The Occurrence of Listeria Monocytogenes in the Mushroom Production Chain and the Use of Bacteriophage for its Control

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The occurrence of Listeria monocytogenes in the mushroom production chain and the use of bacteriophage for its control

Vincenzo Pennone
The occurrence of *Listeria monocytogenes* in the mushroom production chain and the use of bacteriophage for its control

A Thesis Presented to Cork Institute of Technology for the Degree of Doctor of Philosophy

By

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²Department of Biological Sciences, Cork Institute of Technology, Co. Cork, Ireland

Submitted to Cork Institute of Technology, March 2019
DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at Cork Institute of Technology or elsewhere.

Signed: [Redacted]

Date: 26.05.2019
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Acknowledgements
ABSTRACT

The objective of this study was to investigate the routes of *L. monocytogenes* contamination in the mushroom industry and propose control actions. A first survey showed that the raw materials contribute to the introduction of *L. monocytogenes* into the mushroom growing units. Characterisation, by polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE), of the isolates obtained from the survey indicated persistent strains, cross contamination between mushroom producers (probably through shared services) similarities with clinical isolates, and the similarity with global clones of *L. monocytogenes* in several Countries. Subsequently, the current hygiene practices in use in the mushroom industry during the production of compost (pasteurisation), casing (physical barriers) and mushrooms (cookout between mushroom crops) were studied. The results showed re-contamination of the compost after pasteurisation, re-contamination in the casing production facility and ineffectiveness of cookout in the inactivation of *L. monocytogenes* on the floors of growing facilities. Implementation of the hygiene practices has been proposed, but also novel biotechnologies have been studied for future applications. The amidase domain of the listeriophage vB_LmoS_293 endolysin (293-amidase) was cloned and purified. The 293-amidase showed lytic activity against *L. monocytogenes* autoclaved cells and biofilm inhibition. The 293-amidase protein was also displayed on polyhydroxyalkanoate (PHA) bionanoparticles (BNPs) and preliminary tests showed antilisterial activity. The application of 293-amidase or BNPs in a pilot scale mushroom growing unit showed no efficacy of pre-harvest treatments in the casing or on the floor against *L. monocytogenes*. 

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PUBLICATIONS


Pennone V., A. Coffey, O. McAuliffe and K. Jordan. Effectiveness of hygiene practices currently in use for the decontamination of *Listeria monocytogenes* from the mushroom production environment. *To be submitted to Post Harvest Biology and Technology*

Pennone V., M. Sanz Gaitero, A. Coffey, K. Jordan, M.J. van Raaij and O. McAuliffe. Reduction of *L. monocytogenes* by the amidase domain of the phage vB_LmoS_293 endolysin: in-vitro studies. *Submitted to Viruses*

Pennone V., A. Coffey, O. McAuliffe and K. Jordan. Pilot scale trials in a mushroom growing facility to assess the efficacy of bacteriophages against *Listeria monocytogenes* during production of *Agaricus bisporus*. *To be submitted to Field Crops Research*
CONFERENCE ABSTRACTS


ORAL COMMUNICATIONS


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>(cg) MLST</td>
<td>Core genome MLST</td>
</tr>
<tr>
<td>(wg) MLST</td>
<td>Whole genome MLST</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>293-amidase</td>
<td>Amidase domain of the phage vB_LmoS_293 endolysin</td>
</tr>
<tr>
<td>A</td>
<td>Amidase treatment</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AFNOR</td>
<td>Association Française de Normalisation</td>
</tr>
<tr>
<td>ALOA</td>
<td>Agar Listeria according to Ottaviani and Agosti</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of official analytical chemists (now AOAC International)</td>
</tr>
<tr>
<td>B</td>
<td>BNPs treatment</td>
</tr>
<tr>
<td>BAM</td>
<td>Bacteriological analytical manual</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BLA</td>
<td>Brilliance Listeria agar</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BLEB</td>
<td>Buffered Listeria enrichment broth</td>
</tr>
<tr>
<td>BNPs</td>
<td>Bionanoparticles</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPR</td>
<td>Biocidal Products Regulation</td>
</tr>
<tr>
<td>BPW</td>
<td>Buffered Peptone Water</td>
</tr>
<tr>
<td>BS EN ISO</td>
<td>British, European and International Standards</td>
</tr>
<tr>
<td>BZT</td>
<td>Benzethonium chloride</td>
</tr>
<tr>
<td>CBD</td>
<td>Cell wall binding domains</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal complex</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for disease control and prevention</td>
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<tr>
<td>CDD</td>
<td>Conserved Domains Database</td>
</tr>
<tr>
<td>CDP</td>
<td>Cool down pinning</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolt</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>LIPI</td>
<td>Listeria pathogenicity island</td>
</tr>
<tr>
<td>LLO</td>
<td>Listeriolysin O</td>
</tr>
<tr>
<td>Lm</td>
<td>Listeria monocytogenes</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Molar / Mole</td>
</tr>
<tr>
<td>m²</td>
<td>Meter square</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-Assisted-Laser-Desorption/Ionization Time-of-Flight</td>
</tr>
<tr>
<td>Mb</td>
<td>Megabase</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi locus sequence typing</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multi locus variable-number tandem repeat analysis</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimolar</td>
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<tr>
<td>MRD</td>
<td>Maximum recovery diluent</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MVLST</td>
<td>Multi-Virulence-loci-Sequence-Typing</td>
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<tr>
<td>NA</td>
<td>Nutrient agar</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NFC</td>
<td>Non-food contact</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nitrilotriacetic acid charged with Ni(^{2+})</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PALCAM</td>
<td>Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aesculin-Mannitol- agar</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCIA</td>
<td>Phenol chloroform-isoamyl alcohol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoate</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>PMF</td>
<td>Peptide mass fingerprinting</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>Poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>QAC</td>
<td>Quaternary ammonium compound</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>RASFF</td>
<td>Rapid Alert System for Food and Feed</td>
</tr>
<tr>
<td>RBP</td>
<td>Receptor binding protein</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RM</td>
<td>Raw materials</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RoI</td>
<td>Republic of Ireland</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
</tr>
<tr>
<td>SAR</td>
<td>Signal-anchor-release</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SP1</td>
<td>Spawn run 1</td>
</tr>
<tr>
<td>SR2</td>
<td>Spawn run 2</td>
</tr>
<tr>
<td>SR3</td>
<td>Spawn run 3</td>
</tr>
<tr>
<td>ss</td>
<td>Stainless steel</td>
</tr>
<tr>
<td>SSI</td>
<td>Stress survival islet</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific Broth</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tris</td>
<td>Trisaminomethane</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>U/mg</td>
<td>Units per milligram</td>
</tr>
<tr>
<td>UC</td>
<td>Uninoculated control</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group method with averages</td>
</tr>
<tr>
<td>US</td>
<td>United states of America</td>
</tr>
<tr>
<td>USDA</td>
<td>US Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VIDAS</td>
<td>Vitek Immunodiagnostic Assay System</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequence(ing)</td>
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<td>WHO</td>
<td>World health organisation</td>
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<td>89</td>
</tr>
</tbody>
</table>
two sampling occasions in 10 mushroom growing facilities, while raw materials (RM) were analysed as in Survey 1.

Figure 2.2 Hygiene practices guidelines suggested to the mushroom growers between sampling occasions 3 and 4 of Survey 1.

Figure 2.3 Overall prevalence of *L. monocytogenes* in the four growing environment sub-categories and on the mushrooms.

Figure 2.4 Incidence of *L. monocytogenes* during Survey 1 per each mushroom growing facility. The numbers indicate the percentage of positive samples for each company at each sampling occasion.

Figure 2.5 Incidence of *L. monocytogenes* during Survey 1 per each raw material producer. The numbers indicate the percentage of positive samples for each company at each sampling occasion.

Figure 2.6 Incidence of *L. monocytogenes* during Survey 2 per each mushroom growing facility. The numbers indicate the percentage of positive samples for each company at each sampling occasion.

Figure 2.7 Incidence of *L. monocytogenes* during Survey 2 per each raw material producer. The numbers indicate the percentage of positive samples for each company at each sampling occasion.
Chapter 3

Figure 3.1 Minimum spanning tree summarising the data from the PFGE profiles of the 279 isolates tested. Within a circle, each segment represents an isolate, while the circle represents a pulsotype where the isolates show > 90% similarity. The length of the line between the circles represents the distance of the relationship between the pulsotypes/isolates. Pulsotypes with only one isolate are represented by a circle with no segments. The distinction between Lineage I and II is shown and the serogroups are represented by different colours: green, 1/2a 3a; red 4b 4d 4e; violet 1/2b 3b 7.

Figure 3.2 Comparison with isolates from different food sectors in Ireland. M, companies of the mushroom production; RoI Dairy/Fish, dairy and fish producers in the Republic of Ireland.

Figure 3.3 Comparisons showing similarities between mushroom isolates in the Republic of Ireland (RoI M) and isolates from Romania (A, Romania 1 and 2), Australia (B), Turkey (C), Austria and Slovakia (D) and Northern Ireland (E, NI).

Figure 3.4 Comparison between isolates from the mushroom industry (M) and clinical isolates. UCHG, University College Hospital Galway; CSF, Cerebrospinal fluid.
Chapter 4

Figure 4.1 Schematic representation of the sampling plan. In the compost production unit, samples were analysed after phase II and phase III. In the casing production unit, samples were analysed before and after the application of new hygiene practices. In the mushroom production facilities, samples were analysed before and after the first cookout and before the start of a new mushroom crop.

Figure 4.2 Post-harvest hygiene procedures followed by the two mushroom producers.

Figure 4.3 Temperatures recorded during the cookout in Company 1 (A and B) and Company 2 (C and D). Each point is the average of three measurements and the Standard Deviation is represented by the error bars.

Figure 4.4 Dendrograms showing the PFGE profiles of isolates obtained from Company 1 (A) and Company 2 (B). The bars on the left show the percentage of similarity.

Figure 4.5 Similarity between the pulsotype obtained in this study from Company 1 and 3 more isolates obtained from Company 2, 3 and 4, in a previous study (Chapter 2 of this thesis).

Figure 4.6 Dendrogram showing 100% similarity between the PFGE profiles of the 2 L. monocytogenes isolates obtained from Company 1 and one isolate obtained
from a compost producer.

Chapter 5

Figure 5.1 BLAST alignment output showing the region on the ORF 25 of vB_LmoS_293 with homology to conserved amidase domain.

Figure 5.2 Local (A) DNA sequence alignment and (B) amino acid sequence alignment of predicted and cloned 293-amidase generated with CLC Main Workbench. Of note is the SNP in panel A (boxed region) showing a change from GCA to GCG in the cloned fragment of amidase-293.

Figure 5.3 Mass spectrum of the 293-amidase digested with trypsin for identification by peptide mass fingerprint. On the horizontal axis are the masses and on the vertical axis is the abundance. Numbers on the peaks indicate the mass of the fragments obtained from the digestion with trypsin. A correspondence with the predicted sequence of the 293-amidase was found with the fragments: 1034.53, 1293.61, 1307.59, 1373.65, 1668.70, 1853.90 and 3301.27.

Figure 5.4 SDS-PAGE analysis of the 293-amidase purification showing the affinity chromatography fractions. The over-expressed protein band has been highlighted throughout the gel with a white rectangle and is clearly visible in the supernatant of the induced and lysed E. coli cells transformed with the pCri8A-293-amidase (S). After binding to the Ni-NTA resin, the band is less visible in the flow-through (F). L, ladder; S, supernatant.
of lysed cells; F, flow-through; W1-W2-W3, washing steps; 145-245-345-545, elution fractions.

Figure 5.5 MIC of the 293-amidase against autoclaved *L. monocytogenes* 473 cells in microtiter plates. The blue lines represent the controls (*L. monocytogenes* 473 cells incubated with phosphate buffer and no 293-amidase), while the red line represents the treatment (*L. monocytogenes* 473 cells incubated with 293-amidase at different concentrations). On top of the charts are reported the p values of T-tests between the averages of control and treatment. The X-axes are times and the Y-axes are OD620. Each data point is the average of triplicates and the standard deviations are indicated as error bars.

Figure 5.6 Influence of pH values (A) and temperature (B) on amidase activity of the 293-amidase (reported as “Amidase” in the legend) against *L. monocytogenes* 473 (reported as “Control” in the legend). In (A), the X-axes are times and the Y-axes are OD_{620}. Each data point is the average of triplicates and the standard deviations are indicated as error bars. C, Control. In (B), the Y-axis represents the OD_{620} and the histograms show the reduction in turbidity, indicated as percentage of reduction in the “amidase” boxes, at the end of the incubation at 25 °C, 37 °C and 50°C.

Figure 5.7 Biofilm prevention (A) and biofilm removal ability (B) of the 293-amidase against *L. monocytogenes* 473 in microtiter plates assays. Values are the average of triplicates and the standard deviation is represented as error bars. P values are shown below the horizontal...
axes. In C, D, and E are shown images obtained from the stainless steel coupons assays. C, \textit{L. monocytogenes} 473 biofilm after 4 days of incubation at 20 °C. D, Co-inoculation of \textit{L. monocytogenes} 473 and 293-amidase. E, the application of 293-amidase on a 4-day old biofilm. Red stained cells are damaged/dead, while green stained cells are live. The scale bars at the bottom-right in C, D, E, are 10 μm size.

\textbf{Chapter 6}

\textbf{Figure 6.1} The application of PhaC-amidase BNPs resulting in turbidity reduction at different PHA BNPs concentrations, during incubation at 37°C. On the horizontal axis are represented time points and on the vertical axis the absorbance at 600 nm (OD$_{600}$). The asterisks indicate turbidity reductions where p<0.05, with 1 mg/ml PHA BNP concentration after 4 h incubation, and with 5 mg/ml after 24 h incubation.

\textbf{Chapter 7}

\textbf{Figure 7.1} A detailed map of the pilot scale mushroom production unit.

\textbf{Figure 7.2} The plots used to grow the mushroom during the crop trial.

\textbf{Figure 7.3} \textit{L. monocytogenes} counts on the floor with 293-amidase application. On the horizontal axis the three experimental conditions are shown, while on the vertical axis are the mean of \textit{L. monocytogenes} counts, expressed as mean Log$_{10}$ CFU/cm$^2$. The error bars are the standard deviation and the colours, as shown in the
legend, are the sampling time-points, in hours. UC, uninoculated control; IC, inoculated control; A, 293-amidase treatment.

Figure 7.4 *L. monocytogenes* counts during the treatments with 293-amidase and PhaC-amidase BNPs. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as log_{10} CFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the sampling occasions. UC, uninoculated control; IC, Inoculated control; A, casing treated with 293-amidase; B, casing treated with PhaC-amidase BNPs.

Figure 7.5 *L. monocytogenes* counts in the casing soil inoculated with the phage vB_LmoS_293. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as log_{10} CFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the sampling occasions. UC, uninoculated control; IC, Inoculated control; L, casing inoculated with the phage vB_LmoS_293; Ex, casing treated with phosphate buffer.

Figure 7.6 *L. monocytogenes* occurrence on the mushrooms harvested during the 3 flushes treated with 293-amidase and PhaC-amidase BNPs. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as Log_{10} CFU/g. The error bars are the standard deviation and different colours, as shown in
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Chapter 1

Literature review

Listeria monocytogenes overview in the mushroom production industry

1.1 Abstract

Listeria monocytogenes is a foodborne pathogen causing listeriosis and it is of major concern in the food industry, especially regarding the Ready-to-eat foods. Its ability to causing outbreaks represents a risk for both public health and economy. Agaricus bisporus is the most commonly commercially cultivated mushroom. In the mushroom industry, no outbreaks of listeriosis have been recorded, but some recalls happened for the presence of L. monocytogenes on the mushrooms. The mushroom production represents a favourable habitat for L. monocytogenes, for that reason particular hygiene practices are commonly undertaken, both as pre-harvest procedures, i.e. pasteurisation of compost, and post-harvest procedures, i.e. cookout of the growing rooms. Novel biotechnologies for L. monocytogenes control in the food industry are in continuous development and bacteriophages (or phages) are providing promising results. The use of recombinant bacteriophage-encoded enzymes in pathogens control, in place of the phage particles, withdraws some potential issues related to the use of bacteriophages, such as lysogeny and bacterial resistance. Endolysins are a class of enzymes encoded by the bacteriophages, involved in the cell lysis by peptidoglycan cleavage. They are particularly effective against Gram positive bacteria, for the direct contact with the cell wall from the extern of the cell and much research is being undertaken to optimise their effectiveness against pathogens, such as L. monocytogenes. In particular, chimeric enzymes, encapsulation and protein display on the surface of bionanoparticles are being investigated as novel bacteriophage-derived biotechnologies to apply in the food industry.
1.2 Introduction

Listeriosis is a foodborne disease caused by *Listeria monocytogenes* that generally causes self-limiting gastrointestinal illness, but can result in more serious illnesses. It has a mortality rate of 24% (Scallan et al., 2011) and among the foodborne diseases, has the highest hospitalisation rate of about 99% (Buchanan et al., 2017). *L. monocytogenes* has the ability to cross three key barriers: the intestinal barrier, the blood-brain barrier, and the fetoplacental barrier, so that it can infect organs such as the brain or uterus, and cause severe life-threatening infections such as meningitis, encephalitis, spontaneous abortion, or miscarriage. Healthy individuals are normally not susceptible to *L. monocytogenes*, but it can have severe implications for those with compromised immune systems, such as the elderly, newborn infants and pregnant women. From 2008–2016, the number of recorded cases in Europe increased significantly, from a total of 1459 reported cases in 2008 to 2527 reported cases in 2016 (EFSA & ECDC, 2016). The EU notification rate increased from 0.32 cases per 100,000 population in 2008 to 0.47 cases per 100,000 population in 2016 (EFSA & ECDC, 2016).

*L. monocytogenes* is ubiquitous in the environment (McLauchlin & Rees, 2009), therefore, contamination of the food processing environment is inevitable unless stringent efforts are put in place to prevent such contamination. *L. monocytogenes* can survive for long periods of time in a seemingly hostile environment such as a food processing facility. It has been demonstrated that *L. monocytogenes* has the ability to endure various stresses, such as sanitizers, pH and temperature (Moorhead & Dykes, 2004; Zhang et al., 2011) and also has the ability to form biofilm (Cruz & Fletcher, 2011; Latorre et al., 2010), which can lead to its
persistence in the food processing environment (FPE). If the FPE is contaminated, cross-contamination of *L. monocytogenes* to the food itself is a major possible route of food contamination (Pérez-Rodríguez et al, 2008). This is a major concern for the food industry, given the public health risks associated with *L. monocytogenes* contamination of food. In particular, it is a problem for producers of ready-to-eat (RTE) food products, since they are usually minimally prepared once in the hands of the consumer. However, a reduction in the occurrence of listeriosis associated with food products is possible. Measures can be taken to prevent food processing environment contamination in the first instance, then to reduce its occurrence in the food processing environment through adequate hygiene measures, and finally, to reduce cross-contamination to food. Furthermore, it has been calculated that preventing growth of *L. monocytogenes* at retail and onwards, mainly through a better control of temperature and shorter storage times, could lead to a 37% reduction in listeriosis (EFSA, 2018)

Fresh mushrooms, such as *Agaricus bisporus*, are considered RTE foods because of their utilisation in raw salads. Over the past few years, the mushroom industry has been attempting to raise awareness of the risk related to *L. monocytogenes* presence in the production environment to reduce the risk of cross-contamination to the final product (Ekman, 2017; Viswanath et al., 2013). The aim of this chapter is to review the occurrence of *L. monocytogenes* in the food processing environment, with a particular focus on the mushroom industry, with a view to understanding such contamination, and exploring options for its reduction.
1.3 **Current regulations on occurrence of* L. monocytogenes* in food**

The current legislation dealing with the occurrence of *L. monocytogenes* in foods in the EU, Regulation (EC) No 2073/2005, sets the criteria that foods must comply with regarding the presence of *L. monocytogenes* (https://eur-lex.europa.eu/eli/reg/2005/2073/oj). It requires absence (10 x 25 g samples) for foods intended for infants and special medical purposes. In addition, Regulation (EC) No 2073/2005 allows presence at varying different levels depending on the ability of the food to support the growth of the bacterium. For ready-to-eat (RTE) foods unable to support the growth of *L. monocytogenes*, such as fresh whole mushrooms (Leong et al, 2015), the numbers should be <100 CFU/g throughout the shelf-life of the product (5 x 25 g samples). For RTE foods able to support growth, *L. monocytogenes* must be absent in 5 x 25 g samples at the time of leaving the production plant or it may not exceed 100 CFU/g throughout its shelf-life (5 x 25 g samples). Fresh sliced mushrooms would fall into this category (Leong et al, 2013). If the ability of a food to support growth has not been determined, it is assumed that growth will occur and the criterion of absence is applied. There are similar regulations in Canada (Anonymous, 2011a) and in Australia/New-Zealand (Anonymous, 2014c).

In the US, stricter regulations are in place. Absence of *L. monocytogenes* in 5 x 25 g of food, and in the processing environment, is required at all times (Anonymous, 2014a). These ‘zero tolerance’ policies pose a serious challenge to food business operators that are exporting to the US and are required to meet US regulations. In general, companies in the export business use the relevant regulation in the country they export to. Further information on regulations in different
jurisdictions can be found in a special issue of Food Control published in 2011 (Anonymous, 2011b).

1.4 Prevalence of *L. monocytogenes* in the food processing environment and on food

1.4.1 Food processing environment (FPE) occurrence

There have been many surveys published on the prevalence of *L. monocytogenes* in food processing environments, as shown in Table 1.1. However, comparison of the survey results from one survey to another is not always possible. The following variables can influence the observed prevalence:

- Different methodologies for sampling are used, for example the swab size and the area that is swabbed, which can vary from 10 cm$^2$ to 1 m$^2$ (Rodriguez-Lopez et al., 2015; Taguchi et al., 2017a).

- Different methodologies for *L. monocytogenes* analysis are used, for example the ISO, BAM or alternative methods (Roccato et al., 2017; Sala et al., 2016).

- Different frequencies of repeated facility testing, for example Sala et al., (2016) surveyed on one occasion, where another facility is surveyed on several occasions (Bolocan et al., 2015) and several facilities are surveyed on several occasions (Beno et al., 2016).

- A facility that has been shown to be positive is targeted.

- Different sample numbers and different sampling locations can influence the results.
Table 1.1. Some surveys on the occurrence of *L. monocytogenes* in food at retail level and in food processing environments, conducted between 2015 and 2017. Due to the different methodologies used, comparison of the results for occurrence is not appropriate.

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Country</th>
<th>N Samples (N Companies)</th>
<th>Samples Type</th>
<th>Survey period</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retail</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EN ISO 11290-1 + API</td>
<td>India</td>
<td>480 (5R markets)</td>
<td>Vegetables</td>
<td>2013 - 2014</td>
<td>(Mritunjay &amp; Kumar, 2017)</td>
</tr>
<tr>
<td>EN ISO 11290-1, EN ISO 11290-2: 1996</td>
<td>Czech Republic</td>
<td>339 (n.a, Retail)</td>
<td>Vegetables</td>
<td>2014</td>
<td>(Vojkovská et al., 2017)</td>
</tr>
<tr>
<td>EN ISO 11290-1, EN ISO 11290-2</td>
<td>UK</td>
<td>862 (n.a, Retail and Catering)</td>
<td>Meat Pies</td>
<td>2013</td>
<td>(Melachlín et al., 2016)</td>
</tr>
<tr>
<td>EN ISO 11290-1, EN ISO 11290-2: 1996</td>
<td>Italy</td>
<td>778 (n.a, Retail from 12 cities)</td>
<td>Smoked Fish</td>
<td>2011</td>
<td>(Acciai et al., 2017)</td>
</tr>
<tr>
<td>Enrichment + PCR</td>
<td>China</td>
<td>200 (n.a, Retail)</td>
<td>Chilled and frozen animal products</td>
<td>n.a</td>
<td>(Tao, Chen, Bie, Lu, &amp; Lu, 2017)</td>
</tr>
<tr>
<td>Test Method</td>
<td>Country</td>
<td>Numbers &amp; Description</td>
<td>Year(s)</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
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<td>---------------------------------------------------------------------------------------</td>
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<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>GB 4789 30 - 2010</td>
<td>China</td>
<td>648 (16 Retail + 4 Dairy farms)</td>
<td>2013-2014</td>
<td>(W. Wang et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>GB 4789 30-2010 + MPN</td>
<td>China</td>
<td>1036 (134 Retail)</td>
<td>2012-2014</td>
<td>(Wu et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>MPN + API + BAX PCR</td>
<td>USA</td>
<td>27389 (n.a., Retail)</td>
<td>2010-2013</td>
<td>(Luchansky et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>One enrichment + RAPIDEC</td>
<td>Singapore</td>
<td>475 (54 food shops, 24 salad bars, 19 supermarkets)</td>
<td>2011-2015</td>
<td>(Chau et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>OXOID Listeria Précis method, OXOID Biochemical Identification System, OXOID Listeria Test Kit and MICROBACT Listeria 12L system</td>
<td>Nigeria</td>
<td>193 (2 fish markets)</td>
<td>1-Year study</td>
<td>(Esther, Isiaku, &amp; Kolawole, 2017)</td>
<td></td>
</tr>
<tr>
<td>VIDAS kit + API</td>
<td>Chile</td>
<td>850 (n.a., Retail)</td>
<td>2008-2009</td>
<td>(Montero et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>Food Processing environment</td>
<td>Spain</td>
<td>270 environmental samples (21)</td>
<td>Sponges moistened with LPT neutralizing broth 200cm²</td>
<td>2010 - 2011</td>
<td>(Rodriguez-López et al., 2015)</td>
</tr>
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<tr>
<td>50 ml BPW, 1ml + 25 ml HF broth, 30 °C 24 h. 100ul from tubes w/black media in 10ml FF, 37 °C 24 h, 100ul on Chromogenic agar 16s, PFGE, Serotyping PCR, RAPD-PCR, Biofilm (SS), Fluorescence microscopy</td>
<td>USA</td>
<td>183 raw milk samples, 4430 environmental samples (9)</td>
<td>3M Sponges</td>
<td>2013 - 2016</td>
<td>(Sarah M Beno et al., 2016)</td>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>226 environmental and raw materials samples (1)</td>
<td>Sponge swabs</td>
<td>2012 - 2013</td>
<td>(Andrei Sorin Bolocan et al., 2015)</td>
</tr>
<tr>
<td>EN ISO 11290-1</td>
<td>Romania</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 retail food samples (55), 87 production swabs and samples (2)</td>
<td>Pickled Veggies, Cotton swabs (10x10cm)</td>
<td>2013- 2015</td>
<td>(Takuschi et al., 2017)</td>
</tr>
<tr>
<td>EN ISO 11290-1, EN ISO 11290-2:2004, Serotyping, Ribotyping, PFGE</td>
<td>Japan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5869 (54)</td>
<td>Food samples,</td>
<td>2013</td>
<td>(L. Long,</td>
</tr>
<tr>
<td>Country</td>
<td>Sample Type</td>
<td>Year</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portugal</td>
<td>20 Food and 60 Swabs (10)</td>
<td>2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Portugal</td>
<td>Sponge swabs, Liquid No swab description in the methods</td>
<td>2015?</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>Sausages, salami and soppressa samplings from the start to the end of production. 197 swabs analysed for Lm presence</td>
<td>2008-2013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>255 environmental samples NFCS (1)</td>
<td>2012-2013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>Cotton swabs + Sponge swabs</td>
<td>2012-2013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>500ml milk, brine, 500g cheese, swabs and cotton swabs</td>
<td>12/2013</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>Plant A: 1248 environmental samples and 16 food samples. Plant B: 736 environmental samples and 16 food samples (2)</td>
<td>07/2014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>Tilapia sashimi, Cotton swabs, 10x10cm</td>
<td>2-year period</td>
<td>2016</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.a. – not available
The source of processing environment contamination may be determined to some extent by Pulsed Field Gel Electrophoresis (PFGE) analysis of isolates from different locations. For example, an isolate from raw materials with a similar PFGE profile, also called “pulsotype”, to a food processing environment (FPE) isolate indicates that the contamination may have been introduced via the raw materials. Rückler et al. (2014) verified that out of the seven pulsotypes detected in the FPE at the beginning of their survey, four were also isolated from the raw materials. Similarly, identification of a similar pulsotype inside a food processing facility and in the area outside the food processing facility can indicate a potential source of contamination (Fox et al., 2011). In both cases, transfer from the environment to the raw material or from the inside to the outside of the FPE cannot be ruled out.

*Listeria monocytogenes* has been repeatedly isolated from the mushroom production environment, although its presence on the mushrooms themselves has always been reported as low, i.e. 1-2% (Anonymous, 2006; Viswanath et al., 2013). Because of the inability of whole mushrooms to support the growth of *L. monocytogenes*, it is likely that its presence on the mushrooms is a result of cross-contamination (Leong et al, 2013).

**1.4.2 Persistence of *L. monocytogenes* in the processing environment**

Persistence of *L. monocytogenes* in the FPE is generally defined as repeated isolation of an indistinguishable pulsotype from the same facility for longer than 6 months (Ferreira et al, 2014; Fox et al., 2011). This indicates that the strain with that pulsotype can persist in the processing environment, despite the hygiene procedures of the facility. It is possible that repeated occurrence of a strain in a processing environment may result from repeated contamination of the processing facility from a source external to the FPE, but even in that situation, it is necessary for the strain
to survive and persist. The basis of persistence is still unclear. Indeed, a genetic marker for persistence has not been identified, although a correlation between stress-response genes and persistence has been proposed. For example, *qacH* and *pocR*, which are Quaternary Ammonium Compounds (QAC) resistance genes, have been found to be correlated to persistence (Martínez-Suárez et al, 2016; Mazza et al, 2015; Morettrø et al., 2017). Cold temperature adaptation and osmotic stress adaptation genes have been investigated and also proposed as possible persistence markers (Cabrita et al, 2015; Hingston et al, 2015). However, these genotypes do not always correlate with the persistence phenotype as persistent strains that do not have these genes have been identified and transient strains that do not persist have been shown to have these genes. An alternative hypothesis is that the harbourage sites and niches where persistent strains survive give them protection from the cleaning procedures, aided by the formation of biofilms (for review, see Carpentier & Cerf, 2011).

The earthy environment of a mushroom growing unit creates a promiscuous habitat for *L. monocytogenes* survival and persistence. In the mushroom industry, examples of *L. monocytogenes* persistence were reported previously (Leong et al, 2014). In particular, *L. monocytogenes* has been isolated repeatedly from floors, revealing an environment where cross-contamination is a constant risk (Viswanath et al, 2013).
1.4.3 Occurrence of L. monocytogenes at retail level

Awareness of the presence of L. monocytogenes at retail level is relevant because it is the last step before the product reaches the consumer. For that reason, it is important to evaluate the prevalence of L. monocytogenes at retail level. Recently conducted risk assessments for L. monocytogenes in deli meats indicated that the majority of listeriosis cases and deaths associated with deli meats are probably due to contamination of products at retail. Endrikat et al, (2010) estimated that 83% of human listeriosis cases and deaths attributable to deli meats are due to retail-sliced products. By performing a risk assessment using product-specific growth kinetic parameters, Pradhan et al, (2010) demonstrated that 63-84% of human listeriosis deaths linked to deli ham and turkey can be attributed to contamination at retail. Studies on the occurrence and cross-contamination at retail level are not as frequent as processing environment studies, but this route is obviously also an important source of listeriosis. Some examples of surveys on the prevalence of L. monocytogenes at retail level are shown in Table 1.1.

1.4.4 The European Food Safety Authority (EFSA) baseline survey

Surveys conducted over a long period or on thousands of samples usually represent a consistent source of data on the prevalence and persistence of L. monocytogenes and can give the Food Business Operators (FBOs) a greater opportunity to improve their hygiene practices, focusing on specific issues, for example staff workflows, sanitising regimes etc. (Bolocan et al., 2015; Leong et al., 2017; Murugesan et al, 2015; Ortiz et al, 2010; Taguchi et al, 2017a).

In 2010-2011, a European Union-wide baseline survey on the prevalence of L. monocytogenes at retail level was undertaken (EFSA, 2013). Similar methodologies
were used to determine the prevalence of *L. monocytogenes* in packaged (not frozen) hot or cold smoked or gravad fish, packaged heat-treated meat products and soft or semi-soft cheeses in 26 member states and one non-member state. A total of 3,053 batches of fish, 3,530 batches of deli-meat and 3,452 batches of cheese were analysed at the end of their shelf-life for detection and enumeration of *L. monocytogenes*. The organism was detected in 10.3% of fish samples while the prevalence on meat and cheese was 2.07 and 0.47%, respectively. The number of samples with a *L. monocytogenes* count exceeding the level of 100 CFU/g at the end of shelf-life was 1.7%, 0.43% and 0.06% for fish, meat and cheese samples, respectively.
1.5 Growth of *L. monocytogenes* on food

If *L. monocytogenes* is present in the food processing environment, it can transfer to the food being produced. Therefore, in situations where cross-contamination can occur, the ability of the food to support the growth of *L. monocytogenes* becomes important. The survival and growth of *L. monocytogenes* in food depends on factors intrinsic to the food, for example pH and water activity, and extrinsic factors such as competing microflora, relative humidity, storage temperature and packing material. Processing techniques used in food production, temperature flux and the physiological state of cells can cause variations of survival and growth. Previously stressed cells exposed to sub lethal conditions can cause *L. monocytogenes* to be more resistant to additional stressors (Lin & Chou, 2004; Lou & Yousef, 1997).

The EU and other jurisdictions have published guidelines for undertaking challenge studies to determine the ability of food to support the growth of *L. monocytogenes*. (Anonymous, 2011a, 2014c; Beaufort et al., 2014). In general, determining the ability of foods to support the growth of *L. monocytogenes* must be assessed for each individual food type as many RTE foods are traditionally produced in local regions using variable formulations which may have an impact on the growth of *L. monocytogenes*. Also, any changes in the ingredients or processing method, either due to the desire to extend the shelf life of a product or to address the consumer’s demand for the reduction of preservatives in their food may lead to new risks (Rivera et al., 2017) and therefore impact on the ability of *L. monocytogenes* to grow. Challenge studies must therefore be undertaken with every newly formulated food.
In cases where growth potential is demonstrated (≥ 0.5 log increase in numbers from day 0 to day end), the initial numbers and the growth rate determine if the numbers will exceed the limit of 100 CFU/g during shelf-life. The EURL *Lm* Technical Guidance document (Beaufort et al., 2014) includes a section on undertaking challenge studies to determine growth rate. The major differences between the challenge studies to determine growth potential and the challenge studies to determine growth rate are that when testing growth rate, each strain must be tested individually, sampling must be undertaken on at least 10 occasions and food storage must be carried out at a uniform temperature. In contrast, when testing growth potential, a pool of three to five different *L. monocytogenes* strains are used as inoculum, the number of samplings depends on the shelf-life of the product tested and the temperatures tested have to reflect the production, retail and storage temperatures (Beaufort et al., 2014).

Prior to undertaking challenge studies in food, predictive modelling can be used to give an indication of the ability of the food to support growth. Software such as “Combase” (Baranyi & Tamplin, 2004), “Food Spoilage and Safety Predictor” (Technical University of Denmark, 2014) and “Pathogen Modelling Programme” (United States Department, Service, & Research Agricultural, 2019) can be used to facilitate such estimations. It is important to remember that predictive modelling is only an estimation of the ability of the food to support growth. If growth is predicted, there could be other characteristics of the food that would inhibit growth, such as competing microflora, that are not accounted for in the models. An example of this was demonstrated by Schwartzman et al (Schwartzman et al, 2011). Using Combase, the authors determined that in 40% of cases where growth in cheese was predicted, no growth actually occurred in the cheese. Therefore, while predictive
modelling is a good starting point, the results are not definitive. Challenge studies following the EURL Lm Technical Guidance document (Beaufort et al., 2014) revealed that whole mushrooms do not support the growth of *L. monocytogenes*, while RTE sliced mushrooms do (Leong et al., 2015a).
1.6 Food recalls and outbreaks due to *L. monocytogenes*

The occurrence of *L. monocytogenes* in food is treated as a very serious issue by regulatory authorities. Such occurrence leads to recalls/withdrawals of food and can lead to outbreaks of listeriosis.

1.6.1 Recalls

The detection of *L. monocytogenes* in food must lead to the protection of public health and the recall/withdrawal of contaminated food from the market. *L. monocytogenes* contamination of a product can be very low and not evenly distributed on the food, and therefore, testing of the food is important. If any contamination is detected, either before or after product release, then a product recall/withdrawal, either voluntary or compulsory, may be instigated. Such recall/withdrawal can have major consequences for food businesses, especially where a product from one company is used as an ingredient by other companies. Economic loss, combined with reputational damage can be detrimental for companies. As an example the “US recalls, Market Withdrawals & Safety Alerts” website has on file 137 alerts involving *L. monocytogenes* between January 2017 and November 2018 involving a large number of foods and food companies (USFDA, 2018). Where products are used as ingredients by other companies, recalls can be very extensive and can result in hundreds of products being recalled (Grabowski, 2016; Johns, 2017; LaFrance, 2017). In the mushroom industry there have been several recalls in the past years for the presence of *L. monocytogenes*, as shown in Table 1.2, but no outbreaks have been linked to these recalls.
Table 1.2. Mushroom recalls in the European Union since 2013. Data adapted from the RASFF\textsuperscript{a} database

<table>
<thead>
<tr>
<th>Date</th>
<th>Country</th>
<th>Food type</th>
<th>RASFF reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/11/2018</td>
<td>Belgium</td>
<td>Chilled enoki mushrooms</td>
<td>2018.3327</td>
</tr>
<tr>
<td>26/12/2017</td>
<td>France</td>
<td>Enoki mushrooms from South Korea</td>
<td>2017.2213</td>
</tr>
<tr>
<td>21/08/2017</td>
<td>United Kingdom</td>
<td>Lily mushrooms from Thailand</td>
<td>2017-1118</td>
</tr>
<tr>
<td>09/02/2017</td>
<td>The Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2017-0012</td>
</tr>
<tr>
<td>12/05/2016</td>
<td>The Netherlands</td>
<td>Golden enoki mushrooms from South Korea</td>
<td>2015-1421</td>
</tr>
<tr>
<td>23/11/2015</td>
<td>The Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2015-1291</td>
</tr>
<tr>
<td>04/11/2014</td>
<td>The Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2014-1194</td>
</tr>
<tr>
<td>12/06/2014</td>
<td>Greece</td>
<td>Chilled enoki mushrooms from South Korea, via the Netherlands</td>
<td>2014-0326</td>
</tr>
<tr>
<td>24/02/2014</td>
<td>Ireland</td>
<td>Mushrooms from Ireland</td>
<td>2014-0255</td>
</tr>
<tr>
<td>28/01/2014</td>
<td>The Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2014-0037</td>
</tr>
<tr>
<td>10/12/2013</td>
<td>Belgium</td>
<td>Enoki mushrooms from China</td>
<td>2013-1318</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The Rapid Alert System for Food and Feed. https://webgate.ec.europa.eu/rasff-window/portal/
1.6.2 Outbreaks

Despite the efforts of the food industry to prevent and control \textit{L. monocytogenes} both in Europe and USA, listeriosis can still be transmitted through contaminated food, with a current incidence rate of approximately 0.2-0.5 cases/100,000 population (CDC, 2017; European Food Safety Authority, 2017).

In Table 1.3, details of the outbreaks which occurred in the EU from 2013-2015 (European Food Safety Authority, 2017) and in the U.S.A. from 2013-2016 are presented (CDC, 2017). It is however important to keep in mind that the majority of the \textit{L. monocytogenes} cases are sporadic and many are not reported (European Food Safety Authority, 2018).

More recently, the biggest \textit{L. monocytogenes} outbreak in history has been registered in South Africa, causing about 1060 reported cases of listeriosis and 216 deaths (National Department of Health Republic of South Africa, 2017; Spies, 2018; World Health Organization, 2018). The source of contamination was identified in a single manufacturing plant located in Polokwane, Limpopo Province, SA, producing RTE meat products for different companies. \textit{L. monocytogenes} ST-6, identified as the major ST in the outbreak, was isolated from the working environment and food samples. On the 3rd September 2018, the outbreak was officially considered at an end and the World Health Organisation recommended the South African government to focus the attention on the maintenance and improvement of good practices in the production and retail sector, but also the consumers to improve good hygiene practices (World Health Organization, 2018).

Another outbreak of \textit{L. monocytogenes} in 2015 was traced to a company producing frozen vegetables in Hungary (Greenyard Frozen). The export of these products globally involved more than 100 Countries in the outbreak, with 47
hospitalisations and nine deaths (European Food Safety Authority, 2018; Gardiner, 2018; Whitworth, 2018). As a matter of precaution, the same company recalled all the frozen vegetable products manufactured in the Hungarian facility during the period 2016-2018 (Food.gov.uk, 2018). Investigations revealed that the outbreak might be the result of cross-contamination from the working environment to the food products and the production was stopped until the investigation was considered completed (European Food Safety Authority, 2018). Recall of the products from the market and the cessation of production at the Hungarian plant resulted in control of the outbreak.
Table 1.3 - Reported *Listeria monocytogenes* outbreaks in the EU (2013-2015) (European Food Safety Authority, 2017) and in the U.S.A. (2013-2016) (CDC, 2017)

<table>
<thead>
<tr>
<th>Year</th>
<th>Food Vehicle</th>
<th>Reporting Country</th>
<th>Cases</th>
<th>Hospitalised</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Not Available</td>
<td>Austria*</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>Cheese</td>
<td>Belgium</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>Pig meat and products thereof</td>
<td>Belgium</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>Crustaceans, shellfish, molluscs and products thereof</td>
<td>France</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>Vegetables and juices and other products thereof</td>
<td>Germany</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2013</td>
<td>n.a.</td>
<td>Netherlands</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2013</td>
<td>n.a.</td>
<td>Sweden</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
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<td>7</td>
<td>2</td>
</tr>
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<td>25</td>
<td>3</td>
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<td>Mexican style cheese, pasteurized</td>
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<td>9</td>
<td>8</td>
<td>1</td>
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</table>
| Year | Category                                      | Country                      | Count | Count
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<td>0</td>
</tr>
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<td>---------------------</td>
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<td>Product</td>
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<td>Count</td>
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<td>------</td>
<td>----------------------------------------------</td>
<td>-------------------------------</td>
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<tr>
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</tr>
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<td>2016</td>
<td>Salads</td>
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<td>19</td>
<td></td>
</tr>
<tr>
<td>2016</td>
<td>Artisanal soft cheese, unpasteurized</td>
<td>US - Multistate</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

*2 outbreaks, #3 outbreaks, n.a. – not available
1.7 Methodologies of sampling for *L. monocytogenes*

As *L. monocytogenes* is ubiquitous in the environment, there is a risk of contamination of food processing environments. Maintaining the processing environment free from *L. monocytogenes* is important especially in high-risk areas where there is the risk of cross-contamination to the food. In order to monitor the presence of *L. monocytogenes*, applying appropriate Environmental Monitoring Programmes is necessary (Beno et al., 2016). The responsibility for such monitoring lies with the food business owner (FBO). Sampling programmes should target areas where contamination would occur, for example, drains, floors, wet areas, hard to clean niches in the equipment, doors, windows, and air handling systems. Additionally, samples should not be taken directly after cleaning/disinfection as sampling in this manner would severely decrease the chances of recovering *L. monocytogenes*. In order to obtain the most accurate representation of the contamination present, sampling plans must attempt to obtain as many positive samples as possible so that positive areas can be targeted for more rigorous hygiene measures. The European Union Reference Laboratory on *L. monocytogenes* has published a guidance document on sampling that can help with controlling *L. monocytogenes* (Pueyo et al, 2012). A history of testing and sample results should also be established to verify that cleaning methods are sufficient, where positive results should be acted on immediately by cleaning to eradicate contamination, and then confirmed free from *L. monocytogenes* by retesting.

End-product testing is a useful part of controlling *L. monocytogenes*. However, it is not sufficient on its own to advise control procedures against *L. monocytogenes* as low numbers are difficult to detect in large batches where not all units of the batch
will be tested. Process control testing should be undertaken in association with end-product testing.

All samples should be taken and tested immediately or stored at 4 °C and then tested with 24 hours of collection.
1.8 Methodologies for sample analysis of *L. monocytogenes*

Conventional and rapid methods are available for the detection and enumeration of *L. monocytogenes*. The International Standardisation Organisation (ISO) standard method for detection is ISO 11290-1:2017, and for enumeration is ISO 11290-2:2017. The detection method is a two-step enrichment process, using Fraser broth supplemented with ferric ammonium citrate, which augments the growth of *L. monocytogenes*, lithium chloride that inhibits the growth of enterococci, nalidixic acid and acriflavine hydrochloride for inhibiting the competitive microflora (Fraser & Sperber, 1988). The detection of *L. monocytogenes* starts with a pre-enrichment step in half Fraser broth (containing 10 mg/l of nalidixic acid and 12.5 mg/l of acriflavine hydrochloride), incubated at 30 °C for 24 h. Following, 100 μl of the pre-enrichment broth are transferred to 10 ml of full Fraser broth (containing 20 mg/l of nalidixic acid and 25 mg/l of acriflavine hydrochloride) and incubated at 37 °C for 24 h. Each enumeration and detection step is plated on a selective media, such as Agar Listeria according to Ottaviani and Agosti (ALOA), PALCAM, or any other equivalent selective media, for 48 h at 37 °C. A total of four days is required to complete the analysis. The selective media for identification of *L. monocytogenes* are based on chromogenic and selective activity. For ALOA, the chromogenic activity is based on the activities of the β-D-glucosidase and phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes. The β-D-glucosidase activity is detected using the chromogenic substrate X-glucoside, the hydrolysis of which induces the formation of a blue-green colour. Phosphatidylinositol is a substrate used by pathogenic *Listeria* species (*monocytogenes* and *ivanovii*) which, when degraded, produces an opaque halo around the colonies. The selective component of ALOA is
provided by a mixture of lithium chloride, antibiotic and anti-fungal agents (nalidixic acid, ceftazidime, polymyxin B sulphate and cycloheximide). For PALCAM medium, the selective activity is afforded by lithium chloride, ceftazidime, polymyxin B and acriflavine hydrochloride. Similar to ALOA, it has a double indicator system for *L. monocytogenes*: aesculin, hydrolysed by *L. monocytogenes*, results in the formation of a black halo around colonies, indicating the presence of ferrous iron. In addition, mannitol, which is not fermented by *L. monocytogenes*, allows easy differentiation from mannitol-fermenting contaminants by producing a change from red to yellow for the pH indicator phenol red. The enumeration method, ISO 11290-2:2017, consists of resuspension of the sample in a diluent such as Buffered Peptone Water (BPW) or Fraser broth if enumeration and detection method are performed simultaneously, and plating of 100 μl or 1ml on ALOA or other solid media selective for *L. monocytogenes*. The detection limit of this method is 10 cfu/g, but a recent study demonstrated the possibility of reducing this limit to 1 cfu/g by using the pouring method for 10ml of suspension in a 140mm Petri dish plate (Hunt et al, 2017).

The Bacteriological Analytical Manual (BAM) detection method uses a buffered Listeria enrichment broth (BLEB) incubated for 24 h and 48 h enrichment prior to plating on chromogenic selective agars (Hitchins et al, 2017).

Alternative methods exist for rapid testing and screening, according to validated methods (ISO 16140-1:2016, ISO 16140-2:2016), most notability AOAC International and/or Association Francaise de Normalisation (AFNOR: French Standardization Association) validated methods, where the AOAC database alone contains almost 50 alternative protocols for detection and enumeration of *L. monocytogenes*. These methods are based on different technologies, including
molecular and immunological techniques. Molecular methods include DNA hybridisation and Real Time PCR. These methods may result in a more rapid detection of *L. monocytogenes*, although disadvantages include a direct detection limit of about 100 CFU/ml (depending on the test used) and the lack of a bacterial isolate for further characterisation. By using molecular testing in combination with agar plates (Larsen et al., 2014), isolates can be obtained for further characterisation. One advantage of molecular methods is that they provide confirmation of *L. monocytogenes* as they target virulence genes such as *actA* or *hly* (Poimenidou et al., 2018). Immunological methods are based on the antibody-antigen reaction, where antibodies specific for *L. monocytogenes* are used, for example in flow-cytometry assays (Donnelly & Baigent, 1986; Ikeda et al., 2009; Wilkinson et al., 2017). Enzyme-linked immunosorbent assay (ELISA) incorporate fluorescent or colorimetric detection, or a newer method combining immunoassay techniques to real time immunoquantitative PCR (iqPCR). A validated ISO 16140 and AOAC International method based on Enzyme-linked Fluorescent Assay (ELFA) is the Vitek Immunodiagnostic Assay System (VIDAS), which uses recombinant phage-related proteins for rapid detection of *L. monocytogenes* in food matrices. Many phage-related *L. monocytogenes* detection methods have been developed in the past years (Bai et al., 2016; Hagens & Loessner, 2014). For example, in 1997, Loessner et al, inserted a luciferase gene into the capsid of the listeriophage A511, developing a novel detection method that, in pure cultures, had a detection limit of as low as 500-1000 cells per ml (Loessner et al., 1997). Similarly, the same phage A511 was modified by the same group with the celB gene coding for β-glycosidase and a 96-well microplate assay was developed for detection of *L. monocytogenes* in 6 h with a detection limit of $10^3$ cfu/ml (Hagens et al., 2011). Other bacteriophage-based
strategies developed for detection of *L. monocytogenes* include the use of the bacteriophage endolysin cell wall binding domains (CBD), tagged with fluorescent markers (Schmelcher et al., 2010) or coated to paramagnetic beads (Kretzer et al., 2007).
1.9  Characterisation of *L. monocytogenes* isolates

1.9.1 Serotyping/serogrouping

An agglutination method, developed in 1979 by Seeliger and Hohne (Seeliger & Hohne, 1979), allows the sub-species differentiation of *L. monocytogenes* by serotype, based on the reactions of somatic (O) and flagellar (H) antigens with specific antisera. The method allows the identification of a total of 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7), as described by Farber and Peterkin in 1991 (Farber & Peterkin, 1991). In 1990, Garcia demonstrated that the agglutination tests cannot discriminate between 4b and 4e (Garcia et al., 1990).

In 2004, a multiplex PCR-based method was developed (Doumith et al., 2004) and it introduced the concept of the serogroup: a new characterisation method that allowed the grouping of all the known serotypes of *L. monocytogenes* into five phylogenetic groups: 1/2a-3a, 1/2b-3b-7, 1/2c-3c, 4b-4d-4e and 4a-4c. In this way, an isolate of *L. monocytogenes* can be associated with one of the four serotypes commonly associated to outbreaks and disease (1/2a, 1/2b, 1/2c and 4b). This PCR-based assay is a multiplex of five genes: *lmo0737, lmo1118, ORF2819, ORF2110* and *prs*. Currently, a combination of serotyping and serogrouping is used (Burall et al., 2011). In 2013, Vitullo et al., (Vitullo et al., 2013) developed a real-time PCR assay to allow serogrouping of *L. monocytogenes* and differentiate these from other *Listeria* species. It combines the results of two triplex PCRs, targeting *ORF2110, ORF2819*, and *lmo1118*, and then targeting *lmo0737, plc A* and *prs*.

1.9.2 Other sub-typing methods
Beyond the species level, subtyping of *L. monocytogenes* strains is a useful tool to track routes of contamination throughout the processing environment, and to give some indication of the source of such contamination. Typeability, discrimination power, reproducibility, speed, cost and lab capacities are the main factors in deciding the appropriate method to use. DNA fingerprinting-based methods include Pulsed Field Gel Electrophoresis (PFGE), which is the current gold-standard method for assessing the interrelatedness of *L. monocytogenes* strains (Goering, 2010) and for monitoring routes of contamination in food processing environments (Leong et al., 2017). PFGE results in restriction enzyme digestion of the DNA of the tested strain depending on the genome structure and presence of restriction enzyme sites which differ from one strain to the other depending on the genetic relatedness of the strains. Pulsotypes, indicating closely related strains, can be identified and compared using bioinformatics software, such as BioNumerics (Applied Maths, Belgium). The comparison between pulsotypes is shown in a dendrogram, which gives an indication of how related or different the pulsotypes are. The PulseNet International network (PulseNet International, 2016) uses standardised PFGE protocols for the study of *L. monocytogenes* (and other pathogenic bacteria), although this is now moving towards whole genome sequencing (WGS).

Other molecular typing methods include multi-locus sequence typing (MLST), multi locus variable-number tandem repeat analysis (MLVA), Restriction Fragment Length Polymorphism (RFLP), amplified fragment length polymorphism (AFLP) (Lomonaco et al., 2011) and randomly amplified polymorphic DNA (RAPD) (Chambel et al., 2007). A database for MLST contains the reference allele sequences and sequence types for different organisms and for isolate epidemiological data.
Interrogation and analysis software which facilitates a query of the allele sequences and sequence types in the databases are available (Achtman et al., 2016; Institute Pasteur MSLT, 2019; Jolley et al., 2018).

1.9.3 Whole genome sequencing of L. monocytogenes isolates

Whole genome sequencing is becoming increasingly popular as an analysis tool for bacterial strains. In recent years, the cost of WGS has decreased dramatically, and as a result, the number of whole genome sequences for strains of *L. monocytogenes* has increased from 64 in the years 2001-2013 to 1522 in 2018 (Figure 1.1). In 2013, through the Genome TRAKR Network, the Centre for Disease Control and Prevention in the United States of America (CDC) and the Food and Drug Administration (FDA), commenced a study on the use of WGS as a tool for tracking *L. monocytogenes* isolates from food processing environment analysis and during disease outbreaks (FDA, 2016). Similar studies have been undertaken in other countries leading to the availability of thousands of whole genome sequences of *L. monocytogenes* strains. Such data has been used, for example, in the Quargel cheese outbreak in Austria in 2009/2010, where WGS facilitated the identification of two distinct 1/2a *L. monocytogenes* strains (QOC1 and QOC2) which together were responsible for the outbreak (Rychli et al., 2014). The WGS data can also be used for subtyping (Institute Pasteur MSLT, 2019), outbreak detection (FDA, 2016), persistence (Stasiewicz et al., 2015), antimicrobial resistance (AMR) predictions (Köser et al., 2014), etc. Databases on core genome (cg) MLST and whole genome (wg) MLST are available for strain comparisons (Moura et al., 2016, 2017) and analysis of single nucleotide polymorphisms (SNPs) can give information on strain relatedness (Fox et al., 2017; Moura et al., 2016). Such information will provide a large database of well-characterised environmental (food, water, processing facility,
clinical etc.) isolates that will facilitate a better understanding of *L. monocytogenes* characteristics.

Figure 1.1. *L. monocytogenes* genomes published each year on NCBI. Source: GeneBank, accessed 03/01/2019.

However, WGS will only allow accurate and reliable identification of outbreaks and outbreak sources in conjunction with solid epidemiological data on food consumption. Similar strains can be obtained from apparently unrelated locations (Fox et al., 2017). Therefore, epidemiological data collection and analysis is still critical in order to take full advantage of WGS-based subtyping data for foodborne pathogens.

For example, in Denmark in 2017, an outbreak of listeriosis was connected to smoked salmon (Schjørring et al., 2017). The use of epidemiological data and WGS analysis allowed the authorities to link the cgMLST of the Danish outbreak to other listeriosis cases and isolates from salmon samples reported in France. At this point, an international notification was sent on various platforms (European Epidemic
Intelligence Information System for food and waterborne diseases (EPIS-FWD) reference UI-426, Early Warning and Response System (EWRS) reference EWRS20170831DK0001 and RASFF notification RASFF-2017.1319), genetic profiles were compared and a cured salmon product, manufactured in Poland and distributed in France, was matching the cgMLST of the Danish outbreak. After recall of the product, no further cases were registered in France by the end of 2017 (Schjørring et al., 2017).

Several next-generation sequencing platforms are currently available, including MiSeq by Illumina and PacBio by Pacific BioSciences, which has recently been acquired by Illumina. These platforms are becoming very popular, for the low price and good quality of reads given by the MiSeq technology and the long reads obtained by a smaller amount of DNA with PacBio. Based on the outputs needed, the most appropriate technology has to be chosen. First of all, with MiSeq it is possible to obtain 25-50 million reads with lengths of up to 300 base pairs (bp), while with PacBio, the output can be up to 800k reads longer than 10k bp. Furthermore, the error rate declared by MiSeq is about 0.1%, while PacBio is 11-15%. Consequently, with PacBio, it is possible to obtain a closed bacterial chromosome, but the cost is higher and the error rates are higher than MiSeq. More detailed information can be found in (Besser et al., 2018; Vincent et al., 2017).
1.10 Control of *L. monocytogenes* in the food processing environment

1.10.1 Traditional methods for control of *L. monocytogenes* in the food processing environment

Creating and maintaining a completely *L. monocytogenes*-free processing environment is important in ensuring the production of safe food, however, this can be impossible to achieve. The occurrence of *L. monocytogenes* in the processing facility can result from a number of different sources, for example, contaminated incoming raw materials, *L. monocytogenes* carriage by FPE personnel, insufficient cleaning strategies and sampling programmes in place, the facility design and location. Each of these factors must be controlled in order to reduce the occurrence of *L. monocytogenes*, to interrupt the route of transfer to the food. Although expensive, sub-typing of isolates, using methods such as Pulsed Field Gel Electrophoresis (PFGE), can facilitate the identification of putative routes of contamination and persistence (Chen et al., 2016).

Awareness of *L. monocytogenes* (facilitated by detailed documentation) that can be obtained from having an appropriate processing environment sampling plan, sampling appropriate places at the right frequency, is an important step in controlling the organism. There is a need for standardisation of sampling, which should be done with a sponge-type swab, allowing sufficient surface area to be sampled. If occurrence of *L. monocytogenes* is detected, it can be eliminated through targeted intervention strategies that help to prevent product contamination. Adequate sampling will allow problems of contamination to be pre-empted and addressed in a timely manner (Dalmasso & Jordan, 2013).
A number of products are actually used during the cleaning and disinfection processes in the food industry to remove or prevent the presence of *L. monocytogenes* and other pathogens. It is fundamental that the steps of cleaning and disinfection are performed properly, to maximise their effectiveness. Four factors have to be considered when deciding the best practice to use: the type and concentration of product, time of application, temperature and physical actions. Detergents are used for cleaning and they have to be chosen based on the type of surface to clean and also the type of dirt that they need to remove, for example, greasy dirt will need a different detergent than salty dirt, and so, it depends on the type of food produced in the FPE. Some more information for small businesses can be found on the SafeFood website (https://www.safefood.eu/Professional/Food-Science/Resources/Cleaning-and-Hygiene-for-Small-Food-Businesses.aspx).

Biocides are used for disinfection and they are needed to reduce and kill pathogens from the FPE. In the European Union, they are regulated under the Biocidal Products Regulation (BPR) nr 528/2012 (available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32012R0528). A biocide is defined as a chemical or organism capable of killing potentially harmful organisms and biocides are divided into 22 types, grouped into four main groups as shown in Table 1.4.

In the food industry, including mushroom production, sodium hypochlorite and quaternary ammonium compounds are biocides generally used for disinfection, especially of food-contact surfaces. *L. monocytogenes* can be resistant to sanitisers and detergents and can form biofilm, making it difficult to inactivate (Lourenco et al., 2011). This can be compounded by its survival in niches and harborage sites that can be difficult to clean. Therefore, attention to detail in cleaning and disinfection,
hygienic design of equipment and the establishment of critical control areas close to any food contact surface will all contribute to the control of *L. monocytogenes*. Maintaining good hygiene practices during installation of new equipment or during construction at a processing facility can be challenging (Leong et al., 2017).
Table 1.4. Classification of biocides according to the EU 528/2012. Adapted from https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32012R0528, Annex V.

<table>
<thead>
<tr>
<th>Group</th>
<th>Product Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Disinfectant</td>
<td>• PT 1 Human hygiene&lt;br&gt;• PT 2 Disinfectants and algaecides not intended for direct application to humans or animals&lt;br&gt;• PT 3 Veterinary hygiene&lt;br&gt;• PT 4 Food and feed area&lt;br&gt;• PT 5 Drinking water</td>
</tr>
<tr>
<td>Group 2 Preservatives</td>
<td>• PT 6 Preservatives for products during storage&lt;br&gt;• PT 7 Film preservatives&lt;br&gt;• PT 8 Wood preservatives&lt;br&gt;• PT 9 Fibre, leather, rubber and polymerised materials preservatives&lt;br&gt;• PT 10 Construction material preservatives&lt;br&gt;• PT 11 Preservatives for liquid-cooling and processing systems&lt;br&gt;• PT 12 Slimicides&lt;br&gt;• PT 13 Working or cutting fluid preservatives</td>
</tr>
<tr>
<td>Group 3 Pest control</td>
<td>• PT 14 Rodenticides&lt;br&gt;• PT 15 Avicides&lt;br&gt;• PT 16 Molluscicides, vermicides and products to control other invertebrates&lt;br&gt;• PT 17 Piscicides&lt;br&gt;• PT 18 Insecticides, acaricides and products to control other arthropods&lt;br&gt;• PT 19 Repellents and attractants&lt;br&gt;• PT 20 Control of other vertebrates</td>
</tr>
<tr>
<td>Group 4 Other Products</td>
<td>• PT 21 Antifouling products&lt;br&gt;• PT 22 Embalming and taxidermist fluids</td>
</tr>
</tbody>
</table>
1.11 Outline of mushroom industry

Since ancient times, wild mushrooms have been consumed for their nutritional or therapeutic properties, sometimes for religious purposes, especially when psychoactive species were used (Matsushima et al., 2009). Large-scale mushroom production started in France in 1600s, with *Agaricus bisporus*, followed by *Pleurotus* spp. in USA in 1900s (Chang, 2008). *Agaricus bisporus* still retains the largest part of the market nowadays, its global production increased from 495,127 metric tons in 1961 to 10,378,163 metric tons in 2016, with the biggest global production in China, followed by USA and Netherlands (FAO, 2016).

The production of *A. bisporus* is based on the inoculation of pasteurised compost, sometimes called “substrate”, with the mushroom spawn. The formation of fruiting bodies, which represent the reproductive process of the mushroom, is stimulated by the application of casing material on top of the compost, as shown schematically in Figure 1.2 (McGee et al., 2018).
Stages of colonisation of the mushroom growing medium by *Agaricus bisporus*

1. **Phase 2 Semi-pasteurised compost**

2. **Phase 3 compost colonised with *A. bisporus* mycelium**

3. **Start of mushroom cropping process**

4. **Colonisation of the casing layer**

5. **Development of primordia**

6. **First flush of mushrooms**

**Figure 1.2.** Representation of the stages of colonisation of *Agaricus bisporus* during the mushroom production (McGee, 2018)

### 1.1.1 Compost production

In the mushroom industry, composting is a process that requires three phases before the mushroom production can start. Briefly, in phase I and phase II the raw materials are mixed, incubated and pasteurised, while in phase III the mushroom spawn is added.

**Phase I:** The process starts with the storage of raw materials, possibly indoor for good hygiene practices. Straw bales are usually soaked with what is commonly called “goody water”, which consists of recycled water coming from the whole composting process, containing microorganisms useful for the digestion of the materials (Safianowicz et al., 2018). After the soaking process, the straw is mixed with chicken and horse manure, blended and transferred to a room equipped with an aeration system. During the incubation, the temperature reaches about 80 °C due to the microbial activity (X. Zhang et al., 2014). The mix is incubated for 10-13 days...
and materials are moved and mixed frequently to avoid anaerobic decomposition that would produce unwanted toxic compounds and bad smell due to release of sulphur gases. At the end of Phase I, the mix is inoculated with 1% materials that went through Phase II, containing microorganisms, especially actinomycetes, which are deactivated during Phase I by the high temperature reached. The mix is then transferred to another room where the Phase II process will take place.

**Phase II:** In the pasteurisation room, the temperature is raised to 60 °C for one or two days. This process aims to deactivate unwanted organisms, such as insects and pathogens (Straatsma et al., 2000). Following pasteurisation, a 3-day conditioning process starts by lowering the temperature to 45 °C and circulating air through the compost. This allows the growth of thermophilic fungi, like *Scytalidium thermophilum* (Straatsma and Samson 1993), which is involved in the growth of *A. bisporus* (Vos, 2017; Straatsma et al. 1989; Straatsma et al. 1994) by removing ammonia and suppressing *A. bisporus* competitors (Ross and Harris 1983). At this stage, the pH decreases to 7.5 and the compost is ready to be colonised by *A. bisporus*.

**Phase III:** The pasteurised compost is transferred to an aseptic room where it is inoculated with mushroom mycelium and incubated at 25 °C aerobically for 16-19 days (Gerrits 1988). After that period, the compost is fully colonised by *A. bisporus* and the pH reaches 6.5-7 (Vos, 2017). Temperature, oxygen concentration, ammonia, pH and other parameters are constantly monitored during the whole process in order to put in place corrective actions in the case that some parameters deviate the standard range. At this stage, the compost is transported to the growers to start the mushroom production.

1.11.2 *Casing Production*
Casing soil is a mixture of peat and lime which is added on the top of a Phase III compost to stimulate the formation of fruiting bodies by *A. bisporus*. The mechanism behind the induction of the reproductive phase in *A. bisporus* by the casing soil is still fairly unknown, although a number of factors have been demonstrated to influence the mushroom’s development, such as the presence of Pseudomonads and other bacteria able to destroy volatile compounds, produced by *A. bisporus*, that inactivate the fruiting bodies formation (Cai et al., 2009; Miller et al., 1995; Siyoum et al., 2016). The production of casing soil starts with the acquisition and storage of raw materials, usually peat, which maintain high moisture and poor nutrients availability, and lime, which neutralises the pH of the casing.

1.11.3 Phase IV

Once the casing is prepared, it is topped on Phase III compost in trays, bags or shelves and the system is incubated in the mushroom growing tunnel at 22-25 °C, this process is called Phase IV (Vos, 2017). After seven days at 85% humidity and high CO₂ levels, the temperature is lowered to 18-22 °C, the relative humidity is raised to 90% and the CO₂ levels are lowered by venting. This induces the mushroom’s fruit body formation (Visscher 1988). At this point, the harvest (or “picking”) of the mushrooms of the right size can start and it can be done mechanically or manually. Most commonly, mushrooms are harvested manually during a time frame called “flush”. A flush generally lasts one week and the picking is done until all the available mushrooms are harvested. In general, three flushes of mushrooms are produced for each production process, with the 50-70% of yield obtained after the first flush. If the harvest is done mechanically, all the mushrooms of a flush are recovered simultaneously.
1.12 *Listeria monocytogenes* in the mushroom production in Ireland

In Ireland, mushroom production is the largest horticultural sector, with a production of 64000 tonnes in 74 production units, for a value of €112 million in 2013, of which about 75% was exported to the UK (Teagasc Mushroom Stakeholder Consultative Group, 2013). For that reason, the mushroom industry has been particularly affected by the Brexit announcement following the UK referendum in 2016 (Reuters News Agency, 2016), although, many efforts are made by the entrepreneurs to keep their business safe (Allen, 2018).

To date, no reports of listeriosis linked to mushroom consumption have been recorded, although some surveys have shown the possible contamination on mushrooms and mushroom production facilities (Venturini et al., 2011; Viswanath et al., 2013). Recalls have been registered around the world (Table 1.2), including in Ireland in 2014, for the presence of *L. monocytogenes* on mushrooms (EU, 2019). In a mushroom production unit, there are several opportunities to create the perfect environment for *L. monocytogenes* contamination, and with it, possibly, other pathogens. The first possible scenario can result from inadequate pasteurisation, which may not completely remove the pathogens from the phase II compost. Inadequate storage, manipulation and/or transport of the compost and casing in lorries can lead to cross-contamination with potential contamination of the mushrooms (Leong et al., 2014; Leong et al., 2017; Viswanath et al., 2013).
1.13 Novel methods for control of *L. monocytogenes* in food

While methodologies for the control of *L. monocytogenes* in food are necessary, avoiding food contamination by having a *L. monocytogenes*-free processing environment can reduce the risk of food contamination.

New and exciting methodologies include research on the use of lytic bacteriophages and bacteriocins as an alternative, or in addition to, commonly used *L. monocytogenes* control practices (McAuliffe & Jordan, 2012; Strydom & Witthuhn, 2015).

*Use of live phages as biocontrol agents:* The advantage of using bacteriophages as biocontrol agents against *L. monocytogenes* is due to the nature of certain characteristics of the bacteriophages themselves. First of all, bacteriophages are highly specific in targeting their host, without affecting the remaining microflora; they are self-replicating and self-limiting, meaning that the bacteriophage will increase its numbers based on the host availability; they display very low toxicity, being made only of a few proteins and nucleic acids; live bacteriophage preparation are not expensive to produce; they are able to resist common food processing environmental stresses and have a long shelf life (Sillankorva et al., 2012). Bacteriophages are very commonly isolated in foods, suggesting that they are present in the same environment as their bacterial host and they are consumed daily by humans without known side effects (O’Sullivan et al., 2019). On the other hand, bacteria can develop resistance to bacteriophages due to a number of mechanisms that can involve the modification of membrane receptors in presence of the bacteriophage, generating bacteriophage-insensitive mutants, or for genome or plasmid encoded mechanisms, such as acquisition of restriction systems.
and CRISPR/Cas systems (Labrie et al., 2010). To prevent resistance, a cocktail of bacteriophages with different host ranges, including also mutants, is a procedure commonly adopted (Bai et al., 2016; Endersen et al., 2017; Sadekuzzaman et al., 2017). Another potential hurdle to the use of live phages is potential horizontal gene transfer from strain to strain via specific phage types, which could result in lysogenic conversion of the host (Howard-Varona et al., 2017). For that reason, only lytic bacteriophages are suitable as live control agents and the bacteriophage genome must be fully sequenced to prove the absence of lysogenic characteristics and any antibiotic resistance or bacterial virulence genes (Hagens & Loessner, 2010).

From a regulatory perspective, bacteriophages are not specifically regulated and this makes it difficult to obtain authorisation for the use of commercial products in the food industry. Such products need to be approved by the authorities in each country, such as the Food and Drug Administration (FDA) in the United States of America (USA) or the European Food Safety Authority (EFSA) in Europe, and their application can be as a food-processing aid, food additives or surface disinfectants. To date, a number of phage-based products have been approved as food-processing aids in the USA, Canada, Israel, Australia, New Zealand, Switzerland, Norway and the Netherlands. The only antilisterial phage-based products that have been approved in the food production industry are LISTEX™ P100, now called PhageGuard Listex (EBI Food Safety, Wageningen, The Netherlands), and ListShield™, a cocktail of bacteriophages developed and produced by Intralytix Inc. (Baltimore, MD, USA) against L. monocytogenes. PhageGuard Listex was approved by the FDA and the US Department of Agriculture (USDA) in 2007 as a food-processing aid, while the EFSA still retains some concerns about its efficacy in the long term (Anonymous, 2006, 2014b; Gironés, Ru, & Simmons, 2016). ListShield™
has received approval as food-processing aid and food additive from the FDA. As a surface disinfectant, only ListShield™ has been approved by the FDA and the Environmental Protection Agency (EPA) in the USA for non-food contact surfaces and equipment in the food industry. In Europe, to obtain the authorisation as a surface disinfectant, a dossier must be developed under the guidelines of the Biocidal Products Regulation 528/2012, proving not only the efficacy of the product, but also safety for humans and absence of bacterial resistance development (Fernández et al., 2017).

Studies have highlighted the effectiveness of the bacteriophage treatments for the inactivation of *L. monocytogenes* in lettuce, cheese, apples, smoked salmon and frozen foods (Perera et al., 2015). The effectiveness of bacteriophages on food and surfaces is also related to various factors, such as the host range of the bacteriophage, the bacteriophage resistance of some strains, the quantity of the *L. monocytogenes* and phage inoculum and physical factors related to the environment (i.e., temperature) and to the food itself (pH, texture, etc.) (Fister et al., 2015; Gutiérrez et al., 2017; lacumin et al., 2016; Lee et al., 2017; Oliveira et al., 2014; Vongkamjan et al., 2017).

**Use of phage-derived proteins as biocontrol agents:** As only a limited number of lytic bacteriophage are available, purified bacteriophage-derived lytic enzymes, such as endolysins, are being investigated (for review see references (Fenton et al., 2010; Schmelcher & Loessner, 2016). Endolysins are a class of enzymes, also known as peptidoglycan hydrolases, induced and activated by the bacteriophage genome in the host cell cytoplasm, which specifically cleave the peptidoglycan wall of the cells and inducing cell lysis and release of progeny phage. Endolysins generally act in concert with the holin proteins, which creates pores in the
cytoplasmic membrane, allowing the endolysin to reach the residues of peptidoglycan and cleave them. The structure of the endolysin is usually modular, with at least one catalytic domain and a cell wall binding domain. Depending on the sequence of peptidoglycan targeted, the endolysins can be classified in five major groups, as shown in Figure 1.3 (Ajuebor et al., 2016):

1. N-acetyl-β-D-acetylmuramoyl-L-alanine amidase;
2. N-acetyl-β-D-muramidases (lysozyme-like);
3. Lytic transglycosylases;
4. N-acetyl-glucosaminidases;
5. Endopeptidases.

Figure 1.3. Schematic representation of peptidoglycan residues with cleavage position of the five classes of endolysins indicated by the arrows (Ajuebor et al., 2016)

In 2000, a less common class of endolysins was discovered, called SAR-endolysins, which have a N-terminal SAR (signal-anchor-release) domain. Although
with a different metabolic pathway (SAR-endolysins accumulate as inactive form on
the cell membrane and the SAR domain refolds and activates the proteins when the
membrane is depolarised), SAR-endolysins still require the intervention of holins (in
this case called pinholins) to access the peptidoglycan (Catalão et al., 2013; Young,
2014). Recombinant endolysins have been produced and purified starting from the
bacteriophage genome and expressed in E. coli or yeast expression vectors, purified
with affinity chromatography and their antimicrobial activity was assessed by many
in-vitro and in-vivo tests (Fenton et al., 2011; Van Tassell et al., 2017; Zhang et al.,
2012a). More recently, studies have shown the possibility of including phage lytic
enzymes into Poly(N-isopropylacrylamide) (PNIPAM) nanoparticles to trigger their
release according to the temperature (Hathaway et al., 2017a); furthermore, beads of
polyhydroxyalkanoate (PHA) bionanoparticles (BNPs) are produced by a number of
microorganisms and phage-derived lysins can be bound to their surface, for a more
concentrated delivery (Altermann et al., 2018). To increase their effectiveness, high
hydrostatic pressure can be used in combination with endolysins (Misiou et al.,
2017).

There are several advantages of using phage-derived proteins in place of live
bacteriophages including: 1) the possibility of horizontal genetic transfer is null,
because only the recombinant protein is used and its action is only lysis; 2) no
bacterial resistance has ever been reported so far, mainly due to the highly site-
specific nature of the endolysins on the peptidoglycan and a mutation at that level
could be dangerous for the cell wall stability (Fischetti, 2010); 3) lysins can be
manipulated, truncated, chimeric enzymes (formed by subunits from different
enzymes bound together) can be easily produced and their lytic activity has been
reported in literature (Ajuebor et al., 2016; Fischetti, 2018; Ruth et al., 2016).
However, in some cases, the production of recombinant bacteriophage-encoded proteins can encounter solubility and stability problems (Fenton et al., 2010).

The application of endolysins against *L. monocytogenes* has been investigated in various studies. Promising results were shown by the endolysin of the bacteriophage P100, PlyP100, when applied during the production of fresh cheese (Van Tassell et al., 2017). Moreover, PlyP100 combined with nisin showed reduction in *L. monocytogenes* counts under the detection limit (Ibarra-Sánchez et al., 2018).

In soya milk, LysZ5, the endolysin of the bacteriophage FWLLm3, reduced more than 5 log_{10} CFU ml^{-1} *L. monocytogenes*, *L. innocua* and *L. welshimeri*, without affecting *S. aureus* or *E. faecalis* (Zhang et al., 2012).

Endolysins, however, sometimes can be hard to produce for stability issues, but the production of single domains in some cases have shown improved stability, easier production and retain of lytic activity (Horgan et al., 2009a).
1.14 Scope of this thesis

In the following chapters, a project aimed at the reduction of *L. monocytogenes* in the mushroom production chain has been undertaken. This project involved raw materials (casing and compost) producers and mushroom growers. In Chapter 2, a 2-year survey was conducted to assess the occurrence of *L. monocytogenes* in the areas considered at high risk of cross-contamination. In Chapter 3, the isolates obtained from Chapter 2 were characterised by PCR and PFGE and routes of cross contamination were determined. Current hygiene practices in the raw materials and mushroom production were evaluated for their effectiveness against *L. monocytogenes* contamination in Chapter 4. The work done in Chapter 5 was focused on the production and antilisterial activity of a recombinant bacteriophage-encoded amidase from listeriophage vB_LmoS_293. This amidase was then bound with polyhydroxyalkanoate (PHA) bionanoparticles (BNPs) and tested against *L. monocytogenes* in Chapter 6. Finally, in Chapter 7, the bacteriophage vB-LmoS_293, the recombinant amidase and the BNPs were tested in a pilot scale mushroom growing unit to determine their potential efficacy during the mushroom production.
1.15 References


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Chapter 2: Highly confidential

Sources of *Listeria monocytogenes* in *Agaricus bisporus* mushroom production
2.1 Abstract

In *Agaricus bisporus* production, the occurrence of *L. monocytogenes* is still poorly understood. A number of studies have shown the presence of *L. monocytogenes* in the mushroom processing environment, suggesting its source from raw materials storage areas (Viswanath et al, 2013; Murugesan et al, 2015). In this study, 10 mushroom producers and three raw material (compost and casing) producers participated in two surveys on the occurrence of *L. monocytogenes* in the most high-risk areas of the production environment. In the first survey, floors, drains, platforms, crates, raw materials and mushrooms were tested for *L. monocytogenes* presence and the results were used to recommend improvements in hygiene practices, with the aim of reducing occurrence and cross contamination in the mushroom production environment. The second survey was focused on the presence of *L. monocytogenes* on food contact surfaces (compost and casing sampled during the mushroom production), raw materials and mushrooms. The results showed an overall *L. monocytogenes* occurrence of 32.1% and 5.7% on the mushrooms. In the casing, 16% of samples were positive, while in the compost, 7.4% were positive for *L. monocytogenes*. Chi square analysis of the data showed an association between the occurrence of *L. monocytogenes* in the casing soil, the processing environment and on the mushrooms, suggesting a cross contamination scenario. The results described in this chapter suggest that *L. monocytogenes* is naturally present in the raw materials used for mushroom growth, especially in the casing soil, and some improvements are needed in the hygiene practices commonly in use in the mushroom production, to reduce the risk of cross contamination on the mushrooms.
2.2 Introduction

*Listeria monocytogenes* is a Gram positive foodborne pathogen that can cause listeriosis, a disease with a high mortality rate, especially in infants, the elderly and the immunocompromised, that are considered as categories of high-risk individuals (Buchanan et al., 2017). The presence of *L. monocytogenes* is a matter of concern for the Ready to Eat (RTE) food industry due to the absence of an antimicrobial hurdle at the consumer level.

*Agaricus bisporus* is the most common mushroom commercialised in Ireland and globally. Mushrooms are considered a RTE food, as they are sometimes used as a raw ingredient (FSAI, 2006), for example, in salads. No outbreaks or sporadic cases of listeriosis have been associated with commercial mushrooms, but *L. monocytogenes* has been detected on mushrooms at retail level and also at production level (Ekman, 2017). There are a limited number of studies available concerning the occurrence of *L. monocytogenes* in the mushroom industry and they are usually limited to a single facility or to a short timeline (Murugesan et al., 2015; Viswanath et al., 2013). A previous survey of random samples in mushroom production facilities identified floors, drains and other non-food contact surfaces as high-risk sites for the occurrence of *L. monocytogenes* (Jordan, unpublished). Therefore, the aim of this study was to determine the presence of *L. monocytogenes* in such high-risk areas of the mushroom industry in Ireland, and in the raw materials used for mushroom production.
2.3 Materials and methods

2.3.1 Recruitment of participating companies

To determine the presence and persistence of *L. monocytogenes* in the mushroom industry, ten mushroom producers, geographically distributed in the north, south and east of Ireland were recruited to participate in this study. In addition, one casing and two compost producers (raw material suppliers) were also recruited to participate in the project.

2.3.2 Sampling plan

From 2015 to 2017, the 13 companies collected samples to be analysed for *L. monocytogenes* presence. The sampling areas, targeted to find *L. monocytogenes*, were identified as non-food contact areas (NFC: floors, drains, ladders, picking platforms and crates), food contact materials (FC: tap water used for watering, compost and casing sampled from the growing facilities) and food (F: mushrooms). The raw materials were collected from one casing and two compost producers (RM: phase III compost and casing). There was a total of six sampling occasions, which can be divided in two surveys, as shown in Figure 2.1: Survey 1 consisted of 4 sampling times to assess the presence of *L. monocytogenes* in the production environment. Initially, there were 3 sampling occasions (every two months). As the prevalence was high on these 3 occasions, they were followed by a 6-month gap during which some guidelines were implemented for improvement of hygiene practices, with suggestions on minimising *L. monocytogenes* entry and spread into the mushroom production environment and for its removal after the crop production (see Figure 2.2 for more detail). After 6 months, there was a fourth sampling occasion to determine whether the hygiene practices resulted in reduction of *L.
monocytogenes. Survey 2 consisted of two more sampling occasions to assess the presence of *L. monocytogenes* in the casing and compost collected from the mushroom producers during growth of the mushrooms (FC), on the mushrooms (F) at the edge of the growing shelves, where water dripping from the upper shelves or the ceiling was observed and on the raw materials (RM, phase III compost and casing).

At the start of Survey 1, boxes containing 6 sponge swabs (3M Ireland Ltd, Dublin, Ireland) two dippers (VWR International Ltd., Dublin, Ireland) for liquid samples (drain water and water used for watering) and two sterile bags (VWR International Ltd., Dublin, Ireland) for mushroom samples, plus two ice packs (VWR International Ltd., Dublin, Ireland) and instructions for sampling were sent to the mushroom growers. The compost and casing producers received boxes containing 10 sterile bags and 10 pairs of sterile gloves (Fleming Medical Ltd, Limerick, Ireland), plus two ice packs and instructions. Training was provided to the staff of the participating companies on the correct procedure for taking environmental and food samples. Once sampling was completed, samples were returned by the companies and immediately analysed. At the start of Survey 2, all the producers received boxes containing 10 sterile bags and 10 pairs of sterile gloves, plus ice packs and instructions.
Figure 2.1. Schematic diagrams showing the Survey 1 (A) and Survey 2 (B) organisation. In A, swabs for non-food contact surfaces (NFC: floors, drains, ladders, picking platforms and crates), liquid samples of food contact materials (FC: tap water used for watering) and NFC (drain water), and food (F: mushrooms) were analysed for \textit{L. monocytogenes} occurrence in 10 mushroom growing facilities. Raw materials (RM) were analysed in 2 compost producing facilities and one casing producing facility. After 3 sampling occasions, there was a six months gap ("Stop 6
months” box), in which hygiene improvement guidelines were suggested, followed by the sampling occasion 4. In B, the categories of food contact materials (FC: compost and casing sampled from the growing facilities) and food (F) were analysed for *L. monocytogenes* occurrence in two sampling occasions in 10 mushroom growing facilities, while raw materials (RM) were analysed as in Survey 1.
Strategy to help control *L. monocytogenes* during mushroom production

**Minimise its entry into the production environment**

Keep the area surrounding the entrance to the growing room clear of any soil, compost and casing debris. Have an effective disinfectant footbath or foot mat at the entrance to the growing room. Change and/or wash the footbath sponge or mat daily when harvesting is in progress and traffic is heavy and top up daily with fresh disinfectant solution. Clean clothing garments and aprons should be used always.

**Hygiene control during crop production to minimise its spread in the production environment**

All staff, especially harvesters and cleaners, should be aware of *L. monocytogenes* and the reasons for attention to detail in its control. Picking is a high-risk activity when contamination can be transferred to mushrooms. Disposable gloves should be worn during picking and changed every time a harvester leaves the growing room on a break, or if they are damaged. Ensure that crates are kept off the floor using appropriate dollys or pallets. Clean and disinfect the floors of growing rooms between flushes. Clear the floors of large debris using a gentle flow of water and a floor scraper. Use detergents and disinfectants (Product Type 4 only) as instructed. Detergents and disinfectants have different functions: a detergent is for cleaning and biofilm and slime removal, a disinfectant (e.g. hypochlorite, or peracetic acid) is used to kill bacteria.

**Hygiene control post crop production to sterilise the area and inactivate/reduce bacteria**

After harvesting, the growing room floors should be cleared of large debris using a gentle flow of water and a floor scraper. Debris and soil can inactivate detergents and disinfectants. Pay particular attention to drains and floors. Do not wash large volumes of debris into the drainage system in growing rooms. Collect before washing out. 'Cook-out' temperatures should be 60 °C in the compost for a minimum of 8 hours to ensure all substrates have reached this temperature to kill all relevant microbes, pests and diseases. All staff moving from the picking area to the packing area must observe hygiene guidelines.

Figure 2.2. Hygiene practices guidelines suggested to the mushroom growers between sampling occasions 3 and 4 of Survey 1.
2.3.3 Isolation and enumeration of L. monocytogenes from environmental, casing, compost and mushroom samples

The samples were analysed for the presence of L. monocytogenes, as described by the BS EN ISO 11290-1:1997+A1:2004. Casing, compost and mushroom samples were also analysed for enumeration of L. monocytogenes following the BS EN ISO 11290-2:1998. Briefly, to analyse the swabs, 100 ml of Fraser broth (Merck, Dublin, Ireland) was added to the bag containing the swab and, after macerating, left for 1 h at room temperature for resuspension and recovery of the cells. Subsequently, a supplement containing 10 mg/l of nalidixic acid and 12.5 mg/l of acriflavine hydrochloride (Merck, Dublin, Ireland) was added to the Fraser broth and the swabs were incubated at 30 °C for 24 h for pre-enrichment. After incubation, 20 µl of the pre-enrichment culture was plated on a selective medium for L. monocytogenes (ALOA (Agar Listeria acc. to Ottaviani Agosti), Biomerieux, France) while 100 µl of the pre-enriched sample was transferred to a tube containing 10 ml of Full Fraser supplement containing 20 mg/l of nalidixic acid and 25 mg/l of acriflavine hydrochloride (Merck, Dublin, Ireland) which was incubated at 37 °C for 48 h for full enrichment. After incubation, 20 µl of the full enrichment sample was plated on an ALOA plate and incubated at 37 °C for 48 h. One or two presumptive positive colonies (blue-green with an opaque halo) were isolated from ALOA plates, streaked for confirmation on a second selective medium (Brilliance Listeria agar, BLA, Oxoid Ltd., Basingstoke, UK), streaked on brain heart infusion (BHI) agar (Oxoid Ltd., Basingstoke, UK) and the isolate recovered and stored in cryovials containing 20% glycerol at -20 °C for future characterisation.

For liquid samples (drain water and water used for watering), 10 ml of sample was added to 90 ml of Fraser broth which was processed as described above. For casing/compost/mushroom analysis, 25 g of sample was added to 225 ml of Fraser broth,
homogenised and kept for 1 h at room temperature. One millilitre was spread-plated onto three ALOA plates for enumeration of presumptive *L. monocytogenes* colonies and the plates were incubated at 37 °C for 48 h. Pre-enrichment supplement was added to the samples and the procedure continued as described for the swabs. Mushroom samples were analysed after the “best before” date, to avoid issues that would have compromised the participation of the mushroom growers in the project.

### 2.3.4 Confirmation of isolates as *L. monocytogenes*

All of the isolates were subject to species confirmation via PCR, as described by Ryu et al., (2013). Briefly, a single colony, grown in BHI agar at 37 °C overnight, was resuspended in 50 μl of sterile water. After heat treatment at 95 °C for 15 mins, the solution was centrifuged at 900 x g for 10 mins. The cell free extract was used as DNA template for the confirmation PCR using primers for the genes *prs* and *lmo1030*.

### 2.3.5 Statistical analysis

The Kruskal-Wallis Test was performed to determine if there was a statistical difference between the first four sampling times in Survey 1. Chi square tests were performed to evaluate if any statistical association was present between the different categories of data and P values <0.05 were considered to reject the null hypothesis that there were no statistical associations.
2.4 Results

2.4.1 Overall results showing high occurrence of *L. monocytogenes*

There was a variation in the number of samples received from each company. All of the mushroom growers sent samples during survey 1, while one compost producer missed the first sampling occasion. One mushroom grower did not participate in survey 2, while three others missed one sampling occasion.

All of the isolates obtained from the samples were confirmed to be *L. monocytogenes* by PCR. For the purposes of prevalence, if one colony selected from a sample was positive, the sample was counted as positive. The overall results of the prevalence study in the targeted areas are summarised in Table 2.1. In total, during surveys 1 and 2, 744 samples were analysed and 239 were positive for *L. monocytogenes*, resulting in a prevalence of 32.1%. One hundred and forty one mushroom samples were obtained, with a prevalence of 5.7%. The processing environment samples in surveys 1 and 2 numbered 603 and, of these, 37.8% were positive for *L. monocytogenes*. Included in the processing environment category were the raw materials (10.5% positive), food contact materials (19.7% positive) and non-food contact surfaces (63.6%). The raw materials were represented by the Phase III compost (7.4% positive) and the casing soil (16% positive). In Figure 2.3, the prevalence of *L. monocytogenes* in mushrooms and the relevant sub-categories of NFC surfaces is shown. The highest prevalence was observed on the floors (77.8% positive), followed by the drains (76.4% positive) and picking platforms (62.5% positive). The swabs on the bottom of the crates used for transportation of picked mushrooms, showed a *L. monocytogenes* prevalence of 19.2%. All the positive casing, compost and mushroom samples had numbers of *L. monocytogenes* lower than 100 cfu/g.

Chi square tests (Table 2.2) showed that there was no association between the prevalence on mushrooms and raw materials (*p*>0.05), although, significant association
was shown between mushrooms and the processing environment (p<0.05) and between mushrooms and the FC and NFC surfaces (both p<0.05). Table 2.3 describes the results of the Chi square analysis between all types of samples taken during the survey. In particular, this analysis confirms the association between the presence of \textit{L. monocytogenes} in the processing environment and on the mushrooms, but also reveals an association between mushrooms and fresh casing (p<0.05), while no association was observed between Phase III compost and mushrooms (p>0.05). Table 2.4 summarises the Chi square results, showing possible routes of cross-contamination to the mushroom.

Table 2.1. Overall incidence of \textit{L. monocytogenes}. Raw materials (RM) are shown more in detail at the bottom of the table.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>% positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>744</td>
<td>239</td>
<td>32.1</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>141</td>
<td>8</td>
<td>5.7</td>
</tr>
<tr>
<td>Processing environment</td>
<td>603</td>
<td>229</td>
<td>38</td>
</tr>
<tr>
<td>Raw materials</td>
<td>190</td>
<td>20</td>
<td>10.5</td>
</tr>
<tr>
<td>Food Contact</td>
<td>122</td>
<td>24</td>
<td>19.7</td>
</tr>
<tr>
<td>Non Food Contact</td>
<td>291</td>
<td>185</td>
<td>63.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Samples: RM</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>% positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase III compost</td>
<td>121</td>
<td>9</td>
<td>7.4</td>
</tr>
<tr>
<td>Casing soil</td>
<td>69</td>
<td>11</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 2.3. Overall prevalence of *L. monocytogenes* in the four growing environment sub-categories and on the mushrooms.

Table 2.2. Chi square analysis of *L. monocytogenes* in the mushrooms and processing environment categories. P values < 0.05 reject the null hypothesis that there is no association between the categories.

<table>
<thead>
<tr>
<th>Categories</th>
<th>P (0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushrooms and Processing environment</td>
<td>8.1x10^{-22}</td>
</tr>
<tr>
<td>Mushrooms and Food Contact</td>
<td>0.000534</td>
</tr>
<tr>
<td>Mushrooms and Non Food Contact</td>
<td>1.9x10^{-61}</td>
</tr>
<tr>
<td>Mushrooms and Raw Materials</td>
<td>0.116692</td>
</tr>
</tbody>
</table>
Table 2.3. Chi square analysis between the incidence of *L. monocytogenes* in all the subcategories and the mushrooms, ranked per p values. “Mushroom compost” and “mushroom casing” indicate compost and casing sampled during the mushroom growth. “Casing” and “phase III compost” were sampled from casing and compost producers. Yes and No indicate if there was a significative association (p<0.05).

<table>
<thead>
<tr>
<th>P (0.05)</th>
<th>Category 1</th>
<th>Category 2</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.05x10^{-20}</td>
<td>Mushrooms</td>
<td>floors</td>
<td>Yes</td>
</tr>
<tr>
<td>1.05x10^{-26}</td>
<td>Mushrooms</td>
<td>drains</td>
<td>Yes</td>
</tr>
<tr>
<td>3x10^{-26}</td>
<td>Floors</td>
<td>phase III compost</td>
<td>Yes</td>
</tr>
<tr>
<td>7.58x10^{-23}</td>
<td>Drains</td>
<td>phase III compost</td>
<td>Yes</td>
</tr>
<tr>
<td>3.29x10^{-15}</td>
<td>Mushrooms</td>
<td>platforms</td>
<td>Yes</td>
</tr>
<tr>
<td>4.9x10^{-15}</td>
<td>Floors</td>
<td>casing</td>
<td>Yes</td>
</tr>
<tr>
<td>6.45x10^{-13}</td>
<td>Drains</td>
<td>casing</td>
<td>Yes</td>
</tr>
<tr>
<td>1.58x10^{-12}</td>
<td>Platforms</td>
<td>phase III compost</td>
<td>Yes</td>
</tr>
<tr>
<td>3.6x10^{-10}</td>
<td>Floors</td>
<td>mushroom casing</td>
<td>Yes</td>
</tr>
<tr>
<td>6.49x10^{-10}</td>
<td>Floors</td>
<td>mushroom compost</td>
<td>Yes</td>
</tr>
<tr>
<td>8.01x10^{-9}</td>
<td>Drains</td>
<td>mushroom casing</td>
<td>Yes</td>
</tr>
<tr>
<td>1.31x10^{-8}</td>
<td>Drains</td>
<td>mushroom compost</td>
<td>Yes</td>
</tr>
<tr>
<td>4.16x10^{-8}</td>
<td>Floors</td>
<td>crates</td>
<td>Yes</td>
</tr>
<tr>
<td>2.9x10^{-7}</td>
<td>Drains</td>
<td>crates</td>
<td>Yes</td>
</tr>
<tr>
<td>2.36x10^{-6}</td>
<td>Platforms</td>
<td>casing</td>
<td>Yes</td>
</tr>
<tr>
<td>0.000371</td>
<td>Platforms</td>
<td>mushroom casing</td>
<td>Yes</td>
</tr>
<tr>
<td>0.00048</td>
<td>Platforms</td>
<td>mushroom compost</td>
<td>Yes</td>
</tr>
<tr>
<td>0.00048</td>
<td>Mushrooms</td>
<td>mushroom compost</td>
<td>Yes</td>
</tr>
<tr>
<td>0.0006</td>
<td>Mushrooms</td>
<td>mushroom casing</td>
<td>Yes</td>
</tr>
<tr>
<td>0.000936</td>
<td>Platforms</td>
<td>crates</td>
<td>Yes</td>
</tr>
<tr>
<td>0.004124</td>
<td>Mushroom compost</td>
<td>phase III compost</td>
<td>Yes</td>
</tr>
<tr>
<td>0.00497</td>
<td>Mushroom casing</td>
<td>Phase III compost</td>
<td>Yes</td>
</tr>
<tr>
<td>0.014835</td>
<td>Mushrooms</td>
<td>casing</td>
<td>Yes</td>
</tr>
<tr>
<td>0.017752</td>
<td>Mushrooms</td>
<td>crates</td>
<td>Yes</td>
</tr>
<tr>
<td>0.063098</td>
<td>Crates</td>
<td>phase III compost</td>
<td>No</td>
</tr>
<tr>
<td>0.06623</td>
<td>Phase III compost</td>
<td>casing</td>
<td>No</td>
</tr>
<tr>
<td>0.11938</td>
<td>Floors</td>
<td>platforms</td>
<td>No</td>
</tr>
<tr>
<td>0.14490</td>
<td>Platforms</td>
<td>drains</td>
<td>No</td>
</tr>
<tr>
<td>0.314195</td>
<td>Mushroom compost</td>
<td>casing</td>
<td>No</td>
</tr>
<tr>
<td>0.342265</td>
<td>Mushroom casing</td>
<td>casing</td>
<td>No</td>
</tr>
<tr>
<td>0.563282</td>
<td>Mushrooms</td>
<td>phase III compost</td>
<td>No</td>
</tr>
<tr>
<td>0.593181</td>
<td>Crates</td>
<td>mushroom casing</td>
<td>No</td>
</tr>
<tr>
<td>0.67978</td>
<td>Crates</td>
<td>mushroom compost</td>
<td>No</td>
</tr>
<tr>
<td>0.702549</td>
<td>Crates</td>
<td>casing</td>
<td>No</td>
</tr>
<tr>
<td>0.95508</td>
<td>Mushroom compost</td>
<td>mushroom casing</td>
<td>No</td>
</tr>
<tr>
<td>0.996775</td>
<td>Floors</td>
<td>drains</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 2.4. Chi square analysis revealing the statistical association between the presence of *L. monocytogenes* on mushrooms and processing environment categories. The association can be interpreted as “Routes of possible cross-contamination on the mushrooms”. “Mushroom compost” and “mushroom casing” indicate compost and casing sampled in the mushroom production during the mushroom growth. “Casing” and “phase III compost” were sampled from casing and compost producers. P values <0.05 were considered significative, rejecting the null hypothesis that there is no association. Only Phase III compost showed no association with the mushrooms.

<table>
<thead>
<tr>
<th>Routes of possible cross-contamination on the mushrooms</th>
<th>p (0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floors</td>
<td>2.05x10^-20</td>
</tr>
<tr>
<td>Drains</td>
<td>1.05x10^-26</td>
</tr>
<tr>
<td>Platforms</td>
<td>3.29x10^-15</td>
</tr>
<tr>
<td>Mushroom compost</td>
<td>0.00048</td>
</tr>
<tr>
<td>Mushroom casing</td>
<td>0.0006</td>
</tr>
<tr>
<td>Casing</td>
<td>0.014835</td>
</tr>
<tr>
<td>Crates</td>
<td>0.017752</td>
</tr>
<tr>
<td>Phase III compost</td>
<td>0.563282</td>
</tr>
</tbody>
</table>

2.4.2 Survey 1: mushroom production environment sampling

The results from Survey 1 are summarised in Table 2.5. In total, 507 samples were analysed, with an overall prevalence of *L. monocytogenes* of 39.6%. Of the samples tested, mushroom samples had a prevalence of 3.8%, while the processing environment had a prevalence of 46.3%. In the processing environment, *L. monocytogenes* was commonly found on raw materials (10.1%), food contact materials (7.1%) and non-food contact surfaces (63.6%). The Kruskal-Wallis Test showed no statistical differences between each time point, even after the 6 month gap (p>0.05). In more detail, Figure 2.4 shows the results of each sampling occasion at each mushroom growing facility. Company 11 and Company 13 showed a reduction of *L. monocytogenes* prevalence during the four sampling
occasions, however this reduction was not significant (p>0.05). In all the other companies, an increase of *L. monocytogenes* occurrence was shown during the four sampling occasions, or, in Company 4, 7 and 10, a very random occurrence. The raw materials producers’ results are shown in Figure 2.5. Also in this case, sampling occasion 4 did not show any improvement.

Table 2.5. Incidence of *L. monocytogenes* in Survey 1.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>% of positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>507</td>
<td>201</td>
<td>39.6</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>79</td>
<td>3</td>
<td>3.8</td>
</tr>
<tr>
<td>Processing environment</td>
<td>428</td>
<td>198</td>
<td>46.3</td>
</tr>
<tr>
<td>Raw materials</td>
<td>109</td>
<td>11</td>
<td>10.1</td>
</tr>
<tr>
<td>Food contact</td>
<td>28</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td>Non-food contact</td>
<td>291</td>
<td>185</td>
<td>63.6</td>
</tr>
</tbody>
</table>
Figure 2.4. Incidence of *L. monocytogenes* during Survey 1 per each mushroom growing facility. The numbers indicate the percentage of positive samples for each company at each sampling occasion.
Figure 2.5. Incidence of *L. monocytogenes* during Survey 1 per each raw material producer. The numbers indicate the percentage of positive samples for each company at each sampling occasion.
2.4.3 Survey 2: sampling on growing shelves

In Table 2.6, the results of Survey 2 are shown. In this case, 237 samples were collected, with an overall prevalence of *L. monocytogenes* of 16%. Sixty two mushroom samples were collected from the edges of the shelves, with a prevalence of 8.1%. The prevalence on raw materials was 11.1% and food contact materials was 23.4%. Chi square analysis showed an association between *L. monocytogenes* prevalence on mushrooms and food contact materials, in particular casing and compost sampled during mushroom growth (as shown before in Tables 2.2, 2.3 and 2.4).

The results of Survey 2 from the individual mushroom growing companies are shown in Figure 2.6. Samples from Company 11 were not received in both the sampling occasions, while Company 9 and 12 did not send samples on the sampling occasion 5 and Company 10 on the sampling occasion 6. These data represent the occurrence of *L. monocytogenes* in the mushroom growing shelves, with variability between 0% and 33.3%. Figure 2.7 shows the results of the raw materials producers.

Table 2.6. Incidence of *L. monocytogenes* in Survey 2.

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples</th>
<th>No. of positives</th>
<th>% of positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>237</td>
<td>38</td>
<td>16</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>62</td>
<td>5</td>
<td>8.1</td>
</tr>
<tr>
<td>Processing environment</td>
<td>175</td>
<td>31</td>
<td>17.7</td>
</tr>
<tr>
<td>Raw materials</td>
<td>81</td>
<td>9</td>
<td>11.1</td>
</tr>
<tr>
<td>Food contact</td>
<td>94</td>
<td>22</td>
<td>23.4</td>
</tr>
</tbody>
</table>
Figure 2.6. Incidence of *L. monocytogenes* during Survey 2 per each mushroom growing facility. The numbers indicate the percentage of positive samples for each company at each sampling occasion.
Figure 2.7. Incidence of *L. monocytogenes* during Survey 2 per each raw material producer. The numbers indicate the percentage of positive samples for each company at each sampling occasion.
2.5 Discussion

The aim of this study was to determine the presence of *L. monocytogenes* in the mushroom industry in Ireland. A previous study highlighted the areas where a most probable contamination was possible (Jordan, unpublished) and these indications were used to target critical areas for this study. Only a few studies have been conducted so far in the mushroom industry (Murugesan et al., 2015; Viswanath et al., 2013), which represent a precious source of information, but more extensive studies involving a large number of producers, including also raw materials producers, are needed for a better perspective on *L. monocytogenes* contamination.

This study represents the first large-scale survey on *L. monocytogenes* in the Irish mushroom industry and the cooperation with the food business owners was fundamental for creating awareness around the issue of contamination in their facilities. Dirty and damp areas are the best environment for *L. monocytogenes* growth in a mushroom growing facility and the constant presence of residues of raw materials and mushrooms, on the floors, during the preparation of the crop or the harvest, allows the spread of contamination around the facility (LaBorde, 2017). Survey 1 revealed a constant contamination on the floors, platforms and drains, with isolation of *L. monocytogenes*, in 73%, 66% and 77% of the samples tested respectively. The crates were tested because of the absence of dedicated trolleys in some facilities, with the consequence that sometimes they were stored on the floor. Crates were also usually placed on the picking platforms during the harvest of mushrooms. The results showed a contamination on the crates in 16% of cases and a possible cross-contamination scenario from the floor to the platforms and crates.

At the end of the first three sampling occasions, it was realised that the contamination level at the facilities was high at the targeted sites. Therefore, advice on corrective action was provided to the mushroom growers (Figure 2.2). In a previous study,
this approach proved successful in a cheesemaking facility (Dalmasso & Jordan, 2013). Following a period of 6 months to allow implementation of the advice, further samples were taken at the same facilities. However, the prevalence of \textit{L. monocytogenes} on this fourth sampling occasion was reduced only in Company 11 and 13, but the difference was not significant (p>0.05) (Figure 2.4).

A study conducted by Murugesan et al. (2015) showed scenarios of cross contamination inside a mushroom production facility, highlighting the presence of a persistent clone. Multi Virulence Locus Sequence Typing (MVLST) was used to characterise the isolates obtained from that study, although a better discrimination between isolates of \textit{L. monocytogenes} would be given by other technologies, like Pulsed Field Gel Electrophoresis (PFGE) or Whole Genome Sequencing (WGS).

Previous studies by Leong et al. (2017) and Madden et al. (2018) in the Republic of Ireland and Northern Ireland, respectively, also identified potential routes of cross contamination between food processing facilities (including mushroom growing facilities) using PFGE or both PFGE and WGS. In both studies, no known links were identified between facilities. However, common transportation services or common ingredients were shown to contribute to \textit{L. monocytogenes} spread.

Raw materials, casing and Phase III compost represent categories of particular concern in the mushroom production. These materials represent sources of \textit{L. monocytogenes} and are directly in contact with the final product. Thus, if cross contamination occurs at this level, it can be transferred to the consumers. Because of their role in providing the optimal environment for the development of mushrooms e.g. important microflora, further heat treatment of casing and compost is not possible.

Casing soil has sometimes been linked to pathogens transferred to the mushrooms, as in the case of \textit{Salmonella enterica serovar Kedougou} in the republic of Ireland in 2001 (Doran et al., 2005; FSAI, 2001). The presence of \textit{L. monocytogenes} in soils is not
surprising considering its ubiquitous nature (Weis & Seeliger, 1975), but particular care has to be considered to reduce cross contamination to avoid the risk of outbreaks.

Phase III compost is obtained from horse and chicken manure, considered sources of *L. monocytogenes*, however, it undergoes a pasteurisation stage, that has been proven to reduce the pathogenic load of up to 7 logs in 2 hours of treatment (Weil et al., 2013). Viswanath et al. isolated *L. monocytogenes* from compost storage areas, revealing a possible *L. monocytogenes* survival or recontamination post heat treatment (Viswanath et al., 2013). Heat treatment is done in large sheds with several tons of compost so it is difficult to envisage even heat distribution. More studies are needed to determine the reason of *L. monocytogenes* isolation from pasteurised compost.

As a result of the outcome of Survey 1, and the risk of cross contamination demonstrated in other studies (Murugesan et al., 2015; Viswanath et al., 2013), an assessment of *L. monocytogenes* contamination in used casing and compost, and on mushrooms subjected to water drips from the upper shelves was made in Survey 2. The results showed that used casing and compost can be contaminated with *L. monocytogenes*. It is then logical that mushrooms at the edge of the lower shelves, subjected to excess water from the upper shelves, are at higher risk of contamination. This was indeed the case, as 8.1% of the mushrooms on the edge were contaminated whereas only 3.8% of mushrooms randomly sampled were contaminated. Chi square comparison confirmed associations between used casing and compost contamination and occurrence of *L. monocytogenes* on the mushrooms. Studies on *L. monocytogenes* occurrence on mushrooms at production level are scarce, but a previous survey conducted on mushrooms at a retail level, revealed a contamination of 1.1% (Anonymous, 2006). In another study, at retail level, *L. monocytogenes* was isolated from 1% of mushroom samples (Samadpour et al., 2006), while in two other surveys, targeting *Listeria* species, an occurrence of 8.4% (Strapp et al., 2003) and 12% (Heisick et al., 1989) were detected.
Casing soil, the top layer of the mushroom growing substrates, does not receive any heat treatment. For that reason, it is considered a more possible candidate for carrying *L. monocytogenes* contamination on the mushrooms. The results from Survey 1 and Survey 2 revealed 16% *L. monocytogenes* presence in the casing soil provided by the casing manufacturer.

In this study, the high occurrence of *L. monocytogenes* does not necessarily indicate a failure in the hygiene practices, although, in the mushroom industry, a stronger cleaning and sanitising regime, united with physical barriers between the different areas of the facilities, might drastically improve the situation. However, like in all vegetable production facilities, *L. monocytogenes* is commonly found, due to its strong association with soil (Weis & Seeliger, 1975), but more awareness and training of the personnel in the food production facility can reduce the risk of outbreaks.
2.6 Conclusions

*L. monocytogenes* was regularly isolated from the mushroom growing environment and normal hygiene measures were ineffective for its reduction. From the results of Surveys 1 and 2, it was apparent that *L. monocytogenes* is a common part of the flora of the casing soil and is often found in Phase III compost. This is understandable as the raw materials represent the normal habitat of *L. monocytogenes* (e.g. manure, peat and lime), which cannot receive adequate heat treatment. Thus, as hygiene measures eliminate some contamination, the raw materials reintroduce further contamination.

Between crops of mushrooms in a growing facility, there is a hygiene process called 'cookout'. Different facilities have different processes for cookout, but the process generally involves heating of the environment to about 70 °C for 10 h, followed by sanitation and possible heat treatment for 3 h at 60°C.

The effectiveness of cookout at two different facilities was studied in Chapter 4.
2.7 References


scale mushroom production facility. *Journal of Food Protection*, 76(4), 608–615.


Chapter 3

Diversity of *Listeria monocytogenes* strains isolated from *Agaricus bisporus* mushroom production

3.1 Abstract

The aims of this study were to characterise the genetic diversity of *Listeria monocytogenes* isolates obtained from commercial mushroom production, to establish the persistence, re-contamination and the risk of cross-contamination from the working environment to the final products, creating awareness about the presence of *L. monocytogenes* thus helping to prevent the possibility of cross-contamination. From an extensive analysis of commercial mushroom production, analysed with BS EN ISO 11290-1:1996/Amd 1:2004 and BS EN ISO 11290-2:1998/Amd 1:2004, 279 *L. monocytogenes* isolates were obtained. All of the isolates were characterised by pulsed-field gel electrophoresis, species PCR and serogroup PCR. All the isolates were confirmed as *L. monocytogenes*; 30.1% were serogroup 1/2b-3b-7, 40.8% were serogroup 1/2a-3a and 29.1% were serogroup 4b-4d-4e. There were 77 pulsotypes from the 279 isolates, 40 of the pulsotypes had only one strain and 37 had two or more strains, indicating great diversity in the isolates. The high genetic diversity is indicative of the fact that current hygiene practices are successful at removing *L. monocytogenes* but that re-contamination of the production environment is frequent. The results obtained are very valuable in creating awareness of *L. monocytogenes* in mushroom production and for the improvement of hygiene practices.
3.2 Introduction

*Listeria monocytogenes* is a pathogenic, Gram positive bacterium that can cause listeriosis in individuals with a compromised immune system, particularly young children, pregnant women and the elderly (Farber & Peterkin, 1991). Although relatively rare, the hospitalisation rate is about 99% (Scallan et al., 2011) and the mortality rate about 24% (de Noordhout et al., 2014).

Due to its ubiquitous nature and its ability to grow at refrigeration temperatures, low pH, high salt concentrations and in the presence of other stress-inducing factors, it is a concern in the food industry, particularly in ready-to-eat (RTE) foods where there is no bacterial inactivation step between production and consumption.

There have been no outbreaks of listeriosis that have been linked to mushrooms, although there have been a number of product recalls due to the possible presence of *L. monocytogenes*. Examples include recalls of mushrooms in Canada (Anon., 2015) and in the United States (Kieler, 2017). In the European Union, there have been several recalls of mushrooms due to *L. monocytogenes*, mainly in enoki mushrooms from eastern countries (Table 3.1), but no outbreaks of listeriosis have been associated with any of these recall events.
Table 3.1 Mushroom recalls in the European Union since 2013. Data adapted from the RASFF* database

<table>
<thead>
<tr>
<th>Date</th>
<th>Country</th>
<th>Food Type</th>
<th>RASFF reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>21/08/2017</td>
<td>United Kingdom</td>
<td>Lily mushrooms from Thailand</td>
<td>2017.1118</td>
</tr>
<tr>
<td>09/02/2017</td>
<td>Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2017.0012</td>
</tr>
<tr>
<td>12/05/2016</td>
<td>Netherlands</td>
<td>Golden enoki mushrooms from South Korea</td>
<td>2015.1421</td>
</tr>
<tr>
<td>23/11/2015</td>
<td>Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2015.1291</td>
</tr>
<tr>
<td>04/11/2014</td>
<td>Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2014.1194</td>
</tr>
<tr>
<td>12/06/2014</td>
<td>Greece</td>
<td>Chilled enoki mushrooms from South Korea, via the Netherlands</td>
<td>2014.0326</td>
</tr>
<tr>
<td>24/02/2014</td>
<td>Ireland</td>
<td>Mushrooms from Ireland</td>
<td>2014.0255</td>
</tr>
<tr>
<td>28/01/2014</td>
<td>Netherlands</td>
<td>Enoki mushrooms from South Korea</td>
<td>2014.0037</td>
</tr>
<tr>
<td>10/12/2013</td>
<td>Belgium</td>
<td>Enoki mushrooms from China</td>
<td>2013.1318</td>
</tr>
</tbody>
</table>


In Ireland, the majority of commercial mushroom production is of *Agaricus bisporus*, commercially cultivated mainly in its white or brown forms. There is a large export market, mainly to the United Kingdom. While the majority of consumers cook the mushrooms prior to consumption, there are some that consume them uncooked.
In a survey in Ireland in 2006, *L. monocytogenes* was detected in only 1.1% (8/727) of samples. The numbers of *L. monocytogenes* in all samples were <100 cfu/g, and so they were classified as satisfactory according to the microbiological criterion specified in Commission Regulation (EC) No 2073/2005, as the shelf-life was considered less than 5 days (Anon., 2006). A subsequent study showed that *A. bisporus* does not support the growth of *L. monocytogenes* (Leong et al., 2015).

The possibility of disease outbreaks due to cross-contamination of product with *L. monocytogenes* from the production environment makes mushroom producers very concerned about its presence in the production environment, especially as mushrooms are considered an RTE food, where the products are consumed without the cooking process that would normally kill the pathogens.

In order to facilitate prevention of *L. monocytogenes* contamination and to increase the quality of the product, extensive studies on *L. monocytogenes* are becoming more common (Beno et al., 2016; Leong et al., 2017). Such studies accomplish not only an assessment the areas of frequent contamination but also to assess the presence of persistent strains and to test improvements and new strategies in the hygiene practices used to reduce the risk of cross contamination from the work environment to the final product (Murugesan et al., 2015; Viswanath et al., 2013).

Studies on genetic characterisation of the *L. monocytogenes* populations in food producing facilities have been conducted using strain typing methods such as Pulsed Field Gel Electrophoresis (PFGE). These studies provide an opportunity to examine the genetic diversity in the processing facilities and to assess putative routes of cross-contaminations, helping to establish more precise control measures to address issues with *L. monocytogenes* (Leong et al., 2014; Negi et al., 2015; Henriques et al., 2017).

The aim of this study was to characterise 279 isolates of *L. monocytogenes* obtained from the mushroom production chain by PCR and PFGE, to assess the diversity of strains and
identify putative routes of contamination, creating awareness about the presence of *L. monocytogenes* thus helping to prevent the possibility of cross-contamination.
3.3 Materials and methods

3.3.1 L. monocytogenes isolates

*L. monocytogenes* isolates were obtained from an extensive assessment of commercial mushroom production (Chapter 2 of this thesis). Thirteen mushroom growing facilities and raw material producers, representing a wide geographical spread around the south, east and north of the Republic of Ireland, were involved in the sampling programme from June 2015 to June 2016. The results of an initial survey of random samples were used to identify sampling locations that were likely to yield a positive result. Locations that were targeted in the sampling programme included production environment swabs (for example, floors, ladders, platforms and wet spots), drains and raw materials (casing and phase III compost). Mushroom samples were also analysed, primarily to determine if there was cross-contamination from the production environment.

The processing environment samples were analysed for detection according to the International Standard BS EN ISO 11290-1:1996/Amd 1:2004 and the mushrooms according to BS EN ISO 11290-1:1996/Amd 1:2004 and BS EN ISO 11290-2:1998/Amd 1:2004 for detection and enumeration, respectively, using only Agar Listeria according to Ottaviani-Agosti (ALOA, Biomerieux, France) as the plating medium and taking 1 to 3 isolates from each positive sample. The isolates were purified using Brilliance Listeria Agar (BLA) (Oxoid Ltd., Basingstoke, UK) and Brain Hearth Infusion (BHI) agar (Merck KGaA, Darmstadt, Germany) and frozen in 20% glycerol at -20 °C for further analysis. Each isolate was characterised by PFGE, species-specific PCR and serogroup PCR.

3.3.2 PFGE typing and analysis

The PFGE analysis was performed using the International Standard PulseNet protocol (Anon., 2013). *Listeria monocytogenes* isolates were grown overnight on Brain
Hearth Infusion (BHI) agar (Merck KGaA, Darmstadt, Germany) at 37 °C. Subsequently, a suspension of cells in 10 mmol l⁻¹ Tris: 1 mmol l⁻¹ EDTA buffer, pH 8.0 (TE) with an OD₆₅₀ of 1 was prepared. To 400 µl of cell suspension, 20 mg ml⁻¹ of Lysozyme (Merck KGaA, Darmstadt, Germany) was added and incubated at 55 °C for 20 min. After incubation, 20 µl of Proteinase K stock (20 mg ml⁻¹) (VWR International Ltd., Dublin, Ireland) was added, followed by 400 µl of 1% SeaKem Gold agarose (Lonza, Rockland, Maine, USA). Plugs were prepared by dispensing the mix into plug moulds. Solid plugs were then lysed for 2 h in 5 ml Cell Lysis Buffer (50 mmol l⁻¹ Tris, 50mmol l⁻¹ EDTA, pH 8.0 + 1% Sarcosyl) supplemented with 25 µl of 20 mg ml⁻¹ Proteinase K solution. The plugs were then washed 2X in distilled water and 4X in TE buffer.

DNA was digested either with 10 U µl⁻¹ of the restriction enzyme SgsJ FastDigest (Fisher Scientific Ireland Ltd, Dublin, Ireland) or 50 U µl⁻¹ of the restriction enzyme ApaI FastDigest (Fisher Scientific Ireland Ltd, Dublin, Ireland). The restricted DNA was run on a 1% SeaKem Gold agarose gel for 21 h as described in the PulseNet protocol, on a CHEF-DR III (Bio-Rad, Hercules, California, USA). After staining with 1 µg ml⁻¹ ethidium bromide solution, the gels were observed with a AlphalImager 2200 Gel Documentation System (Alpha Innotech, San Leandro, California, USA) and UPGMA (unweighted pair group method with averages) analysis was performed with BioNumerics v7.0 software (Applied Maths, Belgium) using the Pearson coefficient with 1% tolerance.

3.3.3 Species-specific PCR

To confirm that the isolates were *L. monocytogenes*, species-specific PCR was used. A single colony, following overnight on BHI at 37 °C, of each isolate was resuspended in 50 µl of sterile water, heated at 95 °C for 15 min and centrifuged in a benchtop centrifuge at 2000 g for 10 min. The supernatant, containing the template DNA, was used for PCR analysis. The PCR was performed as outlined in the protocol of Ryu et al., (2013).
3.3.4 Serogroup PCR

Each *L. monocytogenes* isolate was grown from a stock stored at -20 °C on a BHI agar plate. A single colony was resuspended in 10 ml of BHI broth and grown overnight. The DNA was extracted using the UltraClean Microbial DNA Kit (CamBio, UK), following the supplier’s instructions, or with the PrepMan™ Ultra (Applied Biosystems, Foster City, California, USA), following the supplier’s instructions. The PCR was performed according to the protocol of Doumith et al, (2004).
3.4 Results

3.4.1 Characterisation of the isolates

From an extensive assessment of *L. monocytogenes* in the mushroom industry (from June 2015 to June 2016), the 279 *L. monocytogenes* isolates were confirmed by PCR and then characterised. Serogrouping PCR showed the isolates belonged to group 1/2a-3a in 40.8% of cases, group 1/2b-3b-7 in 30.1% of cases and group 4b-4d-4e in 29.1% of the cases.

PFGE profiles were obtained from *ApaI* and *SgsI* digestions, yielding 77 pulsotypes. Of these, 40 pulsotypes consisted of only one isolate, while 37 pulsotypes contained two or more isolates. The number of isolates from each of the production units sampled is shown in Table 3.2. Twenty-four pulsotypes were shared between two or more production units and 8 pulsotypes were identified as persistent (data not shown). For this study, persistence was defined as the same pulsotype obtained from one company for at least six months. Six companies out of 13 showed the presence of at least one persistent pulsotype, with a maximum of 5 persistent pulsotypes in one company (Table 3.2).
Table 3.2 Pulsotypes from different food premises. Each Company shares between 1 and 13 pulsotypes with other food premises and persistent pulsotypes were identified in six Companies

<table>
<thead>
<tr>
<th>Company</th>
<th>Number of pulsotypes</th>
<th>Number of persistent pulsotypes</th>
<th>Number of pulsotypes shared with other Companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>17</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>M2</td>
<td>12</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>M3</td>
<td>15</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>M4</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M5</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>M6</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M7</td>
<td>9</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>M8</td>
<td>17</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>M9</td>
<td>7</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>M10</td>
<td>12</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>M11</td>
<td>11</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>M12</td>
<td>11</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>M13</td>
<td>12</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 3.1 shows a Minimum Spanning Tree that was obtained from a comparison of the isolates, using BioNumerics. Within a circle, each segment represents an isolate, while the circle represents a pulsotype where the isolates show > 90% similarity. The distance between the clusters is correlated with the genetic distance obtained from the dendrogram and the colours represent the serogroup of each isolate. Lineages I and II are identified.
Figure 3.1. Minimum spanning tree summarising the data from the PFGE profiles of the 279 isolates tested. Within a circle, each segment represents an isolate, while the circle represents a pulsotype where the isolates show > 90% similarity. The length of the line between the circles represents the distance of the relationship between the pulsotypes/isolates. Pulsotypes with only one isolate are represented by a circle with no segments. The distinction between Lineage I and II is shown and the serogroups are represented by different colours: green, 1/2a 3a; red 4b 4d 4e; violet 1/2b 3b 7.
Within 7 of the 77 pulsotypes there are strains of a different serogroup, and there is one pulsotype that had an isolate from each serogroup. Furthermore, 11 anomalies with the correlation between serogroups and lineages were detected, namely three serogroup 4b-4d-4e and three serogroup 1/2b-3b-7 isolates (which are considered Lineage I) clustered into Lineage II, and five serogroup 1/2a-3a isolates (which are considered Lineage II) clustered into Lineage I.

The overall prevalence of *L. monocytogenes* on mushroom samples was 3.80%. The positive samples were confined to two facilities and the numbers were always <100 cfu/g. The PFGE analysis revealed that the genetic profiles obtained from the mushroom isolates were indistinguishable (>90% similar) from some of the profiles of isolates from the production environment, in particular from floors and drains, thus indicating a possible cross-contamination scenario.

3.4.2 Comparisons with other PFGE profiles

The PFGE profiles of the isolates obtained in this study were compared with 350 isolates from other food premises in the Republic of Ireland (RoI). From the 87 pulsotypes identified in the previous study (Leong et al., 2017), 7 pulsotypes (similarity >90%) were common to the two groups (Figure 3.2). Six of the common pulsotypes were found previously in the dairy industry, and one was found previously in a fish store.
Figure 3.2. Comparison with isolates from different food sectors in Ireland. M, companies of the mushroom production; Rol Dairy/Fish, dairy and fish producers in the Republic of Ireland.
Figure 3.3 shows some comparisons of the PFGE profiles from this study with profiles obtained from food sectors outside the RoI. In particular, similarities > 90% were found with isolates obtained from Romania (Figure 3.3a), Australia (Figure 3.3b), Turkey (Figure 3.3c), Austria, Slovakia (Figure 3.3d) and Northern Ireland (Figure 3.3e). A number of pulsotypes were also shared with profiles from clinical isolates stored in the database (Figure 3.4).
Figure 3.3. Comparisons showing similarities between mushroom isolates in the Republic of Ireland (Rol M) and isolates from Romania (A, Romania 1 and 2), Australia (B), Turkey (C), Austria and Slovakia (D) and Northern Ireland (E, NI).
Figure 3.4. Comparison between isolates from the mushroom industry (M) and clinical isolates. UCHG, University College Hospital Galway; CSF, Cerebrospinal fluid.
3.5 Discussion

The results of this study showed that there is a large diversity of genetically distinct *Listeria monocytogenes* in the mushroom production chain, represented by 77 distinguishable pulsotypes. As shown in Figure 3.1, the isolates were distributed into Lineages I and II, and the serogroup distribution into the lineages is coherent with other studies in the majority of the cases; serogroup 1/2b-3b-7 and serogroup 4b-4d-4e in the Lineage I and serogroup 1/2a-3a in the Lineage II (Brosch et al., 1994), although some exceptions can be seen. This sporadic strain differentiation into different Lineages has been observed in previous studies and is partly explained with theories involving horizontal gene transfer between different *Listeria* species (den Bakker et al., 2013; Nadon et al., 2001). Subsequent studies on, for example, whole genome sequencing would be required to better clarify these evolutionary dynamics.

Agglutination tests with antisera are commonly used to serotype isolates, but serogrouping multiplex PCR was chosen as previous studies have shown good correlation between serogroup and serotype. Leong et al., (2017) and Murugesan et al., (2015) have shown that in 100% of cases, isolates belonging to the serogroup 1/2a-3a were actually serotype 1/2a, isolates belonging to serogroup 1/2b-3b-7 were actually serotype 1/2b and isolates belonging to serogroup 4b-4d-4e were actually serotype 4b. Thus, while not absolutely definitive, serogrouping is a good indication of serotype. In addition, the agglutination serotyping test doesn’t discriminate between serotypes 4b and 4e (Garcia et al., 1990).

The high diversity of strains obtained from this study suggests that *Listeria monocytogenes* is present in the mushroom production, reflecting its ubiquitous presence in the environment in general. Mushroom growing conditions represent a suitable ecological niche for occurrence and survival of *L. monocytogenes*, involving variable
temperatures, moist humid conditions and organic substrates. Even though pasteurisation of some of the most high-risk raw materials is undertaken, the possibility of post-pasteurisation contamination and cross-contamination exist. The distribution of the isolates in and between the facilities showed both the presence of persistent strains and continuous re-contamination of the site with strains of a different PFGE profile. Possible sources of these strains could be raw materials, transport of raw materials or the general production environment.

Many pulsotypes were shared between different facilities, as shown in Table 3.2. This could arise from common visitors to many different sites, transport crates that may be used by multiple facilities or raw material suppliers. This indicates that some strains of *L. monocytogenes* can persist in the facility for months, resisting the regular hygiene treatments. Persistence of *L. monocytogenes* is an issue that needs to be investigated carefully, and PFGE characterisation is a very powerful tool for demonstrating the presence of persistent isolates in the production environment. In some cases persistence have been described with Multi-Virulence-loci-Sequence-Typing (MVLST) (Murugesan et al., 2015), but this subtyping method does not have the discriminatory power of PFGE as it is based on the characteristics of only a few genes, whereas a PFGE profile is based on the whole genome of each isolate.

In mushroom production, there is a variety of hygiene procedures, including one or more ‘cook-out’ steps and the use of sanitisers. Cook-out is a heat process where the temperature of the growing house is brought up to 50 °C to 70 °C for a time of at least 8 hours. The aim is to inactivate all the possible pests and pathogens in the growing environment. However, the time-temperature combination varies and not all facilities perform a cook-out procedure. Despite the high temperature aimed at, it is possible that not all parts of the facility reach the required temperature (floors, for example; Chapter 4 of this thesis), and, therefore, *L. monocytogenes* may survive. *L. monocytogenes* can form
biofilm, which can increase its survival of stress (Frank and Koffi, 1990). Some isolates from this study have shown the ability to produce biofilm (data not shown). It is not very clear if *L. monocytogenes* is able to increase its minimum inhibitory concentration of the most common sanitisers (Lourenco et al., 2009), although it has been described in many studies that the complex matrix forming the biofilm can develop a protective effect, enhancing *L. monocytogenes* resistance to sanitisers, like benzalkonium chloride, hydrogen peroxide, peracetic acid and sodium hypochloride (Pan et al., 2006; van der Veen and Abee, 2011; Henriques and Fraqueza, 2017). The survival cells are possibly able to colonise again the working environment, increasing the risk of cross-contamination.

Serotypes 1/2a, 1/2b and 4b have been implicated in >90% of human listeriosis cases (Farber & Peterkin, 1991; McLauchlin, 1990). In this study, 40.8% of the isolates serogrouped 1/2a-3a, 30.1% were 1/2b-3b-7 and 29.1% were 4b-4d-4e. Lineages I and II contain serotypes that have been implicated in human illness, thus becoming a matter of concern for the food business operators. Many isolates, as shown in Figure 3.4, have highly similar PFGE profiles with some clinical isolates, indicating that these strains have disease-causing potential. Further studies would be required to characterise these particular isolates to determine the relatedness to the clinical isolates. Whole genome sequencing could provide information about the presence of virulence and antibiotic resistance genes, and comparison with other *L. monocytogenes* genomes for Single Nucleotide Polymorphisms (SNPs) analysis could reveal more details about diversity. However, epidemiological data and food attribution studies are necessary in association with WGS to definitively relate strains to illness.

Other isolates showed similarities with those from different companies both in Ireland and abroad, in particular with Turkish, Romanian, and Northern Ireland isolates (Figure 3.3). The existence of the so called “Global Clones” of *L. monocytogenes* has been
already discussed in the past (Chenal-Francisque et al., 2013; Leong et al., 2017), but further studies would be required to understand the origin and evolution of these clones.

Given the diversity and nature of the facilities involved in the mushroom industry, \textit{L. monocytogenes} was commonly found. The risk of cross-contamination to the final product is high, considering the utilisation of ladders and platforms for picking the mushrooms from the upper shelves, the transport boxes and the necessary handling of the final product. The low prevalence of positive mushroom samples indicates that the strategies undertaken by the producers to reduce cross-contamination are successful. These good, but improvable, methods for preventing the occurrence on mushrooms are important as whole closed cap \textit{Agaricus bisporus} do not support the growth of \textit{L. monocytogenes}, although sliced mushrooms may support growth of \textit{L. monocytogenes} (Leong et al., 2013). Actions already in place to reduce cross-contamination are, for example, the regular use of sanitisers and heat treatments at the end of the crop harvest (LaBorde, 2017), plus the pasteurisation of the compost prior to delivery to the production facility (Weil et al., 2013). Additionally, advice on improving hygiene practices is available (Dzingirayi & Korsten, 2016) and the utilisation of novel technologies, like bacteriocins and bacteriophages alone or in addition to other sanitation procedures (Bolocan et al, 2017; Lacumin \textit{et al}., 2016; Schmelcher and Loessner, 2016; Cláudia \textit{et al}., 2017) has been proposed.
3.6 Conclusions

In this study, characterisation of *L. monocytogenes* isolates by PCR and PFGE showed different scenarios in the mushroom industry. There is a variety of pulsotypes, some of them persistent, other are brought by continuous recontamination. The comparison of pulsotypes showed similarities with other facilities, this highlighting possible routes of cross contamination, but also the existence of global clones of *L. monocytogenes*. In conclusion, these results show a good degree of hygiene practices, but improvements need to be done to reduce the possibility of cross contamination to the mushrooms.
3.7 References


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is associated with increased resistance to surfactant sanitizers and heat. *J Food Prot* **53**, 550-554.


Chapter 4

Effectiveness of hygiene practices currently in use for the decontamination of *Listeria monocytogenes* from the mushroom production environment

To be submitted to Post Harvest Biology and Technology
4.1 Abstract

The commercial production of *Agaricus bisporus* is a well-established 3-stage procedure. The first stage is the production of compost, where the mushroom is inoculated. The second stage is the production of casing soil and the third stage is the production of the mushrooms. The compost used by the mushroom industry is based on straw and manure and it is produced in three phases: phase I, mixing and incubation; phase II, pasteurisation; phase III, inoculation and colonisation of the mushroom spawn. Casing soil is a mixture of peat and lime and its natural microflora stimulates the mushroom growth. In a mushroom production facility, casing is layered on top of phase III compost and the manipulation of temperature, humidity and CO₂ content facilitates the colonisation of casing soil and production of mushroom fruiting bodies. Hygiene practices are commonly in place at each step of mushroom production. These include pasteurisation of compost, hygiene practices applied at the production of casing soil, post-harvest cookout and sanitation at the mushroom production level. However, pests, diseases and foodborne pathogens, like *L. monocytogenes*, are often present in the mushroom production environment. In this work, the presence of *L. monocytogenes* was evaluated before and after the application of hygiene practices at each of the three stages of mushroom production. Swabs and samples were taken before and after the procedures were undertaken and the results were compared to assess the efficacy of such treatments. The results showed absence of *L. monocytogenes* in phase II compost samples, while it was detected in 40% of phase III samples. In the casing soil production unit, 31% and 22% of samples were positive before and after corrective actions were undertaken, but no statistically significant difference was shown (p>0.05). In the mushroom growing units, the cookout inactivated *L. monocytogenes* on the shelves, but 13% and 19% of samples, taken after the complete process of sanitation, were positive.
These results show the possibility of *L. monocytogenes* re-contamination of phase III compost, cross-contamination at the phase of casing soil production and possible cookout and sanitation survival of the organism at the mushroom growing facilities.
4.2 Introduction

*Listeria monocytogenes* is a foodborne pathogen of major concern in the food industry, especially in the Ready to Eat (RTE) foods sector (Farber & Peterkin, 1991). Its ability to cause disease and its presence in food leading to product recalls is a threat to public health and for the food production industry (Buchanan et al., 2017). *Agaricus bisporus* is a widely distributed industrially-produced mushroom, considered RTE because of its usage in raw salads (Anonymous, 2006). No outbreaks of listeriosis have been recorded due to *L. monocytogenes* in mushrooms, but there have been several product recalls in recent years, raising awareness among the mushroom producers of this potential hazard (EU, 2019; FSAI, 2006).

Mushroom production can be divided in three stages: 1) phase III compost production, 2) casing soil production and 3) mushroom production, as described in Chapter 1. In Stage 1, the phase III compost used in mushroom production is a mixture of manure and straws, incubated for 12 days in a process called “phase I”, where the microbial aerobic digestion starts and the temperatures can reach 80°C (Zhang et al., 2014). The process continues with a pasteurisation step (phase II), a heat treatment at 60°C for 14 h to remove the unwanted microflora and create the conditions for the mushroom colonisation (Straatsma et al., 2000). After pasteurisation, the compost is inoculated with *A. bisporus* mycelium and incubated aerobically at 25°C for 16-19 days (this stage is called phase III), before being delivered to the mushroom growing facilities (Vos, 2017). In Stage 2, casing soil is produced for use in the mushroom production. It is placed on top of phase III compost and its natural microflora stimulates the growth of the mushroom fruiting bodies (McGee, 2018). Casing soil production consists of mechanically mixing peat and lime. In order to preserve the natural microflora of the raw materials that influences mushroom development, there are no antimicrobial steps during its production (Cai et al., 2009;
McGee, 2018; Siyoum et al., 2010), however, hygiene hurdles for cross-contamination reduction, such as the use of sanitisers and the building of physical barriers between storage areas, are in place. In Stage 2, at the mushroom growing facilities, phase III compost is topped with a layer of casing soil and incubated at 22-25°C for 7 days. Then, by modifying the temperature, humidity and CO$_2$ content of the growing room, the fruiting body of the mushroom is stimulated and formed (Kertesz & Thai, 2018). After harvest of the mushrooms, which usually lasts about 3 weeks per crop, the growing room is heat treated, cleaned and sanitised in a process called ‘cookout’, before a new mushroom crop is started.

While the cleaning and disinfection procedures are not standardised across mushroom growing facilities, in general, there is an initial heat-treatment at 60-70 °C for 18-20 h which is followed by a sanitisation process (often including disinfection with sodium hypochlorite). The growing room is then emptied and cleaned with a power hose. This may be followed by a second heat-treatment at 60-70°C for 3-6 h, to ensure that all the surfaces are sanitised before a new crop was started.

The aim of the work described in this chapter was to assess the efficacy of the hygiene procedures applied at the three stages of mushroom production: pasteurisation of the compost, hygiene practices applied at the production of casing soil, post-harvest cookout and sanitation at the mushroom production level.
4.3 Materials and methods

4.3.1 Sampling plan

One casing, one compost and two mushroom producers agreed to participate in the study of the assessment on the effectiveness of hygiene procedures in the mushroom production chain. The sampling plan is schematised in Figure 4.1.

The casing facility was visited on seven occasions. Three visits were undertaken at the start of the project when there was little attention paid to *L. monocytogenes* at the facility, and four visits after the company implemented some basic hygiene processes:

- Washing the conveyor belts every two months with peracetic acid;
- Loaders washed twice per day with a disinfectant;
- Introduction of pools for boot disinfection at the entrance and exit of all the areas;
- New bays to store all the casing indoor were built;
- Some exit areas between the storage bays were closed, to better organise the traffic of lorries and loaders;
- The lime stock was reduced from a monthly quantity to a quantity usable in one week, with a new batch of lime delivered every week

At each visit, swabs were taken from conveyor belts in the general facility, floors and loaders, while casing samples were taken from the storage bays and conveyor belts in the storage bays.

The compost producer agreed to ship the samples of Phase II and Phase III compost via express courier and the samples were analysed within 24 h of being taken.
Figure 4.1. Schematic representation of the sampling plan. In the compost production unit, samples were analysed after phase II and phase III. In the casing production unit, samples and swabs were analysed before and after the application of new hygiene practices. In the mushroom production facilities, samples and swabs were analysed before and after the first cookout and swabs were analysed before the start of a new mushroom crop.
The two mushroom growers were visited to collect floor swabs and samples of a mixture of casing, compost and mushroom residues from the growing shelves, at the end of mushroom production, before and after the cookout process and before starting a new mushroom crop. Temperature probes (LS Technology, UK) were placed on the shelves and on the floor during cookout.

4.3.2 L. monocytogenes detection and enumeration

All swabs were tested for the presence of *L. monocytogenes*, as described by the BS EN ISO 11290-1:1997+A1:2004. Raw materials (casing and compost from raw materials producers) and samples of used mix of casing and compost taken at the end of the mushroom crop, before and after cookout, were tested for the presence and number of *L. monocytogenes*, as described by the BS EN ISO 11290-2:1998. One presumptive colony (blue-green with an opaque halo) from each positive sample was grown in Brain Hearth Infusion (BHI) and stored in cryovials containing 20% glycerol at -20°C for further characterisation.

4.3.3 Characterisation of isolates

To confirm the isolates as *L. monocytogenes*, Polymerase Chain Reaction (PCR) was performed according to Ryu et al., (2013). The isolates were serogrouped by PCR according to Doumith et al., (2004) and Pulsed Field Gel Electrophoresis was performed according to the PulseNet protocol (Anonymous, 2013). BioNumerics 7.6 (Applied Maths) was used for strain comparison.

4.3.4 Statistical analysis

The data obtained were processed with SPSS (IBM, USA) to determine if there was a significant difference between the prevalence of *L. monocytogenes* before and after on the application of hygiene practices. Briefly, the data were analysed for normality distribution
with descriptive statistics and, based on the results, parametric or non-parametric tests (ANOVA and Kruskal-Wallis, respectively) were performed.
4.4 Results

4.4.1 Occurrence of *L. monocytogenes* in Casing

From the casing company, a total of 96 samples were analysed. In total, the samples taken before the corrective action on hygiene was implemented showed an overall occurrence of *L. monocytogenes* of 31%, whereas the samples taken afterwards were 22% positive (Table 4.1). The casing samples were 34% positive before the corrective actions and 20% positive after the corrective actions. The swabs were 27% positive before and 29% positive after the corrective actions. After corrective action on hygiene was implemented, the first batch of casing samples showed absence of *L. monocytogenes*, but 3 months later the occurrence in the casing increased to 20%. The prevalence of *L. monocytogenes* in casing samples and swabs tested before and after corrective action on hygiene was implemented showed no significant difference (p>0.05).

Table 4.1. Occurrence of *L. monocytogenes* in a casing production facility before and after structural and hygiene practice improvements

<table>
<thead>
<tr>
<th></th>
<th>Before corrective actions</th>
<th>After corrective actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casing Total</td>
<td>26</td>
<td>30</td>
</tr>
<tr>
<td>Casing Positives</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Casing % Positives</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Swabs Total</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>Swabs Positives</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Swabs % Positives</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>Total Samples</td>
<td>59</td>
<td>37</td>
</tr>
<tr>
<td>Total Positives</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>Total Positives %</td>
<td>31</td>
<td>22</td>
</tr>
</tbody>
</table>
4.4.2 Occurrence of L. monocytogenes in Compost

Samples of Phase II compost, obtained immediately after pasteurisation, showed an absence of *L. monocytogenes*, while samples obtained from Phase III compost showed an occurrence of 40% (p<0.05, Table 4.2).

Table 4.2. Occurrence of *L. monocytogenes* in a compost producer after pasteurisation (phase II) and on the compost ready for the mushroom production (phase III).

<table>
<thead>
<tr>
<th></th>
<th>Phase II</th>
<th>Phase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Positives</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Positives %</td>
<td>0</td>
<td>40</td>
</tr>
</tbody>
</table>
4.4.3 The effectiveness of cookout in the mushroom growing facilities

The two mushroom producers used slightly different hygiene processes between crops of mushrooms (Figure 4.2). In Company 1, following the first cookout process, where the room temperature was raised to 70 °C for 12 h, the room was emptied, cleaned with a power hose and sodium hypochlorite (1%) was applied to all the surfaces. A second cookout raised the temperature of the growing room to 70 °C for 3 h, followed by another treatment with sodium hypochlorite. In Company 2, a first cookout process raised the temperature of the growing room to 70 °C for 12 h. The room was then emptied, cleaned with a power hose and disinfected with a quaternary ammonium compound based product (Omnicide 2%). A second cookout of 70 °C for 12 h was done, before starting a new mushroom crop.

Figure 4.2. Post-harvest hygiene procedures applied by the two mushroom producers.
During the first cookout process at Company 1, the temperature reached almost 70 °C for 10 h in the used raw materials on the shelves, but less than 50 °C on the floor (Figure 4.3A and 4.3B). Before cookout in Company 1, the incidence of *L. monocytogenes* was on average 50%, with 100% of the floor swabs and 25% of the spent substrates samples positive (Table 4.3). After the first cookout there was an overall presence of *L. monocytogenes* of 17%, with 56% of the floor swabs positive and no positives in the spent substrates. Floor swabs taken after the entire process of sanitation, just before the start of a new crop, showed 13% of positives (p<0.05).

![Figure 4.3. Temperatures recorded during the cookout in Company 1 (A and B) and Company 2 (C and D). Each point is the average of three measurements and the Standard Deviation is represented by the error bars.](image-url)
Table 4.3. Effect of the cookout and sanitation in Mushroom Company 1. The table shows the occurrence of *L. monocytogenes* before the first cookout (“Before” column), after the first cookout (“After” column) and before starting a new mushroom crop (“Final” column).

<table>
<thead>
<tr>
<th>Company 1</th>
<th>Before</th>
<th>After</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swabs Total</td>
<td>10</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Swabs Positives</td>
<td>10</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Swabs Positives %</td>
<td>100</td>
<td>56</td>
<td>13</td>
</tr>
<tr>
<td>Samples Total</td>
<td>20</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Samples Positives</td>
<td>5</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Samples Positives %</td>
<td>25</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>Total Positives</td>
<td>15</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Total Positives %</td>
<td>50</td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>
During the first cookout process at Company 2, the temperature also reached almost 70°C for 10 h in the used raw materials on the shelves and between 50 and 60°C on the floor for 3 h (Figure 4.3C and 4.3D). Before cookout, the incidence of *L. monocytogenes* was on average 63%, with 75% of the floor swabs and 45% of the spent substrates samples positive (Table 4.4). After the first cookout there was an overall presence of *L. monocytogenes* of 40%, with 67% of the floor swabs positive and no positives in the spent substrates. Floor swabs taken after the second cookout, just before the start of a new crop, were 19% positive (p<0.05).

Table 4.4. Effect of the cookout and sanitation in Mushroom Company 2. The table shows the occurrence of *L. monocytogenes* before the first cookout (“Before” column), after the first cookout (“After” column) and before starting a new mushroom crop (“Final” column).

<table>
<thead>
<tr>
<th>Company 2</th>
<th>Before</th>
<th>After</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swabs Total</td>
<td>16</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Swabs Positives</td>
<td>12</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Swabs Positives %</td>
<td>75</td>
<td>67</td>
<td>19</td>
</tr>
<tr>
<td>Samples Total</td>
<td>11</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Samples Positives</td>
<td>5</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Samples Positives %</td>
<td>45</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Total Positives</td>
<td>17</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Total Positives %</td>
<td>63</td>
<td>40</td>
<td>19</td>
</tr>
</tbody>
</table>
One isolate for each positive sample and swab was taken for further characterisation. All the isolates were confirmed as *L. monocytogenes* by PCR. Five isolates were recovered at the end of the sanitation process, before the start of a new crop, two from Company 1 and three from Company 2. The isolates were serogrouped by PCR, resulting in the 2 isolates from Company 1 being serogroup 1/2a-3a, 2 isolates from Company 2 being serogroup 4b-4d-4e and one isolate from Company 2 being serogroup 1/2b-3b-7.

**4.4.4 Pulsed Field Gel Electrophoresis analysis**

Five isolates in total, obtained from floor swabs after the cleaning and sanitising process in the two mushroom growing facilities, were characterised by PFGE. The profiles obtained are shown in Figure 4.4A and 4.4B. In Company 1, the two isolates were 100% similar, while the three isolates tested from Company 2 showed 3 distinct pulsotypes.

The pulsotype obtained from Company 1 showed about 94% similarity to an isolate obtained from casing sampled during mushroom growth at another facility (Figure 4.5). This pulsotype also showed about 90% similarity to an isolate obtained from a compost sample from Company 2 (which was obtained from a previous study, described in Chapter 2 of this thesis) and to an isolate from mushrooms obtained previously (Chapter 2) from another mushroom growing unit (Figure 4.5). It was 100% similar to the PFGE profile of an isolate obtained previously (Chapter 2) from a compost producer (Figure 4.6).
Figure 4.4. Dendrograms showing the PFGE profiles of isolates obtained from Company 1 (A) and Company 2 (B). The bars on the left show the percentage of similarity.
Figure 4.5. Similarity between the pulsotype obtained in this study from Company 1 and 3 more isolates obtained from Company 2, 3 and 4, in a previous study (Chapter 2 of this thesis).
Figure 4.6. Dendrogram showing 100% similarity between the PFGE profiles of the 2 *L. monocytogenes* isolates obtained from Company 1 and one isolate obtained from a compost producer.
4.5 Discussion

The results of this study indicate that the current hygiene practices employed in mushroom production do not appear to eliminate *L. monocytogenes* from the mushroom growing facilities, and if they do, recontamination occurs. In particular, pasteurisation of the compost, was shown to be efficient in deactivating *L. monocytogenes* as no positives were found immediately after pasteurisation. However, some positives were found in the Phase III samples. The sample size in this work is small, because only one compost producer accepted to participate to this project. Furthermore, after phase II, the procedure for inoculating the compost with mushroom spawn is very strict regarding sterility and obtaining samples during this delicate step was not easy. Weil et al., (2013), conducted a study to establish the efficiency of compost pasteurisation on inactivation of various pathogens, including *L. monocytogenes*. The study showed that only two hours at 60 °C were necessary to completely kill *L. monocytogenes* in Phase II compost. However, the results shown here suggest that the manipulation of Phase II compost is a critical point, where cross contamination can occur during the Phase III or after Phase III, during the transportation. For that reason, more studies are required to clarify the routes of cross contamination during production of Phase III compost before it is delivered to the mushroom growers.

After the first sampling at the casing producer, corrective actions to address the relatively high occurrence of *L. monocytogenes* were suggested. Following the implementation of these corrective actions there was a reduction of *L. monocytogenes* to undetectable levels only on the first sampling occasion. On subsequent sampling occasions there was an overall occurrence of 20%.

Some studies have shown the possibility of *L. monocytogenes* surviving in the casing soil, and suggested pre-harvest treatments for pathogen control on the mushrooms, especially with intervention on the irrigation water (Chikthimmah, 2006). In a casing soil
production facility, it is almost impossible to control the spread of *L. monocytogenes*, because of its ubiquitous presence, especially in the soils (Weis and Seeliger, 1975). The hygiene improvements undertaken by the casing producer reduced the presence of *L. monocytogenes* at the first sampling time, but after three months positive samples were detected again. For that reason, more studies are needed to better establish the routes of cross contamination in a casing production environment.

The PFGE profiles obtained in this study showed a possible cross contamination scenario from the raw materials to the mushroom growing facility (Figure 4.5 and 4.6) and cross contamination to the mushrooms (Figure 4.5). In addition, 3 isolates obtained from 3 different mushroom producers sampled in a previous study (Chapter 2 of this thesis) showed high similarity with the PFGE profile of the isolates obtained after the sanitation process in Company 1 (Figure 4.5). Previous studies have already highlighted *L. monocytogenes* persistence and cross contamination in the mushroom industry in the Republic of Ireland (Leong et al., 2017; Madden et al., 2018; Pennone et al., 2018). However, in this study, no similarities were found between the five isolates characterised by PFGE and isolates obtained from other food sectors or from clinical isolates (data not shown). Further studies with a higher number of samples and isolates might provide a higher number of PFGE profiles with more similarities between isolates obtained in previous studies.

The cookout process used by both the facilities in this study did not totally inactivate *L. monocytogenes*. No isolates were obtained, after cookout, from the used casing/compost mixture on the growing shelves. At these locations, the temperature reached the target value of 70 °C and there was inactivation of *L. monocytogenes* in all cases. However, isolates were obtained, after cookout, from the floor samples at both facilities. The temperature of the floor did not reach the target value (Figure 4.3) that would inactivate *L. monocytogenes*. None of the floors at the facilities were insulated, making it difficult to
achieve the desired temperature. Particular attention should be given, in the construction of new mushroom growing houses, to the insulation of the floors, to achieve the target cookout temperature. In addition to surviving the cookout temperature of the floors, the strains that survived also resisted sanitation and a further heat treatment. It is possible that the strains were resistant to the sanitation process, but it is more likely that they survived in niches where the sanitiser concentration was more diluted (for review see Carpentier & Cerf, 2011), or in areas where the sanitation process was not completed properly, or where biofilms are formed (Lourenco et al., 2011). Further characterisation of the isolates with regard to heat resistance and sanitiser resistance is needed. Either way, the results of this study show that cookout as it is practiced at these two facilities is not as effective as thought. Improvements in the floor disinfection are needed after the cookout in order to achieve fully inactivation of \textit{L. monocytogenes} between crops of mushrooms.

Based on the results of this study, multiple critical points for control of \textit{L. monocytogenes} are recognisable in a mushroom growing facility:

- \textit{L. monocytogenes} can enter in the facility via the raw materials, such as casing and compost;

- \textit{L. monocytogenes} can survive the cookout process, as the floor does not reach the required temperature;

- Biofilm formation, sanitiser resistance and persistence characteristics decrease the possibility of successfully removing \textit{L. monocytogenes} from the processing environment.

Alternative approaches are needed to control \textit{L. monocytogenes} during mushroom production. One such approach is to use bacteriophages and recombinant proteins to control the pathogen at production level (see Chapters 5, 6 and 7). In addition, training in awareness of \textit{L. monocytogenes} at production facilities and reduction of cross contamination, as discussed in Chapter 8, may reduce the risk of mushroom contamination. The efficacy of post-harvest treatments of whole or sliced mushrooms against \textit{L.
monocytogenes has not been addressed in the current study, but treatment with bacteriophages, various sanitisers and UV light has been studied by Murray et al. (2015). However, issues with efficacy, the quality of the mushrooms and the applicability of the processes on large scale need to be addressed.
4.6 Conclusions

In this study it was shown that the efficacy of the actual hygiene practices at the three levels of mushroom production (casing, compost and mushroom production) are not always effective in the removal of *L. monocytogenes*. The main issues are related to the presence of *L. monocytogenes* in the raw materials and the ineffectiveness of the cookout procedure on the floors. More in-depth studies are required to address the issue of cross contamination at every level of the mushroom production, by the individualisation of structural (insulation of the floors) and practical (new hygiene procedures) improvements.
4.7 References


Clinical Microbiology, 42(8), 3819–3822.


Chapter 5

Reduction of *L. monocytogenes* by the amidase domain of the phage vB_LmoS_293 endolysin: *in-vitro* studies

Submitted to Viruses
5.1 Abstract

Listeria monocytogenes is a ubiquitous Gram positive bacterium that is a major concern for food business operators because of its pathogenicity and ability to form biofilms in food production environments. To date, a number of bacteriophages (phages) against L. monocytogenes have been isolated and some have been approved for use in food processing environments (ListShield and PhageGuard Listex). Endolysins are proteins produced by phages in the host cell, that are able to cleave one of the five bonds of the peptidoglycan cell wall, thus allowing release of progeny phage into the environment. In this study, the amidase domain of the phage vB_LmoS_293 endolysin (293-amidase) was cloned and expressed in E. coli. The purified protein was then tested against L. monocytogenes for Minimum Inhibitory Concentration (MIC), lytic activity at different pH and temperature values, lytic spectrum and against biofilm. The results showed activity at three different temperatures (20 °C, 37 °C and 50 °C) and pH values (pH 4, 8 and 10), with a wider lytic spectrum compared to the phage (L. monocytogenes 473 and 3099, a serotype 4b and serogroup 1/2b-3b-7, respectively). The protein also showed the potential to inhibit the biofilm formation on abiotic surfaces. These results show the great potential of using recombinant antimicrobial proteins against pathogens.
5.2 Introduction

In recent years, a number of scientific projects have been focused on finding novel biotechnological approaches to prevent or remove *L. monocytogenes* biofilms (Bolocan et al., 2017; Carpentier & Cerf, 2011; Gutiérrez et al., 2017). Promising results have been obtained with the utilisation of phages for *L. monocytogenes* removal (Gironés et al., 2016; Gutiérrez et al., 2017; Iacumin et al., 2016; Oliveira et al., 2014). Phages can infect host bacteria cells usually with high specificity and they can be divided in two classes: virulent phages with a lytic life cycle, where the phages infect the host cells, using it to produce new progeny that are immediately released, with consequent cell lysis; or temperate phages, where after infecting the host cells the phages, can integrate their DNA into the host genome and release their progeny only when particular conditions trigger the assembly of phage particles (Howard-Varona et al., 2017).

For *L. monocytogenes* biocontrol in the food industry, two products containing phages are currently commercially available, Listex P100 (www.phageguard.com) and ListShield (www.intralytix.com). Listex P100 contains the phage P100, a wide spectrum virulent listeriophage, which has been proven to remove *L. monocytogenes* from foods and biofilms on food processing environment surfaces (Carlton et al., 2005; Iacumin et al., 2016; Oliveira et al., 2014; Soni & Nannapaneni, 2010a, 2010b). ListShield is a cocktail of 6 listeriophages with proven efficacy against a wide spectrum of *L. monocytogenes* strains, both in foods and on biofilms (Bai et al., 2016; Gutiérrez et al., 2017; Perera et al., 2015; Sadekuzzaman et al., 2017; Yang et al., 2017). Both phage preparations have been approved for use by the Food and Drugs Administration (FDA) to be used during food production and are under revision by the European Food Safety Authority (EFSA et al., 2016).
For the perspective of using live phages as biocontrol agents, only virulent phages can potentially find an application (Hagens & Loessner, 2010). The main reason is for the ability of temperate phages to potentially transfer genetic material from one host to another, in a mechanism called “transduction” (Wagner & Waldor, 2002). In this way, virulence genes or antimicrobial resistance genes can potentially be introduced into the host genome. An example is the case of *Vibrio cholerae*, where the *ctxA* and *ctxB* genes, coding for the cholera CTX toxin, are integrated into the bacterial genome by the phage CTXφ (Waldor & Mekalanos, 2006). Furthermore, phage resistance can develop in the bacteria albeit at a very low rate (Fister et al., 2015). For these reasons, alternative applications of phages and phage-derived proteins for *L. monocytogenes* inactivation are being investigated, including the use of phage endolysins (or lysins) (Ajuebor et al., 2016). Endolysins are enzymes encoded by the phage genome involved in the release of phage progeny from the host cell. Their mode of action is through the cleavage of the peptidoglycan of the host cell wall (Loessner, Wendlinger, & Scherer, 1995). They usually act in synergy with other phage encoded proteins, known as holins, a diverse group of small proteins that are involved in the formation of holes in the inner membrane of the host cell, allowing the endolysin access to the peptidoglycan (Wang et al., 2000).

Based on the presence of certain catalytic domains which direct which peptidoglycan bond is cleaved, phage endolysins can be divided in five groups: Endo-β-N-acetylglucosaminidase, N-acetylmuramidase, Endopeptidase, N-acetylmuramoyl-L-alanine amidase and γ-D-glutamyl-L-lysine endopeptidase (Ajuebor et al., 2016). Using recombinant endolysin as an antimicrobial instead of live phages has mainly the advantage of limiting the possibility of development of bacterial resistance to the endolysin, because of the high specificity of the endolysins to the peptidoglycan bonds. A mutation at this level would most likely compromise the fitness of the bacterial cells (Fischetti, 2010).
Studies have already shown reduction of *L. monocytogenes* through the application of endolysins. For example, PlyP100, the endolysin encoded by the listeriophage P100, has shown antilisterial activity when applied to drained curd, during the production of fresh cheese (Van Tassell et al., 2017). The same endolysin, combined with nisin, was applied during the production of queso fresco, showing reduction in *L. monocytogenes* numbers under the detection limit. By contrast, the application of nisin alone showed very little antilisterial activity at the time of application, with consecutive regrowth of *L. monocytogenes* to levels comparable to the untreated controls (Ibarra-Sánchez et al., 2018).

The endolysin LysZ5 of the phage FWLlm3, has shown more than 5 log_{10} CFU ml^{−1} reduction of *L. monocytogenes, L. innocua* and *L. welshimeri*, without affecting *S. aureus* or *E. faecalis* in soya milk (Zhang et al., 2012).

Further studies have shown the possibility of using the catalytic domains of endolysins rather than the full length protein as biocontrol agents. In some cases, this resulted in a display of increased activity compared to the full length endolysin, as shown by the activity of the cysteine- and histidine-dependent amidohydrolase/peptidase (CHAP) domain of the staphylococcal phage endolysin LysK against strains of *S. aureus* (Fenton et al., 2011). Here, the CHAP_{K} domain of LysK showed a broader lytic spectrum compared to the full endolysin and lytic activity at different temperatures, pH and concentrations (Fenton et al., 2011).

The phage vB_LmoS_293 is a temperate *Siphoviridae*, which infects strains of *L. monocytogenes* of the serotypes 4b and 4e (Casey et al., 2015). Previous whole genome sequencing of this phage by our group revealed its lysogenic nature (Casey et al., 2015). Issues associated with employing temperate phages for the purposes of biocontrol led to the pursuit of the endolysin of this phage as a potential biocontrol agent. The endolysin of phage vB_LmoS_293, an N-acetylmuramoyl-L-alanine amidase, was chosen for further study. The aim of this work was to produce a recombinant amidase, the catalytic domain of
the vB_LmoS_293 endolysin, referred to as 293-amidase, for further characterisation of antimicrobial activity.
5.3 Materials and methods

5.3.1 Bacterial strains and culture conditions

The *L. monocytogenes* strains used for this study are detailed in Table 5.1. All the strains were stored at -20 °C and grown in Brain Heart Infusion (BHI, Oxoid Ltd., UK) at 37 °C.

Table 5.1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Serotype/Serogroup</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em> 473</td>
<td>4b</td>
<td>Dairy industry</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 777</td>
<td>1/2c</td>
<td>Dairy industry</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 2075</td>
<td>4b-4d-4e</td>
<td>Mushroom industry</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 2081</td>
<td>1/2a-3a</td>
<td>Mushroom industry</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 3099</td>
<td>1/2b-3b-7</td>
<td>Mushroom industry</td>
</tr>
<tr>
<td><em>E. coli</em> Top 10</td>
<td></td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>E. coli BL21 (DE3)</td>
<td></td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
</table>
5.3.2 Bioinformatics analysis

The listeriophage vB_LmoS_293 genome was previously annotated and deposited in the GeneBank database with the Accession Number KP399678.1 (Casey et al., 2016). The sequence of the Open Reading Frame (ORF) coding for the phage endolysin was analysed with the Basic Local Alignment Search Tool (BLAST) against the Conserved Domains Database (CDD, Marchler-Bauer et al., 2017) for identification of the amidase domain (293-amidase). The primers for the 293-amidase cloning were designed with Serial Cloner 2.6.1 (Perez, 2004) to incorporate an Neol and Hindlll cleavage site at both 5' and 3' ends, respectively. In silico PCR and ligation were performed to predict the absence of dimers and the success of the cloning procedure.

5.3.3 Amidase cloning

**Plasmids used:** The sub-cloning of the 293-amidase was performed using the TOPO-TA cloning kit, which includes the pCR®2.1-TOPO® vector, following the manufacturer’s instructions. pCri-8A was chosen as the expression vector for this study. pCri-8A is a pET-28a based vector which allows the introduction of a His6-tag at the N-terminus of the cloned protein and conferred kanamycin resistance to the cells transformed with it (Goulas et al., 2014).

**DNA extraction:** Phage DNA was extracted as described by Casey et al., (2015). Briefly, 1 ml of cell lysate was incubated at 37 °C for 30 min with RNAse A (1mg/ml, Sigma, Ireland) and 12 U/ul of DNAse I (New England Biolabs, UK). After spinning for 5 min at maximum speed on a top bench centrifuge (Eppendorf, Germany), 100 μl of a mix of 0.5 M Tris-HCl pH 9, 0.25 M EDTA, 2.5 % SDS (Sigma, Ireland) was added to the supernatant and incubated at 65 °C for 5 min. Subsequently, 125 ul of 8 M Potassium
acetate was added and the mix was incubated at -20 °C for 5 min. After centrifugation for 5 min at 17000 X g, 2 X phenol chloroform-isoamyl alcohol (PCIA, Sigma, Ireland) extractions were performed and an equal volume of isopropanol was added to the top layer for DNA precipitation at -20 °C for 5 min. The DNA was then washed twice with 70 % ethanol and resuspended in 20 μl of PCR grade H$_2$O. The DNA was run on a 1 % agarose gel at 70 V for 30 min to verify its integrity.

**293-amidase sub-cloning:** For subcloning, the Polymerase Chain Reaction (PCR) and ligation reaction were performed using the TOPO-TA cloning kit (Thermo Fisher Scientific, US) following the manufacturer’s specifications. The pCR®2.1-TOPO®-293-amidase vector was transformed into chemically competent *E. coli* Top10 cells (Thermo Fisher Scientific, US), following the manufacturer’s instructions. The transformed cells were then plated on Luria Bertani (LB, Oxoid Ltd., UK) agar containing 50 μg/mL kanamycin and 40 μL of 40 mg/mL X-gal (Both supplied by Sigma, Ireland) and incubated at 37 °C overnight. White colonies were selected and screened for the presence of the insert with a colony PCR and grown overnight at 37 °C in LB broth with 50 μg/mL kanamycin, shaking at 250 rpm. Plasmid DNA was extracted from each of the selected colonies using the QIAprep Spin miniprep kit (Qiagen, Germany).

**293-amidase cloning into the pCri8A vector:** The 293-amidase was excised from the pCR®2.1-TOPO® vector by double digestion with the endonucleases *Ncol* and *HindIII* (Thermo Fisher Scientific, US) for 15 min at 37 °C and then loaded on a 1% agarose gel in Tris Borate EDTA (TBE, Sigma, Ireland) and run in an electrophoresis unit (Bio-Rad, US) at 70 V for 30 min. The same procedure was followed to double digest and linearise the expression vector pCri8A. The linearised pCri8A and the 293-amidase band were purified from the gel with the QIAquick gel extraction kit (Qiagen, Germany) and a ligation reaction (T4 DNA Ligase, New England Biolabs, UK) was performed overnight at room
temperature. The ligation product was then transformed into *E. coli* Top10 cells according to the manufacturer’s instructions. The transformed cells were plated on LB agar containing 50 µg/mL kanamycin and incubated at 37 °C overnight. A single colony was grown in LB broth with 50 µg/mL kanamycin, shaking at 250 rpm and the plasmids recovered with a mini prep kit and sequenced by Eurofins GATC, Germany.

**Sequence analysis:** Sequences were analysed with CLC Main workbench 8 (https://www.qiagenbioinformatics.com/products/clc-main-workbench/) and the plasmids containing the 293-amidase gene were transformed into chemically competent *E. coli* BL21(DE3) (Thermo Fisher Scientific, US) following the manufacturer’s instructions and plated on LB agar petri dishes containing 50 µg/mL kanamycin and incubated at 37°C overnight.

### 5.3.4 Protein expression and purification

One colony of *E. coli* BL21(DE3) transformed with pCri8a-293-amidase was incubated overnight in 10 ml of Terrific Broth (TB, 23.6 g/L Yeast Extract, 11.8 g/L Tryptone, 9.4 g/L K₂HPO₄, 2.2 g/L KH₂PO₄, 8 ml/L Glycerol) with 50 µg/mL kanamycin at 37 °C, shaking at 250 rpm. A 3 L flask containing 990 ml of TB containing 50 µg/mL kanamycin was inoculated with 10 ml of an overnight culture of *E. coli* BL21(DE3) transformed with pCri8a-293-amidase and incubated at 37 °C, shaking at 100 rpm. When the culture reached 0.5 OD₆₀₀, the cells were chilled on ice for 10 mins and then induced by adding 1 ml Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, Ireland) 1M (final concentration: 1mM) and incubated overnight at 16 °C. After incubation, the cells were chilled on ice for 10 mins and the cells harvested by centrifugation at 2000 x g for 30 mins at 4 °C (Sorvall™ RC 6 Plus, Fisher Scientific, Canada). The cell pellet was resuspended in 10 ml of lysis buffer (50mM Tris, 300mM NaCl, 10% glycerol) and frozen until lysis was performed. The thawed cell pellet was lysed by sonication (MSE Soniprep 150, Fisher
Scientific, UK) with 5 X medium frequency impulses for 10 secs followed by 10 secs of rest on ice. The lysate was spun on a top bench centrifuge at maximum speed for 30 mins at 4 °C and the clarified supernatant was filtered through a 0.22 μm syringe filter (Sarstedt, Germany). Subsequently, the filtrate was incubated with 2 ml of Immobilised Metal Affinity Chromatography (IMAC) resin containing Nitrilotriacetic acid charged with Ni$^{2+}$ (Ni-NTA) (Bio-Rad, US) for 1 hr on an orbital shaking platform. The mix was transferred to a gravity chromatography column (Econo-Pac, Bio-Rad, US) and affinity chromatography purification was performed according to the manufacturer, using a running buffer composed of 5 mM NaH$_2$PO$_4$, 30 mM Na$_2$HPO$_4$, 300 mM NaCl, 45 mM Imidazole. After three washing steps with running buffer, the 293-amidase was eluted in four fractions containing 145, 245, 345, 545 mM Imidazole respectively. All the fractions were run on a 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) in a Mini-PROTEAN® Tetra vertical electrophoresis cell (Bio-Rad, US) at 180 V for 1 h and stained with Coomassie blue to visualise the bands. The fractions containing the purified 293-amidase were pulled together and dialysed overnight at 4 °C in a dialysis membrane (Cut-off 10kDa, VWR International Ltd., Ireland) in 1 L of phosphate buffer (NaH$_2$PO$_4$ 5mM, Na$_2$HPO$_4$ 30mM, NaCl 300mM) to remove the imidazole. The purified protein was stored at -20 °C in 0.5 ml aliquots. The concentration of the 293-amidase was measured as the Optical Density at 575 nm (OD$_{575}$) using the Pierce BCA protein assay (Thermo Fisher Scientific, US), following the microtiter plate protocol from the manufacturer guidelines.

5.3.5 In-gel digestion and Matrix-Assisted-Laser-Desorption/Ionization Time-of-Flight (MALDI-TOF)

MS grade Pierce Trypsin Protease (Rockford, IL, USA) was dissolved in 50 mM acetic acid to a final concentration of 10 ng/μl. Three μl of trypsin solution were added to
10 µl of 293-amidase sample for digestion. Ten µl of 0.5 mM ammonium bicarbonate was added and the solution digested in a heating block at 37 °C for 3.5 h. Mass spectrometry was performed on the digested sample with an Axima TOF2 MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK). A 0.5 µl aliquot of matrix solution (α-cyano 4-hydroxy cinnamic acid, 10 mg/ml in 50 % acetonitrile-0.1 % (v/v) trifluoroacetic acid) was deposited onto the target and left for 5 secs before being removed. The residual solution was allowed to air-dry and 0.5 µl of the sample solution was deposited onto the pre-coated sample spot. A 0.5 µl aliquot of matrix solution was added to the deposited sample and allowed to air-dry. The sample was subsequently analysed in positive-ion reflectron mode. Protein identification was carried out via peptide mass fingerprinting (PMF) using the Mascot search engine (http://www.matrix-science.com). The monoisotopic, positive ion data +/- 0.25 Da was searched using the following parameters: NCBInr database or Swiss Prot, taxonomy all entries, trypsin digest with one missed cleavage.

5.3.6 Determination of Minimum Inhibitory Concentration (MIC) and specific activity

To determine the MIC, the 293-amidase was tested against L. monocytogenes 473, the strain used to propagate the phage vB_LmoS_293. For the preparation of cells, L. monocytogenes 473 was grown in 100 ml of BHI broth overnight and the cells were subsequently autoclaved at 121 °C for 15 mins (Astell Scientific, UK). The cells were harvested by centrifugation at 2000 x g for 15 mins, washed with 10 ml sterile water and aliquots frozen until required. Luria Bertani (LB) agar plates were overlaid with 5 ml of L. monocytogenes 473 autoclaved cells. To determine the MIC, 10 µl of 293-amidase at increasing concentrations (5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 75 µg/mL and 150 µg/mL) were spotted on a lawn of L.
*L. monocytogenes* 473, dried in a laminar flow cabinet, incubated at 37 °C and checked every 30 mins for the presence of lysis.

The MIC was determined in parallel also in microtiter plates. The autoclaved *L. monocytogenes* 473 cells were resuspended in sterile phosphate buffer (NaH₂PO₄ 5mM, Na₂HPO₄ 30mM, NaCl 300mM) to an OD₆₂₀ of 1. A volume of 100 μl of the cell suspension was aliquoted into a microtiter plate well. The MIC was tested as follows: 100 μl of purified 293-amidase was added to the cells to a final concentration of 5 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL, 75 μg/mL and 150 μg/mL, respectively, and the reduction in turbidity at OD₆₂₀ was recorded with a microplate reader (Synergy HT, Biotek Instruments, USA) at the time 0, 20, 45, 75, 105 mins and after 16 h. Each experiment was performed in triplicate. The differences between controls and treatments were analysed with a Student’s t-test for statistical relevance.

The specific activity of the 293-amidase was calculated as the concentration of protein that gave approximately 50% OD₆₂₀ reduction at the end of incubation, expressed as U/mg of protein.

5.3.7 Influence of temperature on 293-amidase activity

To check the optimal temperature for activity, 1 μg of 293-amidase was spotted on a lawn of *L. monocytogenes* 473, prepared as described in 5.3.6, incubated at 25°C, 37°C and 50°C and checked every 30 mins for lysis. Additionally, in microtiter plates, 100 μl of autoclaved *L. monocytogenes* 473 cells, resuspended in phosphate buffer to an OD₆₂₀ of 1 were added to 100 μl of 293-amidase at a final concentration of 40 μg/mL and incubated at 25°C, 37°C and 50°C. The reduction in turbidity was recorded with a microplate reader at the times 0, 30, 90, 180 mins and 24 h. The experiments were performed in triplicates and the differences between controls and treatments were analysed with a Student’s t-test.
5.3.8 Lytic spectrum of 293-amidase

To check the lytic activity against different *L. monocytogenes* strains, overnight cultures in BHI were prepared for each of the 5 *L. monocytogenes* strains listed in Table 5.1. The cultures were autoclaved and the pellets were resuspended as described in 5.3.6. LB plates were overlaid with 5 ml of LB top-agar 0.7%, containing 1 ml of each autoclaved strains, spotted with 1 μg of 293-amidase and incubated at 37 °C, checking every 30 mins for the presence of lysis.

The lytic spectrum of 293-amidase was tested also as turbidity reduction at OD\(_{620}\). The autoclaved *L. monocytogenes* strains listed in Table 5.1 were resuspended in phosphate buffer to an OD\(_{620}\) of 1. In a microtiter plate, 100 μl of resuspended cells were added to 100 μl of 293-amidase at a final concentration of 40 μg/mL. The reduction in turbidity at OD\(_{620}\) was measured with a microplate reader every 10 mins at an incubation temperature of 37 °C. The tests were performed in triplicates and the differences between means were assessed for statistical differences using the Student’s t-test.

5.3.9 Influence of pH on 293-amidase activity

Buffers at pH 4 (Na-Citrate 20mM, pH 4), pH 8 (Tris-HCl 20mM, pH 8) and pH 10 (Boric acid 20mM, pH 10) were used to resuspend autoclaved cells of *L. monocytogenes* strain 473 to assess the influence of pH on the amidase activity. The cells were resuspended to an OD\(_{620}\) of 1 and, in a microtiter plate, 100 μl of cell suspension were mixed with 100 μl of 293-amidase at a final concentration of 40 μg/mL and the reduction in turbidity at OD\(_{620}\) was recorded every 10 mins by a microplate reader with incubation temperature of 37 °C. The experiment was performed in triplicates and the differences in means were analysed with the Student’s t-test for statistical differences.

5.3.10 Efficacy of 293-amidase against *L. monocytogenes* biofilm
To assess the efficacy of the amidase to prevent adhesion of *L. monocytogenes* to an abiotic surface, 10 µl of an overnight culture of *L. monocytogenes* 473 were added to 90 µl of BHI broth in a microtiter plate well. In the control wells, 100 µl of phosphate buffer were added, while in the treatment wells, 100 µl of 293-amidase at a final concentration of 75 µg/mL were added to the cell suspensions. The microplate was incubated 5 days at 20 °C and the biofilms were stained with crystal violet according to the method used by Bolocan et al., (2017). The absorbance at 595 nm was recorded with a microplate reader. The assay was performed in triplicate and the differences in mean values were analysed with a Student’s t-test.

To test the efficacy of the 293-amidase on *L. monocytogenes* biofilm removal, 20 µl of an overnight culture were added to 180 µl of BHI broth in a microtiter plate wells. After four days of incubation at 20 °C, the wells were washed with Phosphate buffer saline (PBS, Sigma, Ireland) to remove unattached cells and 100 µl of phosphate buffer were added to the control wells, while 100 µl of 293-amidase were added to the treatment wells. After 24 hours of incubation at 20 °C, the microplate was stained with crystal violet and the results recorded as stated previously.

### 5.3.11 Fluorescence microscopy on stainless steel coupons

Stainless steel (ss) coupons (10 by 15 mm, type AISI-304, no. 2b, finish, 3 mm thick) were prepared as described by Bolocan et al., (2017). Briefly, the coupons were treated with acetone, washed with a detergent, rinsed thoroughly with water and autoclaved at 121°C for 15 min. Each coupon was placed in a well of a square Petri dish with 25 compartments (Thermofisher Scientific, US) and tested for the efficacy of the 293-amidase to prevent adhesion and for biofilm removal. To test the anti-adhesion activity, to each well containing a coupon, 500 µl of BHI broth plus 10 µl of an overnight culture of *L. monocytogenes* 473 were added. To the control and the treatment, 500 µl of phosphate
buffer or 75 µg of 293-amidase were added, respectively. After 5 days of incubation at 20 °C, the coupons were washed with PBS to remove unattached cells and stained with Live/Dead fluorescent dye (Thermofisher Scientific, US) containing Propidium Iodide (excitation/emission 535/617nm) and Syto9 (excitation/emission 485/498nm), following the manufacturer instructions. Biofilms were visualised with a Leica DMi8 fluorescent microscope and images processed with the LAS X software (Leica, Germany).

To test the 293-amidase biofilm removal activity, 1 mL of BHI with 10 µl of an overnight culture of *L. monocytogenes* 473 was added to each well containing a coupon. Biofilms were grown for 4 days at 20°C and then the coupons were washed with PBS and 500 µl of phosphate buffer or 75 µg of 293-amidase were added respectively to the control and the treatment. After 24 hours of incubation at 20 °C, the coupons were washed again with PBS, stained with Live/Dead fluorescent dye and observed as described previously.
5.4 Results

5.4.1 Cloning, expression and purification of the 293-amidase

Bioinformatic analysis of the phage vB_LmoS_293 genome showed that the region 19966.20502 (536 bp) of Open Reading Frame (ORF) 25 encoded the 178 aa 293-amidase (Figure 5.1). The fragment was successfully PCR-amplified from the genome of phage 293 and cloned into the expression vector pCri8A. Sequence alignment of the DNA and predicted amino acid sequences of the 293-amidase from phage 293 and that of cloned fragment can be seen in Figure 5.2A. This analysis revealed a Single Nucleotide Polymorphism (SNP) at position 567, changing the corresponding codon from “GCA” to “GCG”. However, since both the codons translate to alanine, this was a silent mutation, with no consequences on the amino acid sequence of the protein, as shown in Figure 5.2B. The predicted length of the cloned protein was 219 amino acids, with predicted mass of approximately 25 kDa and an isoelectric point of 9.3 (pI 9.3). Figure 5.3 shows an SDS PAGE, showing the over-expression of a protein between 25 kDa and 35 kDa. This protein was confirmed as the 293-amidase domain of the endolysin from the phage vB_LmoS_293 by peptide mass fingerprint (Figure 5.4). The concentration of the purified 293-amidase was calculated as 150 µg/ml with the BCA assay.

Figure 5.1. BLAST alignment output showing the region on the ORF 25 of vB_LmoS_293 with homology to conserved amidase domain.
Figure 5.2. Local (A) DNA sequence alignment and (B) amino acid sequence alignment of predicted and cloned 293-amidase generated with CLC Main Workbench. Of note is the SNP in panel A (boxed region) showing a change from GCA to GCG in the cloned fragment of amidase-293.
Figure 5.3. SDS-PAGE analysis of the 293-amidase purification showing the affinity chromatography fractions. The over-expressed protein band has been highlighted throughout the gel with a white rectangle and is clearly visible in the supernatant of the induced and lysed *E. coli* cells transformed with the pCri8A-293-amidase (S). After binding to the Ni-NTA resin, the band is less visible in the flow-through (F). L, ladder; S, supernatant of lysed cells; F, flow-through; W1-W2-W3, washing steps; 145-245-345-545, elution fractions.
Figure 5.4. Mass spectrum of the 293-amidase digested with trypsin for identification by peptide mass fingerprint. On the horizontal axis are the masses and on the vertical axis is the abundance. Numbers on the peaks indicate the mass of the fragments obtained from the digestion with trypsin. A correspondence with the predicted sequence of the 293-amidase was found with the fragments: 1034.53, 1293.61, 1307.59, 1373.65, 1668.70, 1853.90 and 3301.27
5.4.2 Determination of MIC and specific activity of amidase-293

The assays performed on autoclaved *L. monocytogenes* 473 cells showed a MIC of 10 μg/ml. In the microtiter plate assays, this concentration gave a 42% reduction in 16 hours incubation (Figure 5.5, p<0.05), while a 58% and 53% reduction in autoclaved *L. monocytogenes* 473 was observed at 40 μg/ml and 75 μg/ml, respectively (p<0.05). The specific activity of the 293-amidase was calculated as 100 U/mg after 16 h of incubation.
Figure 5.5. MIC of the 293-amidase against autoclaved *L. monocytogenes* 473 cells in microtiter plates. The blue lines represent the controls (*L. monocytogenes* 473 cells incubated with phosphate buffer and no 293-amidase), while the red line represents the treatment (*L. monocytogenes* 473 cells incubated with 293-amidase at different concentrations). On top of the charts are reported the p values of T-tests between the averages of control and treatment. The X-axes are times and the Y-axes are OD_{620}. Each data point is the average of triplicates and the standard deviations are indicated as error bars.
5.4.3 Influence of pH and temperature on activity of 293-amidase

The 293-amidase showed activity at different pH conditions (Figure 5.6A). In particular, the activity in the buffer at pH 4 produced a turbidity reduction of 39% after 3 h of incubation, while at pH 8 and pH 10, the reduction was 49% and 52%, respectively, after 3 h of incubation (p<0.0001 in all the three conditions tested).

The temperature influenced the activity of the 293-amidase (Figure 5.6B), showing 58% of turbidity reduction in microtiter plates, and visible lysis on a lawn of *L. monocytogenes* 473 after 30 mins of incubation at 37 °C. By contrast, at 25 °C and 50 °C, the turbidity reduction was 9% and 35% respectively, and on the spot on lawn assays the lysis was shown only after 3 h of incubation.
Figure 5.6. Influence of pH values (A) and temperature (B) on amidase activity of the 293-amidase (reported as “Amidase” in the legend) against *L. monocytogenes* 473 (reported as “Control” in the legend). In (A), the X-axes are times and the Y-axes are OD<sub>620</sub>. Each data point is the average of triplicates and the standard deviations are indicated as error bars. C, Control. In (B), the Y-axis represents the OD<sub>620</sub> and the histograms show the reduction in turbidity, indicated as percentage of reduction in the “amidase” boxes, at the end of the incubation at 25 °C, 37 °C and 50°C.
5.4.4 Lytic spectrum of 293-amidase

Of the strains tested, described in Table 5.1, the 293-amidase showed lytic activity in the presence of *L. monocytogenes* 473 (serotype 4b) and 3099 (serogroup 1/2b 3b 7), with a statistically significant difference between the controls and treatments, with P-values < 0.05.

5.4.5 Biofilm inhibition and removal

The tests performed on biofilm inhibition and removal are summarised in Figure 5.7. Incubation of 75 µg of 293-amidase with *L. monocytogenes* 473 at 20 °C resulted in a 78% reduction (p<0.0001) in the formation of biofilm on polystyrene microplates tested after 5 days of incubation (Figure 5.7A). The 293-amidase applied to a 4-day old biofilm resulted in a 25% reduction on the pre-formed biofilm (p<0.05, Figure 5.7B).

On stainless steel coupons, the 293-amidase inhibited the formation of a biofilm, while no evidence of removal was observed when the protein was applied on a 4-day old biofilm (Figure 5.7, C-E). Live and damaged cells, stained with the Live/Dead fluorescent dye, were observed in both the controls and treated biofilms.
Figure 5.7. Biofilm prevention (A) and biofilm removal ability (B) of the 293-amidase against *L. monocytogenes* 473 in microtiter plates assays. Values are the average of triplicates and the standard deviation is represented as error bars. P values are shown below the horizontal axes. In C, D, and E are shown images obtained from the stainless steel coupons assays. C, *L. monocytogenes* 473 biofilm after 4 days of incubation at 20 °C. D, Co-inoculation of *L. monocytogenes* 473 and 293-amidase. E, the application of 293-amidase on a 4-day old biofilm. Red stained cells are damaged/dead, while green stained cells are live. The scale bars at the bottom-right in C, D, E, are 10 μm size.
5.5 Discussion

In food safety, the use of phage-derived proteins as antimicrobials and for biofilm removal is currently being investigated (Ajuebor et al., 2016; Gray et al., 2018). Using the catalytic domains only rather than the full length endolysin can facilitate the production of the recombinant protein, by increasing their stability and solubility and, in some cases, can improve the lytic activity (Domingo-Calap & Delgado-Martínez, 2018). The results described in this work, have shown that the 293-amidase is able to reduce *L. monocytogenes* 473 with a MIC of 10 μg/ml. Furthermore, the host range is broader than the phage itself, because of the amidase activity recorded against a 1/2b-3b-7 strain, while vB_LmoS_293 showed activity only against 4b and 4e *L. monocytogenes* strains (Casey et al., 2015). This phenomenon was also reported by Fenton et al, where the characterisation of the CHAP_k domain of the LysK endolysin showed a broader activity than the host range of the full length endolysin LysK (Fenton et al., 2011). Further characterisation of the 293-amidase showed a reduction in turbidity at all the pH values tested, but with highest activity at pH 10. This characteristic is in accordance with other studies where other endolysins showed improved activity at high pH, with optimal activity at pH 8-9 (LeBlanc et al., 2015; Oliveira et al., 2016). The antimicrobial activity of the 293-amidase showed the potential for preventing biofilm formation. Indeed, when 293-amidase was co-incubated with *L. monocytogenes* 473, biofilm formation was inhibited, both in polystyrene microtiter plates and on stainless steel coupons. It is likely that, the damage induced by the 293-amidase to the peptidoglycan of the bacterial cells was enough to inhibit the adhesion on abiotic surfaces. On the contrary, when applied to a four-day old biofilm, the 293-amidase showed a reduction of 25% in polystyrene plates, while on stainless steel coupons no differences were observed between the control and treatment. Possibly, the differences were minimal and could not be visualised, or the biofilm formed on stainless steel coupons was thicker than the biofilm formed on polystyrene plates and
was less affected by the application of the 293-amidase. This would contradict past studies, where differences in *L. monocytogenes* biofilm formation ability related to the surfaces tested were shown, with a stronger biofilm formed on polystyrene surfaces than the one formed on stainless steel coupons (Di Bonaventura et al., 2008; Poimenidou et al., 2016).

To date, anti-listerial activity of endolysins have been mainly focused on in-vitro experiments on planktonic cells or only a few food matrices, as described by Gray et al., (2018). Only the endolysin PlyLM, identified by Simmons et al, (2012) in the genome of a *L. monocytogenes* 4b strain, has been previously tested against *L. monocytogenes* biofilms (Simmons et al., 2012). In that case, a monolayer biofilm was grown 24 h at 37 °C and PlyLM reduced the biofilm by 20 %, while completely removed the biofilm when combined with a protease. Further investigations might be required to assess if the same activity is registered at lower temperatures, such as 20 °C, as tested in this study, which reflect more the food production environment. More studies are required also to further characterise the activity of the 293-amidase and to improve its antilisterial activity on biofilms.
5.6 Conclusions

In this study, the 293-amidase antilisterial activity was characterised for host range, pH, temperature and concentration. The results on biofilms grown on microtiter plates revealed a potential preventive activity against *L. monocytogenes* biofilm and a reduction in a four-day old biofilm. This work shows the great potential of recombinant active domains of endolysins against *L. monocytogenes* biofilms and further studies are required to optimise the process of production and application in the FPE.
5.7 References


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Chapter 6

*Listeria monocytogenes* lysis mediated by a recombinant amidase expressed on polyhydroxyalkanoate bionanoparticles
6.1 Abstract

In recent years, novel biotechnologies for pathogens control in the food processing environment have been developed. One of these approaches consist in binding recombinant enzymes on the surface of polyhydroxyalcanoate (PHA) bionanoparticles (BNPs), as they have been proved to be cost-effective, produced in high yields by engineered microorganisms and environmentally friendly. The aim of this work was to produce PHA BNPs displaying on their surface the amidase domain of the listeriophage vB_LmoS_293 endolysin and to test its efficacy against *L. monocytogenes* in *in-vitro* experiments. The results showed enzymatic activity on autoclaved *L. monocytogenes* 473 cells with BNPs concentration of 1 mg/ml and 5 mg/ml. These results represent a promising study for the potential application of tailored BNPs displaying recombinant listeriophage enzymes as novel antimicrobials in the food industry.
6.2 Introduction

Recombinant bacteriophage-derived proteins represent a valid alternative to the use of phages themselves. Endolysins (lysins) are a class of enzymes, produced by bacteriophages in the host cell, that act by cleaving the peptidoglycan in the cell wall and subsequently lysing the bacterial cell from within, allowing the phage progeny to be released in the surrounding matrix (Ajuebor et al., 2016). In recent years, lysins have been the subject of much research as possible agents for foodborne pathogen reduction (Etobayeva et al., 2018; Larpin et al., 2018; Misiou et al., 2018). There are some advantages in the use of lysins as an alternative to the use of phage particles. For example, there are no potential issues with the transfer of genetic material, no resistance has been recorded so far because they cleave highly specific structures in the peptidoglycan where a mutation to generate resistance might be fatal to the bacterial cell (Fischetti, 2010). The use of single domains rather than the full-length lysin has shown some advantages, like easier production, improved stability and, in some cases, enhanced lytic activity. One such example is the CHAP domain of the S. aureus Phage K endolysin, LysK (Horgan et al., 2009). Studies on the CHAP domain have shown a stronger lytic activity than the original lysin, a broader lytic spectrum and its efficiency against S. aureus biofilms (Fenton et al., 2013; Horgan et al., 2009).

Novel research approaches are employing nanotechnology combined with recombinant endolysins, to enhance their lytic activity (Kashani et al., 2018), for detection purposes (Kretzer et al., 2007) or to trigger the release of the enzyme depending on environmental factors, as in the case of the CHAPk included in Poly(N-isopropylacrylamide) (PNIPAM) nanoparticles for a thermally controlled release in the surrounding matrix (Hathaway et al., 2017a). The Listeria phage endolysin Ply500, for
example, has been conjugated with silica nanoparticles (SNPs) and its efficacy was demonstrated both in cultures and on lettuce (Solanki et al., 2013).

Another novel biotechnology was developed by using polyhydroxyalkanoate (PHA) bionanoparticles (BNPs) to deliver recombinant enzymes (Peters & Rehm, 2006). PHA can be synthesised by various microorganisms, expressing the gene PHA synthase phaC (Yuan et al., 2001), as carbon and energy reservoir, especially under stress conditions (Keshavarz & Roy, 2010). PHA particles aggregate into spherical structures, forming the BNPs. PhaC can be fused to the recombinant proteins at both the C- and N-terminus, forming BNPs that display the proteins on the surface, maintaining their native activities (Rehm, 2010). BNPs are advantageous because they are economically efficient, non-toxic and produce high yields (Choi & Lee, 1997; Philip et al., 2007; Yuan et al., 2001). Furthermore, PHA, which is the main constituent of the BNPs, has been approved by the FDA as a component of food additives (Philip et al., 2007). In a recent study, a lytic enzyme from the Methanobrevibacter ruminantium virus φmr (PeiR) was successfully bounded to PHA BNPs and it showed lytic activity against several methanogen strains (Altermann et al., 2018).

The aim of this work was to produce PHA BNPs displaying the amidase domain of a Listeria phage endolysin and conduct preliminary experiments to assess their lytic activity in vitro.
6.3 Materials and methods

6.3.1 Polyhydroxyalkanoate (PHA) bionanoparticles (BNP) production

**Bioinformatic analysis:** The genome of listeriophage vB_LmoS_293 has been previously annotated and is available in GeneBank database with the Accession Number KP399678.1 (Casey et al., 2016). The Open Reading Frame (ORF) coding the vB_LmoS_293 endolysin was analysed with the Basic Local Alignment Search Tool (BLAST) against the Conserved Domains Database (CDD, Marchler-Bauer et al., 2017) to identify the amidase domain.

**Plasmid construction for PhaC-amidase BNPs production:** The sequence coding for the amidase domain was provided to GeneArt (Regensburg, Germany) to be optimised for expression in *E. coli*, synthesised and cloned into the pET14b vector. Briefly, the gene was amplified with Platinum PCR SuperMix High Fidelity (Life Technologies Auckland, New Zealand) and subcloned using the TA cloning kit with the plasmid pCR2.1, according to the manufacturer's instructions (Life Technologies). The resulting plasmids were sequenced, the plasmids harbouring the right sequence were digested with restriction enzymes and ligated into the pET14b plasmid. The resulting plasmid pKR-amidase was transformed into *E. coli XL1 Blue* cells (Stratagene, La Jolla, CA, United States) for screening.

*Production and purification of PhaC-amidase BNPs:* The PhaC-amidase BNPs were produced and purified by Polybatics LTD (Palmerston North, New Zealand) as described in Altermann et al., (2018). Briefly, the plasmids carrying the amidase-domain-*phaC* gene fusions were transformed into *E. coli BL21 I(DE3)* competent cells, carrying the helper plasmid pMCS69 (Amara & Rehm, 2003). *E. coli BL21 I(DE3)* carrying both the pMCS69 helper plasmid and the respective *phaC*-fusion plasmid, were grown in 1 L LB broth...
supplemented with ampicillin (50 mg/ml), and chloramphenicol (64 mg/ml) and 1% (w/v) glucose at 37°C with shaking (150 rpm). When the OD_{600} reached 0.5, the production of the BNPs was induced by the addition of 1 mM IPTG followed by agitation at 25°C for 48 h. The cells were harvested by centrifugation (6,000 x g, 5 min at 4°C), the pellet resuspended in 50 mM phosphate buffer pH 7.5 and lysed via sonication (Vibracell sonicator, Sonics and Materials, Newtown, CT, United States) on ice with 20 s bursts at medium intensity and 30 s rest intervals over a 10 min time. The BNPs were recovered by ultracentrifugation 21,000 x g for 2 h at 4°C in a Sorvall TH641 swing-out rotor (Thermofisher Scientific, Auckland, New Zealand) over a glycerol gradient as described in Brandl et al., (1988) and resuspended in 20 % ethanol.

6.3.2 Turbidity reduction assay

*L. monocytogenes* 473, a serotype 4b strain used to propagate the phage vB_LmoS_293, was grown in 100 ml of BHI broth overnight at 37 °C and the culture autoclaved at 121 °C for 15 min. The cells were harvested by centrifugation (6000 x g, 5 min at room temperature), the pellet resuspended in 10 ml of 50 mM phosphate buffer pH 7.5 with 300 mM NaCl and stored at -20 °C until needed. The autoclaved and resuspended cells were thawed at room temperature and diluted in phosphate buffer 50 mM pH 7.5 with 300 mM NaCl to an OD_{600} of 1.0. In a 96-well polystyrene plate, 100 μl of resuspended cells were added to each well, plus 100 μl of PhaC-amidase BNPs resuspended in the same phosphate buffer of the bacterial cells in order to obtain final concentrations of 5 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml. The plates were incubated at 37°C and the turbidity was recorded with a Synergy plate reader (Synergy HT, Biotek Instruments, USA) at time 0, 1, 4, 5 and 24 h. The experiments were performed in triplicate and the average of each timepoint was plotted on a chart with its standard deviation. Student’s t-test was performed to assess if there was significant difference between the controls and treatments.
6.4 Results

6.4.1 PHA-amidase cloning and BNPs production

The sequence coding the amidase was identified as the region 19966-20502 (536bp) of Open Reading Frame (ORF) 25 in the phage vB_LmoS_293 genome. The amidase was combined with the PhaC synthase at the N-terminal end of the fusion protein and the expression in *E. coli* produced the tailored BNPs.

6.4.2 Lytic activity assay

The lytic activity of the PhaC-amidase BNPs was assessed by turbidity reduction assays on autoclaved cells of *L. monocytogenes* 473 and the results are shown in Figure 6.1. The cells were incubated in the presence of the PhaC-amidase BNPs at four different concentrations for 24 h at 37 °C. At final concentrations of 1 mg/ml and 5 mg/ml an OD$_{600}$ reduction was observed with the PhaC-amidase BNPs, with reductions of 33.9% and 38%, respectively. The Student’s t-test showed a significant difference between the control and the treatment only after a 4 h incubation with a concentration of 1 mg/ml of PhaC-amidase BNPs and after a 24 h incubation with a concentration of 5 mg/ml (p<0.05) of PhaC-amidase BNPs.
Figure 6.1. The application of PhaC-amidase BNPs resulting in turbidity reduction at different PHA BNPs concentrations, during incubation at 37°C. On the horizontal axis are represented time points and on the vertical axis the absorbance at 600 nm (OD$_{600}$). The asterisks indicate turbidity reductions where $p<0.05$, with 1 mg/ml PHA BNP concentration after 4 h incubation, and with 5 mg/ml after 24 h incubation.
6.5 Discussion

This study investigated a novel biocontrol treatment for *L. monocytogenes* in the food processing environment based on the use of an active domain of an endolysin displayed on PHA BNPs. The results showed the lytic activity of the amidase-PhaC BNPs against autoclaved cells of *L. monocytogenes* 473. This initial investigation is encouraging considering the potential applications that this technology can have in the food production industry, where a major problem is represented by the cross contamination of *L. monocytogenes* from the processing environment to the final product (Bolocan et al., 2015; Jordan et al., 2018; Muhterem-Uyar et al., 2015; Simmons et al., 2014). For example, the formation of biofilms has been associated with sanitisers resistance in *L. monocytogenes* (Lourenco et al., 2011) and the purified 293-amidase has shown anti-biofilm properties (chapter 5 of this thesis). The application of the amidase-PhaC BNPs in the food processing environment could represent a valid weapon for biofilm inhibition and removal, especially because enzymes immobilised on PHA granules surface have been proven to be stable for several months (Peters & Rehm, 2006) and this could be a useful tool to prevent persistence.

Furthermore, due to their biodegradability, PHA BNPs could be released in the food processing environment and be naturally degraded after their use (Philip et al., 2007). Other studies have revealed the antimicrobial activity of BNPs displaying recombinant phage-derived enzymes, as shown, for example, by Altermann et al, (2018) on rumen methanogens. These bacteria are responsible for the methane production in the rumen forestomach, with a strong impact on the environment. In that case, BNPs displaying a lytic enzyme, PeiR encoded by the *Methanobrevibacter ruminantium* M1 prophage φmr, were shown to inhibit a broad range of methanogens, with efficacy against
Methanobacterium formicicum, Methanosphaera stadtnae and four species of Methanobrevibacter.

Further studies are required for BNP large scale production optimisation and to test their efficacy in the food production environment.
6.6 Conclusions

A novel approach to the potential control of *L. monocytogenes* in the food processing environment was adopted by combining the amidase domain of listeriophage vB_LmoS_293 with PHA BNPs. Initial tests have shown their efficacy on *L. monocytogenes* reduction in-vitro. Further studies will be needed to characterise the antilisterial activity of the BNPs and to prove their efficacy against biofilms and a larger scale efficacy *in-vivo*. 
6.7 References


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Chapter 7

Pilot scale trials in a mushroom growing facility to assess the efficacy of bacteriophages against *Listeria monocytogenes* during production of *Agaricus bisporus*

To be submitted to Field Crops Research
7.1 Abstract

The commercial production of *Agaricus bisporus* is a very well established protocol. However, the nature of the raw materials and some gaps in the hygiene practices, such as poor attention to details during the mushroom production and harvest, can lead to *L. monocytogenes* cross contamination on the mushrooms. In this study, bacteriophage-based antimicrobials were tested on the floors and in the casing soil of a pilot scale mushroom growing facility for reduction of deliberately-inoculated *L. monocytogenes*. The results showed the ineffectiveness of these treatments *in-vivo*, although they were effective in previous studies *in-vitro*. Possible reasons leading to these negative results have been hypothesised, such as too low concentration of the treatments, unfavourable environmental conditions or inactivation and are discussed in this chapter.
7.2 Introduction

*Agaricus bisporus* is the most common commercially cultivated mushroom. Its worldwide production increased from 495,127 metric tons in 1961 to 10,378,163 metric tons in 2016, with the biggest global production in China, followed by USA and Netherlands (FAO, 2016). In Ireland, mushroom production represents the largest part of the horticultural sector, accounting for an income of €480 million in 2018 (www.bordbia.ie). The mushroom production process is highly standardised and starts with the production of the raw materials: phase III compost and casing soil. Phase III compost is a mixture of straw and manure, incubated together, pasteurised and then inoculated with mushroom spawn before being used for the mushroom growth. Casing soil is a mixture of peat and lime and it contains a natural microflora that enhances mushroom production. The formation of fruiting bodies is induced by the application of casing soil on top of the compost (McGee et al, 2018).

Mushrooms can be cultivated in trays, bags or shelves and the incubation is undertaken in a mushroom growing tunnel at 22-25 °C. After seven days, the relative humidity is raised and the CO\textsubscript{2} levels are lowered by venting. This induces the mushroom's fruit body formation (Visscher 1988). At this point, harvesting (or “picking”) of the mushrooms commences and it is usually done manually, during a time frame called “flush”, when all the available mushrooms are picked. The growing conditions are maintained, during which further mushrooms grow and are picked in further flushes. Commonly, three flushes of mushrooms are produced in each crop, with the highest yield obtained in the first flush.

*Listeria monocytogenes* is a foodborne pathogen that causes listeriosis, which generally causes self-limiting gastrointestinal illness, but can result in more serious illnesses. Listeriosis has a mortality rate of 24% (de Noordhout et al., 2014) and among the
foodborne diseases, has the highest hospitalisation rate of about 99% (Scallan et al., 2011). To date, listeriosis has never been linked to mushroom consumption, although studies have shown that *L. monocytogenes* can be commonly isolated from the mushroom production environment (Leong et al., 2017; Madden et al., 2018; Pennone et al., 2018; Venturini et al., 2011; Viswanath et al., 2013). Recalls of mushrooms have been recorded around the world (Pennone et al., 2018). In 2014, the presence of *L. monocytogenes* on mushrooms in Ireland was reported (EU, 2019). Critical points for cross contamination in a mushroom production unit are represented by presence of *L. monocytogenes* in the raw materials, contaminated transportation vehicles, cross contamination from sources external to the food production environment (i.e. walking in the mushroom production unit from the yard without changing or cleaning shoes before entering the growing area), persistence, inadequate hygiene practices (Leong et al., 2014; Leong et al., 2017; Pennone et al., 2018; Viswanath et al., 2013). Common hygiene practices in the mushroom industry include pre-harvest actions such as controlling the possible introduction of *L. monocytogenes* to the mushroom growing facility through pasteurisation of compost, the use of footbaths and other physical barriers. Post-harvest actions include the use of sanitisers, such as sodium hypochlorite in the growing room environment to wash the floors, and cookout, a process that consists of steaming the mushroom growing room after each crop. Some of these processes, such as cookout, are not undertaken in all the mushroom growing facilities. Furthermore, these processes have often been shown to be inefficient, i.e. due to floor temperatures not enough high during the cookout process (chapter 4 of this thesis). For that reason, research on new methods to enhance the existing hygiene procedures is always needed.

Bacteriophages are viruses that kill bacteria (Howard-Varona et al., 2017) and their utilisation as pathogen control agents in food production environments has gained much attention in recent years. In agriculture, for example, bacteriophage-based preparations are
available from the US-based phage company OmniLytics (AgriPhage), for the control of the plant pathogens *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* on tomato and pepper crops. Field trials have shown the effectiveness of a cocktail of bacteriophages isolated against *Xanthomonas campestris* pv. *vesicatoria* (AgriPhage, OmniLytics Inc., USA), a Gram negative bacterium that infects tomato plants, producing a disease (Bacterial spot), which causes great losses in the tomato production. The application of bacteriophages in 3 different formulations, namely pregelatinised corn flour, casecrete NH-400 (casein) and skim milk, was studied in greenhouse and field trials. In greenhouse trials, the skim milk formulation showed significant reduction in the disease symptoms on the tomato leaves, while in field trials the best results were obtained with the casecrete and pregelatinised corn flour. For a comprehensive review on the application of bacteriophage treatments in agriculture, see Zaczek et al., (2015).

In food production, a number of companies have targeted common foodborne pathogens. Intralytix produces cocktails of bacteriophages against *L. monocytogenes, Escherichia coli* O157: H7 and highly pathogenic *Salmonella* serotypes. PhageGuard Listex P100, produced by the Dutch company Micreos, has been approved by the United States Food and Drug Administration (FDA) and the United States Department of Agriculture’s (USDA) Food Safety and Inspection Service for *L. monocytogenes* control in both raw and ready-to-eat food products.

Research on new methods for pathogen inactivation is in constant progress and promising results have been obtained by the use of phage endolysins (Ajuebor et al., 2016). Endolysins, or lysins, are enzymes encoded by the bacteriophage that cleave the peptidoglycan of the host cells, causing cell lysis and release of the phage progeny (Loessner et al., 1995). In the control of *L. monocytogenes* in the food production industry, laboratory studies have shown the efficacy of endolysins. For example, the lysin PlyP100, alone or combined with nisin, was effective against *L. monocytogenes* contamination
during the production of fresh cheese (Ibarra-Sánchez et al., 2018; Van Tassell et al., 2017). The use of lysins has the advantage of eliminating the possibility of bacterial resistance and genetic transfer (Fischetti, 2010). Furthermore, in some cases, the use of single active domains in place of the full length lysin confers more stability to the enzyme and facilitates its production (Horgan et al., 2009). Recently, the association of nanotechnologies with the use of endolysins has shown encouraging results for pathogen detection, or to trigger enzyme release or to increase lytic activity (Hathaway et al., 2017; Kashani et al., 2018; Kretzer et al., 2007; Solanki et al., 2013). Promising results have been shown by the development of polyhydroxyalkanoate (PHA) bionanoparticles (BNPs) to deliver recombinant enzymes (Peters & Rehm, 2006). In a recent study, the utilisation of PHA BNPs displaying a lytic enzyme from the Methanobrevibacter ruminantium virus ϕmru (PeiR) was successfully tested against methanogen bacteria (Altermann et al., 2018a). However, no pilot scale or field trials have been undertaken.

The aim of this study was to test the efficacy of a number of bacteriophage-based treatments in reducing L. monocytogenes on the floor and in casing soil during the production of A. bisporus in a pilot scale mushroom growing unit. The treatments consisted of a preparation of live bacteriophages (vB_LmoS_293), a purified preparation of recombinantly-produced 293-amidase (Chapter 5) and the amidase bound to polyhydroxyalcanoate bionanoparticles (PhaC-amidase BNPs) (Chapter 6).
7.3 Materials and methods

7.3.1 Bacterial strains

A streptomycin resistant mutant of *L. monocytogenes* strain 473 (*L. monocytogenes* 473-s) was generated and used for the trial. Briefly, a single colony of the wild type *L. monocytogenes* strain 473 was grown in tryptic soy broth (TSB, Oxoid Ltd, UK) to the early stationary phase. An equal volume of TSB supplemented with 2000 μg/ml Streptomycin sulphate was added (Streptomycin sulphate final concentration: 1000 μg/ml, Sigma, Ireland) and incubated to the early stationary phase. Aliquots of 100 μl were then plated on both Nutrient agar (NA, Oxoid Ltd, UK) and agar Listeria acc. to Ottaviani Agosti (ALOA, Lab M Limited, UK), both supplemented with 1000 μg/ml Streptomycin sulphate and the plates incubated overnight at 37 °C. Streptomycin resistant strains were selected and tested on streptomycin-free NA to ensure stability of the phenotype. Growth curves and phage sensitivity tests were performed to assess if there were any differences between the mutant and wild type strains.

An overnight culture was grown at 37 °C in brain hearth infusion (BHI) broth (Oxoid Ltd, UK) and the culture was diluted in maximum recovery diluent (MRD, Oxoid Ltd, UK) to prepare an inoculum of approximately $10^7$ CFU/ml.

7.3.2 Preparation of Bacteriophage-based Treatments

*Bacteriophage:* The vB_LmoS_293 bacteriophage was propagated as follows: 1 ml of *L. monocytogenes* 473 overnight culture was incubated with 1 ml of BHI and 100 μl of CaCl$_2$ 0.185 M for 30 mins at 37 °C. One hundred μl of phage filtrate and 1 ml of BHI broth were added to the mix and incubated at 37 °C for a further 1 h, then, 10 ml of BHI, 1 ml of overnight bacterial culture and 1 ml of CaCl$_2$ 0.185 M were added to the mix and incubated for 3 h at 37 °C. Subsequently, the mix was transferred into 1 L of BHI
supplemented with 100 ml of CaCl$_2$ 0.185 M and incubated at 37 °C overnight. The cells debris was removed by centrifugation (3000 x g, 10 mins, 4 °C) and the supernatant filtered with 0.45 μm syringe filters (Sarstedt, Germany). The phage titre was assessed by plaque assay on serial dilutions. Briefly, each dilution was filtered through a 0.45 μm syringe filter and 1 ml of filtrate was added to a sterile tube containing 100 μl of CaCl$_2$ and 100 μl of *L. monocytogenes* 473-s overnight culture. To the mix, 4 ml of sterile TSB containing 0.3% agar (TSA 0.3%, Oxoid Ltd, UK) were added, vortexed and overlaid on a TSA plate. The plates were incubated at 37 °C and checked for phage plaques after 18 h. The filtrate was then diluted in MRD to give an inoculum of approximately $10^8$ PFU/ml. The phage concentration added to a bag of casing soil was approximately $10^6$ PFU/g.

**293-amidase:** The 293-amidase was cloned, expressed and purified as described in Chapter 5. One hundred ml of purified amidase in phosphate buffer at a concentration of 90 μg/ml were used to inoculate each bag of casing soil, with a final concentration of 1.3 μg of 293-amidase per g of casing soil.

**PhaC-amidase BNPs:** The BNPs were produced as described in Chapter 6 and diluted in 50 mM phosphate buffer containing NaCl 300 mM, pH 7.5 to obtain a volume of 300 ml at a concentration of 0.5 mg/ml. The quantity used to inoculate each casing soil bag was 100 ml, with a final concentration of 7 μg of PhaC-amidase BNPs per g of casing soil.

### 7.3.3 Pilot scale mushroom growing unit

A schematic representation of the pilot scale unit used for this study is given in Figure 7.1.

A drainage system collects liquid waste from each room and from the middle of the corridor that is sent to a closed tank for treatment of pathogenic waste before disposal.
Figure 7.1. A detailed map of the pilot scale mushroom production unit.
Temperature, ventilation and humidity are regulated from the commands in the office or from a panel at the entrance of the facility. Each room is closed hermetically with sliding doors and is provided with doors facing the yard, for the raw materials collection or waste disposal (after appropriate treatment). A cookout system is available in the facility and each mushroom growing room can be steamed at the end of each crop. For the purpose of the trial, the access to the building was strictly restricted only to personnel trained for working with pathogens and a risk assessment was developed to perform the appropriate health and safety procedures.

7.3.4 Experimental plan

L. monocytogenes reduction on the floor: The 293-amidase was tested against *L. monocytogenes* 473-s on the floor. One mushroom growing room was cleaned, sanitised and a cookout of 12 hours at 70 °C was undertaken before starting the trial. Tiles of 56 x 35 cm were marked on the floor. Three tiles were used for the uninoculated controls (UC), where no *L. monocytogenes* or treatment were applied; 3 tiles for the inoculated controls (IC), where only *L. monocytogenes* 473-s was applied; and 3 tiles were used for the treatment (A), where both *L. monocytogenes* 473-s and 293-amidase were applied. Tiles were covered with a plastic box to minimise the dispersion of aerosol of *L. monocytogenes*. Three spray bottles were cleaned with water, sterilised with ethanol 70% and left to dry in a laminar flow cabinet for 30 mins. One spray bottle was filled with 150 ml of MRD, a second one with 100 ml of *L. monocytogenes* 473-s, $10^7$ CFU/ml, and a third spray bottle was filled with 50 ml of 293-amidase at a concentration of 90 μg/ml. The UC was sprayed with 150 ml of MRD, while the IC was sprayed with 100 ml of *L. monocytogenes* 473-s inoculum plus additional 50 ml of MRD, for moisture consistency, all over the surface of the tile and left 5 mins to rest, to allow the entire aerosol to deposit on the floor. The treatment tile was sprayed with 100 ml of *L. monocytogenes* 473-s inoculum, left 5 mins to
deposit on the floor and then sprayed with 50 ml of 293-amidase. The experiments were conducted in independent triplicate. Sponge-stick swabs containing 10 ml of sterile neutralising buffer (3M, USA) were used to swab portions of tiles measuring 18.5 x 35 cm at 3 sampling occasions: at time 0 (directly after inoculation), at 24 h and 48 h after inoculation.

L. monocytogenes reduction during the mushroom growth: Two mushroom growing rooms were cleaned, sanitised and a cookout of 12 h at 70 °C was undertaken before use for this trial. The mushrooms were grown in plastic crates (plots) of 0.2 m³ volume (Figure 7.2) which were filled with Phase III compost (16 kg) and casing soil (7 kg), obtained from commercial producers. After the plots were filled with Phase III compost (previously inoculated with mushroom mycelium), the casing soil was weighed and aliquoted into sterile plastic bags and inoculated with L. monocytogenes 473-s and the treatments, at the concentrations described previously. While wearing disposable suits and sterile gloves, the casing was mixed manually in the bag after inoculation, and then spread on the top of the compost. The plots were labelled and kept at distance from the mixing environment, to avoid cross contamination. To avoid cross contamination with phages, the controls were set up separately from the phage treatment. From each plot, 3 samples of 10 g were taken at each sampling occasion, on day 0, day 1, day 3, day 6, day 13, day 20, day 27 and day 34.
Figure 7.2. The plots used to grow the mushroom during the crop trial.
The mix was incubated on the shelves in the mushroom growing room, following the parameters used in the mushroom industry, as shown in Table 7.1. Briefly, mushroom growth was obtained in three steps: 1) colonisation of the compost and casing by the mushroom mycelium, 2) growth of mushroom fruiting bodies and 3) harvesting. The colonisation step is divided in three parts: “spawn run 1” (SP1), or phase III compost colonisation (inoculation of the compost with mycelium), which in this case was completed by the compost producer; “spawn run 2” (SR2) and “spawn run 3” (SR3) or casing colonisation, where the mushroom mycelium grows through the casing soil. Temperature, humidity and CO₂ content are regulated to induce the mushroom fruiting body formation (Pinheading) in the “cool down pinning” (CDP) step. After approximately four days, the first mushrooms are ready to be harvested (or picked) by hand. Three flushes of mushroom harvest are usually undertaken in the mushroom industry, each flush divided into two harvest steps. Usually, flush 1 has the highest yield and flush 3 the lowest. The crop finishes when, at flush 3, there are no more mushrooms available, with a maximum time-lapse of 38 days from start to finish. The experiments were conducted in independent triplicate.
Table 7.1. Parameters used for the mushroom growth during the trial. “Phase”: first, compost colonisation (SP1) is followed by casing colonisation (SR2 and SR3). Then a cooling down pinning (CDP) phase triggers the mushroom to produce fruit bodies during the Pinheading phase, which will lead to the Harvest flushes. “Compost” indicates the compost temperature in °C; “Fresh Air Setting” shows the fan position (%); “RH set points”, the relative humidity (%); “CO₂ End Value” represents the CO₂ content threshold, in ppm, that activates the computer for CO₂ regulation; “Desired Air”, is the targeted air temperature, in °C; Spawn run 1 (SP1) in this case happened at the compost producer level, so the trial described here started at Day 0.

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|       | Harvest 4 | 30/70 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 | 30/80 |
|       | Harvest 5 | 89.9 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 | 87.89 |
|       | Fresh Air Setting | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 |
|       | RH set points | 18.5 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 18.5 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |

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In one room (Room 4), the 293-amidase and the PhaC-amidase BNPs were tested against *L. monocytogenes* during mushroom growth, while in a separate room (Room 3), the bacteriophage vB_LmoS_293 was tested, to avoid phage contamination of the other treatments. One single mushroom growing plot in room 3 was tested to assess if the phosphate buffer used to resuspend the BNPs was influencing *L. monocytogenes* survival and the mushroom production. The controls were: uninoculated control, without *L. monocytogenes* or any treatment, and inoculated control, with *L. monocytogenes* and no treatment. The experiments were conducted in independent triplicate.
7.3.5 *L. monocytogenes* detection and enumeration

Sponge swabs or casing samples of 10 g were resuspended in 90 ml of MRD, serially diluted in MRD-filled dilution racks (Hyserve, Germany) and 100 µl of each dilution spread on agar Listeria acc to Ottaviani Agosti (ALOA, Oxoid Ltd, UK), supplemented with 1 mg/ml of streptomycin (ALOA-s). The plates were incubated at 37 °C overnight. Presumptive *L. monocytogenes* were green colonies with an opaque halo. The load of *L. monocytogenes* was recorded as Log_{10} CFU/g. During the mushroom growth trial, when the numbers of *L. monocytogenes* were too low to be detected by direct counts, a pre-enrichment in half-Fraser broth and, if needed, a second enrichment in full-Fraser broth (Merck, Ireland) were undertaken. Briefly, 10 g of casing were suspended in 90 ml of Fraser broth base and, after one hour, the supplement, plus 1 mg/ml streptomycin was added and the samples incubated at 30 °C overnight. Twenty µl were plated on ALOA-s and 100 µl were transferred into tubes containing 10 ml of full Fraser broth, supplemented with streptomycin and incubated overnight at 37 °C. After incubation, 20 µl were spread on ALOA-s plates and incubated at 37 °C overnight. In this case, only presence/absence of *L. monocytogenes* was recorded.

Mushroom samples, 100 g per plot, were homogenised manually in the sampling bag and the juice was serially diluted and plated as described before. At the same time, enrichments were performed as described previously, with the modification that 25 g of mushrooms were added to a stomacher bag and 225 ml of Fraser broth base was added. The sample was homogenised for 3 mins and, after 1 h of incubation at room temperature, the supplements were added and the bag was incubated at 30 °C, overnight, following the same protocol as described before.

7.3.6 Bacteriophage detection and enumeration
The bacteriophages were detected and enumerated as follows: 10 g of casing sample was resuspended in 90 ml of MRD and diluted as described before. Each sample dilution was filtered through a 0.45 μm syringe filter and 1 ml was added to a sterile tube containing 100 μl of CaCl₂ and 100 μl of *L. monocytogenes* 473-s overnight culture. To the mix, 4 ml of TSB containing agar 0.3% (TSA 0.3%, Oxoid Ltd, UK) were added, vortexed and overlaid on a TSA plate. The plates were incubated at 37 °C and checked for phage plaques after 18 h. Phage counts were reported as Log₁₀ PFU/g.

7.3.8 Statistical analysis

The data of *L. monocytogenes* counts, bacteriophage counts and mushroom yield were processed through the software SPSS (IBM, USA) for statistical analysis. Briefly, the normality of the data distribution was analysed with descriptive statistics and, depending on the output, parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis was performed.
7.4 Results

7.4.1 Comparison between L. monocytogenes 473 and L. monocytogenes 473-s

There were no observed differences in the behaviour of L. monocytogenes 473-s and the wild type L. monocytogenes 473 strain in growth experiments, bacteriophage sensitivity, 293-amidase treatment in-vitro, or in stability in casing (data not shown).

7.4.2 L. monocytogenes reduction on the floor

L. monocytogenes counts showed a natural reduction in numbers throughout the floor trial. The inoculated controls showed L. monocytogenes counts with an average from $4.4 \log_{10} \text{CFU/cm}^2$ at time 0 to $2.5 \log_{10} \text{CFU/cm}^2$ at time 48 h, while with the 293-amidase treatment, the average of counts were from $5.6 \log_{10} \text{CFU/cm}^2$ to $2.9 \log_{10} \text{CFU/cm}^2$ at time 48h (Figure 7.3). Analysis of variance showed significant difference between the control and the 293-amidase treatment only at time 0 ($p<0.05$), while at 24 h and 48 h, even though the 293-amidase treatment showed higher counts, no statistically significant differences were shown ($p>0.05$).
Figure 7.3. *L. monocytogenes* counts on the floor with 293-amidase application. On the horizontal axis the three experimental conditions are shown, while on the vertical axis are the mean of *L. monocytogenes* counts, expressed as mean Log_{10} CFU/cm^{2}. The error bars are the standard deviation and the colours, as shown in the legend, are the sampling time-points, in hours. UC, uninoculated control; IC, inoculated control; A, 293-amidase treatment.
7.4.3 293-amidase and PhaC-amidase BNPs effect on L. monocytogenes during the mushroom production

*L. monocytogenes* counts, in the inoculated control, were naturally reducing throughout the trial, from $7.6 \log_{10} \text{CFU/g}$ at day 0 to $0.56 \log_{10} \text{CFU/g}$ at day 34 (Figure 7.4). In the casing treated with 293-amidase, the occurrence of *L. monocytogenes* decreased from $7.2 \log_{10} \text{CFU/g}$ at day 0 to $1 \log_{10} \text{CFU/g}$ at day 34. In the casing inoculated with the PhaC-amidase BNPs the occurrence of *L. monocytogenes* decreased from $7.3 \log_{10} \text{CFU/g}$ at day 0 to $3 \log_{10} \text{CFU/g}$ at day 34 (Figure 7.4). The presence of *L. monocytogenes* in single plot inoculated with the phosphate buffer used to resuspend the BNPs was higher than the inoculated control between day 15 and day 27 ($p<0.05$), while at the other sampling occasions no significant differences were found (Figure 7.5). Kruskal-Wallis tests showed significant differences between the inoculated control and the treatments ($p<0.05$), although these treatments did not bring about a reduction of *L. monocytogenes*, but led to higher counts throughout the crop trial, especially where BNPs were applied, as shown in Figure 7.4.

The mushrooms tested showed the presence of *L. monocytogenes* throughout the 3 harvest flushes, with no significant differences between the controls and treatments ($p>0.05$, Figure 7.6). The plots treated with the 293-amidase were negative on the direct counts in the flush 3, but positive after enrichment (data not shown).
Figure 7.4. *L. monocytogenes* counts during the treatments with 293-amidase and PhaC-amidase BNPs. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as $\log_{10}$ CFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the sampling occasions. UC, uninoculated control; IC, Inoculated control; A, casing treated with 293-amidase; B, casing treated with PhaC-amidase BNPs.
Figure 7.5. *L. monocytogenes* counts in the casing soil inoculated with the phage vB_LmoS_293. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as log_{10} CFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the sampling occasions. UC, uninoculated control; IC, Inoculated control; L, casing inoculated with the phage vB_LmoS_293; Ex, casing treated with phosphate buffer.
Figure 7.6. *L. monocytogenes* occurrence on the mushrooms harvested during the 3 flushes treated with 293-amidase and PhaC-amidase BNPs. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of *L. monocytogenes* expressed as $\log_{10}$ CFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the 3 flushes. UC, uninoculated control; IC, Inoculated control; A, casing treated with 293-amidase; B, casing treated with PhaC-amidase BNPs.
7.4.4 vB_LmoS_293 survival in the casing soil and effect on L. monocytogenes

Bacteriophages were recovered throughout the entire trial from the plots where they were inoculated. However, despite an increase from $2.9 \log_{10}$ PFU/g at day 0 to $3.9 \log_{10}$ PFU/g at day 7, the numbers dropped to $0.8 \log_{10}$ PFU/g at day 34 ($p<0.05$, Figure 7.7). A similar pattern was shown by the *L. monocytogenes* counts in the same plots, where, between day 0 and day 7, numbers between $6.4 \log_{10}$ CFU/g and $5.9 \log_{10}$ CFU/g, respectively, were observed, while after day 7, a progressive decrease to $2.2 \log_{10}$ CFU/g at day 34 was observed ($p<0.05$, Figure 7.5).
Figure 7.7. Phage survival in the casing soil throughout the trial. The horizontal axis represents the experimental conditions, while on the vertical axis the mean of counts of bacteriophages expressed as $\log_{10}$ PFU/g. The error bars are the standard deviation and different colours, as shown in the legend, represent the sampling occasions. UC, uninoculated control; IC, Inoculated control; L, casing inoculated with the phage vB_LmoS_293.
On the mushrooms, the numbers of *L. monocytogenes* in the plots treated with the bacteriophage decreased during the three flushes, from $3.6 \log_{10} \text{CFU/g}$ at flush 1 to $0.1 \log_{10} \text{CFU/g}$ at flush 3. The inoculated control showed no significant differences with the bacteriophage treatment ($p>0.05$), from $4 \log_{10} \text{CFU/g}$ in the flush 1, to $0.5 \log_{10} \text{CFU/g}$ in the flush 3 (Figure 7.8). Two mushroom samples taken in the flush 2 from the inoculated control and one sample taken in the flush 3 from a plot treated with the bacteriophage showed no direct counts of *L. monocytogenes*, but they were positive after enrichment. No bacteriophages were recovered from the mushrooms over the 3 flushes.
Figure 7.8. *L. monocytogenes* counts on the mushrooms harvested from plots inoculated with the bacteriophage. On the horizontal axis are represented the experimental conditions and on the vertical axis are *L. monocytogenes* counts expressed as $\log_{10}$ CFU/g. The error bars are the standard deviation and the colours, as shown in the legend, represent the 3 flushes. UC, uninoculated control; IC, Inoculated control; L, casing inoculated with the phage vB_LmoS_293; Ex, casing treated with phosphate buffer.
7.4.5 Effect of the treatments on the mushroom yields

A total of 98 kg of mushrooms were harvested at the end of the 3 flushes: 40.16 kg in the flush 1, 39.32 in the flush 2 and 18.52 in the flush 3. No significant differences were shown between yields of controls and treatments with 293-amidase and BNPs (p>0.05, Figure 7.9). A difference was shown in the yield of mushrooms grown in the plots inoculated with the bacteriophage, but only in the flush 1, where the yield was significant different compared to the uninoculated control (p<0.05, Figure 7.10).
Figure 7.9. Mushroom yield in room 4. On the horizontal axis are represented the experimental conditions, while on the vertical axis the mean of kg harvested, with the standard deviation in the error bars. The colours, as shown in the legend, indicate the 3 flushes. UC, uninoculated control; IC, inoculated control; A, 293-amidase treatment; B, BNPs treatment.
Figure 7.10. Mushroom yield in room 3. On the horizontal axis are represented the experimental conditions, while on the vertical axis the mean of kg harvested, with the standard deviation in the error bars. The colours, as shown in the legend, indicate the 3 flushes. UC, uninoculated control; IC, inoculated control; L, vB_LmoS_293 treatment; Ex, plot inoculated with phosphate buffer.
7.5 Discussion

In this pilot-scale trial, none of the treatments showed further reduction of *L. monocytogenes* during mushroom growth or in the environment, when compared to the natural reduction occurring in the control experiments. While the treatments showed promising results in laboratory experiments (Chapters 5 and 6), when applied under growing conditions their efficacy was not apparent. In the floor trial, at least three factors could have inhibited the lytic activity of the 293-amidase: 1) Moisture may have not been sufficient: tests performed in laboratory were in liquid culture or on stainless steel coupons submerged in a liquid culture, while on a floor made of concrete, its porous texture quickly adsorbed the treatments applied, possibly causing natural reduction of *L. monocytogenes* due to desiccation and the inactivation of the protein. 2) The concentration of the treatment was not adequate: the optimal concentration of the 293-amidase for lytic activity determined in-vitro (Chapter 5) was not transferable to the pilot scale trial; a higher concentration could have facilitated the contact on the floor between the 293-amidase and *L. monocytogenes*, which was probably hidden by the porous matrix. 3) Adverse environmental conditions: low temperature, humidity and natural microflora able to produce proteases could have contributed to the inactivation of the 293-amidase. It needs to be pointed out that, for practical reasons, the floor experiment was not performed during the mushroom production trials. The space on the floor did not permit the presence of the experimental tiles and the possibility to walk through the growing room during the mushroom production; the watering would cause the washing out of *Listeria* and treatment to the drains, while during the experimental trials it was necessary for the *L. monocytogenes* to stay on the floor; residues of casing and compost falling on the floor could have compromised the potential growth or reduction of *L. monocytogenes*. The procedure of sampling in this case was undertaken with sponge stick swabs. Although
enumeration of bacteria from swabs is not recommended, because of the difficulty in recovering all the cells from the floor surface and from the sponge itself, this procedure was chosen for simplicity and after testing, the consistency in *L. monocytogenes* cells recovery in preliminary assays (data not shown).

In the mushroom growing plots, even more variables could have potentially masked the efficacy of the treatments. A first consideration is the watering during the trial: when the mushroom crop starts to grow, water needs to be applied daily to maintain the right moisture needed for the mushrooms to grow and colonise the casing soil. Each watering occasion could have diluted the treatments, rendering them ineffective. Furthermore, the natural microflora inhabiting the casing soil could have contributed not only to the reduction of *L. monocytogenes* numbers throughout the trial (O’Patchen, 2011), but also to the inactivation of the treatments. The matrix constituting the casing soil could represent an obstacle to bacteriophage-host interactions and protect the bacteria from the treatments or vice-versa. Casing soil has a neutral pH, but lactic acid bacteria that are present in both the compost and casing could acidify the substrates, which could modify the lytic activity of the protein. The mushrooms themselves could cause inhibition of the growth of *L. monocytogenes*, especially the microflora carried on its surface (Xiang et al., 2017). However, challenge tests have shown that the whole mushroom do not support *L. monocytogenes* growth (Leong et al., 2015). Finally, the mushroom mycelium and fruiting bodies could have contributed to the inactivation of the anti-listerial treatments tested in the trial, due to proteolytic activity (Burton et al., 1994). The sporadic isolation of *L. monocytogenes* and bacteriophages from the uninoculated controls (Figure 7.4, 7.5 and 7.7) highlights the difficulty in transferring protocols from a laboratory scale to a pilot scale mushroom production unit.

The treatments applied to the casing soil did not affect the yield of mushroom harvested in the 3 flushes, with the exception of the bacteriophage treatment, where the
yield was significantly lower (p<0.05). To date, pilot scale trials on mushroom cultivation are scarce and the application of bacteriophages on mushrooms have been tested only as a post-harvest treatment (Kim et al., 2011; Murray et al., 2015). The reason for the reduction in yield due to bacteriophage during growth is not clear, as it is not clear the absence of bacteriophage on the mushrooms. An explanation could be some interaction between the mushrooms and the phage, or the bacteriophage infecting part of the microflora in the casing soil that triggers the formation of fruiting bodies. All the mushroom samples tested showed cross contamination of \textit{L. monocytogenes} from the casing soil. In a previous study, O’Patchen et al. (2011) showed that after inoculating casing and compost with \textit{L. monocytogenes}, the cross contamination rate on the mushrooms was 56\%, although, no enrichments were performed in that study. These results highlight the necessity to increase hygiene strategies in order to reduce cross contamination (Chapter 7.8).

Despite the presence of \textit{L. monocytogenes} on the mushroom fruiting bodies, no bacteriophages were isolated from them. Although bacteriophages have been isolated in the past from mushrooms (Casey et al., 2016; Kim et al., 2011), in this case the hurdle created by the mushroom could have kept their level below the limit of detection.

The \textit{L. monocytogenes} 473-s strain used in this study was a natural mutant, selected for its streptomycin resistance. In \textit{in-vitro} experiments, no differences were shown between the wild type \textit{L. monocytogenes} 473 and the mutant. Preliminary studies in casing soil showed the stability of the streptomycin resistant mutant. Further characterisation and whole genome sequence comparison would be needed to show their relatedness.

The results shown of this pilot scale trial highlight the difficulty in transferring results obtained \textit{in-vitro} to \textit{in-vivo} conditions. There are a number of additional variables in a pilot-scale trial, compared to a laboratory trial that cannot be controlled. To overcome these variables more trials are needed to optimise the protocols in order of concentrations, formulations and mode of application of the treatments. An evaluation of the costs implied
in the production of such treatments could also provide an indication of their potential applicability in a commercial scale. For example, the use of bacteriophages in a commercial setting, based on the results shown by this trial, could not be considered as the mushroom yield was reduced. The use of 293-amidase and PhaC-amidase BNPs needs to be optimised.
7.6 Conclusions

A pilot scale mushroom growing unit was used to test bacteriophage-related treatments for *L. monocytogenes* reduction on the floors and during the mushroom growth. The results showed a lack of efficacy of such treatments, despite being effective *in-vitro*. This study highlights the difficulty of transferring the results obtained in laboratory-scale conditions to pilot scale conditions, where a high number of variables can influence the effectiveness of treatments. This indicates the need for more pilot scale studies in complex environments like mushroom production, where pathogens like *L. monocytogenes* are still able to colonise, even with a very well established production protocol.
7.7 References


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Chapter 8

General discussion
8.1 General discussion

The purpose of this study was to assess the occurrence of *L. monocytogenes* in mushroom production and to investigate options for its control using bacteriophage.

The results of Chapter 2 showed a high occurrence of *L. monocytogenes* in the mushroom growing environment and in the raw materials (casing and compost) used for mushroom growth. *Agaricus bisporus* mushrooms are considered a ready-to-eat food as there is no heat treatment or any other microbiological inactivation between production and consumption. Although cooking of the mushrooms is undertaken in most cases, the option of adding them uncooked to, for example, salads is important for the industry. Therefore, the high occurrence of *L. monocytogenes* in the mushroom production environment is an important consideration. One of the possible contamination scenarios highlighted in Chapter 2 is with the raw materials. If *L. monocytogenes* is introduced in the mushroom growing facility with the raw materials, the watering system can transfer *L. monocytogenes* to the lower shelves and, eventually, to the floor. Once on the floor, the spread of *L. monocytogenes* to different areas of the mushroom processing environment happens by walking of personnel or with trolley wheels. If materials (crates, packaging) are stored on the floor, the risk of cross contamination is higher. Contaminated crates and materials can spread contamination to other facilities if transportation services are shared.

The results of Chapter 3 provide evidence to this theory, showing the same pulsotype in the raw materials and all around the mushroom growing facilities, including on the mushrooms. Furthermore, pulsotypes were also shared between mushroom growing facilities, confirming both the possible use of shared transportation services and the contamination coming from the raw materials. One of the hygiene measures used in the mushroom industry to control pathogens after a growth cycle is complete is called ‘cook-out’, a high temperature treatment in the growing environment followed by sanitation. In
Chapter 4, the results showed that while ‘cook-out’ kills *L. monocytogenes* in used casing and compost, the process does not inactivate them on the floors, principally because the floor temperature does not reach the desired temperature of 60°C. Only structural changes can resolve this issue, such as insulation of the floors, although this can be proposed only prior to the building of a mushroom growing unit, because of the high costs and time needed for the renewal of an old growing facility. The results of Chapter 4 provide some advice to better understand the routes of *L. monocytogenes* contamination in the raw materials. In the production of casing soil, for example, no heat treatments are undertaken in order to preserve the natural microflora that is needed to stimulate the growth of *Agaricus bisporus*, such as *Pseudomonas* species, that metabolise volatile compounds that inhibit the development of *Agaricus bisporus* primordia (Cai et al., 2009; McGee, 2018). Furthermore, due to the high amounts of materials involved in casing production, contamination of the casing by the faecal material of wild birds and animals cannot be controlled. Finally, the continuous transportation of materials by lorries, loaders and conveyor belts helps the spreading of contamination. In compost production, the pasteurisation process seemed to inactivate *L. monocytogenes* as no positive samples were found in the compost immediately after pasteurisation. However, due to the large amount of materials involved in compost production (pasteurisation sheds can have several tonnes of compost), cold spots during pasteurisation can permit *L. monocytogenes* survival. Furthermore, post-pasteurisation contamination of the compost is always possible, during the transportation, for example. At mushroom growing facilities, the cookout process is ineffective if the floors do not reach the desired temperature of 65-70 °C. It is important to highlight that improvements in hygiene practices targeting *L. monocytogenes* reduction, can be effective also against other pathogens, such as *S. aureus*, *E. coli*, *Salmonella spp*, reducing the risk of outbreaks and recalls not only due to *L. monocytogenes*. Furthermore, hygiene improvements can lead also to a reduction of diseases affecting the mushrooms.
such as green mold, associated with fungi of the genus *Trichoderma*, cobweb, caused by ascomycetes belonging to the genus *Cladobotryum*, brown cap, generated by various viruses and the dry bubble disease, associated to the mycoparasite *Verticillium fungicola*.

Reduction of *L. monocytogenes* at growing facilities can also involve the use of biotechnologies in synergy with good hygiene practices, such as the application of bacteriophages. As only virulent phages are recommended for use as biocontrol agents (Hagens & Loessner, 2010), the production of recombinant endolysins extends the possibility of also using temperate phage enzymes for that purpose. The production of the 293-amidase, described in Chapter 5, had the advantage of using a temperate phage genome (vB_LmoS_293) against *L. monocytogenes*. The potential application of recombinant endolysins against *L. monocytogenes* has been already documented in the literature (Ibarra-Sánchez et al., 2018; Van Tassell et al., 2017; Zhang et al., 2012), but no studies have been conducted yet regarding their potential application in the mushroom industry. Furthermore, the production of recombinant active domains rather than the full length endolysin, has already been shown to increase the stability of the enzyme and retain its lytic activity (Fenton et al., 2011; Simmons et al., 2012). Polyhydroxyalcanoate bionanoparticles (PHA BNPs) are a novel biotechnology being tested for the delivery of lytic enzymes and, because promising results have been already described in the literature (Altermann et al., 2018), it was chosen for the delivery of the 293-amidase (Chapter 6). The results from the laboratory experiments resulted in *L. monocytogenes* inactivation, however, optimisation is needed in terms of conditions and concentration of the treatments, for the practical application of these biotechnologies, as highlighted by the results of Chapter 7. This chapter involved an attempt to transfer the promising results from the laboratory to a pilot-scale mushroom growing environment, studying inactivation of *L. monocytogenes* on the floor and in casing. No inactivation of *L. monocytogenes* was seen, demonstrating the challenge of transferring positive results from the laboratory to the
mushroom growing environment. In mushroom production, in particular, there are variables that cannot be adjusted to facilitate the treatments, such as the watering system, which is needed for mushroom growth but could dilute or wash off the treatments, the casing soil itself, which could be an obstacle for contact between \textit{L. monocytogenes} and the treatments, or the microflora of the mushroom and the casing that could produce compounds that inactivate the treatments. For these reasons, a better understanding of the processes that lead to the ineffectiveness of these treatments in the pre-harvest environment is needed and optimisation, for example of the concentrations to be used in-vivo, need to be undertaken.

The results of this thesis show that \textit{L. monocytogenes} is a natural component of the microflora of the mushroom production process and its occurrence during the mushroom growth is very difficult to reduce. A more feasible approach, based on the results discussed here, consists of applying hygiene procedures with the aim of reducing cross contamination from the production environment to the mushrooms. For that purpose, hygiene guidelines aimed at reduction of cross contamination were proposed to the mushroom industry. The aim of these guidelines was to create awareness within the industry regarding \textit{L. monocytogenes} pathogenicity and occurrence, by sharing with them the overall results obtained with the surveys undertaken in this thesis, and to give practical advice on how to reduce cross contamination in the mushroom growing environment. For example, the harvester, called the “picker” in the mushroom industry, is a critical person in the whole process, because of the physical contact with the mushrooms during the harvest, which can transfer \textit{L. monocytogenes} to the mushrooms. It was suggested that the pickers change their gloves very frequently, in particular when moving to a different shelf, thus reducing the risk of transferring contamination from one shelf to the next. Furthermore, when climbing ladders to the upper shelves, it was suggested that it would be better to hold to the railings of the ladder rather than touching the steps with the gloved hands, because
L. monocytogenes had been isolated frequently from the steps. Other advice was given on washing and disinfecting the knives and keeping all the materials (packaging, crates, waste) off the floor by using platforms and trolleys. Details on the post-harvest hygiene procedure, such as cleaning, cookout and sanitation, were also included, explaining the issues with the temperature of the floors during cookout, as shown in Chapter 4. With regard to the use of a power hose after the cookout, it was explained that a power hose creates an aerosol that can spread L. monocytogenes all around the growing room and it can be used only if a second heating step is undertaken, as this second heat treatment would inactivate the pathogens transferred to the shelves by the aerosol.

In conclusion, this work has demonstrated that L. monocytogenes occurrence in the mushroom industry is high due to its natural presence in the raw materials and its total elimination from the mushroom growing environment is a target almost impossible to reach. Novel biotechnologies, such as the use of bacteriophage and their recombinant lytic enzymes, can be a powerful tool in cross contamination reduction, but their application needs to be optimised for the mushroom production environment. The focus of the hygiene practices needs to be on the reduction of cross contamination, with implementation of the procedures outlined above. Cross contamination reduction will reduce the occurrence of L. monocytogenes on the mushrooms and if the mushrooms are L. monocytogenes-free, disease outbreaks and recalls can be averted, with benefits for the industry and for public health.
8.2 References


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*Rock'n'Roll
Peace and Love

Vincenzo*