Persistence of *Listeria monocytogenes* in response to a natural antimicrobial, nisin

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In

Food Microbiology

at

Massey University

Palmerston North, New Zealand

Shuyan Wu

2019

Abstract

The persistence of bacteria can be defined by prolonged existence of a proportion of a population in an environment despite antimicrobial treatment. Food contamination by *Listeria monocytogenes* (*L. monocytogenes*) may be due to the persistence of *L. monocytogenes* despite antimicrobial treatments aimed at controlling this pathogen. How *L. monocytogenes* persists and survives in food environments is poorly understood.

The persistence of cells exposed to antibiotics is well recorded however the persistence following treatment with natural antimicrobials like the bacteriocin nisin (used for controlling food pathogens in a variety of food systems across a wide pH range) has not been determined. This study used two *L. monocytogenes* isolates, one from food and one from an animal origin, to optimize methods for obtaining *L. monocytogenes* persisters following nisin treatment. *L. monocytogenes* were treated with nisin, and a biphasic killing pattern was observed for both strains. Persister cells formed a population of cells showing tolerance to the high concentrations of nisin and proved to be non-resistant when regrown and re-exposed to nisin.

Next, this study examined persistent subpopulations of *L. monocytogenes* following exposure to nisin treatment under different environmental conditions (such as rich/poor media and variations in pH) representing different food systems. The pH and nutrient levels influenced the production of persister cells of *L. monocytogenes*. Stationary phase cells re-suspended in a nutrient rich environment (Trypticase Soy broth, TSB) showed greater survival than in a nutrient limiting environment (spent TSB medium) under nisin treatment. However, traditional microbiological techniques such as agar plating are limited in providing information of persister cells exposed to antimicrobial substances.

Flow cytometry has potential to identify persister cells that although they cannot be cultured, can still pose a threat to human health. The *L. monocytogenes* stationary phase populations in spent medium or re-suspended in fresh TSB medium were individually treated

with nisin, and the treated cells were assayed using flow cytometry combined with live/dead staining. The results revealed a reduction in viable populations and the differentiation of living cells and dead/damaged cells within the treated population. This finding was consistent with previous results from plate count experiments. Distinct changes in cell permeability were detected within 2hrs of the nisin treatment in spent medium and re-suspended in fresh medium, indicating possible variations in the mechanisms of persister formation between the two groups.

The persistence of a *L.monocytogenes* stationary phase population when facing nisin treatment was investigated for gene expression with RNA extracts obtained after 90mins of nisin treatment. RNA Seq analysis was used for gene expression profiling of the persister cells in spent (persister N) and rich medium (persister TN) compared with untreated cells. Functional genes associated with the persister populations were identified in multiple systems including heat shock related stress response, cell wall synthesis, ATP-binding cassette (ABC) transport system, phosphotransferase system (PTS system), and SOS/DNA repair. Differences were found in gene regulation between persister N and persister TN populations. Nutrition may be associated with the variations in gene expression resulting in variations in the size or composition of the persistent populations. This study provided information on the formation of persister cells exposed to nisin and provides some insight into possible mechanisms of impeding bacterial persistence.

Acknowledgements

The accomplishment of the thesis is the result of massive supports and caring from many people, including my supervisors, fellow PhD candidates, Lab staff, my family and friends.

First, I would like to thank my main supervisor, Steve Flint, for accepting me as your PhD student. You gave me the chance to fulfil myself. You offered valuable advices and feedbacks on my project. And you cheered for my every progress during this long journey. In our weekly meetings, you always had unique perspectives to interpret my data. You helped me to understand things in a different way.

Second, I would like to thank my co-supervisor, Pak-Lam Yu. I am honoured to be your last PhD student when you retired from Massey University. I appreciate your scrutiny of every detail of my experiments. You helped to revise and improve my experimental methods. And you provided thoughtful advices with your expertise in the natural antimicrobial agent (nisin). I thank you greatly for your instruction.

I learned from Steve and Pak about professional attitudes - hardworking with passion and devotion.

Next, I would like to give a big thank to my fellow PhD candidates and Lab staffs at Massey University. My fellows exchanged wonderful opinions/ideas in the Lab. The lab staffs assisted me getting the right agents/consumables and helped me getting access to many equipments in other Labs (the the Riddet Institute and Science Towers). Special thanks go to the Lab staffs Ann-Marie Jackson and Haoran Wang. Great thanks go to David Wheeler from the Institute of Fundamental Science at Massey University for assisting me to analyse transcriptomic data. Great thanks go to Dr. Fran Wolber (from School of Health Sciences, Massey University) for her help with the flow cytometry analysis.

Finally, I would like to express my sincere gratitude to my family. My family shows me their love and supports for the entire time. This makes me capable of completing the study. Sincere love goes to my parents who always support me unconditionally. Sincere gratitude goes to my endearing grandparents who truly give me faith to overcome what I have to confront.

In memory of my beloved grandmothers and my maternal grandfather in heaven, I wish you can share my achievement and happiness out there. I miss you a thousand times. I love you always.

Declaration

The presented thesis is comprised of seven chapters. Partial results of chapters 3, 4, 5 and 6 are structured as manuscripts that have either been submitted or published. Therefore, sections in the materials and methods are repeated in some chapters, however, results and discussion are different for each chapter.

Table of contents

Abstract i
Acknowledgements iii
Declaration iv
Table of contents v
List of Figures ix
List of Tables xi
List of publications and conference presentations xii
Chapter 1 General introduction 1
1.1 Rationale and importance 1
1.2 Research questions and hypotheses 5
1.2.1 Questions 5
1.2.2 Hypotheses 5
1.3 References 6
Chapter 2 Literature Review 8
2.1 Persistence definition and formation 8
2.1.1 Bacterial persistence 8
2.1.2 *Listeria monocytogenes* persistence 10
2.2 Strategies for controlling the occurrence of persistent *L. monocytogenes* 11
2.2.1 Cleaning and sanitation 11
2.2.2 Physical approaches 12
2.2.3 GRAS Preservatives (Chemical additives) 13
2.2.4 Natural preservatives 15
2.3 Current insights on persistent *Listeria monocytogenes* from food industrials 18
2.4 Mechanism of persister formation 21
2.4.1 Stringent response 22
2.4.2 Toxin-antitoxin (TA) module 24
2.4.3 Efflux pump 26
2.4.4 Other Mechanisms 28
2.4.5 Possible mechanisms for persistence of *L. monocytogenes* 29
2.5 Conclusions 31
2.6 References 32
Chapter 3 Persister cell formation of *Listeria monocytogenes* in response to a natural antimicrobial, nisin 52
Abstract 53
3.1 Introduction 54

3.2 Materials and Methods 56
3.2.1 Bacterial strains and growth conditions 56
3.2.2 Nisin stock preparation and the minimum inhibitory concentration (MIC) test 56
3.2.3 Optimising a method to obtain *L. monocytogenes* persisters following nisin treatment 57
3.2.4 Identification of *L. monocytogenes* persisters from stationary phase cells by measuring the death kinetics curve 58
3.2.5 Identification of the phenotypic susceptibility of persister isolates to nisin 58
3.2.6 Statistical analysis 59
3.3 Results 60
3.3.1 Efficacy of nisin on inhibiting the growth of two *L. monocytogenes* isolates 60
3.3.2 The persister cells of *L. monocytogenes* following treatment with different concentrations of nisin 62
3.3.3 The death kinetics of *L. monocytogenes* exposed to nisin 66
3.3.4 The population of persister cells as sensitive to nisin as the parental strain 68
3.4 Discussion 70
3.5 Acknowledgement 71
3.6 Supplementary information 72
3.7 References 74
Chapter 4 Characterising the persister cells of *Listeria monocytogenes* following nisin treatment 77
Abstract 78
4.1 Introduction 79
4.2 Materials and Methods 81
4.2.1 Evaluating factors impacting on the production of *L. monocytogenes* persisters 81
4.2.2 Evaluating the metabolic level of *L. monocytogenes* persister cells 84
4.2.3 Growth recovery of *L. monocytogenes* persister cells in a nisin-free environment 85
4.2.4 Estimating cell surface charge of *L. monocytogenes* persister cells 86
4.2.5 Statistical analysis 86
4.3 Results 87
4.3.1 The effect of nutrient concentration on the production of *L. monocytogenes* persister cells 87
4.3.2 Impact of pH on the production of *L. monocytogenes* persister cells under lethal nisin 89
4.3.3 Impact of nutrient composition on the production of *L. monocytogenes* persister cells under lethal nisin 92
4.3.4 Metabolic activity of *L. monocytogenes* persister cells by the XTT assay 94
4.3.5 Growth recovery of *L. monocytogenes* persister cells 96
4.3.6 Cell surface charges of *L. monocytogenes* persister cells 98

4.4 Discussion 100
4.5 Conclusion 103
4.6 Acknowledgements 103
4.7 Supplementary Information 104
4.8 References 107
Chapter 5 Flow cytometry investigation on *Listeria monocytogenes* persistence during lethal nisin treatment 112
Abstract 113
5.1 Introduction 114
5.2 Materials and methods 116
5.2.1 Flow cytometry 116
5.2.2 Fluorescence microscopy analysis 117
5.2.3 Free radical production by XTT assay 117
5.2.4 Statistical analysis 118
5.3 Results 119
5.3.1 Flow cytometric assessment of the antimicrobial activity of lethal nisin on *L. monocytogenes* 119
5.3.2 Fluorescence microscopy verification of live/dead staining of *L. monocytogenes* cells during lethal nisin treatment 129
5.3.3 Free radical production on population level of *L. monocytogenes* cells during lethal nisin treatment 135
5.4 Discussion 138
5.5 Acknowledgement 140
5.6 Supplementary materials 141
5.7 References 144
Chapter 6 Transcriptomic study on the persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin 148
Abstract 149
6.1 Introduction 150
6.2 Materials and Methods 152
6.2.1 Bacterial strains and growth conditions 152
6.2.2Total RNA extraction 152
6.2.3 Transcriptome sequencing 153
6.2.4 Differential expression analyses 153
6.2.5 RT-qPCR (Reverse Transcription Quantitative PCR) validation 153
6.3 Results 154
6.3.1 Diagnostic MA (DESeq2) plots visualized the nisin treatment effect on persister formation in different conditions 154

6.3.2 RNA-Seq analysed data on the log2 fold changes over the mean of normalised counts and significant genes from the nisin treatment highlighted in persister N and persister TN. 156
6.3.3 RT-qPCR validation 162
6.4 Discussion 164
6.4.1 Stress response 164
6.4.2 Cell wall synthesis 165
6.4.3 ATP-binding cassette (ABC) system 167
6.4.4 Phosphotransferase (PTS) system 168
6.4.5 SOS response and DNA repair 169
6.4.6 Phage/prophage 171
6.4.7 Other genes 171
6.4.8 Conclusions 173
6.5 Supplementary materials 175
6.6 References 177
Chapter 7 Summary and Final Discussion 185
7.1 *L. monocytogenes* persisting in nature 185
7.2 Presence of *L. monocytogenes* persister cells during biphasic killing by lethal doses of nisin 185
7.3 Increasing the persister fraction under nisin treatment 186
7.4 Persister cells versus viable and un-culturable cells 187
7.5 Molecular mechanisms for persister formation 189
7.6 Future work 190
7.7 Supplementary information 193
7.8 References 195

List of Figures

Chapter 2

Figure 2. 1 Occurrence of a fraction of persister cells within a bacterial population 9

Chapter 3

Figure 3. 1 The effect of low nisin (1.5 µg/ml, 3 µg/ml and 6 µg/ml) treatment on the survival and growth of A1 and M5 strains..... 61
Figure 3. 2 Killing of log phase *L. monocytogenes* cells treated with nisin at a series of concentrations from 0-75µg/ml at 30°C for 24 hrs. 62
Figure 3. 3 Killing of stationary phase (18 h cultures) of *L. monocytogenes* treated with 0-75µg/ml nisin 30°C for 24 hrs 64
Figure 3. 4 Concentration- dependent killing of *L. monocytogenes* stationary phase cells (from 24-hrs overnight culture) treated with 0-75µg/ml nisin at 30°C for 24 hrs 65
Figure 3. 5 Death kinetics of *L. monocytogenes* overnight cultures treated with 75µg/ml nisin 75µg/ml, 30°C for 24 hrs. 67
Figure 3. 6 Six persister strains of *L. monocytogenes* (AP1-AP6) that survived 75µg/ml nisin treatment for 24hrs were re-grown with in fresh TSB and then re-exposed to 75µg/ml nisin at 30°C for 24hrs..... 69

Supplementary Figure S3. 1 Growth curves of *L. monocytogenes* strains (A1 and M5) in TSB at 30ºC. The experiment was performed with three biological replicates, and error bars indicate the standard deviation. 72
Supplementary Figure S3. 2 MIC test with nisin treatments. 72

Chapter 4

Figure 4. 1 Dose-dependent killing of re-suspended cells of *L. monocytogenes*. 88
Figure 4. 2 The effect of nisin on cells re-suspended in TSB at different pH values (pH 5-8) on the production of persister cells. 91
Figure 4. 3 The effect of nisin treatmemt on cells re-suspended in MWB at different pH values (pH 5-8) on the production of persister cells. 93
Figure 4. 4 Metabolic activity of *L. monocytogenes* persister cells 95
Figure 4. 5 Cell surface charges of *L. monocytogenes* persister cells..... 99

Supplementary Figure S4. 1 Death kinetics of TSB re-suspended *L. monocytogenes* cultures treated with 75µg/ml nisin (cell viability was meatured at time0, 1, 2, 3, 4, 12 and 24hrs). 104
Supplementary Figure S4. 2The growth curves for impedance change with time were measured by the BacTrac™ 4000 microorganism growth analyser..... 105
Supplementary Figure S4. 3 Serial 10-fold dilutions of overnight cultures without nisin treatment were determined by the time for impedance change into the exponential phase of cell growth (E=15) to obtain regression equation for each strain 106

Chapter 5

Figure 5. 1 Flow cytometry (FC) dot plots of FITC-A (FL1, green fluorescence) vs. PerCP-Cy5.5A (FL3, red fluorescence) of *L. monocytogenes* control cells..... 120

Figure 5. 2 Flow cytometry profiles showing the cell sorting plots of *L. monocytogenes* stationary phase cells directly treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).... 124
Figure 5. 3 Flow cytometry profiles showing the cell sorting plots of TSB re-suspended *L. monocytogenes* cells treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right)..... 128
Figure 5. 4 Epifluorescence micrograph of *L. monocytogenes* live control (log phase cells, A and B) and dead control (heat inactivated cells, C and D) for both A1 (A and C) and M5 strain (B and D). 129
Figure 5. 5 Epifluorescence micrograph profiles showing TO/PI stainings of *L. monocytogenes* stationary phase cells directly treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).... 131
Figure 5. 6 Epifluorescence micrograph profiles showing TO/PI stainings of TSB re-suspended *L. monocytogenes* cells following with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right)..... 134
Figure 5. 7 Free radical production measured by the XTT assay, of *L. monocytogenes* under exposure to lethal nisin. 137

Supplementary figure S5. 1 Agar plate counting of cell viability under 75µg/ml nisin from different time points (0, 30, 60, 90 and 120 mins)..... 143

Chapter 6

Figure 6. 1 MA plots show the log2 fold changes from the treatment over the mean of normalised counts for the blank versus the persister N sample. 155
Figure 6. 2 MA plots show the log2 fold changes from the treatment over the mean of normalised counts for the blank versus the persister TN sample. 155
Figure 6. 3 KEGG pathway enrichment is found in differentially expressed genes of group N versus TN. The red stars are genes in the dataset that were significantly differentially expressed. 161
Figure 6. 4 RT-qPCR validation in persister of TN group. X axis represents Log2 Ratio of RNA- Seq data, and Y axis represents Log2 Ratio of RT-qPCT results. 163

List of Tables

Chapter 3

Supplementary Table S3. 1 Spread plating and drop plating were used to show consistency of results on enumerating survived cells from nisin treatment with different concentrations..... 73

Chapter 4

Table 4. 1 pH value of liquid environments from different nisin treatment groups for A1 strain (mean of triplicate readings +/- standard deiation) 90
Table 4. 2 pH value of liquid environments from different nisin treatment groups for M5 strain 90
Table 4. 3 Growth recovery of *L. monocytogenes* persister cells compared with the expected impedance detection time for a non-persister population. 97

Chapter 5

Supplementary table S5. 1 Summary of viable cell number estimation from % event detected from H1-3 quadrant by FC analysis and cell counts (log_{10} cfu/ml) obtained from recovery on agar plating. The measurements were performed in duplicate..... 141

Chapter 6

Table 6. 1 a list of interested genes which change in persister N group 157
Table 6. 2 a list of interested genes which change in persister TN 159
Table 6. 3 RT-qPCR validation in persister N and TN group. 163

Supplementary Table 6. 1 Primer pairs used for qRT-PCR validation..... 175

Chapter 7

Supplementary Table S7. 1 Genes that showed contrary expression in persister TN over persister N 193
Supplementary Table S7. 2 Biofilm formation of A1/M5 strains and their persister cells surviving from 75µg/ml lethal nisin treatment 194

List of publications and conference presentations

Shuyan Wu, Pak-Lam Yu, Steve Flint (2019) Flow cytometry investigation on *Listeria monocytogenes* persistence during lethal nisin treatment. Food Control (in review)

Shuyan Wu, Pak-Lam Yu, David Wheeler, Steve Flint (2018) Transcriptomic study on persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin. Journal of Global Antimicrobial Resistance doi:https://doi.org/10.1016/j.jgar.2018.06.003.

Shuyan Wu, Pak-Lam Yu, Steve Flint (Nov. 2018) Flow cytometry investigation on *Listeria monocytogenes* persistence during lethal nisin treatment. NZMS 2018, Dunedin, New Zealand (poster presentation)

Shuyan Wu, Pak-Lam Yu, Steve Flint (Jul. 2018) *Listeria monocytogenes* persistence under natural antimicrobial agent nisin. NZFSSRC (New Zealand Food Safety Science & Research Centre) 2018 Annual Symposium, Hamilton, New Zealand (oral presentation)

Shuyan Wu, Pak-Lam Yu, Steve Flint (2017) Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. Food Control 77:243-250.

Shuyan Wu, Pak-Lam Yu, David Wheeler, Steve Flint (Nov. 2017) Transcriptomic study on persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin. NZMS 2017, Auckland, New Zealand (oral presentation)

Shuyan Wu, Pak-Lam Yu, Steve Flint (Nov. 2016) Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. NZMS 2016, Christchurch, New Zealand (oral presentation)

Chapter 1 General introduction

1.1 Rationale and importance

Maintaining food quality and safety relies on controlling microbial levels through disinfection and sterilization, and the use of preservatives.

Disinfection and sanitization are used to assure the food quality during manufacture. Chemicals (e.g. chlorine, hydrogen peroxide and quaternary ammonium compounds) are currently the most commonly used sanitation methods. However, the use of chemical sanitizers requires minimum concentrations of the sanitizer appropriate for the microbial contamination during the rinse step following cleaning to ensure sanitization and precautions to avoid chemical residues which could be toxic in the final products. Some chemical sanitisers, such as ozone or electrolyzed water, can be corrosive to the surfaces and therefore have limited use. Heat treatment which is used extensively in the food industry for sterilisation, is a useful but expensive approach for the sanitation of food processing plants and is not suitable for some applications such as fresh fruit and vegetables.

Preservatives are used as additives to foods to maintain food safety and quality. There are many approved chemical food preservatives but there is some consumer resistance to the addition of preservatives to foods. The most acceptable preservatives are those regarded as natural preservatives such as bacteriocins.

With all chemical treatments – sanitisers for cleaning food manufacturing plant, treating products and preservatives added to products, a growing concern is the development of persister cells (Wood *et al.*, 2013). Persisters are phenotypic variants of the wild type bacteria strain and genetically identical to susceptible wild type bacteria (Lewis, 2005). Persisters may be formed from the population in response to particular triggers or switches, such as nutrient shift or antimicrobial stresses and become the stubborn minority capable of surviving under harsh environmental stresses over a long term (Lewis, 2005). Persister cells are highly tolerant to antimicrobial treatments used in the food industry (Madden *et al.*, 2018). For those studying

persister cells, the challenge is to obtain sufficient persister cells in their viable and non-culturable state for investigation. This is a particular concern for foodborne pathogens such as *L. monocytogenes* (Allison *et al.*, 2011).

L. monocytogenes is a Gram-positive bacterium which induces the life-threatening food borne disease listeriosis which predominantly affects immuno-compromised people, including elders, neonates and pregnant women (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* uses different strategies to cross human defences, causing listeriosis by septicemia, meningitis, foetal loss, and premature labour with a high mortality rate (around 20– 30%) (Laksanalamai *et al.*, 2012). As a recalcitrant pathogen, *L. monocytogenes* is capable of surviving and proliferating under a wide range of pH, temperatures and salinity, and this enables *L. monocytogenes* to colonize food processing surfaces and contaminate food (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* strains can survive sanitation in food processing and are therefore difficult to control (Carpentier and Cerf, 2011). Listeriosis outbreaks are associated with diverse foods, including fruits, vegetables, raw meat and poultry, seafood, and ready-to-eat (RTE) foods (Carpentier and Cerf, 2011).

The number of listeriosis cases continues to increase globally. A listeriosis case in 2014 due to contaminated sausages caused the deaths of at least 12 people in Denmark (http://barfblog.com/ author/dougpowell/). In 2015, Blue Bell ice cream products caused three deaths in USA and the company recalled eight million gallons of ice cream and suspended operations (https://about-listeria.com/listeria-outbreaks/blue-bell-ice-cream-listeria-outbreak). Between 2017 and 2018, the world's worst recorded listeria outbreak killed 216 people and sickened more than 1,000 by consuming processed meat products in South Africa (https://medicalxpress.com/news/2018-09-safrica-declares-listeria-outbreak.html). In February 2018, 3 deaths and 12 illnesses were associated with *L. monocytogenes* contaminated rock melon in Australia (https://www. stuff.co.nz/world/australia/102370447). New Zealand also has food safety issues with *Listeria*. One particularly notable food safety issue was the contamination of mussels with *L. monocytogenes* which resulted in the death of two babies(Cruz *et al.*, 2014).

Detection with the listeria contamination leads to product recalls (www.recalls.govt.nz). The Leonard's Superior Smallgoods company in Auckland was warned with two recalls of *Listeria*-contaminated meat to shut down all production of ready-to-eat products in 2008 (https://www.nzherald.co.nz /nz/news/article.cfm?c_id=1&objectid=10495434). Whitestone Windsor blue cheese from New Zealand tested positive for *Listeria* contamination by health authorities of Australia in 2014 (http://www.abc.net.au/news/2014-03-07/27urgent-recall27-for-imported-cheese-product/5306_854). LeaderBrand recalled its salad products and suspended operations at its factories after a batch tested positive for *L. monocytogenes* in 2017 (https://www.mpi.govt.nz/food-safety/food-recalls/recalled-food-products/various-salad leader brand-produce/). In July 2018, a frozen vegetable mix was recalled as precaution amid fears it could contain a particularly dangerous strain of *Listeria* that has been linked to nine deaths (https://www.radionz.co.nz/news/national/361524/countdown-withdraws-frozen-veggie product -after-australian-recall). Overall, the New Zealand Public Health Surveillance Report estimates the incidence of listeriosis shows an increase from the previous 12 month period (https://surv.esr.cri.nz).

Food contamination by *L. monocytogenes* is most likely due to the persistence of *L. monocytogenes* despite antimicrobial treatments aimed at controlling this pathogen. How *L. monocytogenes* persists and survives in food environments is poorly understood. *L. monocytogenes* forms biofilms in which cells are preserved in a self-produced polymer matrix and this decreases the efficiency of cleaning and disinfection. Foodborne diseases are associated with bacterial attachment which is the initial process in development of a biofilm(Srey *et al.*, 2013). In some cases of foodborne illness outbreaks by *L. monocytogenes*, the attachment of *L. monocytogenes* is important for its persistence in food environments (Lunden *et al.*, 2000). The formation of a biofilm, however, does not explain the persistence of planktonic cells.

Persistent *L. monocytogenes* from food processing environments and foods, sampled over a long period (up to months and even years) are present in extremely low cell numbers (as low as 30 cfu consumed for susceptible people) that are a food safety hazard. An investigation of

persistent *L. monocytogenes* in cold smoked salmon processing over the period of six years identified the majority isolates as serotypes 1/2a and 1/2b and two main PFGE profiles identified the routes of contamination during processing (Di Ciccio *et al.*, 2012). Ortiz *et al.* examined contamination of *L. monocytogenes* in an Iberian pork-processing plant, and by molecular typing, determined that the same persistent strain of *L. monocytogenes* had survived in the manufacturing area for 3 years (Ortiz *et al.*, 2010). These low numbers of cells are detected by enrichment in a laboratory (Holch *et al.*, 2013). The persistence studies of *L. monocytogenes* need to re-grow the persistent *L. monocytogenes* strains, however the physiological state of persisters is lost during this re-growth (Highmore *et al.*, 2018). The results of such studies do not reflect the original character of persister cells.

Understanding persistent *L. monocytogenes* formation from an *in vitro* study will provide clues about the mechanism of generating *Listeria* contamination, and help in designing improved strategies to prevent *L. monocytogenes* contamination in food.

Nisin is a natural antimicrobial peptide (bacteriocin), produced by certain strains of *Lactococcus lactis* subspecies, that has been successfully applied to control pathogenic microorganisms including *L. monocytogenes*. Nisin has been given a "generally recognized as safe" label for use as a commercial preservative (Guerra *et al.*, 2005), and is marketed under the commercial name Nisaplin®(Cabo *et al.*, 2001). It is approved for use in the USA, Australia and New Zealand(Muriana and Kanach, 1995). The efficacy of nisin has been demonstrated in the inhibition of *L. monocytogenes* growth in different environments (Benkerroum and Sandine, 1988). However *L. monocytogenes* strains isolated from food have been shown to have enhanced nisin tolerance (Rasch and Knöchel, 1998). This tolerance phenotype could be associated with the generation of *L. monocytogenes* persister cells however there is no evidence of the generation of persister cells following nisin treatment.

This PhD project used *L. monocytogenes* isolates from food environments to obtain persistent subpopulations following exposure to the natural antimicrobial (nisin). Nisaplin® (nisin as the effective component) was used in current work to demonstrate the presence of persisting

cells. Stationary phase *L. monocytogenes* were treated with high concentration of nisin to detect the persistent subpopulation. These cells when facing what is regarded as a lethal concentration of nisin were used to investigate the mechanisms resulting in the formation of persister cells.

1.2 Research questions and hypotheses

1.2.1 Questions

- Can a persistent subpopulation be obtained by exposure to the natural antimicrobial nisin?
- What are the conditions that favour the *L. monocytogenes* persister formation in the planktonic form?
- What mechanisms are involved in *L. monocytogenes* persisting under high concentrations of nisin?

1.2.2 Hypotheses

- *L. monocytogenes* persister formation is a response to environmental change, like antimicrobial stress (nisin);
- *L. monocytogenes* persister formation during nisin treatment is dependent on decreasing cell metabolic rate
- *L. monocytogenes* persister formation when facing nisin stress is influenced by specific environmental features, such as nutrients and pH;
- *L. monocytogenes* persister formation following nisin stress is due to the expression of specific genes.

1.3 References

Allison, K. R., Brynildsen, M. P. and Collins, J. J. (2011). Heterogeneous bacterial persisters and engineering approaches to eliminate them. *Current Opinion in Microbiology*, 14, 593-598.

Benkerroum, N. and Sandine, W. E. (1988). Inhibitory Action of nisin Against *Listeria monocytogenes*. *Journal of Dairy Science*, 71, 3237-3245.

Cabo, M., Pastoriza, L., Bernárdez, M. and Herrera, J. (2001). Effectiveness of CO 2 and Nisaplin on increasing shelf-life of fresh pizza. *Food Microbiologyogy*, 18, 489-498.

Carpentier, B. and Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiologyogy*, 145, 1-8.

Cruz, C. D., Pitman, A. R., Harrow, S. A. and Fletcher, G. C. (2014). *Listeria monocytogenes* Associated with New Zealand Seafood Production and Clinical Cases: Unique Sequence Types, Truncated InlA, and Attenuated Invasiveness. *Applied and Environmental Microbiology*, 80, 1489-1497.

Di Ciccio, P., Meloni D., Festino AR., Conter M., Zanardi E., Ghidini S., Vergara A., Mazzette R., Ianieri A.. (2012). Longitudinal study on the sources of *Listeria monocytogenes* contamination in cold-smoked salmon and its processing environment in Italy. *International Journal of Food Microbiologyogy*, 158, 79-84.

Guerra, N. á., Macias, C. á., Agrasar, A. and Castro, L. (2005). Development of a bioactive packaging cellophane using Nisaplin® as biopreservative agent. *Letters in Applied Microbiology*, 40, 106-110.

Highmore, C. J., Warner, J. C., Rothwell, S. D., Wilks, S. A. and Keevil, C. W. (2018). Viable-but-nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson induced by chlorine stress remain infectious. *Mbio*, *9*(2), e00540-18.

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M. and Gram, L. (2013). Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Applied Environmental Microbiology*, 79, 2944-2951.

Laksanalamai, P., Joseph, L. A., Silk, B. J., Burall, L. S., Tarr, C. L., Gerner-Smidt, P., and Datta, A. R. (2012). Genomic characterization of *Listeria monocytogenes* strains involved in a multistate listeriosis outbreak associated with cantaloupe in US. *PloS one*, 7, e42448.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)*, 70, 267-274.

Lunden, J. M., Miettinen, M. K., Autio, T. J. and Korkeala, H. J. (2000). Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *Journal of Food Protection*, 63, 1204-1207.

Madden, R. H., Hutchison, M., Jordan, K., Pennone, V., Gundogdu, O. and Corcionivoschi, N. (2018). Prevalence and persistence of *Listeria monocytogenes* in premises and products of small food business operators in Northern Ireland. *Food Control*, 87, 70-78.

Muriana, P. M. and Kanach, L. (1995). Use of Nisaplin™ to inhibit spoilage bacteria in buttermilk ranch dressing. *Journal of Food Protection*, 58, 1109-1113.

Ortiz, S., Lopez, V., Villatoro, D., Lopez, P., Carlos Davila, J. and Martinez-Suarez, J. V. (2010). A 3-Year Surveillance of the Genetic Diversity and Persistence of *Listeria monocytogenes* in an Iberian Pig Slaughterhouse and Processing Plant. *Foodborne Pathogens and Disease*, 7, 1177-1184.

Rasch, M. and Knöchel, S. (1998). *Variations in tolerance of Listeria monocytogenes to nisin, pediocin PA-1 and bavaricin A*. *Letters in Applied Microbiology*, *27*(5), 275-278

Srey, S., Jahid, I. K. and Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control*, 31, 572-585.

Swaminathan, B. and Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection*, 9, 1236-1243.

Wood, T. K., Knabel, S. J. and Kwan, B. W. (2013). Bacterial Persister Cell Formation and Dormancy. *Applied and Environmental Microbiology*, 79, 7116-7121.

Chapter 2 Literature Review

2.1 Persistence definition and formation

2.1.1 Bacterial persistence

Persistent bacteria were firstly defined in the treatment of disease with antibiotics(Balaban *et al.*, 2004). Persistent bacteria are known to enter a viable-but-nonculturable (VBNC) state in response to environmental stresses. Studies on bacterial persistence have been mainly based on persistent infections from clinical relevance. Non-growing persisters have been reported for *Salmonella enterica* (*S. enterica*) serovar Typhimurium during systemic infection of mice (Helaine and Kugelberg, 2014). Persistent *Mycobacterium marinum* was identified in macrophages of infected zebrafish larvae(Adams *et al.*, 2011), and recurrence of *Staphylococcus aureus* (*S. aureus*) infections on skin, in soft tissue, and in the bloodstream are common despite protracted courses of treatment (Cohen *et al.,* 2013). Contrary to resistant bacteria that carry heritable mutations, persisters represent a small subpopulation with a non-inheritable phenotypic variation and account for the relapse of infections (Helaine and Kugelberg, 2014).

Most research on these cells recognizes that the persisters are not antibiotic resistant in their VBNC status, and their phenotypic tolerance is defined as non-inherited antibiotic resistance(Levin and Rozen, 2006). Nearly 70 years ago, Bigger described persisters as a subpopulation of bacteria that could survive under the prolonged exposure to penicillin, and in an antibiotic free environment, persisters could switch back to a growing state and generate a new population that is as sensitive to antibiotic treatment as was the parental strain (Figure 2.1) (Kint *et al.*, 2012). Persisters are phenotypic variants of the wildtype and genetically identical to susceptible wild type bacteria, in contrast to resistant cells. Persisters may be dormant cells with low metabolic activity (Wood *et al.*, 2013). It has been proposed that bacterial communities in all ecosystems generate dormant cells as a perseverant “seed bank” that enables the population to recover and repopulate the habitat after catastrophic events (Lennon and Jones, 2011).

Main stream studies on persister cells have focused on the clinical significance relating to antibiotic treatment(Dhar and McKinney, 2007). Instead of sampling persisters *in situ*, laboratory models are currently used to study persister cells and this has helped in providing sufficient *L. monocytogenes* persister populations for examination. The consequence of persisters surviving consecutive rounds of antibiotic treatments results in chronic infections and the eventual appearance of antibiotic resistance strains (Kint *et al.*, 2012). However, persisters in the food environment usually yield after cleaning, sanitation and disinfection treatments with different antimicrobial agents rather than the antibiotics used for clinical applications (Carpentier and Cerf, 2011).

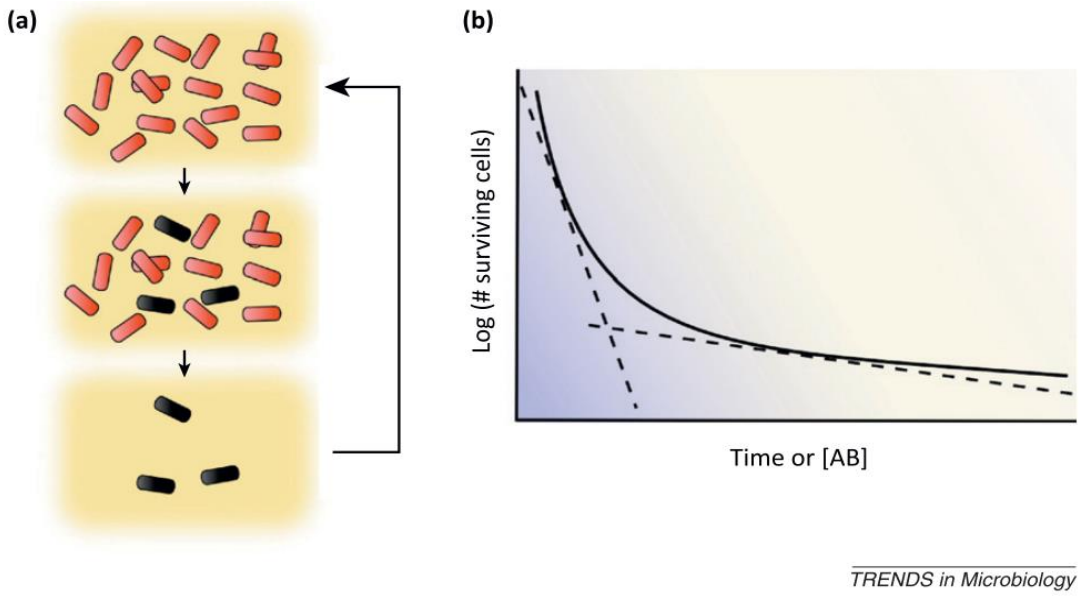


Figure 2. 1 Occurrence of a fraction of persister cells within a bacterial population

(a) The upper panel shows an isogenic population of antibiotic-sensitive bacterial cells (red). Persister cells (black) are phenotypic variants of the wild type that are formed at low frequency in the population (middle panel). Upon antibiotic treatment, the majority of cells are killed off, leaving the tolerant persisters unaffected (lower panel). Removal of the antibiotic pressure (denoted by the arrow alongside the panels) allows the persister cells to resume growth, thereby generating a new population that is genetically identical to the original one. Hence, this new population is again susceptible to the antibiotic. (b) Biphasic killing pattern of a sensitive bacterial population upon antibiotic treatment (solid line). Following antibiotic treatment, the number of cells in the population (y-axis) initially decreases sharply (steep dashed line). In a second phase, however, increasing the treatment length or antibiotic concentration ([AB]) (x-axis) kills the remaining persister cells at a much slower rate (gradually declining dashed line) (Kint *et al.*, 2012).

2.1.2 *Listeria monocytogenes* persistence

L. monocytogenes is a Gram-positive bacterium discovered by Murray in 1926(Murray *et al.*, 1926), and epidemiological studies combined with DNA sequencing have refined *L. monocytogenes* into four genetic lineages (I, II, III, and IV) (Glaser *et al.*, 2001). Developments in molecular biology have followed the prevalence of *L. monocytogenes* in distinct ecological niches and among clinical cases(Hain *et al.*, 2012). Lineage II strains (containing serotypes of 1/2a, 1/2c, and 3a) seem to be widespread isolates in natural environments and are commonly found in foods and food associated environments (Orsi *et al.*, 2011). Serotype 1/2a is a predominant subset in many cases of animal listeriosis and sporadic human clinical cases(Gorski *et al.*, 2006). In 2010, Gaul *et al.* reported their investigation on a baffling outbreak of hospital-acquired listeriosis from patients in Texas, and identified the cause as *L. monocytogenes* serotype 1/2a within the diced celery from a RTE chicken salad (Gaul *et al.*, 2013). Knabel *et al.* confirmed by sequence typing that *L. monocytogenes* serotype 1/2a was a predominant clone responsible for human listeriosis across Canada for more than two decades (Knabel *et al.*, 2012). Lineage II isolates have been found at a high frequency in foods and food processing environments, although some studies showed that lineage I isolates also can be recovered from in food environments(Lomonaco *et al.*, 2015). Overrepresentation of lineage II strains in food environments has been explained due to the high recombination rate of the *L. monocytogenes* genome among the lineage II isolates which has a significant role in survival in a variety of natural environments, compared with lineage I isolates which have a relatively low recombination rate (den Bakker *et al.*, 2008).

According to Bigger's definition of bacterial persister cells (Bigger, 1944), VBNC cells present a problem for the food industry. DNA identification techniques are used to detect pathogen contaminants with a relatively reliable outcome (Highmore *et al.*, 2018), but they do not

specifically identify the persistent populations. To understand the mechanism and development of a persister cell population tolerant to antimicrobial treatments, sufficient persister cells in their viable and non-culturable state are required for investigation.

Knudsen *et al.* determined that *L. monocytogenes* formed a persister subpopulation in an *in-vitro* model as a clinically relevant study. A stationary phase culture of *L. monocytogenes*(8 $\log_{10}$ cfu/ml) was capable of developing a 3 to 4 $\log_{10}$cfu/ml subpopulation that survived 72 hours treatment with 100μg/ml norfloxacin, and higher numbers of persisters(5-6 $\log_{10}$ cfu/ml) were surviving in an older stationary phase culture (3 day old and 6 day old stationary cells) or surface(0.2-μm pore-size polycarbonate filter) associated cells treated with100μg/ml norfloxacin(Knudsen *et al.*, 2013).The re-exposure of the re-grown cells to norfloxacin in refreshed medium showed that the survival was not caused by norfloxacin resistance and the re-grown population was still as sensitive to norfloxacin as the parental strain(Knudsen *et al.*, 2013). This study introduced *L. monocytogenes* to the list of bacterial species capable of producing a persister population which could survive bactericidal antibiotics in a dormant state.

Food borne transmission is the primary source of human infection with *L. monocytogenes* (Linke *et al.*, 2014). *L. monocytogenes* has been isolated from a variety of raw and processed food products, including milk and dairy products(Castro *et al.*, 2018), meat products(Ferreira *et al.*, 2011), seafood(Cruz and Fletcher, 2011), vegetables (Murugesan *et al.*, 2015), and other ready-to-eat (RTE) foods(Chau *et al.*, 2017), and has also been detected from food manufacture and farm environments(Latorre *et al.*, 2011). However, the incidence reports of listeriosis with high mortality (Cruz *et al.*, 2014; Gaul *et al.*, 2013; Gray *et al.*, 2004; Rychli *et al.*, 2014) show a globally increasing health threat by *L. monocytogenes* persistence. How *L. monocytogenes* persists and survives in food environments is still poorly understood.

2.2 Strategies for controlling the occurrence of persistent *L. monocytogenes*

2.2.1 Cleaning and sanitation

Effective cleaning and sanitation is important to control and prevent transmission of

human pathogens during food processing and in final food products (Hammons *et al.*, 2017). The primary purpose of a Clean in Place (CIP) process is to clean food processing surfaces and remove or kill bacteria that threaten the safety and quality of food(Van Houdt and Michiels, 2010). A sanitiser rinse is included in a CIP to minimise the numbers of bacteria(Beltrame *et al.*, 2015; Tan *et al.*, 2014).The chemicals used for sanitizing (disinfection) in food manufacturing plant include chlorine dioxide (CD) (Cruz and Fletcher, 2012), quaternary ammonium compounds (QAC) (Belessi *et al.*, 2011; McEgan and Danyluk, 2015), acidified sodium chlorite based products (Chen and Zhong, 2018; Inatsu *et al.*, 2017)and peracetic acid (Fernandes *et al.*, 2015; Pezzuto *et al.*, 2016). Krysinski *et al.* demonstrated that QAC, peracetic acid and chlorine dioxide were the most effective compounds against *L. monocytogenes* adhering to stainless steel or plastic conveyor belt surfaces(Krysinski *et al.*, 1992).

But the efficiency of various disinfectants against *L. monocytogenes* is dependent on pH, temperature and other factors. QAC are more efficient against surface adhering *L. monocytogenes* at 20°C than 4°C (Mafu *et al.*, 1990); peracetic acid is more effective against surface adhering *L. monocytogenes* than *L. monocytogenes* in suspension(Stopforth *et al.*, 2002).The presence of organic material can reduce the anti-*Listeria* effect of chlorine(El-Kest and Marth, 1988). The effect of disinfectants can be influenced by the character of the surface to which the bacteria are attached. For example, hypochlorite is more effective against *L. monocytogenes* attaching to the smooth surface of stainless steel than on stainless steel with a pitted surface(Mustapha and Liewen, 1989).

2.2.2 Physical approaches

Many other methods have been combined into disinfectants for improved sanitation. Vaporization (Back *et al.*, 2014) and the infrared sensor based aerosolization (Kim *et al.*, 2014) of sanitizers were shown to have stable bactericidal effects, minimizing sanitizer usage. Aerosolized disinfectant applications successfully inactivated a mixed biofilm comprising *Escherichia coli* (*E.coli*) *O157:H7*, *S. typhimurium*, and *L. monocytogenes*, and were more

effective than disinfectants in an aqueous solution for reducing the cells below the detection limit (1.48 $\log_{10}$ cfu/10cm^2)(Park *et al.*, 2012).The efficacy of sanitizer washes can be enhanced against *L. monocytogenes* when coupled with frozen storage (Tadepalli *et al.*, 2018), ultrasound (Gomez-Lopez *et al.*, 2015; Lee *et al.*, 2014) and moist heat (Masuku *et al.*, 2014). In order to maintain the sensory quality of fresh products, it has been found that slightly acidic electrolyzed water combined with chemical (Prado-Silva *et al.*, 2015; Tango *et al.*, 2017) and physical treatments (Dewi *et al.*, 2017; Luo and Oh, 2016) effectively decontaminate *L. monocytogenes* in a food system.

The need to ensure that no sanitiser residues could lead to issues with food safety, sensory or corrosion of stainless steel, alternative antimicrobials are being considered in food processing plants (Simoes *et al.*, 2010). Photodynamic treatment is an environmentally friendly disinfection technique for killing bacteria, especially antibiotic-resistant strains. This treatment involves photo-activating sensitive molecules to form radicals and active oxygen species that are highly toxic to cells(Walther *et al.*, 2009). With a film of photocatalytic titanium dioxide nanoparticles, ultraviolet A light is used to initiate *L. monocytogenes* biofilm inactivation(Chorianopoulos *et al.*, 2011). *L. monocytogenes* biofilm which formed on a packaging surface was inhibited by exposure to an LED-based light (visible light) within 2 hours of exposure(Buchovec *et al.*, 2010).

To date, hurdle technology, the combination of different strategies resulting in a synergistic effect in microbial inactivation, has been effective in inactivating *L. monocytogenes*(Berrang *et al.*, 2008). Examples of hurdle approaches are the combination of sanitizer plus ultrasonication(Berrang *et al.*, 2008; Torlak and Sert, 2013), preservatives combined with a physical treatment(Arevalos-Sánchez *et al.*, 2012; Ban *et al.*, 2012; Tajik *et al.*, 2015), and a mix of sanitizers with natural antimicrobials(Minei *et al.*, 2008).

2.2.3 GRAS Preservatives (Chemical additives)

Chemical additives (such as sodium acetate, sodium bicarbonate, sodium lactate, and potassium sorbate) are approved preservatives applied in food manufacture. They are regarded as “generally recognized as safe” (GRAS) preservatives which allow to be used in a specific

application with additional regulation approval (Wederquist *et al.*, 1994). Use of these additives in food products and processing effectively extends shelf life and enhances flavour (Wederquist *et al.*, 1994). Sodium benzoate, sodium propionate, potassium sorbate, and sodium diacetate have been prepared as dipping or washing solutions to individually investigate their control of *L. monocytogenes* on turkey (Islam *et al.*, 2002), ham (Glass *et al.*, 2007), and fresh poultry (Gonzalez-Fandos and Dominguez, 2007), and the shelf life of the washed/dipped samples is significantly increased in these studies. The effectiveness of acidic calcium sulphate (bactericidal effect) or lactic acid (bacteriostatic effect) were reported as post-processing dipping solutions to inhibit or control the growth of *L. monocytogenes* on vacuum-packaged frankfurters stored at 4.5ºC for up to 12 weeks (NuÑEz de Gonzalez *et al.*, 2004). Use of 2.5% acetic acid dipping combined with sodium lactate and sodium diacetate in the formulation inhibited growth of *L. monocytogenes* on frankfurters and subsequent detectable survival after exposure to simulated gastric fluid (Stopforth *et al.*, 2005). Lauric arginate at concentrations of 800 ppm proved effective in reducing cold growth of *L. monocytogenes* in whole milk, skim milk, and Queso Fresco cheese (QFC) at 4ºC for up to 28 days without affecting the sensory quality (Soni *et al.*, 2010). Anaerobic conditions enhanced the control of *Listeria* with sodium lactate and sodium diacetate alone or in combination (Skandamis *et al.*, 2007)

Incorporation of chemical preservatives in food products is important for control of *L. monocytogenes* when used in combination with other processing techniques. Novel carbohydrate fatty acid derivatives are chemically synthesized and have shown efficacy against *Listeria spp.* and other food spoilage microorganisms (Nobmann *et al.*, 2009). Dipping chicken meat artificially contaminated with *L. monocytogenes* into tri-sodium phosphate for 10mins significantly reduced the population of the pathogen on the surface of meat to a safe limit (Kakey *et al.*, 2016). Combined effects of three GRAS antimicrobials including, bacteriophage P100, lauric arginate, and potassium lactate–sodium diacetate mixture reduced *L. monocytogenes* growth at 4ºC in queso fresco cheese (Soni *et al.*, 2012). Heat (Grosulescu *et al.*, 2011) or nitrate addition (Glass *et al.*, 2007) can facilitate the GRAS preservatives to control *L. monocytogenes*.

2.2.4 Natural preservatives

2.2.4.1 Plant-derived preservatives

Natural antimicrobials are effective in *L. monocytogenes* control. Plant-derived compounds provide some natural antimicrobials for *L. monocytogenes* control (Lin *et al.*, 2016; Upadhyay *et al.*, 2013). Extracts from herbs and medicinal plants contain anti-biofilm activity against *L. monocytogenes*(Sandasi *et al.*, 2010). Essential oils from culinary herbs (such as lemon grass, citronella, cinnamon, thyme oil, and oregano oil) are active against *L. monocytogenes* attachment on abiotic surfaces (de Oliveira *et al.*, 2012; Desai *et al.*, 2012; Oliveira *et al.*, 2010). Essential oils are effective on both young and old biofilms of *L. monocytogenes*(Jadhav *et al.*, 2013; Oliveira *et al.*, 2010; Upadhyay *et al.*, 2013). Kerekes *et al.* found that essential oils of clary sage, juniper, lemon and marjoram interfere with the autoinducer-mediated quorum sensing in some Gram-negative bacteria (Kerekes *et al.*, 2013). Essential oils from herbs applied in combination, are active against *L. monocytogenes* (Desai *et al.*, 2012; Patra and Baek, 2016). Essential oil nano-emulsions show an inhibitory effect against *L. monocytogenes* persisting on red mustard leaves (Kang and Bin Song, 2018).

2.2.4.2 Animal-derived preservatives

Animal derived antimicrobial compounds can reduce up to 4 logCFU/cm^2 of *L. monocytogenes* in biofilms on stainless steel surfaces (Bodur and Cagri-Mehmetoglu, 2012). Chitosan, produced by the deacetylation of chitin (the exoskeleton element of crustaceans, such as crabs and shrimp), has anti-biofilm activity against *L. monocytogenes*(Orgaz *et al.*, 2011). Animal derived antimicrobial compounds (e.g. chitosan) may lessen the potential for *L. monocytogenes* to remain as a long-term resident in food processing plant (Berrang *et al.*, 2014; Orgaz *et al.*, 2013) however cold stress may increase the tolerance of *L. monocytogenes* to this antimicrobial(Puga *et al.*, 2016). Guo *et al.* developed edible chitosan-acid solutions incorporating lauric arginate ester, sodium lactate, and sorbic acid alone or in combination and coated these mixes on polylactic acid packaging films. They found that the antimicrobial films

containing 1.94 mg/cm^2 of chitosan and 1.94 µg/cm^2 of lauric arginate ester were the most effective against *L. innocua*, *L. monocytogenes*, and *S. Typhimurium* in TSB and reduced them to an undetectable level (<0.69 $\log_{10}$ cfu/ml) (Guo *et al.*, 2014).

2.2.4.3 Microbial derived preservatives

Many bacteria are able to compete with *L. monocytogenes* as planktonic cells or biofilms by producing specific antimicrobial compounds (Habimana *et al.*, 2011; Mariani *et al.*, 2011; Speranza *et al.*, 2009) and these microbial derived compounds are potential alternatives to sanitising agents against *L. monocytogenes* in food environments (Camargo *et al.*, 2016; Winkelstroeter *et al.*, 2015). Antimicrobial biosurfactants from microbial sources are a class of natural antimicrobials (de Araujo *et al.*, 2011). Surfactin from *Bacillus subtilis* and rhamnolipids from *P. aeruginosa* reduce adhesion and disrupt biofilms of *L. monocytogenes* on polystyrene surfaces, (do Valle Gomes and Nitschke, 2012). Bacteriophages are used to control seafood-borne *L. monocytogenes* biofilms on stainless steel either clean or soiled with fish protein (Ganegama Arachchi *et al.*, 2013). Lactic acid bacteria in biofilms delay the growth of *L. monocytogenes* in soft cheeses(Speranza *et al.*, 2009).

2.2.4.3.1 Nisin

Bacteriocins (ribosomally synthesized peptides) secreted by lactic acid bacteria can control *L. monocytogenes* proliferation (Winkelstroeter *et al.*, 2011). The most extensively studied bacteriocin is nisin A (here referred to as nisin), a 34-amino-acid class I bacteriocin (lantibiotic) produced by *Lactococcus lactis* strains(Drider *et al.*, 2006). Nisin is a typical microbial derived antimicrobial peptide and has been classified as a natural GRAS preservative (Guerra *et al.*, 2005). It is the most widely used bacteriocin and is capable of effectively inactivating and inhibiting many Gram-positive bacteria including *L. monocytogenes*(Rai and Bai, 2014). Nisin is commercially available as Nisaplin®(Cabo *et al.*, 2001). It is a safe preservative approved for industrial use in the USA, Australia and New Zealand (Muriana and Kanach, 1995). The efficacy

of nisin to control *L. monocytogenes* in cheeses, milk, salads and meat products has been extensively reported (Cotter *et al.*, 2005; Davies *et al.*, 1997; Galvez *et al.*, 2007). Nisin has been combined with other antimicrobials for synergetic activity against *Listeria*. Nisin in combination with 3 or 5 g/100 ml acetic acid or sodium diacetate or 3 g/100 ml potassium benzoate, applied individually or as mixtures, prevented *L. monocytogenes* growth for up to 90 days (Samelis *et al.*, 2005). A nisin-based sanitizer was found to minimise transfer of *L. monocytogenes* from melon rind surfaces to fresh-cut pieces resulting in microbial populations in fresh-cut pieces that were below detection by enrichment (Ukuku *et al.*, 2015).

The antimicrobial mechanisms for nisin are well understood. Nisin adsorbs to the cytoplasmic membrane of vegetative cells, and binds to lipid II which is the main transporter of the peptidoglycan subunits from the cytoplasm to the cell wall(Cotter *et al.*, 2005). The lipid II binding with nisin prevents cell wall synthesis leading to cell death(Drider *et al.*, 2006). Lipid II is also regarded as a docking molecule to initiate pore formation in the cell membrane causing cell death.(Cotter *et al.*, 2005).

Nisin producing strains of lactic acid bacteria inhibit the growth of *L. monocytogenes*, and as a natural antimicrobial agent, nisin may be used to increase the shelf-life and the safety of minimally processed foods (Siroli *et al.*, 2016). The antimicrobial ability of nisin may be variable due to the chemical composition and physical condition of the food system (Jung *et al.*, 1992; Ruiz *et al.*, 2009). Homogenization of milk caused changes in the milk fat and/or milk proteins, which resulted in diminished antilisterial activity of nisin (Bhatti *et al.*, 2004). The antilisterial effect of nisin was improved in the presence of 100% CO_2 and increasing NaCl concentrations (0.5 to 5.0% w/v) (Nilsson *et al.*, 1997). Using nisin, edible films with high hydrophobicity values of 280 to 450 units in an acidic environment increases the inhibitory effect against *L. monocytogenes* (Ko *et al.,* 2001). Thomas reported that an elevated NaCl concentration (7% w/v) potentiated nisin activity against *L. monocytogenes* while the effect of temperature was not so apparent(Thomas and Wimpenny, 1996). Between pH 7.92 and ca. pH 5, a fall in pH appeared to increase nisin's effectiveness against microorganisms, but at more acid pH values (ca, pH 4.5 to 5), the microorganisms showed resistance to nisin at 20 and 25 ºC (Thomas and Wimpenny, 1996).

The antibacterial activity of nisin-loaded solid lipid nanoparticles against *L. monocytogenes* DMST 2871 and *Lactobacillus plantarum* TISTR 850 lasted for up to 20 and 15 days, respectively, compared to only one and three days, respectively, for free nisin (Prombutara *et al.*, 2012).

The use of nisin in controlling food pathogen proliferation has been demonstrated in a variety of food systems, with activity over a wide pH range from acidic to alkali conditions (Gharsallaoui *et al.*, 2016). Nisin is used for the preservation of foods over a wide pH range including seafood(Budu-Amoako *et al.*, 1999), liquid egg product (Delves‐Broughton *et al.*, 2008), processed cheese (Maisnier-Patin *et al.*, 1992)and milk products (Gharsallaoui *et al.*, 2016; Maisnier-Patin *et al.*, 1995).

The efficacy of nisin is influenced by strain variations (Rasch and Knochel, 1998). The molecular mechanisms used by *L. monocytogenes* to cope with nisin are poorly understood. To date, the sigma immune gene appears to be important in nisin resistance (Begley *et al.*, 2010; Palmer *et al.*, 2009). Nisin tolerant strains may harbor much more complex phenotypic and genotypic responses (Tessema *et al.*, 2009). *L. monocytogenes* isolates from cheeses treated with nisin, PlyP100, and a combination of the two did not develop resistance to nisin or PlyP100 while they were showing tolerance to other stressful conditions (Ibarra-Sanchez *et al.*, 2018). When using antimicrobial peptides such as nisin, it is important to understand how environmental factors can impact on antimicrobial resistance (Malekmohammadi *et al.*, 2017).

2.3 Current insights on persistent *Listeria monocytogenes* from food industrials

Cleaning, sanitising and preservatives are not completely effective in eliminating microbial contamination (Tan *et al.*, 2014). Persistence of microbial contamination in a food manufacturing environment can result in the long-term survival of a proportion of a population of a pathogen despite repeated treatments with sanitizer or perservatives.

The persistence of *L. monocytogenes* is defined by its prolonged existence when *L. monocytogenes* are repeatedly detected in many food related environments and processed food products (Wang *et al.*, 2015). A recent 3-year multi-food study in Ireland has identified the

presence and persistence of *L. monocytogenes* in 54 small food businesses (Fox *et al.*, 2017). A diversity of *L. monocytogenes* strains was detected with pulsed-field gel electrophoresis, and whole genome sequencing was used to further differentiate *L. monocytogenes* strains.Pulsed-field gel electrophoresis patterns from this study showed a close relationship between isolates (Fox *et al.*, 2017).

The current genome examination studies offer insight into the *L. monocytogenes* subtypes associated with persistence in food processing environments (Ortiz *et al.*, 2016; Pasquali *et al.*, 2018). The genome sequencing study of Holch *et al.* compared three persistent strains with nonpersistent strains, and all persistent *L. monocytogenes* strains were distinguished by two genome deletions: one, of 2,472 bp, typically containing the gene for inlF, and the other, of 3,017 bp, that includes three genes potentially related to bacteriocin production and transport (lmo2774, lmo2775, and the 3'-terminal part of lmo2776) (Holch *et al.*, 2013).

Li *et al.* reported that 12 persistent *L. monocytogenes* strains from a pork processing plant carried the tetracycline resistance determinant tet(M) gene (Li *et al.*, 2016). A combination study of multiple whole genomic sequencing analyses showed that *L. monocytogenes* cells persistent in a milkshake maker for a year formed a unique clade inside an outbreak cluster, and this clustering was consistent with the cleaning practice after the outbreak, and was initially recognized in late 2014 and early 2015 (Li *et al.*, 2017). In the persistent *L. monocytogenes* from the milkshake maker, putative prophages were conserved among prophage-containing isolates (Li *et al.*, 2017). Accumulating genotyping research results will generate some genetic markers for persistent strains from particular food environments and may become reference markers in future food safety monitoring.

Meantime, investigations on specific characteristics (e.g., surface adhering ability, stress tolerance and virulence) have shown heterogeneity among persister cells of *L. monocytogenes* (Ferreira *et al.*, 2014). Persistent *L. monocytogenes* isolates from a dairy farm milking system (Latorre *et al.*, 2011) and from mussel production facilities (Nowak *et al.*, 2017) have shown increased adherence *in vitro* compared with other isolates. Some researchers demonstrated that the greatest biofilm producer in a population was a persistent strain with significant tolerance to

sanitizers and disinfectants, such as quaternary ammonium compounds (Henriques and Fraqueza, 2017; Poimenidou *et al.*, 2016) with the persistent strains producing more extracellular polymeric substances than other strains (Nakamura *et al.*, 2013).

However, Wang *et al.* reported that there was no significant difference in sanitizer tolerance between persistent and transient *L. monocytogenes* strains from RTE deli meats while the persistent isolates showed enhanced adherence (Wang *et al.*, 2015). From a Brazilian cheese and cheese-production environment, Lee *et al.* detected high variability in biofilm production among *L. monocytogenes* strains, indicating that strong biofilm-formation ability may not be a key factor for persistence of specific isolates in cheese manufacturing plants (Lee *et al.*, 2017). A Japanese scientific survey collected the persistent *L. monocytogenes* strains from chicken samples from a retail shop over six months showing higher biofilm ability was obtained from the persistent strains isolated from December to March than from those isolated from April to June (Ochiai *et al.*, 2014). The persistent strains were found to change their biofilm-forming ability in a temperature-dependent manner, which may suggest that the persistent strains alter their biofilm formation in response to changing environmental factors (Luo *et al.*, 2017).

The ability to outcompete other strains may provide advantageous selection for *L. monocytogenes* persisting in food associated environments(Bruhn *et al.*, 2005), In the study of Heir *et al.* with multispecies cultures, growth competition lead to the dominance of a strong competitor strain of *L. monocytogenes* that was only slightly inhibited by *L. innocua*. *L. monocytogenes* showed persistence and strong competitiveness in mixed cultures with resident Gram-negative bacteria from salmon and ready-to-eat meat production facilities (Heir *et al.*, 2018). Orgaz *et al.* demonstrated that persistent *L. monocytogenes* strains showed a better resuscitation rate after chitosan damage (Orgaz *et al.*, 2013). The recovery of *L. monocytogenes* growth also could be one factor protecting *L. monocytogenes* under multispecies' competition or harsh environments (Shi and Zhu, 2009; Srey *et al.*, 2013). Persistent *L. monocytogenes* subtypes are found in many food environments, and persistent *L. monocytogenes* strains do not appear to be characterized by unique genetic or phenotypic characteristics in current publications (Ferreira *et al.*, 2011).

There is increasing concern with the persistence of *L. monocytogenes* in the food environment and this has not been well studied with a lack of laboratory models for food safety studies. The persistence studies discussed in this section all re-grew the persistent *L. monocytogenes* strains, however the physiological state of persisters is lost during this re-growth. A search for a laboratory model to generate a population of *L. monocytogenes* persister cells following treatment with food grade antimicrobials is a key component of this PhD project. Clarifying the emergence of persistence and the mechanisms of persistence will help in understanding the food contamination of *L. monocytogenes* in order to further develop strategies for the control of persister populations in food and food manufacturing environments.

2.4 Mechanism of persister formation

One theory for persistent cells has been termed the dormant seed theory where the persister cells are regarded as dormant – similar to a plant seed (Balaban, 2011). Cell-sorting experiments show that the dormancy of the non-growing population correlates with persistence as dormant populations have 20 times more persisters than normally growing populations, however this also suggests that dormancy does not entirely explain persistence as many dormant cells are not persisters and many persisters are not dormant (Allison *et al.*, 2011). There may be different valid explanations for the formation of persister cells in any population (Cohen *et al.*, 2013).

The formation of persister cells is also known as a growth phase dependent process, with the maximum number of persister cells produced in stationary phase while there are few or no persisters in the exponential growing phase (Kint *et al.*, 2012). Persisters could induce the phenotypic changes and possible mechanisms involved in the pre-adaption of persisters *in vitro* (Balaban, 2011). Persisters may be formed actively in response to particular triggers or switches, such as nutrient shift or starvation (Lewis, 2005). The persister heterogeneity may be evidence that persisters are not simply non-growing cells and some specific changes in physiology enable survival and resuscitation of persister cells (Maisonneuve and Gerdes, 2014). Persisters are

unlikely to derive from bacteria with low redox activity, and inhibition of respiration during the stationary phase reduces persister levels by up to 1,000-fold(Orman and Brynildsen, 2015). Lower stationary phase respiratory activity could prevent digestion of endogenous proteins and RNA, which yields bacteria that are more capable of translation, replication and concomitantly cell death when exposed to antibiotics(Orman and Brynildsen, 2015). Orman and Byrnildsen's findings support bacterial respiration as a prime target for reducing the number of persisters formed in nutrient-depleted, non-growing populations.

2.4.1 Stringent response

The stringent response is one of the most important global regulator systems that is broadly conserved in bacterial stress response. The stringent response produces several nucleotide messengers (linear nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), combined known as (p)ppGpp) in response to environmental stresses. The RelA–SpoT homologue family of bifunctional proteins are key in synthesizing (p)ppGpp from ATP and either GTP or GDP, and degrading (p)ppGpp to pyrophosphate and either GTP or GDP(Hauryliuk *et al.*, 2015). Stringent response inactivation was confirmed with established mutants by disrupting relA and spoT in many bacteria, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) (Nguyen *et al.*, 2011)and *E. coli*(Germain *et al.,* 2013*)*. Carbon, amino acid, and ion starvation have been reported to activate the stringent response by inducing the relA and spoT gene products to synthesize the alarmone (p)ppGpp and this signal regulates bacterial persister cell formation(Zhang, 2014).

RelA has radically different properties in bacterial survival. Under amino acid– starvation, uncharged tRNAs that bind to the ribosomal A site activate ribosome-associated monofuctional RelA to synthesize ppGpp using ATP and GTP in *E. coli* (Magnusson *et al.*, 2005). A novel single-molecule tracking methodology detected a catalytically active part of the RelA cycle that is turned off rather than on, and that rebinding to the ribosome is not necessary to trigger each ppGpp synthesis event in *E. coli* cells (English *et al.*, 2011). However, RelP and RelQ with (p)ppGpp-synthase activities were found in *Enterococcus faecalis* (Abranches *et al.*, 2009) and the oral

pathogen *Streptococcus mutans*(*S. mutans*) (Lemos *et al.*, 2007). It was demonstrated that while relA is responsible for the rapid accumulation of (p)ppGpp upon amino acid starvation, some other rel-proteins were also responsible for constitutive expression of (p)ppGpp in different conditions (Abranches *et al.*, 2009). In addition to RelA, ppGpp is also produced by SpoT, but the synthase activity of SpoT is not as robust as that of RelA and SpoT also contains ppGpp hydrolase activity (Traxler *et al.*, 2008).

Persister formation depends on the stringent response, like (p)ppGpp signaling, as well as on other processes or induction known as the general stress response. RpoS is one of the regulators mediating bacterial persister formation. An rpoS mutant in *P. aeruginosa* showed that its survival in stationary phase was lower when compared with the parental strain when exposed to antibiotics (biapenem and imipenem) (Murakami *et al.*, 2005). Heat stress further reduced the survival of the mutant, however, under osmotic stress, tolerance to biapenem was not dependent on the rpoS gene (Murakami *et al.*, 2005). The role of rpoS varies in persistence studies. Wu *et al.* reported that rpoS deletion caused a defect in *E. coli* persistence to gentamicin but increased persistence to ampicillin and norfloxacin (Wu *et al.*, 2015). A response regulator of RpoS, hnr, has been shown to be important in persister cell development through hrn mutants reducing persister cell levels compared to the wild type (Hansen *et al.*, 2008).

Obg is a conserved GTPase shown to bind (p)ppGpp and it is involved in important cellular processes that are targeted by antibiotics, such as DNA and protein synthesis. Waldron examined the effect of Obg overexpression on the survival of *E. coli* cultures following treatment with antibiotics and observed a significant dose-dependent increase in persistence (Waldron, 2015). Conversely, decreasing Obg using antisense RNA reduced survival, demonstrating that Obg concentration controls persistence in *E. coli* populations. Overexpressed Obg in mutants lacking (p)ppGpp resulted in no increase in survival demonstrating a requirement for (p)ppGpp in Obg-mediated persistence (Waldron, 2015). In the Obg-mediated persistence pathway, the expression of toxin hokB and Obg is tightly correlated, and Obg causes persistence via the transcriptional activation of hokB (Verstraeten *et al.*, 2015). Consistent with this, Obg does not induce persistence in a mutant lacking hokB, and HokB overexpression results in an increase in

the number of persisters. The mediation by (p)ppGp influences toxin-antitoxin responses regulating several essential cellular functions, such as translation (Waldron, 2015).

2.4.2 Toxin-antitoxin (TA) module

Bacterial toxin-antitoxin modules are genetic elements composed of a toxin protein that inhibits bacterial growth by interfering with essential cellular processes and an antitoxin that prevents or impairs the functionality of the toxin until this inhibition is abrogated in response to cellular signaling (Harms *et al.*, 2016). Almost all free-living bacteria contain a number of toxin–antitoxin (TA) operons which influence regulating cell growth, survival and death. Studies of persisters have identified a toxin/antitoxin (TA) system involved in bacterial persistence by constructing a mutant strain (Kint *et al.*, 2012). These TA systems are classified into three groups (type I, II and III) on the basis of the function of the antitoxin(Yamaguchi and Inouye, 2011). In type I TA systems (e.g. *hokB/sokB*), expression of the toxin gene is regulated by an antisense RNA transcribed from the same toxin module but in the reverse orientation The type II module (e.g. HipA families) has an antitoxin protein that inactivates their cognate toxins in direct protein-protein interactions while the type III TA system was identified with an RNA antitoxin for neutralization of toxicity by forming a complex with the toxin protein (Yamaguchi and Inouye, 2011). Type I and II modules have been extensively studied in the formation of bacterial persistence.

The HipA gene which was first detected in the HipBA TA modules of *E.coli,* affected the frequency of *E. coli* persisters after inhibition of murein synthesis (Balaban *et al.*, 2004; Moyed and Bertrand, 1983). Mutant studies of *E. coli* persisters have shown that persistence occurs when toxic HipA levels reach a threshold (Balaban *et al.*, 2004). Expression of hipA7 in the absence of hipB demonstrated a 20-fold higher frequency of a persistent phenotype in a hipA7 allele mutated strain as cultures approach stationary phase, and a relA knock-out model diminished the high persistent phenotype in hipA7 mutants, suggesting that hipA7 facilitates the establishment of the persister state by inducing (p)ppGpp synthesis (Korch *et al.*, 2003). Overexpression of HipA in *E. coli* triggers persistence (growth arrest) by activating synthesis of (p)ppGpp by the enzyme RelA

when associated with amino acid starvation (Bokinsky *et al.*, 2013). It is reported that hipA triggers the activation and release of RelA by inhibiting the aminoacylation efficiency of GltX rather than the translation factor EF-Tu (Germain *et al.*, 2013). *E. coli* K12, and *Mycobacterium tuberculosis* have been the model organisms in studying the molecular mechanism of bacterial persistence since the microorganisms encode a large number of TA modules. The relBE TA module was identified in *E. coli* K12 and *Mycobacterium tuberculosis* (*M. tuberculosis*) first, and later in *Haemophilus influenzae* and *Vibrio cholera* (Gotfredsen and Gerdes, 1998; Sala *et al.*, 2014).

The TisAB/IstR-1 system has been associated with facilitating *E. coli* persistence to ciprofloxacin treatment by stimulating the SOS response and DNA repair when the TisB toxin affects the cell membrane and reduces ATP levels (Wang and Wood, 2011). Knockout tisB mutants resulted in a sharply decreased frequency of persisters, whereas mild overproduction of the peptide induced persister formation (Dörr *et al.*, 2010). The tisB-dependent persisters are highly tolerant to unrelated antibiotics (Dörr *et al.*, 2010).

In other bacteria, several genes, such as MqsR, RelE, HigB, MazF and HicA have been identified as being associated with toxin-antitoxin (TA) modules (Butt *et al.*, 2014; Wang and Wood, 2011) which may be associated with persistence. MazF-expressing persister cells remain largely viable with altered cell morphologies and internal structures. These cells effectively tolerate different bactericidal antibiotics targeting different cellular machineries. MazE induced the regrowth of ciprofloxacin treated persister cells, which activated cell elongation followed by cell division (Cho *et al.*, 2017). The MazEF/RelBE system which is associated with persistence in *E. coli* and *M. tuberculosis* was also found to regulate *S. mutans* persistence producing a log-fold increase in *S. mutans* persisters in the presence of ofloxaxin(Leung and Lévesque, 2012).The toxins (such as MqsR) are global regulators and have a role in biofilm formation, and it presumed that the MqsR toxin induced bacterial persistence as a result of increasing bacterial attachment (Wang and Wood, 2011).

Recent studies have found more TA modules in different bacteria. The toxin, TacT, is an acetyltransferase that blocks the primary amine group of amino acids on charged tRNA molecules,

thereby inhibiting translation and promoting persister formation (Cheverton *et al.*, 2016). Toxin ζ, is a uridine diphosphate-N-acetylglucosamine (UNAG)-dependent ATPase, and is related to generate a small cell subpopulation persists when transient ζ expression promotes a reversible multi-level response by altering the pool of signaling purine nucleotides (Moreno-del Álamo *et al.*, 2017). Toxin mycrocin C producing cells have increased persistence levels due to a combined effect of Mycrocin C imported from the cultured medium and intracellularly synthesized antibiotic. Induction of Mycrocin C is mediated by (p)ppGpp and requires chromosomally encoded toxin-antitoxin modules(Piskunova *et al.*, 2017).

2.4.3 Efflux pump

Efflux pumps are present in both Gram-positive and Gram-negative bacteria, and the efflux is driven by an energetic primary or secondary transporter. Efflux pumps can transport one specific substrate or a range of structurally dissimilar compounds, e.g. antibiotics of different chemical classes (Piddock, 2006). *E. coli* cells carrying the pump had decreased susceptibility to some antibiotics, disinfectants, dyes and detergents, with enhanced efflux activity (Rajamohan *et al.*, 2010). NaCl induces antibiotic (aminoglycoside and imipenem) tolerance in *Acinetobacter baumannii* with an increased level of expression of efflux pumps, and the efflux pump inhibitor PaβN is able to reduce antibiotic tolerance(Hood *et al.*, 2010). The pumps transporting multiple substrates can be associated with multidrug (antibiotic) resistance. Bacterial drug efflux transporters are classified into several families including the ATPbinding cassette (ABC) superfamily, the major facilitator superfamily, the multidrug and toxic-compound extrusion family, the small multidrug resistance family and the resistance nodulation division (RND) family (Kumar and Schweizer, 2005). The RND family and the ABC superfamily are mainly associated with bacterial persistence when facing with antibiotic treatment (Pu *et al.*, 2017).

The AcrAB-TolC pump is the principle RND type multidrug exporter that confers intrinsic drug tolerance to bacteria. The inner membrane transporter AcrB requires the outer membrane factor TolC and the periplasmic adapter protein AcrA (Seeger *et al.*, 2006). AcrA mediates and intervenes the interaction between TolC and AcrB and that AcrB enhances binding

of AcrA to TolC (Xu *et al.*, 2011). In a study of the temporary suppression of bacterial growth following exposure to an antimicrobial agent, deletion of the multiple-drug efflux pump AcrAB in *E. coli* prolongs the post-antibiotic suppression effects (Stubbings *et al.*, 2005). Activation of the AcrAB-TolC pump by oxidative stress leads to an increase in multidrug-tolerant persister cells (Wu *et al.*, 2012). Time-lapse imaging and mutagenesis studies demonstrated that bacterial persisters, under b-lactam antibiotic treatment, show less cytoplasmic drug accumulation as a result of enhanced efflux activity. Consistently, a number of multi-drug efflux genes, particularly the central component TolC, show high expression in persisters (Pu *et al.*, 2016).

The Mex system (e.g. MexAB-OprM efflux pump) found in *E. coli* and *P. aeruginosa*, respectively, also influences antimicrobial persistance (Venter *et al.*, 2015). Tolerance to the antimicrobial peptide colistin in *P. aeruginosa* biofilms is mediated by mexAB-oprM genes (Pamp *et al.*, 2008). Efflux pumps MexAB-OprM and MexEF-OprN are both induced by the MerR-Like Regulator BrlR, conferring tolerance in *P. aeruginosa* biofilms (Liao *et al.*, 2013). But overexpression of the MexEF-OprN efflux pump could result in a constant influx of protons which may lead to cytoplasmic acidification and have a deleterious effect on cell fitness (Olivares *et al.*, 2014). A tetracycline efflux pump Tet45 was identified to play a role in the dissemination and persistence of a *Bhargavaea cecembensis* strain from poultry-litter-exposed soil (You *et al.*, 2013).

The ATPbinding cassette (ABC) superfamily is also involved in bacterial tolerance and persistence (Holyoak *et al.*, 2000; Seaton *et al.*, 2011). The Sca permease in the oral bacterium *Streptococcus gordonii* is a member of a family of ATP-binding cassette (ABC)-type transporters for manganese (Mn^{2+}) and related cations that are associated with self-protection against oxidative stress (Jakubovics *et al.*, 2002). In *P. putida*, the TtgABC efflux pump is the main antibiotic extrusion system that responds to exogenous antibiotics through the modulation of the expression of this operon mediated by TtgR (Molina-Santiago *et al.*, 2014), and bactericidal compounds lead to cell death in a ttgABC-deficient *P. putida* mutant(Garcia *et al.*, 2010). ABC transportation is required for bacterial tolerance in biofilm (Vanderlinde *et al.*, 2010). ABC transporter DppBCDF is reported to increase biofilm formation in *P. aeruginosa* by repressing the Pf5 prophage (Lee *et*

al., 2018). The MerR-like regulator BrlR functions as an activator of ABC transport systems, contributing to the tolerance of *P. aeruginosa* biofilms exposed to tobramycin treatment (Poudyal and Sauer, 2018). *S. mutans* withstands exposure to methyl viologen (MV)/QAC by constituting a heterodimeric multidrug efflux pump of the ABC family with VltA and VltB (Biswas and Biswas, 2011). The MacABCsm efflux pump (n ABC-type tripartite efflux pump) extruded macrolides, aminoglycosides and polymyxins and contributed to oxidative and envelope stress tolerances and biofilm formation in *Stenotrophomonas maltophilia* (Lin *et al.*, 2014). In summary, these efflux pump systems allow bacteria to survive in their ecological niche through preventing an antibiotic from entering the bacterial cell and actively exporting an antibiotic from the bacterial cell.

2.4.4 Other Mechanisms

Persisters are formed in a growth phase dependent way while cell-cell communication might be necessary to induce a phenotypic switch at the population level (Möker *et al.*, 2010). Quorum-sensing-related signaling molecules (phenazinepyocyanin and acyl-homoserine lactone 3-OC12-HSL) increase the numbers of multidrug-tolerant persisters in *P. aeruginosa* cultures (Möker *et al.*, 2010). A synthetic quorum sensing inhibitor, (Z)-4-bromo-5-(bromomethylene)-3-methylfuran- 2(5H)-one, effectively reduces biofilm associated persistence of *P. aeruginosa* (Pan *et al.*, 2013). Leung and Lévesque obtained increasing multi-drug tolerant persisters of *S. mutans* by exogenously introducing a synthetic quorum sensing molecule(CSP pheromone) into the culture, and establishing a connection between the CSP-ComDE intraspecies quorum-sensing pathway and the development of persisters (Leung and Lévesque, 2012).

Unlike Gram-negative bacteria, the quorum sensing system of Gram-positive bacteria is not yet well understood, and the Agr quorum sensing (QS) system is the signaling peptides of quorum sensing identified for *L. monocytogenes* and other Gram-positive bacteria. The Agr system is reported to be involved in persister formation in *S. aureus*, and mutation of agrCA and agrD resulted in increased persister formation of stationary phase cultures with repression of expression of phenol soluble modulin encoding genes and their transporter genes (Xu *et al.*, 2017).

Current evidence suggests that cell density influences the long term survival of *L. monocytogenes* in planktonic form (Wen *et al.*, 2013). Starved cells of *L. monocytogenes* resuspended in water at 2 $\log_{10}$cfu/ml became unculturable within 3days while 7 $\log_{10}$cfu/ml resuspended cells became unculturable within 24 hrs (Herbert and Foster, 2001).

Persisters to antibiotics maybe formed in a manner dependent on the SOS gene network. SOS-induced persistence can counteract DNA damage and promote survival of *E. coli* on exposure to fluoroquinolones (Dörr *et al.*, 2009). Van der veen *et al.* indicated that the SOS response of *L. monocytogenes* contributes to survival upon exposure to the DNA-damaging agent mitomycin C, and activation of the SOS response happened under a range of stresses for *L. monocytogenes* survival (van der Veen *et al.*, 2010).

Other novel systems are also reported to be involved in bacterial persistence. Persister cell formation in *Streptococcus suis* is affected by the global transcriptional regulator CcpA and the catabolic arginine deiminase system (Willenborg *et al.*, 2014). Homoserine-o-succinyltransferase (MetA), the first enzyme in the methionine biosynthetic pathway, is prone to aggregation in *E.coli* persister formation, and substitution of the native metA gene on the *E. coli* K-12 led to reduction in persisters at an elevated temperature (42°C) and in the presence of acetate, as well as lower aggregation of the mutated MetA (Mordukhova and Pan, 2014). A fatty acid signaling molecule, cis-2-decenoic acid (cis-DA), is able to change the status of *P. aeruginosa* and *E. coli* persister cells from a dormant to a metabolically active state without an increase in cell number. This cell awakening is supported by an increase of the persister cells' respiratory activity together with changes in protein abundance and increases of the transcript expression levels of several metabolic markers, including acpP, 16S rRNA, atpH, and ppx (Marques *et al.*, 2014).

2.4.5 Possible mechanisms for persistence of *L. monocytogenes*

There are some proposed mechanisms for *L. monocytogenes* persistence. Ringus *et al.* examined some persistent and non-persistent strains from fish processing and meat plants, and found that transcript levels for the clpB gene were significantly induced in non-persistent strains

compared to persistent strains after salt shock *in vitro* (Ringus *et al.,* 2012). SigB is important in conferring the phenotypic resistance of *L. monocytogenes* to disinfectants both in planktonic cells and biofilms (van der Veen and Abee, 2010). Transcriptome and proteome analysis have provided complex information on the regulation of metabolic and biosynthetic pathways associated with bacterial persistence (Keren *et al.*, 2011; Nilsson *et al.*, 2012).

The transcriptomic analysis of *L. monocytogenes* isolates from food environments demonstrated that repression of genes associated with carbohydrate metabolism and flagella mediated motility occurred in stressed cells adapted to cold and hyper-osmotic environments (Durack *et al.*, 2013). The transcriptomic response of a persistent strain of *L. monocytogenes* on exposure to a sub-lethal concentration of the benzethonium chloride (BZT) showed that many biological processes such as peptidoglycan biosynthesis, bacterial chemotaxis and motility, and carbohydrate uptake, were involved in *Listeria* persistence (Casey *et al.*, 2014). The studies investigating *L. monocytogenes* persistence mainly utilize *L. monocytogenes* isolates detected in food environments or final food products over a long period of time, regarded as persisters while isolates only detected in the beginning of a sampling survey are called non-persistent cells or transient strains. The persistence studies of *L. monocytogenes* need to re-grow the persistent *L. monocytogenes* strains, however the physiological state of persisters is lost during this re-growth.

L. monocytogenes may form persisters when entering into dormancy by reducing their metabolic activity (Wood *et al.*, 2013). RNA expression profiling of persistent *L. monocytogenes* isolates revealed the down-regulation of transcriptional level energy production and flagellar synthesis with their slow or non-growth phenotype (Balaban *et al.*, 2004). The efflux protein is conserved in *Listeria* species (Mereghetti *et al.*, 2000; Zhu *et al.*, 2008), and the ABC transporter permease with an efflux protein negatively regulates genes encoding cell surface proteins (Dlt), cell surface anchor proteins (SrtA), and transcriptional regulators (GntR) resulting in reduced biofilm formation (Zhu *et al.*, 2011). Resistant strains of *L. monocytogenes* alter their cell membrane properties to survive under high concentrations of cell wall acting antibiotics (Collins *et al.*, 2010), and the ABC transporter permease with an efflux protein may contribute to form *L. monocytogenes* persisters by modifying some properties of the cell membrane as well. The ABC

transporter plays a key role in down-regulating *L. monocytogenes* biofilm (Zhu *et al.*, 2008), indicating that there may be a link between persistence and biofilm formation of *L. monocytogenes*.

There is no information on the persistence of *L. monocytogenes* cells following exposure to food preservatives, including biopreservation systems such as the bacteriocin nisin. The development of persister populations following exposure to nisin may result in the development of highly tolerant/resistant strains decreasing the efficiency of bacteriocins as biopreservatives (Kaur *et al.*, 2011). The impaired glucose transportation and structural changes in the hydrophobic core of their plasma membranes could be associated with potential targets in *L. monocytogenes* persistence following exposure to food perservatives (Masias *et al.*, 2017).

2.5 Conclusions

L. monocytogenes has been isolated from a variety of raw and processed food products from dairy to RTE foods. Persistence of microbial contamination in a food manufacturing environment can result in the long-term survival of a proportion of the population of a pathogen despite repeated treatments with sanitiser. Investigations on specific characteristics (e.g., surface adhering ability, stress tolerance and virulence) have shown heterogeneity among persister cells of *L. monocytogenes*. Persisters are not antibiotic resistant in the VBNC status, and their phenotypic tolerance is defined as non-inherited antibiotic resistance. The challenge for researchers in any of these studies is to derive sufficient persister cells in their VBNC state for *in situ* analysis. Accumulative evidence suggests that dormancy does not entirely explain persistence as many dormant cells are not persisters and many persisters are actively responding to stresses. In order to develop effective control measures for persister inhibition, it is important to understand the diverse environments and environmental factors that influence persister formation, and identify different valid explanations or associated gene expression influencing bacterial persistence. Studies on persister populations of *L. monocytogenes* have not included biopreservatives such as nisin.

2.6 References

Abranches, J., Martinez, A. R., Kajfasz, J. K., Chávez, V., Garsin, D. A. and Lemos, J. A. (2009). The molecular alarmone (p) ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus* faecalis. *Journal of bacteriology*, 191, 2248-2256.

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., and Ramakrishnan, L. (2011). Drug Tolerance in Replicating Mycobacteria Mediated by a Macrophage-Induced Efflux Mechanism. *Cell*, 145, 39-53.

Allison, K. R., Brynildsen, M. P. and Collins, J. J. (2011). Heterogeneous bacterial persisters and engineering approaches to eliminate them. *Current Opinion in Microbiology*, 14, 593-598.

Arevalos-Sánchez, M., Regalado, C., Martin, S., Domínguez-Domínguez, J. and García-Almendárez, B. (2012). Effect of neutral electrolyzed water and nisin on *Listeria monocytogenes* biofilms, and on listeriolysin O activity. *Food Control*, 24, 116-122.

Back, K. H., Ha, J. W. and Kang, D. H. (2014). Effect of hydrogen peroxide vapor treatment for inactivating *Salmonella Typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* on organic fresh lettuce. *Food Control*, 44, 78-85.

Balaban, N. Q. (2011). Persistence: mechanisms for triggering and enhancing phenotypic variability. *Current Opinion in Genetics & Development*, 21, 768-775.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. (2004). Bacterial Persistence as a Phenotypic Switch. *Science*, 305, 1622-1625.

Ban, G.-H., Park, S.-H., Kim, S.-O., Ryu, S. and Kang, D.-H. (2012). Synergistic effect of steam and lactic acid against *Escherichia coli* O157: H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. *International Journal of Food Microbiologyogy*, 157, 218-223.

Begley, M., Cotter, P. D., Hill, C. and Ross, R. P. (2010). Glutamate Decarboxylase-Mediated Nisin Resistance in *Listeria monocytogenes*. *Applied Environmental Microbiology*, 76, 6541-6546.

Belessi, C.-E. A., Gounadaki, A. S., Psomas, A. N. and Skandamis, P. N. (2011). Efficiency of different sanitation methods on *Listeria monocytogenes* biofilms formed under various environmental conditions. *International Journal of Food Microbiologyogy*, 145, S46-S52.

Beltrame, C. A., Martelo, E. B., Mesquita, R. A., Barbosa, J., Steffens, C., Toniazzo, G., and Cansian, R. L. (2015). Adhesion of *Listeria monocytogenes* to cutting board surfaces and removal by different sanitizers. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety*, 10, 41-47.

Berrang, M. E., Frank, J. F. and Meinersmann, R. J. (2008). Effect of chemical sanitizers with and without ultrasonication on *Listeria monocytogenes* as a biofilm within polyvinyl chloride drain

pipes. *Journal of Food Protection*, 71, 66-69.

Berrang, M. E., Hofacre, C. L. and Frank, J. F. (2014). Controlling Attachment and Growth of *Listeria monocytogenes* in Polyvinyl Chloride Model Floor Drains Using a Peroxide Chemical, Chitosan-Arginine, or Heat. *Journal of Food Protection*, 77, 2129-2132.

Bhatti, M., Veeramachaneni, A. and Shelef, L. A. (2004). Factors affecting the anti*Listeria*l effects of nisin in milk. *International Journal of Food Microbiologyogy*, 97, 215-219.

Bigger, J. W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet*, 247, 497-500.

Biswas, S. and Biswas, I. (2011). Role of VltAB, an ABC Transporter Complex, in Viologen Tolerance in *Streptococcus mutans*. *Antimicrobial Agents and Chemotherapy*, 55, 1460-1469.

Bodur, T. and Cagri-Mehmetoglu, A. (2012). Removal of *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 biofilms on stainless steel using scallop shell powder. *Food Control*, 25, 1-9.

Bokinsky, G., Baidoo, E. E., Akella, S., Burd, H., Weaver, D., Alonso-Gutierrez, J., and Keasling, J. D. (2013). HipA-triggered growth arrest and β-lactam tolerance in *Escherichia coli* is mediated by RelA-dependent ppGpp synthesis. *Journal of bacteriology*, JB. 02210-02212.

Bruhn, J. B., Vogel, B. F. and Gram, L. (2005). Bias in the *Listeria monocytogenes* Enrichment Procedure: Lineage 2 Strains Outcompete Lineage 1 Strains in University of Vermont Selective Enrichments. *Applied and Environmental Microbiology*, 71, 961-967.

Buchovec, I., Paskeviciute, E. and Luksiene, Z. (2010). Photosensitization-based inactivation of food pathogen *Listeria monocytogenes* in vitro and on the surface of packaging material. *Journal of Photochemistry and Photobiology B: Biology*, 99, 9-14.

Budu-Amoako, E., Ablett, R. F., Harris, J. and Delves-Broughton, J. (1999). Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in cold-pack lobster meat. *Journal of Food Protection*, 62, 46-50.

Butt, A., Higman V., Williams C., Crump M., Hemsley C., Harmer N., Titball R.. (2014). *The HicA toxin from Burkholderia pseudomallei has a role in persister cell formation*. *Biochemical Journal*, *459*(2), 333-344.

Cabo, M., Pastoriza, L., Bernárdez, M. and Herrera, J. (2001). Effectiveness of CO 2 and Nisaplin on increasing shelf-life of fresh pizza. *Food Microbiologyogy*, 18, 489-498.

Camargo, A. C., Lino de Paula, O. A., Todorov, S. D. and Nero, L. A. (2016). In Vitro Evaluation of Bacteriocins Activity Against *Listeria monocytogenes* Biofilm Formation. *Applied Biochemistry and Biotechnology*, 178, 1239-1251.

Carpentier, B. and Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiologyogy*, 145, 1-8.

Casey, A., Fox, E. M., Schmitz-Esser, S., Coffey, A., McAuliffe, O. and Jordan, K. (2014). Transcriptome analysis of *Listeria monocytogenes* exposed to biocide stress reveals a multi-system response involving cell wall synthesis, sugar uptake, and motility. *Frontier Microbiology*, 5, 68.

Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H. and Lindstrom, M. (2018). Occurrence, Persistence, and Contamination Routes of *Listeria monocytogenes* Genotypes on Three Finnish Dairy Cattle Farms: a Longitudinal Study. *Applied and Environmental Microbiology*, 84, 14.

Chau, M. L., Aung, K. T., Hapuarachchi, H. C., Lee, P. S. V., Lim, P. Y., Kang, J. S. L., and Ng, L. C. (2017). Microbial survey of ready-to-eat salad ingredients sold at retail reveals the occurrence and the persistence of *Listeria monocytogenes* Sequence Types 2 and 87 in pre-packed smoked salmon. *BMC Microbiologyogy*, 17, 13.

Chen, H. Q. and Zhong, Q. X. (2018). Antibacterial activity of acidified sodium benzoate against *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in tryptic soy broth and on cherry tomatoes. *International Journal of Food Microbiologyogy*, 274, 38-44.

Cheverton, A. M.*, et al.* (2016). A *Salmonella* toxin promotes persister formation through acetylation of tRNA. *Mol Cell*, 63, 86-96.

Cho, J., Carr, A. N., Whitworth, L., Johnson, B. and Wilson, K. S. (2017). MazEF toxin-antitoxin proteins alter *Escherichia coli* cell morphology and infrastructure during persister formation and regrowth. *Microbiology*, 163, 308-321.

Chorianopoulos, N., Tsoukleris, D., Panagou, E., Falaras, P. and Nychas, G.-J. (2011). Use of titanium dioxide (TiO 2) photocatalysts as alternative means for *Listeria monocytogenes* biofilm disinfection in food processing. *Food Microbiologyogy*, 28, 164-170.

Cohen, N. R., Lobritz, M. A. and Collins, J. J. (2013). Microbial persistence and the road to drug resistance. *Cell Host Microbe*, 13, 632-642.

Collins, B., Joyce, S., Hill, C., Cotter, P. D. and Ross, R. P. (2010). TelA Contributes to the Innate Resistance of *Listeria monocytogenes* to Nisin and Other Cell Wall-Acting Antibiotics. *Antimicrobial Agents and Chemotherapy*, 54, 4658-4663.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology*, 3, 777-788.

Cruz, C. D. and Fletcher, G. C. (2011). Prevalence and biofilm-forming ability of *Listeria monocytogenes* in New Zealand mussel (Perna canaliculus) processing plants. *Food Microbiologyogy*, 28, 1387-1393.

Cruz, C. D. and Fletcher, G. C. (2012). Assessing manufacturers' recommended concentrations of commercial sanitizers on inactivation of *Listeria monocytogenes*. *Food Control*, 26, 194-199.

Cruz, C. D., Pitman, A. R., Harrow, S. A. and Fletcher, G. C. (2014). *Listeria monocytogenes* Associated with New Zealand Seafood Production and Clinical Cases: Unique Sequence Types, Truncated InlA, and Attenuated Invasiveness. *Applied and Environmental Microbiology*, 80, 1489-1497.

Davies, E. A., Bevis, H. E. and DelvesBroughton, J. (1997). The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. *Letters in Applied Microbiology*, 24, 343-346.

de Araujo, L. V., Abreu, F., Lins, U., Anna, L. M. d. M. S., Nitschke, M. and Freire, D. M. G. (2011). Rhamnolipid and surfactin inhibit *Listeria monocytogenes* adhesion. *Food Research International*, 44, 481-488.

de Oliveira, M. M. M., Brugnera, D. F., do Nascimento, J. A., Batista, N. N. and Piccoli, R. H. (2012). Cinnamon essential oil and cinnamaldehyde in the control of bacterial biofilms formed on stainless steel surfaces. *European Food Research and Technology*, 234, 821-832.

Delves-Broughton, J., Williams, G. C. and Wilkinson, S. (2008). *The use of bacteriocin, nisin, as a preservative in pasteurized liquid whole egg*.

den Bakker, H. C., Didelot, X., Fortes, E. D., Nightingale, K. K. and Wiedmann, M. (2008). Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. *Bmc Evolutionary Biology*, 8.

Desai, M. A., Soni, K. A., Nannapaneni, R., Schilling, M. and Silva, J. L. (2012a). Reduction of *Listeria monocytogenes* biofilms on stainless steel and polystyrene surfaces by essential oils. *Journal of Food Protection*, 75, 1332-1337.

Dewi, F. R., Stanley, R., Powell, S. M. and Burke, C. M. (2017). Application of electrolysed oxidising water as a sanitiser to extend the shelf-life of seafood products: a review. *Journal of Food Science and Technology-Mysore*, 54, 1321-1332.

Dhar, N. and McKinney, J. D. (2007). Microbial phenotypic heterogeneity and antibiotic tolerance. *Current Opinion in Microbiology*, 10, 30-38.

do Valle Gomes, M. Z. and Nitschke, M. (2012). Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. *Food Control*, 25, 441-447.

Dörr, T., Lewis, K. and Vulić, M. (2009). SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS genetics*, 5, e1000760.

Dörr, T., Vulić, M. and Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS biology*, 8, e1000317.

Drider, D., Fimland, G., Hechard, Y., McMullen, L. M. and Prevost, H. (2006). The continuing

story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, 70, 564-+.

Durack, J., Ross, T. and Bowman, J. P. (2013). Characterisation of the transcriptomes of genetically diverse *Listeria monocytogenes* exposed to hyperosmotic and low temperature conditions reveal global stress-adaptation mechanisms. *Plos One*, 8, e73603.

El-Kest, S. E. and Marth, E. H. (1988). Inactivation of *Listeria monocytogenes* by chlorine. *Journal of Food Protection*, 51, 520-530.

English, B. P., Hauryliuk, V., Sanamrad, A., Tankov, S., Dekker, N. H. and Elf, J. (2011). Single-molecule investigations of the stringent response machinery in living bacterial cells. *Proceedings of the National Academy of Sciences*, 108, E365-E373.

Fernandes, M. d. S., Kabuki, D. Y. and Kuaye, A. Y. (2015). Behavior of *Listeria monocytogenes* in a multi-species biofilm with *Enterococcus* faecalis and *Enterococcus faecium* and control through sanitation procedures. *International Journal of Food Microbiologyogy*, 200, 5-12.

Ferreira, V.*, et al.* (2011). Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Applied Environmental Microbiology*, 77, 2701-2715.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection*, 77, 150-170.

Fox, E. M., Casey, A., Jordan, K., Coffey, A., Gahan, C. G. M. and McAuliffe, O. (2017). Whole genome sequence analysis; an improved technology that identifies underlying genotypic differences between closely related *Listeria monocytogenes* strains. *Innovative Food Science & Emerging Technologies*, 44, 89-96.

Galvez, A., Abriouel, H., Lopez, R. L. and Ben Omar, N. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiologyogy*, 120, 51-70.

Ganegama Arachchi, G. J.*, et al.* (2013). Effectiveness of phages in the decontamination of *Listeria monocytogenes* adhered to clean stainless steel, stainless steel coated with fish protein, and as a biofilm. *Journal of Industrial Microbiology & Biotechnology*, 40, 1105-1116.

Garcia, V., Godoy, P., Daniels, C., Hurtado, A., Ramos, J. L. and Segura, A. (2010). Functional analysis of new transporters involved in stress tolerance in *Pseudomonas* putida DOT-T1E. *Environmental Microbiology Reports*, 2, 389-395.

Gaul, L. K., Farag, N. H., Shim, T., Kingsley, M. A., Silk, B. J. and Hyytia-Trees, E. (2013). Hospital-Acquired Listeriosis Outbreak Caused by Contaminated Diced Celery—Texas, 2010. *Clinical Infectious Diseases*, 56, 20-26.

Germain, E., Castro-Roa, D., Zenkin, N. and Gerdes, K. (2013). Molecular mechanism of bacterial

persistence by HipA. *Mol Cell*, 52, 248-254.

Gharsallaoui, A., Oulahal, N., Joly, C. and Degraeve, P. (2016). Nisin as a Food Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses. *Crit Rev Food Sci Nutr*, 56, 1262-1274.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., and Charbit, A. (2001). Comparative genomics of *Listeria* species. *Science*, 294, 849-852.

Glass, K. A., McDonnell, L. M., Rassel, R. C. and Zierke, K. L. (2007). Controlling *Listeria monocytogenes* on sliced ham and turkey products using benzoate, propionate, and sorbate. *Journal of Food Protection*, 70, 2306-2312.

Gomez-Lopez, V. M., Gil, M. I., Allende, A., Vanhee, B. and Selma, M. V. (2015). Water reconditioning by high power ultrasound combined with residual chemical sanitizers to inactivate foodborne pathogens associated with fresh-cut products. *Food Control*, 53, 29-34.

Gonzalez-Fandos, E. and Dominguez, J. L. (2007). Effect of potassium sorbate washing on the growth of *Listeria monocytogenes* on fresh poultry. *Food Control*, 18, 842-846.

Gorski, L., Flaherty, D. and Mandrell, R. E. (2006). Competitive fitness of *Listeria monocytogenes* serotype 1/2a and 4b strains in mixed cultures with and without food in the US Food and Drug Administration enrichment protocol. *Applied and Environmental Microbiology*, 72, 776-783.

Gotfredsen, M. and Gerdes, K. (1998). The *Escherichia coli* relBE genes belong to a new toxin–antitoxin gene family. *Molecular microbiology*, 29, 1065-1076.

Gray, M. J., Zadoks, R. N., Fortes, E. D., Dogan, B., Cai, S., Chen, Y., and Wiedmann, M. (2004). *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Applied and Environmental Microbiology*, 70, 5833-5841.

Grosulescu, C., Juneja, V. K. and Ravishankar, S. (2011). Effects and interactions of sodium lactate, sodium diacetate, and pediocin on the thermal inactivation of starved *Listeria monocytogenes* on bologna. *Food Microbiology*, 28, 440-446.

Guerra, N. á., Macias, C. á., Agrasar, A. and Castro, L. (2005). Development of a bioactive packaging cellophane using Nisaplin® as biopreservative agent. *Letters in Applied Microbiology*, 40, 106-110.

Guo, M. M., Jin, T. Z. and Yang, R. J. (2014). Antimicrobial Polylactic Acid Packaging Films against *Listeria* and *Salmonella* in Culture Medium and on Ready-to-Eat Meat. *Food and Bioprocess Technology*, 7, 3293-3307.

Habimana, O., Guillier, L., Kulakauskas, S. and Briandet, R. (2011). Spatial competition with Lactococcus lactis in mixed-species continuous-flow biofilms inhibits *Listeria monocytogenes* growth. *Biofouling*, 27, 1065-1072.

Hain, T., Ghai, R., Billion, A., Kuenne, C. T., Steinweg, C., Izar, B., and Kärst, U. (2012). Comparative genomics and transcriptomics of lineages I, II, and III strains of *Listeria monocytogenes*. *Bmc Genomics*, 13.

Hammons, S. R.*, et al.* (2017). Evaluation of Third-Party Deep Cleaning as a *Listeria monocytogenes* Control Strategy in Retail Delis. *Journal of Food Protection*, 80, 1913-1923.

Hansen, S., Lewis, K. and Vulić, M. (2008). Role of Global Regulators and Nucleotide Metabolism in Antibiotic Tolerance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, 52, 2718-2726.

Harms, A., Maisonneuve, E. and Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science*, 354, aaf4268.

Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T. and Gerdes, K. (2015). Recent functional insights into the role of (p) ppGpp in bacterial physiology. *Nature reviews microbiology*, 13, 298.

Heir, E., Moretro, T., Simensen, A. and Langsrud, S. (2018). *Listeria monocytogenes* strains show large variations in competitive growth in mixed culture biofilms and suspensions with bacteria from food processing environments. *International journal of Food Microbiologyogy*, 275, 46-55.

Helaine, S. and Kugelberg, E. (2014). Bacterial persisters: formation, eradication, and experimental systems. *Trends Microbiol*, 22, 417-424.

Henriques, A. R. and Fraqueza, M. J. (2017). Biofilm-forming ability and biocide susceptibility of *Listeria monocytogenes* strains isolated from the ready-to-eat meat-based food products food chain. *Lwt-Food Science and Technology*, 81, 180-187.

Herbert, K. C. and Foster, S. J. (2001). Starvation survival in *Listeria monocytogenes*: characterization of the response and the role of known and novel components. *Microbiology*, 147, 2275-2284.

Highmore, C. J., Warner, J. C., Rothwell, S. D., Wilks, S. A. and Keevil, C. W. (2018). Viable-but-Nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson Induced by Chlorine Stress Remain Infectious. *Mbio*, 9, 12.

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M. and Gram, L. (2013). Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Applied Environmental Microbiology*, 79, 2944-2951.

Holyoak, C. D., Thompson, S., Ortiz Calderon, C., Hatzixanthis, K., Bauer, B., Kuchler, K., and Coote, P. J. (2000). Loss of Cmk1 Ca(2+)-calmodulin-dependent protein kinase in yeast results in constitutive weak organic acid resistance, associated with a post-transcriptional activation of the

Pdr12 ATP-binding cassette transporter. *Molecular microbiology*, 37, 595-605.

Hood, M. I., Jacobs, A. C., Sayood, K., Dunman, P. M. and Skaar, E. P. (2010). *Acinetobacter baumannii* increases tolerance to antibiotics in response to monovalent cations. *Antimicrob Agents Chemotherapy*, 54, 1029-1041.

Ibarra-Sanchez, L. A., Van Tassell, M. L. and Miller, M. J. (2018). Antimicrobial behavior of phage endolysin PlyP100 and its synergy with nisin to control *Listeria monocytogenes* in Queso Fresco. *Food Microbiologyogy*, 72, 128-134.

Inatsu, Y., Weerakkody, K., Bari, M. L., Hosotani, Y., Nakamura, N. and Kawasaki, S. (2017). The efficacy of combined (NaClO and organic acids) washing treatments in controlling *Escherichia coli* O157:H7, *Listeria monocytogenes* and spoilage bacteria on shredded cabbage and bean sprout. *Lwt-Food Science and Technology*, 85, 1-8.

Islam, M., Chen, J., Doyle, M. P. and Chinnan, M. (2002). Control of *Listeria monocytogenes* on turkey frankfurters by generally-recognized-as-safe preservatives. *Journal of Food Protection*, 65, 1411-1416.

Jadhav, S., Shah, R., Bhave, M. and Palombo, E. A. (2013). Inhibitory activity of yarrow essential oil on *Listeria* planktonic cells and biofilms. *Food Control*, 29, 125-130.

Jakubovics, N. S., Smith, A. W. and Jenkinson, H. F. (2002). Oxidative stress tolerance is manganese (Mn2+) regulated in *Streptococcus gordonii*. *Microbiology-Sgm*, 148, 3255-3263.

Jung, D.-S., Bodyfelt, F. W. and Daeschel, M. A. (1992). Influence of Fat and Emulsifiers on the Efficacy of Nisin in Inhibiting *Listeria monocytogenes* in Fluid Milk¹. *Journal of Dairy Science*, 75, 387-393.

Kakey, S. I., Ali, J. F. and Alzubaidy, Z. M. (2016). Controlling the Growth of Local Isolates of *Listeria monocytogenes* by Using Some Chemicals Preservatives. *Science Journal of University of Zakho*, 1, 651-658.

Kang, J. H. and Bin Song, K. (2018). Inhibitory effect of plant essential oil nanoemulsions against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella Typhimurium* on red mustard leaves. *Innovative Food Science & Emerging Technologies*, 45, 447-454.

Kaur, G., Malik, R. K., Mishra, S. K., Singh, T. P., Bhardwaj, A., Singroha, G., and Kumar, N. (2011). Nisin and class IIa bacteriocin resistance among *Listeria* and other foodborne pathogens and spoilage bacteria. *Microb Drug Resist*, 17, 197-205.

Kerekes, E. B., Deák, É., Takó, M., Tserennadmid, R., Petkovits, T., Vágvölgyi, C., and Krisch, J. (2013). Anti-biofilm forming and anti-quorum sensing activity of selected essential oils and their main components on food-related micro-organisms. *Journal of applied microbiology*, 115, 933-942.

Keren, I., Minami, S., Rubin, E. and Lewis, K. (2011). Characterization and Transcriptome Analysis of Mycobacterium tuberculosis Persisters. *mBio*, 2, e00100-00111.

Kim, S. O., Ha, J. W., Park, K. H., Chung, M. S. and Kang, D. H. (2014). Infrared sensor-based aerosol sanitization system for controlling *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* on fresh produce. *Journal of Food Protection*, 77, 977-980.

Kint, C. I., Verstraeten, N., Fauvart, M. and Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends Microbiol*, 20, 577-585.

Knabel, S. J., Reimer, A., Verghese, B., Lok, M., Ziegler, J., Farber, J., and Canadian Public Health Laboratory Network (CPHLN. (2012). Sequence Typing Confirms that a Predominant *Listeria monocytogenes* Clone Caused Human Listeriosis Cases and Outbreaks in Canada from 1988 to 2010. *Journal of Clinical Microbiology*, 50, 1748-1751.

Knudsen, G. M., Ng, Y. and Gram, L. (2013). Survival of Bactericidal Antibiotic Treatment by a Persister Subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 79, 7390-7397.

Ko, S., Janes, M. E., Hettiarachchy, N. S. and Johnson, M. G. (2001). Physical and chemical properties of edible films containing nisin and their action against *Listeria monocytogenes*. *Journal of Food Science*, 66, 1006-1011.

Korch, S. B., Henderson, T. A. and Hill, T. M. (2003). Characterization of the hipA7 allele of *Escherichia coli* and evidence that high persistence is governed by (p) ppGpp synthesis. *Molecular microbiology*, 50, 1199-1213.

Krysinski, E., Brown, L. and Marchisello, T. (1992). Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection*, 55, 246-251.

Kumar, A. and Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. *Advanced Drug Delivery Reviews*, 57, 1486-1513.

Latorre, A. A., Van Kessel, J. A. S., Karns, J. S., Zurakowski, M. J., Pradhan, A. K., Boor, K. J., and Schukken, Y. H. (2011). Increased in vitro adherence and on-farm persistence of predominant and persistent *Listeria monocytogenes* strains in the milking system. *Applied Environmental Microbiology*, 77, 3676-3684.

Lee, N. Y., Kim, S. W. and Ha, S. D. (2014). Synergistic effects of ultrasound and sodium hypochlorite (NaOCl) on reducing *Listeria monocytogenes* ATCC19118 in broth, stainless steel, and iceberg lettuce. *Foodborne Pathog Disease*, 11, 581-587.

Lee, S. H.*, et al.* (2017). Biofilm-producing ability of *Listeria monocytogenes* isolates from Brazilian cheese processing plants. *Food Res Int*, 91, 88-91.

Lee, Y., Song, S., Sheng, L. L., Zhu, L., Kim, J. S. and Wood, T. K. (2018). Substrate Binding Protein

DppA1 of ABC Transporter DppBCDF Increases Biofilm Formation in *Pseudomonas aeruginosa* by Inhibiting Pf5 Prophage Lysis. *Frontiers in microbiology*, 9, 11.

Lemos, J. A., Lin, V. K., Nascimento, M. M., Abranches, J. and Burne, R. A. (2007). Three gene products govern (p) ppGpp production by *Streptococcus mutans*. *Molecular microbiology*, 65, 1568-1581.

Lennon, J. T. and Jones, S. E. (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nature reviews microbiology*, 9, 119.

Leung, V. and Lévesque, C. M. (2012). A Stress-Inducible Quorum-Sensing Peptide Mediates the Formation of Persister Cells with Noninherited Multidrug Tolerance. *Journal of Bacteriology*, 194, 2265-2274.

Levin, B. R. and Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat Reviews Microbiology*, 4, 556-562.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)*, 70, 267-274.

Li, L., Olsen, R. H., Ye, L., Wang, W., Shi, L., Yan, H., & Meng, H. (2016). Characterization of Antimicrobial Resistance of *Listeria monocytogenes* Strains Isolated from a Pork Processing Plant and Its Respective Meat Markets in Southern China. *Foodborne Pathog Disease*, 13, 262-268.

Li, Z., Pérez-Osorio, A., Wang, Y., Eckmann, K., Glover, W. A., Allard, M. W., and Chen, Y. (2017). Whole genome sequencing analyses of *Listeria monocytogenes* that persisted in a milkshake machine for a year and caused illnesses in Washington State. *BMC Microbiology*, 17, 134.

Liao, J., Schurr, M. J. and Sauer, K. (2013). The MerR-like regulator BrlR confers biofilm tolerance by activating multidrug efflux pumps in *Pseudomonas aeruginosa* biofilms. *Journal of Bacteriology*, 195, 3352-3363.

Lin, L., Zhang, X. J., Zhao, C. T. and Cui, H. Y. (2016). Liposome containing nutmeg oil as the targeted preservative against *Listeria monocytogenes* in dumplings. *RSC Advances*, 6, 978-986.

Lin, Y. T., Huang, Y. W., Liou, R. S., Chang, Y. C. and Yang, T. C. (2014). MacABCsm, an ABC-type tripartite efflux pump of *Stenotrophomonas maltophilia* involved in drug resistance, oxidative and envelope stress tolerances and biofilm formation. *Journal of Antimicrobial Chemotherapy*, 69, 3221-3226.

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., and Stessl, B. (2014). Reservoirs of *Listeria* Species in Three Environmental Ecosystems. *Applied and Environmental Microbiology*, 80, 5583-5592.

Lomonaco, S., Nucera, D. and Filipello, V. (2015). The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. *Infection, Genetics and Evolution*, 35, 172-183.

Luo, K. and Oh, D. H. (2016). Inactivation kinetics of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium on fresh-cut bell pepper treated with slightly acidic electrolyzed water combined with ultrasound and mild heat. *Food Microbiology*, 53, 165-171.

Luo, L. J*., et al.* (2017). A 12-month longitudinal study of *Listeria monocytogenes* contamination and persistence in pork retail markets in China. *Food Control*, 76, 66-73.

Mafu, A. A., Roy, D., Goulet, J., Savoie, L. and Roy, R. (1990). Efficiency of sanitizing agents for destroying *Listeria monocytogenes* on contaminated surfaces. *Journal of Dairy Science*, 73, 3428-3432.

Magnusson, L. U., Farewell, A. and Nyström, T. (2005). ppGpp: a global regulator in *Escherichia coli*. *Trends in Microbiology*, 13, 236-242.

Maisnier-Patin, S., Deschamps, N., Tatini, S., R. and Richard, J. (1992). Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. *Lait*, 72, 249-263.

Maisnier-Patin, S., Tatini, S., R. and Richard, J. (1995). Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in milk. *Lait*, 75, 81-91.

Maisonneuve, E. and Gerdes, K. (2014). Molecular mechanisms underlying bacterial persisters. *Cell*, 157, 539-548.

Malekmohammadi, S., Kodjovi, K. K., Sherwood, J. and Bergholz, T. M. (2017). Genetic and environmental factors influence *Listeria monocytogenes* nisin resistance. *Journal of Applied Microbiology*, 123, 262-270.

Mariani, C., Oulahal, N., Chamba, J.-F., Dubois-Brissonnet, F., Notz, E. and Briandet, R. (2011). Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. *Food Control*, 22, 1357-1362.

Marques, C. N., Morozov, A., Planzos, P. and Zelaya, H. M. (2014). The fatty acid signaling molecule cis-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial-susceptible state. *Applied Environmental Microbiology*, 80, 6976-6991.

Masias, E., Dupuy, F. G., da Silva Sanches, P. R., Farizano, J. V., Cilli, E., Bellomio, A., and Minahk, C. (2017). Impairment of the class IIa bacteriocin receptor function and membrane structural changes are associated to enterocin CRL35 high resistance in *Listeria monocytogenes*. *Biochimica et Biophysica Acta*, 1861, 1770-1776.

Masuku, S. M., Babu, D., Martin, E. M., O'Bryan, C. A., Crandall, P. G. and Ricke, S. C. (2014). Lethality of moist heat and silver dihydrogen citrate sanitizer combinations on *Listeria* spp. adhered to components of a deli meat slicer. *Food Control*, 44, 227-232.

McEgan, R. and Danyluk, M. D. (2015). Evaluation of aqueous and alcohol-based quaternary ammonium sanitizers for inactivating *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria*

monocytogenes on peanut and pistachio shells. *Food Microbiology*, 47, 93-98.

Mereghetti, L., Quentin, R., Marquet-Van der Mee, N. and Audurier, A. (2000). Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. *Applied and Environmental Microbiology*, 66, 5083-5086.

Minei, C. C., Gomes, B. C., Ratti, R. P., D'Angelis, C. E. M. and De Martinis, E. C. P. (2008). Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *Journal of Food Protection*, 71, 634-638.

Möker, N., Dean, C. R. and Tao, J. (2010). *Pseudomonas aeruginosa* Increases Formation of Multidrug-Tolerant Persister Cells in Response to Quorum-Sensing Signaling Molecules. *Journal of Bacteriology*, 192, 1946-1955.

Molina-Santiago, C., Daddaoua, A., Fillet, S., Duque, E. and Ramos, J. L. (2014). Interspecies signalling: *Pseudomonas* putida efflux pump TtgGHI is activated by indole to increase antibiotic resistance. *Environmental Microbiology*, 16, 1267-1281.

Mordukhova, E. A. and Pan, J.-G. (2014). Stabilization of Homoserine-O-Succinyltransferase (MetA) Decreases the Frequency of Persisters in *Escherichia coli* under Stressful Conditions. *PLoS ONE*, 9, e110504.

Moreno-del Álamo, M., Tabone, M., Lioy, V. S. and Alonso, J. C. (2017). Toxin ζ Triggers a Survival Response to Cope with Stress and Persistence. *Frontiers in microbiology*, 8, 1130.

Moyed, H. S. and Bertrand, K. P. (1983). hipA, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of Bacteriology*, 155, 768-775.

Murakami, K., Ono, T., Viducic, D., Kayama, S., Mori, M., Hirota, K., and Miyake, Y. (2005). Role for rpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance. *FEMS Microbiology Letters*, 242, 161-167.

Muriana, P. M. and Kanach, L. (1995). Use of Nisaplin™ to inhibit spoilage bacteria in buttermilk ranch dressing. *Journal of Food Protection*, 58, 1109-1113.

Murray, E. G. D., Webb, R. A. and Swann, M. B. R. (1926). A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes (n. sp.). *The Journal of Pathology and Bacteriology*, 29, 407-439.

Murugesan, L., Kucerova, Z., Knabel, S. J. and LaBorde, L. F. (2015). Predominance and Distribution of a Persistent *Listeria monocytogenes* Clone in a Commercial Fresh Mushroom Processing Environment. *Journal of Food Protection*, 78, 1988-1998.

Mustapha, A. and Liewen, M. (1989). Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. *Journal of Food Protection*, 52, 306-311.

Nakamura, H., Takakura, K., Sone, Y., Itano, Y. and Nishikawa, Y. (2013). Biofilm formation and resistance to benzalkonium chloride in *Listeria monocytogenes* isolated from a fish processing plant. *Journal of Food Protection*, 76, 1179-1186.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., and Britigan, B. E. (2011). Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science (New York, N.Y.)*, 334, 982-986.

Nilsson, L., Huss, H. H. and Gram, L. (1997). Inhibition of *Listeria monocytogenes* on cold-smoked salmon by nisin and carbon dioxide atmosphere. *International Journal of Food Microbiologyogy*, 38, 217-227.

Nilsson, R. E., Latham, R., Mellefont, L., Ross, T. and Bowman, J. P. (2012). MudPIT analysis of alkaline tolerance by *Listeria monocytogenes* strains recovered as persistent food factory contaminants. *Food Microbiologyogy*, 30, 187-196.

Nobmann, P., Smith, A., Dunne, J., Henehan, G. and Bourke, P. (2009). The antimicrobial efficacy and structure activity relationship of novel carbohydrate fatty acid derivatives against *Listeria* spp. and food spoilage microorganisms. *International Journal of Food Microbiologyogy*, 128, 440-445.

Nowak, J., Cruz, C. D., Tempelaars, M., Abee, T., van Vliet, A. H., Fletcher, G. C., and Flint, S. (2017). Persistent *Listeria monocytogenes* strains isolated from mussel production facilities form more biofilm but are not linked to specific genetic markers. *International journal of Food Microbiologyogy*, 256, 45-53.

NuÑEz de Gonzalez, M. T., Keeton, J. T., Acuff, G. R., Ringer, L. J. and Lucia, L. M. (2004). Effectiveness of Acidic Calcium Sulfate with Propionic and Lactic Acid and Lactates as Postprocessing Dipping Solutions To Control *Listeria monocytogenes* on Frankfurters with or without Potassium Lactate and Stored Vacuum Packaged at 4.5°C. *Journal of Food Protection*, 67, 915-921.

Ochiai, Y., Yamada, F., Mochizuki, M., Takano, T., Hondo, R. and Ueda, F. (2014). Biofilm formation under different temperature conditions by a single genotype of persistent *Listeria monocytogenes* strains. *Journal of Food Protection*, 77, 133-140.

Olivares, J., Alvarez-Ortega, C. and Martinez, J. L. (2014). Metabolic compensation of fitness costs associated with overexpression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemotherapy*, 58, 3904-3913.

Oliveira, M. M. M. d., Brugnera, D. F., Cardoso, M. d. G., Alves, E. and Piccoli, R. H. (2010). Disinfectant action of Cymbopogon sp. essential oils in different phases of biofilm formation by *Listeria monocytogenes* on stainless steel surface. *Food Control*, 21, 549-553.

Orgaz, B., Lobete, M. M., Puga, C. H. and San Jose, C. (2011). Effectiveness of chitosan against mature biofilms formed by food related bacteria. *International journal of molecular sciences*, 12, 817-828.

Orgaz, B., Puga, C. H., Martinez-Suarez, J. V. and SanJose, C. (2013). Biofilm recovery from chitosan action: A possible clue to understand *Listeria monocytogenes* persistence in food plants. *Food Control*, 32, 484-489.

Orman, M. A. and Brynildsen, M. P. (2015). Inhibition of stationary phase respiration impairs persister formation in *E. coli*. *Nature Communications*, 6, 7983.

Orsi, R. H., den Bakker, H. C. and Wiedmann, M. (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*, 301, 79-96.

Ortiz, S., Lopez-Alonso, V., Rodriguez, P. and Martinez-Suarez, J. V. (2016). The Connection between Persistent, Disinfectant-Resistant *Listeria monocytogenes* Strains from Two Geographically Separate Iberian Pork Processing Plants: Evidence from Comparative Genome Analysis. *Applied Environmental Microbiology*, 82, 308-317.

Palmer, M. E., Wiedmann, M. and Boor, K. J. (2009). sigma(B) and sigma(L) Contribute to *Listeria monocytogenes* 10403S Response to the Antimicrobial Peptides SdpC and Nisin. *Foodborne Pathogens and Disease*, 6, 1057-1065.

Pamp, S. J., Gjermansen, M., Johansen, H. K. and Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. *Mol Microbiol*, 68, 223-240.

Pan, J., Song, F. and Ren, D. (2013). Controlling persister cells of *Pseudomonas aeruginosa* PDO300 by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. *Bioorganic & Medicinal Chemistry Letters*, 23, 4648-4651.

Park, SH., Cheon, HL. Park, KH. Chung, MS. Choi, SH. Ryu, S. and Kang, DH. (2012). Inactivation of biofilm cells of foodborne pathogen by aerosolized sanitizers. *International Journal of Food Microbiologyogy*, 154, 130-134.

Pasquali, F., Palma, F., Guillier, L., Lucchi, A., De Cesare, A. and Manfreda, G. (2018). *Listeria monocytogenes* Sequence Types 121 and 14 Repeatedly Isolated Within One Year of Sampling in a Rabbit Meat Processing Plant: Persistence and Ecophysiology. *Frontier Microbiology*, 9, 596.

Patra, J. K. and Baek, K. H. (2016). Anti-*Listeria*l Activity of Four Seaweed Essential Oils Against *Listeria monocytogenes*. *Jundishapur J Microbiol*, 9, e31784.

Pezzuto, A., Belluco, S., Losasso, C., Patuzzi, I., Bordin, P., Piovesana, A., and Ricci, A. (2016). Effectiveness of Washing Procedures in Reducing *Salmonella enterica* and *Listeria*

monocytogenes on a Raw Leafy Green Vegetable (Eruca vesicaria). *Frontier Microbiology*, 7, 1663.

Piddock, L. J. (2006). Multidrug-resistance efflux pumps - not just for resistance. *Nat Reviews Microbiologybiol*, 4, 629-636.

Piskunova, J., Maisonneuve, E., Germain, E., Gerdes, K. and Severinov, K. (2017). Peptide–nucleotide antibiotic Microcin C is a potent inducer of stringent response and persistence in both sensitive and producing cells. *Molecular microbiology*, 104, 463-471.

Poimenidou, S. V., Chrysadakou, M., Tzakoniati, A., Bikouli, V. C., Nychas, G. J. and Skandamis, P. N. (2016). Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. *International journal of Food Microbiologyogy*, 237, 164-171.

Poudyal, B. and Sauer, K. (2018). The ABC of Biofilm Drug Tolerance: the MerR-Like Regulator BrlR Is an Activator of ABC Transport Systems, with PA1874-77 Contributing to the Tolerance of *Pseudomonas aeruginosa* Biofilms to Tobramycin. *Antimicrobial Agents and Chemotherapy*, 62, 19.

Prado-Silva, L., Cadavez, V., Gonzales-Barron, U., Rezende, A. C. and Sant'Ana, A. S. (2015). Meta-analysis of the effects of sanitizing treatments on *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* inactivation in fresh produce. *Applied Environmental Microbiology*, 81, 8008-8021.

Prombutara, P., Kulwatthanasal, Y., Supaka, N., Sramala, I. and Chareonpornwattana, S. (2012). Production of nisin-loaded solid lipid nanoparticles for sustained antimicrobial activity. *Food Control*, 24, 184-190.

Pu, Y., Ke, Y. and Bai, F. (2017). Active efflux in dormant bacterial cells - New insights into antibiotic persistence. *Drug Resistance Updates*, 30, 7-14.

Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., and Ge, H. (2016). Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Mol Cell*, 62, 284-294.

Puga, C. H., SanJose, C. and Orgaz, B. (2016). Biofilm development at low temperatures enhances *Listeria monocytogenes* resistance to chitosan. *Food Control*, 65, 143-151.

Rai, V. R. and Bai, J. A. (2014). *Microbial food safety and preservation techniques*: CRC Press.

Rajamohan, G., Srinivasan, V. B. and Gebreyes, W. A. (2010). Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother*, 65, 1919-1925.

Rasch, M. and Knochel, S. (1998). Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. *Letters in Applied Microbiology*, 27, 275-278.

Ringus, D. L., Ivy, R. A., Wiedmann, M. and Boor, K. J. (2012). Salt Stress-Induced Transcription

of sigma(B)- and CtsR-Regulated Genes in Persistent and Non-persistent *Listeria monocytogenes* Strains from Food Processing Plants. *Foodborne Pathogens and Disease*, 9, 198-206.

Ruiz, A., Williams, S., Djeri, N., Hinton Jr, A. and Rodrick, G. (2009). Nisin, rosemary, and ethylenediaminetetraacetic acid affect the growth of *Listeria monocytogenes* on ready-to-eat turkey ham stored at four degrees Celsius for sixty-three days. *Poultry science*, 88, 1765-1772.

Rychli, K., Müller, A. Zaiser, A. Schoder, D. Allerberger, F. Wagner, M. and Schmitz-Esser, S. (2014). Genome Sequencing of *Listeria monocytogenes* "Quargel" Listeriosis Outbreak Strains Reveals Two Different Strains with Distinct In Vitro Virulence Potential. *Plos One*, 9.

Sala, A., Bordes, P. and Genevaux, P. (2014). Multiple toxin-antitoxin systems in Mycobacterium tuberculosis. *Toxins*, 6, 1002-1020.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A. and Smith, G. C. (2005). Combinations of nisin with organic acids or salts to control *Listeria monocytogenes* on sliced pork bologna stored at 4 degrees C in vacuum packages. *Lwt-Food Science and Technology*, 38, 21-28.

Sandasi, M., Leonard, C. and Viljoen, A. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Letters in applied microbiology*, 50, 30-35.

Seaton, K., Ahn, S. J., Sagstetter, A. M. and Burne, R. A. (2011). A Transcriptional Regulator and ABC Transporters Link Stress Tolerance, (p)ppGpp, and Genetic Competence in *Streptococcus mutans*. *Journal of bacteriology*, 193, 862-874.

Seeger, M. A., Schiefner, A., Eicher, T., Verrey, F., Diederichs, K. and Pos, K. M. (2006). Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. *Science*, 313, 1295-1298.

Shi, X. and Zhu, X. (2009). Biofilm formation and food safety in food industries. *Trends in Food Science & Technology*, 20, 407-413.

Simoes, M., Simoes, L. C. and Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *Lwt-Food Science and Technology*, 43, 573-583.

Siroli, L., Patrignani, F., Serrazanetti, D. I., Vannini, L., Salvetti, E., Torriani, S., and Lanciotti, R. (2016). Use of a nisin-producing Lactococcus lactis strain, combined with natural antimicrobials, to improve the safety and shelf-life of minimally processed sliced apples. *Food Microbiologyogy*, 54, 11-19.

Skandamis, P. N., Stopforth, J. D., Yoon, Y., Kendall, P. A. and Sofos, J. N. (2007). Modeling the Effect of Storage Atmosphere on Growth–No Growth Interface of *Listeria monocytogenes* as a Function of Temperature, Sodium Lactate, Sodium Diacetate, and NaCl. *Journal of Food Protection*, 70, 2329-2338.

Soni, K. A., Desai, M., Oladunjoye, A., Skrobot, F. and Nannapaneni, R. (2012). Reduction of

Listeria monocytogenes in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. *International journal of Food Microbiologyogy*, 155, 82-88.

Soni, K. A., Nannapaneni, R., Schilling, M. W. and Jackson, V. (2010). Bactericidal activity of lauric arginate in milk and Queso Fresco cheese against *Listeria monocytogenes* cold growth. *J Dairy Sci*, 93, 4518-4525.

Speranza, B., Sinigaglia, M. and Corbo, M. R. (2009). Non starter lactic acid bacteria biofilms: a means to control the growth of *Listeria monocytogenes* in soft cheese. *Food Control*, 20, 1063-1067.

Srey, S., Jahid, I. K. and Ha, S.-D. (2013). Biofilm formation in food industries: A food safety concern. *Food Control*, 31, 572-585.

Stopforth, J., Samelis, J., Sofos, J., Kendall, P. and Smith, G. (2002). Biofilm formation by acid-adapted and nonadapted *Listeria monocytogenes* in fresh beef decontamination washings and its subsequent inactivation with sanitizers. *Journal of Food Protection*, 65, 1717-1727.

Stopforth, J. D., Yoon, Y., Barmpalia, I. M., Samelis, J., Skandamis, P. N. and Sofos, J. N. (2005). Reduction of *Listeria monocytogenes* populations during exposure to a simulated gastric fluid following storage of inoculated frankfurters formulated and treated with preservatives. *International journal of Food Microbiologyogy*, 99, 309-319.

Stubbings, W., Bostock, J., Ingham, E. and Chopra, I. (2005). Deletion of the multiple-drug efflux pump AcrAB in *Escherichia coli* prolongs the postantibiotic effect. *Antimicrob Agents Chemotherapy*, 49, 1206-1208.

Tadepalli, S., Bridges, D. F., Driver, R. and Wu, V. C. H. (2018). Effectiveness of different antimicrobial washes combined with freezing against *Escherichia coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* inoculated on blueberries. *Food Microbiology*, 74, 34-39.

Tajik, H., Naghili, H., Ghasemmahdi, H., Moradi, M. and Badali, A. (2015). Effects of Zataria multiflora boiss essential oil, ultraviolet radiation and their combination on *Listeria monocytogenes* biofilm in a simulated industrial model. *International Journal of Food Science and Technology*, 50, 2113-2119.

Tan, S. Y.-E., Chew, S. C., Tan, S. Y.-Y., Givskov, M. and Yang, L. (2014). Emerging frontiers in detection and control of bacterial biofilms. *Current opinion in biotechnology*, 26, 1-6.

Tango, C. N., Khan, I., Ngnitcho Kounkeu, P. F., Momna, R., Hussain, M. S. and Oh, D. H. (2017). Slightly acidic electrolyzed water combined with chemical and physical treatments to decontaminate bacteria on fresh fruits. *Food Microbiology*, 67, 97-105.

Tessema, G. T., Moretro, T., Kohler, A., Axelsson, L. and Naterstad, K. (2009). Complex

Phenotypic and Genotypic Responses of *Listeria monocytogenes* Strains Exposed to the Class IIa Bacteriocin Sakacin. *Applied Environmental Microbiology*, 75, 6973-6980.

Thomas, L. V. and Wimpenny, J. W. T. (1996). Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. *Applied Environmental Microbiology*, 62, 2006-2012.

Torlak, E. and Sert, D. (2013). Combined effect of benzalkonium chloride and ultrasound against *Listeria monocytogenes* biofilm on plastic surface. *Letters in applied microbiology*, 57, 220-226.

Traxler, M. F., Summers, S. M., Nguyen, H. T., Zacharia, V. M., Hightower, G. A., Smith, J. T., and Conway, T. (2008). The global, ppGpp‐mediated stringent response to amino acid starvation in *Escherichia coli*. *Molecular microbiology*, 68, 1128-1148.

Ukuku, D. O., Huang, L. and Sommers, C. (2015). Efficacy of Sanitizer Treatments on Survival and Growth Parameters of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on Fresh-Cut Pieces of Cantaloupe during Storage. *Journal of Food Protection*, 78, 1288-1295.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A. and Venkitanarayanan, K. (2013). Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. *Food Microbiologyogy*, 36, 79-89.

van der Veen, S. and Abee, T. (2010). Importance of SigB for *Listeria monocytogenes* Static and Continuous-Flow Biofilm Formation and Disinfectant Resistance. *Applied and Environmental Microbiology*, 76, 7854-7860.

van der Veen, S., van Schalkwijk, S., Molenaar, D., de Vos, W. M., Abee, T. and Wells-Bennik, M. H. J. (2010). The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. *Microbiology-Sgm*, 156, 374-384.

Van Houdt, R. and Michiels, C. W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of applied microbiology*, 109, 1117-1131.

Vanderlinde, E. M., Harrison, J. J., Muszynski, A., Carlson, R. W., Turner, R. J. and Yost, C. K. (2010). Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in Rhizobium leguminosarum bv. viciae 3841. *Fems Microbiology Ecology*, 71, 327-340.

Venter, H., Mowla, R., Ohene-Agyei, T. and Ma, S. (2015). RND-type drug e ffl ux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Frontier Microbiology*, 6, 377.

Verstraeten, N., Knapen, W. J., Fauvart, M. and Michiels, J. (2015). Membrane depolarization-triggered responsive diversification leads to antibiotic tolerance. *Microbial Cell*, 2, 299-301.

Waldron, D. (2015). Obg controls bacterial persistence. *Nature reviews microbiology*, 13, 457.

Walther, J., Bröcker, M. J., Wätzlich, D., Nimtz, M., Rohde, M., Jahn, D., & Moser, J. (2009). Protochlorophyllide: a new photosensitizer for the photodynamic inactivation of Gram-positive

and Gram-negative bacteria. *Fems Microbiology Letters*, 290, 156-163.

Wang, J., Ray, A. J., Hammons, S. R. and Oliver, H. F. (2015). Persistent and Transient *Listeria monocytogenes* Strains from Retail Deli Environments Vary in Their Ability to Adhere and Form Biofilms and Rarely Have inlA Premature Stop Codons. *Foodborne Pathogens and Disease*, 12, 151-158.

Wang, X. and Wood, T. K. (2011). Toxin-Antitoxin Systems Influence Biofilm and Persister Cell Formation and the General Stress Response. *Applied and Environmental Microbiology*, 77, 5577-5583.

Wederquist, H. J., Sofos, J. N. and Schmidt, G. R. (1994). *Listeria*-Monocytogenes Inhibition in Refrigerated Vacuum-Packaged Turkey Bologna by Chemical Additives. *Journal of Food Science*, 59, 498-&.

Wen, J., Karthikeyan, S., Hawkins, J., Anantheswaran, R. C. and Knabel, S. J. (2013). *Listeria monocytogenes* responds to cell density as it transitions to the long-term-survival phase. *International Journal of Food Microbiologyogy*, 165, 326-331.

Willenborg, J., Willms, D., Bertram, R., Goethe, R. and Valentin-Weigand, P. (2014). Characterization of multi-drug tolerant persister cells in *Streptococcus suis*. *BMC Microbiology*, 14, 120.

Winkelstroeter, L. K., Gomes, B. C., Thomaz, M. R., Souza, V. M. and De Martinis, E. C. (2011). *Lactobacillus* sakei 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. *Food Control*, 22, 1404-1407.

Winkelstroeter, L. K., Tulini, F. L. and De Martinis, E. C. P. (2015). Identification of the bacteriocin produced by cheese isolate *Lactobacillus paraplantarum* FT259 and its potential influence on *Listeria monocytogenes* biofilm formation. *Lwt-Food Science and Technology*, 64, 586-592.

Wood, T. K., Knabel, S. J. and Kwan, B. W. (2013). Bacterial persister cell formation and dormancy. *Applied and Environmental Microbiology*, 79, 7116-7121.

Wu, N., He, L., Cui, P., Wang, W., Yuan, Y., Liu, S., and Zhang, Y. (2015). Ranking of persister genes in the same *Escherichia coli* genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. *Frontiers in microbiology*, 6.

Wu, Y., Vulic, M., Keren, I. and Lewis, K. (2012). Role of oxidative stress in persister tolerance. *Antimicrob Agents Chemotherapy*, 56, 4922-4926.

Xu, T., Wang, X.-Y., Cui, P., Zhang, Y.-M., Zhang, W.-H. and Zhang, Y. (2017). The agr quorum sensing system represses persister formation through regulation of phenol soluble modulins in *Staphylococcus aureus*. *Frontiers in microbiology*, 8, 2189.

Xu, Y., Lee, M., Moeller, A., Song, S., Yoon, B. Y., Kim, H. M., and Ha, N. C. (2011). Funnel-like

hexameric assembly of the periplasmic adapter protein in the tripartite multidrug efflux pump in gram-negative bacteria. *Journal of Biological Chemistry*, 286, 17910-17920.

Yamaguchi, Y. and Inouye, M. (2011). Regulation of growth and death in *Escherichia coli* by toxin–antitoxin systems. *Nature reviews microbiology*, 9, 779.

You, Y., Hilpert, M. and Ward, M. J. (2013). Identification of Tet45, a tetracycline efflux pump, from a poultry-litter-exposed soil isolate and persistence of tet(45) in the soil. *J Antimicrob Chemother*, 68, 1962-1969.

Zhang, Y. (2014). Persisters, persistent infections and the Yin–Yang model. *Emerging microbes & infections*, 3, e3.

Zhu, X., Liu, W., Lametsch, R., Aarestrup, F., Shi, C., She Q., Shi, X., Knøchel, S. (2011). Phenotypic, Proteomic, and Genomic Characterization of a Putative ABC-Transporter Permease Involved in *Listeria monocytogenes* Biofilm Formation. *Foodborne pathogens and disease*, 8, 495-501.

Zhu, X., Long, F., Chen, Y., Knochel, S., She, Q. and Shi, X. (2008). A Putative ABC Transporter Is Involved in Negative Regulation of Biofilm Formation by *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 74, 7675-7683.

Chapter 3 Persister cell formation of *Listeria monocytogenes* in response to a natural antimicrobial, nisin

Partial data was published in *Food Control* 77 (2017) 243-250

Original publication available at http://dx.doi.org/10.1016/j.foodcont.2017.02.011

Shuyan Wu [a], Pak-Lam Yu [b], Steve Flint [a, *]

[a] *Massey Institute of Food Science and Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

[b] *School of Engineering & Advanced Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

Abstract

The persistence of bacteria can be defined by prolonged existence in an environment despite antimicrobial treatment. When this occurs with a food pathogen such as *L. monocytogenes* in a food manufacturing environment, this poses a risk to food safety. The persistence of cells exposed to antibiotics is well recorded however the persistence following treatment with natural antimicrobials like the bacteriocin nisin has not been determined. This study used two *L. monocytogenes* isolates, one from food and one from an animal origin to optimize methods for obtaining *L. monocytogenes* persisters following nisin treatment. Overnight cultures of *L. monocytogenes* were treated with nisin (up to 75µg/ml), and a biphasic killing pattern was observed for both strains. Persister cells became a population of cells showing tolerance to the high concentrations of nisin in the tests and proved to be non-resistant when regrown and re-exposed to nisin.

3.1 Introduction

L. monocytogenes is a Gram-positive mobile and rod-shaped bacterium, and it induces the life-threatening food borne disease listeriosis which predominantly affects immuno-compromised people, including elders, neonates and pregnant women(Swaminathan and Gerner-Smidt, 2007). As a recalcitrant pathogen, *L. monocytogenes* is capable of proliferating and contaminating food (Swaminathan and Gerner-Smidt, 2007). It survives sanitation in food processing and is therefore difficult to control (Carpentier and Cerf, 2011). Listeriosis outbreaks (*L. monocytogenes* infection) are associated with diverse foods, including fruits, vegetables, raw meat and poultry, seafood, and ready-to-eat (RTE) foods (Carpentier and Cerf, 2011). Food contamination by *L. monocytogenes* is most likely due to the persistence of *L. monocytogenes* despite antimicrobial treatments aimed at controlling this pathogen(Wang *et al.*, 2015).

The persistence of *L. monocytogenes* in a food environment is defined by its prolonged existence when *L. monocytogenes* are repeatedly detected in many food related environments and in processed food products (Wang *et al.*, 2015). Investigations on specific characteristics (e.g., serotypes, surface adhering ability, stress tolerance and virulence) have shown heterogeneity among persister cells of *L. monocytogenes*(Ferreira *et al.*, 2014).

Currently, persistent *L. monocytogenes* from food processing environments and foods, sampled over a long period are present in low cell numbers that are a food safety hazard. These low number cells are detected on enrichment in a laboratory (Holch *et al.*, 2013). The persistence studies of *L. monocytogenes* need to re-grow the persistent *L. monocytogenes* strains, however the physiological state of persisters is lost during this re-growth (Highmore *et al.*, 2018). The results in such studies does not reflect the original character of persister cells.

The problem of persister cells is not new with reports from nearly 70 years ago on the survival following prolonged exposure to penicillin that in an antibiotic free environment revert to a growing state and generate a new population that is as sensitive to antibiotic treatment as the parental strain(Bigger, 1944). Persisters are currently defined as the surviving population when a microbial culture is exposed either to an increasing concentration of bactericidal antibiotics or to

a fixed concentration over a long time (Kint *et al.*, 2012). Persisters are phenotypic variants of the wildtype and genetically identical to susceptible wild type bacteria, in contrast to resistant cells (Kint *et al.*, 2012). So, the persisters are not antibiotic resistant, and their phenotypic tolerance is defined as non-inherited antibiotic resistance(Levin and Rozen, 2006).

This study used two *L. monocytogenes* isolates, one from food and one from an animal origin to optimise methods to obtain *L. monocytogenes* persisters following nisin treatment.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Two *L. monocytogenes* strains were used in this study. One labelled A1, was obtained from a food manufacturing environment (AsureQuality Limited, New Zealand). This was identified as serotype O1/2 and deposited in the New Zealand reference culture bank as NZRM 4734 by the Environmental and Scientific Research Ltd. (ESR) Special Bacteriology and Culture Collection Laboratory. The actual food environment that provided the A1 strain from AsureQuality Limited is confidential and cannot be disclosed to a third party. The other strain, labelled M5 (originally named NCTC 7963), originated from a guinea pig mesenteric lymph node.

Both strains were confirmed as *L. monocytogenes* by 16S r DNA PCR, stored under -80˚C and revived in BHI (brain heart infusion) broth (Merck Millipore, German) at 30˚C 18 hrs and streaked on BHI agar plates to obtain pure colonies for use. Colonies were freshly picked for each experiment and the cultures were prepared in Tryptic Soy Broth (TSB) for subsequent experiments as TSB medium showed better reproducibility for nisin antimicrobial evaluation than BHI in the preliminary work.

The growth of two strains was investigated in a 96 well plate with each well containing 200µl of TSB medium inoculated with a single colony. The plate was incubated at 30˚C. The turbidity of the wells was measured using a microplate reader (Spectrostar, Bio-Tek, USA) at 595nm at given time points. The experiments were repeated in triplicate and averaged reading values were used to produce growth curves against measured time points (Supplementary figure 3.1).

3.2.2 Nisin stock preparation and the minimum inhibitory concentration (MIC) test

Nisaplin® containing 2.5% nisin was used in the study. All the antimicrobial treatments were reported using the actual concentrations of the active component, nisin. The nisin stock solution (750µg/ml) was prepared from Nisaplin® by dissolving in 0.2% bovine serum albumin

(BSA) and 0.01% acetic acid. For both strains (A1 and M5), the MIC of nisin to *L. monocytogenes* was determined by the protocol from Nguyen with some modifications (Nguyen *et al.*, 2008). 50µl fresh tryptic soy (TSB) broth (BD™, USA) was added to each well of a 96 well plate (Costar, USA) and 50µl of fresh overnight culture containing ~5 $\log_{10}$ cfu/ml cells was added to each well. After overnight incubation at 37˚C, the MIC was defined as the concentration of nisin in the last well in which culture growth did not occur by direct observation.

Efficacy of nisin in inhibiting the growth of *L. monocytogenes* (A1 and M5 strains) was further investigated using an overnight culture (5ml) mixed with 4ml fresh TSB and 1ml nisin of different concentrations in a glass bottle. The negative control consisted of 0.2% BSA and 0.01% acetic acid. The glass bottles were incubated at 30˚C for 24hrs, shaken at 150rpm. Experiments were done in triplicate. The bacteria in nisin-treated and control tests were sampled over the first four hours and at 24hrs to confirm the number of viable *L. monocytogenes* using spread plating (Hoben and Somasegaran, 1982).

3.2.3 Optimising a method to obtain *L. monocytogenes* persisters following nisin treatment

The A1 and M5 cultures were individually prepared from a single colony inoculated into 15ml TSB medium. A1 and M5 were individually inoculated in TSB at 30˚C for 24 hrs to obtain log phase cells and stationary phase cells (Supplementary figure 3.2). Log phase cultures (6~7$\log_{10}$ cfu/ml) were collected from 10hrs incubation at 30˚C, and concentrated by centrifugation up to 8 $\log_{10}$ cfu/ml of log phase culture for a subsequent nisin treatement. The 18hrs-old and 24hrs-old stationary phase cells were adjusted to approximately 8 $\log_{10}$ cfu/ml for the following nisin treatment as well.

For each strain dose-dependent killing test, 900ul of 8 $\log_{10}$ cfu/ml cells were treated with 100ul nisin solution or blank solution(0.2% BSA and 0.01% acetic acid) at final nisin concentrations of 0, 6, 12, 18, 24, 30, 40, 50, 60, 75 µg/ml and incubated for 30˚C for 24hrs shaken at 150rpm. The cell number in the blank control was counted before incubation (labelled

as time 0 in the figures) using a drop plating technique on BHI agar (Hoben and Somasegaran, 1982). All samples were centrifuged and re-suspended in 0.1% peptone water and then serially diluted, and the viable count was enumerated on BHI agar plates by 10µl drop plating. Nisin treatment experiments were performed in triplicate for each strain.

3.2.4 Identification of *L. monocytogenes* persisters from stationary phase cells by measuring the death kinetics curve

The death kinetics of *L. monocytogenes* following nisin treatment were estimated. A1 and M5 strains were individually grown in 9ml TSB at 30˚C for 18 hrs, and 1ml of nisin stock solution was then added into each 9ml of overnight culture to reach a final concentration of 75µg/ml nisin. A control group was included with 1ml of 0.2% BSA and 0.01% acetic acid solution. The nisin treatment done with shaking at 150rpm and samples were taken at 1, 4, 8 and 24hrs for cell enumeration with the viable count enumerated on BHI agar plates using the 10µl drop plating method. Death kinetic measurements were performed in triplicate for each strain.

3.2.5 Identification of the phenotypic susceptibility of persister isolates to nisin

The cells surviving the highest concentration (75µg/ml) of nisin were revived on the BHI agar plates and used for a repeat exposure to 75µg/ml nisin. The colonies from the survivors at 75µg/ml nisin were picked and grown in 10ml TSB at 30˚C for 18 hrs shaken at 150rpm. The culture of survivors was subjected again to 75µg/ml nisin (9ml overnight culture treated with 1ml nisin solution to reach the final concentration at 75µg/ml nisin) and sampled at 1, 4, 8 and 24hrs for cell enumeration. Blank group was added with 1ml of 0.2% BSA and 0.01% acetic acid solution. The re-exposure experiment was carried out with 6 persister isolates for each strain.

3.2.6 Statistical analysis

The mean and standard deviations of triplicate experiments in this study were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

3.3 Results

3.3.1 Efficacy of nisin on inhibiting the growth of two *L. monocytogenes* isolates

The MIC for nisin treatment of strains A1 and M5 was 1.5μg/ml and 3μg/ml respectively (Supplementary Figure S3.2), which indicated that nisin is an effective antimicrobial agent for inhibiting the two *L. monocytogenes* isolates. In the tests using three low concentrations of nisin treatment (under 1.5μg/ml, 3μg/ml and 6μg/ml) (Figure 3.1), the phenotypic responses of A1 and M5 strains were very similar, and nisin showed anti-*Listeria* activity from the beginning of incubation. All cultures produced a large drop in cell number after the first hour. The cell numbers dropped as the nisin concentration increased. For 1.5μg/ml and 3μg/ml nisin treatments, stationary phase cultures of both strains decreased by 3 and 5 $\log_{10}$cfu/ml respectively and the remaining cells were able to recover and grow during the 24hrs incubation.

The cell culture was regarded as a mixed community composed of non-persister cells and persister cells. Non-persister cells are commonly susceptible to stresses. In the above nisin treatment tests (Figure 3.1), it was assumed that a fair amount of non-persister cells exposed to 1.5μg/ml and 3μg/ml treatment remained viable and were able to grow again by utilizing the fresh medium within the incubation system. For 6μg/ml nisin treatments, the cell number dropped to 2 $\log_{10}$ cfu/ml during the first four of incubation, and only reached to between 4-5 $\log_{10}$ cfu/ml the next day. This indicates a greater non-persister population had been killed at 6μg/ml nisin. The population surviving 6μg/ml nisin treatments comprised of cells growing at a lower growth rate in 24 hrs incubation compared with cells treated at lower concentrations of nisin. The persister cells could become the predominant population with 6μg/ml nisin treatment. Since persister cells are a population of cells showing tolerance to high concentrations of antimicrobial agents, the 6 μg/ml nisin was used to obtain a persister cell population in the following tests.

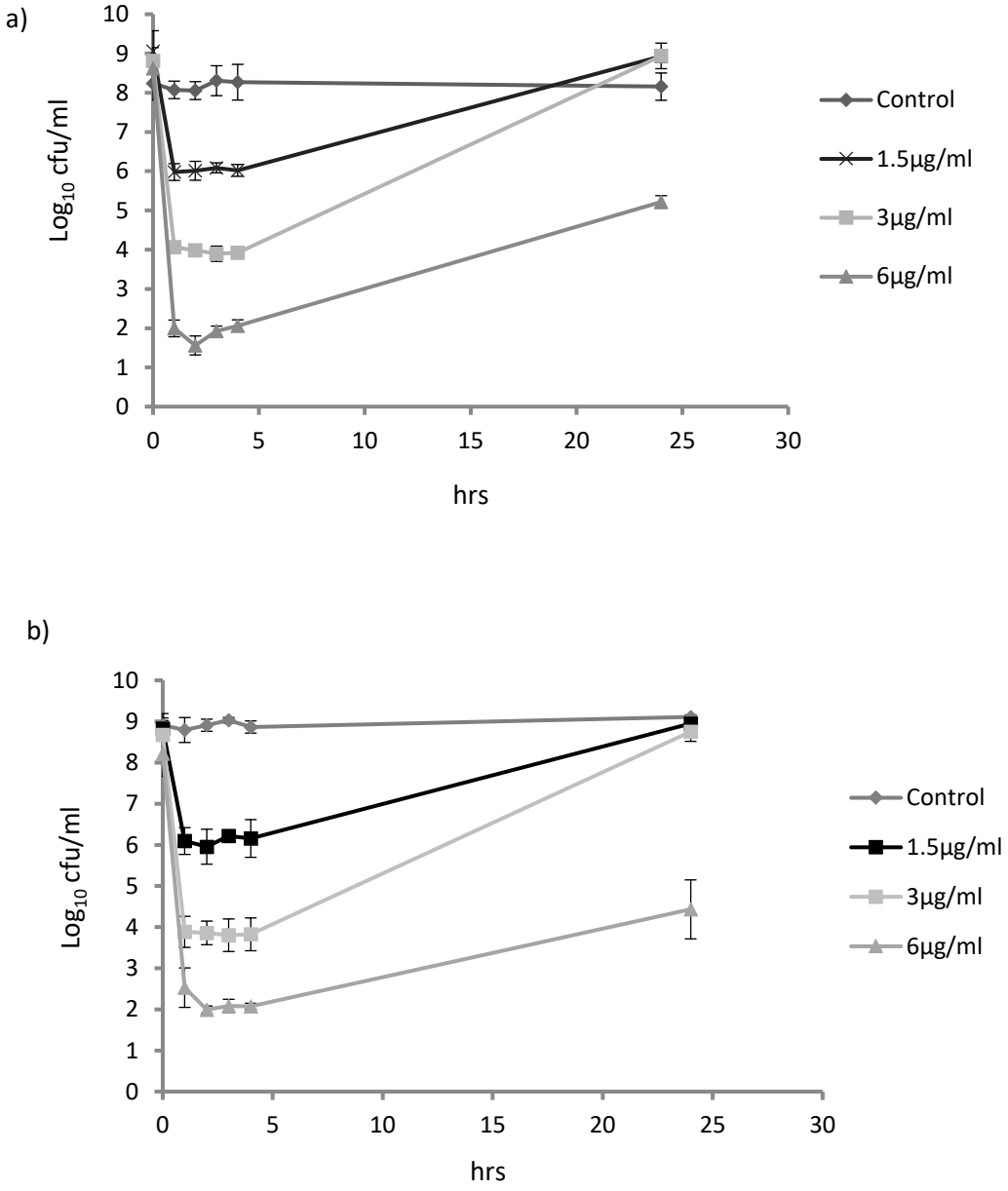


Figure 3. 1 The effect of low nisin (1.5 µg/ml, 3 µg/ml and 6 µg/ml) treatment on the survival and growth of A1 and M5 strains.

a) Overnight cultures of A1 treated with different concentrations of nisin over 24 hrs. b) Overnight cultures of M5 (reference strain) treated with different concentrations of nisin over 24 hrs. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

3.3.2 The persister cells of *L. monocytogenes* following treatment with different concentrations of nisin

Log phase cells of *L. monocytogenes* A1 and M5 strains were exposed to increasing concentrations of nisin (0-75µg/ml) at 30°C for 24 hrs (Figure 3.2). Log phase cells were susceptible to nisin treatment with survivors hard to detect at nisin concentrations >40 µg/ml for A1 and >24 µg/ml for M5. Nisin treatment was very effective in reducing viable cells of *L. monocytogenes.* However, active growing log phase cells used in this trial could not generate sufficient persister cells for further study. In a food manufacturing environment, where conditions are note ideal for growth, the population of *L. monocytogenes* is likely to be dominated by slow growing or cells in the stationary phase of a growth cycle.

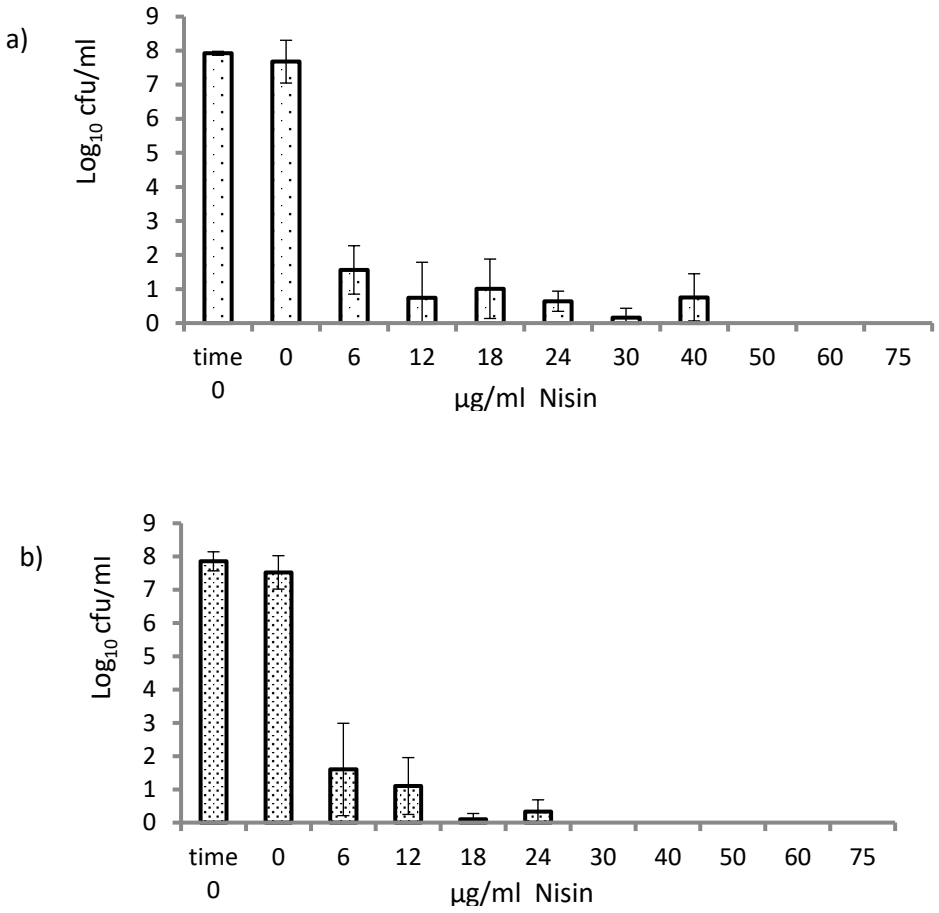


Figure 3. 2 Killing of log phase *L. monocytogenes* cells treated with nisin at a series of concentrations from 0-75µg/ml at 30°C for 24 hrs.

a) *L. monocytogenes* A1 b) *L. monocytogenes* M5. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

It has been suggested that the number of persisters surviving antibiotic treatment compared to the initial total population is influenced by cell physiology and environmental conditions such as the growth phase of the bacteria (Luidalepp *et al.*, 2011).

The stationary phase cells (from 18hrs incubation) of *L. monocytogenes* A1 and M5 strains were exposed to increasing concentrations of nisin (0-75µg/ml) at 30°C for 24 hrs. The number of cells of the A1 strain decreased as sharply at a nisin concentration of 12µg/ml (Figure 3.3 a) while the number of cells of the M5 strain decreased as sharply at a nisin concentration of 18µg/ml (Figure 3.3 b). The majority of the population (nearly 6-7 $\log_{10}$ cfu/ml) was killed rapidly, leaving a plateau of surviving cells < 3 $\log_{10}$cfu/ml. Biphasic killing patterns (Kint *et al.*, 2012), typical for populations with persister cells, were observed (Figure 3.3). The level of persisters produced in stationary phase cells exposed to lethal concentrations of nisin showed a similar killing pattern to that detected in other bacteria surviving antibiotic treatments (Balaban *et al.*, 2004) .

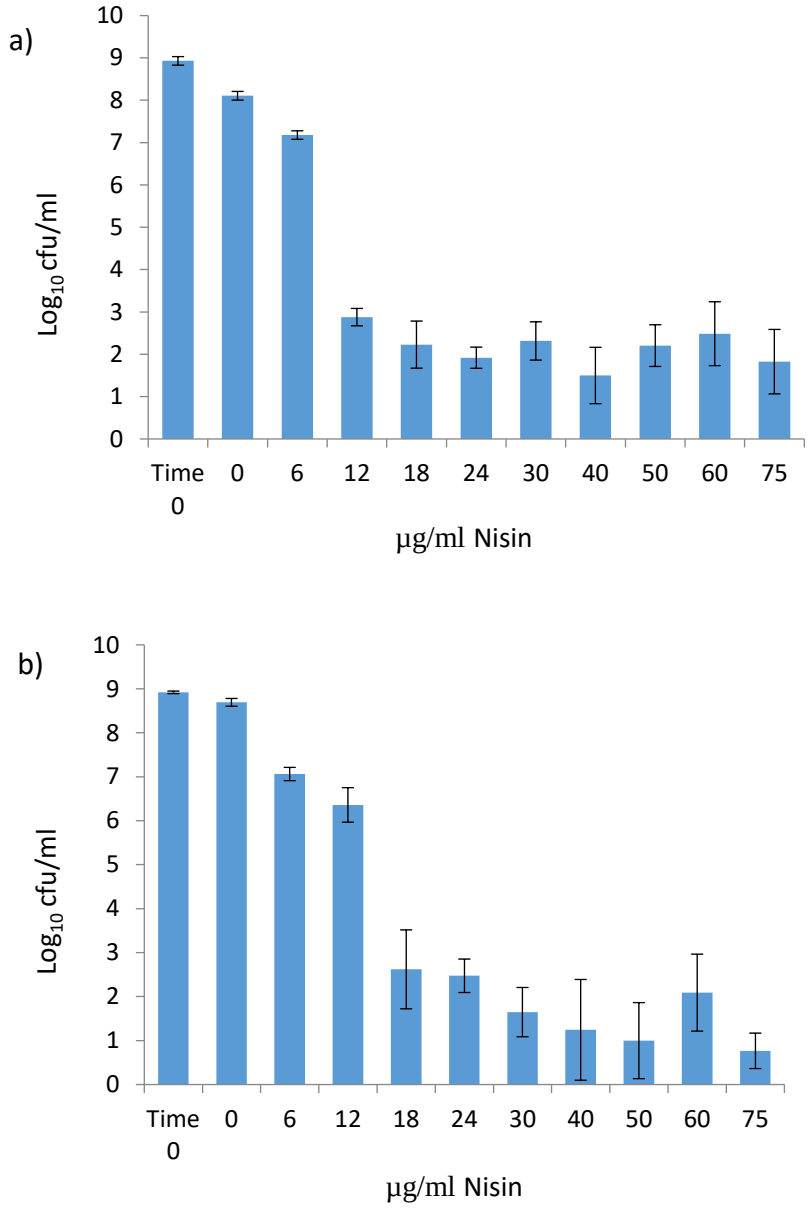


Figure 3. 3 Killing of stationary phase (18 h cultures) of *L. monocytogenes* treated with 0-75µg/ml nisin 30°C for 24 hrs

a) *L. monocytogenes* A1 b) *L. monocytogenes* M5. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

The level of persisters surviving antibiotic treatment is also influenced by cell physiology such as the length of stationary phase (Klapper *et al.*, 2007). The stationary phase cells (from 24hrs incubation) of *L. monocytogenes* A1 and M5 strains were exposed to increasing concentrations of nisin (0-75µg/ml) at 30˚C for 24 hrs. The stationary phase cells (from 24hrs incubation) of *L. monocytogenes* A1 and M5 strains show similar survival patterns in test results from 18hrs-old stationary phase cells when exposed to increasing concentrations of nisin (0-50µg/ml) (Figure 3.4). Persister cells were not detected at nisin concentrations > 50 µg/ml in 24hrs-old stationary phase cells of either strain, which indicates 18hrs-old stationary phase cells are better for isolating persister cells under high concentration of nisin treatment in the subsequent experiments.

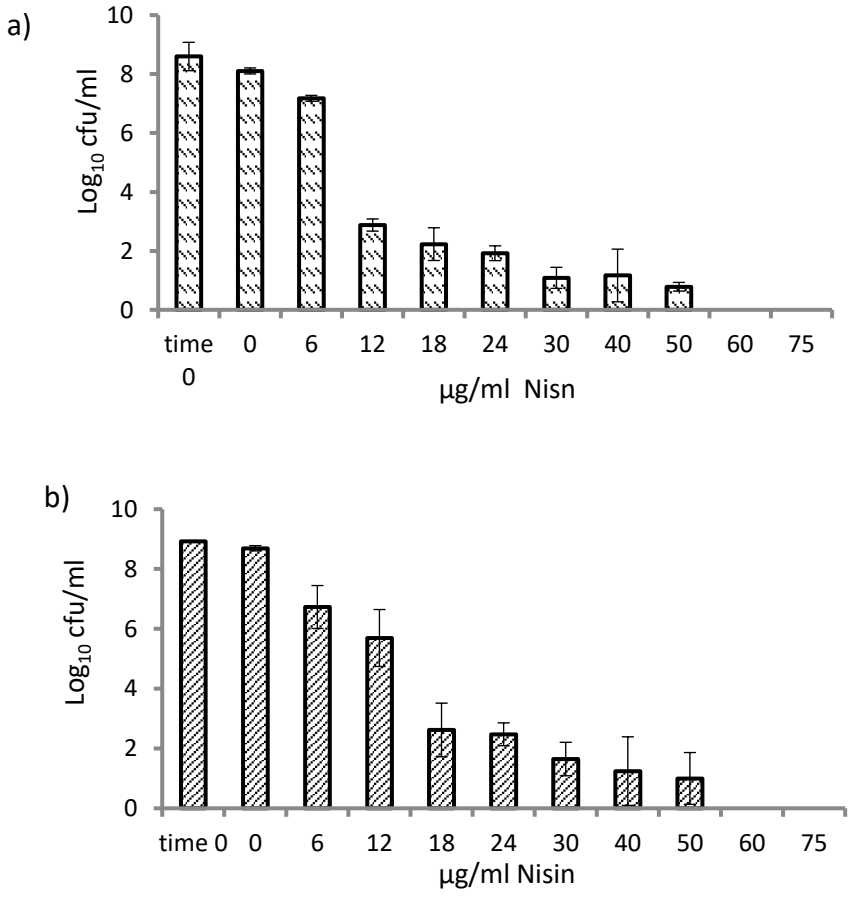


Figure 3. 4 Concentration- dependent killing of *L. monocytogenes* stationary phase cells (from 24-hrs overnight culture) treated with 0-75µg/ml nisin at 30°C for 24 hrs

a) *L. monocytogenes* A1 b) *L. monocytogenes* M5 The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

3.3.3 The death kinetics of *L. monocytogenes* exposed to nisin

L. monocytogenes overnight cultures were treated with 75μg/ml of nisin over 24 hrs, and a similar killing pattern was observed for both A1 and M5 strains (Figure 3.5). The killing curve against time showed that the bulk population (at least 6 $\log_{10}$ cfu/ml) was killed within the first hour, and the remaining population was killed at a much slower rate over the remaining 23 hrs. This indicates that a major part of the non-persister population had been killed rapidly at the beginning, and persister cells become a population of cells showing tolerance to the high concentrations of nisin in the 24 hrs tests.

Persisters are known as the surviving population tolerant to a lethal concentration of bactericidal antibiotics or to a fixed concentration over long time. In this study, the cells survived beyond 24hrs in this time dependent killing test, however, after 24 hrs, at 30hrs and 48hrs of treatment, no cells could be recovered in agar plates. This reflects cell injury or death or persister cells that have become viable but non culturable (Besnard *et al.*, 2000).

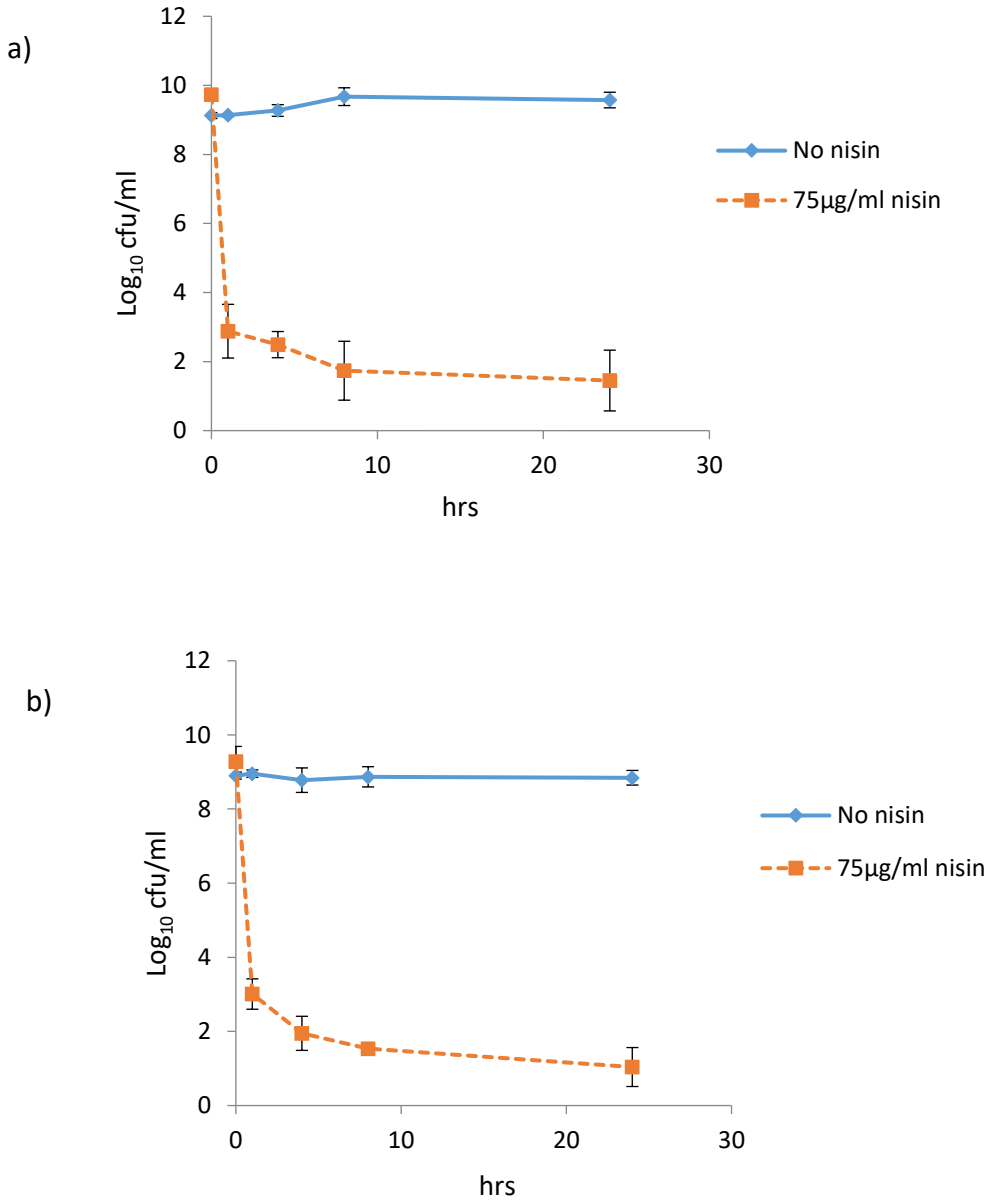


Figure 3. 5 Death kinetics of *L. monocytogenes* overnight cultures treated with 75µg/ml nisin 75µg/ml, 30°C for 24 hrs.

a) *L. monocytogenes* A1 b) *L. monocytogenes* M5 . The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

3.3.4 The population of persister cells as sensitive to nisin as the parental strain

In order to confirm that the surviving bacteria were not resistant, the persisters that survived for 24hrs at 75μg/ml nisin were revived on nisin-free BHI agar plates at 30°C after 24hrs incubation. The persister isolates were inoculated into fresh TSB medium and grown to stationary phase cultures, and then re-treated with 75μg/ml nisin at 30°C for 24 hrs. For persisters of A1 and M5, both remained as susceptible to nisin treatment as the parental strain (Figure 3.6a and 3.6b). This re-exposure test showed that the persisters had not acquired resistance and have the persisters phenotype that persisters can survive under high concentrations of antibiotics, and are able to switch back to a growing state and generate a new population that is as sensitive to antibiotic treatment as their parental strain(Cohen *et al.*, 2013).

The colonies from the survivors at 75μg/ml nisin were picked and grown for re-exposure tests. However, there were variations among the nisin sensitivity of persister isolates for both A1 and M5 strains. The re-exposure experiment was carried out with 6 persister isolates for each strain (AP1-6 and MP1-6). Some persister isolates (AP6, MP2, and MP6) showed much more sensitivity to 75μg/ml nisin than their mother strains while other isolates (AP1, AP2, AP5, and MP3) had a comparatively higher persistence than the mother strains (Figure 3.6a and 3.6b). The persister isolates with higher persistence showed a relatively reduced rate of killing during the first 10hrs' incubation. In general, by comparing the number of persisters (≤ 3 $\log_{10}$ cfu/ml) with the initial total population (~8 $\log_{10}$ cfu/ml), the consequence of persisters surviving at lethal nisin treatments remained as a sensitive bactericidal response in which the dominant bulk population were inactivated during the nisin treatment (Kint *et al.*, 2012).

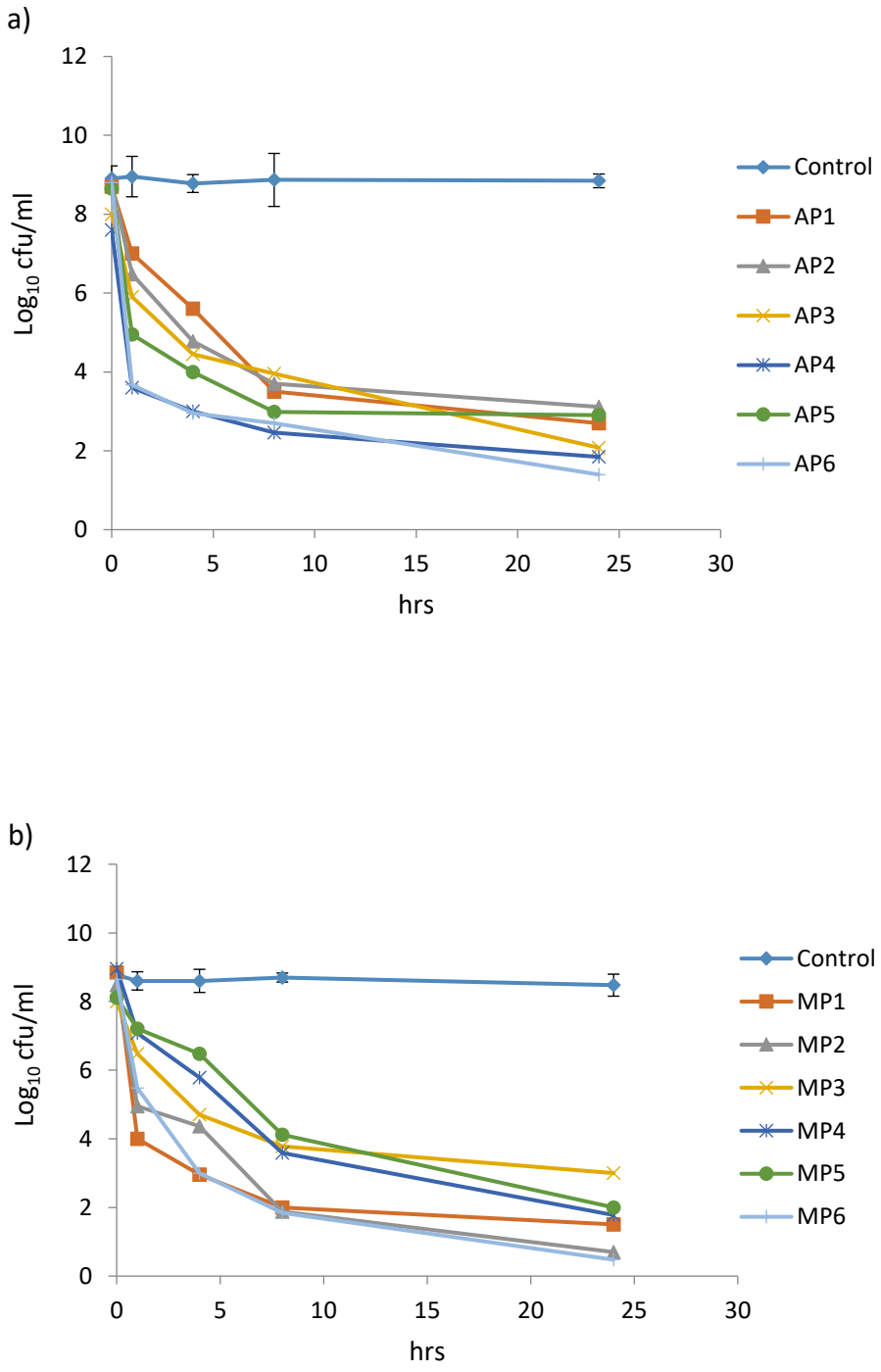


Figure 3. 6 Six persister strains of *L. monocytogenes* (AP1-AP6) that survived 75µg/ml nisin treatment for 24hrs were re-grown with in fresh TSB and then re-exposed to 75µg/ml nisin at 30°C for 24hrs

a) A1 . b) M5 The experiments were performed with three biological replicates, and error bars indicate the standard deviation.

3.4 Discussion

An investigation of a persister population of *L. monocytogenes* following treatment with nisin has not been reported. Nisin was added to a stationary phase population of *L. monocytogenes* to identify whether the nisin treatment results in persisters and whether the persister cells could be distinguished from the non-persister cells. A biphasic killing pattern is characteristic of a bacterial population to sensitive to an antibiotic treatment, represented by the first phase, with the second phase representing the persisters within a bacterial community (Kint *et al.*, 2012).

Persister cells reach maximum numbers in the stationary phase of growth and antimicrobial treatment at this point can isolate the persister cells from non-persister cells (Kint *et al.*, 2012). Knudsen *et al.* (Knudsen *et al.*, 2013)determined that *L. monocytogenes* formed a persister subpopulation in an *in vitro* model: a stationary phase culture of *L. monocytogenes* (8 $\log_{10}$ cfu/ml) developed a 3-4 $\log_{10}$ cfu/ml culture of survivors following 72 hrs treatment with100µg/ml norfloxacin a DNA synthesis inhibitor).

Nisin inactivates *L. monocytogenes* by disturbing cell membrane structure and cell wall synthesis. Nisin treatments on *L. monocytogenes* overnight culture cells showed high and rapid efficiency in killing the bulk population (non-persisters). By comparing with the outcomes from 24hrs-old stationary phase cells, 18hrs-old stationary phase cells produced a more stable level of persisters following the treatments with the serial concentrations of nisin (Figure 3.3 and 3.4).

Persister cells were counted using a 10µl drop plating technique in the study since it is a convenient method to count cells surviving the nisin treatments. In initial tests, the reliability of this method was confirmed by comparing with the traditional spread plating (Supplementary table S3.1). According to the results, a similar killing pattern can be obtained by both methods for *L. monocytogenes* A1 and M5 strains which were exposed to increasing concentrations of nisin (0-75µg/ml) at 30°C for 24 hrs. Basically, the two methods show consistent results.

The persister cells surviving <75µg/ml lethal nisin can be used for studies to understand the persistence formation of *L. monocytogenes*. The low level of the persister population is a

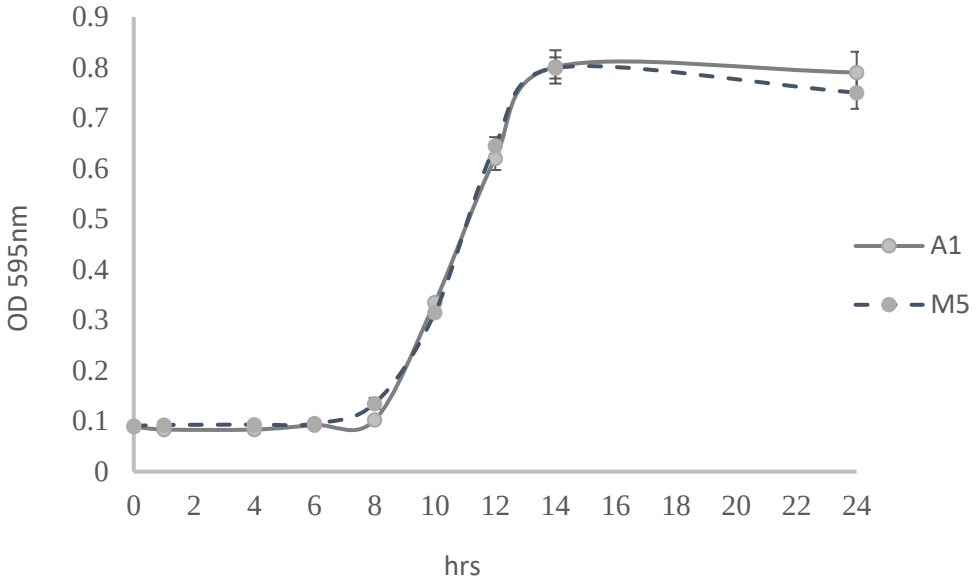
challenge for current research since the persister cells were embedded within a large number of dead non-persister cells and their debris after nisin treatment. The concentration of persister cells by normal centrifugation does not facilitate the isolation of sufficient numbers of the persister population. Another method is needed to provide sufficient persister cells for subsequent investigation.

In this study, populations of *L. monocytogenes* cells surviving nisin treatment agree with the definitions of persister populations (Balaban *et al.*, 2004, Kint *et al.*, 2012, Cohen *et al.*, 2013.) This is the first report of persister populations of *L. monocytogenes* following nisin treatment.

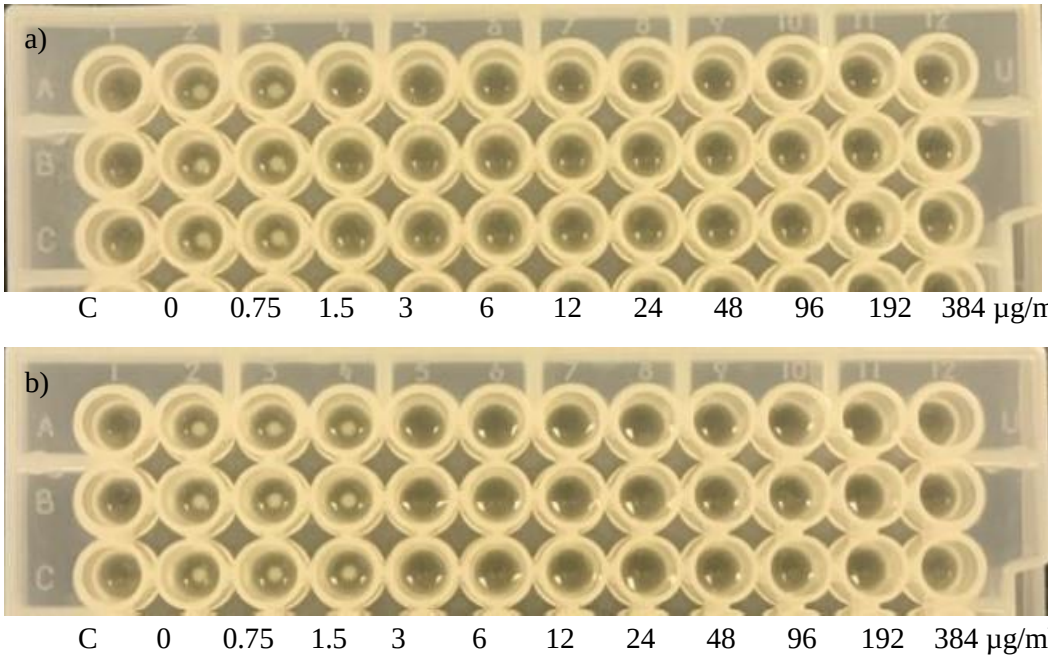
3.5 Acknowledgement

We thank AsureQuality Limited, New Zealand for the kind provision of *L. monocytogenes* strains used in this study. This work was supported by postgraduate research support funding of School of Food and Nutrition.

3.6 Supplementary information



Supplementary Figure S3. 1 Growth curves of *L. monocytogenes* strains (A1 and M5) in TSB at 30ºC. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.



Supplementary Figure S3. 2 MIC test with nisin treatments.

a) A1 b) M5 *N, negative control, adding TSB medium only without *L. monocytogenes* cells

Supplementary Table S3. 1 Spread plating and drop plating were used to show consistency of results on enumerating survived cells from nisin treatment with different concentrations.

Cell counting data	Overnight culture treated with nisin (Figure 3.3a and 3.3b)			
	A1 strain (Log_{10} cfu/ml)		M5 strain (Log_{10} cfu/ml)	
Nisin (µg/ml)	Spread plating	Drop plating	Spread plating	Drop plating
0(0h)	9.03 – 8.82	8.94 - 8.70	8.95 - 8.89	8.70 - 8.31
0(24h)	8.20 – 8.00	8.46 - 8.25	8.81 - 8.64	8.99 - 8.28
6	7.27 – 7.07	7.91 - 7.85	7.20 - 6.90	7.89 - 6.66
12	3.09 – 2.60	3.19 - 2.41	6.75 - 5.98	6.81 - 6.05
18	2.81 – 1.79	2.78 - 1.85	3.84 - 1.92	3.08 - 2.02
24	2.14 – 1.88	2.32 - 1.01	2.87 - 2.00	2.80 - 2.29
30	2.80 – 1.97	2.91 - 1.85	2.06 - 1.23	1.93 - 1.10
40	2.14 – 0.98	2.97 - 0.51	2.22 - 0.39	2.52 - 1.20
50	2.74 – 1.84	2.68 - 2.04	1.59 - 0.83	1.82 - 1.26
60	2.15 – 1.90	2.06 - 1.07	2.92 - 1.51	2.91 - 0.00
75	2.66 – 1.28	1.13 - 0.00	1.08 - 0.43	1.78 - 0.00

3.7 References

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science*, 305, 1622-1625.

Besnard, V., Federighi, M. and Cappelier, J. M. (2000). Evidence of Viable But Non-Culturable state in *Listeria monocytogenes* by direct viable count and CTC-DAPI double staining. *Food Microbiologyogy*, 17, 697-704.

Bigger, J. W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet*, 247, 497-500.

Carpentier, B. and Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiologyogy*, 145, 1-8.

Cohen, Nadia R., Lobritz, Michael A. and Collins, James J. (2013). Microbial Persistence and the Road to Drug Resistance. *Cell Host & Microbe*, 13, 632-642.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection*, 77, 150-170.

Highmore, C. J., Warner, J. C., Rothwell, S. D., Wilks, S. A. and Keevil, C. W. (2018). Viable-but-Nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson Induced by Chlorine Stress Remain Infectious. *Mbio*, 9, 12.

Hoben, H. and Somasegaran, P. (1982). Comparison of the pour, spread, and drop plate methods for enumeration of Rhizobium spp. in inoculants made from presterilized peat. *Applied and Environmental Microbiology*, 44, 1246-1247.

Holch, A., Webb, K., Lukjancenko, O., Ussery, D., Rosenthal, B. M. and Gram, L. (2013). Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Applied Environmental Microbiology*, 79, 2944-2951.

Kint, C. I., Verstraeten, N., Fauvart, M. and Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends in Microbiology*, 20, 577-585.

Klapper, I., Gilbert, P., Ayati, B. P., Dockery, J. and Stewart, P. S. (2007). Senescence can explain microbial persistence. *Microbiology*, 153, 3623-3630.

Knudsen, G. M., Ng, Y. and Gram, L. (2013). Survival of Bactericidal Antibiotic Treatment by a Persister Subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 79, 7390-7397.

Levin, B. R. and Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat Reviews Microbiology*, 4, 556-562.

Luidalepp, H., Jõers, A., Kaldalu, N. and Tenson, T. (2011). Age of Inoculum Strongly Influences Persister Frequency and Can Mask Effects of Mutations Implicated in Altered Persistence. *Journal of bacteriology*, 193, 3598-3605.

Nguyen, V. T., Gidley, M. J. and Dykes, G. A. (2008). Potential of a nisin-containing bacterial cellulose film to inhibit *Listeria monocytogenes* on processed meats. *Food Microbiologyogy*, 25, 471-478.

Swaminathan, B. and Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection*, 9, 1236-1243.

Wang, J., Ray, A. J., Hammons, S. R. and Oliver, H. F. (2015). Persistent and Transient *Listeria monocytogenes* Strains from Retail Deli Environments Vary in Their Ability to Adhere and Form Biofilms and Rarely Have inlA Premature Stop Codons. *Foodborne Pathogens and Disease*, 12, 151-158.

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Shuyan Wu

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Shuyan Wu, Pak-Lam Yu, Steve Flint (2017) Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. Food Control 77:243-250.

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: and / or
- Describe the contribution that the candidate has made to the Published Work: The candidate carried out laboratory work, data collection and analysis, and manuscript preparation guidance of direction and editorial help from the co-authors and supervisors.

01/12/2019

Candidate's Signature — Date

01/12/2019

Principal Supervisor's signature — Date

GRS Version 3– 16 September 2011

Chapter 4 Characterising the persister cells of *Listeria monocytogenes* following nisin treatment

Partial data was published in *Food Control* 77 (2017) 243-250

Original publication available at http://dx.doi.org/10.1016/j.foodcont.2017.02.011

Shuyan Wu [a], Pak-Lam Yu [b], Steve Flint [a, *]

[a] *Massey Institute of Food Science and Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

[b] *School of Engineering & Advanced Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

Abstract

Nisin is used for controlling food pathogens in a variety of food systems across a wide pH range. This study examined persistent subpopulations of *L. monocytogenes* following exposure to nisin treatment under different environmental conditions (such as rich/minimum medium, and different pH values) representative of different food systems. The pH and nutrient levels influenced the production of persister cells of *L. monocytogenes*. Stationary phase cells re-suspended in nutrient rich environment, Trypticase Soy broth (TSB) showed the best survival under lethal nisin treatment 75µg/ml for 24hrs incubation. The full TSB suspension was the preferred approach to obtain an adequate number of persister cells for the following analyses. XTT assays showed that a reduced level of metabolism maybe an important requirement for the *L. monocytogenes* persistence and the persister population which could survive at high concentration of nisin was probably related to a less negative cell surface charge. These data provide some understanding of the mechanism of persistence under lethal nisin exposure and provide some fundamental information for investigating gene expression of persister cells.

4.1 Introduction

Studies on persister cells have focussed on the clinical significance relating to antibiotic treatment (Dhar and McKinney, 2007). The consequence of persisters surviving antibiotic treatment is believed to result in chronic infections and the eventual appearance of antibiotic resistant strains (Kint *et al.*, 2012). Knudsen *et al.* determined that *L. monocytogenes* formed a persister subpopulation under antibiotic exposure (100µg/ml norfloxacin) in an *in-vitro* model (Knudsen *et al.*, 2013). The re-exposure of the re-grown cells to norfloxacin in refreshed medium showed that the survival was not caused by norfloxacin resistance and the re-grown population was still as sensitive to norfloxacin as the parental strain. However, other factors could affect this increase in persisters. Knudsen *et al.* added fermentable carbohydrates and gentamicin to norfloxacin persisters of *L. monocytogenes EGDe* and found that addition of 10 mM glucose to 10 µg/ml of gentamicin killed two more log units of the *L. monocytogenes* persisters compared to the *L. monocytogenes* persisters to which no carbohydrate was added during treatment with 10 µg/ml of gentamicin (Knudsen *et al.*, 2013).

Microbial persisters are a challenge to food safety. Persistence of microbial contamination can result in the long-term survival of a proportion of the population of a pathogen despite treatments with various antimicrobial agents (Carpentier and Cerf, 2011). *L. monocytogenes* persisters in the food environment usually result following treatments with different disinfectants and other non-toxic antimicrobials rather than the antibiotics used for clinical applications. For *L. monocytogenes* in food environments, sanitizers and natural antimicrobials which are derived from animals(Bodur and Cagri-Mehmetoglu, 2012), plants (Sandasi *et al.*, 2010) and microbes(Camargo *et al.*, 2016; Winkelstroeter *et al.*, 2015) have been reported to be effective in inhibiting and inactivating this pathogen. However, preservatives used in the food industry to control the growth of heat resistant bacteria cannot kill persister cells (Wood *et al.*, 2013). Nisin is a typical microbial derived antimicrobial peptide used commercially as a natural preservative(Guerra *et al.*, 2005), called Nisaplin® (Cabo *et al.*, 2001), used for the

control of Gram-positive pathogens, especially *L. monocytogenes*. Nisin can effectively control *L. monocytogenes* in foods by initiating pore formation in the cell membrane and hindering cell wall synthesis (Cotter *et al.*, 2005), and the application of nisin in controlling food pathogen proliferation is used in a variety of food systems, and is active over a wide pH range from acidic to alkali conditions (Gharsallaoui *et al.*, 2016). Nisin is often used for the preservation of neutral and slight alkali foods, including seafood(Budu-Amoako *et al.*, 1999), liquid egg (Delves-Broughton *et al.*, 2008), processed cheese (Maisnier-Patin *et al.*, 1992)and milk products (Gharsallaoui *et al.*, 2016; Maisnier-Patin *et al.*, 1995). Nisin, however, is not capable of eradicating persistent food pathogens. The persistence of *L. monocytogenes* in the food environment is reported annually but the persistence formation has not been well studied (Ferreira *et al.*, 2014). There is a lack of information about how persister cells of this bacterium form under different conditions within the food industry. Miyaue *et al.* discovered that persisters can maintain antibiotic-resistant phenotypes for up to 4 weeks at 37◦C in a fresh, nutrient-rich, antibiotic containing medium, suggesting that bacterial memory relating to the persister state is high (Miyaue *et al.*, 2018). In rich medium, antibiotic-forced dormancy of cell functions was speculated as the main mechanism resulting in this phenomenon(Miyaue *et al.*, 2018).

This study used *L. monocytogenes* isolates, one reference strain and one isolated from the food industry, to obtain a persistent subpopulation generated by exposure to a high concentration of the natural antimicrobial nisin. Clarifying the conditions leading to the emergence of persistence under non-antibiotic antimicrobial treatment (nisin) will help in understanding the persister population the risk to food safety and how intrinsic or extrinsic factors may affect the number of persisters in the population.

The mechanism of *L. monocytogenes* persister formation is difficult to investigate when persister isolates in nature may exist as non-culturable cells. Understanding the mechanism of persistence using an *in vitro* study will help in developing strategies to control this food safety risk. In order to study these persister cells, this following method obtained persistent subpopulations following exposure to a natural antimicrobial treatment under different conditions.

Survival of the persister subpopulations was used to evaluate the possible mechanisms resulting in the formation of persisters.

4.2 Materials and Methods

4.2.1 Evaluating factors impacting on the production of *L. monocytogenes* persisters

4.2.1.1 The effect of nutrient concentration on the production of *L. monocytogenes* persister cells

To examine the effect of nutrients on persister cell formation, trials were done on full strength TSB and 10% TSB solutions. For each strain (A1 and M5 strain), cultures were prepared from a single colony used to inoculate 25ml TSB medium and incubated at 30˚C for 18 hrs..

20ml of the 18hrs-old overnight cultures were centrifuged at 4500×g for 10mins to remove the culture supernatant, and then washed three times with 0.1% peptone water. Half of the washed cells were collected by centrifugation and re-suspended in 10ml fresh TSB and another 10ml cells was re-suspended in diluted TSB (1:9 ratio of dilution with distilled water). Every 10ml of re-suspended cells was used for an immediate dose-dependent killing test. To do this, 900ul of re-suspended culture was treated with 100ul nisin solution at the desired concentration (0, 6, 12, 18, 24, 30, 40, 50, 60, 75µg/ml) or a blank solution (0.2% BSA with 0.01% acetic acid buffer) and incubated at 30˚C for 24 hrs shaking at 150rpm. The test samples were centrifuged and re-suspended in 0.1% peptone water and then serially diluted, and the viable count was enumerated on BHI agar plate by drop plating (Hoben and Somasegaran, 1982). Experiments were performed in triplicate for each strain.

The death kinetics of TSB re-suspended *L. monocytogenes* following 75µg/ml nisin treatment were estimated (Supplementary Figure S4.1). 9ml of 18hrs old stationary phase A1 and M5 strains were individually re-suspended into the same volume of TSB medium, and 1ml of nisin stock solution/blank solution was then added into each 9ml of re-suspended culture to reach a final concentration of 75µg/ml nisin. The nisin treatment was done with shaking at 150rpm and

samples were taken at 1, 4, 8, 12 and 24hrs for cell enumeration with the viable count enumerated on BHI agar plates using the 10µl drop plating method. Death kinetic measurements were performed in triplicate for each strain.

4.2.1.2 The effect of pH/nutrient composition on the production of *L. monocytogenes* persister cells

4.2.1.2.1 pH measurements

In order to determine the effect of pH on the development of persister cells, three experimental groups were set up: first, overnight culture cells were directly treated with nisin (see methods 3.2.4 in chapter 3) ; second, overnight culture cells were washed and re-suspended in fresh TSB medium then treated with nisin; and third, overnight culture cells were washed and re-suspended in fresh 10% TSB medium followed by nisin treatment; all the nisin treatments took place at 30°C for 24 hrs shaking at 150rpm. The incubated cultures were collected before (time 0) and after (time 24) the nisin treatment by centrifugation and 0.22µm-filtration for measuring pH using a SevenCompact pH meter (Mettler Toledo, Greifensee, Switzerland).

4.2.1.2.2 The effect of TSB under varied pH on the production of *L. monocytogenes* persister cells

5ml fresh TSB medium (pH ~7.3) was adjusted to various pH values (~5.2 and ~6.3) with 1N HCL solution and adjusted to pH 8.1 with 1M NaOH. Washed 18hrs-stationary phase cells were re-suspended in the same volume of fresh TSB at different pH values, to which 75µg/ml nisin was subsequently added. Control preparations were prepared with0.2% BSA with 0.01% acetic acid added instead of nisin. Since the nisin solvent buffer is acidic (pH ~4.4), re-suspended cells in fresh TSB with varied pHs were added to nisin or blank solutions reaching final pH values of 5.0, 6.0, 7.0, and 8.0 respectively at the beginning of incubation as measured by a

SevenCompact pH meter (Mettler Toledo, Greifensee, Switzerland). 1ml of re-suspended cultures in the nisin or blank solutions were incubated at 30˚C for 24 hrs shaking at 150rpm.

The cell number in the blank control was always counted before the treatment by drop plating (labelled as time 0)(Hoben and Somasegaran, 1982). At the end of incubation, the test samples were centrifuged and re-suspended in 0.1% peptone water and then serially diluted, and the viable count was enumerated on BHI agar plate by drop plating. Experiments were performed in triplicate for each strain.

4.2.1.2.3 The effect of minimal nutrients under varied pH on the production of *L. monocytogenes* persister cells

Modified Welshimer's broth (MWB) is a chemically defined minimal medium (Premaratne *et al.*, 1991) used in this study to evaluate the effect of nutrients on the production of *L. monocytogenes* persister cells. For each strain (A1 and M5 strain), cultures were prepared from a single colony used to inoculate 25ml TSB medium and incubated at 30˚C for 18 hrs. The cells from 18 h cultures were washed and re-suspended in fresh full strength MWB medium followed by nisin treatment; all the nisin treatments took place at 30˚C for 24 hrs shaken at 150rpm.

5ml fresh MWB medium (pH ~7.23) was adjusted pH values of ~5.1, ~6.2 and ~7.1 with 1N HCL solution and adjusted to pH 8.1 with 1M NaOH. Washed 18hrs-stationary phase culture cells were re-suspended into the same volume of fresh MWB at various pH values, with 75µg/ml nisin or control solution (0.2% BSA with 0.01% acetic acid buffer) added. Prior to adding the cells, the pH of the media after adding nisin or buffer was determined using a SevenCompact pH meter (Mettler Toledo, Greifensee, Switzerland) and the final pH adjusted to 5.0, 6.0, 7.0 and 8.0 at the beginning of incubation. 1ml of re-suspended cultures with nisin treatment or with a control solution were incubated for at 30˚C for 24hrs shaken at 150rpm.

The cell number in the control was always counted before the treatment by drop plating (labelled as time 0)(Hoben and Somasegaran, 1982). At the end of the treatment time, the test samples were centrifuged and re-suspended in 0.1% peptone water and then serially diluted, and the viable count was enumerated on BHI agar plate by drop plating and spread plating. Experiments were performed in triplicate for each strain.

4.2.2 Evaluating the metabolic level of *L. monocytogenes* persister cells

The XTT assay was used to compare the metabolic activity of *L. monocytogenes* overnight culture cells with persister cells following nisin treatment (Koban *et al.*, 2012). To obtain overnight culture cells, A1 and M5 colonies were individually grown in 9ml TSB at 30°C for 18 hrs, and serial dilutions of cells prepared in 0.1% peptone water to give approximately 8 to 4 $\log_{10}$ cfu/ml.

For obtaining *L. monocytogenes* persister cells, overnight culture cells were re-suspend in fresh TSB and each 900µl re-suspended cells was given an immediate nisin treatment at final concentration of 75µg/ml for 24hrs at 30°C, and the nisin test was replicated in multiple 2.0ml Eppendorf tubes. After 24hrs incubation, cells were centrifuged and re-suspended in 0.1% peptone water to a cell density >6 $\log_{10}$ cfu/ml. Serial dilutions of overnight cells and persister cells were centrifuged to remove the supernatant, and each cell pellet was mixed with 200ul XTT (1mg/ml sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) containing electron coupling agents PMS (25nM phenazine methosulfate), and incubated in the dark at 37°C for 3hrs. Active cells reduce XTT to a highly colored formazan dye, and the optical density was read at 450nm by a microtiter plate reader (Bio-tek Inc.) with a reference wavelength of 690nm for correction. The obtained values were blank corrected and experiments were performed in triplicate for each strain.

4.2.3 Growth recovery of *L. monocytogenes* persister cells in a nisin-free environment

The BacTrac™ 4000 microorganism growth analyser was used to measure cell growth of *L. monocytogenes* persister cells in a nisin-free TSB medium. This BacTrac™ 4000 measuring system enables the detection of cell growth in real-time by using two simultaneous impedance detection systems - M-value and E-value. The M-value represents the change in the impedance of the growth medium and is expressed as the percentage decrease from the initial value; The E-value represents the same for the electrode surface (Wang *et al.*, 2016). In order to assess if there is growth recovery of *L. monocytogenes* persister cells, serial 10-fold dilutions of overnight cultures without nisin treatment were firstly measured to determine the time for impedance change into the exponential phase of cell growth (E=15) to obtain a standard growth curve for each strain (Supplementary Figure 4.2 and 4.3).

The overnight cultures of each strain in TSB at 30 °C were serially diluted in 0.1% peptone water (with the dilution factors from 10^{-1} to 10^{-6}). For each dilution, 20 µl of bacteria was inoculated into each vial of the BacTrac™ 4000 containing 10 ml of TSB. Measurements of cell growth at 30 °C for 24 hrs from the inocula were performed in three independent tests for both strains. The impedance changes were measured and recorded every 20mins. Each dilution of culture was enumerated on BHI agar by the 10µl drop plating method and incubated at 30 °C for 24 hrs. The standard curve for impedance change with time against the plate count was calculated by using the Microsoft Excel programme. The theoretical growth (expected impedance change time) for a known population of *L. monocytogenes* persister cells can be determined by using the calibration equation.

L. monocytogenes persister cells were collected as mentioned previously (Method 4.2.2) and 20 µl of persisters were inoculated into each vial of the BacTrac™ 4000 containing 10 ml of TSB. Three independent persister samples were tested by the BacTrac™ 4000 and each persister sample was run in triplicate. The actual growth time (reach impedance change E=15) can be obtained and compared with the theoretical growth time of *L. monocytogenes* persister cells according to the calibration.

4.2.4 Estimating cell surface charge of *L. monocytogenes* persister cells

The surface charge of the A1 and M5 strains was expressed as the Zeta potential in a Malvern Zeta Sizer IV system (Malvern Instruments Ltd, UK). For each strain, the overnight cultures were centrifuged at 4500 x g for 10min to remove the culture supernatant, and cell pellets were washed and re-suspended into fresh TSB. Re-suspended cells were treatment with nisin 75ug/ml for 24hrs as mentioned previously. Persister cells of each strain were recovered by centrifugation from replicates of 1ml nisin treatments and added to a 5mM NaCl solution. Cells were concentrated to 6 $\log_{10}$ cfu/ml in pH solutions (pH 2, 3, 4, 5, 6, 7, 8 solutions prepared by combining 0.01M citric acid and 0.02M Na_2HPO4) and added to the Zeta Sizer cuvettes for testing. In order to compare results with non persister populations, an overnight culture of each strain without adding nisin was washed in 5mM NaCl solution and then diluted to 6 $\log_{10}$ cfu/ml in the pH solutions as described and tested in cuvettes. Measurements were performed in triplicate.

4.2.5 Statistical analysis

The mean and standard deviation of triplicate experiments in this study were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

4.3 Results

4.3.1 The effect of nutrient concentration on the production of *L. monocytogenes* persister cells

Persister cells become highly tolerant to antibiotics and it is believed that the persistence of cells is an active process impacted by nutrient availability (Nguyen *et al.*, 2011). Persisters adapt to survive under high concentrations of antimicrobial agents by down-regulating biosynthetic pathways while the cells show a dormant slow or no-growth phenotype (Wood *et al.*, 2013). The present study investigated how nutrient levels affected *L. monocytogenes* persister production. Stationary phase *L. monocytogenes* cells were re-suspended in fresh or diluted TSB followed by an immediate nisin treatment (Figure 4.1). The re-suspension in full TSB showed greater survival than in the dilute TSB. The persister cells of the A1 strain in full TSB were up to 4.71 $\log_{10}$ cfu/ml at 75µg/ml nisin while in diluted TSB group the persister number was around 2.56 $\log_{10}$ cfu/ml at 75µg/ml nisin(Figure 4.1a). The persisters of the M5 strain were enumerated between 3.82 $\log_{10}$ cfu/ml at 75µg/ml nisin in full TSB while in diluted TSB the persister numbers were <1 $\log_{10}$ cfu/ml from 50µg/ml (Figure 3b). Generally, diluted TSB groups had relatively lower persistence with the immediate nisin treatments in this study, and the re-suspended cells in full TSB group produced a high number of persister cells which were viable under high concentration of nisin. In 75µg/ml nisin treatment on full TSB re-suspended groups, biphasic killing patterns were detected as well during 24hrs of incubation (Supplementary Figure 4.1). The fresh medium in the incubation could not induce survived cells back to active growing state, and the results indicated that persisters from nisin treatments of the re-suspended cells retained a non-growing phenotype in the presence of fresh medium. The very low numbers of persister cells in previous nisin treatment of overnight cultures (Chapter 3 Figure 3.3) is a challenge to obtain sufficient persister cells for analysis. Other studies have used centrifugation to concentrate enough cells for analysis. However, concentrated cell pellets in this present study produced a large portion of dead cells and debris (Chapter 3 Figure 3.3). Using persister cells prepared in this chapter following re-suspension of persister cells in full strength TSB and re-exposure to nisin

appears to be a useful approach to obtain an adequate number of persister cells for subsequent analysis.

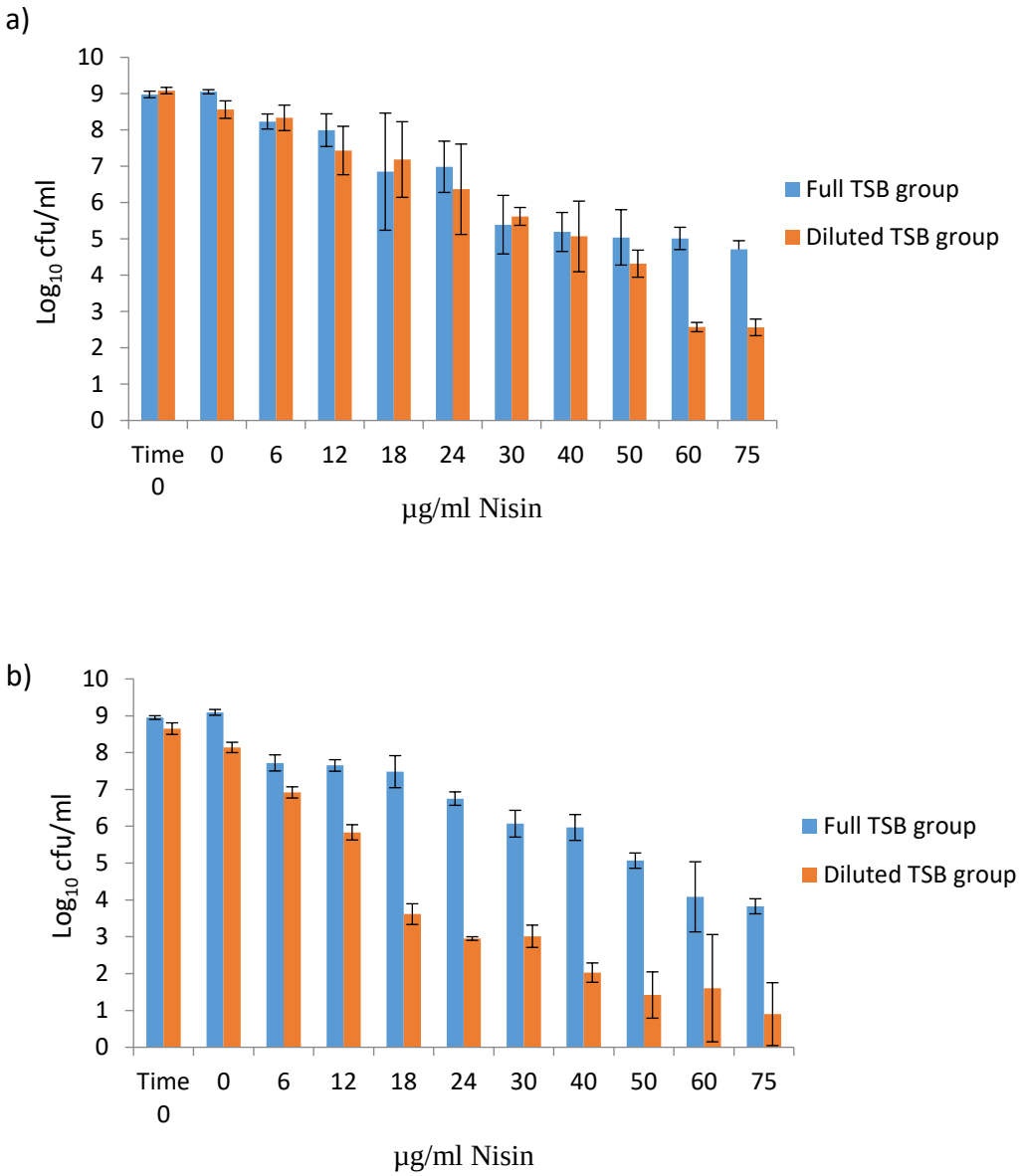


Figure 4. 1 Dose-dependent killing of re-suspended cells of *L. monocytogenes*.

a) The blue bars represent an overnight culture of the A1 strain re-suspended in TSB followed by an immediate nisin treatment at concentrations of 0-75µg/ml at 30˚ C for 24 hrs. The orange bars represent an overnight culture of the A1 strain re-suspended in diluted TSB (1:9 ratio) followed by an immediate nisin treatment at a concentration of 0-75µg/ml at 30˚C for 24 hrs. b) The blue bars represent an overnight culture of the M5 strain re-suspended in TSB followed by an immediate nisin treatment at concentrations of 0-75µg/ml at 30˚ C for 24 hrs. The orange bars represent an overnight culture of the M5 strain re-suspended in diluted TSB (1:9 ratio) followed by an immediate nisin treatment at a concentration of 0-75µg/ml at 30˚C for 24 hrs. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

4.3.2 Impact of pH on the production of *L. monocytogenes* persister cells under lethal nisin

The pH of the liquid phase of nisin treated cultures showed similar changes for both strains : first, 18hrs-old stationary phase cells were treated with direct nisin treatment for 24 hrs in Tables 4.1 and 4.2. The spent medium of the stationary phase cells was very acidic pH near at 5.1 at the beginning of nisin treament, slightly lowering to around 5.0 after 24hrs incubation; second, cells re-suspended in full TSB containing lethal nisin showed neutral pH at 7.1 at the start of incubation, with the pH dropping to 7.0 at the end of incubation; third, cells re-suspended in diluted TSB lethal nisin showed simlarly neutral pH at 7.2 at the start of inclubation and a pH drop to 6.99 at the end of incubation. The number of persister cells of A1 strain were enumerated as ~1.9 $\log_{10}$ cfu/ml , ~4.4 $\log_{10}$ cfu/ml and ~2.95 $\log_{10}$ cfu/ml respectively in the three groups while persister cells of M5 strain were enumerated as ~0.8 $\log_{10}$ cfu/ml , ~3.9 $\log_{10}$ cfu/ml and ~1.55 $\log_{10}$ cfu/ml respectively in the three groups. In the re-suspended cells with neutral pH , the full strength TSB re-suspended group generated a higher number of persister cells, which implies that the strength of nutrients could be involved in persister formation under lethal nisin stress. It was suspected that nisin performed better as an antimicrobial in the acidic environment of the spent medium. Studies have reported that the efficacy of nisin and other bacteriocins increases in acidic pH (Thomas and Wimpenny, 1996). Nisin shows antimicrobial activity against a wide range of Gram positive bacteria including *L.monocytogenes* in a pH-dependent fashion (Delves-Broughton *et al.,* 1996). To prove it, TSB medium with varied pH values were investigated to determine the effect on persister formation under 75μg/ml nisin treatment.

For the A1 strain, the nisin treatment of re-suspended cells in full TSB at pH of 5, 6, 7 and 8 produced 3.24±0.15, 3.69±0.26 4.14±1.15 and 4.79±0.51 $\log_{10}$ cfu/ml respectively of persister cells (Figure 4.2a). For the M5 strain, the nisin treatment on re-suspended cells in full TSB at pH of 5, 6, 7 and 8 produced <10 cfu/ml, 2.75±0.30, 3.82±0.78, and 4.66±0.29 $\log_{10}$ cfu/ml of persister cells respectively (Figure 4.2b). Note that the control cell numbers dropped from8.37±0.0.19 to 6.57±0.81 $\log_{10}$ cfu/ml over 24hrs at pH 5 (Figure 4.2b).

The results demonstrated that the pH value of resuspension TSB medium had an impact on persister formation under lethal nisin exposure. The level of persister cells increased from acidic to alkaline groups. The acidic environment facilitated the antimicrobial effect of nisin, and the number of persister cells reduced in acidic TSB compared with the persister level in neutral and alkaline TSB. The M5 strain was more sensitive to pH than the A1 strain as the control cells showed a loss of approximately 2 $\log_{10}$ cfu/ml when re-suspended in TSB at pH 5 with <10 cfu/ml persister cells following nisin treatment. However, approximately 3 $\log_{10}$ cfu/ml persister cells of A1strain were obtained following nisin treatment in TSB at pH 5 and 2-3 $\log_{10}$ cfu/ml of persister cells were produced following nisin treatment in TSB at pH 6 for both A1 and M5 strains. Apart from the impact of pH, nutrient content within TSB medium could play a role in supporting the formation of persister cells.

Table 4. 1 pH value of liquid environments from different nisin treatment groups for A1 strain (mean of triplicate readings +/- standard deiation)

Liquid	At beginning of nisin treatment (time 0)		At end of nisin treatment (time 24)	
phases	pH	Cell number (Log_{10} cfu/ml)	pH	Persister number (Log_{10} cfu/ml)
1*	**5.15±0.199**	**8.08±0.173**	**5.10±0.121**	**1.90±0.225**
2*	**7.18±0.225**	**8.30±0.604**	**7.05±0.158**	**4.42±0.431**
3*	**7.25±0.025**	**8.22±0.146**	**6.99±0.193**	**2.95±0.38**

*1, spend medium from nisin test on 18hrs-old stationary phase cells; 2, full TSB in which cells were re-suspended followed nisin treatment; 3, 10-fold diluted TSB in which cells were re-suspended followed nisin treatment.

Table 4. 2 pH value of liquid environments from different nisin treatment groups for M5 strain

Liquid	At beginning of nisin treatment (time 0)		At end of nisin treatment (time 24)	
phases	pH	Cell Number (Log_{10} cfu/ml)	pH	Persister Number (Log_{10} cfu/ml)
1*	**5.08±0.129**	**8.48±0.511**	**4.99±0.203**	**0.83±1.04**
2*	**7.07±0.104**	**8.26±0.133**	**6.98±0.093**	**3.91±0.253**
3*	**7.19±0.033**	**8.126±0.275**	**6.95±0.100**	**1.55±0.386**

*1, spend medium from nisin test on 18hrs-old stationary phase cells; 2, full TSB in which cells were re-suspended followed nisin treatment; 3, 10-fold diluted TSB in which cells were re-suspended followed nisin treatment.

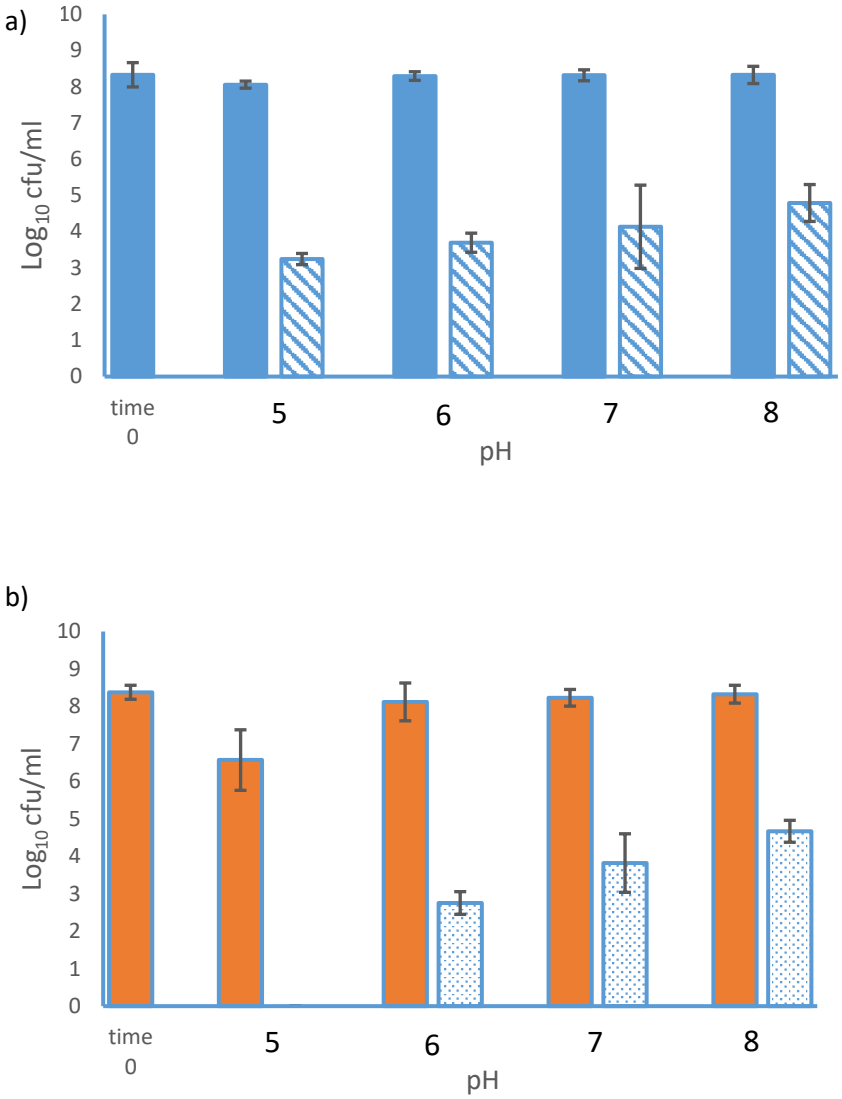


Figure 4. 2 The effect of nisin on cells re-suspended in TSB at different pH values (pH 5-8) on the production of persister cells.

a) Stationary phase cells of the A1 strain were re-suspended in TSB at different pH values from 5 to 8 and exposed to 75µg/ml nisin at 30ºC for 24 hrs shaken at 150rpm. Bars with pattern ■ represents control group in which cells were not treated with nisin; Bars with pattern ▨ represents persister cells suviving from 75µg/ml nisin treatment. b) Stationary phase cells of M5 strain were re-suspended into TSB at different pH values from 5 to 8 and applied with 75µg/ml nisin at 30ºC for 24 hrs under 150rpm shaking condition. Bars with pattern ■ represents control group in which cells were treated with blank buffer; Bars with pattern ▨ represents persister cells suviving from 75µg/ml nisin treatments. Error bars = Standard deviation.

4.3.3 Impact of nutrient composition on the production of *L. monocytogenes* persister cells under lethal nisin

For the A1 strain, MWB at pH of 5 resulted in some loss in viable cells even without nisin treatment (Figure 4.3a). This presumably reflects the stress on the cells in this acidic environment in a minimal nutrient environment and is in contrast to the high survival seen for this strain in the richer TSB medium (Figure 4.2a). No detectable persister cells (<10 cfu/ml) were seen for this strain at this pH when exposed to nisin, in contrast to the same strain in TSB where 3.24±0.15 $\log_{10}$ cfu/ml persister cells were produced (Figure 4.2a). At pH of 6, 7 and 8, persister populations were 1.69±1.49, 2.88±0.63 and 4.51±0.20 $\log_{10}$ cfu/ml respectively in MWB. There was a significant difference (p<0.05) in the number of persister cells between cells resuspended in MWB and TSB at pH 5, 6, and 7. In summary, under minimal nutrient conditions such as MWB, fewer persister cells are produced compared with the rich TSB medium. This supports the earlier findings that the number of persister cells was affected by the nutrient strength in full strength and 10% TSB.

For the M5 strain, full MWB at pH of 5 caused cell loss in the control group as was seen for strain A1. This reflects the sensitivity to the low pH in a weak MWB medium 6.16±0.63 $\log_{10}$ cfu/ml compared with the greater survival seen in rich TSB medium 6.57±0.81 $\log_{10}$ cfu/ml. The M5 strain appeared to be more sensitive than the A1 strain to pH. No viable cells (<10 cfu/ml) of strain M5 were detected at pH of 5 following nisin treatment. At pH 6, 7, and 8 of MWB re-suspensions, 2.59±0.81, 2.58±0.64 and 2.39±0.60 $\log_{10}$ cfu/ml persister cells respectively were detected (Figure 4.3b). The numbers of persister cells surviving nisin treatment in MWB at pH 7 and 8 (2.58±0.64 and 2.39±0.60 $\log_{10}$ cfu/ml) were statistically significantly different (p<0.05) to the numbers of persister cells surviving in TSB at the same pH (3.82±0.72 and 4.67±0.30 $\log_{10}$ cfu/ml) respectively. In neutral and alkali conditions, a medium rich in nutrients could be an advantage in forming persister cells following nisin treatment.

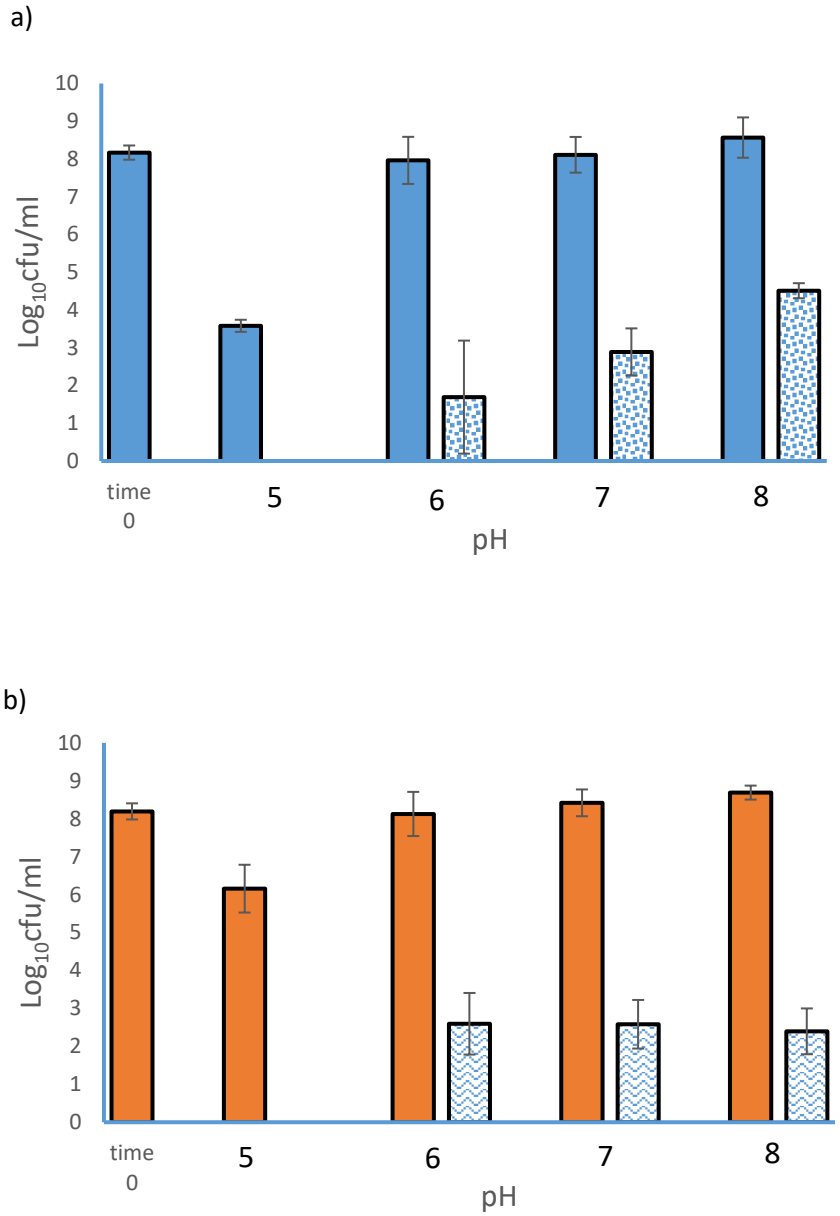


Figure 4. 3 The effect of nisin treatmemt on cells re-suspended in MWB at different pH values (pH 5-8) on the production of persister cells.

a) Stationary phase cells of A1 strain re-suspended in MWB at different pH 5 to 8 and exposed to 75µg/ml nisin at 30ºC for 24 hrs shaken at 150rpm. Bars with pattern represent the control group in which the cells were not treated with nisin. ; Bars with pattern represent persister cells suviving 75µg/ml nisin treatment. b) Stationary phase cells of M5 strain re-suspended in MWB at different pH values from 5 to 8 and exposed to 75µg/ml nisin at 30ºC for 24 hrs shaken at 150rpm. Bars with pattern represent the control group in which the cells were not treated with nisin; Bars with pattern represent persister cells suviving the 75µg/ml nisin treatment. Error bars = Standard deviation.

4.3.4 Metabolic activity of *L. monocytogenes* persister cells by the XTT assay

The persister cells in these nisin treatment tests showed a non-growing status under the high concentration of nisin stress. Whether the persisters following nisin treatment are dormant cells was determined by measuring the cellular metabolism using the XTT assay (Koban *et al.*, 2012). For the A1 strain, three independent persister samples at 6 $\log_{10}$ cfu/ml, showed absorbance at 450nm (OD_{450}) with values of 0.231, 0.135 and 0.127, while A1 overnight culture cells at 6 $\log_{10}$ cfu/ml showed OD_{450nm} of 0.493±0.072 (Figure 4.4a). For the M5 strain, three independent persister samples with cell numbers reaching at 6 $\log_{10}$ cfu/ml showed OD_{450} of 0.097, 0.095 and 0.111, while M5 overnight culture cells at 6 $\log_{10}$ cfu/ml showed OD_{450nm} of 0.215±0.017(Figure 4.4b). The persister cells showed lower metabotic activity than the equivalent number of cells from an overnight culture. It is hard to obtain a representative sample of persister and non persister cells for study. In any natural environment, there will be a mixture of persister and non persister cells so even in the situation where a culture is taken that has not been exposed to antimicrobial treatment, the assumption is that all the cells are non-persister phenotypes is not strictly correct. However the non-persisters will dominate compared with the population of viable cells. In samples that have been treated with an antimicrobial, the persister cells will dominate, although there will still be non-viable non-persister cells present. In previous concentration- dependent nisin treatments on *L. monocytogenes* overnight cultures (Chapter 3 Figure 3.3 and 3.4), the main population was killed rapidly by nisin and this sensitive group is assumed to be non-persister cells. In the XTT assay, *L. monocytogenes* overnight culture cells were taken as a typical population which is predominantly non-persisters, to compare with *L. monocytogenes* persister cells. The A1 non-persisters showed higher metabolic activity than M5 non-persisters and the low metabolism levels of their persister cells indicate that the formation of a persister cell for these two strains could be associated with the shutting down of biosynthetic pathways.

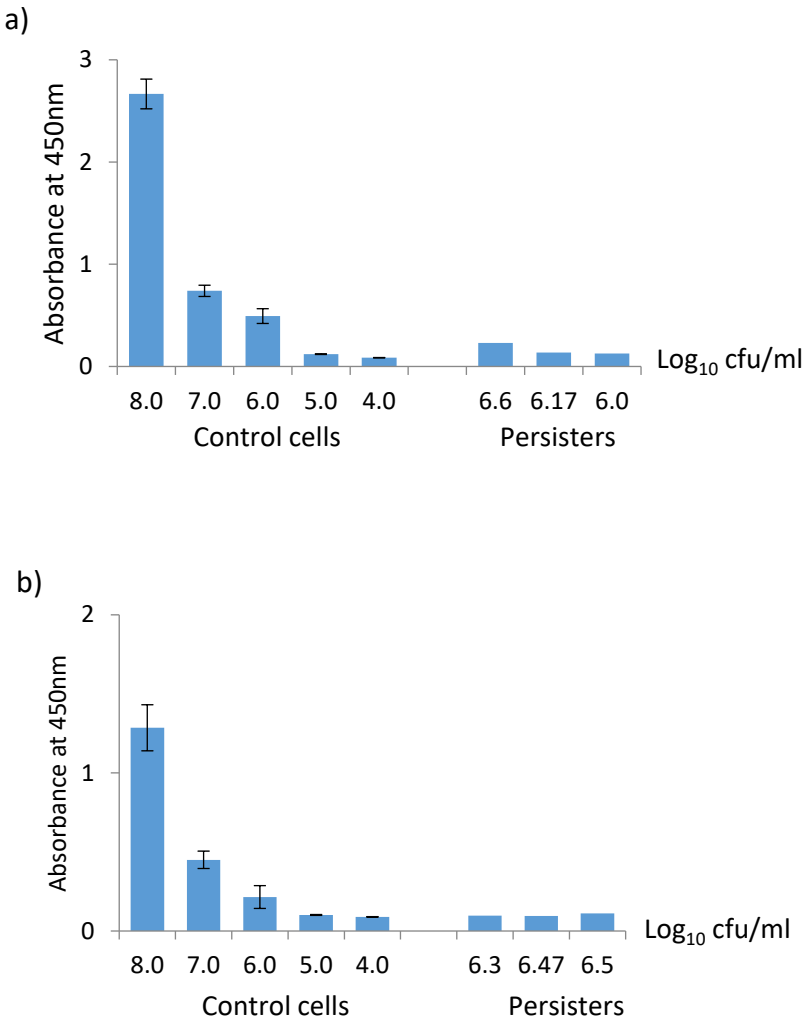


Figure 4. 4 Metabolic activity of *L. monocytogenes* persister cells

a) Serial 10- fold dilutions of A1 overnight culture in 0.1% peptone water from 8 to 4 $\log_{10}$ cfu/ml (control cells) and concentrated A1 persister cells (three independent collections) from 24hrs exposure to 75µg/ml nisin at 30˚C were used to measure the metabolic activity by the XTT assay. b) Serial dilutions of M5 overnight culture in 0.1% peptone water from 8 to 4 $\log_{10}$ cfu/ml (control cells) and concentrated M5 persister cells (three independent collections) from 24hrs exposure to 75µg/ml nisin at 30˚C were used to measure the metabolic activity by the XTT assay. The experiments consisted of three biological replicates, and error bars indicate the standard deviation.

4.3.5 Growth recovery of *L. monocytogenes* persister cells

The XTT assay showed that *L. monocytogenes* persister cells of both strains demonstrated lower metabolic activity than the overnight cultures(Figure 4.3), and the low metabolism is believed to be a phenotype of persister cells with a dormant status (Wood *et al.*, 2013). Once the antimicrobial stress is removed, persister cells are known to be capable of reviving in fresh medium (Lewis, 2005). In order to further compare the metabolism of the persister and non persister phenotypes, the rate of impedance change for the persister cells was compared with the impedance calibration for a non-persister population. Impedance change is proportional to the number of microbial cells assuming that they are all equally metabolically active. If there is a difference in metabolic activity, we should see a change in the time taken for a change in impedance. The persister cells of both strains from 75µg/ml nisin treatment were individually inoculated into fresh TSB medium for growth recovery. Regression equations ($Y=-2.3333X + 22.5500$ $r^2=0.9936$ for A1 strain; $Y= -3.1592 X + 30.9167$ $r^2= 0.9974$ for M5 strain) were calculated from the time for an impedance change against cell numbers of *L. monocytogenes* overnight cultures (non persister cells) (Table 4.3). For the persiser samples of both strains the rate of impedance change for the CFU(colony forming unit) of the persister cells was compared with the expected rate of impedance change for an equivalent non-persister population. Impedance times are shown in hours (Table 4.3). In all but one result, the detection time for the persister cells was longer than that for the expected value for the same number of non-persister cells (or more correctly, the general population of cells). Generally, persisters of M5 strains showed longer delays compared with A1 in the change in impedance compared with the general population of the respective strain (Table 4.3). This delay is important as it indicates a lower metabolic rate in the persister cells compared with the non persister cells. This is the first time an impedance assay has been used to assess the metabolic activity of a population of persister cells.

Table 4. 3 Growth recovery of *L. monocytogenes* persister cells compared with the expected impedance detection time for a non-persister population.

Persisters	Cell number by plate counting (CFU)	Expected impedance time calculated from linear regression equation (hrs)	Actual detection impedance time(hrs)	Regression equation* Y(hrs) X(Log_{10} cfu) (Supplementary Figure S4.3)
A1				
persister a	1.09×10^4 - 2.40×10^4	12.73 ± 0.56	13.11 ± 0.82	Y=-2.3333 X + 22.5500 r^2=0.9936
persister b	2.76×10^4 - 6.14×10^4	11.78 ± 0.57	13.00 ± 0.88	
persister c	2.96×10^5 - 8.45×10^5	9.25 ± 0.75	8.89 ± 0.77	
M5				
persister a	2.29×10^4- 6.92×10^4	16.40±1.07	17.28 ± 0.63	Y=-3.1592 X + 30.9167 r^2= 0.9974
persister b	4.47×10^3- 8.91×10^3	18.92±0.67	20.39 ± 0.25	
persister c	7.08×10^3 - 1.41×10^4	18.29±0.67	20.28 ± 0.25	

4.3.6 Cell surface charges of *L. monocytogenes* persister cells

Cell membrane fluidity and cell surface charge have been reported to attribute to the bacteriocin-resistance of *L. monocytogenes*(Kaur *et al.*, 2011). According to the zeta potential protocol for assessing surface charge, the various pHs (from 2 to 8) could influence the dissociation of acidic groups (such as amino groups) on the bacterial surface (Martinez *et al.*, 2008). The electrophoretic mobility of strains A1 and M5, measured in 2-8 pH buffers, showed a decrease in zeta potential with increasing pH, representative of a pH-dependent charge at the bacterial surface in Figure 4.5. The zeta potential demonstrated a similar isoelectric point near pH 4 for both strains. In tests of the pH 7 buffer, the zeta potential indicates electrophoretic mobility of the cells. For both strains, all carried a negative charge at pH7 but the persister cells had a lower negative charge than the overnight cultures. The zeta potential suggested that persisters of *L. monocytogenes* showed a change in cell surface charge that agrees with the results from Kaur *et al.* 2011.

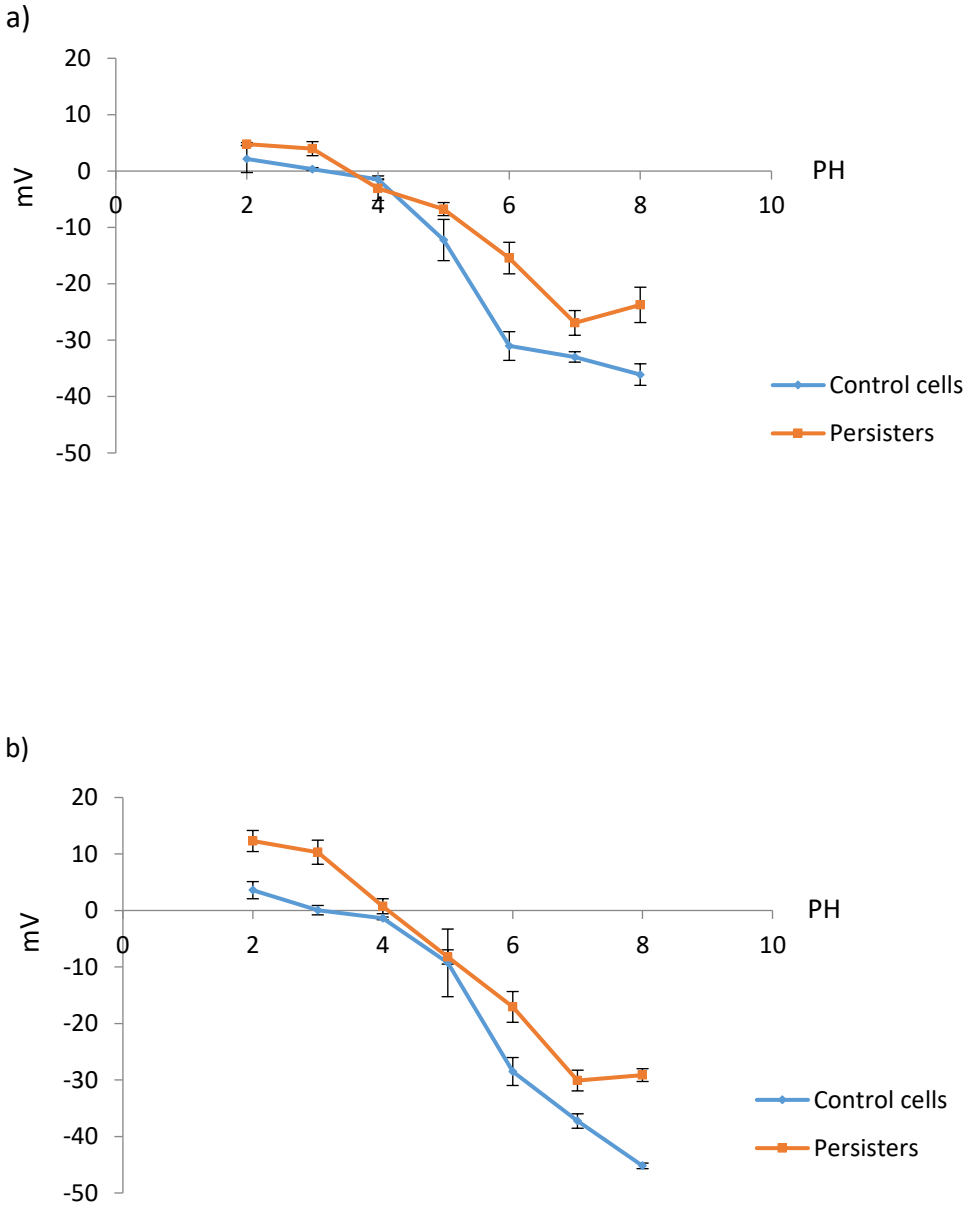


Figure 4. 5 Cell surface charges of *L. monocytogenes* persister cells

a) zeta potential of A1 overnight culture and its persister cells at 6 $\log_{10}$ cfu/ml; b) zeta potential of M5 overnight culture and its persister cells at 6 $\log_{10}$ cfu/ml. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.

4.4 Discussion

In the previous study, *L. monocytogenes* overnight cultures were treated with 75µg/ml of nisin for 24 hrs, and the killing curve against time showed that the bulk of the population was killed initially with the remaining population killed at a much slower rate (Chapter 3). The dormancy of persisters is often explained by slow or no growth of the cells or a slow metabolism (Wood *et al.*, 2013). Accordingly, supressed metabolic level contributes to bacterial tolerance to stress. Nutrient limitation is a condition in which bacteria may enter a dormant state that may be associated with increased persistence (Gardner *et al.*, 2007). Planktonic cells of *L. monocytogenes* Scott A grown in diluted TSB could be more resistant to benzalkonium chloride than cells grown in full strength TSB (Tyh-Jenq and Frank, 1993) because their strain in diluted TSB went through a slower growth rate than cells growing in TSB at 21ºC during 48hrs incubation. For their study sanitizer inactivation involved treatment for a short time (60s) after the grown cells were washed and re-suspended in PBS buffer.

In the present study, a high cell density of stationary phase cells (grown population) resuspended in different conditions was treated with a high concentration of nisin. Stationary phase cells of *L. monocytogenes* were resuspended in full TSB, diluted TSB or MWB. Exposing re-suspended cells to nisin showed that the bulk of the population was killed rapidly and the remaining cells were killed at a slower rate. The persister population could not utilize the fresh medium following re-suspension while under the high concentration of nisin. This demonstrates that the re-suspended stationary phase cells were not actively growing during nisin treatment. The resuspended cells in an extremely slow/ non-growth state could represent a dormant phenotype. The reduced metabolism could be an important requirement for the *L. monocytogenes* persistence (Knudsen *et al.*, 2013). As the low number of persister cells from immediate nisin treatment is too low for analysis (Figure 3.3 in Chapter 3), persister cells re-suspended in full TSB were used as this produced a higher number of persister cells. Persister cells re-suspended in diluted TSB or MWB produced a low number of persister cells (Figure 4.1 and 4.2). The physiological state of the whole population may be important in explaining the stronger persistence of the treated cells

re-suspended in full strength TSB. In the weak medium there may be insufficient nutrients to stimulate a strong stress response to protect the cells against the nisin challenge and therefore the number of persister cells is less than in the rich medium. Further studies are needed to investigate more fully the effect of the environment on the survival of persister cells. Under conditions of low nutrients and acid pH, the physiological activity of the cells and their response to challenge with antimicrobials is likely to be different compared to a rich and pH neutral environment.

The persisters can switch to be non-persisters once the antimicrobial stresses are removed and complete optimal growth in an enriched environment (Wood *et al.*, 2013). For food safety, the persistence of *L. monocytogenes* is a concern and may explain why *L. monocytogenes* is repeatedly detected in many food related environments and processed food products (Wang *et al.*, 2015). Before this present study, all studies on *L. monocytogenes* persistence from food environments just isolated persisters at extremely low cell number and revived the sample persisters in rich medium for further analysis. Using this approach for a study of persister cells of *L. monocytogenes* is difficult as the phenotype will have been changed during the laboratory enrichment. In the present study, persister cells of *L. monocytogenes* become highly tolerant to nisin treatment, and the persisters which were isolated from nisin treatment were concentrated for direct analysis and measurements without any enrichment step. Maintaining the persister phenotype is key and a challenge for persistence studies (Balaban *et al.*, 2004). The main strategy for isolating persister cells is applying antimicrobial agents, but dead cell debris is an unavoidable event and makes the isolation of persister and non persister populations difficult.

In this study the XTT results indicate that the persisters of *L. monocytogenes* enter dormancy by reducing metabiolic activity. The results from the impedance study support this conclusion. In another report, RNA expression profiling of persistent *L. monocytogenes* isolates following ampicillin treatment revealed down-regulation of transcriptional level energy production and flagellar synthesis with a slow or non-growth phenotype (Balaban *et al.*, 2004). Dormant persisters of *E. coli* under β-lactam antibiotic treatment have been found to show less cytoplasmic drug accumulation as a result of enhanced efflux activity(Pu *et al.*2016).The efflux

protein is conserved in *Listeria* species(Mereghetti *et al.*, 2000; Zhu *et al.*, 2008), and the ABC transporter permease with an efflux protein negatively regulates genes encoding cell surface proteins (Dlt), cell surface anchor proteins (SrtA), and transcriptional regulators (GntR)resulting in reduced biofilm formation(Zhu *et al.*, 2011). It is known that resistant strains of *L. monocytogenes* alter their cell membrane properties to survive under high concentrations of cell wall acting antibiotics(Collins *et al.*, 2010), and the ABC transporter permease with an efflux protein could be involved in the formation of *L. monocytogenes* persisters by modifying some properties of the cell membrane. The ABC transporter plays key role in down-regulating *L. monocytogenes* biofilm (Zhu *et al.*, 2008), indicating that there may be a link between persistence and biofilm formation of *L. monocytogenes*. Persister cells isolated from food environments are able to adhere to food processing surfaces and produce biofilms (Alessandria *et al.*, 2010).The attached persisters should have a distinct phenotype (e.g. produce more EPS) and life style (enhanced biofilm) compared with the persisters in planktonic form. In future work the persister cells which were successfully collected following re-suspension in fresh medium will be used to extract RNA for gene profiling of persister cells under nisin stress.

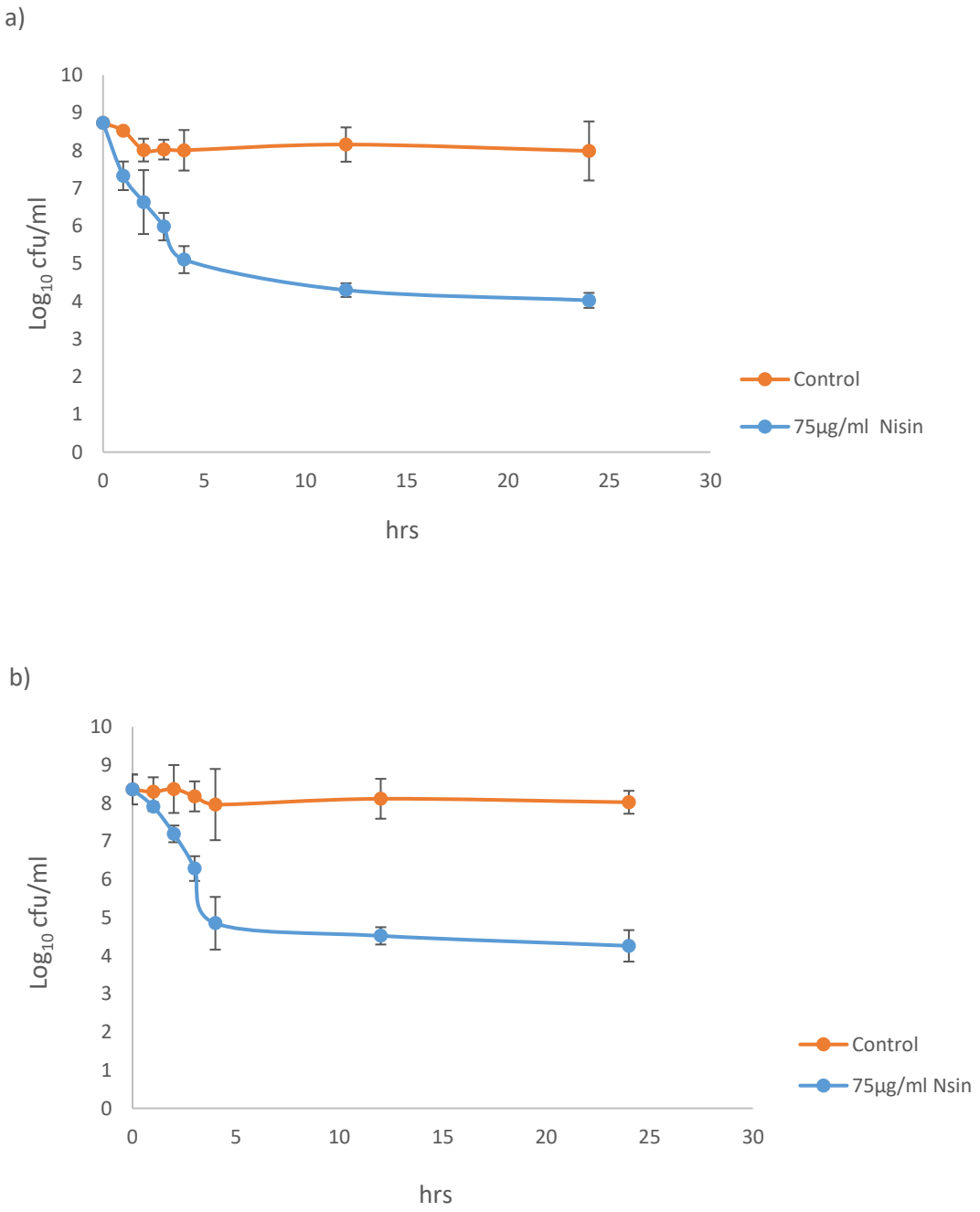
4.5 Conclusion

Persisters are currently defined as the population surviving when a microbial culture is exposed either to increasing concentrations of bactericidal antibiotics or to a fixed concentration over a long time (Kint *et al.*, 2012). Persister cells exist as a population of cells showing tolerance to the high concentrations of nisin with greater numbers surviving following re-suspension in fresh medium. Using cultures in a full TSB suspension is a successful approach to obtain adequate numbers of persister cells for analysis. A reduced metabolism is an important requirement for the *L. monocytogenes* persistence, as seen in the XTT assays and impedance assays, and the persister population has a lower negative cell surface charge than the general population. Persisters generated from re-suspended cells exposed to high concentrations of nisin depend on both pH and nutrients.

4.6 Acknowledgements

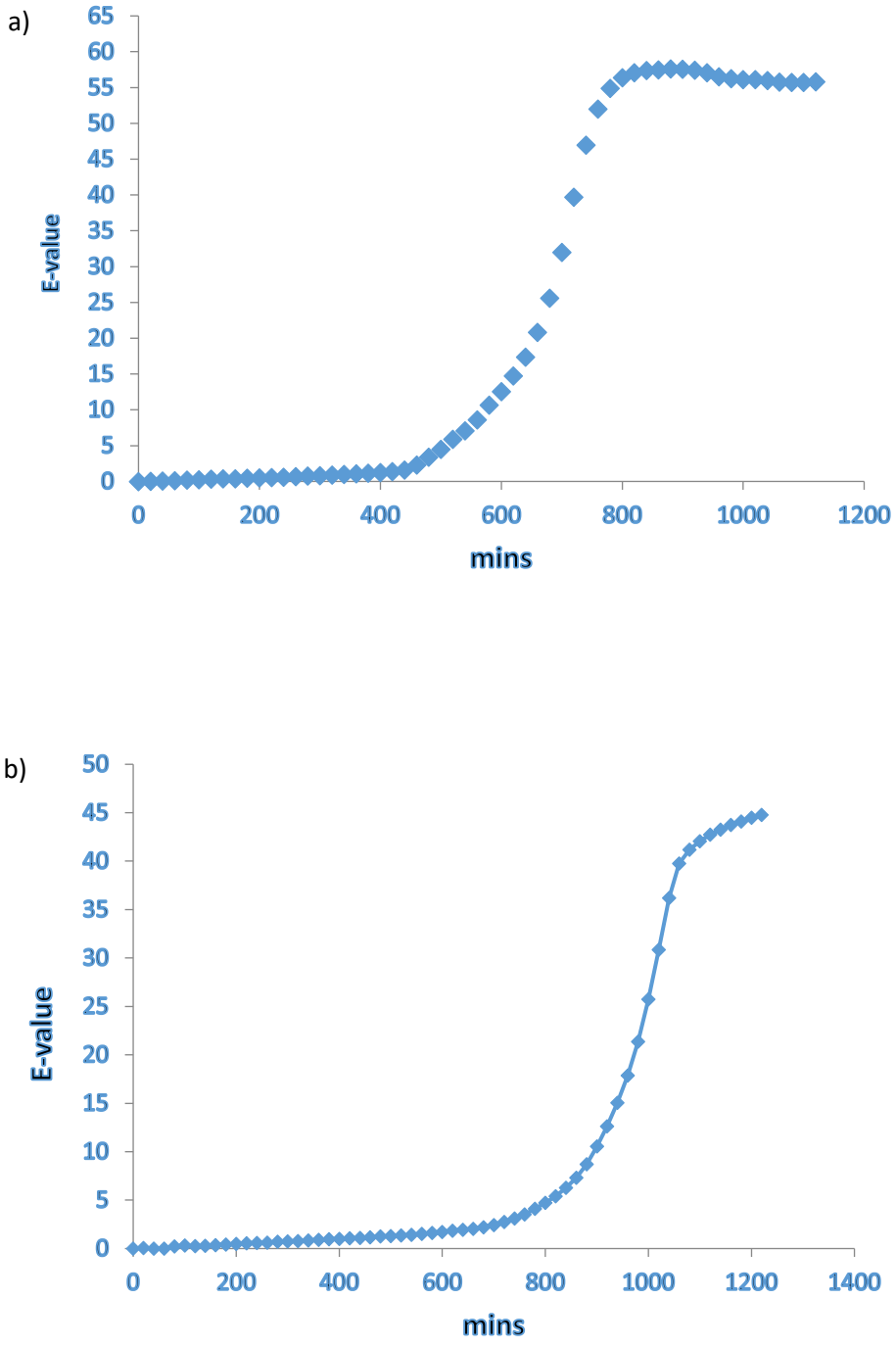
We thank AsureQuality Limited, New Zealand for the kind provision of *L. monocytogenes* strains used in this study. This work was supported by postgraduate research support funding of School of Food and Nutrition.

4.7 Supplementary Information



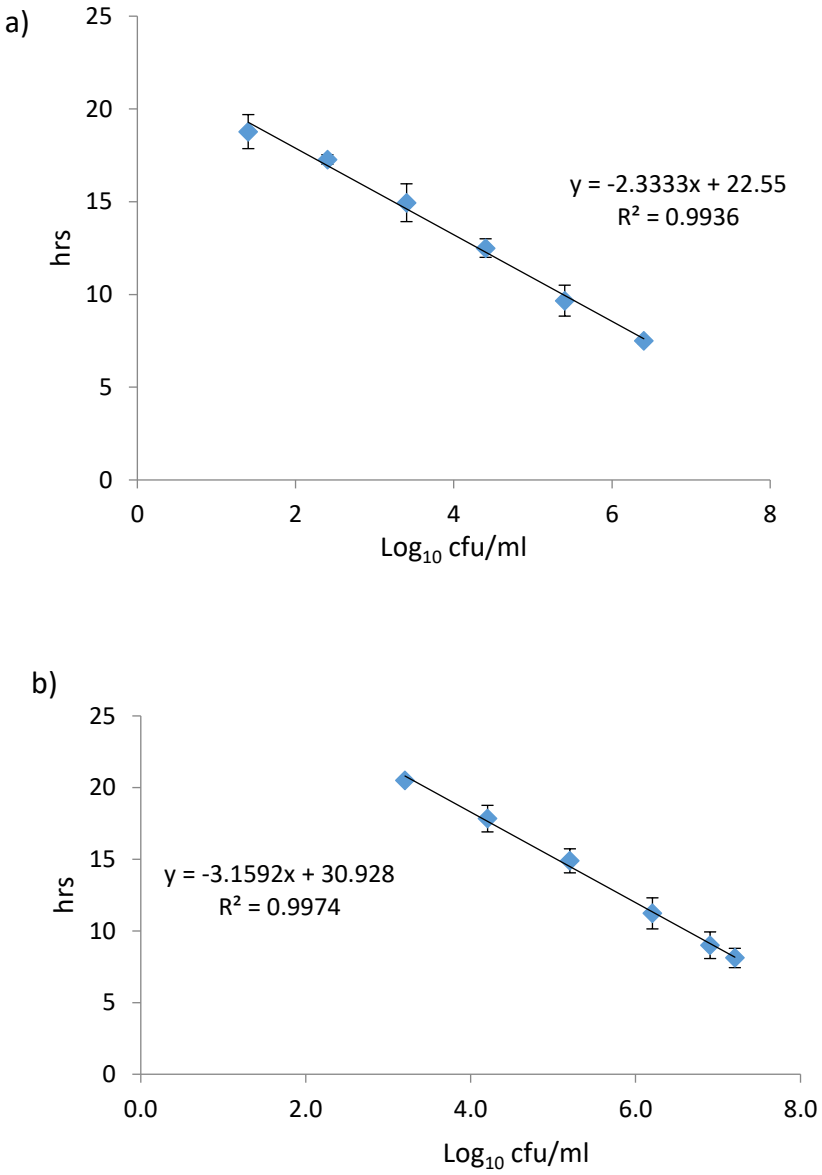
Supplementary Figure S4. 1 Death kinetics of TSB re-suspended *L. monocytogenes* cultures treated with 75µg/ml nisin (cell viability was meatured at time0, 1, 2, 3, 4, 12 and 24hrs).

a) Time-dependent killing of TSB re-suspended *L. monocytogenes* A1 culture with nisin at 75µg/ml, 30˚C for 24 hrs. b) Time-dependent killing of TSB re-suspended *L. monocytogenes* M5 culture with nisin at 75µg/ml, 30˚C for 24 hrs. The experiment was performed with three biological replicates, and error bars indicate the standard deviation.



Supplementary Figure S4. 2The growth curves for impedance change with time were measured by the BacTrac™ 4000 microorganism growth analyser.

a) The growth curve of one A1 persister sample' inocula; b) The growth curve of one M5 persister cells' inocula.



Supplementary Figure S4. 3 Serial 10-fold dilutions of overnight cultures without nisin treatment were determined by the time for impedance change into the exponential phase of cell growth (E=15) to obtain regression equation for each strain

a) A1; b) M5.

4.8 References

Alessandria, V., Rantsiou, K., Dolci, P. and Cocolin, L. (2010). Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. *International Journal of Food Microbiologyogy*, 141, S156-S162.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science*, 305, 1622-1625.

Bodur, T. and Cagri-Mehmetoglu, A. (2012). Removal of *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 biofilms on stainless steel using scallop shell powder. *Food Control*, 25, 1-9.

Budu-Amoako, E., Ablett, R. F., Harris, J. and Delves-Broughton, J. (1999). Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in cold-pack lobster meat. *Journal of Food Protection*, 62, 46-50.

Cabo, M., Pastoriza, L., Bernárdez, M. and Herrera, J. (2001). Effectiveness of CO 2 and Nisaplin on increasing shelf-life of fresh pizza. *Food Microbiologyogy*, 18, 489-498.

Camargo, A. C., Lino de Paula, O. A., Todorov, S. D. and Nero, L. A. (2016). In Vitro Evaluation of Bacteriocins Activity Against *Listeria monocytogenes* Biofilm Formation. *Applied Biochemistry and Biotechnology*, 178, 1239-1251.

Carpentier, B. and Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiologyogy*, 145, 1-8.

Collins, B., Joyce, S., Hill, C., Cotter, P. D. and Ross, R. P. (2010). TelA Contributes to the Innate Resistance of *Listeria monocytogenes* to Nisin and Other Cell Wall-Acting Antibiotics. *Antimicrobial Agents and Chemotherapy*, 54, 4658-4663.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology*, 3, 777-788.

Delves-Broughton, J., Blackburn, P., Evans, R. and Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek*, 69, 193-202.

Delves‐Broughton, J., Williams, G. C. and Wilkinson, S. (2008). *The use of bacteriocin, nisin, as a preservative in pasteurized liquid whole egg*.

Dhar, N. and McKinney, J. D. (2007). Microbial phenotypic heterogeneity and antibiotic tolerance. *Current Opinion in Microbiology*, 10, 30-38.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection*, 77, 150-170.

Gardner, A., West, S. A. and Griffin, A. S. (2007). Is Bacterial Persistence a Social Trait? *Plos One*, 2.

Gharsallaoui, A., Oulahal, N., Joly, C. and Degraeve, P. (2016). Nisin as a Food Preservative: Part 1: Physicochemical Properties, Antimicrobial Activity, and Main Uses. *Crit Rev Food Sci Nutr*, 56, 1262-1274.

Guerra, N. á., Macias, C. á., Agrasar, A. and Castro, L. (2005). Development of a bioactive packaging cellophane using Nisaplin® as biopreservative agent. *Letters in Applied Microbiology*, 40, 106-110.

Hoben, H. J. and Somasegaran, P. (1982). COMPARISON OF THE POUR, SPREAD, AND DROP PLATE METHODS FOR ENUMERATION OF RHIZOBIUM SPP IN INOCULANTS MADE FROM PRE-STERILIZED PEAT. *Applied and Environmental Microbiology*, 44, 1246-1247.

Kaur, G., Malik, R. K., Mishra, S. K., Singh, T. P., Bhardwaj, A., Singroha, G., and Kumar, N. (2011). Nisin and Class IIa Bacteriocin Resistance Among *Listeria* and Other Foodborne Pathogens and Spoilage Bacteria. *Microbial Drug Resistance*, 17, 197-205.

Kint, C. I., Verstraeten, N., Fauvart, M. and Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends in Microbiology*, 20, 577-585.

Knudsen, G. M., Ng, Y. and Gram, L. (2013). Survival of Bactericidal Antibiotic Treatment by a Persister Subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 79, 7390-7397.

Koban, I., Matthes, R., Hübner, N. O., Welk, A., Sietmann, R., Lademann, J., and Kocher, T. (2012). XTT assay of ex vivo saliva biofilms to test antimicrobial influences. *GMS Krankenhaushygiene interdisziplinär*, 7.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry (Moscow)*, 70, 267-274.

Maisnier-Patin, S., Deschamps, N., Tatini, S., R. and Richard, J. (1992). Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. *Lait*, 72, 249-263.

Maisnier-Patin, S., Tatini, S., R. and Richard, J. (1995). Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in milk. *Lait*, 75, 81-91.

Martinez, R. E., Pokrovsky, O. S., Schott, J. and Oelkers, E. H. (2008). Surface charge and zeta-potential of metabolically active and dead cyanobacteria. *Journal of Colloid and Interface Science*, 323, 317-325.

Mereghetti, L., Quentin, R., Marquet-Van der Mee, N. and Audurier, A. (2000). Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. *Applied and Environmental Microbiology*, 66, 5083-+.

Miyaue, S., Suzuki, E., Komiyama, Y., Kondo, Y., Morikawa, M. and Maeda, S. (2018). Bacterial Memory of Persisters: Bacterial Persister Cells Can Retain Their Phenotype for Days or Weeks After Withdrawal From Colony–Biofilm Culture. *Frontiers in Microbiology*, 9, 1396.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., and Britigan, B. E. (2011). Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria. *Science*, 334, 982-986.

Premaratne, R. J., Lin, W. J. and Johnson, E. A. (1991). Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Applied Environmental Microbiology*, 57, 3046-3048.

Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., and Ge, H. (2016). Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. *Molecular Cell*, 62, 284-294.

Sandasi, M., Leonard, C. and Viljoen, A. (2010). The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. *Letters in applied microbiology*, 50, 30-35.

Thomas, L. V. and Wimpenny, J. W. (1996). Investigation of the effect of combined variations in temperature, pH, and NaCl concentration on nisin inhibition of *Listeria monocytogenes* and *Staphylococcus aureus*. *Applied Environmental Microbiology*, 62, 2006-2012.

Tyh-Jenq, R. and Frank, J. H. (1993). Susceptibility of starved planktonic and biofilm *Listeria monocytogenes* to quaternary ammonium sanitizer as determined by direct viable and agar plate counts. *Journal of Food Protection*, 56, 573-576.

Wang, H., Palmer, J. and Flint, S. (2016). A rapid method for the nonselective enumeration of Yersinia enterocolitica, a foodborne pathogen associated with pork. *Meat Science*, 113, 59-61.

Wang, J., Ray, A. J., Hammons, S. R. and Oliver, H. F. (2015). Persistent and Transient *Listeria monocytogenes* Strains from Retail Deli Environments Vary in Their Ability to Adhere and Form Biofilms and Rarely Have inlA Premature Stop Codons. *Foodborne Pathogens and Disease*, 12, 151-158.

Winkelstroeter, L. K., Tulini, F. L. and De Martinis, E. C. P. (2015). Identification of the bacteriocin produced by cheese isolate *Lactobacillus paraplantarum* FT259 and its potential influence on *Listeria monocytogenes* biofilm formation. *Lwt-Food Science and Technology*, 64, 586-592.

Wood, T. K., Knabel, S. J. and Kwan, B. W. (2013). Bacterial Persister Cell Formation and Dormancy. *Applied and Environmental Microbiology*, 79, 7116-7121.

Zhu, X., Liu, W., Lametsch, R., Aarestrup, F., Shi, C., She Q., Shi, X., Knøchel, S. (2011). Phenotypic, Proteomic, and Genomic Characterization of a Putative ABC-Transporter Permease

Involved in *Listeria monocytogenes* Biofilm Formation. *Foodborne pathogens and disease*, 8, 495-501.

Zhu, X., Long, F., Chen, Y., Knochel, S., She, Q. and Shi, X. (2008). A Putative ABC Transporter Is Involved in Negative Regulation of Biofilm Formation by *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 74, 7675-7683.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)
We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Shuyan Wu
Name/Title of Principal Supervisor: Steve Flint
Name of Published Research Output and full reference:
Shuyan Wu, Pak-Lam Yu, Steve Flint (2017) Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. Food Control 77:243-250.

In which Chapter is the Published Work: Chapter 4
Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: and / or
- Describe the contribution that the candidate has made to the Published Work: The candidate carried out laboratory work, data collection and analysis, and manuscript preparation guidance of direction and editorial help from the co-authors and supervisors.

01/12/2019

Candidate's Signature — Date

01/12/2019

Principal Supervisor's signature — Date

GRS Version 3– 16 September 2011

Chapter 5 Flow cytometry investigation on *Listeria monocytogenes* persistence during lethal nisin treatment

Partial data was submitted to *Food Control* in Jan 2019

Shuyan Wu [a], Pak-Lam Yu [b], Steve Flint [a, *]

[a] *Massey Institute of Food Science and Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

[b] *School of Engineering & Advanced Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

Abstract

Traditional microbiological techniques such as agar plating are limited in providing information of persister cells exposed to antimicrobial substances. Flow cytometry can identify metabolically active cells that may not be able to be cultured and therefore has potential to identify persister cells that although they cannot be cultured, can still pose a threat to human health. The *L. monocytogenes* stationary phase populations in spent medium or re-suspended in fresh TSB medium were individually treated with 75μg/ml nisin for 24hrs at 30ºC, and the treatments were assayed using flow cytometry combined with live/dead staining. The results revealed a reduction in viable populations and the differentiation of living cells and dead/damaged cells within the treated population. This finding was consistent with previous results from plate count experiments. Changes in cell permeability were detected during the nisin treatment in spent medium and re-suspended in fresh medium, indicating possible variations in the mechanisms of persister formation between the two groups.

5.1 Introduction

Bacterial persistence is responsible for long term contamination in food processing environments. Proportions of clonal bacterial populations can survive lethal doses of antimicrobial agents while the population as a whole remains susceptible (Cañas-Duarte *et al.*, 2014). Although this persister phenotype is characterized by cells entering a transient state of arrested or reduced growth, the mechanism of bacterial persistence is not completely understood (Shah *et al.*, 2006).

Persister cells are believed to be a minority group in a population with most studies focussing on antibiotic tolerance (Radzikowski *et al.*, 2017). Persister cells can be either slow growing or viable but non-culturable cells (Highmore *et al.*, 2018). Traditional agar plate counting is inadequate to examine the true state of persister cells following exposure to what are considered lethal concentrations of antimicrobials as the persister population is regarded as viable but non culturable. Flow cytometry (FC) is a flow-based techenique for measuring and sorting cells (Tracy *et al.*, 2010), and the simultaneous measurement of certain properties of cells has been used to evaluate physiological states following different physical and chemical antimicrobial treatments in bacterial survival studies (Léonard *et al.*, 2016).

Flow cytometry has been used to assess the antimicrobial activity of several essential oils (Munoz *et al.*, 2009; Paparella *et al.*, 2008) against *L. monocytogenes* by clearly discriminating different subpopulations (including viable, dead and injured cells), and significant differences were observed between plate count results and flow cytometric data, which suggests the presence of a sublethally stressed subpopulation unable to form colonies on agar plates. The combination of FC with additional methods (including plate counting and microscopy) can help to elucidate the mechanism of antimicrobial activity and also to display differences in microbial sub-populations in response to antimicrobial treatments (Léonard *et al.*, 2016). Fluorescence activated cell sorting techniques have determined physiological change in *L. monocytogenes* when exposed to sublethal concentrations of bacterioncins such as nisin and leucocin) (Swarts *et al.*, 1998;

Weeks *et al.*, 2006). In this chapter FC using viable/non-viable flouoresent staining was used to investigate the persister population of *L. monocytogenes* resulting from lethal nisin treatment.

Persisters are tolerant to antimicrobial treatments over long exposures times, and studies show that any treatment that lasts more than 45 minutes results in activation of a stress response mechanism and possible induction of persistency (Cañas-Duarte *et al.*, 2014). The ability of persister cells to survive antibiotic treatment, without being genetically resistant, is likely to be a key factor in many persistent bacterial infections (Fisher *et al.*, 2017). Prokaryotes are capable of inducing suicide through the production of free radicals and subsequent uncontrolled metabolic activity (an imbalance between anabolism and catabolism causes significant damage to intracellular components) during the sublethal stress (Dodd *et al.*, 1997). Metabolic imbalance following biocidal and other stresses has been postulated to lead to free radical production and self-destruction of bacteria (Denyer and Stewart, 1998).

When respiring bacterial cells are subjected to certain stresses (such as osmotic stress), the suicide response is accompanied by growth arrest (Aldsworth *et al.*, 1999). As a consequence, a burst of excess free radicals occurs, and it is this burst of free radicals which is lethal to the cell, not the action of the stress *per se* (Aldsworth *et al.*, 1999). Membrane active compounds, such benzalkonium chloride (BAC) (Luppens *et al.*, 2001) and eugenol(Gill and Holley, 2006), are reported to result in ions and ATP efflux in different bacteria including *L. monocytogenes*, and membrane damage companied with a burst of free radicals occurs as the initial response to bacterial stress (Dodd *et al.*, 1997). Stationary phase cells under mild stress are protected against free-radical production through decreasing the level of metabolism (Rees *et al.*, 1995). The present study investigates how *L. monocytogenes* deals with the free-radical generation during lethal nisin treatment under two different conditions and whether free-radical production has an influence on persister formation.

5.2 Materials and methods

5.2.1 Flow cytometry

20ml of 18hrs-old stationary phase was obtained from single inoculation for both the A1 and M5 strains. For each strain, 10ml of 18hrs-old stationary phase cells of *L. monocytogenes* were treated with 75µg/ml nisin for 24hrs at 30ºC with 150prm shaking. Another 10ml of 18hrs-old stationary cells were re-suspended in fresh TSB medium, followed by treatment with 75µg/ml nisin for 24hrs using the same conditions as described previously. From each of the two treatment groups, cells were harvested at 0mins, 10mins, 30mins, 1hrs, 2hrs, 4hrs and 24hrs during the treatment. Each harvest of cells was collected by centrifugation (7000 ×g, 5 min), washed in filtered PBS (phosphate buffered saline) to remove excess antimicrobial compounds, and finally re-suspended in filtered PBS for subsequent analysis. Prior to FC analysis, aliquots of each sample were prepared as unstained cells and live/dead stained cells. Cells were stained for 5mins at room temperature with thiazole orange (TO) and propidium iodide (PI) fluorescent dyes (3µl of each dye in 500µl of PBS re-suspended cells) from the BC^{TM} Cell Viability Kit, and all samples were analyzed using the BD Accuri™ C6 Flow Cytometer (BD Biosciences, USA). Samples were excited with 488 nm and green TO fluorescence was measured with the FL1 detector (FITC-A channel, 530 ± 30 nm), and red PI fluorescence was measured at with FL3 detector (PerCP-Cy5.5-A channel, > 670 nm). Sample flow rate was adjusted to slow mode, and a total of 10,000 events were collected per sample for analysis. The cell population was selected by gating on forward scatter (FSC) and side scatter (SSC) plot. In order to eliminate background noise from the flow cytometry signals, samples of unstained cells were run to set thresholds using forward and side scatter channels. The "LIVE" regions were set up using log phase cells as control cells, and "dead" regions were set up using heat-killed cells (90ºC for 30mins) (Paparella *et al.*, 2008). Quantitative assessment of each bacterial subpopulation was performed by counting the number of events included inside the corresponding gate, and results compared with cell counts from drop plating.

5.2.2 Fluorescence microscopy analysis

L. monocytogenes treated with 75µg/ml nisin was examined using fluorescence microscopy. 18hrs-old stationary phase cells of *L. monocytogenes* were treated with nisin for 24hrs at 30ºC with150prm shaking while the same stationary cells in a separate group were re-suspended into fresh TSB medium and treated with nisin in the same incubation conditions. The two treatment groups, were sampled at 0mins, 10mins, 30mins, 1hrs, 2hrs, 4hrs and 24hrs during the treatment. Cells were collected from the samples by centrifugation (7000 ×g, 5 min), washed in filtered PBS to remove excess antimicrobial compounds, and re-suspended in filtered PBS for TO/PI staining with BC™ Cell Viability Kit. 500µl cell culture was stained with 3 µL of each dye, and incubated at room temperature for 5 min. The stained microbial samples were mounted onto microscope slides, and examined using an Olympus BX53 epifluorescent microscope (Olympus, USA). U-RFL-T mercury lamp was used as the excitation source, and the fluorescence signals were collected by two separate emission filters for TO (WB, 450–480 nm) and PI (WG, 510–550 nm). Images were visualized and analyzed using Image-Pro software (Olympus, USA).

5.2.3 Free radical production by XTT assay

XTT is reduced to soluble formazans by superoxide radical anions, and XTT reduction indicates free radical production (Sutherland and Learmonth, 1997). Free radical production and release from cells following lethal nisin treatment was examined by the XTT assay. 18hrs-old stationary phase cells of *L. monocytogenes* were treated with nisin for 24hrs at 30ºC with 150 rpm shaking while the same stationary cells in a separate group were re-suspended into fresh TSB medium and treated with nisin in the same incubation conditions. The two treatment groups were sampled at 10mins, 30mins, 1hrs, 2hrs, 4hrs and 24hrs during the treatment. The cells from the samples were harvested at each time point by centrifugation (7000x, 5mins). Each cell pellet from centrifugation was mixed with 200ul XTT containing the electron coupling agents 25nM PMS. Meanwhile, 100µl supernatant of each sample after centrifugation was used to mix with 200ul XTT containing electron coupling agent 25nM PMS. All cell pellets and their supernatant samples

were incubated in the dark at 37°C for 3hrs. Reduced XTT with a highly coloured formazan dye from each sample was detected by a microtiter plate reader (Bio-tek Inc), and the optical density was read at 450nm. The values obtained were blank corrected and experiments were performed in triplicate for each strain.

5.2.4 Statistical analysis

The mean and standard deviation of triplicate experiments in this study were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

5.3 Results

5.3.1 Flow cytometric assessment of the antimicrobial activity of lethal nisin on *L. monocytogenes*

The BD Cell Viability Kit was used for staining live and dead cells of *L.monocytogenes*. TO is a permeant dye and enters all cells, live and dead, and was detected under the FITC-A channel. Live cells have intact membranes and are impermeable to dyes such as PI, which leaks into cells with compromised membranes. PI stained cells were detected for differentiating dead cells from live cells in a mixed population. In Figure 5.1, log phase cells that stained by both dyes appeared in the quadrant H1-3 and correspond to living cells. Heat inactivated cells stained by both dyes appeared in the quadrant H1-1 and correspond to the dead cells. These results provided reference plots for live and dead cells of both *L. monocytogenes* strains.

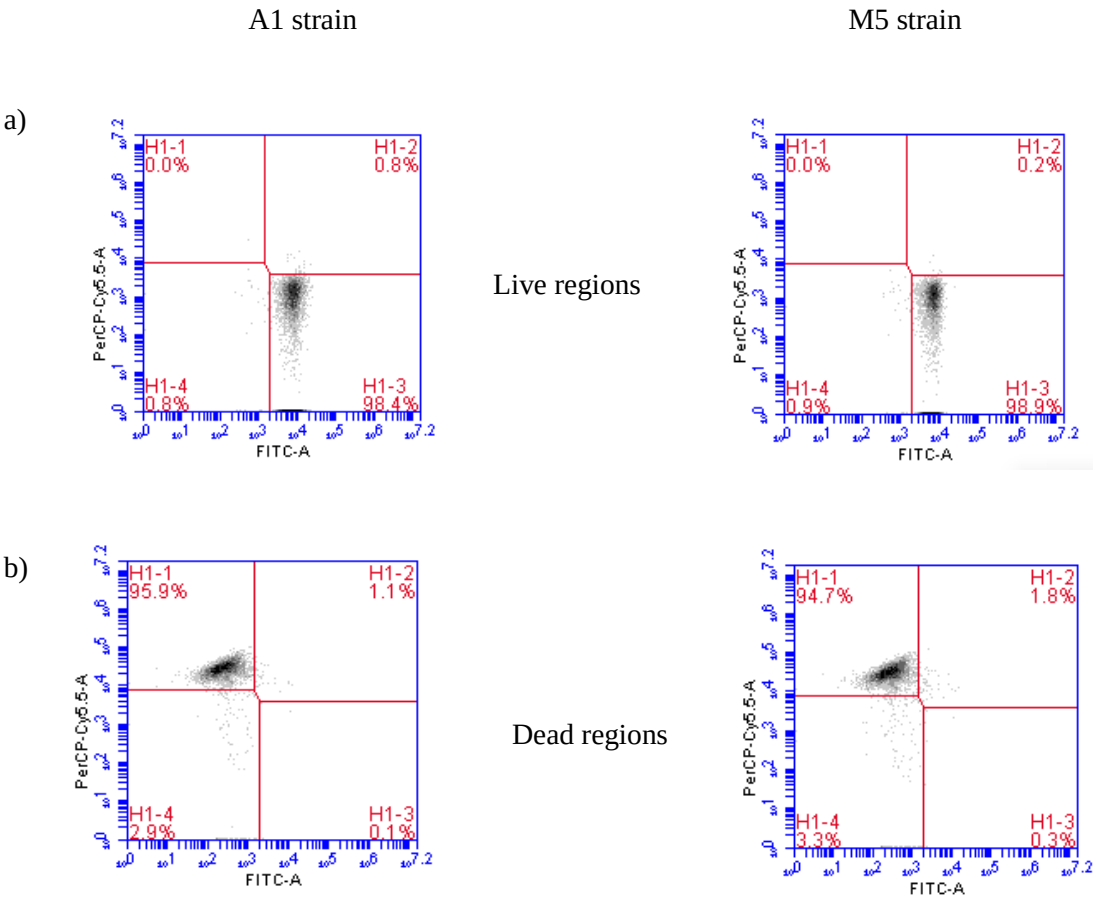


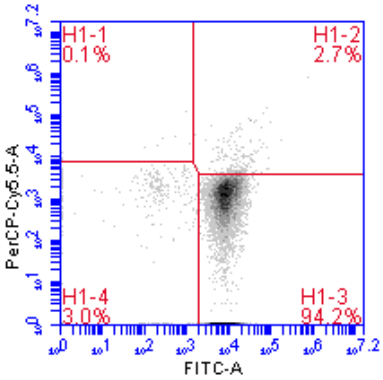
Figure 5. 1 Flow cytometry (FC) dot plots of FITC-A (FL1, green fluorescence) vs. PerCP-Cy5.5A (FL3, red fluorescence) of *L. monocytogenes* control cells.

a) Live regions of log phase *L. monocytogenes* of A1 strain (left) and M5 strain(right). b) Dead regions of heat inactivated *L. monocytogenes* of A1 strain (left) and M5 strain(right).

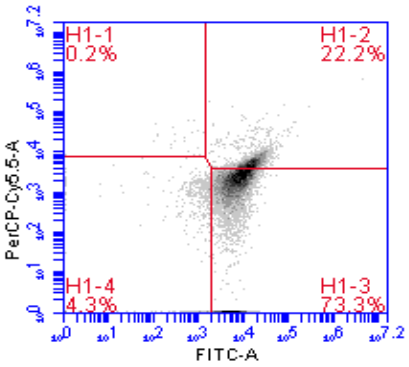
L. monocytogenes stationary phase cells treated with 75μg/ml nisin at different time points were detected using FC by live/dead staining (Figure5.2). Nisin acts on the cell membrane by interupting the membrane structure with pore formation. After 30mins of nisin treatment, 55% of the collected cells in quadrant H1-3 stained only with TO for the A1 strain, with the remaining 45% of cells penetrated by the PI dye and detected in quadrants H1-1 and H1-2. Cell numbers in the "live region" H1-3 reduced as the treatment time extended. After 24 hrs following nisin treatment 91.7% of the cells were sorted into the H1-1 and H1-2 quadrants. For the M5 strain, half of the cells were detected in the PI channel (H1-1 and H1-2) 10 mins following nisin treatment, and the percentage in the live region (H1-3) reduced as the treatment time continued. And 95.2% of the cells were detected as PI stained at the end of nisin treament (24hrs). Cells in the H1-2 quadrant are those stained by both TO and PI so are deteced under both fluorescence channels. Some studies refer to the uptake of PI as representing injuried or damaged cells (Paparella *et al.*, 2008). Flow cytometric data allowed an estimate of the impact of nisin on *L. monocytogenes* cell damage from quadrants H1-1 and H1-2. At 24hrs of nisin treament, the A1 strain had 50.2% of cells sorted into the "dead region" and 41.5% of cells sorted in H1-2 region representing damaged cells while the M5 strain generated 79.3% of cells in the "dead region" and only 15.9% cells in the "damaged" region. The percentage of cells in quadrants H1-3 at different time points were calculated as alive cells. Culturable cells following nisin treatment were recovered and enumerated by agar plating (supplementary table S5.1). The cell number from FC detection was comparable to agar plating after nisin treatment times of 0, 10mins, 30mins, and 1hrs, and was higher than plate counting at time points of 2, 4, 6, 24hrs. This difference between enumerations was assumed to represent "viable but not culturable cells" cells(Afari and Hung, 2018). FC analysis demonstrated the killing dynamics of nisin on stationary phase cells.

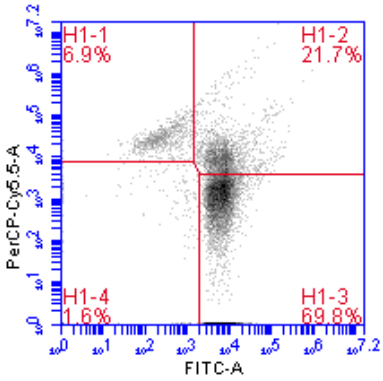
A1 strain

M5 strain

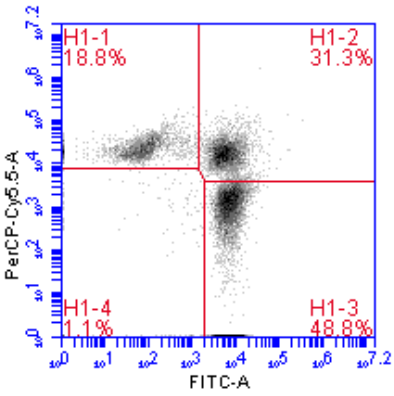


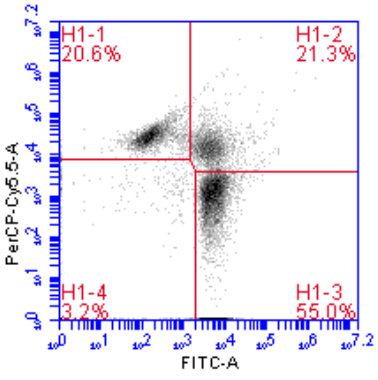
Time 0



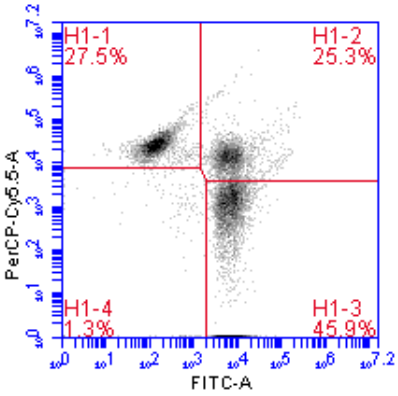


Time 10mins



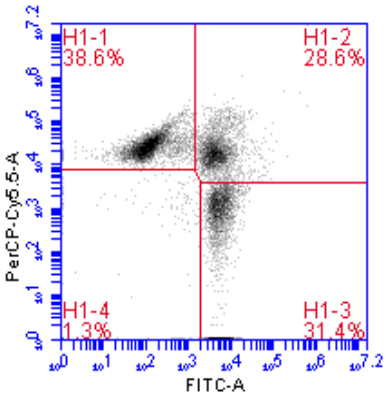


Time 30mins

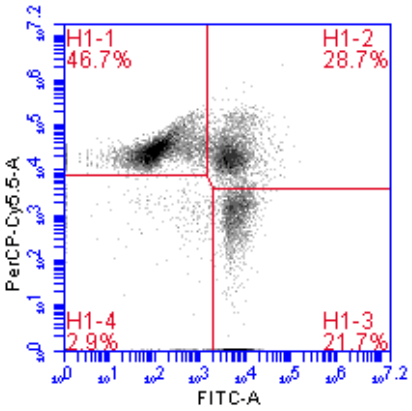


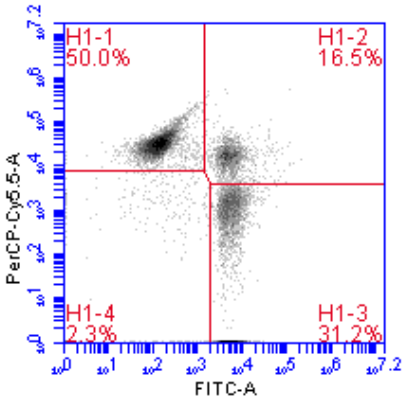
A1 strain

M5 strain

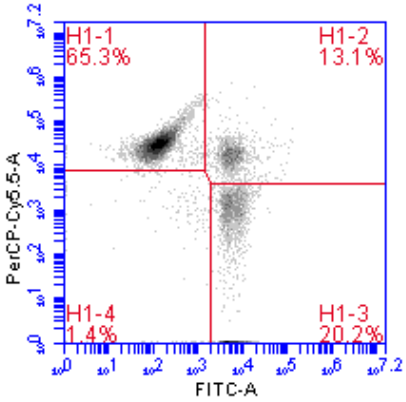


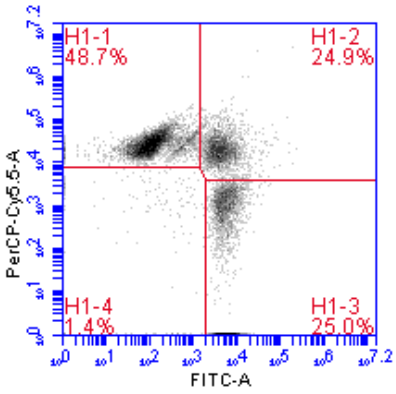
Time 1hrs



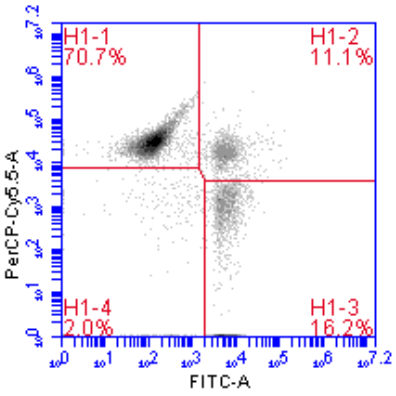


Time 2hrs





Time 4hrs



A1 strain M5 strain

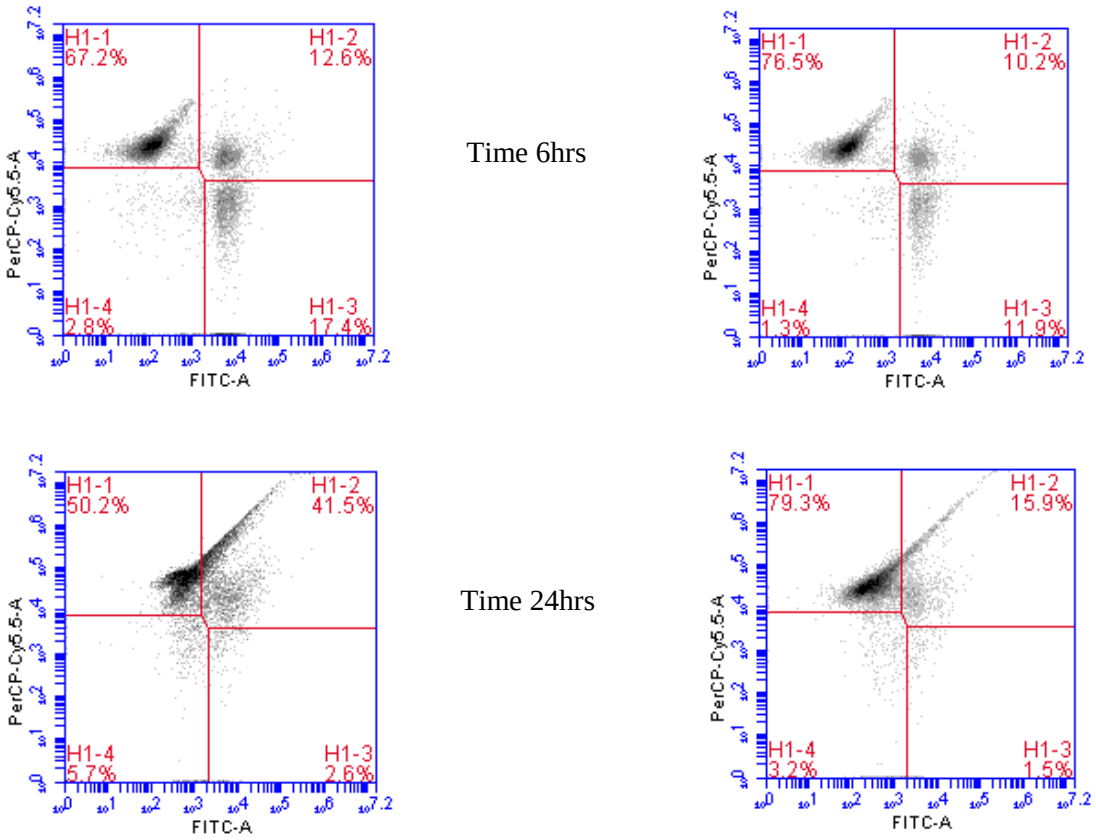
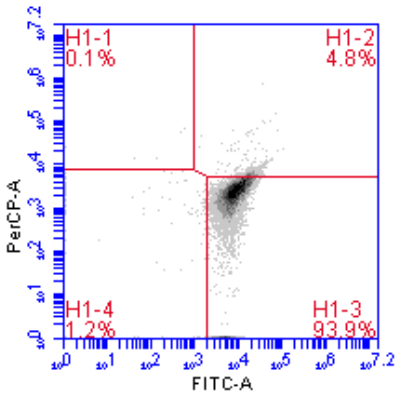


Figure 5. 2 Flow cytometry profiles showing the cell sorting plots of *L. monocytogenes* stationary phase cells directly treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).

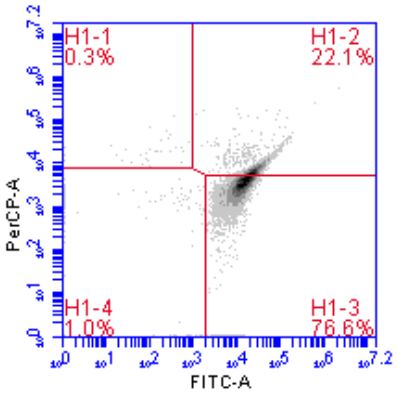
L. monocytogenes grown in TSB, and re-suspended in fresh TSB before treatment with 75µg/ml nisin at different time points was also examined using FC (Figure 5.3). The re-suspended cells were predominantly stained by TO dye demonstrating the "living" population before the nisin treatment. For both strains, >88% the collected cells were detected in the PI channel (H1-1 and H1-2) 10mins after nisin treatment while agar plating 10mins following nisin treatment group retrieved the bulk of the population of ~8 $\log_{10}$ cfu/ml (supplementary table S5.1). This suggests that the majority of the population could have permealized cell membrane as result of nisin treatment leading to the uptake of the PI dye, but the cells with damaged membranes were still able to replicate and grow. However, as a result of the PI uptake, almost all treated cells appeared within the "dead" region in FC analysis, even though these cells were able to be revived on culture. The permealization state continued after 30mins, 1hrs and 2hrs of nisin treatment. After 2hrs of nisin treatment of the M5 strain, the number of detected in the H1-4 quadrant increased with low fluorescence most likely representing as lysed cells as indicated in another study (Amor *et al.*, 2002). The A1 strain started to generate more "lysed" collects with low fluoresence in quatrant H1-4 4hrs after nisin treatment while counts in H1-1 and 1-2 decreased. 24hrs after nisin treatment, the "lysed" cells detected in quadrant H1-4 represented 12.6% of the population for A the1 strain and 5.8% for the M5 strain. The subpopulations representing viable cells in quadrant H1-3 grew from 4hrs, 6hrs and 24hrs of nisin treatment. Although the FC analysis did not achieve the real differeniation of live/dead cells in the early stage of nisin treament (at time 10mins, 30mins, 1hrs and 2hrs) , the results suggested that permeability of cell membrane changes during nisin treament and up to 4hrs of nisin treament persister cells were detected as "intact" structures capable of the uptake of TO dye only.

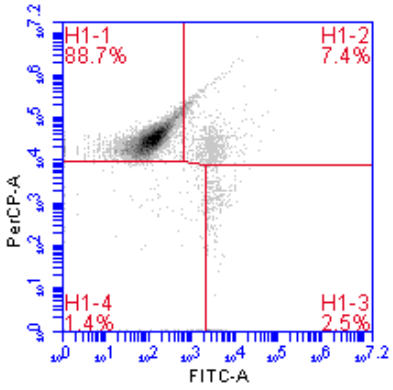
A1 strain

M5 strain

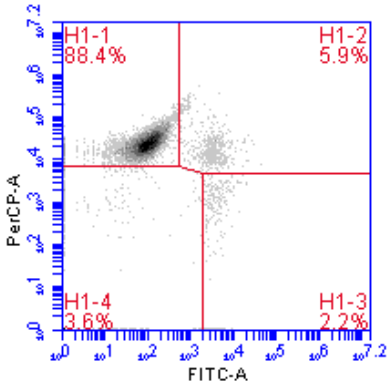


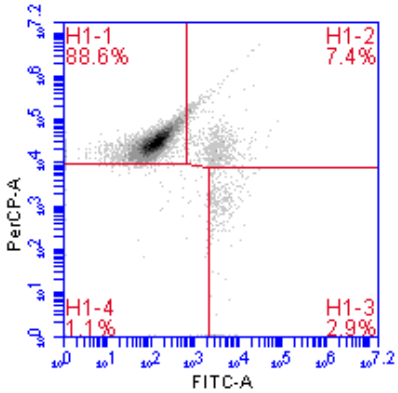
Time 0



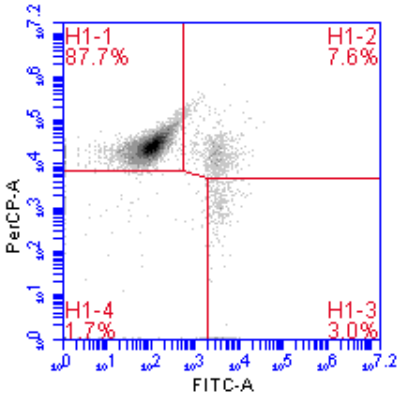


Time 10mins



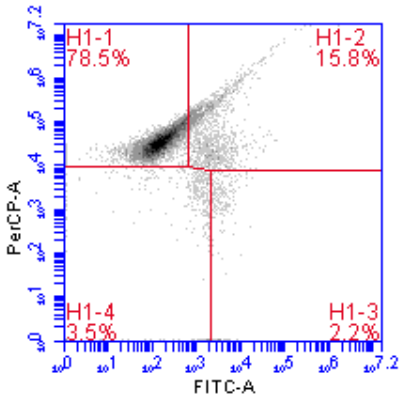


Time 30mins

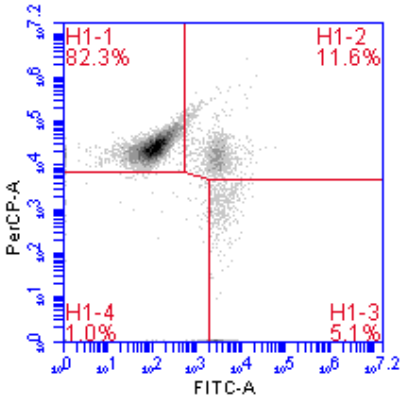


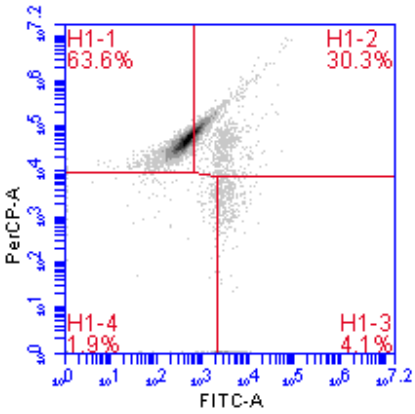
A1 strain

M5 strain

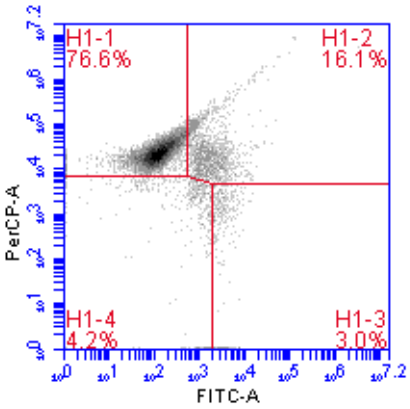


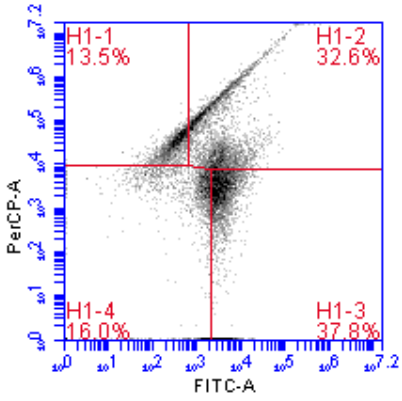
Time 1hrs



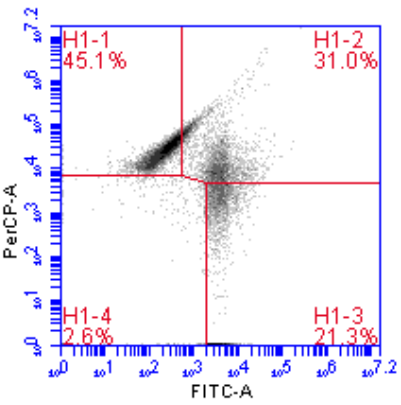


Time 2hrs





Time 4hrs



A1 strain M5 strain

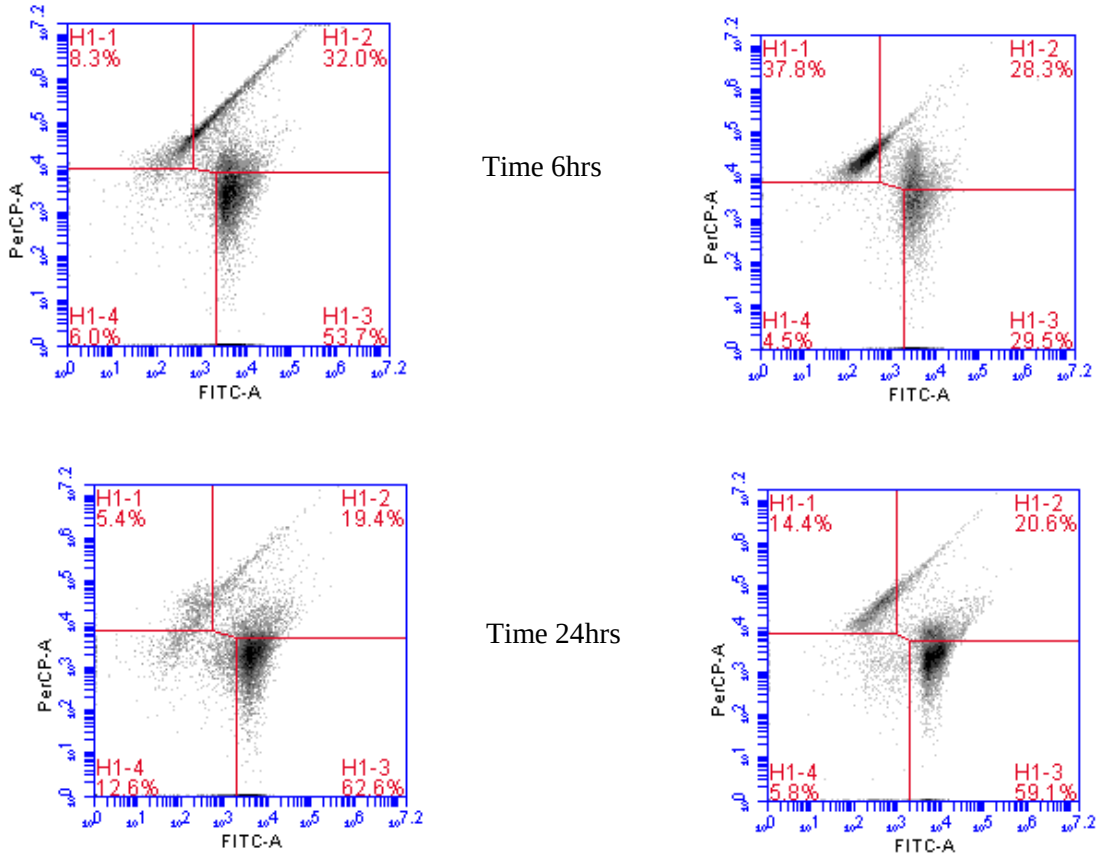


Figure 5. 3 Flow cytometry profiles showing the cell sorting plots of TSB re-suspended *L. monocytogenes* cells treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).

5.3.2 Fluorescence microscopy verification of live/dead staining of *L. monocytogenes* cells during lethal nisin treatment

The fluorescence microscopy observed that log phase cells (live control) predominantly stained green, and heat inactivated cells (dead control) accepted the PI dye resulting in all red staining.

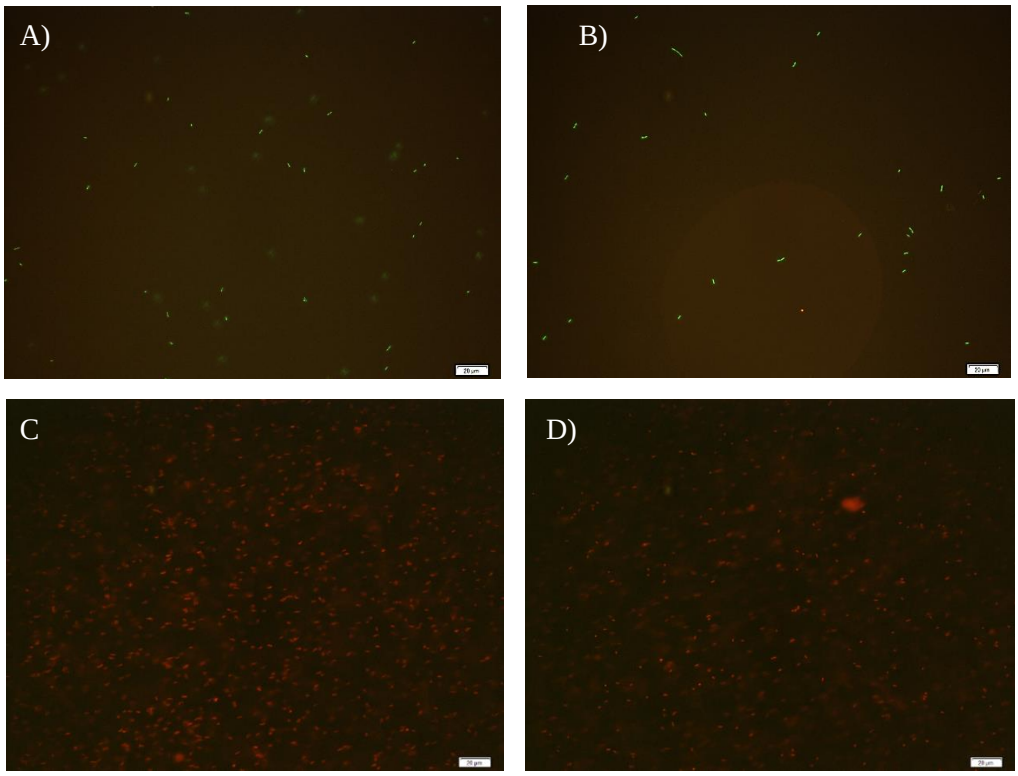
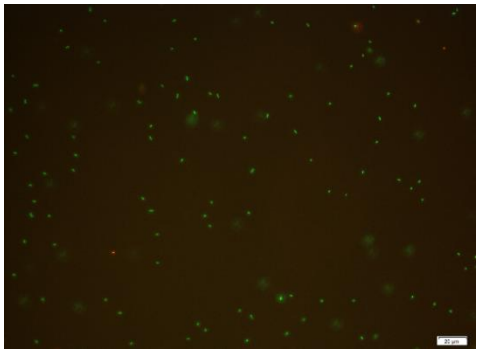
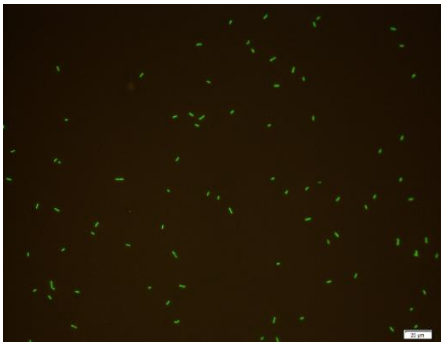
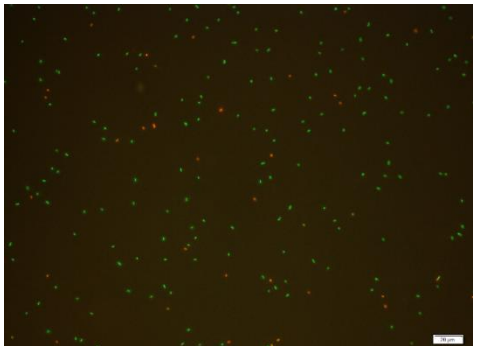
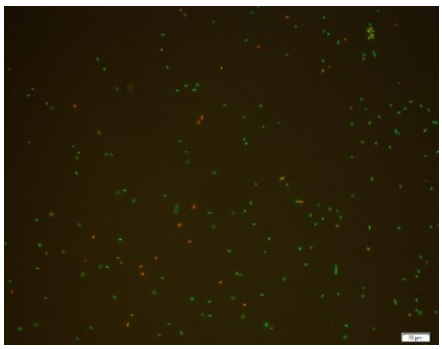
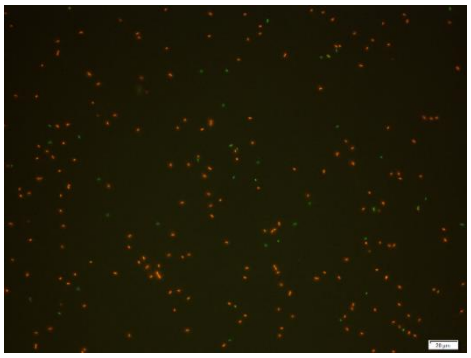
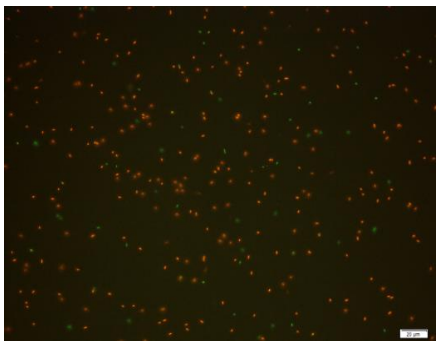
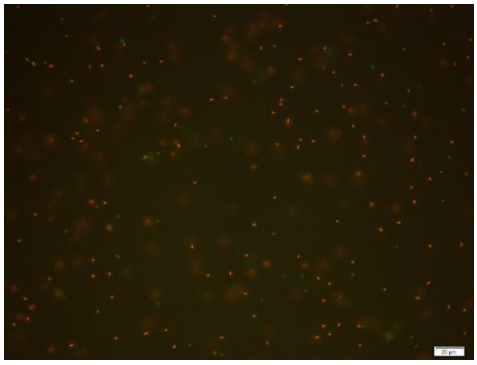
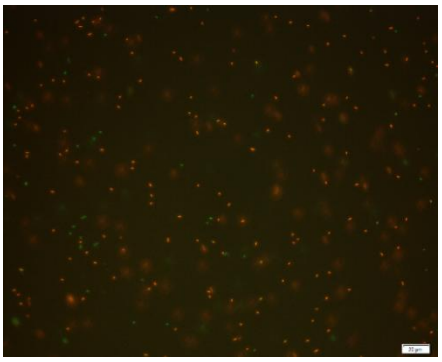


Figure 5. 4 Epifluorescence micrograph of *L. monocytogenes* live control (log phase cells, A and B) and dead control (heat inactivated cells, C and D) for both A1 (A and C) and M5 strain (B and D).

For *L. monocytogenes* stationary phase cells in spent media treated with 75µg/ml nisin at different time points, exposure to lethal nisin resulted in membrane damage after 30mins of treatment, as indicated by the red color of the cells in Figure 5.5. For both strains, the dominant population was stained with PI dye after 2hrs of treatment, and the live cells with green fluorescence were hardly seen under microscopy. The observation indicated that the cell membrane lost integrity during the nisin treatment. The microscopic observation showed consistency with the results from the FC analysis in Figure 5.2.

A1 strain		M5 strain
	Time 0	
	Time 10mins	
	Time 30mins	
	Time 1hrs	

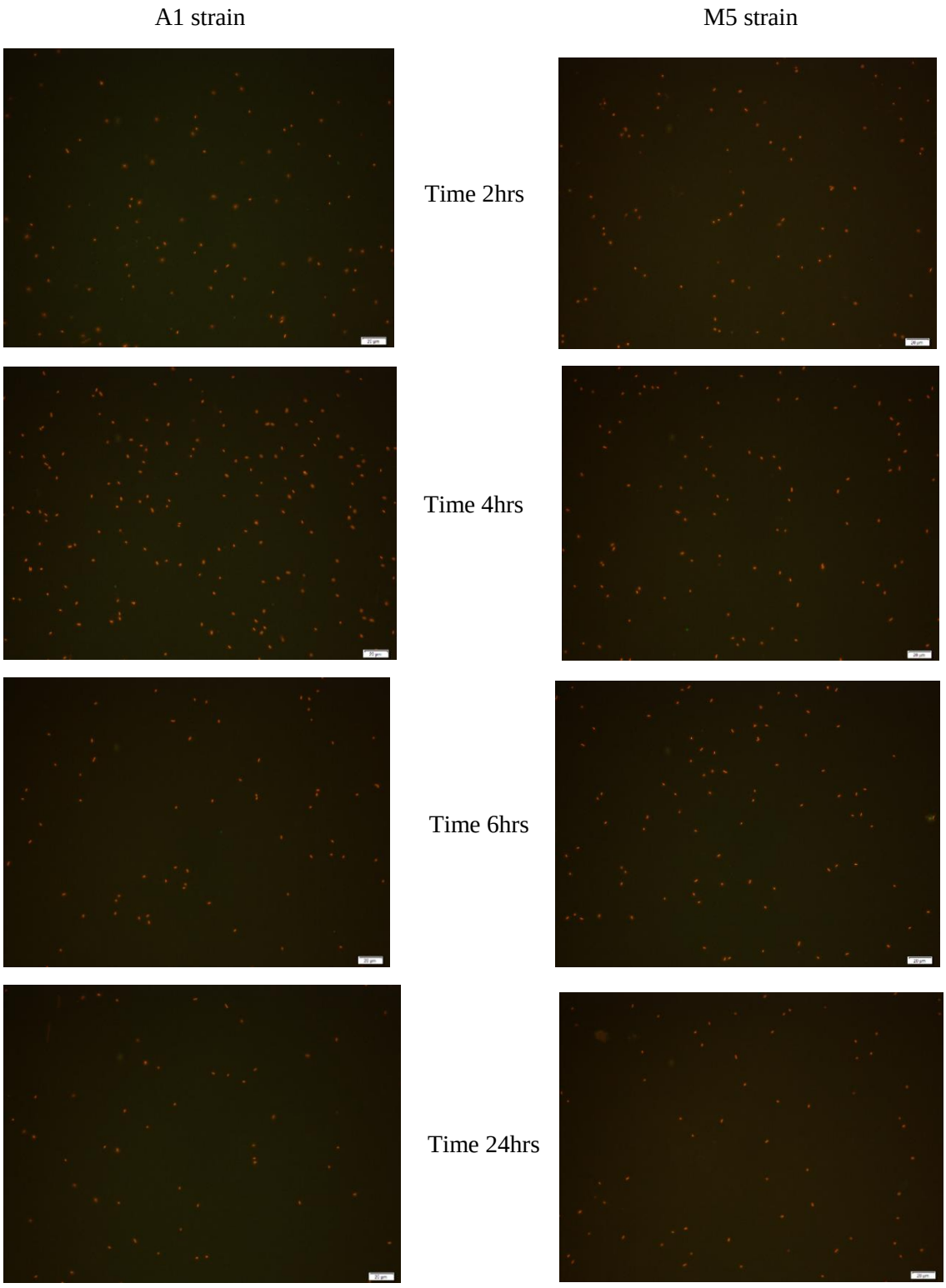
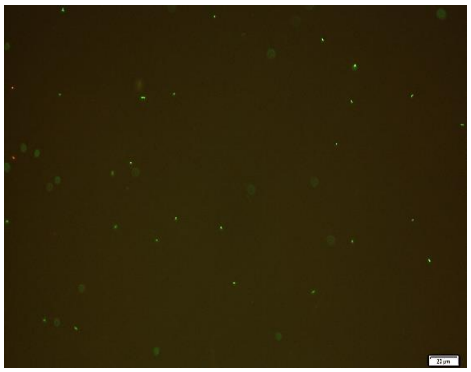
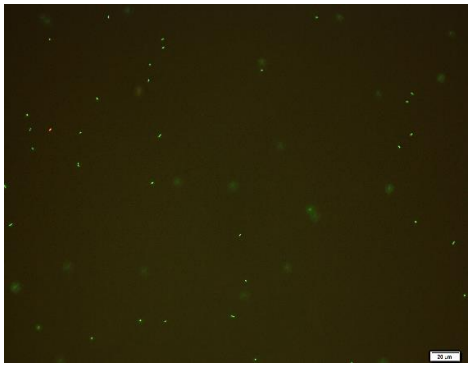
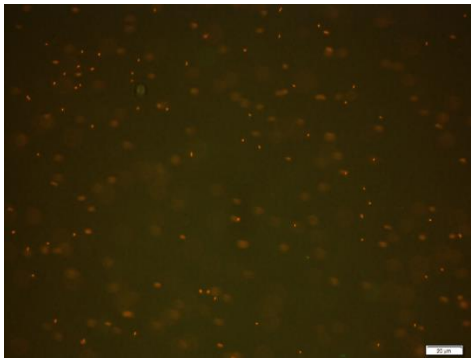
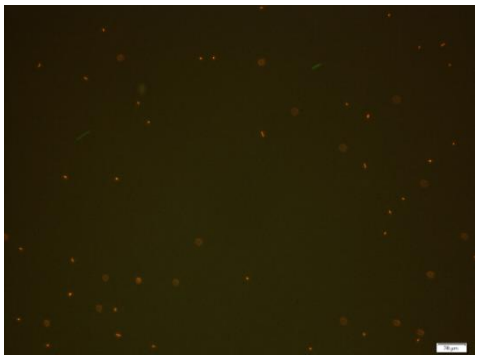
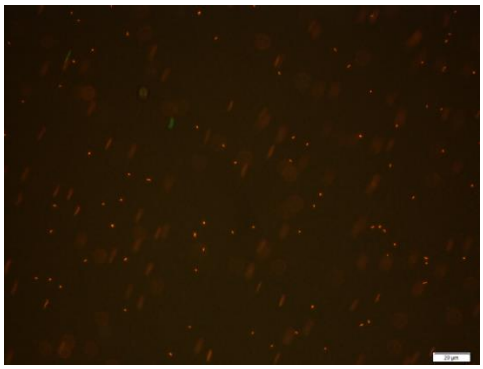
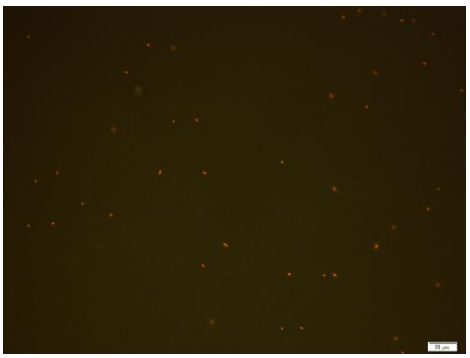
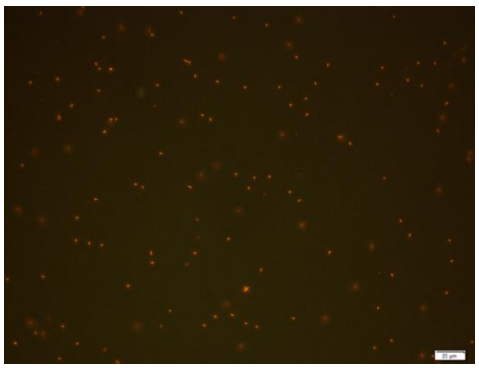
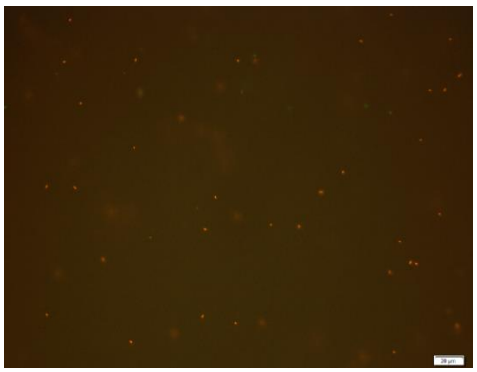


Figure 5. 5 Epifluorescence micrograph profiles showing TO/PI stainings of *L. monocytogenes* stationary phase cells directly treated with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).

When the stationary phase cells were re-suspended in fresh TSB before exposure to lethal nisin, rapid and extensive membrane damage in *L. monocytogenes*, appeared after 10-min exposure resulting in a shift of cell population (>88%) to the "dead" region (Figure 5.3). Microscopy results agreed with the FC results showing 100% PI-positive cells stained with fluorescent red cells (Figure 5.6). However, the cell viability data showed that the PI positive cells during the early stage of nisin treatment were viable and able to be revived when the nisin stress was removed (supplementary table S5.1). After 2hrs of treament, some live cells started to be observed, and at the end of treatment (24hrs) live cells with green fluoresence could been seeen by microscopy, agreeing with the cell differetiation from the FC analysis in Figure 5.3.

A1 strain		M5 strain
	Time 0	
	Time 10mins	
	Time 30mins	
	Time 1hrs	

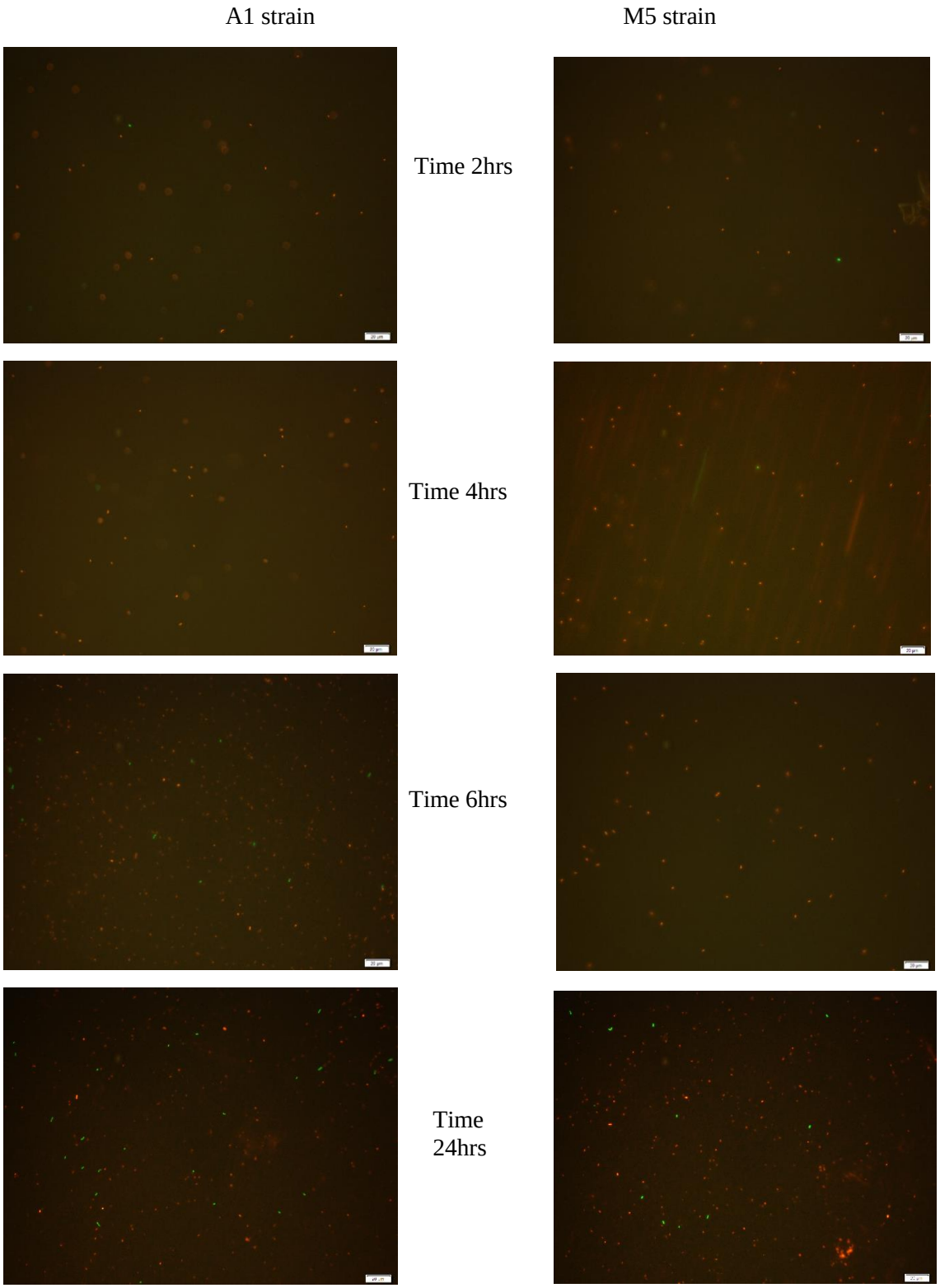


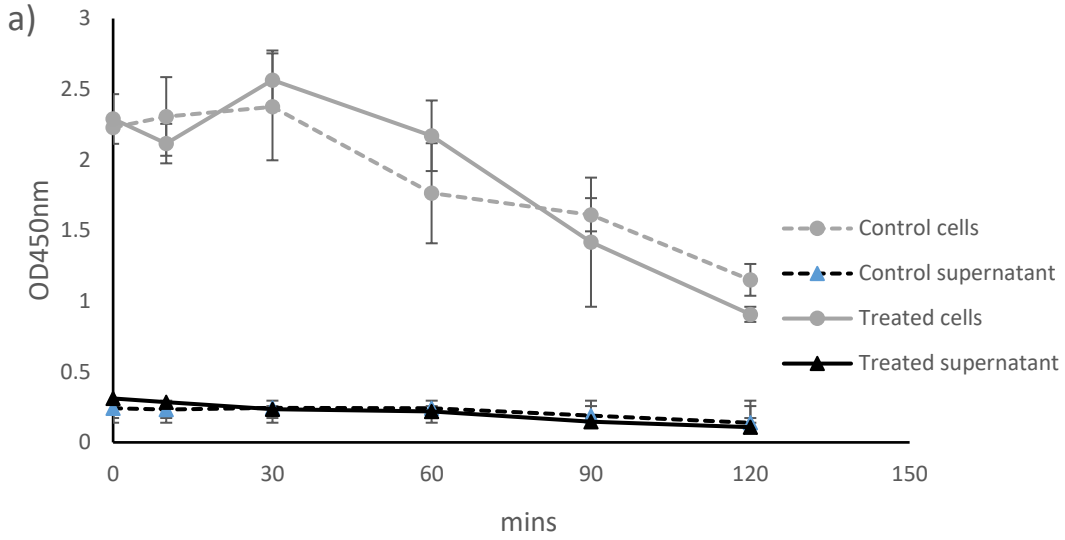
Figure 5. 6 Epifluorescence micrograph profiles showing TO/PI stainings of TSB re-suspended *L. monocytogenes* cells following with 75µg/ml nisin at different time points (time 0, 10mins, 30mins, 1hrs, 2hrs, 4hrs, 6hrs and 24hrs) for A1 strain (left) and M5 strain(right).

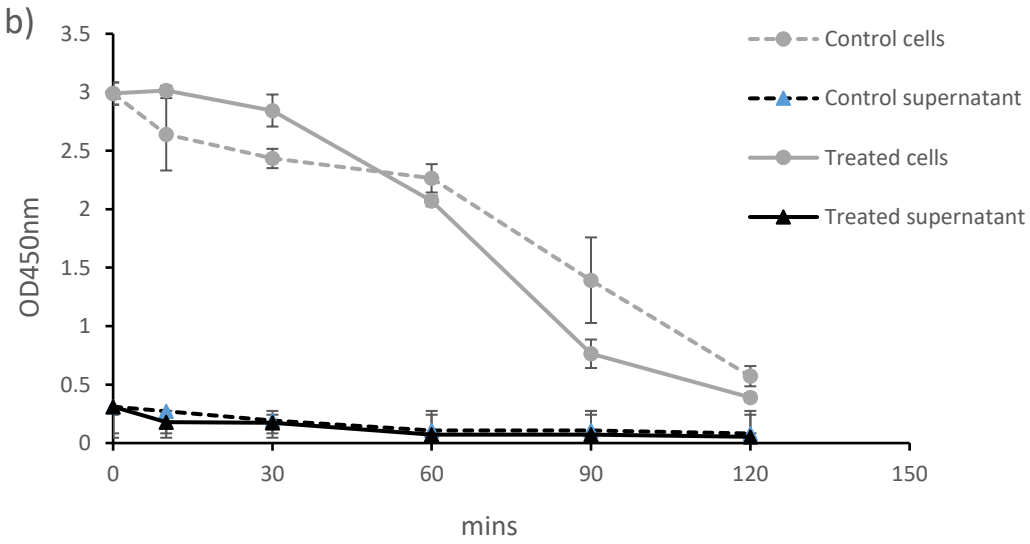
5.3.3 Free radical production on population level of *L. monocytogenes* cells during lethal nisin treatment

Nisin is known to create permeability in the cell membrane allowing the release of the cell constituents. XTT detection allows the viability of cells to be determined and the quantification of superoxide radicals released by the cells. Stationary phase cells of both strains of *L. monocytogenes* treated immediately with 75µg/ml nisin, produced free radicals within 30mins of treatment and the free radicals decreased representing cell death in the bulk population (Supplementary figure S5.1). The culture supernatants from the control and the nisin treated groups did not contain detectable free radicals (Figure 5.7 a and b). Free radicals and hydrogen peroxide may be restrained intracellularly and induce a bactericidal effect through damage to the nucleic acids within cells. Agar plating results showed the numbers of stationary phase cells were inactivated rapidly during nisin treatment while the control group of the whole population remained viable (Supplementary figure S5.1 a and b).

In the cells re-suspended in fresh TSB before exposure to nisin, a high level of free radical production was detected from the cells and the culture supernatants for both strains at time 0 (Figure 5.7 c and d). This indicates that the fresh TSB re-suspension facilitates *L. monocytogenes* existing as a fully respiring cell actively exchanging with the environment. In the A1 strain, the fresh TSB may support the respiratory activity of re-suspended control cells for up to 30mins following nisin treatment (Figure 5.7 c). As the nisin treatment continued, the free radical production from the cells and the supernate both decreased for both stains (Figure 5.7 c and d). This shows the sensitivity of the *L. monocytogenes* to the high concentration of nisin when re-suspended in fresh TSB. The control cells (resuspended in fresh TSB but not exposed to nisin) slowed a gradual decrease in cellular free radical production but a rapid reduction in free radicals in the supernate. Agar plating showed that re-suspended cells were inactivated during the nisin treatment while the control group remained viable (Supplementary Figure S5.1 c and d). In the treated group, the re-suspended A1 cells remained for up to 1hrs of nisin treatment, although the decrease in free radicals indicated a suppression of metabolism under the effect of nisin. The re-

suspended cells of the M5 strain were more sensitive than the re-suspended A1 strain indicated by the earlier cell death in the M5 strain (Supplementary Figure S5.1 c and d).





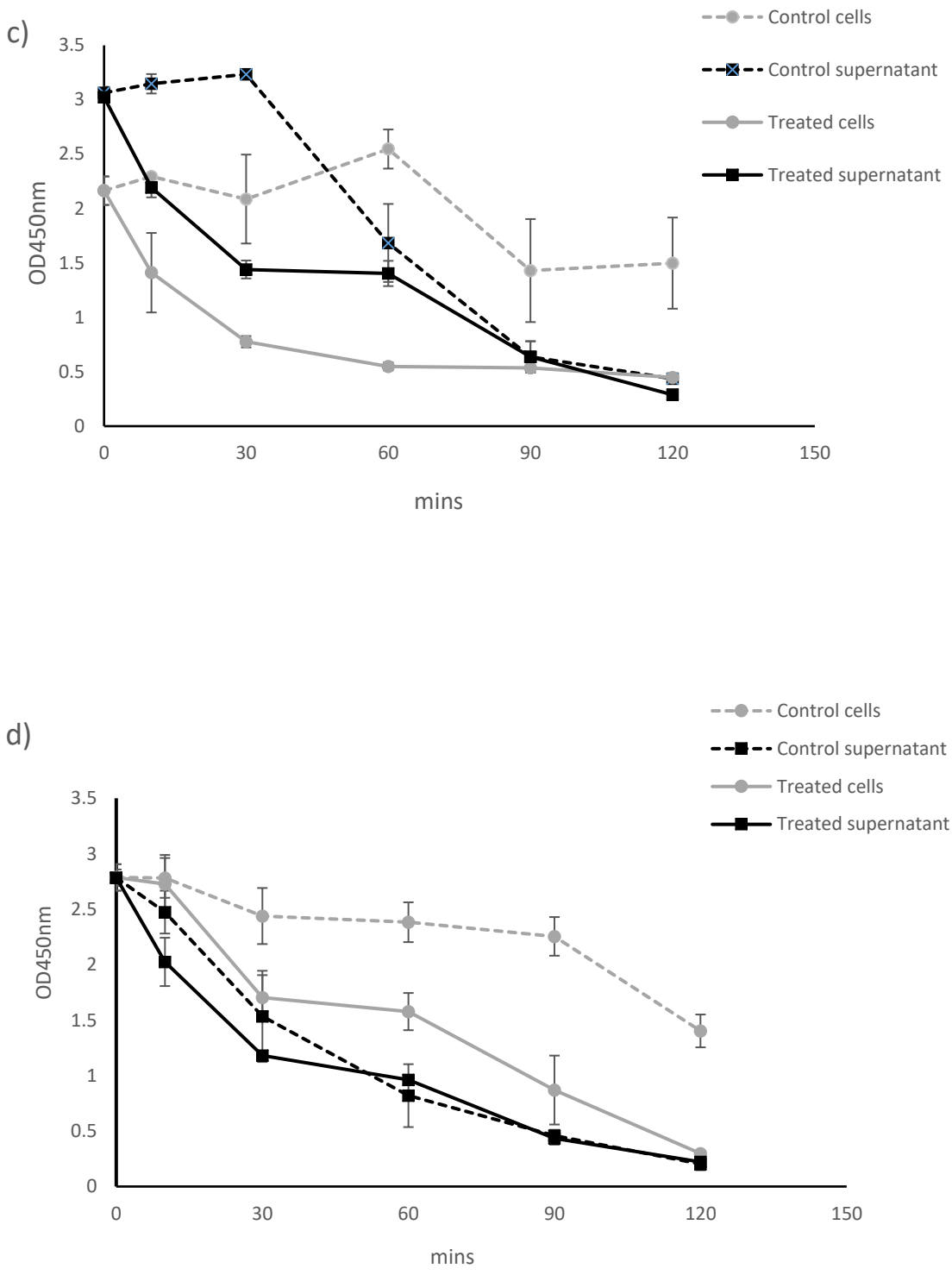


Figure 5. 7 Free radical production measured by the XTT assay, of *L. monocytogenes* under exposure to lethal nisin.

a) A1 stationary phase cells were treated with 75µg/ml nisin; b) M5 stationary phase cells were treated with 75µg/ml nisin; c) fresh TSB re-suspended cells of the A1strain were treated with 75µg/ml nisin; d) fresh TSB re-suspended cells of the M5 strain were treated with 75µg/ml nisin; XTT assays were conducted at times 0, 30, 60, 90, 120mins.

5.4 Discussion

Flow cytometry is a rapid technique with high numerical resolution, capable of measuring the heterogeneity within a population for the rapid quantification of microbial flora (Munoz *et al.*, 2009). The effectiveness of many antimicrobial agents has been examined with FC. For instance, chlorine-based sanitizers (Afari and Hung, 2018), electrolyzed oxidizing water (Zhang *et al.*, 2018), essential oil extract from plants (Paparella *et al.*, 2008; Yuan *et al.*, 2017) and bacteriocins (Swarts *et al.*, 1998) have been used to treat different pathogens including *L. monocytogenes*. These FC analyses have produced estimated compositions of the mixed population (such as intact cells and inactivated cells) when exposed to environmental and chemical conditions that are unfavourable for cell proliferation. The antimicrobial properties of oregano, rosemary and laurel extracts have revealed three different cell populations: living, dead and compromised cells. These were revealed using FC staining with SYTO 9 and PI of *L. monocytogenes*, and live cell percentage was seen to decrease within 8hrs of treatment (Munoz *et al.*, 2009).

The present FC study found that the H1-3 quadrant (as live region) showed a reduction in the percentage of counts during 24hrs of exposure of *L. monocytogenes* to nisin. The H1-2 quadrant collected counts which were dual stained (Figure 5.2) showing a similar staining property compared with the compromised cells from Muñoz 's study (Munoz *et al.*, 2009). The dual staining of cells suggests this subgroup responded the lethal nisin treatment in a different way to the cells in the H1-1 quadrant, representing dead cells (Figure 5.2). The current FC studies were able to find a distinct population, viable but non-culturable cells (VBNC), in the H1-2 quadrant which were stressed and lost their ability to grow on agar medium (Nebe-von-Caron *et al.*, 2000). The culturable population was detected by direct plating in our study. Nisin treatment (24hrs) of a stationary phase population of cells in spent culture medium generated 2.10±0.15 $\log_{10}$ cfu/ml persister cells for the A1 strain and 1.75±0.71 $\log_{10}$ cfu/ml persister cells for the M5 strain. The FC analysis obtained a higher persister cell level at 3.89±0.95 $\log_{10}$ cfu/ml and 3.65±0.13 $\log_{10}$ cfu/ml for the A1 and M5 strains respectively (Supplementary table S5.1). The

re-suspended cell group generated (4.45±0.29 and 4.08±0.16 log_{10} cfu/ml) persister cells for each strain determined by plate counting while the FC analysis produced 5.27±0.39 and 5.25±0.09 log_{10} cfu/ml of persister cells respectively for each strains A1 and M5 (Supplementary table S5.1). Kennedy *et al.* found that intact cells of *L. monocytogenes* discriminated with SYTO 9 and PI staining by FC analysis resulted in 11% - 20% growth on agars (Kennedy *et al.*, 2011). Consistently, the results of the present trial showed that the number of bacteria recovered by plate counting always showed approximately 1 log_{10} cfu/ml less compared with the numbers estimated by FC analysis (Supplementary table S5.1).

The presence of "injured cells" is a concern for microbiologists, as this subpopulation can recover and revert to their physiologically active state in a food or in the human body (Paparella *et al.*, 2008). Multiparametric FC studies have demonstrated that cell permeability, as monitored by PI, is a sensitive marker of cell damage, yet it is a poor indicator of cell death of stressed bacteria (Amor *et al.*, 2002; Joux and Lebaron, 2000). Swarts *et al.* used FC to study the effect of the bacteriocin leucocin B-TA11a on *L. monocytogenes*, and the uptake of the live/dead Baclight dyes suggested that cells remained viable but became leaky, possibly indicating bacteriocin-induced pore formation in the target membranes (Swarts *et al.*, 1998). The present study, found that the re-suspended population became leaky or permealized indicated by the uptake of the PI dye immediately after adding nisin while agar plating proved the whole population to be viable up to 30mins following nisin treatment. At the end of nisin treatment, persister cells were discriminated by FC as intact cells staining with TO suggesting that permeability changed in the population during the 24hrs of nisin treatment which may contribute to boosting persister formation (Figure 5.3 and Supplementary table S5.1). In the re-suspended cells, *L. monocytogenes* possibly triggered alternative pathways as a mechanism to induce the persister formation.

Free radical production is a phenomenon which readily differentiates highly metabolically active cells with a detectable level of cellular respiration (Denyer and Stewart, 1998). Free radical accumulation is one of the mechanisms which leads to an autocidal process when bacteria face environmental stress, as a burst of excess free radicals is lethal to the cell,

not the action of the stress *per se* (Denyer and Stewart, 1998). However, bacterial cells in the stationary phase of growth have restricted metabolic activity and express a survival response which may facilitate cells pre-adapting to stresses (Loewen and Hengge-Aronis, 1994). The stationary phase cells in spent medium in the present trial appear to have been inclined to induce autocide by harboring intracellularly enclosed free radicals, and it is suspected that this contributed to death of the bulk of the population during the lethal nisin treatment. Essentially it appears that the bulk of the population of stationary phase cells in spent medium are unable to adapt to the stress of nisin challenge. The stationary phase cells re-suspended in fresh TSB medium showed active release of free radicals into the external environments prior to the lethal nisin treatment. Membrane damage induced by nisin is likely to result in triggering a stress response (like universal stress proteins, shock proteins) to shut down the respiratory activity (free radical production) and metabolic rate (bio-molecules' exchange with the environment) for retaining vital metabolites which are capable of impeding nisin damage to the cell membrane. Hence, the next step was to examine gene expression of *L. monocytogenes* when facing lethal nisin stress under the two different nutrient conditions (spent medium/ fresh TSB re-suspended medium) which is the subject of the next chapter to clarify possible mechanisms of persister formation at the transcriptional level.

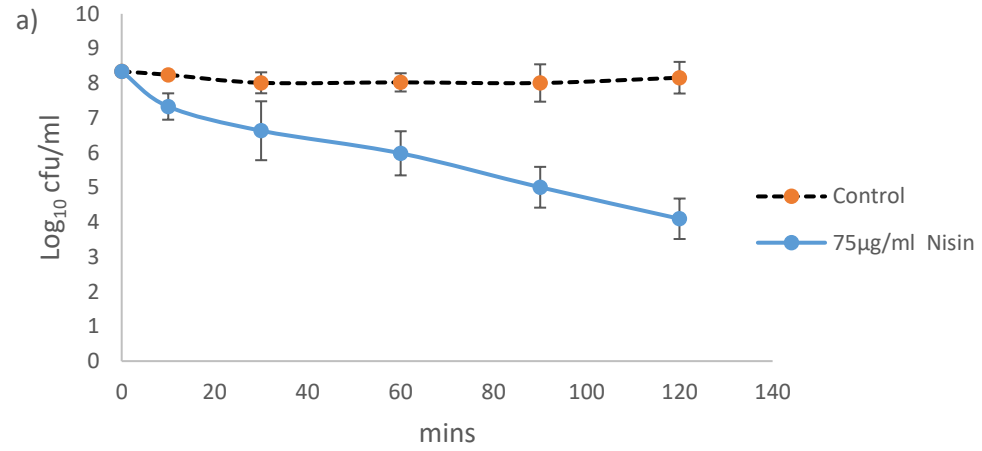
5.5 Acknowledgement

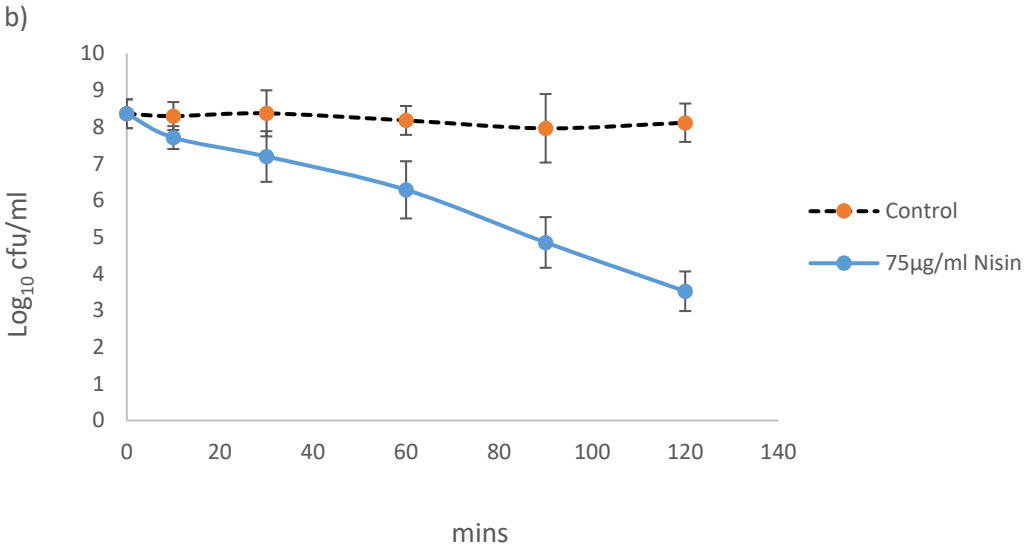
We thank AsureQuality Limited, New Zealand for the kind provision of *L. monocytogenes* strains used in this study. And we thank to Dr. Fran Wolber (from School of Health Sciences, Massey University) for her help with the flow cytometry analysis. This work was supported by postgraduate research support funding of School of Food and Nutrition.

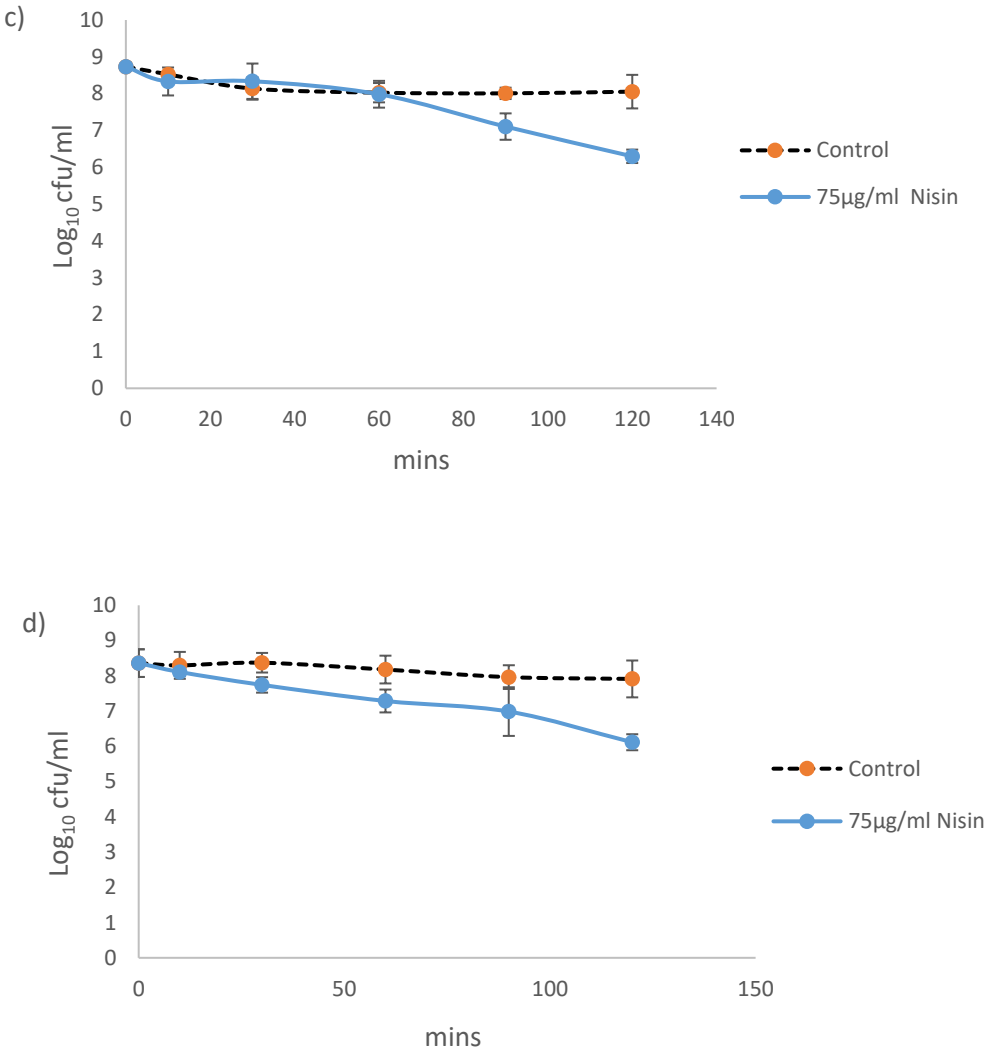
5.6 Supplementary materials

Supplementary table S5. 1 Summary of viable cell number estimation from % event detected from H1-3 quadrant by FC analysis and cell counts (log_{10} cfu/ml) obtained from recovery on agar plating. The measurements were performed in duplicate.

	Stationary phase cells with nisin treatment				TSB re-suspended cells with nisin treatment			
	FC detection (H1-3 quadrant)		Agar counting		FC detection (H1-3 quadrant)		Agar counting	
Time	A1	M5	A1	M5	A1	M5	A1	M5
0	8.67±0.21	7.56±0.74	8.34±0.10	8.59±0.39	8.16±0.36	8.08±0.59	8.73±0.11	8.79±0.23
10mins	7.54±0.25	7.39±0.39	7.33±0.38	7.97±0.31	6.10±0.91	6.04±0.93	8.33±0.37	8.10±0.12
30mins	6.74±0.63	6.66±0.13	6.63±0.85	6.94±0.62	5.46±0.98	5.48±0.21	8.33±0.48	7.74±0.21
1hrs	6.48±0.43	6.34±0.46	5.98±0.64	6.08±0.33	5.34±0.23	5.71±0.57	7.84±0.36	7.28±0.32
2hrs	6.49±0.55	6.31±0.51	4.09±0.58	3.52±0.54	5.61±0.84	5.48±0.21	6.29±0.18	6.10±0.22
4hrs	5.40±0.94	5.21±0.15	2.57±0.26	2.25±0.39	5.58±0.92	5.33±0.38	5.58±0.25	5.17±0.37
6hrs	4.72±0.71	4.55±0.68	2.08±0.21	1.95±0.36	5.21±0.72	5.17±0.69	5.13±0.32	4.39±0.52
24hrs	3.89±0.95	3.65±0.13	2.10±0.15	1.75±0.71	5.27±0.39	5.25±0.09	4.45±0.29	4.08±0.16


a)
Log10 cfu/ml
mins
Control
75µg/ml Nisin


b)
Log10 cfu/ml
mins
Control
75µg/ml Nisin



Supplementary figure S5. 1 Agar plate counting of cell viability under 75µg/ml nisin from different time points (0, 30, 60, 90 and 120 mins)

a) A1 stationary phase cells treated with 75µg/ml nisin; b) M5 stationary phase cells treated with 75µg/ml nisin; c) fresh TSB re-suspended cells of A1strain were with 75µg/ml nisin; d) fresh TSB re-suspended cells of M5 strain treated with 75µg/ml nisin.

5.7 References

Afari, G. K. and Hung, Y.-C. (2018). Detection and Verification of the Viable but Nonculturable (VBNC) State of *Escherichia coli* O157:H7 and *Listeria monocytogenes* Using Flow Cytometry and Standard Plating. *Journal of Food Science*, 83, 1913-1920.

Aldsworth, T. G., Sharman, R. L. and Dodd, C. E. R. (1999). Bacterial suicide through stress. *Cellular and Molecular Life Sciences CMLS*, 56, 378-383.

Amor, K. B., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D., De Vos, W. M., and Abee, T. (2002). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Applied Environmental Microbiology*, 68, 5209-5216.

Cañas-Duarte, S. J., Restrepo, S. and Pedraza, J. M. (2014). Novel Protocol for Persister Cells Isolation. *PLOS ONE*, 9, e88660.

Denyer, S. P. and Stewart, G. S. A. B. (1998). Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation*, 41, 261-268.

Dodd, C. E., Sharman, R. L., Bloomfield, S. F., Booth, I. R. and Stewart, G. S. (1997). Inimical processes: bacterial self-destruction and sub-lethal injury. *Trends in Food Science & Technology*, 8, 238-241.

Fisher, R. A., Gollan, B. and Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology*, 15, 453.

Gill, A. O. and Holley, R. A. (2006). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus* sakei cellular membranes by plant oil aromatics. *International journal of Food Microbiologyogy*, 108, 1-9.

Highmore, C. J., Warner, J. C., Rothwell, S. D., Wilks, S. A. and Keevil, C. W. (2018). Viable-but-Nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson Induced by Chlorine Stress Remain Infectious. *Mbio*, 9, 12.

Joux, F. and Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteriaat single-cell level. *Microbes and infection*, 2, 1523-1535.

Kennedy, D., Cronin, U. P. and Wilkinson, M. G. (2011). Responses of *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* to simulated food processing treatments, determined using fluorescence-activated cell sorting and plate counting. *Applied Environmental Microbiology*, 77, 4657-4668.

Léonard, L., Bouarab Chibane, L., Ouled Bouhedda, B., Degraeve, P. and Oulahal, N. (2016). Recent Advances on Multi-Parameter Flow Cytometry to Characterize Antimicrobial Treatments. *Frontiers in Microbiology*, 7.

Loewen, P. C. and Hengge-Aronis, R. (1994). The role of the sigma factor sigmas (KatF) in bacterial global regulation. *Annual Reviews in Microbiology*, 48, 53-80.

Luppens, S. B., Abee, T. and Oosterom, J. (2001). Effect of benzalkonium chloride on viability and energy metabolism in exponential- and stationary-growth-phase cells of *Listeria monocytogenes*. *Journal of Food Protection*, 64, 476-482.

Munoz, M., Guevara, L., Palop, A., Tabera, J. and Fernandez, P. S. (2009). Determination of the effect of plant essential oils obtained by supercritical fluid extraction on the growth and viability of *Listeria monocytogenes* in broth and food systems using flow cytometry. *Lwt-Food Science and Technology*, 42, 220-227.

Nebe-von-Caron, G., Stephens, P. J., Hewitt, C. J., Powell, J. R. and Badley, R. A. (2000). Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *J Microbiol Methods*, 42, 97-114.

Paparella, A., Taccogna, L., Aguzzi, I., Chaves-Lopez, C., Serio, A., Marsilio, F., and Suzzi, G. (2008). Flow cytometric assessment of the antimicrobial activity of essential oils against *Listeria monocytogenes*. *Food Control*, 19, 1174-1182.

Radzikowski, J. L., Schramke, H. and Heinemann, M. (2017). Bacterial persistence from a system-level perspective. *Curr Opin Biotechnol*, 46, 98-105.

Rees, C. E., Dodd, C. E., Gibson, P. T., Booth, I. R. and Stewart, G. S. (1995). The significance of bacteria in stationary phase to *Food Microbiology*ogy. *International Journal of Food Microbiologyogy*, 28, 263-275.

Shah, D., Zhang, Z., Khodursky, A. B., Kaldalu, N., Kurg, K. and Lewis, K. (2006). Persisters: a distinct physiological state of *E. coli*. *BMC Microbiologyogy*, 6, 53.

Sutherland, M. W. and Learmonth, B. A. (1997). The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. *Free Radic Res*, 27, 283-289.

Swarts, A. J., Hastings, J. W., Roberts, R. F. and von Holy, A. (1998). Flow cytometry demonstrates bacteriocin-induced injury to *Listeria monocytogenes*. *Current Microbiology*, 36, 266-270.

Tracy, B. P., Gaida, S. M. and Papoutsakis, E. T. (2010). Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. *Current Opinion in Biotechnology*, 21, 85-99.

Weeks, M. E., Nebe von Caron, G., James, D. C., Smales, C. M. and Robinson, G. K. (2006). Monitoring changes in nisin susceptibility of *Listeria monocytogenes* Scott A as an indicator of growth phase using FACS. *Journal of Microbiological Methods*, 66, 43-55.

Yuan, W., Lee, H. W. and Yuk, H. G. (2017). Antimicrobial efficacy of Cinnamomum javanicum plant extract against *Listeria monocytogenes* and its application potential with smoked salmon. *International journal of Food Microbiologyogy*, 260, 42-50.

Zhang, C., Chen, X., Xia, X., Li, B. and Hung, Y.-C. (2018). Viability assay of *E. coli* O157: H7 treated with electrolyzed oxidizing water using flow cytometry. *Food Control*, 88, 47-53.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)
We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Shuyan Wu

Name/Title of Principal Supervisor: Steve Flint

Name of Published Research Output and full reference:

Shuyan Wu, Pak-Lam Yu, Steve Flint (2019) Flow cytometry investigation on *Listeria monocytogenes* persistence during lethal nisin treatment. Food Control (submitted)

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: and / or
- Describe the contribution that the candidate has made to the Published Work: *The candidate carried out laboratory work, data collection and analysis, and manuscript preparation guidance of direction and editorial help from the co-authors and supervisors.*

01/12/2019

Candidate's Signature — Date

01/12/2019

Principal Supervisor's signature — Date

GRS Version 3– 16 September 2011

Chapter 6 Transcriptomic study on the persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin

Partial data was published in *Journal of Global Antimicrobial Resistance* 15(2018)25-31

Original publication available at https://doi.org/10.1016/j.jgar.2018.06.003

Shuyan Wu [a], Pak-Lam Yu [b], Dave Wheeler [c], Steve Flint [a, *]

[a] *Massey Institute of Food Science and Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

[b] *School of Engineering & Advanced Technology, Massey University, Riddet Road, 4442, Palmerston North, New Zealand*;

[c] *Institute of Fundamental Science, Massey University, Palmerston North, New Zealand*;

Abstract

The persistence of a *L. monocytogenes* stationary phase population when facing 75μg/ml nisin treatment was determined and RNA Seq analysis was used for gene expression profiling of the persister cells in spent (persister N) and rich (persister TN) media compared with untreated cells. Functional genes associated with the persister populations were identified in multiple systems including heat shock related stress response, cell wall synthesis, ATP-binding cassette (ABC) transport system, phosphotransferase system (PTS system), and SOS/DNA repair. Differences were found in gene regulation between persister N and persister TN populations. Nutrition may be associated with the variations in gene expression resulting in variations in the size or composition of the persistent populations. This study provided information on the gene regulation of persister cells exposed to lethal nisin and provides some insight into possible mechanisms of impeding bacterial persistence.

6.1 Introduction

Persistence of microbial contamination in a food manufacturing environment can result in the long-term survival of a proportion of the population of a pathogen despite repeated treatments with antimicrobials (Nilsson *et al.*, 2012). After sanitation and disinfection, different natural antimicrobials, used as preservatives in food processing and final products can control the growth of heat resistant bacteria but cannot eradicate and kill the persister cells. How *L. monocytogenes* persists and survives in food environments is poorly understood and is important given the low infective dose and high mortality rate associated with this bacterium.

Information on the mechanism of *L. monocytogenes* persistence is limited. Studies investigating persistent *L. monocytogenes* have focussed on sampling *L. monocytogenes* isolates from food environments or food products over a long period of time. The transcriptomic analysis of *L. monocytogenes* isolates from food environments demonstrated that gene regulation associated with carbohydrate metabolism and flagella mediated motility occurred in cells adapted to stress (Soni *et al.*, 2011). Persisters are defined as a subpopulation of bacteria that can survive under the prolonged exposure to antibiotics, and in an antibiotic free environment persisters can switch back to a growing state and generate a new population that is as sensitive to antibiotic treatment as the parental strain (Kint *et al.*, 2012). However, the persistence studies for *L. monocytogenes* using populations that have re-grown from the persistent *L. monocytogenes* population are not a true reflection of the physiological state of persisters. Transcriptomic analyses of *L. monocytogenes* are mainly focused on tolerance to sublethal concentrations of treatments with disinfectants and preservatives and these are not a true reflection of a persister population following exposure to what are assumed to be lethal concentrations of antimicrobials. Nisin is a microbial derived antimicrobial peptide used commercially as a natural preservative in food. This study investigated the lethal antimicrobial impact of nisin on persister formation of *L. monocytogenes*.

Persister cells represent a sub-population of cells that are "temporarily" resistant to antimicrobials. The persister phenotype is believed to be due to distinct changes in gene expression following an antimicrobial challenge, hence this is different from resistant cells (Radzikowski *et al.*, 2017). A previous study (Wu *et al.*, 2017) collected persister cells from *L. monocytogenes* stationary cells exposed to lethal concentrations of nisin (75µg/ml). Persister cells that survived lethal nisin exposure showed heterogeneity in terms of the numbers of persister cells, recovery time, and change in cell permeability under different conditions (spend medium and resuspended in fresh medium) as shown in the previous chapter. Possible mechanisms involved in persister adaption (Balaban, 2011) have used low concentrations of antimicrobial agents, however there does not appear to be any transcriptional level studies that attempt to explore the gene expression associated with *L. monocytogenes* persisters under what are considered lethal concentrations of nisin. The aim of this study was to assess *L. monocytogenes* persisters following exposure to lethal concentrations of nisin by using transcriptome sequencing and subsequent RNA-Seq analysis.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth conditions

The *L. monocytogenes* strain used in the transcriptomic study was from a food manufacturing environment, provided by AsureQuality Limited, New Zealand (labeled as strain A1 in our earlier publication) (Wu *et al.*, 2017). A culture of *L. monocytogenes* A1 strain was obtained from a single colony inoculated into 20 ml tryptic soy broth (TSB) and incubated at 30 °C for 18 h.

Half of the overnight culture was treated with 75μg/ml nisin at 30 °C for 90 mins. The other half of the overnight culture was centrifuged and re-suspended into fresh TSB medium, and then treated with 75μg/ml nisin treatment at 30 °C for 90 mins. The surviving cells from the treated overnight culture were labelled as "**persister N"**. The surviving cells from the treated re-suspended culture were labelled as "**persister TN"**. Stationary phase cells that had not been exposed to nisin treament were used as control (non-persisters) to compare with the persister population.

6.2.2Total RNA extraction

RNA was extracted from the persister N/persister TN cells and the untreated control cells in three independent preparations (in triplicate) using the following procedure. The culture (4 ml) was centrifuged for 10min at 4000 × g and the resultant pellets re-suspended in 800 μl RNAnase free water containing 5 mg/ml lysozyme (Sigma Aldrich) for a 10 mins incubation at 37 °C. The re-suspended cells were mixed by vortex with acid washed glass beads (Sigma Aldrich) for 10 mins, and then centrifuged for 1min at 5000 × g to obtain the supernatant. Total RNA was isolated and purified from the supernatant using the Nucleospin RNA II kit (Macherey–Nagel, Germany). RNA purity was assessed using the Nano Drop (Titertek Berthold, Germany), while RNA integrity was checked by electrophoresis (Invitrogen, USA).

6.2.3 Transcriptome sequencing

With three biological replicates for each group, there were 9 RNA samples in total sent for sequencing on the Illumina sequencing platform. The Otago Genomics & Bioinformatics Facility (University of Otago, Dunedin, New Zealand) prepared the cDNA libraries for the treatment group and the control, and performed the sequencing of cDNA libraries with the Illumina MiSeq system. Output data was sequencing adapter cleaned and base quality trimmed at an error probability of less than 0.01 for subsequent analysis. Mapping of reads to the reference genome *L. monocytogenes EGD-e* (Lim *et al.*, 2016) was performed with Bowtie2 (version 2.3.0).

6.2.4 Differential expression analyses

DESeq2 was used to identify differential expression between the persister cells and the controls (persister N versus control, and persister TN versus control). The false discovery rate approach produced multiple testing corrections for significantly differentially expressed genes. The total read count was determined for each transcript by combining data from sequencing runs for three replicate experiments. Differential expression was determined based on a fold change >1.5. The adjusted *p*-values <0.05 were considered significant. GO (gene ontology) term enrichment was identified by DAVID analysis (https://david.ncifcrf.gov/chartReport.jsp).

6.2.5 RT-qPCR (Reverse Transcription Quantitative PCR) validation

Thirteen differentially expressed genes were selected for RT-qPCR analysis to verify gene expression of RNA-seq. Total RNAs were isolated with the same method mentioned previously and tested using one step RT-qPCR (Luna® Universal One-Step RT-qPCR Kit, Biolab). Quantitation of each transcript was conducted by the Light Cycler 480 platform (Roche Diagnostics) in triplicate. The 16S gene of *L. monocytogenes* was used as the house keeping gene. The primers are listed in Supplementary Table 6.1. The specificity of primers was assessed by using the dissociation curve method and relative gene expressions were calculated by the comparative -ΔΔCp method.

6.3 Results

6.3.1 Diagnostic MA (DESeq2) plots visualized the nisin treatment effect on persister formation in different conditions

The overnight culture of the isolate was exposed to 75μg/ml nisin in spent medium and re-suspended fresh medium for 90mins. The overnight culture before treatment was used as control cells for gene expression comparisons. Total RNA was extracted from 3 replicates of each untreated and treated groups for cDNA libraries construction and sequencing. On average, 2.08 million high quality reads were obtained for each library. Successfully aligned reads covered around 91.05% of the reference genome of *L. monocytogenes EGD-e* (serotype 1/2a, GenBank accession numbers NC_003210). This study used the closely related *L. monocytogenes EGD-e* genome as a reference since the genome of the tested food isolate has not been sequenced. The differential expression analysis identified total 296 genes above a log2 fold change of 1.5 in persister N compared to the control (Figure 6.1). In total, 103 genes were selected as they had a known function with 49 genes up-regulated and 54 genes down-regulated. There were 356 genes above a log2 fold change of 1.5 in persister TN compared to the control (Figure 6.2). 182 genes were selected out with their known functions with 85 genes up-regulated and 97 genes down-regulated.

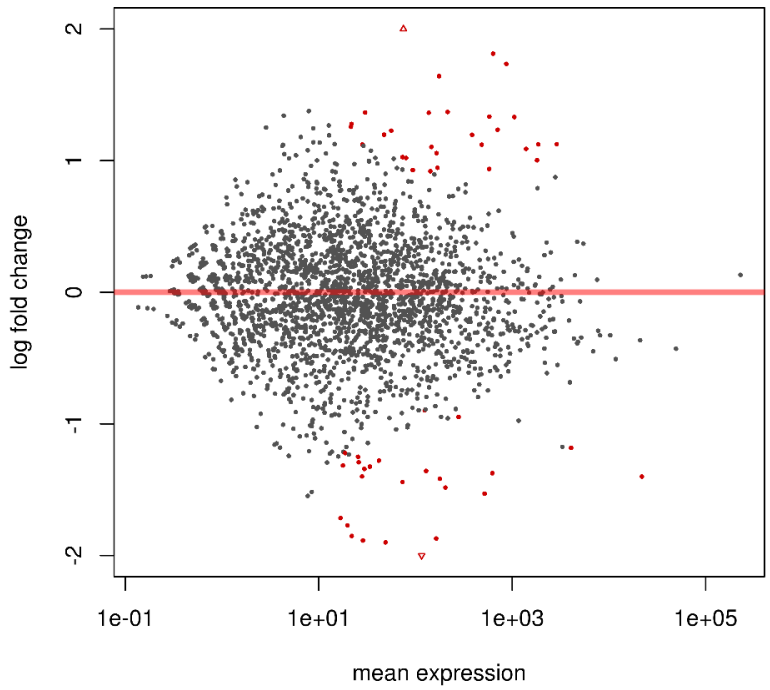


Figure 6. 1 MA plots show the log2 fold changes from the treatment over the mean of normalised counts for the blank versus the persister N sample.

Values are represented by black dots (not significantly differentially expressed) and red dots (significant) and triangles (fall outside the x-axis range)

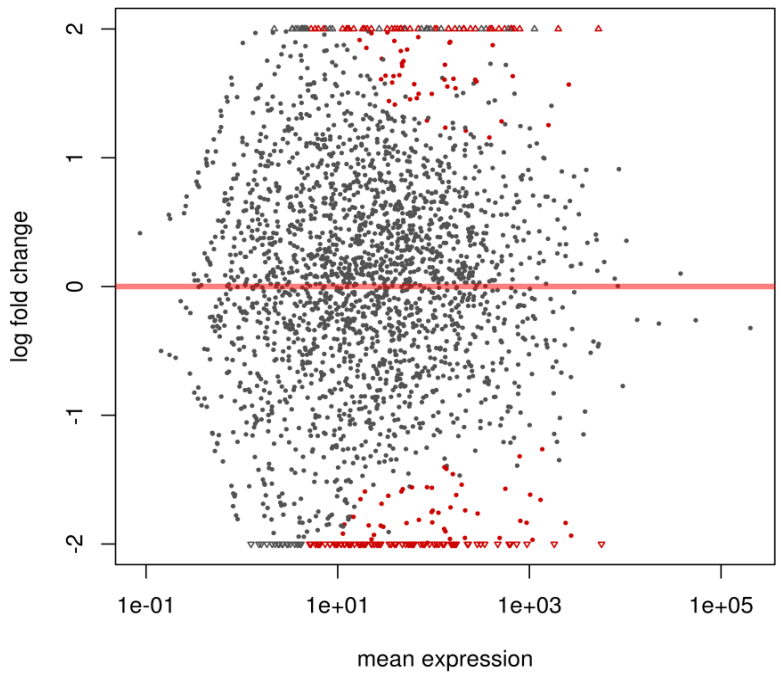


Figure 6. 2 MA plots show the log2 fold changes from the treatment over the mean of normalised counts for the blank versus the persister TN sample.

Values are represented by black dots (not significantly differentially expressed) and red dots (significant) and triangles (fall outside the x-axis range)

6.3.2 RNA-Seq analysed data on the log2 fold changes over the mean of normalised counts and significant genes from the nisin treatment highlighted in persister N and persister TN.

Significantly differentially expressed genes were detected in both pairwise comparisons. The N versus TN comparison had the stronger treatment effect with enrichment for translation functional annotations. There was a complex gene expression difference between persister N and persister TN when compared to control cells, and functional groups of genes were identified as being involved in multiple systems, such as heat shock related stress response, cell wall synthesis, ATP-binding cassette (ABC) transport system, phosphotransferase system (PTS system), and SOS/DNA repair. Differences were found within the multi-systematic gene regulation between persister N (Table 6.1) and persister TN (Table 6.2). GO term enrichment is identified by DAVID analysis, and the translation (Ribosome) term is shown as a significant result in KEGG ((Kyoto Encyclopedia of Genes and Genomes) enrichment (Figure 6.3). The precision of the reads result is not fully obtained when using *L. monocytogenes EGD-e* genome as a reference. A reasonably high number of SNPs (single nucleotide polymorphisms) in the reads were detected in our strain versus the reference, which may indicate the food isolate used in the study is somewhat divergent from the reference strain in the analysis. The results that are limited in that genes specific to the food isolate would not have been examined in this transcriptomic study. Using the food isolate's own fully sequenced genome as a reference would produce more comprehensive image of how this *L. monocytogenes* food environmental isolate survivied under lethal nisin exposure. However, the results from this study are realtively useful in identifying responses that may help in understanding the pathways *L. monocytogenes* may commonly utilize for persister cell formation when facing a lethal dose of the antimicrobial nisin. Common responses identified here may facilitate targeting control of these persistent survivors.

Table 6. 1 a list of interested genes which change in persister N group

Gene accession number[a]	Gene name[b]	Functional Protein	Fold change[c]
Stress response			
CAC99658	lmo1580	Usp, Universal stress protein	2.89
CAC99021	fri	non-heme iron-binding ferritin	2.79
CAD00094	cspB	cspB, cold-shock protein	2.10
CAC98654	lmo0575	GntR family transcriptional regulator	-2.00
CAD00546	clpP	ATP-dependent Clp protease proteolytic subunit	-2.79
CAC99041	lmo0963	HtpX, heat shock protein	-2.91
CAC99075	clpE	ATP-dependent protease	-3.20
CAD00284	clpB	Clp protease subunit B	-3.21
CAC99216	lmo1138	ATP-dependent Clp protease proteolytic subunit	-3.49
CAD00082	lmo2004	GntR family transcriptional regulator	-4.91
Cell wall synthesis			
CAC98344	lmo0129	N-acetylmuramoyl-L-alanine amidase	4.09
CAC99050	dltC	D-alanine--poly(phosphoribitol) ligase subunit 2	2.79
CAC99162	lmo1084	DTDP-L-rhamnose synthetase	2.44
CAC99158	lmo1080	GgaB, teichoic acid biosynthesis protein	2.32
CAC99369	lmo1291	acyltransferase	2.18
CAD00596	lmo2518	hypothetical protein LytR family transcriptional regulator	-2.30
CAD00927	lmo2714	pepdidoglycan bound protein	-3.12
ATP-binding cassette (ABC) transport system			
CAC99714	lmo1636	ABC transporter ATP-binding protein	3.58
CAC99502	lmo1424	manganese transporter	3.16
CAC99466	tcsA	Substrate-binding compound of unspecified monosaccharide ABC transport system	-3.94
CAC99808	lmo1730	sugar ABC transporter substrate-binding protein	-3.49
CAD00805	lmo0278	sugar ABC transporter ATP-binding protein	-2.64
Phosphotransferase system(PTS system)			
CAD00881	lmo2668	BglG	3.33
CAD00880	lmo2667	PTS galacticol transporter subunit IIA	2.92
CAC98242	lmo0027	PTS beta-glucoside transporter subunit IIABC	2.01
CAD00946	lmo2733	PTS fructose transporter subunit IIABC	-2.12
CAC99797	lmo1719	PTS lichenan transporter subunit IIA	-1.96

DNA repair and damage			
CAD00053	lmo1975	DNA polymerase IV	-4.03
CAC99400	nusA	transcription elongation factor (DNA repair and damage tolerance pathways)	-2.57
CAC99380	lmo1302	hypothetical protein LexA family transcriptional regulator	-2.54
CAD00567	uvrB	excinuclease ABC subunit B	-2.42
CAC99476	recA	RecA	-2.29
CAC98221	gyrB	DNA gyrase subunit B	-2.10
CAC99643	polA	DNA polymerase I (repair)	-1.98
Phage/prophage			
CAC98336	lmo0121	hypothetical phage tail protein (membrane protein)	3.86
CAC98338	lmo0123	hypothetical prophage tail protein; prophage endopeptidase tail	3.69
CAC98337	lmo0122	Phage tail protein	3.42
Other			
CAC98264	lmo0049	Hypothetical AgrD protein	8.38
CAC98266	lmo0051	Response regulator	4.47
CAC98332	lmaB	antigen B	3.31
CAC98331	lmaC	antigen C	2.94
CAC99494	lmo1416	VanZ, Glycopeptide antibiotics resistance protein	2.78
CAC99626	mreB	rod shape-determining protein	2.46
CAC99377	glnA	glutamine synthetase	-2.61
CAC99931	lmo1853	hypothetical protein HMA	-2.56
CAC99492	lmo1414	acetyl-CoA:acetyltransferase	-2.42
CAC99293	lmo1215	Flagellum-specific peptidoglycan hydrolase FlgJ	-1.96

[a] All identified genes that showed significantly differential expression in persister N over blank cells(stationary phase cells without nisin treatment) are listed with an adjusted P value <0.05 and a fold-change of ≥ 1.50.

[b] Listed gene names correspond to the gene designations for the reference strain *L. monocytogenes EGD-e* from the ensembl genomes repository (ftp://ftp.ensemblgenomes.org/pub/bacteria/release-34/fasta/bacteria_0_collection/*Listeria*_monocytogenes_egd_e/dna/).

[c] Positive changes indicate genes that have higher transcript levels in the persister N; negative changes indicate genes that have lower transcript leves in the persister N.

Table 6. 2 a list of interested genes which change in persister TN

Gene accession number[a]	Gene name[b]	Functional Protein	Fold change[c]
Stress response			
CAD00814	lmo0287	two-component response regulator	3.94
CAD00747	ftsH	cell division protein FtsH	2.07
CAC99455	lisR	two-component response regulator	-4.12
CAC99658	lmo1580	Usp, Universal stress protein family	-3.65
CAD00094	cspB	cold-shock protein	-2.92
CAC99021	fri	non-heme iron-binding ferritin	-2.76
CAC99216	lmo1138	ATP-dependent Clp protease proteolytic subunit	-2.51
CAD00147	groES	co-chaperonin GroES	-2.17
Cell wall synthesis			
CAD00600	lmo2522	cell wall-binding protein	3.72
CAC99051	dltB	DltB protein for D-alanine esterification	-3.03
CAD00927	lmo2714	pepdidoglycan bound protein	-2.26
CAC99052	dltA	D-alanine--poly(phosphoribitol) ligase subunit 1	-2.13
CAC98344	lmo0129	N-acetylmuramoyl-L-alanine amidase	-5.45
ABC transport system			
CAC99509	lmo1431	ABC transporter ATP-binding protein	4.34
CAC99525	zurA	metal (zinc) transport protein(ABC transporter,ATP-binding protein)	3.61
CAC98997	lmo0919	antibiotic ABC transporter ATP-binding protein	3.42
CAC98367	lmo0152	peptide ABC transporter substrate-binding protein (KEGG quorum sense)	2.39
CAC98350	lmo0135	peptide ABC transporter substrate-binding protein (KEGG quorum sense)	2.27
CAC98937	lmo0859	sugar ABC transporter substrate-binding protein	-6.63
CAC99808	lmo1730	sugar ABC transporter substrate-binding protein	-5.24
CAD00805	lmo0278	sugar ABC transporter ATP-binding protein	-2.50
CAC99466	tcsA	Substrate-binding compound of unspecified monosaccharide ABC transport system	-1.99
PTS system			
CAC98979	lmo0901	PTS cellbiose transporter subunit IIC	-6.19

CAD00896	lmo2683	PTS cellbiose transporter subunit IIB	-6.07
CAD00897	lmo2684	PTS cellbiose transporter subunit IIC	-4.16
CAC98311	lmo0096	PTS mannose transporter subunit IIAB	-3.75
CAC98862	lmo0784	PTS mannose transporter subunit IIB	-3.29
CAC99797	lmo1719	PTS lichenan transporter subunit IIA	-2.30
DNA Repair			
CAC98377	lmo0162	DNA polymerase III subunit delta'	3.82
CAC98220	recF	RecF protein SOS response	3.36
CAD00773	nusG	transcription antitermination protein NusG	2.77
CAC99400	nusA	transcription elongation factor NusA	2.71
CAD00915	recR	recombination protein RecR	2.65
CAD00053	lmo1975	DNA polymerase IV	2.11
CAC98379	lmo0164	YabA, DNA replication intiation control protein	2.09
CAC99959	lmo1881	5'-3' exonuclease	2.06
CAC99660	lmo1582	hypothetical protein YtxK, Adenine-specific DNA methylase	1.99
CAD00379	lmo2301	terminase	-7.1**5**
CAD00012	hup	DNA-binding protein HU	-5.91
CAD00889	lmo2676	DNA polymerase IV	-3.06
CAC99380	lmo1302	LexA family transcriptional regulator	-1.96
Phage/prophage			
CAC98336	lmo0121	phage tail protein	-6.71
CAC98338	lmo0123	hypothetical protein Prophage_tail; Prophage endopeptidase tail	-5.56
CAC98337	lmo0122	hypothetical protein Phage tail protein	-5.45
CAC98343	lmo0128	hypothetical protein Phage-related holin	-4.78
CAD00381	lmo2303	hypothetical protein phage transcriptional regulator, ArpU family	-4.45
Other			
CAD00511	lmo2433	acetylesterase	3.57
CAC98378	lmo0163	hypothetical protein YaaT Cell fate regulator	2.10
CAD00634	fbaA	fructose-1,6-bisphosphate aldolase	1.99
CAC99371	glpD	glycerol-3-phosphate dehydrogenase	-3.50
CAC98768	flaA	flagellin protein	-3.29
CAC99864	inlC	internalin C	-2.92
CAD00727	prfA	listeriolysin positive regulatory protein	-2.22
CAC98332	lmaB	antigen B	-6.46
CAC98333	lmaA	antigen A	-6.28

[a] All identified genes that showed significantly differential expression in persister TN over blank cells(stationary phase cells without nisin treatment) are listed with an adjusted P value <0.05 and a fold-change of ≥ 1.50.

[b] Listed gene names correspond to the gene designations for the reference strain *L. monocytogenes EGD-e* from the ensemble genomes repository (ftp://ftp.ensemblgenomes.org/pub/bacteria/release-34/fasta/bacteria_0_collection/*Listeria*_monocytogenes_egd_e/dna/).

[c] Positive changes indicate genes that have higher transcript levels in the persister TN; negative changes indicate genes that have lower transcript leves in the persister TN.

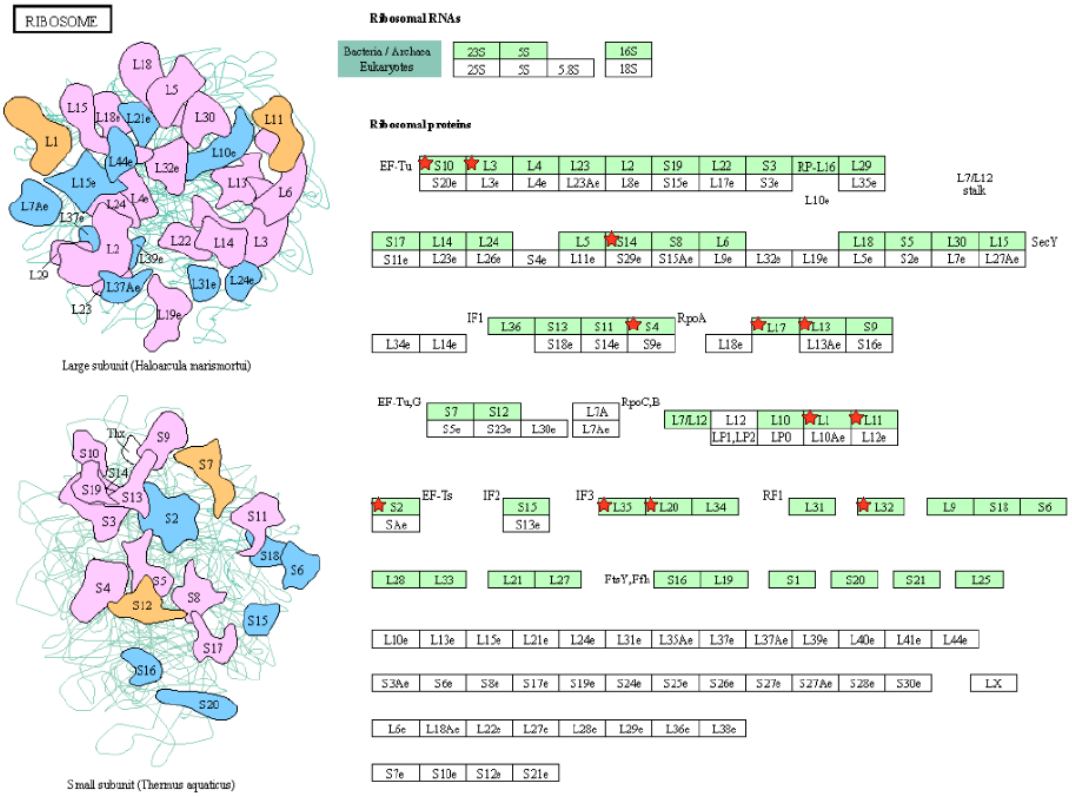


Figure 6. 3 KEGG pathway enrichment is found in differentially expressed genes of group N versus TN. The red stars are genes in the dataset that were significantly differentially expressed.

6.3.3 RT-qPCR validation

This analysis manually selected 13 significantly differential genes which were detected in RNA Seq DEGs data both in the persister N and TN groups. RT-qPCR validation in the persister N group showed that four (csp, lmo1580, lmo2714 and lmo0278) out of 13 genes had agreed regulating effects from RNA-Seq while the other 9 genes failed to validate the RNA-Seq results (Table 6.3). Either extracting more RNA samples or selecting other candidate genes is needed to confirm the results for the persister N group. In the persister N group, spent medium became an inevitable and stressful condition when persister N cells were facing lethal nisin treatment. The cells in the nutrient stressed environment struggled and therefore the response is likely to be inconsistent compared with a less stressful environment. This could explain the discrepancy between RNA-Seq and RT-PCR in persister N group.

Meanwhile, RT-qPCR validation of the persister TN group showed that the expression of all 13 genes was consistent with that shown by RNA Seq. As shown in Figure 6.4, RT-qPCR data correlated well with the RNA-seq data (R^2= 0.8715) according to the fold change and expression level. Performing qPCR on a new set of RNA samples gives more confidence in the response in the persister TN group.

Table 6. 3 RT-qPCR validation in persister N and TN group.

	Persister N		**Persister TN**	
	RNA Seq	RT	RNA Seq	RT
fri	2.79	0.673617	-2.76	0.705475
csp	2.1	7.412704	-2.92	0.612734
lmo1138	-3.49	2.094588	-2.51	0.585064
lmo1580	2.89	1.918528	-3.65	0.570382
lmo2714	-3.12	0.270431	-2.26	0.66281
lmo0129	4.09	0.884745	-5.445	0.332171
lmo1730	-3.49	2.08012	-5.24	0.339151
lmo0278	-2.64	0.004158	-2.5	0.110338
tcsA	-3.94	1.33484	-1.99	0.388683
lmo1719	-1.96	2.244924	-2.3	0.368567
LmaB	3.31	0.99539	-6.46	0.317171
lmo1975	-4.03	2.525671	2.11	2.770219
nusA	-2.57	6.852672	2.71	1.183724

*Positive value presents gene up-regulation and negative value means down-regulation in RNA Seq; value >1 presents gene up-regulation when 0< value<1 means down-regulation in RT-PCR.

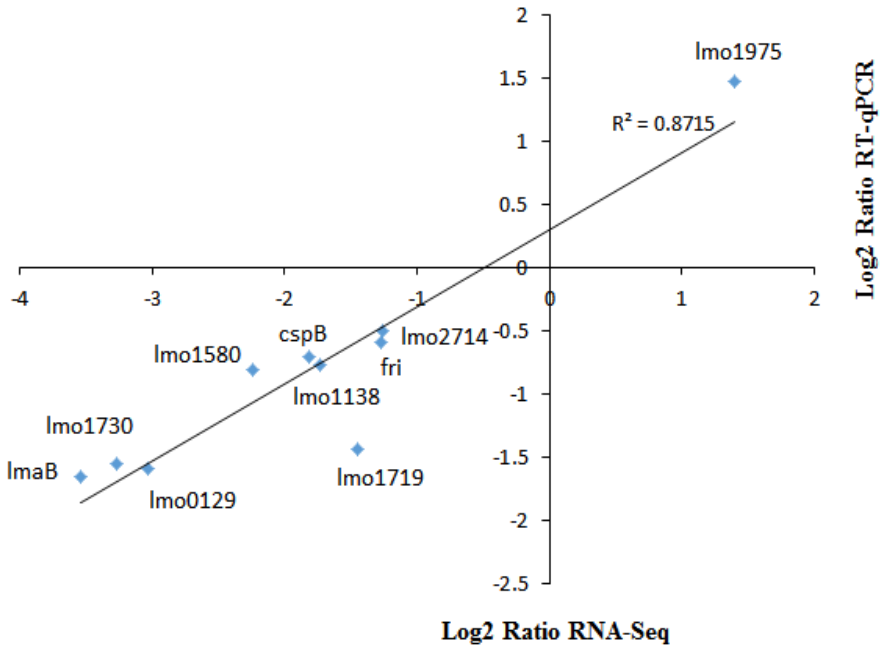


Figure 6. 4 RT-qPCR validation in persister of TN group. X axis represents Log2 Ratio of RNA-Seq data, and Y axis represents Log2 Ratio of RT-qPCT results.

6.4 Discussion

6.4.1 Stress response

Stress proteins Usp, Fri, and Csp are important for bacteria to survive under different stress conditions. Usp and Fri in *L. monocytogenes* have been shown to respond to oxidative conditions and intracellular survival within macrophages (Dussurget *et al.*, 2005; Gomes *et al.*, 2011). CspB of *L. monocytogenes*, is a main protein in the Csp family, associated with promoting host cell invasion, adapting to oxidative stress and osmotic stress (Loepfe *et al.*, 2010; Schmid *et al.*, 2009; Wemekamp-Kamphuis *et al.*, 2002). Tasara reported that the csp gene transcripts were induced under nisin tolerance in *L. monocytogenes* (Tasara *et al.*, 2015). The persister N in our study showed significant up-regulation in usp, fri and cspB gene expression when exposed to a lethal concentration of nisin (Table 6.1). Meanwhile, the persister TN showed down-regulation of these three genes as the surviving TN cells endured the lethal nisin exposure in fresh TSB medium. The fresh nutrient might physiologically contribute to the production of more persister TN cells by discriminately expressing usp, fri and cspB genes.

The generation of persister N or persister TN cells could also be associated with expression of other regulators and heat shock genes. GntR regulators and the heat shock gene (htpX) were detected in persister N. The GntR family is one of the global regulatory systems to adapt and respond to a wide variety of different environments in *L. monocytogenes* (Buchrieser *et al.*, 2003). HtpX is a heat inducible shock gene regulated by sigma factor found in many bacteria (Sakoh *et al.*, 2005; Vickerman *et al.*, 1997). Heat shock genes (ftsH and groES) and the specific two-component regulatory systems (lmo0287 and LisR) were transcriptionally expressed within the persister TN population. The class III heat shock protein gene ftsH (cell division protease) could be responsible for the control of cell growth since the persister cells have been defined as a non-growing sub-populaion (Balaban *et al.*, 2004). The co-chaperonin groES (Class I heat shock protein) is reported to help in rapid refolding of degraded protein (Rangeshwaran *et al.*, 2013), and its down-regulation may relate to the fate of some proteins in persister TN survivors. The

two-component system containing lmo0287 was reported to express during cell growth at 4°C (Chan *et al.*, 2007), and the two-component signal transduction system lisR/KdpE (LisRK) has phenotypically confirmed its adaptative role in response to multiple stresses(Nielsen *et al.*, 2012; Soni *et al.*, 2011). Cotter *et al.* demonstrated that a *L. monocytogenes* mutant (lacking LisK histidine kinase sensor within the LisR/K transduction system) displays significantly enhanced resistance to nisin stress (Cotter *et al.*, 2002). A high concentration of nisin induced 4.71 $\log_{10}$ cfu/ml of persister TN cells while persister N was limited to below 2 $\log_{10}$ cfu/ml (Wu *et al.*, 2017). Transcriptomic analysis detected significant down-regulation of the lisR gene in persister TN implying that this gene or the LisR/K signal transduction system, may play an essential role in increasing the nisin tolerance of *L. monocytogenes*.

The class III heat shock clp genes appear to be important in the persister populations. The Clp proteins are known to fold and cleave proteins. The elevated Clp proteases specifically degrade misfolded proteins generated by stress(Liu *et al.*, 2011). The activity of ClpE proteins is required for cell division (Nair *et al.*, 1999)and clpP is reported to play a role in stress resistance (Frees *et al.*, 2007; Michel *et al.*, 2006). However, this transcriptomic study showed overall suppression of Clp genes, which suggests low transcription of protein metabolism within persisters of *L. monocytogenes* under nisin exposure.

6.4.2 Cell wall synthesis

This study detected changes in gene expression which have a role in the cell wall synthesis of *L. monocytogenes.* Genes, lmo0129 and lmo2714, involved in biosynthesis of peptidoglycan, showed differential expression in persistser N and persister TN. The lmo0129 gene was expressed differently in the two persister populations. The influence of cell wall associated lipoteichoic acids has been shown when *L, monocytogenes* responds to diverse environmental stresses, such as antimicrobial exposure, low temperature, high osmolarity and alkali conditions (Brooks, 2014; Giotis *et al.*, 2010; Nizet, 2006). The dlt operon that comprises putative four genes (dltABCD) is responsible of catalyzing D-alanine residues on cell wall associated lipoteichoic acids (Kovács *et*

al., 2006).The D-alanine residues can facilitate the repulsion of cationic antimicrobial peptides (CAMPs), including nisin (Collins *et al.*, 2010). D-alanine esterification of teichoic acids has been shown to be associated with nisin resistance in *L. monocytogenes* (Vadyvaloo *et al.*, 2004). In this study, persister N cells survived exposure to nisin by amplifying dltC and lmo1080 expression, however, the dlt genes showed down-regulated expression of dltB and dltA in the persister TN population. This indicates that nisin repulsion by D-alanine residues is not a mechanism for persister TN tolerance to nisin.

Transporters of the peptidoglycan subunits from the cytoplasm to the cell wall are the competing targets for nisin entering the bacteria cells (Cotter *et al.*, 2005). The peptidoglycan bound protein gene lmo2714 was down-regulated both in persiser N and TN populations. In the persister N population lmo1084 was up-regulated. This gene is involved in the production of L-rhamnosylation of cell wall teichoic acids and Carvalho *et al.* reported that the L-rhamnose residues on the cell wall could delay antimicrobial peptide interaction with the cell membrane (Carvalho *et al.*, 2015), which fits with the persistence of this population to nisin. The gene lmo0129 (N-acetylmuramoyl -L-alanine amidase, similar to autolysin) was up-regulated in persister N and it is localized downstream of the lmaDCBA operon which influences the virulence of *L. monocytogenes* and encodes a bacteriophage-related protein (Arous *et al.*, 2004a). Virulence has been associated with changing cell membrane integrity (Arous *et al.*, 2004a). In this study, the increasing gene expression of lmaB and lmaC in persister N by 3.31fold and 2.94 fold respectively (Table 6.1) and the subsequent effect on the downstream gene lmo0129, could have an impact on cell membrane fluidity. However, lmo0129 and its upstream genes lmaA and lmaB are down-regulated in persister TN, and the opposite gene expression of lmo0129 suggests a difference in the intra-cellular metabolism of persister N and TN populations. Persister TN showed an up-regulation of the cell wall-binding protein gene lmo2522. Another study demonstrated that lmo2522 conferred a low level of resistance to cephalosporins in *L. monocytogenes* (Nielsen *et al.*, 2012) and it is characterized as a resuscitation promoting factor of *L. monocytogenes* for jump-starting the growth in a dormant status (Krawczyk-Balska and

Markiewicz, 2016). The results obtained from this gene expression study are consistent with the phenotypic observations of persister TN in the present study, showing nisin tolerance and a non-growing phenotype in rich medium.

6.4.3 ATP-binding cassette (ABC) system

Several genes of ABC system were differentially expressed in both persister groups following nisin exposure. Sugar ABC transporter system genes (tcsA, lmo1730, and lmo0278) were down-regulated in both the persister N and TN groups. The ABC transport system utilizes ATP to transport various substrates across cellular membranes and ABC transporters in *L. monocytogenes* are involved in resistance (Collins *et al.*, 2010; Liu *et al.*, 2017), virulence (Fraser *et al.*, 2000) and biofilm formation (Suo *et al.*, 2012; Zhu *et al.*, 2008). The sugar ABC system is important in the energy metabolism of cells. The persisters from nisin treatment may be dormant cells with low metabolic activity (Wu *et al.*, 2017). The down-regulation of sugar ABC transporter genes in both persister N and persister TN were consistent with their low metabolic character from the phenotypic assays (Wu *et al.*, 2017).

The genes responsible for other ABC transporter systems were also differentially expressed in the persister populations. For ion ABC transporters, the manganese transporter lmo1424 was induced in *L. monocytogenes* persister N while zinc transporter (zurA) was promoted in persister TN. Manganese transportation is known to be associated with efflux systems in *Bacillus subtilis* (Huang *et al.*, 2017), and efflux systems can confer increased tolerance of bacteria to multiple drugs (Chitsaz and Brown, 2017). The differential expression of metal ion transporters suggests that the efflux systems involved in persister formation vary between persister N and persister TN. The antibiotic ABC transporter ATP-binding protein gene (lmo0919) and peptide ABC transporter substrate-binding protein genes (lmo0152 and lmo0135) were up-regulated in persister TN. The antibiotic-regulator gene lmo0919 is capable of mediating the expression of the full-length mRNA that encodes the resistance genes, but the transcription regulated by lmo0919 is terminated in the absence of the antibiotic (Dar *et al.*, 2016). The lmo0919 gene was significantly

induced in persister TN in line with the increased tolerance (phenotypic resistance) shown in the presence of nisin in this study. The transcription on/off regulation that is a feature of lmo0919 is likely to play a key role in the persister TN population since the persister cells will return back to the sensitive status like the mother strain when nisin stress is removed. It is considered that the phenotypic resistance of persisters should be conferred by such a transcriptional switch. Two peptide ABC transporter substrate-binding protein genes, lmo0152 and lmo0135, are structurally related to OppA which is required for bacterial survival under a variety of stresses (Borezee *et al.*, 2000). OppA mediates the transport of oligopeptides across the cytoplasmic membrane (Bierne and Cossart, 2007) as *L. monocytogenes* is known to use small peptides for cell-cell signalling (Dickschat, 2010). In the present study, the RNA-Seq analysis also from the KEGG pathway suggests that lmo0152 and lmo0135 are involved in quorum sensing. It is suspected that these two peptide ABC transporter genes are crucial for signalling communications among persister TN cells under a high concentration of nisin.

6.4.4 Phosphotransferase (PTS) system

PTS systems play a role on survival of *L. monocytogenes* with PTS genes showing higher transcript levels in cold adaptation during the stationary phase of growth (Chan *et al.*, 2007). Piveteau *et al.* identified increased transcription of lmo2668 (transcriptional anti-terminator gene) and lmo2667 in *L. monocytogenes* after incubation in a soil environment where nutrients are scarce (Piveteau *et al.*, 2011). Up-regulation of lmo2668 (3.33 fold) and lmo2667 (2.92 fold) were detected in Persister N which is a subgroup of stationary phase cells surviving nisin treatment in this study. Gravesen *et al.* reported that pediocin resistant *L. monocytogenes* mutants showed consistent overexpression of a putative beta-glucoside-specific PTS system (Gravesen *et al.*, 2000). Persister N cells showed a substantial increase in the expression of a beta-glucoside transporter subunit IIABC (lmo0027) which is induced in association with *L. monocytogenes* resistance to class IIa bacteriocins (Gravesen *et al.*, 2004). Class IIa bacteriocins are known to target sensitive cells by using the mannose PTS system as a receptor (Kjos *et al.*, 2010), and there

are studies reporting that the interruption of mannose PTS corresponding genes led to an increase the resistance of *L. monocytogenes* to bacteriocins (Dalet *et al.*, 2001; Katla *et al.*, 2003). The nisin resistance of *L. monocytogens* is linked to reduced expression of a mannose permease of the phosphotransferase system (Crandall and Montville, 1998). The PTS mannose transporter subunit genes (lmo0096 and lmo0784 (Arous *et al.*, 2004b)) were down-regulated in the persister TN population which was tolerant to a high concentration of nisin in the present study. The down regulation of PTS genes is related to decreased cell growth, as these genes are involved in the utilization of various sugars (Bae *et al.*, 2012). Persister N had decreased expression of the fructose PTS transporter (lmo2733) and lichenan PTS transporter (lmo1719) genes while persister TN shoewed decreased expression of cellbiose PTS transporter (lmo0901, lmo2683, lmo2684), mannose PTS transporter (lmo0096 and lmo0784) and lichenan transporter (lmo1719) genes. Down-regulation of these sugar uptake PTS systems could be related to multiple receptors impeding nisin binding to target cells in both persister N and persister TN populations. The different environments (spent and fresh media) appear to influence gene expression between persiser N and persister TN. Sugar uptake was impeded in *L. monocytogenes* persister cells with more effect in persister TN than persister N. This is consistent with the XTT assays showing a low metabolic rate in the persister cells (Wu *et al.*, 2017).

6.4.5 SOS response and DNA repair

The persisters turn up/down the SOS response and DNA repair coding genes under high concentration of nisin in this study. Persister N showed an overall suppression of the SOS response related genes. Persister TN produced a more complex SOS gene response with SOS regulators (recF and recR), DNA polymerases (lmo0162 and lmo1975) and other DNA repair genes (nusG, nusA, lmo0164, lmo1881 and lmo1582) up-regulated.

Persister cells are a heterogeneous population with adaptive tolerance to survive adverse conditions (Radzikowski *et al.*, 2017). The SOS response in bacterial persistence is suspected to be involved in DNA repair important for persister resuscitation (Harms *et al.*, 2016). This is

consistent with a previous study (Wu *et al.*, 2017)that the population of both persister N and persister TN have characteristics of the mother cells once the stress has been removed. The SOS response pathway is reported to be a complementary stress signalling that modulates persister formation(Harms *et al.*, 2016). The number of the persister TN population was significantly greater than the persister N under nisin treatment(Wu *et al.*, 2017). This phenotype could partly result from the differential gene expression of the SOS response between persister TN and persister N.

The SOS repressor lmo1302 was restrained both in persister N and persister TN, which shows its importance in persister formation. However, the protective SOS response may increase stress-induced errors and gene recombination processes under low fidelity DNA polymerases during DNA repair (Žgur-Bertok, 2013). Persister N showed inhibition of the gene expression of DNA polymerase IV (lmo1975) and DNA polymerase I (polA). This implies that the persister N avoided genetic errors by suppressing the SOS response genes in order to maintain consistency in genomic information. Accumulated mutation has been regarded as adaptive outcome which could be essential to produce a resistant strain if persisters are exposed to cyclic antimicorbial treatments (Levin-Reisman *et al.*, 2017; McMahon *et al.*, 2007). The persister TN population had a higher occurrence of variation in gene expression than the persister N population as the SOS response genes were significantly up-regulated. The SOS activator recF and recR, and DNA polymerases (lmo0162 and lmo1975) were induced when the SOS repressor lmo1302 was inhibited. Stress-induced mutagenesis is also linked with the transcription elongation factor NusA in bacteria(Mondal *et al.*, 2016). Stationary-phase adaption under stress conditions has been referred to as a nusA dependent process which is found in *Escherichia coli* (Cardinale *et al.*, 2008). The persister N and the persister TN both were the persisting survivors from stationary phase *L. monocytogenes* following nisin treatment. The increased transcription of nusA and nusG in persister TN supports the hypothesis that persister TN is more likely to involve adaptive mutagenesis as a precursor of a resistant strain. RNA Seq analysis showed that there was a reasonably high number of SNPs (single nucleotide polymorphism) in sample reads from persister

TN versus the reference (*L. monocytogenes EGD-e*). The distinct regulation of the SOS response and DNA repair may lead to a SNP difference in the genes of the persister cells.

6.4.6 Phage/prophage

The food environmental strain of *L. monocytogenes* in this study is the same as majority of *Listeria* strains carrying prophages or phage remnants. Phage gene transcriptions had opposing results for persister N and persister TN. Phage protein genes are not only associated with *Listeria* genome plasticity and evolution, but also offer a versatile genetic insertion impacting on the *Listeria* host (Gilmour *et al.*, 2010). Phage proteins have been suspected to play roles in host takeover, nucleotide metabolism, and transcription regulation on the host survival mechanisms under stress yet specific functions of most phage coding genes have not been examined (Klumpp and Loessner, 2013). Casey *et al.* demonstrated that *L. monocytogenes* exposed to biocide stress showed significant down-regulation of phage/prophage coding genes in transcriptome analysis (Casey *et al.*, 2014). Similarly, persister N revealed suppression of the expression of phage genes when exposed to a high concentration of nisin. Verghese *et al.* reported a *comK*-integrated prophage display influencing the persistence of *L. monocytogenes* in food processing plants (Verghese *et al.*, 2011). Persister TN showed overall up-regulation of phage coding genes, which resulted in the increased persistence of *L. monocytogenes* in the present study. The role of phage/prophage genes in bacterial tolerance is not fully understood however current evidence suggests that phage related genes regulate persistence in varied ways for persister N and persister TN.

6.4.7 Other genes

Quorum sensing (QS) gene regulation was detected in this transcriptomic analysis. Persister N had up-regulated the auto-inducer peptide gene of the Agr system (lmo0049, agrD) and QS response regulator gene (lmo0051). No previous evidence has shown that stress could induce QS

and have an influence on the persistence in *L. monocytogenes.* AgrD-dependent QS does have an effect on biofilm formation, invasion, and virulence (Riedel *et al.*, 2009). However, studies have reported that stress inducible QS molecules were associated with persister cells resulting in noninherited multidrug tolerance populations of *Streptococcus mutans* (Leung and Lévesque, 2012) and *Pseudomonas aeruginosa* (Möker *et al.*, 2010). Persister TN showed up-regulation of QS related genes (lmo0152 and lmo0135) and a signal transduction gene (lmo0163). Evidence from both persister N and persister TN indicates that *L. monocytogenes* is capable of conveying sophisticated QS messages in response to a high concentration of nisin resulting in the production of persister cells.

Genes involved in flagellar biosynthesis showed decreased levels of expression both in persister N and persister TN. In persister N, the flagellum-specific peptidoglycan hydrolase gene lmo1215 (-1.96 fold) was inhibited while persister TN showed suppression of the flagellin coding gene flaA (-3.29 fold). The genes which code for flagellum are influenced when *L. monocytogenes* is grown in the presence of sanitizers, including benzethonium chloride (Casey *et al.*, 2014), chlorine dioxide (Pleitner *et al.*, 2014), and sodium lactate and sodium diacetate (Liu *et al.*, 2017). Flagellar and motility protein overexpression is also detected in *L. monocytogenes* upon 10^{-3} mg/mL exposure to nisin , which is indicative of increased bacterial motility in response to sub-lethal bacteriocin treatment (Miyamoto *et al.*, 2015). However, persister N and persister TN exhibited down-regularion of flagellar assembly which might have an alternative function in modifying hydrophobicity of the cell wall (Brown *et al.*, 2007) as disturbance of cell membrane/wall structure will influence nisin antimicrobial effects. The suppression of flagellum related motility is likely to be associated with the non-growing status of the persister N and persister TN populations.

Alterations in fatty acyl composition of the cell membrane results in increased resistance of *L. monocytogenes* to high concentrations of nisin, and cell memebrane biosynthesis of the resistant *L. monocytogenes* strain produces more phosphatidyl glycerol and less diphosphatidyl glycerol (Verheul *et al.*, 1997). A stress response network under sodium lactate and sodium

diacetate was discovered in *L. monocytogenes*, which is involved with fructose-1,6-bisphosphate aldolase (fbaA) gene regulation (Liu *et al.*, 2017). The tolerance of persister TN to a high concentration of nisin may be attributed to the up-regulation of lmo2433 and fbaA, and inhibition of glpD. Down-regulation of the fatty acid metabolism gene lmo1414 and cell wall resistance gene glnA occurred in the persister N population. The persister N cells showed up-regulation of the antibiotics resistance protein encoding gene lmo1416 and the rod shape-determining gene mreB, and down-regulation of the heavy metal transport gene lmo1853. The gene mreB is known to define the morphological character of a cell through cell wall synthesis and maintenance of *L. monocytogenes* in diverse environments (Fox *et al.*, 2011). Gottschalk *et al.* demonstrated that lmo1416, one of genes controlled by the two-component CesRK system, was induced three fold in *L. monocytogenes*'s response to cell wall-acting antibiotics (Gottschalk *et al.*, 2008). The evidence in the present trial supports the involvement of a number of distinct mechanisms in the formation of persister N and persister TN when exposed to harsh nisin stress.

6.4.8 Conclusions

Nutrition availability has been postulated as a key element influencing bacterial persistence when facing stress. The effect of the media in the persister TN population may produce increased cytoplasmic buffering capacity and decreased proton permeability of the cell membrane compared with the persister N population. RNA expression profiling of the persister isolates revealed the down-regulation at the transcriptional level of genes involved in energy production and flagellar synthesis associated with their slow or non-growth phenotype. However, the details in the transcriptomic results implied that the possible triggers (e.g. quorum sensing relating molecules) driving the occurrence of persisters could vary genetically between those in spent medium (lmo0049/0051) and in re-suspended medium (lmo0152/0135). Variable drivers in gene expression may help understand how the nutrient rich environment stimulates persister frequency. Alternatively, it may be possible that persister cells could be formed before the application of the antimicrobial treatment under different physiological conditions (Harms *et al.*, 2016), resulting in

a heterogeneous group within the bacterial population. Environmental nutrition may be associated with a stochastic change which could define the size or composition of the persistent group during bacterial survival. Beyond the general idea of dormancy, this study provided the genetic regulation evidence for persister cell survival in the presence of lethal nisin and suggest molecular routes of impeding/controlling bacterial persistence.

6.5 Supplementary materials

Supplementary Table 6. 1 Primer pairs used for qRT-PCR validation

Gene name	Gene annotation	Primer pair	Reference
16S	16S ribosomal RNA gene	F: 5'-GATGCATAGCCGACCTGAGA-3' R: 5'- TGCTCCGTCAGACTTTCGTC-3'	(van der Veen *et al.*, 2007)
fri	non-heme iron-binding ferritin	F:5'-CAATCAACTCAGTAGACACAAAGA-3' R: 5'-AGTGAAGAAGTTGTGGCCTCTC-3'	(Cabrita *et al.*, 2015)
cspB	cspB, cold-shock protein	F:5'-CAAACAGGTACAGTTAAATGGTTTA-3' R:5'- ACGATTTCAAATTCAACGCTTTGA-3'	(Schmid *et al.*, 2009)
lmo1138	ATP-dependent Clp protease proteolytic subunit	F: 5'-CATCCTTACCCAAAAATTGATTGAT-3' R:5'- CGGCTAACTCCTGATTGATTTCC-3'	(Ringus *et al.*, 2012)
lmo1580	Usp, Universal stress protein	F: 5'-GGCGCTTGGTCTTGTACATG-3' R:5'-TTCGTCTGCATATTCGGTAGC-3'	Designed by Primer3 Plus online tools (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi)
lmo2714	pepdidoglycan bound protein	F: 5'-ACAAAACCATTCGCCAGTGT-3' R:5'-GTTGTCGCCCATTCTGCATA-3'	
lmo0129	N-acetylmuramoyl-L-alanine amidase	F: 5'-TGGTAAGGATTCAGGTGCGA-3' R: 5'- ATTCCCACTTCAAAACCGGC-3'	
lmo1730	sugar ABC transporter substrate-binding protein	F: 5'-AACTCCATGCCAGCTCGTAT-3' R: 5'-AAGTGGCATTGGTTCAGCTG-3'	
lmo0278	Sugar ABC transporter ATP-binding protein	F: 5'-ACAGAGGCTTCGACGGTAAA-3' R: 5'- CGGCAACAATAGTAGTCGCT-3'	
tcsA	Monosaccharide ABC transporte	F: 5'- TGGCGTTATCCTAGGTGCTT-3' R: 5'- TCAACGCCACCAGTATCTGT-3'	

	r substrate-binding protein		
lmo1719	PTS lichenan transporter subunit IIA	F: 5'-GACGCAATGCTAGCAATCGA-3' R: 5'-TGTGAATGATGCGCCTCAAG-3'	Designed by Primer3 Plus online tools (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi)
lmaB	antigen B	F: 5'-ATGAAACGGACTTAGCGGGA-3' R: 5'-GTTTTCCGTAATGTCGAACAGC-3'	
lmo1975	DNA polymerase IV	F: 5'-CATTCAGCTATGCCCACCAG-3' R: 5'-TCTGGAAAAGATCTCGCGGA-3'	
nusA	Transcription elongation factor	F: 5'- CGCCTTGAAATCTCGATGGA-3' R: 5'- GAGCAGCAATACGTCCGAAA-3'	

6.6 References

Arous, S., Buchrieser, C., Folio, P., Glaser, P., Namane, A., Hebraud, M., and Hechard, Y.. (2004a). Global analysis of gene expression in an rpoN mutant of *Listeria monocytogenes*. *Microbiology*, 150, 1581-1590.

Arous, S., Dalet, K. and Héchard, Y. (2004b). Involvement of the mpo operon in resistance to class IIa bacteriocins in *Listeria monocytogenes*. *FEMS Microbiology Letters*, 238, 37-41.

Bae, D., Liu, C., Zhang, T., Jones, M., Peterson, S. N. and Wang, C. (2012). Global gene expression of *Listeria monocytogenes* to salt stress. *Journal of Food Protection*, 75, 906-912.

Balaban, N. Q. (2011). Persistence: mechanisms for triggering and enhancing phenotypic variability. *Current Opinion in Genetics & Development*, 21, 768-775.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science*, 305, 1622-1625.

Bierne, H. and Cossart, P. (2007). *Listeria monocytogenes* Surface Proteins: from Genome Predictions to Function. *Microbiology and Molecular Biology Reviews*, 71, 377-397.

Borezee, E., Pellegrini, E. and Berche, P. (2000). OppA of *Listeria monocytogenes*, an oligopeptide-binding protein required for bacterial growth at low temperature and involved in intracellular survival. *Infect Immun*, 68, 7069-7077.

Brooks, I. T. (2014). Influence of Lipoteichoic Acid on *Listeria monocytogenes* Biofilm Formation and Cellular Stress.

Brown, P. N., Terrazas, M., Paul, K. and Blair, D. F. (2007). Mutational analysis of the flagellar protein FliG: sites of interaction with FliM and implications for organization of the switch complex. *Journal of bacteriology*, 189, 305-312.

Buchrieser, C., Rusniok, C., The *Listeria*, C., Kunst, F., Cossart, P. and Glaser, P. (2003). Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria* innocua: clues for evolution and pathogenicity. *FEMS Immunology & Medical Microbiology*, 35, 207-213.

Cabrita, P., Trigo, M. J., Ferreira, R. B. and Brito, L. (2015). Differences in the expression of cold stress–related genes and in the swarming motility among persistent and sporadic strains of *Listeria monocytogenes*. *Foodborne Pathogens and Disease*, 12, 576-584.

Cardinale, C. J., Washburn, R. S., Tadigotla, V. R., Brown, L. M., Gottesman, M. E. and Nudler, E. (2008). Termination factor Rho and its cofactors NusA and NusG silence foreign DNA in *E. coli*. *Science*, 320, 935-938.

Carvalho, F., Atilano, M. L., Pombinho, R., Covas, G., Gallo, R. L., Filipe, S. R., and Cabanes, D. (2015). L-Rhamnosylation of *Listeria monocytogenes* wall teichoic acids promotes resistance to

antimicrobial peptides by delaying interaction with the membrane. *Plos Pathogens*, 11, e1004919.

Casey, A., Fox, E. M., Schmitz-Esser, S., Coffey, A., McAuliffe, O. and Jordan, K. (2014). Transcriptome analysis of *Listeria monocytogenes* exposed to biocide stress reveals a multi-system response involving cell wall synthesis, sugar uptake, and motility. *Frontiers in Microbiology*, 5, 68.

Chan, Y. C., Raengpradub, S., Boor, K. J. and Wiedmann, M. (2007). Microarray-based characterization of the *Listeria monocytogenes* cold regulon in log- and stationary-phase cells. *Applied Environmental Microbiology*, 73, 6484-6498.

Chitsaz, M. and Brown, M. H. (2017). The role played by drug efflux pumps in bacterial multidrug resistance. *Essays In Biochemistry*, 61, 127-139.

Collins, B., Curtis, N., Cotter, P. D., Hill, C. and Ross, R. P. (2010). The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various β-lactam antibiotics. *Antimicrobial agents and chemotherapy*, 54, 4416-4423.

Cotter, P. D., Guinane, C. M. and Hill, C. (2002). The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. *Antimicrobial agents and chemotherapy*, 46, 2784-2790.

Cotter, P. D., Hill, C. and Ross, R. P. (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology*, 3, 777-788.

Crandall, A. D. and Montville, T. J. (1998). Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. *Applied and Environmental Microbiology*, 64, 231-237.

Dalet, K., Cenatiempo, Y., Cossart, P. and Héchard, Y. (2001). A σ54-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. *Microbiology*, 147, 3263-3269.

Dar, D., Shamir, M., Mellin, J. R., Koutero, M., Stern-Ginossar, N., Cossart, P., and Sorek, R. (2016). Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science*, 352.

Dickschat, J. S. (2010). Quorum sensing and bacterial biofilms. *Natural Product Reports*, 27, 343-369.

Dussurget, O., Dumas, E., Archambaud, C., Chafsey, I., Chambon, C., Hébraud, M., and Cossart, P. (2005). *Listeria monocytogenes* ferritin protects against multiple stresses and is required for virulence. *FEMS Microbiol Lett*, 250, 253-261.

Fox, E. M., Leonard, N. and Jordan, K. (2011). Physiological and transcriptional characterisation of persistent and non-persistent *Listeria monocytogenes* isolates. *Applied and Environmental Microbiology*, AEM. 05529-05511.

Fraser, K. R., Harvie, D., Coote, P. J. and O'Byrne, C. P. (2000). Identification and Characterization of an ATP Binding Cassette l-Carnitine Transporter in *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 66, 4696-4704.

Frees, D., Savijoki, K., Varmanen, P. and Ingmer, H. (2007). Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. *Molecular Microbiology*, 63, 1285-1295.

Gilmour, M. W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K. M., and Nadon, C. (2010). High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics*, 11, 120.

Giotis, E. S., Muthaiyan, A., Natesan, S., Wilkinson, B. J., Blair, I. S. and McDowell, D. A. (2010). Transcriptome analysis of alkali shock and alkali adaptation in *Listeria monocytogenes* 10403S. *Foodborne Pathogens and Disease*, 7, 1147-1157.

Gomes, C. S., Izar, B., Pazan, F., Mohamed, W., Mraheil, M. A., Mukherjee, K., and Hain, T. (2011). Universal stress proteins are important for oxidative and acid stress resistance and growth of *Listeria monocytogenes* EGD-e in vitro and in vivo. *PloS one*, 6, e24965-e24965.

Gottschalk, S., Bygebjerg-Hove, I., Bonde, M., Nielsen, P. K., Nguyen, T. H., Gravesen, A., and Kallipolitis, B. H. (2008). The two-component system CesRK controls the transcriptional induction of cell envelope-related genes in *Listeria monocytogenes* in response to cell wall-acting antibiotics. *Journal of bacteriology*, 190, 4772-4776.

Gravesen, A., Kallipolitis, B., Holmstrøm, K., Høiby, P. E., Ramnath, M. and Knøchel, S. (2004). pbp2229-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. *Applied and Environmental Microbiology*, 70, 1669-1679.

Gravesen, A., Warthoe, P., Knøchel, S. and Thirstrup, K. (2000). Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative β-glucoside-specific PTS system. *Microbiology*, 146, 1381-1389.

Harms, A., Maisonneuve, E. and Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science*, 354, aaf4268.

Huang, X., Shin, J. H., Pinochet-Barros, A., Su, T. T. and Helmann, J. D. (2017). Bacillus subtilis MntR coordinates the transcriptional regulation of manganese uptake and efflux systems. *Molecular Microbiology*, 103, 253-268.

Katla, T., Naterstad, K., Vancanneyt, M., Swings, J. and Axelsson, L. (2003). Differences in susceptibility of *Listeria monocytogenes* strains to sakacin P, sakacin A, pediocin PA-1, and nisin. *Applied and Environmental Microbiology*, 69, 4431-4437.

Kint, C. I., Verstraeten, N., Fauvart, M. and Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends in Microbiology*, 20, 577-585.

Kjos, M., Salehian, Z., Nes, I. F. and Diep, D. B. (2010). An extracellular loop of the mannose phosphotransferase system component IIC is responsible for specific targeting by class IIa bacteriocins. *Journal of bacteriology*, 192, 5906-5913.

Klumpp, J. and Loessner, M. J. (2013). *Listeria* phages: Genomes, evolution, and application. *Bacteriophage*, 3, e26861.

Kovács, M., Halfmann, A., Fedtke, I., Heintz, M., Peschel, A., Vollmer, W., and Brückner, R. (2006). A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. *Journal of bacteriology*, 188, 5797-5805.

Krawczyk-Balska, A. and Markiewicz, Z. (2016). The intrinsic cephalosporin resistome of *Listeria monocytogenes* in the context of stress response, gene regulation, pathogenesis and therapeutics. *Journal of Applied Microbiology*, 120, 251-265.

Leung, V. and Lévesque, C. M. (2012). A Stress-Inducible Quorum-Sensing Peptide Mediates the Formation of Persister Cells with Noninherited Multidrug Tolerance. *Journal of bacteriology*, 194, 2265-2274.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N. and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science*, 355, 826-830.

Lim, S. Y., Yap, K. P. and Thong, K. L. (2016). Comparative genomics analyses revealed two virulent *Listeria monocytogenes* strains isolated from ready-to-eat food. *Gut Pathogen*, 8, 65.

Liu, X., Basu, U., Miller, P. and McMullen, L. M. (2017). Differential gene expression and filamentation of *Listeria monocytogenes* 08-5923 exposed to sodium lactate and sodium diacetate. *Food Microbiologyogy*, 63, 153-158.

Liu, Y., Ream, A., Joerger, R. D., Liu, J. and Wang, Y. (2011). Gene expression profiling of a pressure-tolerant *Listeria monocytogenes* Scott A ctsR deletion mutant. *Journal of Microbiology and Biotechnology*, 38, 1523-1533.

Loepfe, C., Raimann, E., Stephan, R. and Tasara, T. (2010). Reduced Host Cell Invasiveness and Oxidative Stress Tolerance in Double and Triple csp Gene Family Deletion Mutants of *Listeria monocytogenes*. *Foodborne Pathogens and Disease*, 7, 775-783.

McMahon, M. A. S., Xu, J., Moore, J. E., Blair, I. S. and McDowell, D. A. (2007). Environmental stress and antibiotic resistance in food-related pathogens. *Applied and Environmental Microbiology*, 73, 211-217.

Michel, A., Agerer, F., Hauck, C. R., Herrmann, M., Ullrich, J., Hacker, J., and Ohlsen, K. (2006). Global regulatory impact of ClpP protease of *Staphylococcus aureus* on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. *Journal of bacteriology*, 188, 5783-5796.

Miyamoto, K. N., Monteiro, K. M., da Silva Caumo, K., Lorenzatto, K. R., Ferreira, H. B. and Brandelli, A. (2015). Comparative proteomic analysis of *Listeria monocytogenes* ATCC 7644 exposed to a sublethal concentration of nisin. *Journal of Proteomics*, 119, 230-237.

Möker, N., Dean, C. R. and Tao, J. (2010). *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *Journal of bacteriology*, 192, 1946-1955.

Mondal, S., Yakhnin, A. V., Sebastian, A., Albert, I. and Babitzke, P. (2016). NusA-dependent transcription termination prevents misregulation of global gene expression, 1, 15007.

Nair, S., Frehel, C., Nguyen, L., Escuyer, V. and Berche, P. (1999). ClpE, a novel member of the HSP100 family, is involved in cell division and virulence of *Listeria monocytogenes*. *Molecular Microbiology*, 31, 185-196.

Nielsen, P. K., Andersen, A. Z., Mols, M., van der Veen, S., Abee, T. and Kallipolitis, B. H. (2012). Genome-wide transcriptional profiling of the cell envelope stress response and the role of LisRK and CesRK in *Listeria monocytogenes*. *Microbiology*, 158, 963-974.

Nilsson, R. E., Latham, R., Mellefont, L., Ross, T. and Bowman, J. P. (2012). MudPIT analysis of alkaline tolerance by *Listeria monocytogenes* strains recovered as persistent food factory contaminants. *Food Microbiologyogy*, 30, 187-196.

Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Current issues in molecular biology*, 8, 11.

Piveteau, P., Depret, G., Pivato, B., Garmyn, D. and Hartmann, A. (2011). Changes in gene expression during adaptation of *Listeria monocytogenes* to the soil environment. *PloS one*, 6, e24881.

Pleitner, A. M., Trinetta, V., Morgan, M. T., Linton, R. L. and Oliver, H. F. (2014). Transcriptional and Phenotypic Responses of *Listeria monocytogenes* to Chlorine Dioxide. *Applied and Environmental Microbiology*, 80, 2951-2963.

Radzikowski, J. L., Schramke, H. and Heinemann, M. (2017). Bacterial persistence from a system-level perspective. *Current Opinion in Biotechnology*, 46, 98-105.

Rangeshwaran, R., Ashwitha, K., Sivakumar, G. and Jalali, S. K. (2013). Analysis of Proteins Expressed by an Abiotic Stress Tolerant *Pseudomonas* putida (NBAII-RPF9) Isolate Under Saline and High Temperature Conditions. *Current microbiology*, 67, 659-667.

Riedel, C. U., Monk, I. R., Casey, P. G., Waidmann, M. S., Gahan, C. G. and Hill, C. (2009). AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. *Molecular Microbiology*, 71, 1177-1189.

Ringus, D. L., Ivy, R. A., Wiedmann, M. and Boor, K. J. (2012). Salt stress-induced transcription of σB-and CtsR-regulated genes in persistent and non-persistent *Listeria monocytogenes* strains from food processing plants. *Foodborne Pathogens and Disease*, 9, 198-206.

Sakoh, M., Ito, K. and Akiyama, Y. (2005). Proteolytic activity of HtpX, a membrane-bound and stress-controlled protease from *Escherichia coli*. *Journal of Biological Chemistry*, 280, 33305-33310.

Schmid, B., Klumpp, J., Raimann, E., Loessner, M. J., Stephan, R. and Tasara, T. (2009). Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. *Applied and Environmental Microbiology*, 75, 1621-1627.

Soni, K. A., Nannapaneni, R. and Tasara, T. (2011). The contribution of transcriptomic and proteomic analysis in elucidating stress adaptation responses of *Listeria monocytogenes*. *Foodborne Pathogens and Disease*, 8, 843-852.

Suo, Y., Huang, Y., Liu, Y., Shi, C. and Shi, X. (2012). The expression of superoxide dismutase (SOD) and a putative ABC transporter permease is inversely correlated during biofilm formation in *Listeria monocytogenes* 4b G. *PloS one*, 7, e48467.

Tasara, T., Freitag, J. and Stephan, R. (2015). Proteins of the Cold Shock Domain Family (Csps) contribute to Nisin and Benzalkonium chloride stress tolerance in *Listeria monocytogenes*. *Journal of Food Protection*, 78, 208-208.

Vadyvaloo, V., Arous, S., Gravesen, A., Hechard, Y., Chauhan-Haubrock, R., Hastings, J. W., and Rautenbach, M. (2004). Cell-surface alterations in class IIa bacteriocin-resistant *Listeria monocytogenes* strains. *Microbiology*, 150, 3025-3033.

van der Veen, S., Hain, T., Wouters, J. A., Hossain, H., de Vos, W. M., Abee, T., and Wells-Bennik, M. H. (2007). The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. *Microbiology*, 153, 3593-3607.

Verghese, B., Lok, M., Wen, J., Alessandria, V., Chen, Y., Kathariou, S., and Knabel, S. (2011). comK Prophage Junction Fragments as Markers for *Listeria monocytogenes* Genotypes Unique to Individual Meat and Poultry Processing Plants and a Model for Rapid Niche-Specific

Adaptation, Biofilm Formation, and Persistence. *Applied and Environmental Microbiology*, 77, 3279-3292.

Verheul, A., Russell, N. J., Hof, R. V. T., Rombouts, F. M. and Abee, T. (1997). Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. *Applied and Environmental Microbiology*, 63, 3451-3457.

Vickerman, M. M., Mather, N. M., Minick, P. E., Edwards, C. A. and Clewell, D. B. (1997). Disruption of htpX affects the *Streptococcus gordonii* cell surface. *Journal of Dental Research*, 76, 725-725.

Wemekamp-Kamphuis, H. H., Karatzas, A. K., Wouters, J. A. and Abee, T. (2002). Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. *Applied and Environmental Microbiology*, 68, 456-463.

Wu, S., Yu, P.-L. and Flint, S. (2017). Persister cell formation of *Listeria monocytogenes* in response to natural antimicrobial agent nisin. *Food Control*, 77, 243-250.

Žgur-Bertok, D. (2013). DNA damage repair and bacterial pathogens. *Plos Pathogens*, 9, e1003711.

Zhu, X., Long, F., Chen, Y., Knochel, S., She, Q. and Shi, X. (2008). A putative ABC transporter is involved in negative regulation of biofilm formation by *Listeria monocytogenes*. *Applied Environmental Microbiology*, 74, 7675-7683.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)
We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Shuyan Wu
Name/Title of Principal Supervisor: Steve Flint
Name of Published Research Output and full reference:
Shuyan Wu, Pak-Lam Yu, David Wheeler, Steve Flint (2018) Transcriptomic study on persistence and survival of *Listeria monocytogenes* following lethal treatment with nisin. Journal of Global Antimicrobial Resistance doi:https://doi.org/10.1016/j.jgar.2018.06.003.

In which Chapter is the Published Work: Chapter 6
Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: and / or
- Describe the contribution that the candidate has made to the Published Work: *The candidate carried out laboratory work, RNA collection and raw sequencing data analysis, and manuscript preparation guidance of direction and editorial help from the co-authors and supervisors.*

01/12/2019

Candidate's Signature — Date

01/12/2019

Principal Supervisor's signature — Date

GRS Version 3– 16 September 2011

Chapter 7 Summary and Final Discussion

7.1 *L. monocytogenes* persisting in nature

Studies on *L. monocytogenes* persister cells have focussed on the clinical significance relating to antibiotic treatment like norfloxacin (Knudsen *et al.*, 2013). However, the mechanism of *L. monocytogenes* persister formation in response to food preservatives is limited.

L. monocytogenes causes a life-threatening food borne disease, listeriosis, affecting immuno-compromised people, including elders, neonates and pregnant women (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* from various environments usually dies after treatments with different disinfectants and other non-toxic antimicrobials. However, *L. monocytogenes* is repeatedly detected in many food related environments and processed food products (Ferreira *et al.*, 2014; Wang *et al.*, 2015). The natural preservative, nisin, is regarded as one of the most acceptable preservatives but it cannot eradicate bacteria within food products. Several mechanisms have been proposed for the persistence of *L. monocytogenes* following treatment with antimicrobials with some genes identified that sustain bacterial tolerance when facing different bactericidal treatments (Kint *et al.*, 2012). However, the investigations into persistence of *L. monocytogenes* often relies on re-growing the persister population to obtain enough cells for study and this has problems as the physiological state of persisters is lost during re-growth (Kastbjerg and Gram, 2012).

7.2 Presence of *L. monocytogenes* persister cells during biphasic killing by lethal doses of nisin

To overcome the issues relating to the re-growth of persister cells for study, this PhD project took a different approach. Overnight cultures of *L. monocytogenes* were treated with lethal nisin (up to 75µg/ml), with a biphasic killing pattern observed during 24hrs of treatment. Nearly 10^6–10^7CFU/ml of the overnight culture in stationary phase was killed within the first 4hrs,

followed by a plateau of surviving cells $<10^2$CFU/ml. The lethal nisin effect on the remaining persisting cells was much slower than the initial rate of killing.

Patra *et al.* demonstrated that the persister level is several orders of magnitude larger in stationary phase compared with the persister cells in exponential growth (Patra and Klumpp, 2013). In the present study stationary phase cells (18hrs and 24hrs of overnight culture) generated more persister cells than the exponential phase cells. The conventional plate counting methods revived a consistent level of persister cells from 18hrs old stationary phase populations treated with 75µg/ml nisin. Older stationary phase cells (24hrs of overnight culture) showed an undetectable level of persister cells following treatment with 60µg/ml nisin. It is suspected that physiological variations and fitness differences among stationary phase populations may influence the persister fractions following lethal nisin treatment. Stepanyan *et al.* provided strong support for the hypothesis that variation in persistence in bacteria is not only shaped by differences in the frequency of antibiotic exposure, but also by the fitness of the cells as well as the stage in the growth cycle of a population (Stepanyan *et al.*, 2015).

7.3 Increasing the persister fraction under nisin treatment

The persister population of *L. monocytogenes* surviving nisin treatment is a minority of the population, mixed with an overwhelming quantity of non-persister cells (dead cells). This very low level of persister cells provides insufficient cells for analysis. Deriving sufficient persister cells is key to enable biochemical, physiological and gene expression studies on the persister population. The present study generated a much larger number of persister cells from the stationary phase cells when re-suspended in full TSB medium. The antimicrobial activity in the re-suspended population showed a similar biphasic killing pattern to the treatment of cells in spent medium but the number of persister cells recovered was greater in the re-suspended population. These cells were unable to divide in the presence of fresh medium while the nisin was present. The regrowth of these cells in medium without nisin and subsequent nisin treatment, showed that

these cells were not a resistant population. That is, the re-suspended and treated population was demonstrating the persistent phenotype.

The persister cells from re-suspended cells enable the collection of an adequate number of persisters for analysis for investigating cell membrane potential, metabolic rate and resuscitation. Persister levels are believed to change depending on stochastic and deterministic factors in a bacterial population (Michiels *et al.*, 2016). Persistence appeared to be dependent on certain proteins, such as those associated with transcription, anti-toxin and nucleoid-associated proteins (Maisonneuve and Gerdes, 2014). Where certain persister protein in cells exceed a certain threshold concentration, the cells will switch to the persister state and become tolerant to antibiotics. When environmental factors increase the average expression level of the persister proteins, additional persisters are induced because more cells attain the threshold level required for switching to the persister state (Maisonneuve and Gerdes, 2014). Although persisters are known to be predominantly dormant, there may be a gradient in metabolic activity of persister cells with associated change in gene expression (Kim and Wood, 2016). In this study, a focus was on understanding the development of persister cells from the transcriptional changes.

7.4 Persister cells versus viable and un-culturable cells

Flow cytometry (FC) enables the differentiation of viable, dead and viable non-culturable cells. This technique can be used to differentiate persisters cells from the bulk of the non-persister population following nisin treatment (at 24hrs) using a live/dead staining. In this study, FC was able to show differences in the population of persister N cells (overnight culture treated with nisin) compared with persister TN cells (TSB re-suspended cells with nisin treatment). The results were compared with the plate count results. FC analysis showed more persister cells than conventional plating for each treatment group and this is explained by a viable population that cannot be resuscitated on the agar plate. The results suggest that there is a group in each nisin treated population that contains recoverable cells (with potential of showing reproductive capacity) and un-culturable cells (a “withered” state) after the lethal nisin treatment. The persister and the viable

but non-culturable (VBNC) state share common features, and are like dormant phenotypes. Persister cells co-exist with VBNCs, regarded as the more dormant of the two states (Kim *et al.*, 2018). The key feature that has been suggested to distinguish persister cells from VBNC cells is that VBNC cells are reported to not be resuscitated under normal conditions while persister cells can be easily converted to normal cells that are sensitive to antibiotics or other stresses (Ayrapetyan *et al.*, 2015).

The number of persisters (combined with VBNC) in this study was estimated from the "living cell" quadrant in the FC analysis, and they were determined as surviving cells according to the TO stain. This implies that the persisters that survived 24hrs of lethal nisin treatment possibly regained cell membrane integrity, as the PI dye in the live/dead staining kit only enters cells with permealized or damaged membranes. Kim *et al.* 2018 reported that rifampicin induced persisters of *E. coli* generating limited membrane damage and cells could resuscitate immediately while most of the VBNC cells shrank and finally became dead since they contained little cytosolic content. In the work of Kim *et al.*, the VBNC cells did not stain with PI, which indicates their membranes were not damaged (Kim *et al.*, 2018). Green fluorescent protein expressing *L. monocytogenes* have recently been used to generate VBNC cells by exposure to ppm levels of chlorine, allowing the VBNC cells to be enumerated by direct viable counting under microscopy (Highmore *et al.*, 2018). The VBNC cells of *L. monocytogenes* injected into *Caenorhabditis elegans* , a nematode, were as infectious as the culturable counterpart, which suggests no change in the pathogenicity of VBNC cells as they can be resuscitated once inside a host (Highmore *et al.*, 2018). The decontamination of fresh produce using chemicals such as chlorine based sanitisers, may generate persister cells, undetected by standard methods and, as with VBNC cells, may still be able to cause food-borne disease, explaining why in some food poisoning outbreaks, no food vehicle can be identified (McFarland *et al.*, 2017). Persister cells and VBNC cells are a potential food safety risk, therefore understanding the threshold for VBNC cell development in a food system and developing methods of detecting such cells are important.

7.5 Molecular mechanisms for persister formation

In this study, the responses of the *L. monocytogenes* strain from food associated with persister formation following nisin treatment were detected by the RNA-Seq analysis. The results showed multiple systems associated with persister formation including stress response, cell wall synthesis and the ABC system. This implies that the ability to form persister cells primarily relates to physiological changes associated with the dormant phenotype. Hofsteenge *et al.* found variation in a fraction of persisters in a subset of 11 isolates from 450 environmental *E.coli* isolates, and there was no correlation among variations of persister fractions across two antibiotics, even for antibiotics with nearly identical modes of action (ciprofloxacin and nalidixic acid) (Hofsteenge *et al.*, 2013). They assumed that there is no single physiological change that determines persistence in a population of cells, and the fraction of cells that survive antibiotic treatment (persister) depends on the bacterial strain and the specific antibiotic that is used (Hofsteenge *et al.*, 2013). Recent studies have begun to unravel the complex molecular mechanisms underlying persister formation (e.g. stress responses and toxin–antitoxin modules) to reveal insights into the evolutionary and adaptive aspects of this phenotype (Michiels *et al.*, 2016).

Henry *et al.* developed persister-FACSeq, which is a method that uses fluorescence-activated cell sorting, antibiotic tolerance assays, and next generation sequencing to interrogate persister physiology and its heterogeneity (Henry and Brynildsen, 2016). They found that non-growing *E. coli* persistence to ofloxacin is inversely correlated with the capacity of non-growing cells to synthesize protein (Henry and Brynildsen, 2016). The conserved features of the *hip* toxin–antitoxin modules from *E. coli* suggest that this toxin–antitoxin module might control the level of persistence through redundant mechanisms, in which phenotypic variability may be a general property regulated by threshold levels of toxin proteins, whose function would be to differentiate bacterial populations into distinct subpopulations to cope with stress (Rotem *et al.*, 2010). The expression of enzymes (such as catalases and superoxide dismutases) are reported in *E. coli* (Nguyen *et al.*, 2011) and *P. aeruginosa* (Khakimova *et al.*, 2013)persisters to protect them from

antibiotics (Van den Bergh *et al.*, 2017). However, according to the heterogeneity of persister physiology, the mechanisms involved during persister formation are still enigmatic with debates from recent findings. The alarmone, guanosine tetraphosphate (ppGpp), which has been determined as an important metabolite of bacterial persistence, has recently been disproved with a finding that persister cells formed in absence of ppGpp and are more linked with cell growth arresting proteins (like YihS and PntA) in *E. coli* during ciprofloxacin treatment (Chowdhury *et al.*, 2016). Not long ago, the modelling study by Day suggested that many of the empirically documented features of phenotypic antibiotic tolerance and the formation of bacterial persister cells can be explained by evolution via epigenetic inheritance (Day, 2016), and this could be extend as a new direction for understanding the phenotypic resistance of persisters.

7.6 Future work

In this study, one food environmental isolate (A1) and one reference strain (M5) from animal origin were examined for persister formation following lethal nisin treatment. As strains of *L. monocytogenes* in nature will vary, evaluating the persistence phenotype within a number of strains will be important for future work. Persistence variation among 37 strains of *Acinetobacter baumannii* was examined when stationary cultures faced treatment with polymyxin B and tobramycin (Barth *et al.*, 2014). A high heterogeneity of persister cells formation among isolates was observed. Polymyxin B or tobramycin -treated cultures produced variable percentages of persister cells that varied with the strains corresponding to 0.0007% to 10.1% and 0.0003%–11.84% of the initial population. For this study, a group of 47 food/food environments isolates of *L. monocytogenes* were provided from several analytical testing laboratories in New Zealand and these would be a useful resource to continue exploring the variation of persistence in food related *L. monocytogenes*.

Persister formation of *L. monocytogenes* following nisin treatment under different environmental conditions indicates that nutrient composition of the environment plays a role in the production of persister cells of *L. monocytogenes*. Knudsen *et al.* reported that fermentable

carbohydrates accelerated killing *L.monocytogenes* persisters with gentamicin (Knudsen *et al.*, 2013). In this study, stationary phase cells re-suspended in a nutrient rich environment (TSB) showed greater survival under lethal nisin compared with cells in spent (nutrient deficient) medium. In the rich medium there may be some key nutrient factors to stimulate regulation/reponse to protect the cells against the nisin challenge. Nutrient factors that could affect this differential production of persisters will be considered for investigation in future work.

The flow cytometry analysis using live/dead fluorescent staining showed changes in cell permeability following nisin treatment depending on the nutrient levels in the environment. The cell membrane appeared to be more compromised by increasingly taking up fluorescent PI dyes in the rich nutrient re-suspended group, even though there was a greater proportion of persister cells in this group compared with the group in spent medium. This suggests that the PI stain can enter living cells and this has been demonstrated in another study (Gill and Holley, 2006). PI staining is used as a dye for evaluating cell membrane damage (Cossu *et al.*, 2017) and the results of the present study, suggest that this does not necessarily mean loss in cell viabilty. This suggests some variability in the degree of cell permeability determining whether cells live or die. Permeable cells could be further examined to explore in more detail the structural integrity and morphological traits using transmission electron microscopy and alternative assays to explore the release of cytoplasmic content should be considered for measuring the degree of cell integrity (Ahmed *et al.*, 2018; Oussalah *et al.*, 2006).

Cui *et al.* reported a bacteriostatic agent induced antagonism model using rifampicin and tetracycline and screened the entire single gene deletion mutant library of *E. coli* to identify the molecular basis of this phenomenon (Cui *et al.*, 2018). They found deletion mutants with defects in genes that belong to the DNA repair and recombination pathway responsible for persister cell formation (Cui *et al.*, 2018). In the present study, the nutrient rich environment was associated with variations in gene expression resulting in variations in the size of the persistent population following lethal nisin stress. Comparing the differential gene expression between the persister N and persister TN group in the transcriptomic study, 10 genes (cspB, lmo0129, lmaB, Usp,

lmo1975, lmo0121, lmo0122, lmo0123, fri, and nusA) showed contrary expression in persister TN over persister N (Supplementary Table 7.1). Future work should screen a *L. monocytogenes* single gene deletion mutant library based on the RNA sequencing results for investigating the molecular basis of persister formation. Differentially expressed genes were detected as significant for KEGG enrichment in the ribosome which indicated that the translational level may play the key role of regulating persistence following lethal nisin treatment. The protein profiles of *L. monocytogenes* ATCC 7644 cells treated with a sublethal concentration of nisin (10^{-3} mg/mL) have been studied showing proteins associated with oxidative stress response, production of cell membrane lipids and flagella associated with the response to nisin treatment (Miyamoto *et al.*, 2015). Future work may include a proteomic comparison of persister N and persister TN, which may provide more insights into the antimicrobial effects of nisin and the development of the persister phenotype. Significant differences in gene expression of the phage tail protein genes (lmo121, lmo122, and lmo123) detected in the transcriptomic analysis comparing persister TN over persister N supports the suggestion for a detailed proteomic study. Bacteria are protected against invading nucleic acids (for instance from phages) because of CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated proteins) systems, which are a RNA-guided prokaryotic adaptive immune system (Liu *et al.*, 2017). The CRISPR/Cas system can function as a prokaryotic immune system that confers resistance to foreign genetic elements. Hence, the relationship between the CRISPR/Cas immune system and *L. monocytogenes* persistence under lethal nisin could be another investigation in the future work.

Persister cells isolated from food environments are able to adhere to food processing surfaces and produce biofilm (Alessandria *et al.*, 2010). The two isolates (A1 and M5 strain) used in this study showed a strong ability to produce biofilm. Their persisters surviving 75μg/ml lethal nisin treatment have shown a consistent ability to form good biofilm when compared with the mother strain (Supplementary Table S7.2). Singh *et al.* reported that the high resistance of *S. aureus* biofilm to multiple antibiotics (oxacillin, cefotaxime, ciprofloxin and vancomycin) is largely depend on the presence of persister cells (Singh *et al.*, 2009). Knudsen *et al.* used persister

cells of *L. monocytogenes* attached and grown on polycarbonate filter surfaces exposed to 100µg/ml norfloxacin but there was no significant difference compared with the sensitivity of the planktonic cultures (Knudsen *et al.*, 2013). A more detailed study into the role of biofilm and the link with persistence of *L. monocytogenes* is a topic for future study.

7.7 Supplementary information

Supplementary Table S7. 1 Genes that showed contrary expression in persister TN over persister N

Gene accession number[a]	Gene name[b]	Functional Protein	Fold change[c]
CAD00094	cspB	cold-shock protein	-5.04
CAC98344	lmo0129	N-acetylmuramoyl-L-alanine amidase Cationic antimicrobial peptide (CAMP) resistance, organism-specific biosystem (from KEGG)	-8.15
CAC98332	lmaB	Antigen B	-8.80
CAC99658	Usp	Universal stress protein	-5.32
CAD00053	lmo1975	DNA polymerase IV	5.08
CAC98336	lmo0121	phage tail protein	-9.30
CAC98337	lmo0122	hypothetical protein Phage tail protein	-7.74
CAC98338	lmo0123	hypothetical protein Prophagetail; Prophage endopeptidase tail	-7.63
CAC99021	fri	non-heme iron-binding ferritin	-4.85
CAC99400	nusA	transcription elongation factor NusA	4.06

[a] Identified genes that showed contrary expression in persister TN over persister N are listed with an adjusted P value <0.05 and a fold-change of ≥ 1.50.

[b] Listed gene names correspond to the gene designations for the reference strain *L. monocytogenes* EGD-e from the ensembl genomes repository (ftp://ftp.ensemblgenomes.org/pub/bacteria/release-34/fasta/bacteria_0_collection/*Listeria*_monocytogenes_egd_e/dna/).

[c] Positive changes indicate genes that have higher transcript levels in the persister N; negative changes indicate genes that have lower transcript leves in the persister N.

Supplementary Table S7. 2 Biofilm formation of A1/M5 strains and their persister cells surviving from 75μg/ml lethal nisin treatment

Strain	Biofilm OD 595nm		BFI	
	mean OD 595nm	standard deviation	mean BFI	standard deviation
A1 mother strain	**0.7**	0.192	**1.517**	0.415
A1 persisters	**0.686**	0.126	**1.380**	0.253
M5 mother strain	**0.668**	0.106	**1.537**	0.257
M5 persisters	**0.72**	0.014	**1.560**	0.023

*A microtiter plate assay was used to determine the biofilm formation for all the *L. monocytogenes* strains as a measurement of the isolates' attachment ability (Djordjevic *et al.*, 2002). To correct for background staining when screening for biofilm formation, the mean optical density obtained for the blank controls was subtracted from the mean optical density for each of the test strains in every test. The biofilm formation index (BFI) was used to express biofilm formation by *L. monocytogenes* strains (Naves *et al.*, 2008). The biofilm formation index takes into account the biofilm and the planktonic cell growth in the microtiter plate and is determined by applying the formula: BFI = (AB-CW)/G; AB is the optical density of the attached bacteria with crystal violet staining, CW is the optical density of the blank wells, and G is the optical density of cells growth in suspended culture. Naves *et al.* classified the biofilm production semi-quantitatively based on the BFI in four categories: strong ($\geqslant$1.10), moderate (0.70–1.09), weak (0.35–0.69), and negative (below 0.35)(Naves *et al.*, 2008). The *L. monocytogenes* isolates tested in the project showed strong ability of biofilms formation.

7.8 References

Ahmed, B., Hashmi, A., Khan, M. S. and Musarrat, J. (2018). ROS mediated destruction of cell membrane, growth and biofilms of human bacterial pathogens by stable metallic AgNPs functionalized from bell pepper extract and quercetin. *Advanced Powder Technology*, 29, 1601-1616.

Alessandria, V., Rantsiou, K., Dolci, P. and Cocolin, L. (2010). Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. *International Journal of Food Microbiologyogy*, 141, S156-S162.

Ayrapetyan, M., Williams, T. C. and Oliver, J. D. (2015). Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends in microbiology*, 23, 7-13.

Barth Jr, V. C., Rodrigues, B. Á., Bonatto, G. D., Gallo, S. W., Pagnussatti, V. E., Ferreira, C. A. S., and de Oliveira, S. D. (2014). Heterogeneous Persister Cells Formation in *Acinetobacter baumannii*. *PLOS ONE*, 8, e84361.

Chowdhury, N., Kwan, B. W. and Wood, T. K. (2016). Persistence Increases in the Absence of the Alarmone Guanosine Tetraphosphate by Reducing Cell Growth. *Sci Rep*, 6, 20519.

Cossu, A., Le, P., Young, G. M. and Nitin, N. (2017). Assessment of sanitation efficacy against *Escherichia coli* O157:H7 by rapid measurement of intracellular oxidative stress, membrane damage or glucose active uptake. *Food Control*, 71, 293-300.

Cui, P., Niu, H., Shi, W., Zhang, S., Zhang, W. and Zhang, Y. (2018). Identification of Genes Involved in Bacteriostatic Antibiotic-Induced Persister Formation. *Frontier Microbiology*, 9, 413.

Day, T. (2016). Interpreting phenotypic antibiotic tolerance and persister cells as evolution via epigenetic inheritance. *Mol Ecol*, 25, 1869-1882.

Ferreira, V., Wiedmann, M., Teixeira, P. and Stasiewicz, M. J. (2014). *Listeria monocytogenes* Persistence in Food-Associated Environments: Epidemiology, Strain Characteristics, and Implications for Public Health. *Journal of Food Protection*, 77, 150-170.

Gill, A. O. and Holley, R. A. (2006). Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus* sakei cellular membranes by plant oil aromatics. *International journal of Food Microbiologyogy*, 108, 1-9.

Henry, T. C. and Brynildsen, M. P. (2016). Development of Persister-FACSeq: a method to massively parallelize quantification of persister physiology and its heterogeneity. *Sci Rep*, 6, 25100.

Highmore, C. J., Warner, J. C., Rothwell, S. D., Wilks, S. A. and Keevil, C. W. (2018). Viable-but-Nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson Induced by Chlorine Stress Remain Infectious. *Mbio*, 9(2), e00540-18.

Hofsteenge, N., van Nimwegen, E. and Silander, O. K. (2013). Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. *BMC Microbiology*, 13(1), 25.

Kastbjerg, V. G. and Gram, L. (2012). Industrial disinfectants do not select for resistance in *Listeria monocytogenes* following long term exposure. *International journal of Food Microbiologyogy*, 160, 11-15.

Khakimova, M., Ahlgren, H. G., Harrison, J. J., English, A. M. and Nguyen, D. (2013). The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. *Journal of Bacteriology*, 195, 2011-2020.

Kim, J.-S., Chowdhury, N., Yamasaki, R. and Wood, T. K. (2018). Viable but non-culturable and persistence describe the same bacterial stress state. *Environmental Microbiology*, 20, 2038-2048.

Kim, J.-S. and Wood, T. K. (2016). Persistent persister misperceptions. *Frontiers in microbiology*, 7, 2134.

Kint, C. I., Verstraeten, N., Fauvart, M. and Michiels, J. (2012). New-found fundamentals of bacterial persistence. *Trends in Microbiology*, 20, 577-585.

Knudsen, G. M., Ng, Y. and Gram, L. (2013). Survival of Bactericidal Antibiotic Treatment by a Persister Subpopulation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 79, 7390-7397.

Liu, L., Li, X., Wang, J., Wang, M., Chen, P., Yin, M., and Wang, Y. (2017). Two Distant Catalytic Sites Are Responsible for C2c2 RNase Activities. *Cell*, 168, 121-134.e112.

Maisonneuve, E. and Gerdes, K. (2014). Molecular Mechanisms Underlying Bacterial Persisters. *Cell*, 157, 539-548.

McFarland, N., Bundle, N., Jenkins, C., Godbole, G., Mikhail, A., Dallman, T., and Dabke, G. (2017). Recurrent seasonal outbreak of an emerging serotype of Shiga toxin-producing *Escherichia coli* (STEC O55:H7 Stx2a) in the south west of England, July 2014 to September 2015. *Eurosurveillance*, 22, 30610.

Michiels, J. E., Van den Bergh, B., Verstraeten, N. and Michiels, J. (2016). Molecular mechanisms and clinical implications of bacterial persistence. *Drug Resistance Updates*, 29, 76-89.

Miyamoto, K. N., Monteiro, K. M., da Silva Caumo, K., Lorenzatto, K. R., Ferreira, H. B. and Brandelli, A. (2015). Comparative proteomic analysis of *Listeria monocytogenes* ATCC 7644 exposed to a sublethal concentration of nisin. *Journal of Proteomics*, 119, 230-237.

Naves, P., Del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., and Soriano, F. (2008). Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. *Journal of applied microbiology*, 105, 585-590.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., and Britigan, B. E. (2011). Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria. *Science*, 334, 982-986.

Oussalah, M., Caillet, S. and Lacroix, M. (2006). Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Journal of Food Protection*, 69, 1046-1055.

Patra, P. and Klumpp, S. (2013). Population dynamics of bacterial persistence. *PLOS ONE*, 8, e62814.

Rotem, E., Loinger, A., Ronin, I., Levin-Reisman, I., Gabay, C., Shoresh, N., and Balaban, N. Q. (2010). Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 12541-12546.

Singh, R., Ray, P., Das, A. and Sharma, M. (2009). Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an in vitro study. *Journal of Medical Microbiology*, 58, 1067-1073.

Stepanyan, K., Wenseleers, T., Duéñez-Guzmán, E. A., Muratori, F., Van den Bergh, B., Verstraeten, N., and Michiels, J. (2015). Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen *Pseudomonas aeruginosa*. *Molecular Ecology*, 24, 1572-1583.

Swaminathan, B. and Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes and Infection*, 9, 1236-1243.

Van den Bergh, B., Fauvart, M. and Michiels, J. (2017). Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. *FEMS Microbiol Rev*, 41, 219-251.

Wang, J., Ray, A. J., Hammons, S. R. and Oliver, H. F. (2015). Persistent and Transient *Listeria monocytogenes* Strains from Retail Deli Environments Vary in Their Ability to Adhere and Form Biofilms and Rarely Have inlA Premature Stop Codons. *Foodborne Pathogens and Disease*, 12, 151-158.