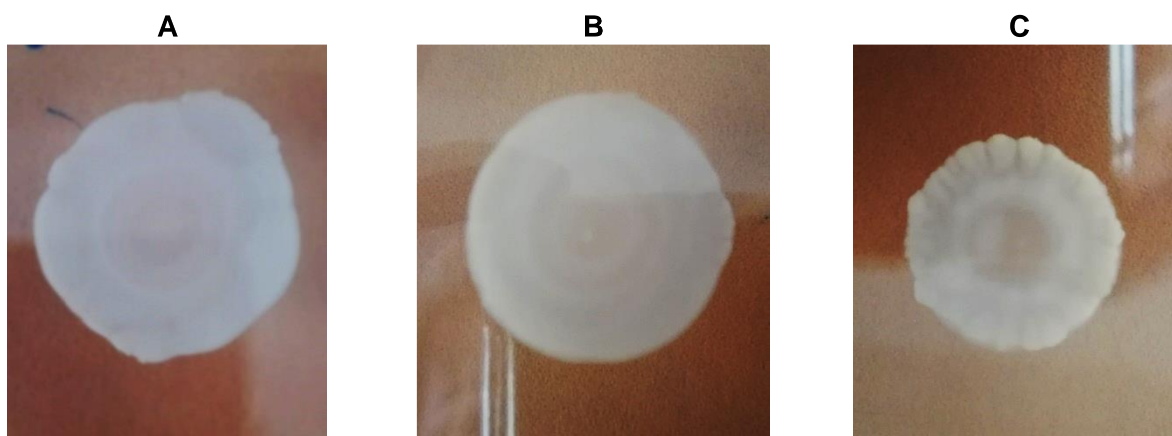

Figure 4.7. Images on *L. monocytogenes* cells motility on semisolid culture medium, incubated at 25°C for 120 hours. **A** - *L. monocytogenes* EGDe; **B** - *L. monocytogenes* EGDe Δ*lmo1013*; **C** - *L. monocytogenes* EGDe Δ*lmo2229*

The wild type strain, *L. monocytogenes* EGDe, and the mutant strain, *L. monocytogenes* EGDe Δ*lmo1013*, formed smooth colonies, in which the formation of concentric rings associated with the circadian migration rhythm, could be observed (**Figure 4.7**). Concentric rings are determined by the alternation of areas with opaque biomass with areas where the biomass has a translucent appearance. Opaque biomass corresponds to the production of extracellular polymeric substances, substances whose production does not take place in the case of cells that form translucent biomass (Kaval *et al*., 2015).

Ability of *L. monocytogenes* mutant strains to form biofilm

The present study aimed to evaluate the ability of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, to form biofilm, compared to the wild type strain, *L. monocytogenes* EGDe. The cultivation of biofilms was performed in static conditions, in polystyrene microtiter plates, at three different temperatures (25, 30 and 37°C), and the amount of the formed biofilm was quantified by staining its matrix with crystal violet.

In general, studies have shown that *L. monocytogenes* has a weak to moderate ability to form biofilm (Barbosa *et al*., 2013), but, nevertheless, some strains can strongly adhere to abiotic surfaces (Borucki *et al*., 2003). According to the study conducted by Doijad *et al*. (2015), depending on the ability to form biofilm at 37°C, *L. monocytogenes* strains can be classified as follows: weak biofilm-forming strains (OD_{595} = 0÷0.3), moderate biofilm-forming strains (OD_{595} = 0.4 ÷ 0.6) and strong biofilm-forming strains (OD_{595} = 0.7÷0.9). In the present case, both wild type strain, *L. monocytogenes* EGDe, and mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* Δ*lmo2229*, indicated a moderate capacity to form biofilm, at 37°C. At this temperature, the difference between mutant cells, Δ*lmo1013* and Δ*lmo2229*, and the wild type ones, in terms of biofilm formation capacity, was not significant (p = 0.12 and 0.07, respectively).

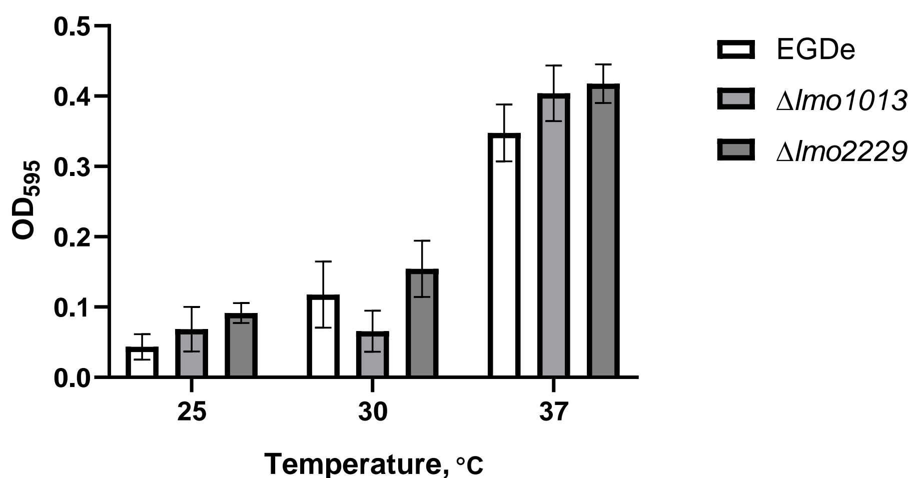

Figure 4.8. The ability of mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, to form biofilm, compared to the wild type strain, *L. monocytogenes* EGDe. Quantification of the biofilm amount was performed by staining with 1% CV and reading the optical density at 595 nm. The values represent the average of three independent experiments ± standard deviation. Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test where the wild-type strain, *L. monocytogenes* EGDe, represented the control

The incubation temperature influenced significantly ($p < 0.001$) the amount of biofilm formed (**Figure 4.8**), especially that of 37°C. The influence of incubation temperature has been also indicated by other studies conducted in this direction (Briandet *et al*., 1999; Di Bonaventura *et al*., 2008), which observed that with increasing incubation temperature, the level of cell surface hydrophobicity increases, which favors the adhesion of bacteria to abiotic surfaces. Thus, the fact that, after incubation at temperatures of 30 and 25°C, respectively, the strains formed less biofilm is explicable. In this case, the amount of biofilm produced by the mutant strains did not differ significantly ($p > 0.05$) from that produced by the wild type strain.

Conclusions

The mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, generated by deletion of the *lmo1013* and *lmo2229* genes, respectively, from the genome of *L. monocytogenes* EGDe, revealed different phenotypic aspects compared to the wild type strain in terms of growth, morphological characteristics and motility. Thus:

- Although *L. monocytogenes* EGDe Δ*lmo1013* mutant strain shows a growth behavior relatively similar to that of the wild type strain, in exponential phase of growth, it becomes more sensitive with the installation of the stationary phase of growth, highlighted by a more pronounced cell death;
- The *L. monocytogenes* EGDe Δ*lmo2229* mutant strain grows more slowly compared to the wild type strain, an aspect that also results from the calculation of the cells generation time;

- Δ*lmo1013* mutant cells have a different morphology compared to the wild type ones, being characterized by a larger diameter;
- Δ*lmo2229* mutant cells differ, from the morphological point of view, from the wild type ones, in terms of length and diameter, having the tendency to form filaments, as a result of a possible division defect;
- The motility of the mutant strains becomes lower, over time, compared to that of the wild type strain. Motility of the Δ*lmo2229* mutant is much more affected as result, most likely, of the cell chaining phenotype.

In terms of biofilm formation capacity, mutant strains behave similarly to the wild type strain, regardless of the temperature at which the cells were incubated.

CHAPTER V

Resistance of the mutant strains, *Listeria monocytogenes* EGDe Δ*lmo1013* and *Listeria monocytogenes* EGDe Δ*lmo2229*, to high pressure and other stress factors associated with food products

The present experimental studies aimed to evaluate the resistance of the genetically modified *L. monocytogenes* strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, compared to the wild type strain, at high pressure treatment, in order to determine the contribution of the proteins encoded by the deleted target genes at barotolerance and/or molecular mechanisms by which the bacterium can recover. The resistance of the constructed mutant strains to other stress factors associated with food, such as osmotic stress (hyperosmotic or hypoosmotic stress) and the presence of nisin, was also assessed.

Resistance of *L. monocytogenes* mutant strains to high pressure treatment

The present research aimed to evaluate the resistance of the constructed mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, compared to the wild type strain, *L. monocytogenes* EGDe, to HPP. The results regarding the viability of Δ*lmo1013* and Δ*lmo2229* mutant cells after treatments at 300, 350 and 400 MPa, 8°C, 5 min, compared to that of the wild type cells, are shown in **Figure 5.1**.

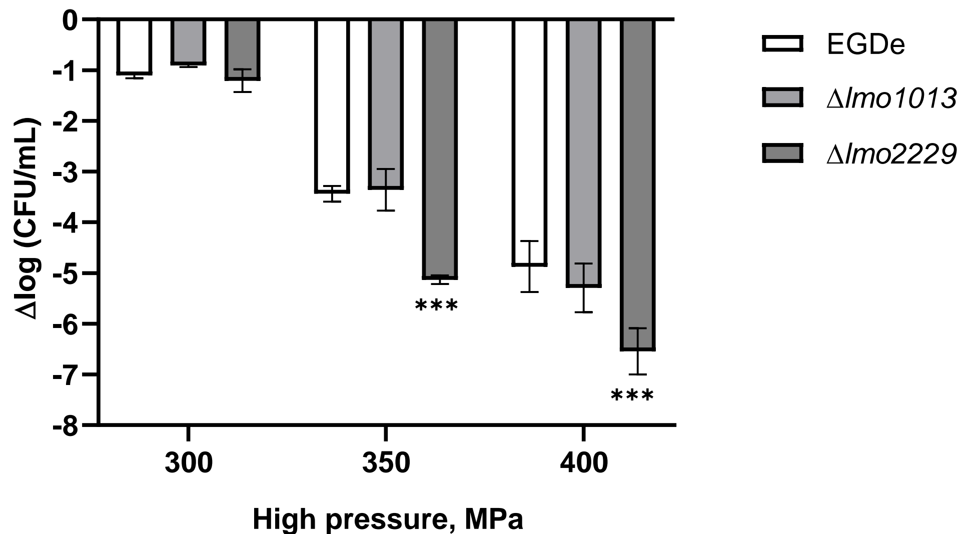

Figure 5.1. Resistance of *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* to HPP (300, 350 and 400 MPa, 8°C, 5 min). The values represent the mean ± standard deviation of three replicates. Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test, where the wild-type strain, *L. monocytogenes* EGDe, represented the control (***$p < 0.001$)

The treatment at 300 MPa, 8°C, 5 min, determined the reduction of the number of wild type and mutant cells, Δ*lmo1013* and Δ*lmo2229*, respectively, by approximately 1 log CFU/mL compared to the afferent control, the resistance of the mutant strains to this treatment being, therefore, similar to that of the wild type strain ($p > 0.9$). Following treatment at 350 MPa, the viability of wild type cells as well as Δ*lmo1013* mutant cells decreased by approximately 3 log CFU/mL compared to control. On the other hand, the number of viable cells, regarding the Δ*lmo2229* mutant, decreased by approximately 5 log CFU/mL compared to the control, the mutant cells being affected to a significantly greater extent ($p < 0.001$) by the treatment at 350 MPa compared to the wild type ones. Treatment at 400 MPa reduced the number of wild type cells and that of the Δ*lmo1013* mutant cells by approximately 5 log CFU/mL, Δ*lmo2229* mutant cells being affected to a greater extent ($p = 0.002$), in which case their number decreased by ~ 6 log CFU/mL.

Starting from the morphological and physiological characterization of *L. monocytogenes* cells exposed to high pressure (Ritz *et al*., 2001), induction of cell wall and membrane susceptibility is a promising strategy to prevent bacteria recovery. Depending on its intensity, high pressure can induce mechanical stress on the cell wall, damaging its structure (Woldemariam and Emire, 2019). In this context, the stability of the cell wall is an important factor in the resistance of the bacterial cells to HPP (Manas and Mackey, 2004). Thus, the increased susceptibility of Δ*lmo2229* mutant cells to HPP could be determined by an inhibition of the repair process of the HPP induced lesions on the cell wall level, in the absence of PBP A2, with a role in new peptidoglycan layers incorporation. Recent studies indicate the essential

function of class A PBPs for the mechanical integrity of the cell wall and the active repair of the lesions on its level (Lai *et al*., 2017; Vigouroux *et al*., 2020).

Deletion of the gene that encodes for MscS did not induce a higher susceptibility of the mutant cells to the HP treatments compared to that of the wild type strain, suggesting that these mechanosensitive channels are not directly involved in the resistance of listerial cells to HPP. Therefore, the resistance of Δ*lmo1013* mutant cells to HPP, similar to that of the wild type cells, could be determined by the activation of large mechanosensitive ion channels present on the cell membrane level. To confirm this hypothesis, it is necessary to eliminate the gene that encodes for the large mechanosensitive ion channels and to test the resistance of the newly constructed mutant strain to HPP in comparison to the wild type strain.

Resistance of *L. monocytogenes* mutant strains to osmotic stress

Resistance to hyperosmotic stress

This study aimed to evaluate the tolerance of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, compared to that of the wild type strain, *L. monocytogenes* EGDe, to hyperosmotic stress induced by addition of 1 to 10% (w/v) NaCl in the culture medium. **Figure 5.2** presents the ability of the strains to proliferate under hyperosmotic conditions, considering their growth in culture medium without supplementary addition of NaCl (negative control). The graph shows that as the NaCl concentration increases, the ability of listerial cells to proliferate decreases.

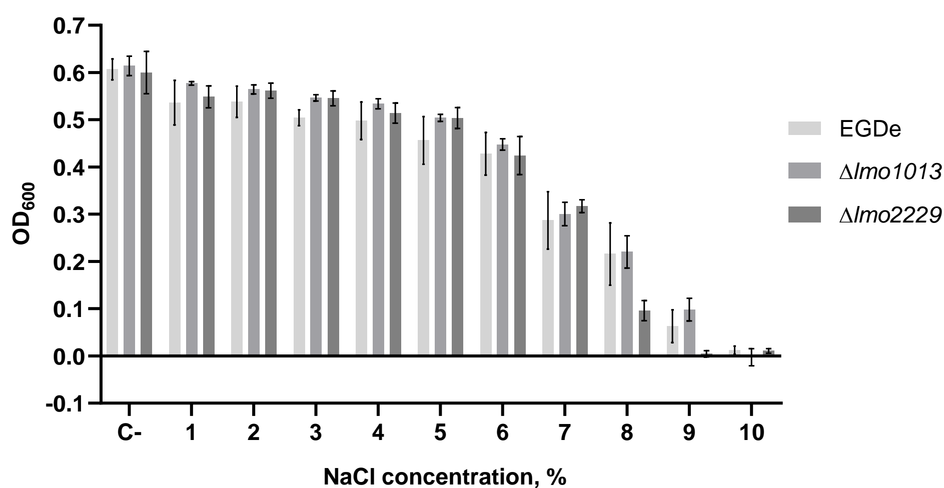

Figure 5.2. Optical density (OD_{600}) of the *L. monocytogenes* EGDe, *L. monocytogenes EGDe* Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* microcultures under hyperosmotic conditions induced by the addition of 1 to 10% NaCl in the growth medium; C- (negative control). The values represent the mean ± standard deviation of three replicates

As can be seen, all three strains were inhibited at a concentration of 10% NaCl. Interestingly, *L. monocytogenes* EGDe Δ*lmo2229* mutant strain indicated a higher sensitivity to hyperosmotic stress compared to the wild type strain, tolerating NaCl concentrations of up to

8%. Regarding the growth of this mutant strain in the presence of 8% NaCl, a significantly lower proliferation capacity ($p < 0.05$) than that of the wild type strain was noticed. Previous studies have indicated changes in the cell wall structure as a strategy of *L. monocytogenes* to resist hyperosmolarity (Bergholz *et al.*, 2012; Burall *et al.*, 2015). Moreover, the study conducted by Durack *et al.* (2015) highlighted the overexpression of the genes associated with the cell wall biosynthesis, as a result of *L. monocytogenes* exposure to hyperosmotic stress. All these aspects suggest the importance of the *L. monocytogenes* ability to adjust the mechanical properties of the cell wall to tolerate high concentrations of NaCl in the extracellular environment. Therefore, the increased sensitivity of the *L. monocytogenes* EGDe Δ*lmo2229* mutant strain, deficient in the PBP A2, to increased hyperosmotic stress suggests the contribution of the eliminated protein to the osmotolerance mechanism of the bacterium.

On the other hand, the *L. monocytogenes* EGDe Δ*lmo1013* mutant strain showed a similar behavior ($p > 0.05$) with that of the wild type strain, both strains tolerating a NaCl concentration of maximum 9%. This result suggests that the *lmo1013* gene, which encodes for the small mechanosensitive ion channels, is dispensable for the growth of *L. monocytogenes* EGDe cells under high osmolarity conditions. A similar phenotype, determined by the elimination of the small mechanosensitive ion channels in *Bacillus* (*B.*) *subtilis*, was observed by Hoffmann *et al.* (2008).

Resistance to hypoosmotic shock

To test the ability of *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* mutant strains to survive, compared to the wild type strain, following severe hypoosmotic shock, listerial cells were grown in the presence of 0.8 M NaCl to middle exponential phase (OD_{600} = ~ 0.5), the cultures being then suddenly diluted in culture medium without supplementary addition of NaCl (0.12 M NaCl).

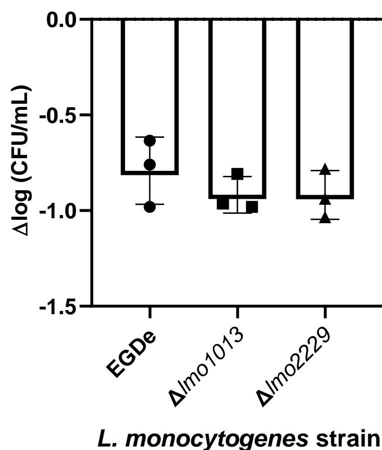

Figure 5.3. Resistance of *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* to hypoosmotic shock. The values represent the mean ± standard deviation of three replicates. Statistical analysis of the experimental data was performed by analysis of variance (ANOVA), followed by the multiple comparison Dunnett's test, where the wild type strain, *L. monocytogenes* EGDe, represented the control

After induction of the hypoosmotic shock, the initial number of cells was reduced by approximately 1 log CFU/mL in all strains, with no significant difference ($p > 0.5$) regarding the behavior of wild type and mutant cells, Δ*lmo1013* and Δ*lmo2229*, respectively (**Figure 5.3**).

The similar survival capacity of Δ*lmo1013* mutant cells with that of the wild type cells indicates the presence of other types of mechanosensitive ion channels in *L. monocytogenes* cells, with a role in the efflux of osmolytes accumulated from the external environment or synthesized during growth under elevated osmolarity. Most likely, the larger mechanosensitive ion channels are the emergency systems by which *L. monocytogenes* cells eliminate water and solutes following a hypoosmotic shock, as observed by Hoffmann *et al*. (2008) in the case of *B. subtilis*. Moreover, previous studies have shown the presence in the genome of *L. monocytogenes* EGDe of two genes, *glpF* and *lmo1539*, which show significant similarity with the genes encoding for aquaporins, specific channels for water transport with an important role in the resistance of listerial cells to hypoosmotic shock (Sleator *et al*., 2003).

The result regarding Δ*lmo2229* mutant cells resistance to hypoosmotic shock, similar to that of the wild type strain, indicates that cell wall resistance is not affected by PBP A2 elimination, which is rigid enough to prevent cell lysis following the accumulation of a massive volume of water. This is not surprising, given the presence in *L. monocytogenes* EGDe cells of other PBPs with transpeptidase activity (PBP A1, PBP B1, PBP B2, PBP B3), which ensure the rigidity of the peptidoglycan by cross-linking the glycan chains. .

Resistance of *L. monocytogenes* mutant strains to nisin

The present study aimed to evaluate the resistance of the mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, compared to that of the wild type strain, *L. monocytogenes* EGDe, to nisin A, a bacteriocin produced by *Lactisococcus lactisococcus* spp. *lactis*. Sensitivity to nisin was evaluated by determining the substance minimum concentration that inhibits cell growth during incubation at 37°C, for 18 hours. Nisin used in this study was a commercial product with 2.5% active substance, equivalent to ≥ 1,000,000 I.U./g, the rest of it being composed of NaCl (75%) and denatured milk dry matter (22.5%).

Initially, the binary microdilution technique was applied, testing the sensitivity of the *L. monocytogenes* strains to nisin concentrations ranging from 5.85 to 3,000 I.U./mL (**Figure 5.4**). In this case, inhibition of the *L. monocytogenes* EGDe Δ*lmo2229* mutant strain growth at the concentration of 3,000 I.U./mL nisin was observed. The growth of the other mutant strain, *L. monocytogenes* EGDe Δ*lmo1013,* as well as that of the wild type strain, were much less affected ($p < 0.05$) by the presence of the nisin in this concentration.

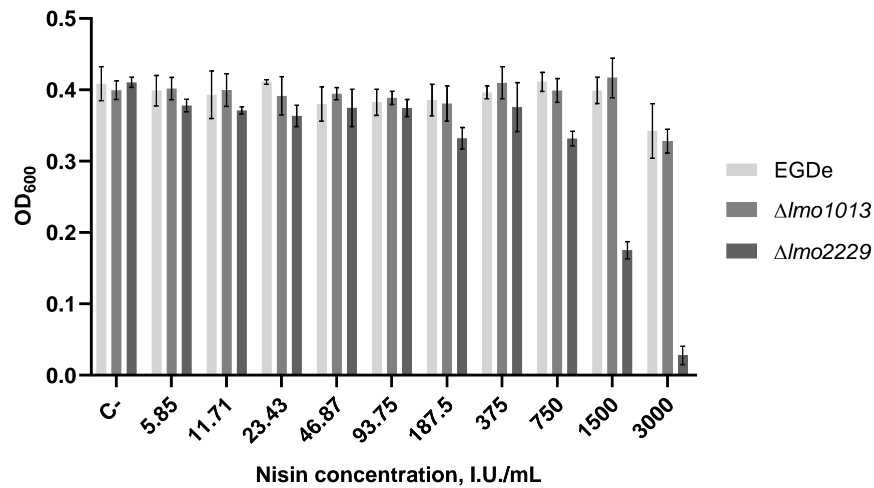

Figure 5.4. Optical density (OD_{600}) of *L. monocytogenes* EGDe, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* microcultures treated with nisin at concentrations ranging from 5.85 to 3,000 I.U./mL; C- (negative control). The values represent the mean ± standard deviation of three replicates

For these reason, it was decided to test the sensitivity of the strains at higher concentrations of nisin, in the range of 3,000 - 4,000 I.U./mL (**Figure 5.5**). Thus, growth of the wild type strain and *L. monocytogenes* EGDe Δ*lmo1013* mutant strain was inhibited at a nisin concentration of 3,700 I.U./mL. Therefore, the elimination of small mechanosensitive ion channels, Lmo1013, does not significantly influence the resistance of the pathogenic bacterium to this bacteriocin.

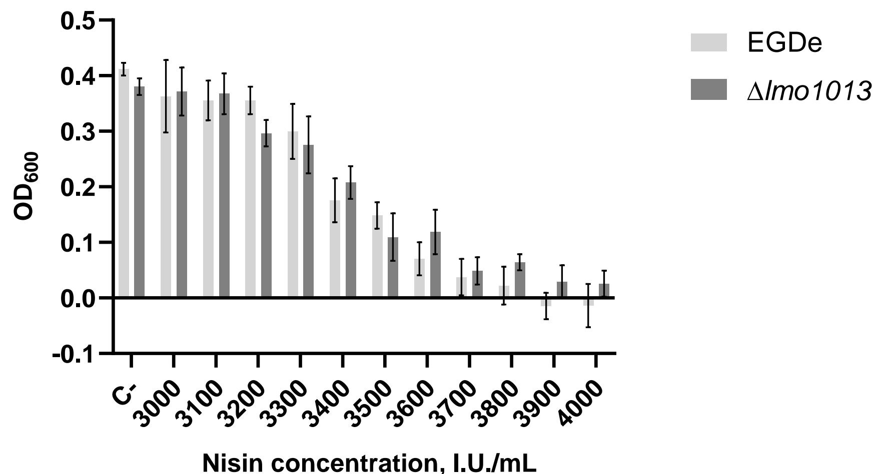

Figure 5.5. Optical density of *L. monocytogenes* EGDe and *L. monocytogenes* EGDe Δ*lmo1013* microcultures treated with nisin of concentrations ranging from 3,000 to 4,000 I.U./mL; C- (negative control). The values represent the mean ± standard deviation of three replicates

Further, to determine more accurately the concentration of nisin that inhibits the growth of Δ*lmo2229* mutant cells, their resistance was tested in the range of 1,000 - 2,000 I.U./mL of nisin (**Figure 5.6**). During this stage, the growth of the mutant cells was inhibited at a nisin concentration of 1,500 I.U./mL, *L. monocytogenes* EGDe Δ*lmo2229* mutant strain being, therefore, approximately 2.5 times more sensitive to this antimicrobial peptide than the wild type strain

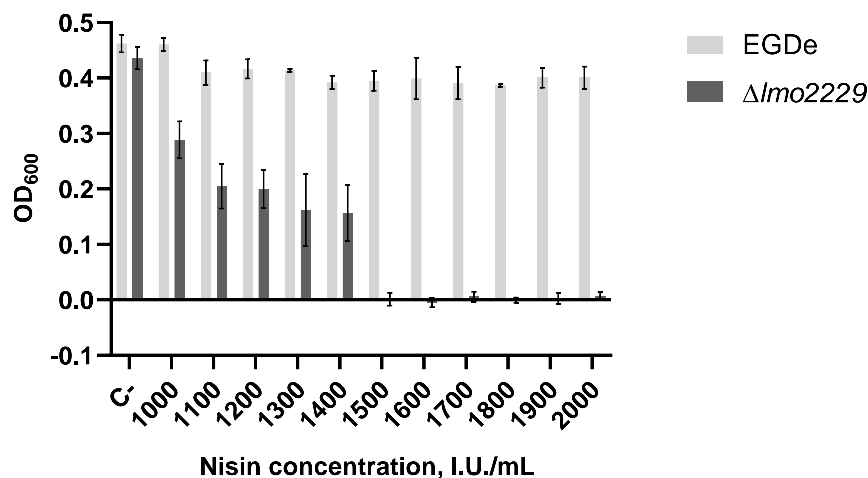

Figure 5.6. Optical density of *L. monocytogenes* EGDe and *L. monocytogenes* EGDe Δ*lmo2229* microcultures treated with nisin of concentrations ranging from 2,000 to 1,000 I.U./mL; C- (negative control). The values represent the mean ± standard deviation of three replicates

The results regarding the sensitivity of *L. monocytogenes* EGDe Δ*lmo2229* mutant strain to nisin are in agreement with the observations of other studies performed in this direction. For instance, Gravesen *et al*. (2004) showed that the expression of *pbp2229* gene, regulated by the molecular signal transduction system encoded by *hpk1021-rrp1022*, is directly involved in the nisin resistance mechanism of *L. monocytogenes* 412N strain. In this case, inactivation of the *pbp2229* and *hpk1021* genes, by insertional mutagenesis, led to the abolition of the nisin resistance phenotype. Subsequently, Collins *et al*. (2012) also confirmed that *lmo2229* gene, whose expression is regulated by LiaR protein, the histidine kinase homologue of HPK1021, is a key element in the resistance of *L. monocytogenes* LO28 strain to nisin.

Conclusions

- Deletion of *lmo1013* gene from the genome of *L. monocytogenes* did not induce the susceptibility of the bacterium to HPP, suggesting that the small mechanosensitive ion channels, Lmo1013, do not contribute to the resistance of the bacterium to this stress;
- Deletion of *lmo2229* gene from the genome of *L. monocytogenes* influenced significantly the resistance of the bacterium to HPP (350 and 400 MPa, 8°C, 5 min), suggesting that the role of PBP A2 is important for cell recovery from exposure to this stress;

- Small mechanosensitive ion channels are dispensable for the growth of *L. monocytogenes* cells under hyperosmotic conditions, while the PBP A2 protein contributes to listerial cella osmotolerance;
- The deleted genes, *lmo1013* and *lmo2229*, encoding for the proteins Lmo1013 and PBP A2, respectively, do not intervene in the resistance of *L. monocytogenes* cells to hypoosmotic stress, the behavior of mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229* being similar to the wild type strain, *L. monocytogenes* EGDe;
- *L. monocytogenes* EGDe Δ*lmo2229* mutant strain is approximately 2.5 times more sensitive to nisin than the wild type strain, indicating the contribution of PBP A2, encoded by the *lmo2229* gene, to bacterium resistance to bacteriocins belonging to lantibiotic class (nisin), which was also noticed in other strains of *L. monocytogenes*.

CHAPTER VI

Assessment of the combined effect of high pressure processing and nisin on *Listeria monocytogenes* survival capacity in ready-to-eat ham

The novelty of the study consists in the use of a *L. monocytogenes* strain with increased barotolerance and resistance to nisin for artificial contamination of the food matrix subjected to HPP. The study proposes the combination of HPP, at a lower intensity, with the addition of nisin, as a possible alternative to the treatment practiced in industry, in order to reduce the costs associated with the process and to obtain a food product safe for consumption. Another objective of the present research was the development of a predictive model, based on logistic regression, in order to estimate the probability of recovery of *L. monocytogenes* after HPP of RTE ham, with or without nisin.

Results and discussions

Selection of a *L. monocytogenes* strain with increased barotolerance

The resistance of *L. monocytogenes* strains treated at 400 MPa, RT, 1 min, is shown in **Figure 6.1**. The strains selected for HPP resistance testing were isolated from foods (strains AB100, AB80, ABS43, RO15 and RO4) and food processing environment (strains AB120, AB199, AB204, AB24 and ABS45).

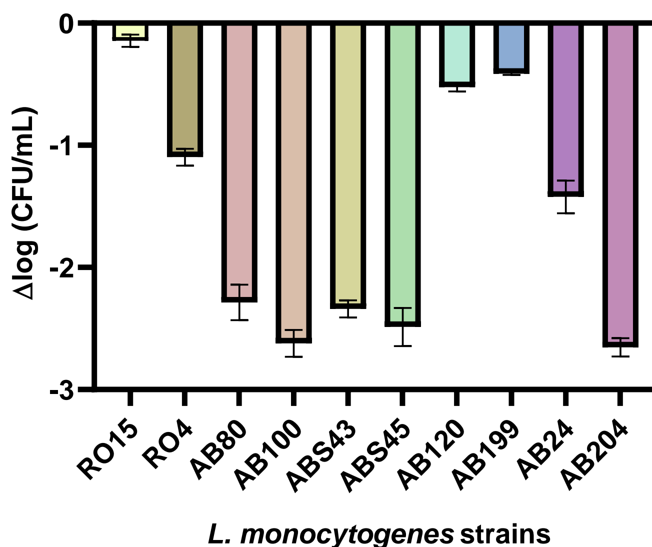

Figure 6.1. Resistance of *L. monocytogenes* strains selected from UGAL collection to high pressure treatment (400 MPa, RT, 1 min). The values represent the mean of two independent experiments ± standard deviation

The strains that showed increased resistance to treatment, in which case the initial number of listerial cells was reduced by less than 1 log CFU/mL, were RO15, AB199, AB120 and RO4. The less resistant strains, whose initial cell number was reduced by ~ 1.5 - 2.5 log CFU/mL, were AB24, AB80, ABS43, ABS45, AB100 and AB204. The obtained results indicated RO15 strain (serotype 4b), an isolate from spicy herring, as the most tolerant to HPP ($p < 0.05$) among the *L. monocytogenes* strains tested in this experiment.

Evaluation of *L. monocytogenes* resistance to nisin

L. monocytogenes RO15, the strain selected after barotolerance test, was evaluated for resistance to nisin, by agar diffusion method. The characterization of the inhibition zones produced by the nisin solutions of concentrations ranging from 0.01 to 2.5 mg/mL is given in **Table 6.1**.

Table 6.1. *Characterization of inhibition zones produced by nisin solutions of concentrations ranging from 0.01 to 2.5 mg/mL on culture medium inoculated with L. monocytogenes RO15*

Nisin concentration (mg/mL)	Inhibition type
0.01	-
0.19	-
0.039	-
0.078	-
0.156	Partial
0.312	Partial
0.625	Partial
1.25	Complete
2.5	Complete

The minimum concentration of nisin, which produced a clear inhibition zone on the culture medium inoculated with *L. monocytogenes* RO15, was 1.25 mg/mL, equivalent to 1,250 I.U./mL.

Characterization of the food matrix

Table 6.2 shows some properties of the food matrix (Prague ham) used in the high pressure experiments. Prague ham is a food product that can support the growth of *L. monocytogenes* during storage under refrigeration conditions, given its almost neutral pH (6.725 ± 0.007), the optimal value of a_w (0.987 ± 0.001) and the reduced content of salt (2%) (EFSA, 2018).

Table 6.2. *Properties of the food matrix (Prague ham) used in high pressure experiments*

Property	Value
pH	6.725±0.007
Water activity	0.987±0.001
Salt content, %	2*
Proteins, %	14*
Carbohydrates, %	3*
Lipids, %	3*

Note: *According to the producer

Growth of *L. monocytogenes*, in the presence or absence of nisin, on the Prague ham stored under refrigeration conditions

The growth of *L. monocytogenes* on Prague ham, treated or not with nisin (25 mg/kg), during 3 weeks of samples storage at 4°C, is shown in **Figure 6.2.**

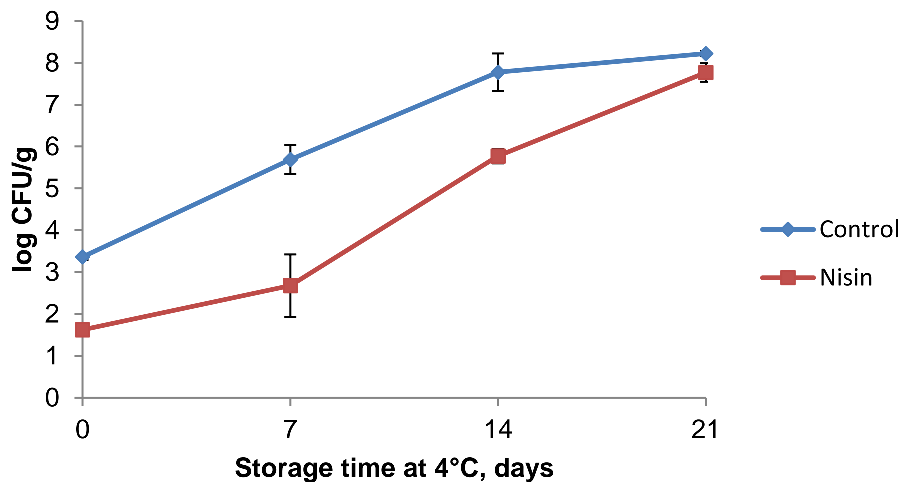

Figure 6.2. Growth of *L. monocytogenes* on Prague ham stored at 4°C in the absence or presence of nisin (25 mg/kg)

As expected, the growth of *L. monocytogenes* on Prague ham (control), from inoculation day until the end of the 21 day storage at 4°C, was significant ($p < 0.05$), the population of listerial cells reaching finally the value of 8.22 ± 0.06 log CFU/g.

Compared to control, nisin treatment reduced the initial *L. monocytogenes* population by 1.74 ± 0.1 log CFU/g ($p < 0.05$). However, at the end of the storage period at 4°C, taking into

account the evolution of the concentration of *L. monocytogenes* cells (7.76 ± 0.22 log CFU/g), it can be stated that nisin had a modest contribution to the control of pathogen growth on the product.

Effect of high pressure treatment, with or without nisin, on *L. monocytogenes* survival on Prague ham

The behavior of *L. monocytogenes* cells on Prague ham, after high pressure treatments application, with or without nisin, and storage at 4°C, for 21 days, is shown in **Figure 6.3**.

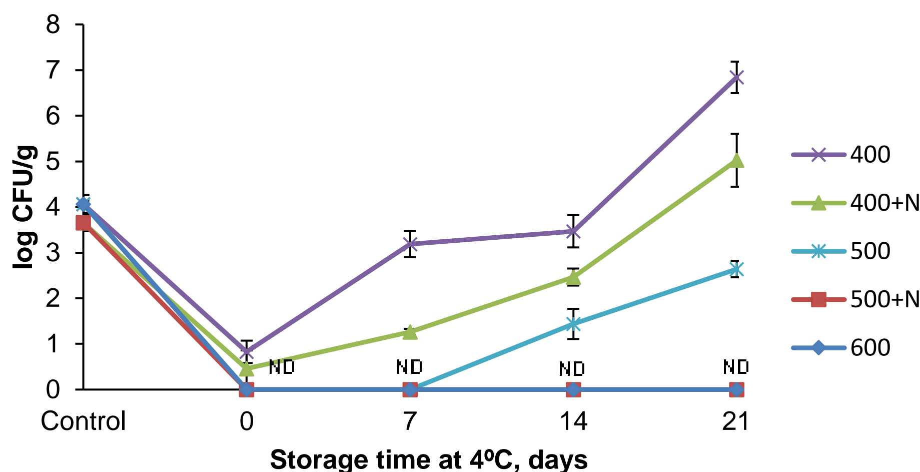

Figure 6.3. Inactivation, after high pressure treatments, and recovery of *L. monocytogenes* on Prague ham, during storage at 4°C. The tests include control with or without nisin (samples not treated at high pressure), samples treated at 400 MPa, 8°C, 3 min (400), samples treated at 400 MPa, 8°C, 3 min, in the presence of nisin (25 mg/kg; 400 + N), samples treated at 500 MPa, 8°C, 3 min (500), samples treated at 500 MPa, 8°C, 3 min, in the presence of nisin (25 mg/kg; 500 + N) and samples treated at 600 MPa, 8°C, 3 min (600); ND - undetectable. The values represent the mean of two replicates ± standard deviation

Immediately after application of the control treatment (600 MPa, 8°C, 3 min), the number of *L. monocytogenes* cells decreased below the detection limit of the employed counting method. Similarly, the application of treatments at 500 MPa, with or without nisin, reduced the listerial population to an undetectable level. Treatments at 400 MPa, with or without nisin, resulted in a significant reduction ($p < 0.05$) of the *L. monocytogenes* population by 3.62 ± 0.39 log CFU/g and 3.26 ± 0.24 log CFU/g, respectively.

During storage under refrigeration conditions of the Prague ham samples treated at 600 MPa, the presence of viable but not culturable *L. monocytogenes* cells was not noticed.

Although treatments at 400 MPa, with or without nisin, reduced significantly the number of *L. monocytogenes* cells, the treated samples were not microbiologically stable over the storage under refrigeration conditions, the initiation of *L. monocytogenes* recovery occurring after 14 and 7 days, respectively, of storage ($p < 0.05$). In this case, at the end of the refrigeration storage of

Prague ham samples, the number of *L. monocytogenes* cells exceeded the limit allowed by the legislation, so that the treatments are not effective from the food safety perspective.

Although immediately after treatment at 500 MPa and over the first 7 days of storage at 4°C, the number of *L. monocytogenes* cells was below the detection limit, the recovery of listerial cells was noticed over the next 14 days of storage. The concentration of *L. monocytogenes* cells reached a higher value than that allowed by the legislation (2.63 ± 0.18 log CFU/g). On the other hand, the addition of bacteriocins prevented the recovery of the pathogen over the storage period, the treatment at 500 MPa in the presence of nisin being, therefore, the most effective variant of combined treatment, among those tested, against *L. monocytogenes*.

Predictive modelling application for determination of *L. monocytogenes* recovery probability on treated Prague ham

Determination of the recovery probability of pathogenic bacteria, after high pressure processing, is extremely important for the safety of food products during their distribution and storage.

The results of the HPP experiments showed that, although reduced to a low or undetectable level immediately after treatment, *L. monocytogenes* cells can recover and subsequently multiply during storage of the meat product under refrigeration conditions. The recovery of the pathogenic bacterium was defined, in the present study, as the detection of a concentration of at least 2 log CFU/g, taking into account the microbiological criteria of the European legislation regarding food safety. In this context, *L. monocytogenes* is considered to pose a low risk to consumers health if, at the time of consumption, the concentration of the bacterium in the food is less than 10^2 CFU/g (Little *et al.*, 2009).

The results of the experimental study were analyzed by applying the developed logistic regression model, described by **Equation 6.1**. The parameters of the variables were estimated using the SPSS Statistics program, by applying the logistic procedure.

$$[\ln P/(1-P)] = 3{,}627 - 0.018 \cdot \text{Pressure} - 0.116 \cdot \text{Nisin} + 0.356 \cdot \text{Storage} \quad \textbf{(6.1)}$$

The standard errors of the estimated regression parameters, together with the statistical significance, are presented in **Table 6.3**. According to them, high pressure and storage period have a significant effect ($p < 0.05$) in the model. The marginal significant effect ($p = 0.099$) of the nisin variable can be explained by testing, in the experimental studies, of a single concentration of nisin, namely the maximum concentration allowed in meat products. However, the backward analysis procedure, which tests the importance of each variable in the system, indicated that removing the nisin variable does not significantly improve the prediction ability of the model. According to the Hosmer – Lemeshow statistical test, there is a good fit of the experimental data on the model ($p = 0.995$), the general percentage regarding the correct predictions being 88.9%.

Table 6.3. *Parameters used in the logistic regression model predicting the probability of recovery of L. monocytogenes on Prague ham after HPP, in the absence or presence of nisin, during storage at 4˚C*

Parameter	Estimated value	SE	*p*
Intercept	3.627	2.272	0.110
Pressure	-0.018	0.007	0.012
Nisin	-0.116	0.070	0.099
Storage	0.356	0.167	0.033

Pressure = High pressure (MPa); Nisin = Amount of nisin added (mg/kg); Storage = Product storage period at 4˚C (days); Hosmer – Lemeshow goodness-of-fit (p = 0.995); Concordance = 88.9%;

The predictions regarding the probability of *L. monocytogenes* recovery on Prague ham, depending on the high pressure treatment, amount of nisin and storage period at 4˚C, calculated by applying **Equation 6.1**, are presented in **Figure 6.4.**

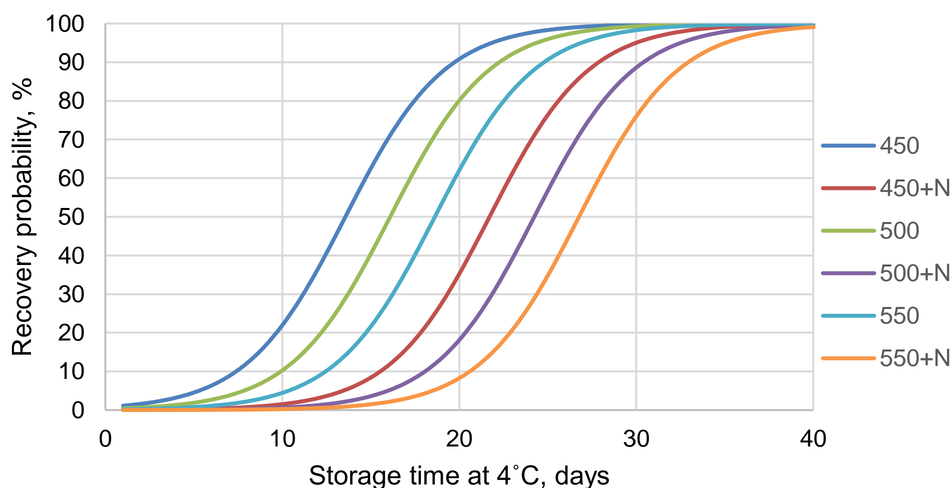

Figure 6.4. Predictions of the developed logistic regression model regarding the probability of *L. monocytogenes* recovery over the refrigerated storage of Prague ham treated at 450-550 MPa, for 3 min, in the absence or presence of nisin (N; 25 mg/kg), at 4˚C

The logistic regression model was applied to estimate the time required for the pathogenic bacterium to recover on the meat product stored at 4˚C, in case of high pressure treatments in the range 450 - 550 MPa, with or without nisin. According to the calculated estimates, a probability of ~ 50% that *L. monocytogenes* will recover after treatments at 450, 500 and 550 MPa, for 3 min, appears after refrigerated storage of the processed product for ~ 13, 15 and 18 days, respectively. The combination of high pressure treatments with the addition of nisin (25 mg/kg) extends the time required for *L. monocytogenes* to recover by ~ 8 days.

Conclusions

- Testing the high pressure resistance (400 MPa, RT, 1 min) of 10 *L. monocytogenes* strains isolated from either food or food processing environment, indicated RO15 strain as being the most barotolerant. The selected strain has also indicated resistance to nisin;

- Nisin addition (25 mg/kg), as additional hurdle, allows the reduction of the high pressure treatment intensity to 500 MPa, without compromising the safety of the meat product, during storage at 4˚C, for 21 days;
- Application of hurdle technology can be a strategy to reduce the costs associated with the high pressure treatment applied by the industry, given the possibility of reducing the intensity of HPP, without compromising food safety;
- A logistic regression model has been developed, which can be used to estimate the probability of *L. monocytogenes* recovery on RTE meat products as function of high pressure treatment, nisin addition and storage period under refrigeration conditions.

Final conclusions

The documentary study of the thesis highlighted that *L. monocytogenes* is a pathogenic bacterium capable of activating various mechanisms to resist the stress factors associated with food processing, both traditional and alternative, which can compromise food safety. Therefore, the main objective of the experimental studies within the doctoral thesis was to reveal some molecular mechanisms involved in the resistance of *L. monocytogenes* to HPP, an alternative technology increasingly used by industry.

The present research is an original and complex approach to the problem represented by the ability of the pathogenic bacterium *L. monocytogenes* to withstand HPP, the final conclusions being as follows:

- The main objective of the doctoral thesis was to reveal some of the resistance mechanism of *L. monocytogenes* to HPP. For this, the deletion of the target genes, *lmo1013* and *lmo2229*, respectively, from the genome of the *L. monocytogenes* EGDe model strain was required, an experimental step performed successfully by using the lactococcal system consisting of the plasmidial vectors *p*ORI280 and *p*VE6007, adapted for site directed mutagenesis;
- By the phenotypic characterization of the constructed *L. monocytogenes* mutant strains, the role of the proteins encoded by the target genes, *lmo1013* and *lmo2229*, in the growth dynamics of listerial cells, their morphology, motility and biofilm forming capacity was revealed:
    - Although penicillin binding protein (PBP) A2, encoded by the *lmo2229* gene, is dispensable to *L. monocytogenes* cells viability, its elimination affects cell growth;
    - The different growth dynamics of the *L. monocytogenes* EGDe Δ*lmo2229* mutant strain can be attributed to the abnormal morphology of the cells, which have the tendency to form filaments, as a result of an imbalance regarding the coordination between elongation and septation of the cells;
    - The higher sensitivity of Δ*lmo1013* mutant cells, in the stationary phase of growth, indicates the importance of the mechanosensitive ion channels in the physiological

processes associated with this phase of growth, as observed previously in the case of other bacteria species;

- The morphology of Δ*lmo1013* mutant cells can contribute to the previous statement, presenting a larger diameter compared to that of the wild type cells;

- The results of the phenotypic study showed that deletion of PBP A2 coding gene, *lmo2229*, affects significantly the motility of *L. monocytogenes*. However, the deletion of the gene has shown that the role of the encoded protein is not important for the pathogen ability to form biofilm;

- The deletion of the gene that encodes for the small mechanosensitive ion channels, *lmo1013*, influenced to a small but significant extent, the motility of *L. monocytogenes*. Although the literature discusses the possible role of the mechanosensitive channels in the process of biofilm formation, experiments on *L. monocytogenes* EGDe Δ*lmo1013* cells have not shown their contribution to this phenotype.

- Testing, in comparison to the wild type strain, the resistance of the constructed mutant strains, *L. monocytogenes* EGDe Δ*lmo1013* and *L. monocytogenes* EGDe Δ*lmo2229*, to high pressure revealed the following aspects:

  - The role of the small mechanosensitive ion channels in the resistance of *L. monocytogenes* cells to high pressure is not significant. Large mechanosensitive ion channels could be responsible, to a greater extent, for the resistance of the listerial cells to HPP;

  - PBP A2 contributes to one of the mechanisms of *L. monocytogenes* cells recovery after exposure to high pressure, which consists in the repairing of the lesions occurred on the cell wall level. In this context, inhibition of peptidoglycan biosynthesis is a promising strategy to prevent *L. monocytogenes* recovery after HPP.

- The results regarding the resistance of the *L. monocytogenes* EGDe Δ*lmo1013* mutant strain to osmotic stress or nisin did not indicate an important role of the small mechanosensitive ion channels in the bacterium's tolerance to these types of stress.

- On the other hand, the importance of PBP A2 in the resistance of *L. monocytogenes* to severe hyperosmotic stress was highlighted, being suggested the contribution of the protein to the osmotolerance mechanism of the bacterium.

- The much lower resistance of the *L. monocytogenes* EGDe Δ*lmo2229* mutant strain to nisin than that of the wild type strain indicates PBP A2 as an important element in the bacterium resistance mechanism to this antimicrobial peptide, as observed in other listerial strains.

- The results regarding the combined effect of HPP and nisin indicate the treatment at 500 MPa, 8ºC, 3 min, applied in the presence of nisin at a concentration of 25 mg/kg, as a good alternative and possibly less expensive to the working regime adopted by the food industry.

- The developed logistic regression model can be a useful tool in estimating the shelf life of ready to eat meat products and optimal processing conditions, given the possibility of predicting the recovery of the pathogenic bacteria *L. monocytogenes*.

Original contributions

The results of the present doctoral thesis contribute to the extension of knowledge in the approached field, namely the resistance of the pathogenic bacterium *L. monocytogenes* to stress conditions associated with food industry, through the following aspects:

- Synthesizing the current research on the resistance of *L. monocytogenes* to stressors associated with either traditional or alternative food processing methods, for an in depth understanding of the survival mechanisms used by the bacterium, to help food industry specialists in implementing strategies effective in combating the pathogen;
- Contribution to the development of a site directed mutagenesis method, based on the lactococcal system formed of the *p*ORI280 and *p*VE6007 plasmidial vectors, which can be successfully applied in the deletion of non-essential genes from the genome of *L. monocytogenes* or other bacteria. The method is a useful genetic editing tool in the study of *L. monocytogenes* resistance mechanisms;
- Bringing new information on the role of the proteins encoded by the target genes, *lmo1013* and *lmo2229*, in the physiology and morphology of *L. monocytogenes* cells;
- Contribution to the knowledge expansion on the mechanisms activated by *L. monocytogenes* following HPP to survive, and the resistance of the bacterium to other stress factors encountered in the food matrices, such as osmotic stress and bacteriocins (nisin);
- Identifying a possible alternative to the high pressure treatment used by industry, by applying hurdle technology;
- Development of a predictive model based on logistic regression, which can estimate the recovery probability of the pathogenic bacterium *L. monocytogenes* on ready-to-eat meat products treated at different combinations of high pressure and nisin, during storage under refrigeration conditions;
- The present doctoral thesis offers new research directions on the resistance of *L. monocytogenes* to HPP and other stress factors encountered by the bacterium in the food industry, so that the incidence of listeriosis caused by the consumption of contaminated food can be reduced.

Perspectives for research continuation

- Investigation, at the transcriptome level, of the high pressure effect on the constructed *L. monocytogenes* mutant strains, in order to determine the genes expressed to compensate the function of the proteins encoded by the deleted genes;

- Biochemical analysis of the cell wall, in the case of *L. monocytogenes* EGDe Δ*lmo2229* cells;
- Deletion or, depending on the role in cell viability, repression by CRISPRi, of other genes possibly involved in the resistance of the bacterium to high pressure, such as *lmo2064* gene (encoding for the large mechanosensitive ion channels), *clp* genes (encoding for the proteases involved in the degradation of denatured proteins), class I heat-shock genes (encoding for chaperones) and cold-shock genes (encoding for RNA chaperones);
- Characterization of the newly constructed mutant strains and testing their resistance to high pressure and other stress factors associated with food and food processing environment;
- Combining HPP with the use of bacteriophages, as a strategy to eliminate *L. monocytogenes* from foods.

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Dissemination of the results obtained during doctoral studies

Articles published in ISI journals

Bucur, F. I., Grigore-Gurgu, L., Crauwels, P., Riedel, C. U., Nicolau, A. I. (2018). Resistance of *Listeria monocytogenes* to stress conditions encountered in food and food processing environments. *Frontiers in Microbiology*, 9, 2700. https://doi.org/10.3389/fmicb.2018.02700. **Impact factor: 4.272**

Zetzmann, M., **Bucur, F. I.**, Crauwels, P., Borda, D., Nicolau A. I., Grigore-Gurgu, L., Seibold, G. M., Riedel, C. U. (2019). Characterization of the biofilm phenotype of a *Listeria monocytogenes* mutant deficient in *agr* peptide sensing. *MicrobiologyOpen*, 8:e826. https://doi.org/10.1002/mbo3.826. **Impact factor: 2.71**

Duru, I. C., Andreevskaya, M., Laine, P., Rode, T. M., Ylinen, A., Løvdal, T., Bar, N., Crauwels, P., Riedel, C. U., **Bucur, F. I.**, Nicolau, A. I., Auvinen, P. (2020). Genomic characterization of the most barotolerant *Listeria monocytogenes* RO15 strain compared to reference strains used to evaluate food high pressure processing. *BMC Genomics*, 21:1, 455. https://doi.org/10.1186/s12864-020-06819-0. **Impact factor: 3.527**

Articles published in IBD journals

Bucur, F. I., Borda, D., Nicolau, A. I., Grigore-Gurgu, L. (2020). Assessment of *L. monocytogenes* cells' capacity to recover in cucumber juice after high pressure treatment. *The Annals of the University Dunarea de Jos of Galati, Fascicle VI – FOOD TECHNOLOGY*, 44:1. https://doi.org/10.35219/foodtechnology.2020.1.03

Articles in review at the time of doctoral thesis defense

Duru, I. C.**, **Bucur, F. I.****, Andreevskaya, M.**, Nikparvar, B., Ylinen, A., Grigore-Gurgu, L., Rode, T. M., Crauwels, P., Laine, P., Paulin, P., Løvdal, T., Riedel, C. U., Bar, N., Borda, D.***, Nicolau, A. I.***, Auvinen, P.***. HPP-induced transcriptome response during recovery of *Listeria monocytogenes*. (**First authors; ***Last authors) – *BMC Genomics* **Impact factor: 3.527**

International conferences

Crauwels, P., Zetzmann, M., Seow, M., **Bucur, F. I.**, Schäfer, L., Chandramohan, R., Nicolau, A., Riedel, C. Towards a functional CRISPRi system for *Listeria monocytogenes*. **Annual Conference of the Association for General and Applied Microbiology**, 15-18.04.2018, Wolfsburg, Germany

Grigore-Gurgu, L., **Bucur, F. I.**, Nicolau, A. I. Progresses in biotechnology supported by CRISPR/Cas based-technology. **Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology**, 5-7.09.2018, Bucharest, Romania

Ribeiro, T. G., **Bucur, F. I.**, Borda, D., Novais, C., Antunes, P., Nicolau, A. I., Bar, N. Peixe, L. Multiple molecular components of *Listeria monocytogenes* affected by high pressure processing treatments: Fourier transform-infrared spectroscopy insights. **FEMS Congress**, 7-11.07.2019, Glasgow, Scotland

Grigore-Gurgu, L., Duru, I. C., Andreevskaya, M., Ylinen, A., **Bucur, F. I.**, Borda, D., Auvinen, P., Nicolau, A. I. From gene sequencing to proteins expression in high pressure treated *Listeria monocytogenes*. **International Symposium EuroAliment-Innovative Minds for Future Food**, 5-6.09.2019, Galaţi, Romania

National conferences with international participation

Bucur F. I., Grigore-Gurgu, L., Nicolau, A. I. Survival mechanisms of the foodborne pathogen *Listeria monocytogenes*. **Scientific Conference Of Doctoral Schools - Perspectives and challenges in doctoral research**, 5th edition, 8-9.06.2017, Galaţi, Romania

Bucur, F. I., Grigore Gurgu, L., Nicolau, A.I. Applying CRISPRi system in baroresistant *Listeria monocytogenes* strains. **Scientific Conference Of Doctoral Schools - Perspectives and challenges in doctoral research**, 6th edition, 7-8.06.2018, Galaţi, Romania

Bucur, F. I., Grigore-Gurgu, L., Borda, D., Nicolau, A. I. *Listeria monocytogenes* survival and recovery capacity in cucumber juice after high pressure treatment. **Scientific Conference Of Doctoral Schools - Perspectives and challenges in doctoral research**, 7th edition, 13-14.06.2019, Galaţi, Romania

Bucur, F. I., Grigore-Gurgu, L., Borda, D., Nicolau, A. I. Assessment of *Listeria monocytogenes* resistance to food processing techniques and its capacity to form biofilm. **Scientific Conference Of Doctoral Schools - Perspectives and challenges in doctoral research**, 8th edition, 18-19.06.2020, Galaţi, Romania

Grigore-Gurgu, L., **Bucur, F. I.** CRISPR – CAS9 mediated genome editing, a cutting-edge tool for microbial metabolic engineering. **Multidisciplinary Conference on Sustainable**

Development - Section: Food Chemistry, Engineering & Technology, 8-9.10.2020, Timişoara, Romania

Awards

Third prize for the oral presentation of the paper **Bucur F. I.**, Grigore-Gurgu, L., Nicolau, A. I. "Survival mechanisms of the foodborne pathogen *Listeria monocytogenes*" în cadrul celei de-a 5-a ediţii a **SCDS - UDJG - Perspectives and challenges in doctoral research**, 8-9.06.2017, Galaţi, Romania.

First prize for the oral presentation of the paper **Bucur, F. I.,** Grigore Gurgu, L., Nicolau, A.I. "Applying CRISPRi system in baroresistant *Listeria monocytogenes* strains" în cadrul celei de-a 6-a ediţii a **SCDS - UDJG - Perspectives and challenges in doctoral research**, 7-8.06.2018, Galaţi, Romania.

First prize for the oral presentation of the paper **Bucur, F. I.**, Grigore-Gurgu, L., Borda, D., Nicolau, A. I. "*Listeria monocytogenes* survival and recovery capacity in cucumber juice after high pressure treatment" în cadrul celei de-a 7-a ediţii a **SCDS - UDJG - Perspectives and challenges in doctoral research**, 13-14.06.2019, Galaţi, Romania.

Prof. G. M. Costin prize offered by prof. dr. ing. Carmen Moraru and Romanian dairy sector for the reseach results presented at **SCDS - UDJG - Perspectives and challenges in doctoral research**, 13-14.06.2019, Galaţi, Romania.

Honorable mention for poster presentation **Bucur, F. I.**, Grigore-Gurgu, L., Borda, D., Nicolau, A. I. "Assessment of *Listeria monocytogenes* resistance to food processing techniques and its capacity to form biofilm" în cadrul celei de-a 8-a ediţii a **SCDS - UDJG - Perspectives and challenges in doctoral research**, 18-19.06.2020, Galaţi, Romania.

International research projects

26.01.2017-31.10.2019 - Proiectul internaţional ERA NET/ ERA IB 2: „Development of a novel industrial process for safe, sustainable and higher quality foods" (**SafeFood**)

Research internships

11.09.-25.11.2017 – Application of CRISPRi technology and site directed mutagenesis for genetic modification of the pathogenic bacterium *Listeria monocytogenes*. (Mobility funded by the SafeFood project - Institute of Microbiology and Biotechnology, University of Ulm, Ulm, Germany)

National and international workshops

„Completion of the Next-Generation Scientists" organized by Illumina & ELTA 90 on Library Preparation, Sequencing and Bioinformatics Techniques. 26.03.2019, Pravets, Bulgaria.

„PhageUgal Summer School" organized by Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galaţi and APC Microbiome Ireland, 16-19.07.2019, Galaţi, Romania

Other activities associated with the doctoral studies period

Book chapters

Grigore-Gurgu, L., **Bucur, F. I.**, Borda, D., Alexa, E.-A., Neagu, C., Nicolau, A. I. (2019). Biofilms formed by pathogens in food and food processing environments. In Bacterial Biofilms, IntechOpen. http://dx.doi.org/10.5772/intechopen.90176

Research articles

Mihalcea, L., **Bucur, F. I.**, Cantaragiu, A. M., Gurgu, L., Borda, D., Iordăchescu, G. (2016). Temperature influence on the *Agaricus bisporus* mushrooms dehydration process. *Scientific Study & Research: Chemistry & Chemical Engineering, Biotechnology, Food Industry*, 17:4, 323-333. ISSN 1582-540X

Conferences

Oniciuc, E. A., **Bucur, F. I.**, Rodriguéz-Lázaro, D., Barbu, V., Hernandéz, M., Nicolau, A. I. Correlation between biofilm formation and composition and molecular aspects of Methicillin-Resistant *Staphylococcus aureus.* **FEMS Congress**, 9-13.07.2017, Valencia, Spain

Bucur, F. I., Mihalache, O. A., Neagu, C., Nicolau, A. I. Faith of salmonellae in mayonaisse during storage under improper refrigeration or refrigeration combined with occasional storage at room temperature. **International Symposium EuroAliment-Innovative Minds for Future Food**, 5-6.09.2019, Galaţi, Romania

Projects

08.06.2017 – Present H2020 international project: „Safer food through changed consumer behaviour: Effective tools and products, communication strategies, education and food safety policy reducing health burden from foodborne illnesses” (SafeConsume)

10.09.2019 – Present Burse pentru educaţia antreprenorială în rândul doctoranzilor şi cercetătorilor postdoctorat (Be Antreprenor!)

Awards

Gold medal obtained at the International Student Creation Contest "ECOTROPHELIA", international phase (2018), by coordinating the Whey4Fun team, participant in the contest with the Whoopie Ice product (Paris, France)

First prize for the results obtained at the National Competition for Awareness and Development of the Entrepreneurial Spirit - ENTREPRENEURIAL PORT, held in Galaţi, between 30.10.-1.11.2018.