Characterizing the culturable bacteria isolated from imported, ready-to-eat (RTE) foods for their ability to control *Listeria monocytogenes*

by

Krishna Sen Gelda

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In partial fulfilment of requirements
for the degree of
Master of Science
in
Food Science

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ABSTRACT

CHARACTERIZING THE CULTURABLE BACTERIA ISOLATED FROM IMPORTED, READY-TO-EAT (RTE) FOODS FOR THEIR ABILITY TO CONTROL LISTERIA MONOCYTOGENES

Krishna Sen Gelda
University of Guelph, 2019

Advisor:
Dr. Jeff Farber

Listeria monocytogenes, an important foodborne pathogen, remains a significant threat to public health. This thesis investigated the culturable microbiota of select imported, RTE foods to see whether the existing bacterial microflora could inactivate, inhibit the growth and/or cause a reduction in the virulence of L. monocytogenes. Among all the foods tested (dried apple slices, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed), the date fruit microbiota displayed the most promise for harbouring antagonistic properties against L. monocytogenes. Of the 191 isolates recovered from five different date fruits, 36 (19%) produced zones of inhibition against L. monocytogenes that ranged from 0.3 to 5.8 mm. The inhibitory strains were all identified as Bacillus spp. Among those Bacillus spp. that were tested for their ability to inhibit PrfA, all caused a significant reduction in the activation of the PrfA protein (p-value < 0.05). In addition, the anti-Listeria compound(s) produced by B. altitudinis DS11 were found to be proteinaceous in nature, acid and alkali-tolerant and resistant to temperature treatments up to 100°C.
DEDICATION

To the past and present members of my loving and devoting family – they truly are an inspiration. Always and forever.
FORWARD

All of the work presented in this dissertation was completed at the University of Guelph in the Canadian Research Institute for Food Safety (CRIFS) from the Department of Food Science in the Ontario Agriculture College (OAC). Financial support was provided by the Canada First Research Excellence Fund (CFREF) through the Food from Thought (FFT) program at the University of Guelph. In addition, all images and graphics presented were either all produced by the primary author, Krishna Gelda, or downloaded from the internet with a creative commons license that required no attribution.

Chapter 1. A review of literature providing background on necessary information needed to understand this thesis.

Chapter 2. Our first data chapter, providing a survey of the microbiota obtained from imported ready-to-eat (RTE) foods and whether their culturable bacteria possess antagonistic properties against the foodborne pathogen *Listeria monocytogenes*. This chapter contains original and unpublished work. Bacterial isolates from the retail pistachios were collected, sub-cultured and stocked by Vivian Ly. The bacterial strain, *Enterobacter tabaci* 3E7, was acquired from Dr. Manish Raizada from the Department of Plant Agriculture from the University of Guelph. Inspiration for the methods and experimental design used were from Dr. Valeria Parreira. All other experiments were performed by the author, Krishna Gelda. An adaptation to this chapter is in preparation to be published in the Journal of Food Protection.

Chapter 3. Our second data chapter, describing how *Bacillus* spp. isolated from imported RTE date fruits can be used to control *L. monocytogenes*. This chapter also contains original and unpublished work completed primarily by the author, Krishna Gelda. *Bacillus pumilus* 10CP18 and 10CP20 were provided as a gift from Atinuke Akinpelu from Dr. Gisèle LaPointe’s laboratory at the University of Guelph. The following *L. monocytogenes* strains (10403S, DP-L4137, NF-L1177, DP-L6508, DP-L5061 and DP-L6562) were acquired from Dr. Daniel Portnoy from the University of California, Berkeley. Inspiration for the methods and experimental design used were from Dr. Valeria Parreira. An adaptation to this chapter is in preparation to be published in the Journal of Food Control.
ACKNOWLEDGEMENTS

This thesis was completed with the direction, help and support of many people.

I would first like to express my deepest gratitude to Dr. Jeff Farber and Dr. Valeria Parreira for giving me the opportunity to work with them on this thesis project. I will always be grateful to their guidance, critique, patience and kindness in helping me bring this thesis project to life. To Dr. Farber in particular for sharing his wealth of knowledge, wisdom and networks with me – I couldn’t have gotten my dream job without his help. I further appreciated the calm, cool and collected nature he would show – it was very inspiring to be around. His technical presentations and panel talks were always some of my favourites to listen to. And to Valeria for being that guardian angel for me and the rest of our group. Her troubleshooting skills were some of the most inspired and her management style is something I truly admired. She was that beacon of light providing us with a constant stream of calm and security throughout this whole process – we are so blessed to have her. My sincerest thank you to the both of them – their feedback and encouragements have pushed me to become a more effective communicator and an overall better researcher.

I thank Dr. Gisèle LaPointe for being my co-advisor and mentor for this project. I appreciated how her door was always open to us and the wealth of knowledge she had to share. I further appreciated the workshops she helped coordinate for us, including trainings for ddPCR, primer design and qPCR. I strive for my memory to be as impeccable as hers. I also thank Nafiseh Jam, our laboratory manager at CRIFS, for filling our work environment with mounds of positive energy. I appreciated all the life she brought to us including the succulent garden in the
kitchen, the delicious ice-cream from food science, the fresh flowers at reception, her vegan chili and for the garden she helped create at the back of our building. She truly is a den-mother to all of us at CRIFS.

I further convey my sincerest gratitude to all the past and current members of Dr. Farber’s lab and CRIFS. I enjoyed chatting, bonding and building relationships with each and every one of them and listening to all of their unique backgrounds and interesting perspectives they offered to share. They are some of my most favourite people I had the privilege to meet and I felt so blessed to share my journey with them. They truly made the work environment more vibrant and additionally helped foster a sense of community and family for me at CRIFS.

I also thank the statistical expertise from both Dr. Steve Bowley and Dr. Michelle Edwards. To Dr. Bowley for teaching PLNT*6170 (Statistics in Plant Agriculture) – it was one of the most useful courses I took throughout this graduate degree. Dr. Bowley was a terrific professor that made the subject of statistics less daunting and dreadful, teaching us practical knowledge and skills on correlation and regression, distributions, error control, experimental design, factorials, residual analysis and variance analysis. To Michelle for her patience and compassion she offers to everyone in our college during her statistical consultations and plethora of workshops. I always enjoyed learning and chatting about statistics and data management with her – she made those subjects more enjoyable and less intimidating.

I further acknowledge our generous funding source, the Canada First Research Excellence Fund (CFREF) through the Food from Thought (FFT) research program at the University of Guelph. I also acknowledge all of the generous scholarships I was blessed to
receive throughout this journey. This included NSERC’s Canada Graduate Scholarship – Master’s Program (CGS-M) and the following from the University of Guelph: the Guelph Food Technology Centre (GFTC) Legacy Fund, the M. Frances Hucks Memorial Research Scholarship and the Soden Memorial Graduate Scholarship in Agriculture. These sources of financial support made it a much more comfortable experience completing this graduate degree and made it easier to conduct great research in the field of food microbiology.

I also thank the University of Guelph for offering tremendously fruitful experiences and many opportunities for professional development. Special thanks to the UofG Library services for their Brain-Food workshops, Dissertation Boot Camp, Grammar Tune-Up workshops, Presentation Boot Camp and writing consultations; to Open Education for their workshops on teaching and for offering the Graduate Student University Teaching Conference and Day (GSUTC/D); and to the University in general for fostering an environmentally-conscious outlook to its green space and for offering delicious plant-based meals.

To my Mississauga family, I thank them from the bottom of my heart for their endless stream of support through their prayers, encouragements, delicious food and for always believing in me – without their tremendous love, I would not be the person whom I am today. And finally, I thank my Bhua. Although she was my biological aunt, I came to see her as a grandmother figure and overall best friend. I appreciate that she still looks after me even though she is no longer with us – another true guardian angel for me.

Thank you all sincerely,

Krishna Gelda
TABLE OF CONTENTS

Abstract ................................................................................................................................. ii
Dedication ........................................................................................................................... iii
Forward ............................................................................................................................... iv
Acknowledgements .......................................................................................................... v
Table of Contents .............................................................................................................. viii
List of Tables ...................................................................................................................... xii
List of Figures .................................................................................................................... xiii
List of Abbreviations ........................................................................................................ xiv
List of Appendices ............................................................................................................. xvii
Research rationale .......................................................................................................... 1
  Hypothesis......................................................................................................................... 2
  Research objectives.......................................................................................................... 3
Chapter 1 – Literature Review .......................................................................................... 4
  1.1 Listeria monocytogenes .............................................................................................. 5
    1.1.1 Microbiology ......................................................................................................... 5
    1.1.2 Clinical manifestations: Listeriosis ....................................................................... 6
    1.1.3 Epidemiology ........................................................................................................ 8
    1.1.4 Pathogenesis ......................................................................................................... 12
    1.1.5 Virulence factors ................................................................................................. 13
    1.1.6 Regulation of pathogenesis ............................................................................... 21
    1.1.7 Fluorescence reporter assay ............................................................................... 23
  1.2 Link to food safety ...................................................................................................... 25
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.1 Ready-to-eat (RTE) foods</td>
<td>25</td>
</tr>
<tr>
<td>1.2.2 Risk assessment of RTE foods</td>
<td>26</td>
</tr>
<tr>
<td>1.3 Control measures</td>
<td>27</td>
</tr>
<tr>
<td>1.3.1 Bacteriocin proteins</td>
<td>28</td>
</tr>
<tr>
<td>Chapter 2 – An examination of the culturable microbiota from imported RTE foods for antibacterial activity against <em>Listeria monocytogenes</em></td>
<td>33</td>
</tr>
<tr>
<td>Abstract</td>
<td>34</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>35</td>
</tr>
<tr>
<td>2.2 Materials and methods</td>
<td>38</td>
</tr>
<tr>
<td>2.2.1 Bacterial strains and growth conditions</td>
<td>38</td>
</tr>
<tr>
<td>2.2.2 Isolation of bacteria from the imported RTE foods</td>
<td>38</td>
</tr>
<tr>
<td>2.2.3 Growth inhibition plate assay</td>
<td>39</td>
</tr>
<tr>
<td>2.2.4 Identification of the inhibitory strains</td>
<td>40</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>43</td>
</tr>
<tr>
<td>2.3.1 Rationale and overview of the foods used</td>
<td>43</td>
</tr>
<tr>
<td>2.3.2 Survey of the bacterial growth from the foods</td>
<td>43</td>
</tr>
<tr>
<td>2.3.3 Growth inhibition plate assay</td>
<td>44</td>
</tr>
<tr>
<td>2.3.4 Overview of the identification of the inhibitory strains</td>
<td>45</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>51</td>
</tr>
<tr>
<td>2.5 Conclusion</td>
<td>55</td>
</tr>
<tr>
<td>Chapter 3 – The use of <em>Bacillus</em> spp. isolated from the microbiota of imported RTE date fruits to control <em>Listeria monocytogenes</em></td>
<td>56</td>
</tr>
<tr>
<td>Abstract</td>
<td>57</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>59</td>
</tr>
<tr>
<td>3.2 Materials and methods</td>
<td>63</td>
</tr>
</tbody>
</table>
3.2.1 Bacterial strains and growth conditions ........................................... 63
3.2.2 Isolation of bacteria from the date fruits ........................................... 63
3.2.3 Identification of the food isolates ..................................................... 64
3.2.4 Growth inhibition plate assay ......................................................... 65
3.2.5 Detection of the pumilacidin toxin from the inhibitory strains ............ 69
3.2.6 Virulence reporter assay ................................................................. 69
3.2.7 Characterization of the anti-Listeria compound(s) ............................ 72
3.2.8 Statistical analysis ........................................................................... 73

3.3 Results ................................................................................................. 74
3.3.1 Overview of the imported date fruits collected .................................. 74
3.3.2 Microbial composition of the date fruits ........................................... 74
3.3.3 Identification of the date fruit isolates .............................................. 75
3.3.4 Impact of the bacterial isolates from date fruits on L. monocytogenes growth .... 76
3.3.5 Screening of the inhibitory strains for safety and novelty ................. 78
3.3.6 Impact of the inhibitory strains on L. monocytogenes virulence expression ...... 79
3.3.7 Characterization of the anti-Listeria compound(s) ............................ 81

3.4 Discussion .......................................................................................... 92
3.4.1 Overview of the imported date fruits collected .................................. 92
3.4.2 Microbial composition of the date fruits ........................................... 92
3.4.3 Identification of the date fruit isolates .............................................. 93
3.4.4 Impact of the bacterial isolates from date fruits on L. monocytogenes growth .... 95
3.4.5 Screening of the inhibitory strains for safety and novelty ................. 97
3.4.6 Impact of the inhibitory strains on L. monocytogenes virulence expression ...... 99
3.4.7 Characterization of the anti-Listeria compound(s) ............................ 100
3.5 Conclusion .................................................................................................................. 103

General conclusion ........................................................................................................... 104

Future Steps ....................................................................................................................... 105

References ......................................................................................................................... 107

Appendix A – iLSM recipe ............................................................................................... 124

Appendix B – Sample statistical analysis ........................................................................ 125

Appendix C – Conference abstracts .................................................................................. 128
  C1 Ontario Food Protection Association (OFPA) Traceability Symposium .................. 128
  C2 Guelph Food Safety Seminars (GFSS) Symposium .................................................... 129
  C3 Plant Sciences Symposium: Agriculture with Less ..................................................... 130
  C4 International Association for Food Protection (IAFP)’s European Symposium ...... 131
  C5 Centre for Public Health and Zoonoses (CPHAZ) Annual Symposium ............... 132
  C6 Agri-Food Excellence Symposium .......................................................................... 133
  C7 International Association for Food Protection (IAFP)’s Annual Meeting ............ 134

Appendix D – Scientific posters ....................................................................................... 135

Appendix E – Data management plan .............................................................................. 141
  Data collection .................................................................................................................. 141
  Documentation and metadata ......................................................................................... 142
  Storage and backup ......................................................................................................... 144
  Preservation ...................................................................................................................... 144
  Sharing and reuse ............................................................................................................ 145
  Ethical and legal compliance .......................................................................................... 145
  Responsibilities and resources ......................................................................................... 146
LIST OF TABLES

Table 1.1. Summary of listeriosis outbreaks in Canada................................................................. 10
Table 1.2. Summary of the recent listeriosis outbreaks (2014 – current) reported worldwide..... 11
Table 1.3. List of the core virulence factors from L. monocytogenes directly linked to PrfA...... 15
Table 1.4. List of bacteriocins with reported efficacy against L. monocytogenes ................. 30

Table 2.1. List of the bacterial strains used in this study.............................................................. 38
Table 2.2. List of the primers used in this study ............................................................................ 41
Table 2.3. Summary of the bacterial strains collected from the various imported, RTE foods that inhibit Listeria monocytogenes ......................................................................................... 49
Table 2.4. Overview of details of the six inhibitory genera............................................................ 50

Table 3.1. List of all Listeria monocytogenes strains used in the study ..................................... 66
Table 3.2. List of all other strains used in this study ................................................................. 67
Table 3.3. Overview of the imported date fruits used in this study ............................................. 68
Table 3.4. List of primers used in this study ................................................................................. 71
Table 3.5. Summary of the bacterial community screened from the date fruits ...................... 83
Table 3.6. Effects of protease enzymes, pH and temperature on the anti-Listeria activity of the B. altitudinis DS11 supernatant (N=3, ± STD) ................................................................. 91
LIST OF FIGURES

Figure 1.1. Path of infection for *Listeria monocytogenes* through the human body ....................... 14

Figure 1.2. Overview of the *L. monocytogenes* infection cycle based on those virulence factors (in red) directly linked to PrfA ........................................................................................................................................................................ 17

Figure 1.3. Schematic representation of the virulence assay using red fluorescent protein (RFP) as a reporter in recombinant *L. monocytogenes* strains........................................................................................................ 24

Figure 2.1. Overview of the imported RTE foods used in this study ................................................. 41

**Figure 2.2.** Flow chart summarizing the steps used to recover bacterial isolates from the imported RTE foods tested............................................................................................................................................... 42

Figure 2.3. Overview of how bacterial isolates were collected from the imported, RTE foods... 47

Figure 2.4. Sample plates from the growth inhibition assay of bacterial isolates, acquired from the Chinese, Tunisian, and U.A.E. date fruits, tested against *Listeria monocytogenes* 4b (Lm4b).... 48

Figure 3.1. Overview of the imported RTE date fruits used in this study ......................................... 68

Figure 3.2. PCR amplification of the 16S rRNA gene from the date fruit inhibitory strains...... 84

Figure 3.3. Bacterial profile of the date fruits based on the 16S rRNA signatures of their cultured bacteria.................................................................................................................................................... 85

Figure 3.4. Summary of the inhibition zones produced by the inhibitory strains isolated from the date fruits against *L. monocytogenes* (N=3, + STD).................................................................................................. 86

Figure 3.5. PCR amplification of the orfY gene against all date fruit inhibitory strains using the orfyF7700/orfyR8122 primer set ................................................................................................................................................. 87

Figure 3.6. Assessing RFP expression (under the control of the PrfA-dependent actA promoter) of *L. monocytogenes* (N=3, + STD)........................................................................................................ 88

Figure 3.7. Fluorescence (RFU, ex/em: 550/580 nm) and growth (OD$_{600}$) from strains of *L. monocytogenes* expressing RFP under the control of the PrfA-dependent actA promoter post treatment with cell-free-supernatant ........................................... 89

Figure 3.8. Characterization of the active supernatant from *B. altitudinis* DS11 using a growth inhibition plate against *L. monocytogenes* 4b .................................................................................................................................................. 90
## LIST OF ABBREVIATIONS

### A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC</td>
<td>Advanced Analysis Centre (University of Guelph Genomics Facility)</td>
</tr>
<tr>
<td>ActA</td>
<td>Actin polymerization inducing protein</td>
</tr>
<tr>
<td>AICC</td>
<td>Akaike information criterion (biased corrected)</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>a\textsubscript{w}</td>
<td>Water activity</td>
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### B

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<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
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<tr>
<td>BIOHAZ</td>
<td>EFSA Panel on Biological Hazards</td>
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<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>Bs</td>
<td>Bacillus species group</td>
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<th>Abbreviation</th>
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<tr>
<td>cAMP</td>
<td>cyclic Adenosine monophosphate</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
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<tr>
<td>CFIA</td>
<td>Canadian Food Inspection Agency</td>
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<tr>
<td>CFREF</td>
<td>Canada First Research Excellence Fund</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>CFS</td>
<td>Cell-free-supernatant</td>
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<tr>
<td>CHL</td>
<td>Chloramphenicol</td>
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<tr>
<td>CPHAZ</td>
<td>Centre for Public Health and Zoonoses</td>
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<td>CRIFS</td>
<td>Canadian Research Institute in Food Safety</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DC</td>
<td>Chinese date fruits</td>
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<td>DI</td>
<td>Iranian date fruits</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide Triphosphates</td>
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<tr>
<td>DP</td>
<td>Palestinian date fruits</td>
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<td>DS</td>
<td>Saudi-Arabian date fruits</td>
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<td>DT</td>
<td>Tunisian date fruits</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine triacetic acid</td>
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<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
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<td>FDA</td>
<td>Federal Drug Administration</td>
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<td>FFT</td>
<td>Food from Thought</td>
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<tr>
<td>G6PT</td>
<td>Glucose-6-Phosphate Transporter</td>
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<td>GFSS</td>
<td>Guelph Food Safety Seminars</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<td>GHP</td>
<td>Good Hygiene Practices</td>
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<tr>
<td>GMP</td>
<td>Good Manufacturing Practices</td>
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<tr>
<td>GRAS</td>
<td>Generally Regarded as Safe</td>
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<tr>
<td>gshF</td>
<td>Glutathione synthase</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td><strong>H</strong></td>
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<tr>
<td>HACCP</td>
<td>Hazard Analysis Critical Control Point</td>
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<td>Hly</td>
<td>Hemolysin</td>
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<tr>
<td>Hpt</td>
<td>Hexose Phosphate Transporter</td>
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<td>HTS</td>
<td>High-throughput sequencing</td>
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<td><strong>I</strong></td>
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<tr>
<td>IAFP</td>
<td>International Association for Food Protection</td>
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<tr>
<td>iap</td>
<td>Invasion associated protein</td>
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<tr>
<td>iLSM</td>
<td>Incomplete <em>Listeria</em> synthetic media</td>
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<tr>
<td>InlA</td>
<td>Internalin A</td>
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<td>InlB</td>
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<td>LB</td>
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<td>LIPI-1</td>
<td>Listeria pathogenicity island 1</td>
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<td>LLO</td>
<td>Listeriolysin O</td>
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<td>Lm4b</td>
<td><em>Listeria monocytogenes</em> FSL J1-110</td>
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<td>Mpl</td>
<td>Zinc metalloproteinase protein</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>NICD</td>
<td>National Institute for Communicable Diseases</td>
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<td>NGS</td>
<td>Next generation sequencing</td>
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<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density at 600 nm</td>
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<td>O/N</td>
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<td><strong>P</strong></td>
<td></td>
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<td>PI-PLC</td>
<td>Phosphatidylinositol-specific phospholipase C</td>
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<td>PHAC</td>
<td>Public Health Agency of Canada</td>
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<tr>
<td>PrfA</td>
<td>Positive regulatory factor A</td>
</tr>
<tr>
<td>PW</td>
<td>Peptone water</td>
</tr>
<tr>
<td><strong>R</strong></td>
<td></td>
</tr>
<tr>
<td>RCBD</td>
<td>Randomized complete block design</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTE</td>
<td>Ready-to-eat</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis Software</td>
</tr>
<tr>
<td>STD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>STR</td>
<td>Streptomycin</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td></td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td><strong>U</strong></td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td><strong>W</strong></td>
<td></td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

Appendix A – iLSM recipe ........................................................................................................................................ 124
Appendix B – Sample statistical analysis ............................................................................................................. 125
Appendix C – Conference abstracts ...................................................................................................................... 128
Appendix D – Scientific posters .......................................................................................................................... 135
Appendix E – Data management plan .................................................................................................................. 141
Research rationale

Illnesses caused by foodborne pathogens continue to remain a major health and economic concern. According to the Government of Canada (2016b), 1 in 8 Canadians are affected by a foodborne illness each year, resulting in around 11,500 hospitalizations and 250 deaths. Due to this high prevalence and risk, there is a great need to discover novel means for controlling these pathogens. One approach to help control these pathogens is to better understand the interactions(s) between the microbiome of foods and the foodborne pathogens. Member(s) of the food microbiome may help inactivate pathogens and/or prevent their growth based on several factors, such as competitive exclusion.

One high-risk product class in Canada is ready-to-eat (RTE) foods, as many of them can support the rapid growth of foodborne pathogens such as *L. monocytogenes* (Health Canada, 2010). This pathogen is one of the most severe, as it is associated with a case-fatality rate of approximately 20 – 30% (Government of Canada, 2011; Swaminathan and Gerner-Smidt, 2007), the highest of any foodborne pathogen (Bennion et al., 2008). In contrast, those RTE foods that do not support the growth of *L. monocytogenes* throughout their shelf-life (category 2B), may instead support the survival of a microflora that possesses beneficial, antimicrobial bacteria/traits that naturally prevent pathogen growth and/or virulence. This is particularly of interest to those RTE foods that have not yet been associated with a foodborne outbreak. Additionally, investigating imported RTE foods that are popular in only certain regions of the world or in certain populations, may increase the probability of finding novel, beneficial bacteria that have not been previously studied for these applications. This approach could lead to the discovery of bacteria that produce
anti-Listeria biocontrol agents/bacteriocins that are effective against a larger breadth of food matrices in our supply chain.

**Hypothesis**

This research thesis work proposes to investigate the culturable microbiota of select imported, RTE foods to see whether the existing bacterial microflora could inactivate and/or inhibit the growth and/or cause a reduction in the virulence of *L. monocytogenes*. These foods include dried apple slices, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed. **Thus, the hypothesis for this thesis is that the microbiota of these foods will contain culturable bacterial members that possess antagonistic properties against the foodborne pathogen *L. monocytogenes*.**
**Research objectives**

The specific objectives of this study are to:

1. Select among the imported RTE foods collected (cumin seeds, date fruits, dried apples, fennel seeds, pistachios, pollen, raisins, and seaweed), a specific food to further pursue in investigations to find bacteria which have antagonistic properties against *L. monocytogenes*.

2. Assess whether the culturable bacteria that are isolated from the food selected in objective 1, can inactivate and/or inhibit the growth of *L. monocytogenes*.

3. Assess whether the inhibitory strains identified in objective 2 would be considered novel and safe to use for biocontrol applications in the food industry.

4. Assess whether the strains identified from objective 3 can also impact the virulence of *L. monocytogenes* based on the inhibition of PrfA expression.

5. Characterize the anti-*Listeria* compound(s) produced from the most promising inhibitory strain identified from objective 3.
Chapter 1 – Literature Review

The purpose of this review is to provide a background on the necessary information needed to understand the thesis. Specifically, this includes an overview of the foodborne pathogen *Listeria monocytogenes* in relation to its microbiology, epidemiology, pathogenesis, virulence factors, regulation of its virulence factors and an overview of a fluorescence reporter assay to study virulence. Next a discussion of this pathogen’s link to food safety is provided, specifically to ready-to-eat (RTE) foods and how risk assessments are done on these foods. Last, a critical analysis of the most widely used and adopted control measures for this pathogen are discussed, with an emphasis on bacteriocin proteins.
1.1 *Listeria monocytogenes*

1.1.1 Microbiology

*Listeria monocytogenes* is a Gram-positive, rod-shaped bacteria that is facultatively anaerobic, non-spore forming, and non-encapsulated. They are small in size, roughly 1 – 2 µm in length by 0.5 µm in diameter, and are additionally found to be catalase-positive, oxidase-negative and motile at low temperatures (Low and Donachie, 1997). They are a member of the phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, and the family *Listeriaceae* (Low and Donachie, 1997). The genus *Listeria* currently contains 17 species (Weller et al., 2015), and while two are considered pathogenic, namely *L. monocytogenes* and *L. ivanovii*, only *L. monocytogenes* is considered to be a significant threat to human public health (Swaminathan and Gerner-Smidt, 2007).

*L. monocytogenes* can be further divided into 12 serotypes based on somatic (O-antigen) and flagellar (H-antigen) antigens: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7 (Seeliger, 1984). However, its pathogenicity is most often associated with four serotypes, mainly, 1/2a, 1/2b, 1/2c, and 4b, as they are the cause of more than 98% of infections in humans (Roche et al., 2003). More specifically, among these human pathogenic serotypes, serotype 4b has been found to cause many of this pathogen’s reported cases worldwide (Boerlin and Piffaretti, 1994). However, strains of the antigenic group 1/2 (1/2a, 1/2b, and 1/2c) are found to predominate more in food isolates (Schönberg et al., 1989). Based on this information, this would suggest that serotype 4b strains may be more virulent and/or better adapted to mammalian host tissues than strains from serogroup 1/2.
*L. monocytogenes* was first isolated and discovered by Murray et al. (1926) during a septicemic outbreak that affected rabbits and guinea pigs in their laboratory at Cambridge, England. Since then, it has proven to be a very hardy pathogen. In nature, *Listeria* spp. live as saprophytes, deriving nutrients from dead and decaying organic matter (Vázquez-Boland et al., 2001b). It can grow and survive outside a host at a wide range of pH values from 4.3 to 9.1 (CDC, 2011), at high salt concentrations of 10% wt/v (Mcclure et al., 1989), and the most concerning, at refrigeration temperatures that span -0.5 to 9.3°C (Walker et al., 1990). It can further multiply at temperatures between 1 and 45°C, where its optimal temperature range is 30 – 37°C (Low and Donachie, 1997). However, organisms grown at 37°C show little or no motility, while tumbling motility has been demonstrated during growth at room temperature (22 – 25°C) via organisms displaying peritrichous flagella (Peel et al., 1988).

### 1.1.2 Clinical manifestations: Listeriosis

Due to its ubiquitous and resilient nature, *L. monocytogenes* can be found in a number of reservoirs such as soil, untreated water, and a variety of food matrices. Its predominant mode of transmission to humans is by ingestion of contaminated food or water. This pathogen has not been found to spread from person-to-person. However, listeriosis can occur after contact with infected animals, so-called cutaneous listeriosis (Government of Canada, 2016a). As a result, farmers and veterinarians must be cautious when handling potentially infected animals, especially during the birthing and/or butchering of the animals.

Once transmitted to humans, this intracellular pathogen can cause listeriosis; a severe, invasive, foodborne disease that leads to intestinal, blood, brain and fetal infections. This is due
to the versatility of the pathogen as it is capable of crossing three tight human barriers: the intestinal barrier, the blood-brain barrier, and the feto-placental barrier (Bierne et al., 2018). It all begins with *L. monocytogenes* invading the intestinal epithelial cells. Once there, it can gain access to the lymphatic system and blood stream, ultimately resulting in dissemination to other internal organs like the liver, spleen, central nervous system, and placenta (Swaminathan and Gerner-Smidt, 2007). Listeriosis can start as early as 20 h after eating contaminated food and can cause symptoms of mild gastroenteritis, fever, headaches and muscle aches (Government of Canada, 2016a). However, for cases of invasive listeriosis, infection can range from 1 – 67 days, with a median incubation period that can differ significantly based on the clinical form of the disease (Goulet et al., 2013). Sequelae such as brain infections and/or blood poisoning can also occur with listeriosis (Government of Canada, 2016a).

Although the incidence of reported listeriosis cases remain low, i.e., 1 – 10 cases per million per year (Goulet et al., 2012), once diagnosed with this disease, the infection can result in a case-fatality rate as high as 30%, despite antibiotic treatment (Swaminathan and Gerner-Smidt, 2007); the highest of any foodborne pathogens (Bennion et al., 2008). This is why *L. monocytogenes* is considered a significant threat to public health; its pathogenic nature is one of the most severe when it comes to food safety.

The dose response of *L. monocytogenes* for humans is still unknown (Buchanan et al., 2017). However, various risk assessments have clearly demonstrated that preventing the growth of *L. monocytogenes* in RTE foods reduces the risk of listeriosis cases by 100- to 1000-fold on a per serving basis (FAO, 2004). Furthermore, recent quantitative modelling conducted by EFSA suggests that more than 90% of invasive listeriosis is caused by ingestion of RTE food
containing > 2,000 colony forming units (CFU)/g, and that one-third of cases are due to growth present at the consumer level (BIOHAZ, 2018). Those at a higher risk of becoming sick are those individuals with a weakened immune system related to aging, underlying diseases, and/or immunosuppressive therapies, as well as pregnant women. Even though the pregnant mothers typically remain asymptomatic, *L. monocytogenes* can invade their placenta and birth canal to cause severe infection (or even death) to the unborn or newborn baby (Charlier et al., 2017).

### 1.1.3 Epidemiology

Human listeriosis can occur as sporadic cases or outbreaks, where an outbreak is defined as two or more people acquiring the same illness from the same contaminated food or drink (CDC, 2018a). Additionally, listeriosis can occur worldwide; however, it is often reported as an outbreak in developed countries. This is due to how outbreaks are reported: the limited surveillance methods in developing countries results in fewer cases being reported by them. Nonetheless, for those countries with better surveillance methods, the incidence is typically 1 – 5 cases annually per million people (Ferreira et al., 2014). Additionally, because listeriosis outbreaks are rarely reported (<10 outbreaks worldwide per year), the annual incidence is suspected to be higher due to small clusters and/or sporadic cases that go unreported (Ferreira et al., 2014).

Detection of listeriosis outbreaks and their respected sources is a complicated process. This is due to the common presence of *L. monocytogenes* in various environments, the long incubation period required (~ 1 week – 2 months) before onset of illness, and the multiple foods that can serve as vehicles in listeriosis outbreaks (Todd and Notermans, 2011. The first
confirmed foodborne outbreak of listeriosis occurred in 1981 in the Maritime Provinces of Canada, where 41 cases and 18 deaths were reported due to the consumption of contaminated cabbage in coleslaw. It was not until this outbreak in Canada that listeriosis became recognized as a serious concern for public health in Canada (Schlech et al., 1983). Since then, Canada has also encountered outbreaks linked to cheese, deli-meat, and packaged salad food matrices. Their largest listeriosis outbreak to-date occurred in 2002, where over 130 foodborne illnesses were linked to the consumption of contaminated soft-ripened cheese in British Columbia (McIntyre et al., 2015). A summary of these Canadian outbreaks can be found in Table 1.1.

When looking at the recent listeriosis outbreaks across the globe (Table 1.2), they have primarily been associated with dairy (cheese, ice cream, raw milk), meat (sausage, pâté, pork products, ham), and produce (bean sprouts, caramel apples, packaged salads, frozen vegetables). The most noteworthy and largest outbreak to date occurred in South Africa from 2017 – 2018, where the source of the outbreak was traced back to Polony, a RTE processed meat product. This outbreak involved nine South African provinces and resulted in 1024 cases, and over 200 deaths. Neonates (≤ 28 days of age) were the most affected age group, followed by adults aged 15 – 49 years of age (NICD, 2018). In 2014, there was another large listeriosis outbreak, this time in Denmark, which was associated with Rullepølse, another RTE processed meat product. In this incident, 41 cases and 17 deaths were recorded (Jensen et al., 2016).

For both large outbreaks, although the route of contamination was unclear, the introduction of L. monocytogenes was thought to have occurred during the process of pressing, cutting, and wrapping the meat before distribution, after the meat was boiled (NICD, 2018;
Jensen et al., 2016). This demonstrates that listeriosis outbreaks are often due to poor sanitation practices followed during the manufacturing, storage or handling of the final product. This further emphasizes the need for food processing plants to follow Good Hygiene Practices (GHP), Good Manufacturing Practices (GMP), and a food safety management system based on the principles of Hazard Analysis and Critical Control Points (HACCP).

Table 1.1. Summary of listeriosis outbreaks in Canada

<table>
<thead>
<tr>
<th>Year</th>
<th>Food</th>
<th>Locationa</th>
<th>Number of cases (deaths)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>Cheese</td>
<td>British Columbia</td>
<td>135 (0)</td>
<td>McIntyre et al., 2015</td>
</tr>
<tr>
<td>2008</td>
<td>Cheese</td>
<td>Quebec</td>
<td>40 (2)</td>
<td>Gaulin and Ramsay, 2010</td>
</tr>
<tr>
<td>2008</td>
<td>Meat (deli)</td>
<td>Nationwide (7)</td>
<td>57 (24)</td>
<td>Currie et al., 2015</td>
</tr>
<tr>
<td>2015-2016</td>
<td>Packaged salad</td>
<td>Nationwide (5)</td>
<td>14 (3)</td>
<td>PHAC, 2016</td>
</tr>
</tbody>
</table>

a Number in brackets refers to the number of provinces implicated in the outbreak.
Table 1.2. Summary of the recent listeriosis outbreaks (2014 – current) reported worldwide

<table>
<thead>
<tr>
<th>Food Matrix</th>
<th>Type</th>
<th>Year</th>
<th>Locationa</th>
<th>Number of cases (death)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy</td>
<td>Cheese (soft)</td>
<td>2014</td>
<td>USA (2)</td>
<td>8 (1)</td>
<td>CDC, 2014a</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>2014</td>
<td>USA (4)</td>
<td>5 (1)</td>
<td>CDC, 2014b</td>
</tr>
<tr>
<td></td>
<td>Cheese (soft)</td>
<td>2015</td>
<td>USA (10)</td>
<td>30 (3)</td>
<td>CDC, 2015a</td>
</tr>
<tr>
<td></td>
<td>Ice Cream</td>
<td>2015</td>
<td>USA (4)</td>
<td>10 (3)</td>
<td>CDC, 2015b</td>
</tr>
<tr>
<td></td>
<td>Milk (raw)</td>
<td>2016</td>
<td>USA (2)</td>
<td>2 (1)</td>
<td>CDC, 2016a</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>2017</td>
<td>USA (4)</td>
<td>8 (2)</td>
<td>CDC, 2017</td>
</tr>
<tr>
<td>Meat</td>
<td>Rullepølse (sausage)</td>
<td>2014</td>
<td>Denmark</td>
<td>41 (17)</td>
<td>Jensen et al., 2016</td>
</tr>
<tr>
<td></td>
<td>Pâté</td>
<td>2016</td>
<td>Switzerland</td>
<td>5 (0)</td>
<td>Althaus et al., 2017</td>
</tr>
<tr>
<td></td>
<td>Polony (sausage)</td>
<td>2017-2018</td>
<td>South Africa (9)</td>
<td>1024 (200)</td>
<td>NICD, 2018</td>
</tr>
<tr>
<td></td>
<td>Pork products</td>
<td>2018</td>
<td>USA (4)</td>
<td>4 (0)</td>
<td>CDC, 2019a</td>
</tr>
<tr>
<td></td>
<td>Ham (deli)</td>
<td>2018</td>
<td>USA (2)</td>
<td>4 (1)</td>
<td>CDC, 2018b</td>
</tr>
<tr>
<td>Mixed</td>
<td>Meat &amp; cheese (deli)</td>
<td>2019</td>
<td>USA (4)</td>
<td>8 (1)</td>
<td>CDC, 2019b</td>
</tr>
<tr>
<td>Produce</td>
<td>Bean sprouts</td>
<td>2014</td>
<td>USA (2)</td>
<td>5 (2)</td>
<td>CDC, 2015c</td>
</tr>
<tr>
<td></td>
<td>Caramel apples</td>
<td>2014</td>
<td>USA (12)</td>
<td>35 (7)</td>
<td>CDC, 2015d</td>
</tr>
<tr>
<td></td>
<td>Packaged salad</td>
<td>2015-2016</td>
<td>USA (9)</td>
<td>19 (1)</td>
<td>CDC, 2016b</td>
</tr>
<tr>
<td></td>
<td>Packaged salad</td>
<td>2015-2016</td>
<td>Canada (5)</td>
<td>14 (3)</td>
<td>PHAC, 2016</td>
</tr>
<tr>
<td></td>
<td>Frozen vegetables</td>
<td>2015-2018</td>
<td>Austria, Denmark, Finland, Sweden, UK</td>
<td>32 (6)</td>
<td>EFSA, 2018a</td>
</tr>
<tr>
<td></td>
<td>Frozen vegetables</td>
<td>2016</td>
<td>USA (4)</td>
<td>9 (3)</td>
<td>CDC, 2016c</td>
</tr>
<tr>
<td>Seafood</td>
<td>Salmon</td>
<td>2016-2018</td>
<td>Denmark, Germany, France</td>
<td>12 (4)</td>
<td>EFSA, 2018b</td>
</tr>
</tbody>
</table>

a Number in brackets refers to the number of provinces/states implicated in the outbreak.
1.1.4 Pathogenesis

After the ingestion of contaminated food, the primary localization point of \textit{L. monocytogenes} is believed to be the intestinal epithelium (Rácz et al., 1972). However, before reaching this epithelium, the ingested organisms must withstand the adverse environments of the stomach, an environment typically lethal for most bacteria. To do so, \textit{L. monocytogenes} has a series of systems in place to combat acid resistance, nutrient acquisition and low oxygen tolerance (Ferreira et al., 2003). However, the precise mechanisms needed to withstand these stresses and ultimately translocate past the stomach remains unknown (Barbuddhe and Chakraborty, 2009). Nonetheless, for most individuals, their gastric acidity is believed to destroy the majority (if not all) of \textit{L. monocytogenes} organisms ingested with their contaminated food (Schuchat et al., 1992; Ho et al., 1986).

If these pathogenic cells manage to survive passage through the stomach, the preferential site of their bacterial replication are the Peyer’s Patches: the lymphoid structures of the small intestines. It is here where \textit{L. monocytogenes} establishes its active site of local infection (Barbuddhe and Chakraborty, 2009). From there, it parasitizes macrophages via its intracellular lifecycle, and actively begins to invade a range of non-phagocytic cells (Barbuddhe and Chakraborty, 2009). Once this intestinal barrier is breached, \textit{L. monocytogenes} can disseminate throughout the body. Its next preferential site of infection is the liver and spleen, where this pathogen begins to multiply inside their respective cells (Vázquez-Boland et al., 2001b; Waite et al., 2011).
For most individuals that remain infected at this point, their anti-
Listeria memory T-cells come into play and the infection is resolved by their cell-mediated immune response. However, in immunocompromised individuals, uncontrolled proliferation of *L. monocytogenes* occurs in the liver/spleen, which could then result in low-levels of bacteremia (Vázquez-Boland et al., 2001b). Patients who experience bacteremia due to *L. monocytogenes*, most likely have a physiological or pathological defect in their T-cell-mediated immunity (Schlech et al., 2005). The bacteremia could then lead to invasion of secondary target organs, such as the brain and uterus (Barbuddhe and Chakraborty, 2009). Once at these secondary sites, further complications could ensue, such as meningitis or even death. A schematic of this infection pathway can be seen in Fig 1.1.

### 1.1.5 Virulence factors

Many virulence factors contribute to the pathogenicity of *L. monocytogenes*, and the severity of the clinical manifestations which can occur. For the context of this dissertation, a review of the major virulence factors linked to PrfA, its master virulence regulator, will be discussed in further detail and a list of these virulence factors, summarized with function, can be found in Table 1.3. Additionally, a schematic of the infection cycle due to these virulence factors can be found in Fig 1.2.
Figure 1.1. Path of infection for *Listeria monocytogenes* through the human body. (a) ingestion of food contaminated with *L. monocytogenes*; (b) passage through the stomach; (c) localization to the Peyer’s Patches of the small intestine – primary site for local infection; (d) intracellular movement through the intestinal barrier; (e) localization to the liver and spleen – preferential site for systemic infection; (f) dissemination through the bloodstream – potential invasion of secondary sites, such as the brain and uterus.
<table>
<thead>
<tr>
<th>Virulence Gene</th>
<th>Encoded Protein</th>
<th>Definition</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>actA</td>
<td>ActA</td>
<td>Actin polymerization inducing protein</td>
<td>Facilitates actin polymerization to generate sufficient force to cause <em>L. monocytogenes</em> to spread from cell-to-cell.</td>
<td>Travier et al., 2013</td>
</tr>
<tr>
<td>hpt</td>
<td>Hpt</td>
<td>Hexose phosphate transporter</td>
<td>Exploits sugars from the host cell cytoplasm to promote the intracellular growth of <em>L. monocytogenes</em>.</td>
<td>Chico-Calero et al., 2002</td>
</tr>
<tr>
<td>hly</td>
<td>LLO</td>
<td>Listeriolysin O</td>
<td>Produces pores in the vacuole via oligomerization of cholesterol to permit entry into the host cytosol; an exotoxin.</td>
<td>Dramsi and Cossart, 2002</td>
</tr>
<tr>
<td>inlA</td>
<td>InlA</td>
<td>Internalin A</td>
<td>Permits receptor-mediated endocytosis of eukaryotic cells displaying the E-cadherin receptor.</td>
<td>Bonazzi et al., 2008</td>
</tr>
<tr>
<td>inlB</td>
<td>InlB</td>
<td>Internalin B</td>
<td>Permits receptor-mediated endocytosis of eukaryotic cells displaying the Met receptor.</td>
<td>Shen et al., 2000</td>
</tr>
<tr>
<td>inlC</td>
<td>InlC</td>
<td>Internalin C</td>
<td>Promotes cell-to-cell spread and interferes with the innate immune responses from the host.</td>
<td>Rajabian et al., 2009</td>
</tr>
<tr>
<td>Gene</td>
<td>Location</td>
<td>Description</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>-------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>mpl</td>
<td>Mpl</td>
<td>Zinc metalloproteinase protein</td>
<td>Maturates the proenzyme PC-PLC (Phosphatidylcholine phospholipase C) via cleavage of its N-terminal propeptide.</td>
<td>Yeung et al., 2005</td>
</tr>
<tr>
<td>plcB</td>
<td>PC-PLC</td>
<td>Phosphatidylcholine phospholipase C</td>
<td>Catalyzes the cleavage of host membrane lipid phosphatidylcholine (PC).</td>
<td>Poussin et al., 2009</td>
</tr>
<tr>
<td>prfA</td>
<td>PrfA</td>
<td>Positive regulatory factor A</td>
<td>Regulates the transcription of most genes implicated in the intracellular life cycle of this pathogen; master virulence regulator.</td>
<td>Freitag et al., 2009</td>
</tr>
</tbody>
</table>
Figure 1.2. Overview of the *L. monocytogenes* infection cycle based on those virulence factors (in red) directly linked to PrfA. (I) Entry into the target cell is facilitated by either receptor-mediated endocytosis [via (a) InlA – E-cadherin or (b) InlB – Met interactions] or (c) phagocytosis. (II) The pathogen is then internalized inside a single-membrane cell vacuole. (III) Escape from the vacuole and entry into the cytosol is mediated by the expression of an exotoxin (LLO) and two phospholipases (PI-PLC, PC-PLC), with the latter phospholipase requiring processing by a metalloprotease (Mpl). (IV) In the cytosol, ActA is expressed to facilitate motility by polymerizing actin filaments at the bacterial surface. (V) Motility can then permit (a) replication with the aid of the Hpt sugar transporter and (b) engulfment by neighbouring cells with the aid of InlC to relax the junctional tension between host cells.
In general, the \textit{L. monocytogenes} infection cycle can be divided into three broad stages: entry into the target cell, escape from the vacuole to enter the cytosol, and movement within and between cells (Portnoy et al., 1992).

\textbf{1.1.5.1 Entry into the target cell}

\textit{L. monocytogenes} can be internalized by both phagocytic and non-phagocytic cells (Radoshevich and Cossart, 2017). Entry into phagocytic cells, such as macrophages, is mediated directly by the phagocyte. Examples of this entry are seen by the uptake of \textit{L. monocytogenes} by M cells at the site of Peyer’s patches. This entry contributes to invasion of the intestines (Jensen et al., 1998).

For non-phagocytic cells, such as the intestinal epithelial cells, entry is facilitated by receptor-mediated endocytosis (Radoshevich and Cossart, 2017). This form of entry is the most important pathway used by \textit{L. monocytogenes} to cause invasive disease (Marquis et al., 2015). This is done through expression of the internalin A (InlA) and internalin B (InlB) proteins from the respective genes \textit{inlA} and \textit{inlB}. Both surface proteins confer specificity for different cell membrane receptors. InlA binds specifically to the E-cadherin receptor (Bonazzi et al., 2008), an interaction associated with the invasion of goblet cells from the intestinal mucosa (Nikitas et al., 2011). InlB binds specifically to the Met receptor, an interaction associated with the invasion of hepatocytes from the liver (Shen et al., 2000).

Upon binding of the internalin to its respective cell membrane receptor, a phosphorylation cascade is induced, resulting in a signal transduction pathway that allows for the uptake of \textit{L. monocytogenes} into the target host cell. This uptake then internalizes the pathogen inside a single-membrane cell vacuole (Lecruit, 2005).
1.1.5.2 Entry into the cytosol

The next step is to escape the vacuole to permit the entry of *L. monocytogenes* into the target cell’s cytosol. This is facilitated by the expression of three proteins: Listeriolysin O (LLO) and two phospholipases, phosphatidylinositol-specific phospholipase C (PI-PLC), and phosphatidylcholine phospholipase C (PC-PLC) (Radoshevich and Cossart, 2017).

LLO, expressed by the *hyl* gene, is a hemolysin exotoxin, belonging to the pore-forming family of cholesterol-dependent cytolysins. Its function is to bind to and oligomerize the cholesterol of cellular membranes to form large pores, up to 35 nm in diameter (Hamon et al., 2012). However, expression of this protein is regulated by pH, and is further triggered by acidic pH (Bavdek et al., 2012). This in turn restricts expression of LLO to the inside of the vacuole, as the pH of the vacuole is more acidic than the cytosol of the cell. Thus, *L. monocytogenes* does not form these pores in the cellular membrane of its target cell (Bavdek et al., 2012).

Once LLO is expressed, both PC-PLC and PI-PLC (expressed from the genes *plcA* and *plcB*, respectively) act synergistically with LLO to permit complete vacuolar rupture and escape of *L. monocytogenes* into the cytosol of the target cell. Both PLCs cleave their target phospholipids from the cellular membrane to aid LLO in the rupturing of the vacuole (Griffith and Ryan, 1999; Poussin et al., 2009). For PC-PLC, it exists as a proenzyme and needs to be activated. This is facilitated through a zinc metalloproteinase protein (Mpl), encoded by the *mpl* gene. This protein maturates the PC-PLC proenzyme via cleavage of its N-terminal propeptide (Yeung et al., 2005).
1.1.5.3 Cell-to-cell spread

Once inside the cytosol of the target cell, *L. monocytogenes* finds itself in a new environment in which it can survive, thrive and multiply. It does so by taking advantage of the nutrients abundant in the cytosol. One way it accomplishes this is through the action of the hexose phosphate transporter (Hpt) via the *hpt* gene. This transporter mimics the function of the mammalian glucose-6-phosphate transporter (G6PT) by exploiting the sugars from the host cell cytoplasm to promote its intracellular growth (Chico-Calero et al., 2002). As a result, *L. monocytogenes* can induce changes in the morphology and function of the host cell’s organelles to promote infection (Radoshevich and Cossart, 2017). This adaptation was seen by Marquis et al. (1993) who observed that the doubling time of *L. monocytogenes* inside *in vitro* cytosolic cells was similar to this pathogen’s doubling time in rich bacterial growth media (~ 40 min).

To permit motility and intracellular spread from cell-to-cell, *L. monocytogenes* synthesizes a membrane anchored protein called ActA, the actin polymerizing inducing protein, expressed by the *actA* gene (Travier et al., 2013). ActA can polymerize host cell actin filaments at the bacterial surface to form an actin tail, and synthesis of this tail is what permits motility. Once motile, the tail forms protrusions that can be engulfed by neighboring cells to permit intracellular spread (Travier et al., 2013). Once engulfed by a new cell, *L. monocytogenes* becomes encapsulated by another cell-membrane vacuole. As before, this vacuole can then be lysed by the joint activities of the LLO and PLCs to permit *L. monocytogenes* to begin a new intracellular infection cycle in a neighboring cell (Radoshevich and Cossart, 2017). Internalin C (InlC) has also been found to aid in the cell-to-cell spread. This protein relaxes the junctional
tension between host cells and acts by interfering with the host’s innate immune responses by targeting enzyme complexes linked to the signaling of inflammation (Rajabian et al., 2009).

The combination of LLO and ActA results in an almost exclusive intracellular lifecycle. This lifestyle of L. monocytogenes helps it avoid the extracellular defenses of the host, to permit the survival of its own cells. The loss of expression from either of these proteins has been shown to fully attenuate the virulence of this pathogen, thereby demonstrating the importance of LLO and ActA in the virulence of L. monocytogenes (Portnoy et al., 1988; Kocks et al., 1992).

1.1.6 Regulation of pathogenesis

L. monocytogenes can exist in two contrasting lifestyles: a harmless saprophyte and a virulent pathogen. To switch between the two, a regulatory protein, known as PrfA (positive regulatory factor A; expressed by the prfA gene), cues this transition (Radoshevich and Cossart, 2017). PrfA is known as the master regulator of L. monocytogenes and is tightly linked to the virulence of this pathogen; L. monocytogenes strains that lack prfA are found to be completely avirulent (Xayarath and Freitag, 2012; Chakraborty et al., 1992).

PrfA is a member of the cAMP receptor protein family of bacterial transcription factors. To prompt this lifestyle transition of L. monocytogenes, PrfA integrates several environmental cues that activate a set of key virulence factors during host infection (Freitag et al., 2009). One such signal is temperature. Specifically, at 37°C (human body temperature) an RNA hairpin obscuring the prfA ribosome binding site is destabilized, enabling translation of the prfA gene (Johansson et al., 2002). From this thermoregulation, prfA selectively becomes activated upon
entry into the host cell and this proper temporal expression of PrfA is critical for bacterial
invasion. If not controlled, inappropriate expression of PrfA will occur that will cause loss of L.
monocytogenes fitness, both inside and outside of the host (Bruno and Freitag, 2010;
Vasanthakrishnan et al., 2015). As a result, PrfA acts an energy-saving switch that prevents the
wasteful production of virulence factors, thereby maximizing the bacterial fitness of L.
monocytogenes throughout its lifecycle (Scortti et al., 2007).

PrfA is directly responsible for the transcription of 9 core virulence genes (actA, hpt, hly,
inlA, inlB, inlC, mpl, plcA, plcB) and has further been found to indirectly affect the expression of
over 140 other genes, many of which are important in virulence (de las Heras et al., 2011). Some
of these regulated virulence genes belong to the LIPI-1 (Listeria pathogenicity island 1), a 9-kb
chromosomal island that is responsible for the expression of 6 key virulence factors, prfA, plcA,
hly, mpl, actA, and, plcB (Vázquez-Boland et al., 2001a). Within this island, actA is one of the
most upregulated PrfA-dependent genes, where its expression was found to increase 200-fold in
the host cytosol as compared to levels in broth culture (Shetron-Rama et al., 2003).

It was also found that glutathione synthase (gshF), via its production of glutathione
(GSH), is a critical determinant in PrfA activation. GSH is a small, antioxidant peptide used by a
variety of cells (e.g., eukaryotes, cyanobacteria and proteobacteria) as a redox buffer to protect
against oxidative damage (Masip et al., 2006). In the context of PrfA, GSH acts as a cofactor and
the affinity of PrfA for the promoter regions of hly (encodes LLO) and actA are dependent on the
oxidation levels of GSH (Reniere et al., 2015).
1.1.7 Fluorescence reporter assay

For the context of this thesis, the virulence of *L. monocytogenes* was studied using methods proposed by Portman et al., 2017. Specifically, fluorescence spectroscopy was used on reporter strains of *L. monocytogenes* in a reducing environment with GSH. These strains use red fluorescent protein (RFP) as a fluorescent reporter where RFP can be induced upon expression of this pathogen’s virulence regulator, PrfA. These reporter strains include a wild-type indicator (WT), a negative control devoid of a functional PrfA protein (ΔprfA), and a positive control that constitutively expresses the PrfA protein (PrfA*).

The RFP gene was cloned downstream of a PrfA-dependent *actA* promoter in a pPL2 vector. To obtain expression of RFP from the WT strain, complete activation of the PrfA protein must be achieved. To do so, the strain must be grown in a reducing environment that mimics the redox potential of the host cytosol. This reducing environment is obtained by using exogenous glutathione (GSH) along with an in-house limiting media, incomplete *Listeria* synthetic media (iLSM) (Whiteley et al., 2017).

Once WT-RFP expression was optimized to match the PrfA*-RFP expression, these *L. monocytogenes* reporter strains were tested against culture supernatants of inhibitory strains obtained from the RTE foods. In particular, the culture supernatants from these inhibitory strains were tested against the recombinant *L. monocytogenes* strains to assess whether expression of the PrfA protein through the RFP reporter, could be modulated in the reporter WT strain. A schematic of this experimental design can be found in Fig 1.3.
Figure 1.3. Schematic representation of the virulence assay using red fluorescent protein (RFP) as a reporter in recombinant L. monocytogenes strains. Culture supernatants produced from anti-Listeria inhibitory strains were used to monitor the expression of the PrfA master virulence regulator in a reducing environment through the actA promoter fused to the red fluorescence protein (RFP) gene.
1.2 Link to food safety

Due to its hardy nature, *L. monocytogenes* can survive for long periods of time in the environment of food processing plants, and potentially multiply in food products. In addition, its frequent presence in different environments and its ability to adapt and survive stressful conditions makes the control of *L. monocytogenes* in the food processing environment a considerable challenge (Ferreira et al., 2014). Although this pathogen can be inactivated by thermal treatments used in food production, post-processing contamination from equipment and the environment represents a major concern (Lappi et al., 2004). While *L. monocytogenes* cannot totally be eliminated from the food chain, the objective that industry and regulatory bodies strive for is to reduce the risk of contamination occurring, i.e., strive for continuous improvement. Thus, if poor sanitation and improper HACCP practices are followed, this pathogen will likely remain problematic for that food processing plant.

1.2.1 Ready-to-eat (RTE) foods

Since low numbers of *L. monocytogenes* are unlikely to cause infection in most individuals, the growth of this pathogen to higher numbers in food can have a serious impact on food safety. One high-risk product class that is of concern to food processing plants in Canada is RTE foods such as deli-meats, vegetables, seafood and dairy products. This is because many RTE foods can support the rapid growth of foodborne pathogens such as *L. monocytogenes* (Health Canada, 2010). RTE foods are defined as those foods not requiring any further preparation before consumption, except perhaps washing/rinsing, thawing, or warming (Government of Canada, 2011). *L. monocytogenes* can grow in a RTE food under the following conditions:
• Temperature of the food is between -0.4 to 45°C
• pH value of the food is 4.4 or higher
• Water activity (a_w) value is at 0.92 or higher

A RTE food will not support the growth of *L. monocytogenes* when the product formulation has a pH and a_w outside the range mentioned above. Historically, RTE foods contaminated with *L. monocytogenes* at levels exceeding 100 CFU/g of food have been implicated in outbreaks of listeriosis (CFIA, 2018). Due to the large number of outbreaks associated with these foods, a risk assessment was developed to help control *L. monocytogenes* in RTE foods (Government of Canada, 2011).

### 1.2.2 Risk assessment of RTE foods

Based on the risk assessment provided by the Government of Canada (2011), RTE foods are classified into two categories. In addition, validation data from industry needs to be provided to the CFIA to support the risk categorization of their RTE food. If insufficient, inadequate or no data is provided, the respective RTE food is considered by default as a category 1 RTE food. However, validation is not required if the physiochemical properties (i.e., pH and/or a_w) of the RTE food fall within the range that does not support the growth of *L. monocytogenes*. These same procedures apply if the food product is imported.

Category 1 RTE foods are those which can support the growth of *L. monocytogenes* throughout the shelf life and should therefore receive a high priority for industry and regulatory oversight. Some of these foods include deli-meats, soft cheeses, hot dogs and pâté.
Category 2 RTE foods are sub-divided into two subgroups. Category 2A foods can support limited growth of \textit{L. monocytogenes} (≤ 100 CFU/g) throughout their stated shelf life. In addition, these RTE products are known to occasionally contain low levels of \textit{L. monocytogenes} and do not have a kill step, and/or have a refrigerated shelf life of ≤ 5 days. Some of these foods include refrigerated cold-smoked rainbow trout/salmon and fresh-cut produce. Category 2B foods do not support the growth of \textit{L. monocytogenes} throughout their shelf life and should therefore receive low priority for industry and regulatory oversight. Some of these foods include ice cream, hard cheese, dry salami and dried-salted fish.

For the purposes of this thesis, all RTE products investigated (dried apple slices, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed) appear to fall under category 2B of RTE foods. Thus, it is expected that these imported RTE foods will support the survival, but not the growth of \textit{L. monocytogenes}. However, they may contain a diverse source of culturable bacteria which possess antagonistic properties against \textit{L. monocytogenes}.

1.3 Control measures

Food industries must be aware that the presence or contamination of \textit{L. monocytogenes} in their food or food-processing environments can have a significant impact on the overall safety of their RTE foods. However, when effective, safe and reliant measures to control this pathogen are implemented, the burden of \textit{L. monocytogenes} to industry and public health authorities can be reduced. The purpose of these control measures is to inactivate or control the growth of \textit{L. monocytogenes} throughout the whole food chain. Some of these measures include the use of \textit{Listeria} inhibitors (such as chemicals, bacteriocins, bacteriophages, etc.) and/or post-lethality
treatments such as high-pressure processing and/or heat (Government of Canada, 2011). For the context of this thesis, only bacteriocin proteins as *Listeria* inhibitors will be further investigated as these relate the most to our research questions.

### 1.3.1 Bacteriocin proteins

Bacteriocins are ribosomally synthesized peptides or proteins produced by certain bacteria (typically lactic acid bacteria) that act to inhibit various bacterial groups (Gálvez et al., 2007). They are known as bioprotective agents that demonstrate activity against a wide range of pathogenic and spoilage bacteria (Cotter et al., 2005). Bacteriocins are additionally recognized as being ‘friendly’ antimicrobial agents, as they are highly prevalent in nature and are generally produced by food-grade organisms (Camargo et al., 2018). As a result, they are not typically associated with any public health risk. To evoke these antimicrobial properties, bacteriocins typically act through a mechanism of forming pores in the bacterial cytoplasmic membrane, which then leads to the death of that microorganism (Camargo et al., 2018; Gálvez et al., 2007). However, the spectrum of inhibition is dependent on the producing bacterial strain and the type of bacteriocin (Holzapfel et al., 1995).

When applied to Gram-positive bacteria like *L. monocytogenes*, the bacteriostatic or bactericidal effects of these bacteriocins can result in growth delay, total inhibition/inactivation, and/or overall reductions in cell counts (Zilelidou and Skandamis, 2018). Examples of bacteriocins that have demonstrated efficacy against *L. monocytogenes* can be found summarized in Table 1.4. These bacteriocins range in sizes from 1170 – 7140 Da and are all found to be
derived from lactic acid bacteria that include *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Pediococcus* and *Streptococcus* spp.

The effectiveness of these bacteriocins against *L. monocytogenes* varies. Enterocin B3A-B3B, BM1157, nisin, plantaricin, and sakacin were found to be successful against *L. monocytogenes* in a biofilm model (Al-Seraih et al., 2017; Yi et al., 2018; Camargo et al., 2018; Winkelströter et al., 2015, 2011); carnobacteriocin, enterocin AS-48, nisin, pediocin PA-1, and piscicosin CS526 were found to be effective against *L. monocytogenes* in meat (Health Canada, 2017; Ananou et al., 2005; Gharsallaoui et al., 2016; Rodríguez et al., 2002; Azuma et al., 2007); divergicin M35 and piscicosin were found to be successful in seafood (Health Canada, 2016; Yamazaki et al., 2003); and BM1157, lacticin and nisin were found to be useful in dairy foods (Yi et al., 2018; Morgan et al., 2001, 1999; Gharsallaoui et al., 2016). Among these bacteriocins, nisin and pediocin PA-1 have received certification as commercial bioprotective agents (Balciunas et al., 2013), sakacin and plantaricin are commercially available (Chikindas et al., 2018), and carnobacteriocin and divergicin M35 have received novel food additive approval from Health Canada to limit or inhibit the growth of *L. monocytogenes* in various seafood, meat, and poultry products (Health Canada, 2016, 2017).
<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Size (Da)</th>
<th>Producer</th>
<th>Source</th>
<th>Effectiveness</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM1157</td>
<td>1770</td>
<td><em>L. crustorum</em></td>
<td>Dairy, koumiss</td>
<td>Bactericidal activity against <em>Listeria</em> growth in milk through biofilm destruction and pore formation</td>
<td>Yi et al., 2018</td>
</tr>
<tr>
<td>carnobacteriocin</td>
<td>5070</td>
<td><em>C. maltaromaticum</em></td>
<td>Meat, raw</td>
<td>Approved by Health Canada to inhibit the growth of <em>Listeria</em> in various meat and poultry products</td>
<td>Health Canada, 2017; Casaburi et al., 2011</td>
</tr>
<tr>
<td>divergicin M35</td>
<td>4520</td>
<td><em>C. divergens</em></td>
<td>Seafood, mussels</td>
<td>Approved by Health Canada to inhibit the growth of <em>Listeria</em> in various RTE seafood products</td>
<td>Health Canada, 2016; Tahiri et al., 2009</td>
</tr>
<tr>
<td>enterocin AS-48</td>
<td>7140</td>
<td><em>Enterococcus</em> spp.</td>
<td>Cheese</td>
<td>Displayed growth inhibition against <em>Listeria</em> on sausages</td>
<td>Ananou et al., 2005; Grande Burgos et al., 2014</td>
</tr>
<tr>
<td>enterocin B3A-B3B</td>
<td>5180</td>
<td><em>E. faecalis</em></td>
<td>Faeces, infant</td>
<td>Impeded <em>Listeria</em> biofilm formation on stainless steel</td>
<td>Al-Seraih et al., 2017</td>
</tr>
<tr>
<td>lacticin</td>
<td>7080</td>
<td><em>L. lactis</em></td>
<td>Dairy, kefir</td>
<td>Growth inhibition of <em>Listeria</em> in yogurt, cottage cheese, soup, and infant formula</td>
<td>Morgan et al. 1999, 2001</td>
</tr>
<tr>
<td>nisin</td>
<td>3150</td>
<td><em>Lactococcus</em> spp.</td>
<td>Meat, sausage</td>
<td>First bacteriocin approved as a food preservative worldwide to control pathogens, including <em>Listeria</em></td>
<td>Gharsallaoui et al., 2016; Camargo et al., 2018</td>
</tr>
<tr>
<td>Pediocin PA-1</td>
<td>4630</td>
<td><em>Pediococcus</em> spp</td>
<td>Meat products</td>
<td>Strong activity against <em>Listeria</em>, particularly in RTE meat products</td>
<td>Rodríguez et al., 2002</td>
</tr>
<tr>
<td>---------------</td>
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<td>--------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Piscicosin CS526</td>
<td>4430</td>
<td><em>C. piscicola</em></td>
<td>Fish, surimi</td>
<td>Growth inhibition of <em>Listeria</em> in cold-smoked salmon and ground meat</td>
<td>Azuma et al., 2007; Yamazaki et al., 2003</td>
</tr>
<tr>
<td>Plantaricin</td>
<td>3225</td>
<td><em>L. paraplantarum</em> / <em>plantarum</em></td>
<td>Cheese</td>
<td>Reduced planktonic and sessile growth of <em>Listeria</em> in a biofilm model</td>
<td>Winkelströter et al., 2015; Guerrieri et al., 2009</td>
</tr>
<tr>
<td>Sakacin</td>
<td>4640</td>
<td><em>L. sakei</em></td>
<td>Meat, sausage</td>
<td>Inhibited early stages of <em>Listeria</em> biofilm formation on stainless steel surfaces</td>
<td>Winkelströter et al., 2011;</td>
</tr>
</tbody>
</table>

*As reviewed by Jordan and McAuliffe (2018), Camargo et al., (2018) and Muriana (1996).*
However, what appears to be lacking in the existing body of literature is the use of these antilisterial bacteriocins for foods other than dairy, meat, and seafood. This is of concern because outbreaks of listeriosis can also occur in other foods such as bean sprouts (CDC, 2015c), caramel apples (CDC, 2015d), frozen vegetables (EFSA, 2018a; CDC, 2016c), and packaged salad (CDC, 2016b; PHAC, 2016). This gap of knowledge could perhaps be due to these bacteriocins being isolated from products other than produce. Thus, their effectiveness may be limited to solely the foods from which they were isolated. Therefore, it is of interest to discover antilisterial bacteriocin-producing bacteria from foods that have not previously been investigated, such as the foods used in this study. In doing so, this can better protect our food supply system from the threats of *L. monocytogenes*. 
Chapter 2 – An examination of the culturable microbiota from imported RTE foods for anti-bacterial activity against *Listeria monocytogenes*
Abstract

Listeria monocytogenes, a resilient and ubiquitous foodborne pathogen, is associated with a high case-fatality rate in humans. This study investigated the culturable microbiota of imported, ready-to-eat (RTE) foods to see how well bacteria isolated from these foods could inhibit/inactivate the growth of L. monocytogenes. Imported RTE foods were acquired from various supermarkets in the Greater Toronto Area (ON, Canada). The foods included dried apples, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed. Bacterial strains were isolated from the foods using blood agar and then screened using an in-house designed growth inhibition plate assay against L. monocytogenes. The inhibitory strains detected were then identified using 16S rRNA sequencing.

A wide diversity of bacteria was recovered from the foods, in that a total of 236 isolates belonging to 122 observed phenotypes were obtained. From the inhibition plate assay, 10 out of the 11 imported RTE foods harboured inhibitory strains against L. monocytogenes, whereby 48 of the collected isolates (20%) were found to produce a zone of inhibition against this pathogen. The inhibitory strains belonged to 6 different genera (Acinetobacter, Aerococcus, Bacillus, Lysinibacillus, Paenibacillus and Sporosarcina) and 15 unique species. Among all the foods tested, the date fruit microbiota displayed the most promise for harbouring antagonistic properties against L. monocytogenes. Overall, it was found that the culturable microbiota of imported RTE foods possess bacterial members that can inhibit the growth of L. monocytogenes. These results could lead to the discovery of either novel antimicrobial metabolites or beneficial anti-Listeria bacteria that could be added to foods to inactivate and/or control L. monocytogenes.
2.1 Introduction

Foodborne pathogens present a major public health and economic concern worldwide, as it is estimated that 1 in 10 people in the world fall ill after eating contaminated food on a yearly basis. This can result in around 600 million cases/year, and an estimated 420,000 deaths (WHO, 2017). Due to this high prevalence and risk, it is very important to study different and novel methods for controlling foodborne pathogens. One approach to help control these foodborne pathogens is to better understand the microbiota of foods in the market; that is, to understand the community of microorganisms that naturally inhabit our foods. These members of the food microbiota may help inactivate foodborne pathogens and/or prevent their growth based on a number of factors, such as competitive exclusion and the production of antimicrobial compounds.

Among these foodborne pathogens, one of the most important is *Listeria monocytogenes* due to its high hospitalization rate and high case-fatality rate in susceptible populations (Government of Canada, 2011). Upon infection, this pathogen can cause invasive listeriosis – a severe foodborne disease that can cause intestinal, blood, brain, and fetal infections. This is due to the versatility of the pathogen as it is capable of crossing three tight human barriers: the intestinal, blood-brain and the feto-placental barrier (Bierne et al., 2018). Listeriosis can start as early as 20 h after eating contaminated food and can cause symptoms of mild gastroenteritis, fever, headaches and muscle aches (Government of Canada, 2016a). However, for cases of invasive listeriosis, infection can range from 1 – 67 days (Goulet et al., 2013) and can result in sequelae such as brain infections and/or blood poisoning (Government of Canada, 2016a). Those at a higher risk of becoming sick are individuals with a weakened immune system related to aging, underlying
diseases and/or immunosuppressive therapies, as well as neonates and pregnant women (Charlier et al., 2017).

Although the incidence of reported listeriosis cases remain low, i.e., 1 – 10 cases per million per year (Goulet et al., 2012), it is associated with an average case-fatality rate of 20 – 30%, that despite antimicrobial treatment (Swaminathan and Gerner-Smidt, 2007), is the highest of any foodborne pathogens (Bennion et al., 2008). This is why *L. monocytogenes* is considered a significant threat to public health, as its pathogenic nature is one of the most severe when it comes to food safety. Since low numbers of *L. monocytogenes* are unlikely to cause infection in healthy individuals, the growth of this pathogen to high numbers in food can have a serious impact on food safety. One high-risk product class that is of concern to food processors in Canada is refrigerated RTE foods as they can support the rapid and progressive growth of foodborne pathogens such as *L. monocytogenes* (Government of Canada, 2011). Some of these foods include deli-meats, vegetables, seafood and dairy products as they do not require any further preparation before consumption, except perhaps washing/rinsing, thawing or warming (Government of Canada, 2011).

Presently, Health Canada has approved the use of food additives to control the growth of *L. monocytogenes* in RTE foods, including certain bacteria (*Carnobacterium maltaromaticum* CB1) and organic acids such as lactate and acetate derivatives (Government of Canada, 2011). Furthermore, other reports in the literature have showed the use of bacteriocins (Camargo et al., 2018) and bacteriophages (Jordan and McAuliffe, 2018) as biocontrol agents for *L. monocytogenes*. However, the natural bacterial community of RTE foods may present a novel approach of preventing/controlling pathogen growth by potentially harbouring beneficial (e.g.,
anti-Listeria) bacteria. Additionally, investigating the microbiota of imported RTE foods may increase the probability of finding novel, beneficial bacteria that have not been previously studied for these applications.

Thus, this study investigated the culturable microbiota of select imported, RTE foods to examine their potential to inactivate and/or inhibit the growth of *L. monocytogenes*. The RTE foods included dried apple slices, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed. It was hypothesized i) that the RTE foods used in this study would contain a diverse microbiota of culturable bacteria which possess antagonistic properties against *L. monocytogenes* and ii) that this microbiota may possess novel bacteria that can be used for biocontrol applications.

The primary goal of this study was to select among the imported RTE foods collected, a specific food to further pursue in our investigations to find bacteria which possess antagonistic properties against *L. monocytogenes*. To aid in this investigation, an overview of our blood agar collection method will be discussed to explain how we collected bacteria from these foods. Additionally, our in-house designed growth inhibition plate assay will be discussed to explain how we assessed whether our collected bacteria could impact the growth of this pathogen. This study will then end off describing which imported RTE food matrix showed the most promise for potential applications in controlling *L. monocytogenes*. 
2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

All strains (Table 2.1) and food isolates were grown at 37°C in brain heart infusion (BHI; Fisher Scientific, Mississauga, ON). Bacterial stocks were frozen and stored at -80°C in freezing media (12% w/v skim milk, 1% v/v glycerol, 1% v/v DMSO).

Table 2.1. List of the bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acronym</th>
<th>Description</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em> FSL</td>
<td>Lm4b</td>
<td>Serovar 4b; food isolate acquired from Jalisco cheese outbreak (LA, USA, 1985)</td>
<td>CRIFS culture collection, University of Guelph</td>
</tr>
<tr>
<td>J1-110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter tabaci</em></td>
<td>3E7</td>
<td>Endophyte isolated from wild maize</td>
<td>Dr. Manish Raizada (Plant Agriculture, University of Guelph)</td>
</tr>
<tr>
<td>3E7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Isolation of bacteria from the imported RTE foods

Imported RTE foods were acquired from various supermarkets in the Greater Toronto Area (ON, Canada) (Table 2.3 and Figure 2.1). These foods had 10 g portions weighed off into a filter Stomacher® bag (Fisher Scientific, Mississauga, ON) containing 10 mL of 0.1% (w/v) peptone water. Contents of the bag were massaged by hand for 2 min and the residual liquid was aspirated off and subjected to differential centrifugation at 1000 x g for 2 min to remove any food particulates, followed by 14,000 x g for 2 min to recover bacterial cells. The bacterial pellet was suspended in 250 μL and subjected to standard serial dilutions (10⁻¹ to 10⁻²) using 0.1% (w/v) peptone water.
peptone water, then spread-plated onto blood agar (tryptic soy agar enriched with 5% v/v defibrinated sheep blood; Oxoid, Nepean, ON). Plates were then incubated overnight at 37°C. When no growth was detected, the food was enriched overnight in tryptic soy broth (TSB; Fisher Scientific) at room temperature (RT) and 37°C. Post-enrichment, the bacterial pellet was suspended in 1 mL, serially diluted (10\(^{-1}\) to 10\(^{-5}\)), and then spread-plated onto blood agar. All colonies were counted to determine the total viable counts and bacterial isolates were collected in triplicate per observable phenotype, per batch of food. Phenotypes were distinguished from one another based on the colony morphology protocol (Breakwell et al., 2007; Gerhardt et al., 1994). Individual colonies were sub-cultured onto blood agar in order to check for purity and then stored in freezing media at -80°C. This isolation procedure was repeated three times per batch of food. An overview of this method can be found in Figure 2.2.

### 2.2.3 Growth inhibition plate assay

Screening for anti-	extit{Listeria} activity from the food isolates was determined using a growth inhibition plate assay against \textit{L. monocytogenes} FSL J1-110 (Lm4b) as modified by the following (Ahn and Stiles, 1990; Mante et al., 2003; Shehata et al., 2017). Lm4b, along with each collected food isolate, were grown overnight at 37°C in BHI broth. A lawn of an overnight culture of Lm4b was then plated onto BHI agar using a cotton swab (~ 100 μL). The top of the Lm4b lawn was then inoculated with a 5 μL drop of an overnight bacteria culture of each food isolate. The drops were left to dry completely before plates were left to incubate overnight at 37°C. The following day, the plates were inspected for the presence of any zones of clearing (inhibition zones) around the isolate. Food isolates that produced a zone were classified as anti-	extit{Listeria} inhibitory strains. The test was validated using \textit{Enterobacter tabaci} 3E7 as a positive control.
2.2.4 Identification of the inhibitory strains

Bacterial food isolates were identified using 16S rRNA sequencing. Genomic DNA was extracted from the bacterial isolates using a boiling method as follows. An isolated colony was placed in a tube containing 400 μL of TE buffer (1 M Tris, 0.5 M EDTA, pH 8.0) and subjected to boiling at 100°C in a water bath for 15 min. The samples were centrifuged at 14,000 x g for 2 min to pellet out the bacterial cells and the upper aqueous phase was aspirated off to acquire the DNA template. The DNA was subjected to PCR amplification using the V3kl/V6r primer set (Table 2.2) to amplify the V3 to V6 regions of the 16S rRNA operon (Gloor et al., 2010).

Each PCR tube had a final volume of 25 μL, using 10X ThermoPol Reaction Buffer (New England BioLabs-NEB, Mississauga, ON), 200 μM of dNTPs (Invitrogen, Nepean, ON), 0.8 pmol/μL of each primer pair (see Table 3.4), 2.5U Taq DNA Polymerase (NEB, Whitby, ON) and 5 μL of template DNA. PCR was carried out in a Biometra Tprofessional Basic Gradient 96 (Montreal Biotechnologies Inc., Dorval, QC), with the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C/30 s, 60°C/30 s, 72°C/30 s and final extension at 72°C for 5 min. PCR product sizes were determined by 1.5 % (w/v) agarose gel electrophoresis and visualization was by EZ-Vision® DNA dye staining (VWR, Mississauga, ON).

The PCR amplified products were then sent for sequencing at the University of Guelph Advanced Analysis Centre (AAC) Genomics Facility. Amplicon sequences were compared to the GenBank database (NCBI) using nucleotide Basic Local Alignment Search Tool (BLASTn) and the resulting hits allowed for approximate speciation (> 97% identity match) for the strains according to their closest match in the database.
Table 2.2. List of the primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Expected amplicon size (bp)</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3kl-F</td>
<td>ACTCCTACGGGAGGCAGCAGT</td>
<td>800</td>
<td>16S rRNA operon, V3-V6</td>
<td>Gloor et al., 2010</td>
</tr>
<tr>
<td>V6r-R</td>
<td>ACAACACGAGCTGACGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.1. Overview of the imported RTE foods used in this study
**Figure 2.2.** Flow chart summarizing the steps used to recover bacterial isolates from the imported RTE foods tested.
2.3 Results

2.3.1 Rationale and overview of the foods used

A total of 11 different RTE foods were acquired for this study from 9 different supermarkets located around the Greater Toronto Area (ON, Canada) and originating from 9 different countries: Argentina (dried apples), China (date fruits and seaweed), India (cumin and fennel seeds), Japan (seaweed), Poland (pollen), South Africa (raisins), Tunisia (date fruits), U.A.E. (date fruits) and the USA (pistachios). Of these food matrices, a few were acquired from multiple areas – seaweed from both China and Japan, along with date fruits from China, Tunisia and U.A.E. All foods investigated fall under Health Canada’s definition of a category 2B RTE food. That is, a RTE food that does not support the growth of *L. monocytogenes* throughout its shelf life. These foods are typically low moisture in nature (water activity, *a*_<sub>W</sub>, < 0.85; Young et al., 2015) and are given low priority for industry and regulatory oversight (Government of Canada, 2011). Although the above RTE foods may support the survival of *L. monocytogenes*, it is not expected that they will support its growth.

2.3.2 Survey of the bacterial growth from the foods

To assist in the survey of the food microbiota, bacteria were collected by washing them off the foods and plating the wash onto blood agar. For those foods that displayed an absence of an aerobic plate count, the food was subjected to enrichment in TSB at RT and 37°C. Based on the growth observed on the blood agar plates, bacteria were sub-cultured in triplicate per observed phenotype. Phenotypes were discriminated subjectively from one another based on the colony morphology protocol (Breakwell et al., 2007; Gerhardt et al., 1994). This included discrimination based on the colony’s appearance, elevation, hemolytic properties, margin, optical properties,
pigmentation, shape, size and texture. Representative blood agar plates for this collection method can be found in Figure 2.3.

A rich diversity of bacteria was obtained from these foods, with a total of 236 isolates belonging to 122 different phenotypes being observed (Table 2.3). Total aerobic bacterial counts on these foods ranged from $10^3$ to $10^5$ CFU/g, with the exception of fennel seeds for which no bacteria could be recovered until after the food was enriched. Among the selected foods, the largest aerobic plate count was seen with cumin seeds, Chinese seaweed and raisins ($4.7 \times 10^5$, $1.2 \times 10^5$ and $9.6 \times 10^4$ CFU/g, respectively) followed by U.A.E. date fruits, pollen and Tunisian date fruits ($3.1 \times 10^4$, $1.1 \times 10^4$ and $1.0 \times 10^4$ CFU/g, respectively). The foods containing the lowest aerobic plate counts were observed from the Chinese date fruits, dried apples, pistachios and Japanese seaweed ($5.2 \times 10^3$, $4.4 \times 10^3$, $3.0 \times 10^3$ and $2.7 \times 10^3$ CFU/g, respectively).

2.3.3 Growth inhibition plate assay

In total, 48 isolates belonging to 33 observed phenotypes were found to produce a zone of inhibition against *L. monocytogenes* 4b (Table 2.3). The greatest number of inhibitory strains (phenotypes enclosed in brackets) were isolated from U.A.E. date fruits (12 [7]), followed by Tunisian date fruits (6 [5]), raisins (6 [4]), Chinese seaweed (6 [4]), fennel seeds (5 [2]), Chinese date fruits (4 [4]) and pistachios (4[4]). The least number of inhibitory strains were recovered from pollen (3 [1]), cumin seeds (1) and dried apples (1). Additionally, it was found that the Japanese seaweed did not harbour any inhibitory strains. A representative result of the anti-Listeria activity observed from the food isolates can be seen in a sample of this growth inhibition plate (Figure 2.4).
2.3.4 Overview of the identification of the inhibitory strains

Sanger sequencing was used to identify the inhibitory strains which were detected. This involved using universal primers that targeted the V3 to V6 variable regions of the 16S rRNA gene. Species level identity was disclosed if the query sequence had an identity match of >97% on NCBI’s database. Due to how phylogenetically close some species are, some inhibitory strains identified with two species that could not be distinguished with confidence to a single species. For these cases, those inhibitory strains were delineated as both species, separated by a dash.

The inhibitory strains identified belong to six different genera: *Acinetobacter, Aerococcus, Bacillus, Lysinibacillus, Paenibacillus* and *Sporosarina* (Table 2.3). Of these six, *Bacillus* spp. was the most common, being identified from inhibitory strains acquired from all foods except for dried apples, where the inhibitory strain was identified as a *Lysinibacillus* spp. Additionally, many of the foods (Chinese seaweed, cumin seeds, fennel seeds, pistachios, pollen and U.A.E. date fruits) had all of their inhibitory strains identified as being *Bacillus* spp. The greatest diversity of the inhibitory strains was found from the Tunisian date fruits, where the inhibitory strains belonged to four genera: *Acinetobacter, Aerococcus, Bacillus* and *Lysinibacillus* spp. The remaining foods displayed diversity in their inhibitory strains across two genera: Chinese date fruits with *Bacillus* and *Sporosarcina* spp. and raisins with *Bacillus* and *Paenibacillus* spp.

Additionally, the inhibitory strains belonged to 15 unique species identities (IDs) – *Acinetobactor baylyi/soli; Aerococcus viridans/urinaeequi; Bacillus amyloliquefaciens/velezensis, B. cohnii, B. filamentosus/endophyticus, B. flexus/megaterium, B. haikouensis/marisflavi, B. mojavensis/halotolerans, B. mycoides, B. pumilus/safensis, B. subtilis; Lysinibacillus fusiformis, L.
macrolides/pakistanensis; Paenibacillus illinoisensis; and Sporosarcina luteola/koreensis). When broken down by food, the Tunisian date fruits displayed the most diversity with 5 unique species IDs, followed by Chinese seaweed with 4 and raisins with 3. The least diversity of the inhibitory strains was seen with dried apples, cumin seeds and pollen, whereby each displayed a single unique species. The remaining foods, Chinese date fruits, U.A.E. date fruits, fennel seeds and pistachios, contained two unique species.
Figure 2.3. Overview of how bacterial isolates were collected from the imported, RTE foods. Food washes were plated on blood agar and isolates were collected in triplicate per observable phenotype.
Figure 2.4. Sample plates from the growth inhibition assay of bacterial isolates, acquired from the Chinese, Tunisian, and U.A.E. date fruits, tested against *Listeria monocytogenes* 4b (Lm4b). Isolates were drop-plated from an overnight culture onto the agar in the absence (A) or presence (B) of a Lm4b lawn. 3E7: *Enterobacter tabaci* 3E7 (positive control); *: non-inhibitory strain; **: inhibitory strain.
Table 2.3. Summary of the bacterial strains collected from the various imported, RTE foods that inhibit *Listeria monocytogenes*

<table>
<thead>
<tr>
<th>Food</th>
<th>Country of origin</th>
<th>[CFU/g]a</th>
<th>No. Isolatesb</th>
<th>No. Inhibitory strainsb,c</th>
<th>Inhibitory genus identitiesd</th>
<th>Inhibitory species identitiesd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apples (dried)</td>
<td>Argentina</td>
<td>$4.4 \times 10^3$</td>
<td>8 (7)</td>
<td>1</td>
<td><em>Lysinibacillus</em> spp.</td>
<td><em>L. macrolides/pakistanensis</em></td>
</tr>
<tr>
<td>Cumin seeds</td>
<td>India</td>
<td>$4.7 \times 10^5$</td>
<td>24 (13)</td>
<td>1</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. filamentosus/endophyticus</em></td>
</tr>
<tr>
<td>Date fruits</td>
<td>China</td>
<td>$5.2 \times 10^3$</td>
<td>22 (11)</td>
<td>4 (4)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. pumilus/safensis</em></td>
</tr>
<tr>
<td></td>
<td>Tunisia</td>
<td>$1.0 \times 10^4$</td>
<td>20 (12)</td>
<td>6 (5)</td>
<td><em>Sporosarcina</em> spp.</td>
<td><em>S. luteola/koreensis</em></td>
</tr>
<tr>
<td></td>
<td>U.A.E.</td>
<td>$3.1 \times 10^4$</td>
<td>35 (15)</td>
<td>12 (7)</td>
<td><em>Acinetobacter</em> spp.</td>
<td><em>A. baylyi/soli</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Aerococcus</em> spp.</td>
<td><em>A. viridans/urinaeaequi</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. cohnii; B. flexus/megaterium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Lysinibacillus</em> spp.</td>
<td><em>L. fusiformis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. pumilus/safensis; B. subtilis</em></td>
</tr>
<tr>
<td>Fennel seeds</td>
<td>India</td>
<td>0</td>
<td>11 (4)</td>
<td>5 (2)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. filamentosus/endophyticus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>B. mojavensis/halotolerans</em></td>
</tr>
<tr>
<td>Pistachios</td>
<td>USA</td>
<td>$3.0 \times 10^3$</td>
<td>11 (8)</td>
<td>4 (4)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. amyloliquefaciens/velezensis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>B. mojavensis/halotolerans</em></td>
</tr>
<tr>
<td>Pollen</td>
<td>Poland</td>
<td>$1.1 \times 10^4$</td>
<td>40 (19)</td>
<td>3 (1)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. pumilus/safensis</em></td>
</tr>
<tr>
<td>Raisins</td>
<td>South Africa</td>
<td>$9.6 \times 10^4$</td>
<td>22 (13)</td>
<td>6 (4)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. flexus/megaterium</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>B. pumilus/safensis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Paenibacillus</em> spp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>P. illinoisensis</em></td>
</tr>
<tr>
<td>Seaweed</td>
<td>China</td>
<td>$1.2 \times 10^5$</td>
<td>33 (14)</td>
<td>6 (4)</td>
<td><em>Bacillus</em> spp.</td>
<td><em>B. haikouensis/marisflavi;</em></td>
</tr>
<tr>
<td></td>
<td>Japan</td>
<td>$2.7 \times 10^3$</td>
<td>10 (6)</td>
<td>0</td>
<td>—</td>
<td><em>B. mojavensis/halotolerans;</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td><em>B. pumilus/safensis;</em> B. mycoides*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9 countries</td>
<td>$0 – 10^5$</td>
<td>236 (122)</td>
<td>48 (33)</td>
<td>6 different genera</td>
<td>15 unique species identities</td>
</tr>
</tbody>
</table>

---

a Concentration without enrichment.
b Number of observed phenotypes enclosed in brackets.
c Strains detected using the growth inhibition plate assay against *L. monocytogenes*.
d Identified using 16S rRNA sequencing.
e Food was enriched at RT and 37°C to obtain aerobic growth.
<table>
<thead>
<tr>
<th>Genus</th>
<th>Classification</th>
<th>No. of species</th>
<th>Reservoir</th>
<th>Pathogenesis</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Acinetobacter | Proteobacteria < Gammaproteobacteria < Pseudomonadales < Moraxellaceae < Acinetobacter | 20             | Saprophytic (raw vegetables, sewage, soil, water) | Normally non-pathogenic. Associated with some nosocomial infections:  
• A. baumannii | Degradation of crude oil. | Juni, 2015 |
| Aerococcus    | Firmicutes < Bacilli < Lactobacillales < Aerococcaceae < Aerococcus | 5              | Diverse (air, bodily fluids, dust, marine sources, soil, vegetation) | Normally non-pathogenic. Associated with some nosocomial infections:  
• A. urinae | None mentioned; GRAS status from being a lactic acid bacteria. | Collins and Falsen, 2015 |
| Bacillus      | Firmicutes < Bacilli < Bacillales < Bacillaceae < Bacillus | > 140          | Primarily soil                | Most have little or no pathogenic potential.  
• B. anthracis  
• B. cereus  
• B. thuringiensis  
• B. licheniformis | Biotechnology (bioremediation, pharmaceutical production). | Logan and De Vos, 2015 |
| Lysinibacillus| Firmicutes < Bacilli < Bacillales < Bacillaceae < Lysinibacillus | 21             | Diverse (air, aquatic, animal waste, food, insects, soil) | None documented. | Mosquitocidal; bioremediation, biotechnology. | Failor et al., 2019 |
| Paenibacillus | Firmicutes < Bacilli < Bacillales < Paenibacillaceae < Paenibacillus | > 80           | Soil                          | No association with human or mammalian pathogenicity. | Composting of soil; endophytes. | Priest, 2015 |
| Sporosarcina  | Firmicutes < Bacilli < Bacillales < Planococcaceae < Sporosarcina | 9              | Soil and water reservoirs     | None documented. | None mentioned. | Editorial Board, 2015 |

a Phylum < Class < Order < Family < Genus.
b Pathogenic species are listed.
c Adapted from the Bergey’s Manual of Systematics of Archaea and Bacteria.
2.4 Discussion

What appears to be lacking in the existing body of food safety literature are papers on the use of anti-
*Listeria* biocontrol measures for foods other than dairy, meat and seafood (Jordan and McAuliffe, 2018; Camargo et al., 2018). This is of concern, as outbreaks of listeriosis can occur in other foods such as bean sprouts (CDC, 2015c), caramel apples (CDC, 2015d), frozen vegetables (EFSA, 2018a; CDC, 2016c) and packaged salad (CDC, 2016b; PHAC, 2016). This gap of knowledge could perhaps be due to these biocontrol agents being isolated from products other than agricultural produce. Thus, their effectiveness may be limited to solely the foods from which they were isolated. As a result, it is of interest to discover anti-
*Listeria* bacteria from foods that have not been previously investigated, such as the foods used in this study.

This study shows that the culturable microbiota of imported RTE foods possess bacteria that can inhibit the growth of *L. monocytogenes*. Of the 11 foods tested, 10 contained strains which were inhibitory against this pathogen. It was only the Japanese seaweed that did not contain any inhibitory strains found in this study. This could be due to this product having been heavily processed prior to being placed on the market, i.e., its natural microbiota may have been eliminated. This could also explain why its total aerobic plate count was low or the isolation media was not appropriated. Nonetheless, these findings suggest that the natural bacterial community found in imported RTE foods can contribute to preventing *L. monocytogenes* growth.

Additionally, the growth inhibition plate assay proposed was modified from previous methods (Ahn and Stiles, 1990; Mante et al., 2003; Shehata et al., 2017) with the following improvements in mind. The first was the removal of an agar overlay or pathogen-infused agar (Ahn
and Stiles, 1990; Mante et al., 2003; Shehata et al., 2017) by co-inoculating the collected bacterial isolates from its overnight culture directly on top of a fresh lawn of the pathogen. This reduced the duration of the test by one day. The second was an optimization of the assay to work for a smaller drop volume of 5 μL compared to the 15 uL (Shehata et al., 2017), 50 uL (Ahn and Stiles, 1990) and 100 uL (Mante et al., 2003) drop volumes proposed. This allowed for testing or more bacterial isolates, up to 16 different isolates on a single plate (using a 4 x 4 grid), for any potential anti-Listeria activity. The last was a change in the testing medium to BHI. The BHI medium is less selective than the other lactobacilli testing mediums previously proposed: All Purpose TWEEN® - APT (Ahn and Stiles, 1990) and de Man, Rogosa and Sharpe - MRS (Mante et al., 2003); and is more nutrient rich compared to the Lysogeny Broth previously proposed (Shehata et al., 2017). The BHI medium as a result made it easier to acquire sufficient growth of both the target pathogen and the diverse collection of the food bacterial isolates used in this study.

This assay was additionally found to work with a Gram-negative foodborne pathogen, Cronobacter sakazakii (data not shown) and was validated using Enterobacter tabaci 3E7, an endophytic strain acquired from Dr. Manish Raizada that had been validated by their group as a known positive control for L. monocytogenes. E. tabaci 3E7 was selected over other potential positive controls that included Carnobacterium divergens (Duffes et al., 1999), Lactobacillus reuteri (Kuleasan and Cakmakci, 2002), and Lactococcus lactis (Stecchini et al., 1992), because it was found to grow well and consistently produce distinct, large inhibition zones against L. monocytogenes. The modifications of the growth inhibition plate assay proposed can serve as a beneficial tool to detect unconventional anti-Listeria bacteria isolated from RTE foods. It can further perform this detection in a quicker and more robust manner compared to the other methods that exist and can thus serves as a valuable contribution to the field.
To the best of our knowledge, the genera and associated species we isolated have not been documented in the literature to possess anti-
Listeria properties (Table 2.5). As reviewed (Camargo et al., 2018; Government of Canada, 2011; Health Canada, 2016; Health Canada, 2017; Jordan and McAuliffe (2018); and Muriana, 1996), anti-
Listeria genera and species are most commonly associated with lactic acid bacteria that include Carnobacterium spp. (C. divergens; C. maltaromaticum; C. piscicola), Enterococcus spp. (E. faecalis), Lactobacillus spp. (L. crustorum; L. paraplantarum; L. plantarum; L. sakei), Lactococcus spp. (L. lactis), Pediococcus spp. and Streptococcus spp. Thus, the inhibitory strains isolated in this study may all possess novel antagonistic mechanisms against L. monocytogenes that have not yet been identified. Additionally, these 6 detected genera do not contain any human pathogens (Lysinibacillus, Paenibacillus, Sporosarcina), are normally non-pathogenic (Acinetobacter, Aerococcus) or contain only a few species that may be considered pathogenic for humans, i.e., Bacillus spp (Whitman and Bergey’s Manual Trust, 2015). In fact, none of the unique species we found have been associated with human illness. Thus, the inhibitory strains could presumptively be considered safe to use for biocontrol applications in the food industry, although definitive safety testing should be carried out.

One limitation of this study was the use of solely a culture-dependent method with blood agar as the sole medium. This approach cannot capture the complete picture of all the microorganisms that inhabit the specific food menstruum being studied. Additionally, not all microorganisms can be cultured on agar or in broth. As well, those bacteria that are low in abundance or that grow very slowly can be missed. Thus, many bacteria that are part of the food microbiome could have been missed. Notwithstanding this, our choice of blood agar medium proved to work out well in our investigation, as 48 out of the 236 collected isolates (i.e., 20%) were found to inhibit the growth of L. monocytogenes. In addition, 122 different observed phenotypes were isolated, and of
the inhibitory strains, they belonged to 15 unique species across 6 different genera. This success could be due to the composition of the blood agar – its tryptic soy agar base enriched with 5% v/v defibrinated sheep blood resulted in an enriched growth medium that permitted the growth of a wide variety of organisms, including those which are more fastidious in nature. The blood in particular supplies many nutrients and growth factors to permit the growth of species that typically do not grow easily (Buxton, 2005; Gerhardt et al., 1994).

Another limitation that arises with the study of the food microbiota, is trying to distinguish between whether the microbiota recovered was from the food itself or from the environment as the food was shipped, handled, etc. This could be a concern for the foods used in this study, as they were imported from regions outside of North America and needed to be shipped a long distance. However, this should not have been a major factor in the study, as the true origins of the bacterial isolates obtained from the food is less important – what is most significant is that the isolate has the potential to display antibacterial properties against *L. monocytogenes*.

Among all the foods tested, it appears that the date fruit matrix shows the most promise to further investigate the microbiota for antagonistic properties against *L. monocytogenes*. As seen from this study, date fruits could be acquired from a number of different countries, where all date fruit types contained a large enough number of bacteria that could be washed off the food without the need for enrichment (10³ – 10⁴ CFU/g). Additionally, a large number of isolates could be recovered from the dates coming from all countries tested using the blood agar collection method, of which 18 to 34% of these isolates were found to inhibit the growth of *L. monocytogenes*. Furthermore, these inhibitory strains belonged to a broad diversity of genera (5 out of the 6 detected) and species (8 out of the 15 detected).
In summary, the date fruit microbiota represents a food matrix that warrants further investigations in order to study how its bacterial microbiota can help to control *L. monocytogenes*. These investigations could include conducting whole genome sequencing on the inhibitory strains, characterizing the anti-Listeria compounds and determining whether these inhibitory strains can also impact the virulence of *L. monocytogenes*. In addition, the safety and novelty of these inhibitory strains could be better assessed for biocontrol applications in the food industry.

2.5 Conclusion

Overall, this study showed that the culturable microbiota from imported RTE foods, when profiled using blood agar, possess a broad diversity of bacteria that can inhibit the growth of *L. monocytogenes*. Of the 11 foods tested, 10 contained strains which were inhibitory against this pathogen. These foods included dried apples from Argentina; cumin and fennel seeds from India; date fruits from China, U.A.E. and Tunisia; pistachios from USA; pollen from Poland; raisins from South Africa; and seaweed from China. In addition, of the 236 bacterial strains recovered, 20% (i.e., 48 strains) were found to produce a zone of inhibition against *L. monocytogenes* using the high-throughput growth inhibition plate assay developed in this study. The inhibitory strains belonged to 6 different genera (*Acinetobacter, Aerococcus, Bacillus, Lysinibacillus, Paenibacillus* and *Sporosarcina*) and 15 unique species, and none of these unique species have been documented with human illness. Thus, these inhibitory strains could be considered safe to use for biocontrol applications in the food industry. Lastly, the date fruit microbiota was found to display the most promise for harbouring antagonistic properties against *L. monocytogenes* and therefore warrants further investigations of its microbiota for the control of this pathogen.
Chapter 3 – The use of *Bacillus* spp. isolated from the microbiota of imported RTE date fruits to control *Listeria monocytogenes*
Abstract

*Listeria monocytogenes*, an important foodborne pathogen, remains a significant threat to public health as the invasive form of infection, listeriosis, can result in high case-fatality rates in humans. The purpose of this work was to profile the culturable microbiota of different imported, ready-to-eat (RTE) date fruits and to see whether any of their bacterial microbiota possess antagonistic properties against *L. monocytogenes*. Date fruits were acquired from local retail stores from five different geographical regions that included China (DC), Iran (DI), Palestine (DP), Saudi-Arabia (DS) and Tunisia (DT). Bacterial strains were isolated from these dates using blood agar and then screened using a growth inhibition plate assay against *L. monocytogenes*. The inhibitory strains detected were then identified using 16S rRNA sequencing. The most promising inhibitory strains which have no association with human clinical illness were then assessed for their impact on *L. monocytogenes* virulence using a fluorescence spectroscopy model linked to this pathogen’s virulence regulator, PrfA. In addition, the best inhibitory strain (*B. altitudinis* DS11), had its anti-*Listeria* compound(s) further characterized.

This study showed that a large diversity of bacteria could be recovered from these date fruits, as a total of 191 isolates belonging to 91 different phenotypes were isolated. These isolates belonged to 15 different genera and 50 unique species. The profile was predominantly populated by Gram-positive Firmicutes from the order Bacillales, where *Bacillus* spp. were found to represent 70 – 85% of the cultured isolates. From the inhibition plate assay, 36 isolates (i.e., 19%) were found to produce a zone of inhibition against *L. monocytogenes*, with zone sizes ranging from 0.3 to 5.8 mm. Sequencing revealed these inhibitory strains to all be *Bacillus* spp. Among those *Bacillus* spp. that were tested for their ability to inhibit PrfA, all caused a significant reduction in the activation of the...
PrfA protein (p-value < 0.05). In addition, the anti-Listeria compound(s) produced by *B. altitudinis* DS11 were found to be proteinaceous in nature, acid and alkali-tolerant and resistant to temperature treatments up to 100°C. Taken together, these results show the potential of an unconventional RTE food matrix to harbour anti-Listeria properties that could be added to high-risk RTE foods to inactivate and/or control *L. monocytogenes*. 
3.1 Introduction

Illnesses caused by foodborne pathogens continue to remain a major health and economic concern. According to the Government of Canada (2016b), 1 in 8 Canadians are affected by a foodborne illness each year, resulting in around 11,500 hospitalizations and 250 deaths. Due to this high prevalence and risk, there is a great need to discover novel means for controlling these pathogens. One approach to help control these pathogens is to better understand the interaction(s) between the microbiota of foods and the foodborne pathogens. The bacteria which are part of the food microbiota, may be able to control foodborne pathogens based on their antimicrobial properties. This is particularly of interest for those foods that have not yet been associated with a foodborne outbreak.

One noteworthy foodborne pathogen is *Listeria monocytogenes*, a pathogen that remains difficult to control due to its adaptive, resilient and ubiquitous lifestyle. Its main clinical manifestation is listeriosis – a severe, invasive foodborne disease that is associated with one of the highest case-fatality rates among foodborne pathogens, an average rate of 20 – 30% (Swaminathan and Gerner-Smidt, 2007; Bennion et al., 2008). Its predominant mode of transmission to humans is by ingestion of contaminated food or water, and populations that are at the highest risk of infection are immunocompromised individuals, pregnant women and the elderly (Government of Canada, 2016a). For these individuals, uncontrolled proliferation of *L. monocytogenes* can occur to cause bacteremia (Vázquez-Boland et al., 2001b). This blood poisoning could then result in the invasion of the brain and uterus, whereby complications such as miscarriage, meningitis, or even death can occur (Barbuuddhe and Chakraborty, 2009). Due to these complications, *L. monocytogenes* continues to remain a significant threat to public health.
The virulence of *L. monocytogenes* can exist in two contrasting lifestyles: a harmless saprophyte and a human pathogen. To switch between the two, a regulatory protein, known as PrfA (positive regulatory factor A; expressed by the *prfA* gene), cues this transition (Radoshevich and Cossart, 2017). PrfA is known as the master regulator of *L. monocytogenes* and is tightly linked to the virulence of this pathogen; *L. monocytogenes* strains that lack the *prfA* gene are found to be completely avirulent (Xayarath and Freitag, 2012; Chakraborty et al., 1992). This master regulator is directly responsible for the regulation of 9 core virulence genes (actA, hpt, hly, inlA, inlB, inlC, mpl, plcA, plcB) and has further been found to indirectly affect the expression of over 140 other genes, many of which are important in the pathogenesis of *L. monocytogenes* (de las Heras et al., 2011). Thus, investigating this PrfA regulator is an ideal biomarker to target when studying the virulence of *L. monocytogenes*.

While *L. monocytogenes* cannot totally be eliminated from the food chain, the objective that industry and regulatory bodies strive for is to reduce the risk of contamination occurring, particularly for ready-to-eat (RTE) foods that can support the growth of the organism (Health Canada, 2010). These control measures include the use of organic acids, biocontrol agents (such as live bacteria, bacteriocins and bacteriophages) and/or post-lethality treatments such as high-pressure processing and/or heat (Government of Canada, 2011). However, what appears to be lacking in the food safety literature are papers on the use of anti-*Listeria* biocontrol measures for foods other than dairy, meat and seafood (Jordan and McAuliffe, 2018; Camargo et al., 2018). This is of concern as outbreaks of listeriosis can occur in other foods such as bean sprouts (CDC, 2015c), caramel apples (CDC, 2015d), frozen vegetables (EFSA, 2018a; CDC, 2016c) and packaged salad (CDC, 2016b; PHAC, 2016).
The absence of *Listeria* biocontrol in the agriculture-based foods listed above could be due to the current biocontrol agents not being isolated from the same foods for which one wants to apply the biocontrol measures. That is, the effectiveness of the antilisterial biocontrol may be limited to the foods from which they were isolated. For example, *C. divergens* M35 isolated from mussels (Tahiri et al., 2009), was approved by Health Canada as an antimicrobial preservative to inhibit the growth of *Listeria* solely in seafood products (Health Canada, 2016). As well, *C. maltaromaticum* CB1 isolated from raw meat (Casaburi et al., 2011), received similar approval by Health Canada to inhibit the growth of *Listeria* specifically in meat and poultry products (Health Canada, 2017). As seen from both examples, the bacterial strains were applied successfully in a similar food from which they were isolated. As a result, it is of interest to discover anti-*Listeria* bacteria from foods that have not been previously investigated, such as the foods used in this study. This could then expand the effectiveness of anti-*Listeria* biocontrol measures being used in the food supply chain.

Our prior study (Chapter 2, unpublished data) has shown that imported date fruits possess a microbiota whose bacterial members harbour antagonistic properties against *L. monocytogenes*, more so than the other imported RTE foods tested (dried apples, cumin seeds, fennel seeds, pistachios, pollen, raisins and seaweed). It was then hypothesized that the date fruit microbiota represented a food matrix that warranted further investigations to study how its bacterial members can control *L. monocytogenes*.

To the best of our knowledge, this type of food safety application using the date fruit microbiota has not been previously investigated. Instead, studies have looked at the extraction of different bioactive compounds (i.e., phenolic compounds) from either date fruit flesh (Abuharfeil et al., 1999; Kchaou et al., 2016; Ravishanker and Raut, 2016; Saleh and Otaibi, 2013) or date fruit
syrup (Dhaouadi et al., 2011; Taleb et al., 2016b), rather than the microbiota itself to investigate antibacterial properties against different microorganisms. In addition, any antibacterial testing that was done, was not performed on *L. monocytogenes* (Taleb et al., 2016a).

Thus, the present study investigated the culturable microbiota of imported, ready-to-eat (RTE) date fruits to examine how well bacteria isolated from date fruits could control/inhibit the growth of *L. monocytogenes*. Furthermore, this study addressed the following objectives: 1) to assess whether the *L. monocytogenes* inhibitory strains, isolated from the date fruits, would be considered novel and safe to use for biocontrol applications in the food industry; 2) to assess whether the inhibitory strains selected from objective 1 could also impact the virulence of *L. monocytogenes* based on the inhibition of PrfA expression and 3) to characterize the anti-*Listeria* compound(s) produced from the most promising inhibitory strain identified from objective 1.
3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

All strains (Table 3.1 and 3.2) and bacterial food isolates were grown at 37°C in brain heart infusion (BHI; Fisher Scientific, Mississauga, ON) or a defined medium specific for studying L. monocytogenes virulence (iLSM; recipe in Table A1 of Appendix A, adapted from Whiteley et al., 2017). Strains with antibiotic resistance were supplemented with chloramphenicol (CHL, 7.5 μg/mL) and/or streptomycin (STR, 200 μg/mL). Bacterial stocks were frozen and stored at -80°C in freezing media (12% w/v skim milk, 1% v/v glycerol, 1% v/v DMSO). All chemicals were purchased from Millipore Sigma (Oakville, ON) unless otherwise stated.

3.2.2 Isolation of bacteria from the date fruits

Date fruits were acquired from various supermarkets in Mississauga (ON, Canada) (Table 3.3 and Figure 3.1). A total of 10 g of date fruits were weighed into a filter Stomacher® bag (Fisher Scientific, Mississauga, ON) containing 10 mL of 0.1% (w/v) peptone water. Contents of the bag were massaged by hand for 2 min and the residual liquid was aspirated off and subjected to differential centrifugation at 1000 x g for 2 min to remove any food particulates, followed by 14,000 x g for 2 min to pellet out bacterial cells. The bacterial pellet was suspended in 250 μL and subjected to standard serial dilutions (10⁻¹ to 10⁻²) using 0.1% (w/v) peptone water, then spread-plated onto blood agar (tryptic soy agar enriched with 5% v/v defibrinated sheep blood; Oxoid, Nepean, ON). Plates were then incubated overnight at 37°C. All bacterial colonies were counted to determine total viable counts and bacterial isolates were collected in triplicate per observable phenotype per batch of food. Phenotypes were distinguished from one another based on the colony morphology protocol.
(Breakwell et al., 2007; Gerhardt et al., 1994). Individual colonies were sub-cultured on blood agar in order to check their purity and then stored in freezing media at -80°C. Isolation was repeated in triplicate, per batch of food.

### 3.2.3 Identification of the food isolates

All bacterial food isolates were identified using 16S rRNA sequencing. Genomic DNA was extracted from the bacterial isolates using a boiling method as follows. An isolated colony was placed in a tube containing 400 μL of TE buffer (1 M Tris, 0.5 M EDTA, pH 8.0) and was then subjected to boiling at 100°C in a water bath for 15 min. The samples were centrifuged at 14,000 x g for 2 min to pellet out the bacterial cells and the upper aqueous phase was aspirated off to acquire the DNA template. The DNA was then subjected to PCR amplification using the V3kl/V6r primer set (Table 3.4) to amplify the V3 to V6 regions of the 16S rRNA operon (Gloor et al., 2010).

Each PCR tube had a final volume of 25 μL, using 10X ThermoPol Reaction Buffer (New England BioLabs-NEB, Mississauga, ON), 200 μM of dNTPs (Invitrogen, Nepean, ON), 0.8 pmol/μL of each primer pair (Table 3.4), 2.5U Taq DNA Polymerase (NEB, Whitby, ON) and 5 μL of template DNA. PCR was carried out in a Biometra Tprofessional Basic Gradient 96 (Montreal Biotechnologies Inc., Dorval, QC), with the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C/30 s, 60°C/30 s, 72°C/30 s and final extension at 72°C for 5 min. PCR product sizes were determined by 1.5 % (w/v) agarose gel electrophoresis using the GeneRuler 100 bp DNA Ladder (Fisher Scientific, Mississauga, ON) and visualization was by EZ-Vision® DNA dye staining (VWR, Mississauga, ON).

The PCR products were sent for sequencing at the University of Guelph Advanced Analysis
Centre (AAC) Genomics Facility. Amplicon sequences were compared to the GenBank database (NCBI) using nucleotide Basic Local Alignment Search Tool (BLASTn) and the resulting hits allowed for approximate speciation (> 97% identity match) for the strains according to their closest match in the database.

3.2.4 Growth inhibition plate assay

Screening for anti-*Listeria* activity from the food isolates was determined using the growth inhibition plate assay against *L. monocytogenes* FSL J1-110 (Lm4b). Lm4b, along with each collected food isolate, were grown overnight at 37°C in BHI broth. A lawn of an overnight culture of Lm4b was then plated onto BHI agar using a cotton swab (~ 100 μL). The top of the Lm4b lawn was then inoculated with a 5 μL drop of an overnight bacteria culture of each food isolate. The drops were left to dry completely before plates were left to incubate overnight at 37°C. The following day, the plates were inspected and the inhibition zones detected were measured and reported in mm. Food isolates that produced a zone were classified as anti-*Listeria* inhibitory strains. The assay was repeated in triplicate for each isolate.
<table>
<thead>
<tr>
<th><strong>Strain</strong></th>
<th><strong>Acronym</strong></th>
<th><strong>Serotype</strong></th>
<th><strong>Description</strong></th>
<th><strong>Antibiotic</strong></th>
<th><strong>Reference/Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>FSL J1-110</td>
<td>Lm4b</td>
<td>4b</td>
<td>Food isolate from Jalisco cheese outbreak (LA, USA, 1985)</td>
<td>—</td>
<td>CRIFS culture collection, University of Guelph</td>
</tr>
<tr>
<td>10403S</td>
<td>WT</td>
<td>1/2a</td>
<td>Wild-type</td>
<td>STR(^a)</td>
<td>Bécavin et al., 2014</td>
</tr>
<tr>
<td>DP-L4137</td>
<td>ΔprfA</td>
<td>1/2a</td>
<td>ΔprfA (prfA in frame deletion of aa 34 – 146)</td>
<td>STR</td>
<td>Cheng and Portnoy, 2003</td>
</tr>
<tr>
<td>NF-L1177</td>
<td>PrfA*</td>
<td>1/2a</td>
<td>PrfA* [encoded by prfA(G145S)]</td>
<td>STR</td>
<td>Miner et al., 2008</td>
</tr>
<tr>
<td>DP-L6508</td>
<td>WT::pPL2</td>
<td>1/2a</td>
<td>Wild-type strain bearing pPL2_P_actA_RFP</td>
<td>STR, CHL(^b)</td>
<td>Zeldovich et al., 2011</td>
</tr>
<tr>
<td>DP-L5061</td>
<td>ΔprfA::pPL2</td>
<td>1/2a</td>
<td>ΔprfA strain bearing pPL2_P_actA_RFP</td>
<td>STR, CHL</td>
<td>Portman et al., 2017</td>
</tr>
<tr>
<td>DP-L6562</td>
<td>PrfA*::pPL2</td>
<td>1/2a</td>
<td>PrfA* strain bearing pPL2_P_actA_RFP</td>
<td>STR, CHL</td>
<td>Portman et al., 2017</td>
</tr>
</tbody>
</table>

\(^a\) Streptomycin (200 μg/mL); \(^b\) Chloramphenicol (7.5 μg/mL).
Table 3.2. List of all other strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acronym</th>
<th>Description</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus amyloliquefaciens</em> ATCC 23842</td>
<td>ATCC 23842</td>
<td>Acquired from the CRIFS culture collection(^a)</td>
<td>Welker and Campbell, 1967</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> 10CP18</td>
<td>10CP18</td>
<td>Food isolates from cheese curds; gift from Atinuke Akinpelu (PhD student)</td>
<td>Dr. Gisèle LaPointe (Food Science, University of Guelph)</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> 10CP20</td>
<td>10CP20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> subsp. <em>spizizenii</em> ATCC 6633</td>
<td>ATCC 6633</td>
<td>Acquired from the CRIFS culture collection(^a)</td>
<td>Nakamura et al., 1999</td>
</tr>
</tbody>
</table>

\(^a\) Canadian Research Institute for Food Safety (CRIFS), Department of Food Science, University of Guelph.
Table 3.3. Overview of the imported date fruits used in this study

<table>
<thead>
<tr>
<th>Origin</th>
<th>Acronym</th>
<th>Common name</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>DC</td>
<td>Jujube</td>
<td>Ziziphus jujuba</td>
</tr>
<tr>
<td>Iran</td>
<td>DI</td>
<td>Mazafati/Mozafati</td>
<td>Phoenix dactylifera L.</td>
</tr>
<tr>
<td>Palestine</td>
<td>DP</td>
<td>Medjoul</td>
<td></td>
</tr>
<tr>
<td>Saudi-Arabia</td>
<td>DS</td>
<td>Khudary/Khudri</td>
<td></td>
</tr>
<tr>
<td>Tunisia</td>
<td>DT</td>
<td>Deglet Noir</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1. Overview of the imported RTE date fruits used in this study. Date fruits were acquired from five different geographical regions from supermarkets in Mississauga (ON, Canada).
3.2.5 Detection of the pumilacidin toxin from the inhibitory strains

DNA from the identified inhibitory strains was tested for the presence of the orfY gene (geneID: 3666985; locus tag: UP12_RS02020), a biomarker that encodes for a nonribosomal peptide synthetase which was found to be specific to pumilacidin-producing strains of Bacillus (Saggese et al., 2018). The DNA was subjected to touch-down PCR amplification using the orfyF7700/orfyR8122 primer set (Table 3.4) designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Genomic DNA from B. pumilus 10CP18 and B. pumilus 10CP20 was used as a positive control, while the genomic DNA from B. amyloliquefaciens ATCC 23842 and B. subtilis subsp. spizizenii ATCC 6633 was used as a negative control. The PCR preparation was identical to the description under 3.2.3 Identification of the food isolates.

Amplification was initiated with a 60°C annealing temperature that dropped to 50°C in 30 increments followed by 10 cycles of the following: denaturation (94°C, 30 s), annealing (50°C, 30 s) and extension (72°C, 60 s). A final extension step was carried out at 72°C for 5 min. PCR product sizes were determined by 1.5 % (w/v) agarose gel electrophoresis using the GeneRuler 100 bp DNA Ladder (Fisher Scientific, Mississauga, ON) and visualization was by EZ-Vision® DNA dye staining (VWR, Mississauga, ON).

3.2.6 Virulence reporter assay

The virulence reporter assay used was adapted as per the method described by Portman et al. (2017). Strains of L. monocytogenes (Table 3.1) and selected inhibitory strains were grown overnight at 37°C with agitation (250 rpm) in iLSM (with applicable antibiotics throughout). L. monocytogenes strains were then diluted 1:10 into either fresh iLSM or the cell-free supernatant of
the selected inhibitory strains. Diluted cultures were then subjected to 10 mM of glutathione (GSH) and grown overnight at 37°C with agitation (250 rpm) in a 200 mL glass bottle, loosely capped (to permit adequate aeration for the strains). The following day, 500 μL was taken from each culture, transferred into a clear 24-well flat-bottom plate and subsequently read for relative fluorescence units (RFU) on a Victor3 multi-label counter (Wallac, PerkinElmer Life Sciences Canada, Woodbridge, ON, Canada) with the following parameters: 550/580 (excitation/emission), bottom read, optimal flashes, optimal gain. The OD$_{600}$ was taken with a handheld spectrophotometer (Beckman DU®520 general purpose UV/Vis) in parallel for normalization. Fluorescence was then expressed as relative fluorescence units by OD$_{600}$ (RFU/OD$_{600}$).
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Expected</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3kl-F</td>
<td>ACTCCTACGGGAGGCAAGCTGACGAGT</td>
<td>800</td>
<td>16S rRNA operon, V3-V6</td>
<td>Gloor et al., 2010</td>
</tr>
<tr>
<td>V6r-R</td>
<td>ACAACACGAGCTGACGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>orfyF7700</td>
<td>GACTCATCATTTTAGATACGGTCCC</td>
<td>423</td>
<td>orfY gene, specific to pumilacidin-producing strains of <em>Bacillus</em></td>
<td>This study</td>
</tr>
<tr>
<td>orfyR8122</td>
<td>CTCTTTGATGTGATAATACGAGCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2.7 Characterization of the anti-Listeria compound(s)

3.2.7.1 Production of the compound(s)

Production of the anti-Listeria compound(s) was done using the inhibitory strain, *Bacillus altitudinis* DS11, which showed one of the largest inhibition zones. It was grown in BHI broth overnight at 37°C (250 rpm), then diluted 1:10 the following day in fresh BHI broth to grow an additional day at 37°C (250 rpm). The bacterial culture was centrifuged (9000 x g for 10 min at room temperature) and the supernatant was filter-sterilized with a 0.22 μm filter (Millipore Sigma, Oakville, ON, Canada). The cell-free-supernatant (CFS) was size-fractionated and concentrated 25X at 4°C using a 10-kDa cutoff spin column (Amicon Ultra Centrifugal Filters, Millipore Sigma). Biological activity of the 25X concentrated CFS was assessed by testing a 100 μL drop volume as per the growth inhibition plate assay described under 3.2.4 Growth inhibition plate assay. To accommodate this larger drop volume, a hole in the agar plate was made using a P1000 pipette tip.

3.2.7.2 Sensitivity of the compound(s) to enzymes, pH and temperature

Sensitivity of the active CFS (from *B. altitudinis* DS11) was determined as per methods adapted from Khochamit et al. (2015) and Saggese et al. (2018). Proteolytic enzymes (1 mg/mL of either pepsin, proteinase K or trypsin) were added to the active CFS and samples were then incubated for 1 h at 37°C. The effects of pH (2, 4, 10 and 13) were tested by adjusting the pH of the active CFS with HCl (0.2 M for pH 2; 0.1 M for pH 4) and NaOH (0.1 M for pH 10; 0.2 M for pH 13), and then incubating those pH-adjusted samples for 1 h at 37°C. The effects of temperature were tested by incubating the active CFS for 20 min at either 60, 80, 100 or 121°C. Post-incubation, all samples were tested using a 100 μL drop volume as per the modified growth inhibition plate assay.
described under 3.2.7.1 Production of the compound(s). Inhibition zones detected were reported as a percentage of the zone produced relative to the control sample.

3.2.8 Statistical analysis

All experiments were carried out in triplicate (N=3) where the experimental unit was defined as a purified, isolated bacterial colony; mean ± standard deviation (STD) values are represented in tables and figures. Outliers in the dataset were not observed; all observations had an absolute value for their studentized residual of < 3.4 units.

A randomized completed block design (RCBD) was used with a generalized linear mixed model to analyze the fluorescence of *L. monocytogenes* (RFU/OD_{600}) and OD_{600} plots. Variance was partitioned into fixed effects of treatment or strain and tested using the F-test; random effects were block and tested using the log-likelihood test. A normal, Gaussian distribution with a homogeneous covariance structure, was selected based on the plots of the studentized residuals and Akaike information criterion (AICC) fit statistics. A Kenward-Roger correction for the denominator was applied if the model required a heterogeneous error structure (Bowley, 2015).

Comparison of responses were conducted using a means comparison with a Tukey-Kramer multiplicity adjustment for strain comparisons, and a Dunnett multiplicity adjustment for treatment comparisons. A type I error rate of 0.05 (p < 0.05) was applied for all statistical tests. All statistical computations were carried out using the SAS system Proc Glimmix (SAS Studio, SAS release 9.04, SAS Institute, Carry NC). A sample of the variance analysis, studentized residual plots and coding for the *L. monocytogenes* WT::pPL2 fluorescence data (RFU/OD600) post-exposure to the inhibitory strain treatments, can be found in Appendix B.
3.3 Results

3.3.1 Overview of the imported date fruits collected

A total of five date fruits were acquired from different countries from supermarkets located in Mississauga (ON, Canada) (Table 3.3). These imported regions were China (DC), Iran (DI), Palestine (DP), Saudi-Arabia (DS) and Tunisia (DT). Additionally, these date fruits were all found to be associated with different varieties – Jujube for China dates, Mazafati/Mozafati for Iran dates, Medjoul for Palestine dates, Khudary/Khudri for Saudi-Arabia dates and Deglet Noir for Tunisia dates. Lastly, when looking at their botanical scientific name, all date fruits were Phoenix dactylifera L. except for the China dates, which were Ziziphus jujuba.

3.3.2 Microbial composition of the date fruits

To recover the food microbiota, bacteria were washed off the foods and then plated onto blood agar plates. Bacteria were isolated on blood agar based on appearance, with the collection of the isolates being done in triplicate per observable phenotype. Phenotypes were distinguished subjectively from one another based on the colony morphology protocol (Breakwell et al., 2007; Gerhardt et al., 1994). This included discrimination based on the colony’s appearance, elevation, hemolytic properties, margin, optical properties, pigmentation, shape, size and texture.

A rich diversity of bacteria was obtained from the date fruits, with a total of 191 isolates belonging to 91 different phenotypes being observed (Table 3.5). Total aerobic bacterial counts on the date fruits ranged from $10^3$ – $10^5$ CFU/g, where the greatest plate counts were observed from the Palestinian dates ($9.7 \times 10^4$ CFU/g), followed by the Chinese dates ($3.6 \times 10^4$ CFU/g). Plate
counts observed from the Saudi-Arabian dates (2.7 x 10^4 CFU/g) and Tunisian dates (2.5 x 10^4 CFU/g) were fairly similar, while the lowest bacterial plate count was observed from the Iranian dates (2.0 x 10^3 CFU/g), although the latter was heavily populated with fungi. Additionally, the greatest numbers of isolates (observed phenotypes enclosed in brackets) collected using this recovery method were from the Chinese and Palestinian dates with 65 (27) and 52 (24) isolates, respectively. The bacterial isolates recovered from Saudi-Arabian and Tunisian dates were fairly similar with 33 (15) and 28 (16) isolates, respectively, followed by Iranian dates with 13 (9), which had least number of bacterial isolates available for this collection.

3.3.3 Identification of the date fruit isolates

16S rRNA sequencing was used to identify the date fruit microbiota isolated from the blood agar profile using primers that targeted the V3 to V6 variable regions of the 16S rRNA operon system. A sample of the 16S rRNA amplified products can be seen on an agarose gel in Figure 3.2.

The date fruit isolates belonged to 15 different genera: *Ammoniphilus, Bacillus, Brevibacillus, Erwinia, Fictibacillus, Lysinibacillus, Moraxella, Oceanobacillus, Paenibacillus, Pantoea, Rothia, Solibacillus, Streptomyces, Terribacillus* and *Virgibacillus* spp (Figure 3.3 and Table 3.5). Among these 15, *Bacillus* spp. was found across all five date fruits and was found to be the most abundant, representing 70 – 85% of the culturable microbiota from the blood agar profile. Of the other genera, *Brevibacillus* spp. was isolated from date fruits originating from China and Palestine in proportions ranging from 1 to 2%; *Solibacillus* spp. was isolated from date fruits coming from Saudi-Arabia and Tunisia in proportions ranging from 4 to 6%; and *Terribacillus* spp. was
isolated from date fruits originating from China, Palestine and Saudi-Arabia in proportions ranging from 9 to 15%. All other genera observed were unique to date fruits originating from one country.

The greatest diversity of isolates came from the Tunisian dates, i.e., bacteria recovered belonged to seven different genera, namely Bacillus, Erwinia, Lysinibacillus, Moraxella, Pantoea, Rothia and Solibacillus spp. This was followed by the Chinese (Bacillus, Brevibacillus, Fictibacillus, Terribacillus and Virgibacillus spp.) and Palestinian dates (Ammoniphilus, Bacillus, Brevibacillus, Oceanobacillus, and Terribacillus spp.) where the recovered bacteria belonged to five genera, whereas the Iranian (Bacillus, Paenibacillus and Streptomyces spp.) and the Saudi-Arabian dates (Bacillus, Solibacillus and Terribacillus spp.) contained isolates belonging to three genera. In total, the date fruit isolates belonged to 50 unique species identities (IDs). When broken down by food, the Chinese dates displayed the most diversity with 27 unique species, followed by Tunisian dates with 18, Palestinian dates with 15, Saudi-Arabian dates with 14 and Iranian with 10 unique IDs.

3.3.4 Impact of the bacterial isolates from date fruits on *L. monocytogenes* growth

Bacterial isolates were screened using the growth inhibition plate assay against *L. monocytogenes*. Close to 20% of the collected isolates (36 out of 191) produced a zone of inhibition against *L. monocytogenes*, with zone sizes ranging from 0.3 to 5.8 mm (Figure 3.4 and Table 3.5). Broken down by origins of the date fruits, the figures were 11% for the Chinese dates (7 out of 65), 8% for the Iranian dates (1 out of 13), 33% for the Palestinian dates (17 out of 52), 30% for the Saudi-Arabian dates (10 out of 33) and 4% for the Tunisian dates (1 out of 28). Sequencing of the
inhibitory strains revealed them all to be *Bacillus* spp., belonging to six unique species IDs. These *Bacillus* spp. groups (Bs) were as follows: *B. zhangzhouensis/pumilus/safensis*; *B. altitudinis*; *B. flexus*; *B. velezensis/amyloliquefaciens/vallismortis*; *B. subtilis/tequilensis* and *B. halosaccharovorans*. Due to how phylogenetically close these species are, some inhibitory strains identified across multiple species could not be distinguished with confidence to a single species. For these cases, the inhibitory strains were delineated as multiple species, separated by a dash.

The bulk of the inhibitory strains were identified as *B. zhangzhouensis/pumilus/safensis* with 13 of the isolates coming from four date fruits (China, Palestine, Saudi-Arabia and Tunisia). This was followed by *B. altitudinis* with 11 of the isolates coming from three date fruits (China, Palestine and Saudi-Arabia); *B. velezensis/amyloliquefaciens/vallismortis* with 8 of the isolates coming from two date fruits (Palestine and Saudi-Arabia); *B. subtilis/tequilensis* with 2 of the isolates coming from two date fruits (Palestine and Saudi-Arabia); and *B. flexus* and *B. halosaccharovorans* with 1 isolate from Iranian dates and Palestinian dates, respectively.

Among the inhibitory strains, the greatest diversity was seen with the Palestinian dates where the inhibitory strains belonged to 5 out of the 6 *Bacillus* groups (*B. zhangzhouensis/pumilus/safensis*, *B. altitudinis*, *B. velezensis/amyloliquefaciens/vallismortis*, *B. subtilis/tequilensis*, and *B. halosaccharovorans*). This was followed by the Saudi-Arabian dates whose strains belonged to four *Bacillus* groups (*B. zhangzhouensis/pumilus/safensis*, *B. altitudinis*, *B. velezensis/amyloliquefaciens/vallismortis*, and *B. subtilis/tequilensis*); and the Chinese dates whose strains belonged to two *Bacillus* groups (*B. zhangzhouensis/pumilus/safensis*; and *B. altitudinis*). The single inhibitory strains recovered from the Iranian and Tunisian dates were
identified as *B. flexus* and *B. zhangzhouensis/pumilus/safensis*, respectively. The inhibitory strain isolated from the Iranian dates was the only identified as *B. flexus*.

### 3.3.5 Screening of the inhibitory strains for safety and novelty

The pumilacidin toxin gene was investigated by PCR using primers targeting the *orfY* gene (geneID: 3666985; locus tag: UP12_RS02020) that encodes for a non-ribosomal peptide synthetase. Saggese et al. (2018) had found this gene to be specific to pumilacidin-producing strains of *Bacillus* (such as *B. pumilus* and *B. safensis*). In the PCR reactions, *B. pumilus* 10CP18 and *B. pumilus* 10CP20 was used as positive controls, while *B. amyloliquefaciens* ATCC 23842 and *B. subtilis* subsp. *spizizenii* ATCC 6633 as negative controls, along with all 36 inhibitory strains. The control strains worked as expected, whereby only the positive controls produced a band at the expected 423-bp amplicon size (Figure 3.5). In addition, all 13 strains from *B. zhangzhouensis/pumilus/safensis* tested positive for this gene target, whereas inhibitory strains from all other *Bacillus* groups tested negative. As a result, the 13 inhibitory strains from *B. zhangzhouensis/pumilus/safensis* were not further pursued.

For the amylosin toxin, no information on the gene sequence was found, therefore, the species reported to produce this toxin were discarded from the selection of inhibitory strains. This particularly applied to the 8 inhibitory strains from *B. velezensis/amyloliquefaciens/vallismortis*, as they could be putatively identified as *B. amyloliquefaciens*, a reported producer of this toxin (Mikkola et al., 2007; Domingos et al., 2015). Thus the *B. amyloliquefaciens* species was screened by PCR by targeting the *pheS* gene that encodes for phenylalanyl-tRNA synthase, a gene target found to be specific to this species (Chien-Hsun et al., 2017). PCR performed with these species-specific primers (spBamyphes-171F1/353R1) with our 8 inhibitory strains from the *B.*
velezensis/amyloliquefaciens/vallismortis group, showed amplification products to the pheS gene for four of the strains (DP9B, DS3A, DS3B and DS4A). Therefore, it was decided not to further pursue these four inhibitory strains.

Lastly, for the two inhibitory strains that identified as B. subtilis/tequilensis, it was decided not to further pursue them due to their lack of novelty, as these strains have been well studied. A more detailed discussion of this decision can be found in section 3.4.5.

3.3.6 Impact of the inhibitory strains on L. monocytogenes virulence expression

The virulence of L. monocytogenes was studied using methods developed by Portman et al. (2017). Specifically, fluorescence spectroscopy was used on reporter strains of L. monocytogenes in a reducing environment with glutathione (GSH). These strains use red fluorescent protein (RFP) as a fluorescent reporter where RFP can be induced upon expression of PrfA, the virulence regulator of L. monocytogenes. These reporter strains include a wild-type indicator (WT::pPL2), dysfunctional PrfA mutant (ΔprfA::pPL2) as a negative control, and a recombinant strain that constitutively expresses the PrfA protein (PrfA*::pPL2) used as a positive control. The rfp gene was cloned downstream of a PrfA-dependent actA promoter in a pPL2 vector. Thus, to obtain expression of rfp on the WT strain, complete expression and activation of the PrfA protein must be achieved. To do so, the strain must be grown in a reducing environment that mimics the redox potential of the host cytosol. This reducing environment is obtained by using exogenous glutathione (GSH) along with an in-house limiting media, incomplete Listeria synthetic media (iLSM) (Appendix A; Whiteley et al., 2017). The GSH in this environment allosterically activates PrfA in the WT::pPL2 strain to thus allow L. monocytogenes to confer its virulence through PrfA.
The fluorescence reporter assay of Portman et al. (2017) was tested for seven strains of *L. monocytogenes* (Table 3.1). This was done to confirm (in our laboratory setting) whether expression of the PrfA protein through the RFP reporter (PrfA-RFP expression) was due to the transformation of these strains with the pPL2 plasmid that contains the *rfp* gene. PrfA-RFP expression was most predominantly observed in the WT::pPL2 and PrfA*::pPL2 strains (Fig. 3.6). All other strains (Lm4b, WT, ΔprfA, PrfA*, and ΔprfA::pPL2) displayed a basal level of PrfA-RFP expression that was significantly lower than the WT::pPL2 and PrfA*::pPL2 strains (p-value < 0.05). In addition, the PrfA-RFP expression of the WT::pPL2 indicator strain was found to match that of the PrfA*::pPL2 positive control strain, while the respective negative control strain (ΔprfA::pPL2) displayed basal levels of PrfA-RFP expression. This confirmed that the complete activation of the PrfA protein from the WT::pPL2 strain was obtained by simulating a reducing environment that mimics the redox potential of the host mammalian cytosol.

The culture supernatants of selected inhibitory strains (*B. flexus* DI3, *B. halosaccharovorans* DP18B, *B. velezensis/vallismortis* DS6C and *B. altitudinis* DS11) were then tested to see if they could impact PrfA-RFP expression in both the WT::pPL2 indicator and PrfA*::pPL2 control strains. The culture supernatant from all inhibitory strains was found to significantly (p-value < 0.05) reduce the PrfA-RFP expression in the WT::pPL2 relative to the untreated control (Fig. 3.7). The observed reduction was 69 ± 23%, 62 ± 22%, 82 ± 7%, and 70 ± 12% for the *B. flexus* DI3, *B. halosaccharovorans* DP18B, *B. velezensis/vallismortis* DS6C and *B. altitudinis* DS11 treatments, respectively. These supernatant treatments did not, however, significantly impact the PrfA-RFP expression in the PrfA*::pPL2 strain, relative to the untreated control (p-value > 0.05). In addition, when measuring the OD$_{600}$ of the various strains, the *B. flexus* DI3 treatment was found to significantly reduce the OD$_{600}$ of both the WT::pPL2 and PrfA*::pPL2
strains by 41 ± 11% and 34 ± 8%, respectively (p-value < 0.05). The *B. halosaccharovorans* DP18B treatment was also found to significantly reduce the observed OD$_{600}$ in the PrfA*:pPL2 by 23 ± 6% (p-value < 0.05). All other treatments were found to have no significant impact on the OD$_{600}$ observed with the WT:pPL2 and PrfA*:pPL2 strains (p-value > 0.05).

### 3.3.7 Characterization of the anti-*Listeria* compound(s)

Among the four selected inhibitory strains to further pursue (*B. flexus* DI3, *B. halosaccharovorans* DP18B, *B. velezensis/vallismortis* DS6C and *B. altitudinis* DS11), a more detailed characterization was performed on the anti-*Listeria* compound(s) produced by *B. altitudinis* DS11, due to this strain displaying the greatest inhibition against *L. monocytogenes* (as per the growth inhibition plate assay). To extract its anti-*Listeria* compound(s), *B. altitudinis* DS11 was grown in BHI broth at 37°C (with agitation) for two days until an approximate OD$_{600}$ of > 1.2. The culture was then filter sterilized and size-fractionated using various molecular weight cut-off filter sizes (3, 10, 50, and 100 kDa) to concentrate the supernatant by roughly 25X. This concentration step was done, as the unconcentrated cell-free supernatant did not display any anti-*Listeria* activity when subjected to the growth inhibition plate assay. The concentrated supernatant displayed activity when using cut-off filters of 3 and 10 kDa, but lost activity with cut-off filters ≥ 50 kDa ([Fig. 3.8A](#fig3.8a)). These results suggest that i) the anti-*Listeria* compound(s) can be secreted by *B. altitudinis* DS11 and further captured in the culture supernatant, and ii) the native conformation of the antimicrobial compound(s) is somewhere between the sizes of 10 – 50 kDa. Thus, the 10 kDa concentrated supernatant fraction was used to further characterize the anti-*Listeria* compound(s).
To obtain a preliminary characterization of the anti-
Listeria compound(s) from DS11, its stability was analyzed across a wide range of proteolytic enzyme, pH and temperature treatments (Figure 3.8 and Table 3.6). The compound(s) was fully active against L. monocytogenes after treatment with pepsin, however, loss and complete absence of activity was observed after treatment with trypsin and proteinase K, respectively. In addition, the compound(s) showed full activity after being exposed to acidic pH values (pH 2 and 4). However, exposure of the compound(s) to alkaline pH values showed heightened activity of 141 ± 8% and 176 ± 21%, respectively, for pH values of 10 and 13. These effects were found to be specific to the active supernatant under treatment, as the treatments on their own did not impact the growth of L. monocytogenes when subjected to the same growth inhibition plate assay (Figure 3.8 B1 and C1). Lastly, when testing temperature tolerance, the compound(s) displayed full activity after treatment at 60 and 80°C, but displayed loss (53 ± 6%) and complete absence of activity after exposure to temperatures of 100 and 121°C, respectively.
Table 3.5. Summary of the bacterial community screened from the date fruits

<table>
<thead>
<tr>
<th>Date origin</th>
<th>[CFU/g]</th>
<th>Isolates(^a)</th>
<th>Isolate homologies(^b)</th>
<th>Inhibitory strains(^c)</th>
<th>Inhibitory strain identities(^b)</th>
<th>Number of unique spp. identities(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>$3.6 \times 10^4$</td>
<td>65 (27)</td>
<td><em>Bacillus</em> spp., <em>Brevibacillus</em> spp., <em>Fictibacillus</em> spp., <em>Terribacillus</em> spp., <em>Virgibacillus</em> spp.</td>
<td>7 (3)</td>
<td><em>Bacillus</em> spp.</td>
<td>27 (2)</td>
</tr>
<tr>
<td>Iran</td>
<td>$2.0 \times 10^3$</td>
<td>13 (9)</td>
<td><em>Bacillus</em> spp., <em>Paenibacillus</em> spp., <em>Streptomyces</em> spp.</td>
<td>1</td>
<td><em>Bacillus</em> spp.</td>
<td>10 (1)</td>
</tr>
<tr>
<td>Palestine</td>
<td>$9.7 \times 10^4$</td>
<td>52 (24)</td>
<td><em>Ammoniphilus</em> spp., <em>Bacillus</em> spp., <em>Brevibacillus</em> spp., <em>Oceanobacillus</em> spp., <em>Terribacillus</em> spp.</td>
<td>17 (10)</td>
<td><em>Bacillus</em> spp.</td>
<td>15 (5)</td>
</tr>
<tr>
<td>Saudi-Arabia</td>
<td>$2.7 \times 10^4$</td>
<td>33 (15)</td>
<td><em>Bacillus</em> spp., <em>Solibacillus</em> spp., <em>Terribacillus</em> spp.</td>
<td>10 (6)</td>
<td><em>Bacillus</em> spp.</td>
<td>14 (4)</td>
</tr>
<tr>
<td>Tunisia</td>
<td>$2.5 \times 10^4$</td>
<td>28 (16)</td>
<td><em>Bacillus</em> spp., <em>Erwinia</em> spp., <em>Lysinibacillus</em> spp., <em>Moraxella</em> spp., <em>Pantoea</em> spp., <em>Rothia</em> spp., <em>Solibacillus</em> spp.</td>
<td>1</td>
<td><em>Bacillus</em> spp.</td>
<td>18 (1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>$10^3$ – $10^5$</td>
<td>191 (91)</td>
<td>15 different genera</td>
<td>36 (21)</td>
<td><em>Bacillus</em> spp.</td>
<td>51 (6)</td>
</tr>
</tbody>
</table>

\(^a\) Number of bacterial isolates collected; observed phenotypes enclosed in brackets.

\(^b\) Identified using 16S rRNA sequencing.

\(^c\) Strains detected using the growth inhibition plate assay against *L. monocytogenes*; observed phenotypes enclosed in brackets.

\(^d\) Based on the top accession number for that isolate’s FASTA sequence; unique identities for inhibitory strains enclosed in brackets.
Figure 3.2. PCR amplification of the 16S rRNA gene from the date fruit inhibitory strains. M: DNA marker; NTC: no template control.
Figure 3.3. Bacterial profile of the date fruits based on the 16S rRNA signatures of their cultured bacteria. Number in brackets represents the total number of isolates recovered from that date fruit.
Figure 3.4. Summary of the inhibition zones produced by the inhibitory strains isolated from the date fruits against *L. monocytogenes* (N=3, + STD). Inhibitory strains identified across six *Bacillus* species (Bs) groups. Bs1: *B. zhangzhouensis/pumilus/safensis*; Bs2: *B. altitudinis*; Bs3: *B. fleixu*; Bs4: *B. velezensis/amyloliquefaciens/vallismortis*; Bs5: *B. subtilis/tequilensis*; Bs6: *B. halosaccharovorans.*
Figure 3.5. PCR amplification of the *orfY* gene against all date fruit inhibitory strains using the orfyF7700/orfyR8122 primer set. M: DNA marker; NTC: no template control; PC: positive control; NC: negative control; DC: Chinese dates; DI: Iran dates; DP: Palestine dates; DS: Saudi-Arabia dates; DT: Tunisia dates; Bs1: *B. zhangzhouensis/pumilus/safensis*; Bs2: *B. altitudinis*; Bs3: *B. flexus*; Bs4: *B. velezensis/amyloliquefaciens/vallismortis*; Bs5: *B. subtilis/tequilensis*; Bs6: *B. halosaccharovorans*; 1: *B. pumilus* 10CP18; 2: *B. pumilus* 10CP20; 3: *B. subtilis* subsp. *spizizenii* ATCC 6633; 4: *B. amyloliquefaciens* ATCC 23842.
Figure 3.6. Assessing RFP expression (under the control of the PrfA-dependent actA promoter) of L. monocytogenes (N=3, + STD). Strains were grown in incomplete Listeria synthetic media (iLSM) stimulated with glutathione (GSH) at 37°C, 250 rpm. Fluorescence (RFU) was monitored at ex/em= 550/580 nm. * p-value < 0.05.
Figure 3.7. Fluorescence (RFU, ex/em: 550/580 nm) and growth (OD$_{600}$) from strains of *L. monocytogenes* expressing RFP under the control of the PrfA-dependent actA promoter post treatment with cell-free-supernatant. Strains were treated with the supernatant of various inhibitory strains, DI(3), DP(18B), DS(6C), and DS(11), and compared back to the untreated control (N=3, + STD; * p-value (fluorescence/OD$_{600}$) < 0.05; # p-value (OD$_{600}$) < 0.05). Strains were grown in ‘incomplete Listeria synthetic media’ (iLSM) stimulated with glutathione (GSH). DI3: *B. flexus*; DP18B: *B. halosaccharovorans*; DS6C: *B. velezensis/vallismortis*; DS11: *B. altitudinis.*
Figure 3.8. Characterization of the active supernatant from *B. altitudinis* DS11 using a growth inhibition plate against *L. monocytogenes* 4b. (A) Supernatant was concentrated across various molecular weight cut-off columns – 10 kDa cutoff was selected for the remaining tests. Various protease enzymes were tested as a treatment in the absence (B1) or presence (B2) of the active supernatant; various pH values were tested as a treatment in the absence (C1) or presence (C2) of the active supernatant; and various temperatures were tested as a treatment against the active supernatant (D). Control= active supernatant, in the absence of treatment; O/N= overnight culture of the inhibitory strain.
Table 3.6. Effects of protease enzymes, pH and temperature on the anti-*Listeria* activity of the *B. altitudinis* DS11 supernatant (N=3, ± STD)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>CFS&lt;sup&gt;a&lt;/sup&gt; activity</th>
<th>pH</th>
<th>CFS activity</th>
<th>Temperature</th>
<th>CFS activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.7 ± 0.6 mm</td>
<td>Control</td>
<td>5.0 ± 1.0 mm</td>
<td>Control</td>
<td>5.7 ± 0.6 mm</td>
</tr>
<tr>
<td>Pepsin</td>
<td>100 ± 0 %</td>
<td>pH 2</td>
<td>106 ± 10 %</td>
<td>60°C</td>
<td>94 ± 10 %</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>0 ± 0 %</td>
<td>pH 4</td>
<td>106 ± 10 %</td>
<td>80°C</td>
<td>94 ± 10 %</td>
</tr>
<tr>
<td>Trypsin</td>
<td>100 ± 0 %&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pH 10</td>
<td>141 ± 8 %</td>
<td>100°C</td>
<td>53 ± 6 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 13</td>
<td>176 ± 21 %</td>
<td>121°C</td>
<td>0 ± 0 %</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell-free-supernatant.

<sup>b</sup> Faint zone.
3.4 Discussion

3.4.1 Overview of the imported date fruits collected

To the best of our knowledge, this study represents the first to profile the bacterial microbiota from a wide variety of date fruits, which was comprised of dates from five different varieties and two different botanicals. There has been some research done investigating the endophytic microbiome of date palm roots grown under different levels of salt stress (Yaish et al., 2016), along with the fungal root microbiome from healthy and diseased date palm trees (Mefteh et al., 2017).

This study also represents the first to assess whether the bacterial microbiota from date fruits possesses antibacterial properties against *L. monocytogenes*. Some studies have used date fruits for antimicrobial testing on other foodborne pathogens including *B. cereus, E. coli, Salmonella* spp., *Shigella flexeneri, Staphylococcus aureus* and *Yersinia enterocolitica* (as reviewed by Taleb et al., 2016a). In addition, to investigate the antimicrobials, these studies looked specifically at the extraction of different bioactive compounds (i.e., phenolic compounds) from either date fruit flesh (Abuharfeil et al., 1999; Kchaou et al., 2016; Ravishanker and Raut, 2016; Saleh and Otaibi, 2013) or date fruit syrup (Dhaouadi et al., 2011; Taleb et al., 2016b), rather than looking at the date fruit microbiota.

3.4.2 Microbial composition of the date fruits

With the exception of the Iranian dates, the bacterial growth observed on blood agar plates from the date fruits suggested that this food matrix could harbour a sufficient number of bacteria that could be washed off the food without the need for enrichment (10^4 – 10^5 CFU/g). In addition,
a large number of different bacterial isolates were isolated using the blood agar selection method, i.e., close to 50% of the isolates were of a different phenotype. The inadequate total bacterial growth and the abundance of fungal growth seen on blood agar plates from Iranian dates could be attributed to that particular date fruit, Mazafati/Mozafati, favouring a microbiota that is more fungal-based. Alternatively, that particular lot of date fruits could have been contaminated with fungal spores somewhere along the lines of it being handled, processed or stored. The fungal spores may have outcompeted the natural bacterial microbiota of the date fruits, causing the Iranian dates to harbour a microbiota that was predominantly fungal in nature.

3.4.3 Identification of the date fruit isolates

From the 15 identified genera, 11 were Gram-positive (Bacillus, Brevibacillus, Fictibacillus, Lysinibacillus, Oceanobacillus, Paenibacillus, Rothia, Solibacillus, Streptomyces, Terribacillus and Virgibacillus), three were Gram-negative (Erwinia, Moraxella and Pantoea) and one was Gram-variable (Ammoniphilus).

All three Gram-negative genera come from the phylum Proteobacteria, a grouping known to harbour a wide variety of pathogens (Garrity et al., 2015), and which were solely isolated from the Tunisian dates. Of these three genera, Moraxella and Pantoea spp. can be opportunistic pathogens and cause nosocomial infections (Grimont and Grimont, 2015; Juni and Bøvre, 2015;), while Erwinia and Pantoea spp., often considered as saprophytes or ubiquitous, are classified as plant pathogens (Hauben and Swings, 2015; Grimont and Grimont, 2015). In addition, since Moraxella spp. are often considered as a commensal of the mucosal surfaces of humans (Juni and Bøvre, 2015), they could have contaminated the Tunisian dates during their handling and processing.
Of the remaining genera, 10 come from the phylum Firmicutes and order Bacillales (Ammoniphilus, Bacillus, Brevibacillus, Fictibacillus, Lysinibacillus, Oceanobacillus, Paenibacillus, Solibacillus, Terribacillus and Virgibacillus), a phylum and order known for its hardy spore-forming bacteria and endospores (Ludwig et al., 2015; De Vos, 2015). Of this grouping, these genera either have no documented clinical relevance or harbour species with little or no human pathogenic potential, i.e., Bacillus spp. (Whitman and Bergey’s Manual Trust, 2015). In addition, these genera harbour species that are found predominantly in the natural environment from air, decaying matter, plants, soil and water (Whitman and Bergey’s Manual Trust, 2015). Species from these genera were also found to exclusively make up the culturable microbiota observed for date fruits from China, Palestine and Saudi-Arabia. Thus, the cultured bacteria recovered from the three date fruits were most probably coming from the food’s own natural microbiota, as opposed to a contaminant that may have arisen during their handling and processing.

The remaining two Gram-positive genera (Rothia and Streptomyces) both come from the phylum Actinobacteria, a grouping known for harbouring some of the richest sources of natural products, particularly clinically useful antibiotics (Goodfellow, 2015). Rothia spp. can be opportunistic pathogens and are most commonly found in the mouth and respiratory tracts of humans (Austin, 2015). Thus, the presence of this genus in the Tunisian date microbiota could be seen as a contaminant arising from the handling and processing of the date fruits. Streptomyces spp. on the other hand, although not associated with clinical illness, are considered pathogenic to plants and are also known as being one of the largest producers of antimicrobials (Kämpfer, 2015). Species from this genus are predominantly isolated from soil.
Overall, the majority of the genera isolated from date fruits in this study appeared to arise from the natural environment, are Gram-positive, and come from the phylum Firmicutes and order Bacillales. This observation was also exclusively seen with all collected bacterial isolates from the date fruits which came from China, Palestine and Saudi Arabia. Note that the Iranian date microbiota that was recovered may not be representative, as few bacterial isolates were recovered. Additionally, the bulk of the growth observed from the Iranian dates was primarily fungal in nature, which could have arisen from the natural microbiota of the fruit, or from external contamination. However, for the bacterial genera observed (Bacillus, Paenibacillus and Streptomyces), due to their origins being the soil and the surrounding environment, it is likely that the bacteria isolated are part of the natural Iranian date fruit microbiota. Furthermore, while the Tunisian date fruits appeared to display the most bacterial diversity, three of the seven observed genera could harbour opportunistic pathogens (i.e., Moraxella, Pantoea and Rothia), of which two are likely to have originated from the handling and processing of the date fruits (Moraxella and Rothia).

3.4.4 Impact of the bacterial isolates from date fruits on *L. monocytogenes* growth

General conclusions about a specific date fruit type harbouring more anti-Listeria bacteria than others, cannot be made definitively, as only one lot from a specific distributor of each date fruit was investigated. The results may have been different if this study had examined multiple lots or distributors of the date fruits. For example, variances could arise from: i) the time of year the date fruits were harvested; ii) the soil conditions in which the date palms were grown in; iii) the region of the country the date fruits were coming from; and iv) the handling, processing and transport that the date fruits received. Nonetheless, it is apparent that the date fruit matrix harbours a rich
culturable microbiota with bacterial members that possess antagonistic properties against *L. monocytogenes*.

More specifically, it was observed that only specific *Bacillus* spp. harbour anti-*Listeria* behaviour (*B. zhangzhouensis/pumilus/safensis; B. altitudinis; B. flexus; B. velezensis/amyloliquefaciens/vallismortis; B. subtilis/tequilensis; and B. halosaccharovorans*). In addition, varying degrees of anti-*Listeria* activity were observed, as zone sizes ranged widely (i.e., no overlapping confidence intervals), even for inhibitory strains that identified across the same species. The reasons for this large variation are difficult to explain. However, one possible reason could be that the anti-*Listeria* properties may be encoded on a plasmid and the copy number of the plasmids could dictate how much of the antilisterial compound(s) is produced.

In addition, it was found that not all *Bacillus* spp. belonging to the same species possess anti-*Listeria* activity. This was the case for inhibitory strains coming from *B. zhangzhouensis/pumilus/safensis; B. flexus; B. velezensis/amyloliquefaciens/vallismortis; B. subtilis/tequilensis; and B. halosaccharovorans*, where *Bacillus* isolates that did not possess any anti-*Listeria* activity were found to belong to the same species. One of the reasons for this phenomenon could be that the anti-*Listeria* properties are encoded on a plasmid that could be transmitted to similar *Bacillus* spp. through horizontal gene transfer. It would be of interest to compare the whole genome sequences of a non-producing strain with that of an anti-*Listeria* strain from the same species. A comparison of their genomes using a subtraction-based approach, may help to uncover specific antilisterial gene targets.

In contrast, the 11 date fruit isolates that identified as *B. altitudinis* were all found to be anti-*Listeria* inhibitory strains. This perhaps suggests that the anti-*Listeria* activity of this species is
chromosomally-encoded. Thus, it is plausible that many strains belonging to the species *altitudinis*, may naturally harbour anti-*Listeria* properties.

### 3.4.5 Screening of the inhibitory strains for safety and novelty

A search in the literature and Bergey’s manual for *Bacillus* (Logan and De Vos, 2015) for potential association with human clinical illness, was done to see if any of the species identified from the inhibitory strains were potentially pathogenic. From this search, it was determined that only the following were listed and/or suggested to be pathogenic *Bacillus* spp.: *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. licheniformis*; none of which correlated with the inhibitory strains identified (Logan and Vos, 2015). However, *B. amyloliquefaciens*, *B. pumilus*, *B. safensis* and *B. subtilis*, *Bacillus* species associated with the inhibitory strains, were found to be producers of human toxins (Domingos et al., 2015; From et al., 2007; Apetroaie-Constantin et al., 2009; Mikkola et al., 2007; Rasimus-Sahari et al., 2015).

*B. pumilus* and *B. safensis* were found to be associated with the production of the pumilacidin toxin, a roughly 1000 Da lipopeptide that contains a peptide moiety of 7 – 15 residues and a fatty acid tail of 8 – 10 hydrocarbons (Domingos et al., 2015; From et al., 2007; Saggsese et al., 2018). *B. pumilus* strains that produced this toxin were found to be the cause of a food poisoning outbreak with three cases occurring from the consumption of rice at a Chinese restaurant in Norway (From et al., 2007). Additionally, *B. pumilus* was found to be the likely cause of a case of septic arthritis in a healthy child from Canada – bacterial growth in synovial fluid was confirmed on more than one occasion using MALDI-TOF mass spectrometry and 16S rRNA sequencing (Shivamurthy...
et al., 2016). Thus, these two species, which harbour the potential to confer human pathogenicity, should warrant further investigation before considering them as potential biocontrol agents in food.

*B. amyloliquefaciens* and *B. subtilis* were found to be associated with the production of the amylosin toxin, a roughly 1200 Da non-ribosomally produced lipopeptide that contains a chromophore polyene structure (Apetroaie-Constantin et al., 2009; Mikkola et al., 2007; Rasimus-Sahari et al., 2015). *B. subtilis* strains producing this toxin were found to be the cause of food poisoning with five cases that occurred from the consumption of pumpkin curry in Finland and one case from Indian take-out in the UK (Apetroaie-Constantin et al., 2009). The toxin has been found to depolarize the plasma membrane and the mitochondria inside live mammalian cells, due to its ability to form cation-permeant ion channels with high selectivity for K+ ions in lipid membranes (Saris et al., 2009). This in turn was found to disrupt cellular ion homeostasis, mitochondrial function and energy metabolism (Mikkola et al., 2004; Mikkola et al., 2007). Thus, isolates which harbour this toxin would be undesirable to further pursue as biocontrol agents.

To assess the novelty of all the inhibitory strains, they were screened against BACTIBASE (http://bactibase.hammamilab.org/main.php#) – a database dedicated to the properties of over 230 bacteriocins produced by both Gram-positive and Gram-negative bacteria. Upon looking up *Bacillus* in this database, the search engine identified 23 bacteriocins produced by this genus from seven species (*B. cereus, B. circulans, B. coagulans, B. halodurans, B. licheniformis, B. subtilis* and *B. thuringiensis*), of which six were bacteriocins produced by *B. subtilis*. In addition, *B. tequilensis* was recently found to produce an anti-*Listeria* peptide, subtilosin A (Parveen Rani et al., 2016). Hence, it was concluded that the two inhibitory strains that belonged to these two species may lack novelty if they were further pursued.
With regards to *B. amyloliquefaciens, B. pumilus, B. safensis* and *B. subtilis*, Health Canada has approved the use of *B. amyloliquefaciens* and *B. subtilis* for a number of industrial uses, such as for fungicidal pest control (Health Canada, 2018) and bioremediation/biodegradation (Government of Canada, 2015). As well, these four species are generally regarded as safe organisms (GRAS) by the Federal Drug Administration (FDA) for specific applications such as enzyme production (Sanders et al., 2003). However, based on the studies mentioned previously, it is suggested that one needs to carefully assess the use of these *Bacillus* spp. in industrial applications due to their potential status as newly-emerging pathogens (Apetroaie-Constantin et al., 2009; From et al., 2007; Rasimus-Sahari et al., 2015). Taking into account the above recommendations, the list of candidate inhibitory strains was narrowed from 36 down to 17 strains from four different *Bacillus* groups (*B. altitudinis; B. flexus; B. velezensis/vallismortis; and B. halosaccharovorans*). Of these 17, it was decided to pursue the top inhibitory strains (i.e., the strains that produced the largest zone of inhibition against *L. monocytogenes*) from the four *Bacillus* groups. This left *B. altitudinis* DS11, *B. flexus* DI3, *B. velezensis/vallismortis* DS6C and *B. halosaccharovorans* DP18B. Of these four, *B. altitudinis* DS11 appeared to be the most promising inhibitory strain, due to its zone of inhibition being the largest (5.0 ± 0.0 mm).

### 3.4.6 Impact of the inhibitory strains on *L. monocytogenes* virulence expression

The results from virulence testing demonstrated that the compounds produced and/or metabolized by the inhibitory strains can significantly impact the activation of the PrfA master virulence regulator in *L. monocytogenes*, but not necessarily its transcription or translation. This is because a significant reduction in PrfA-RFP was only observed in the WT::pPL2 strain that requires complete activation of PrfA with GSH (in a reducing environment) in order to see RFP expression.
The same was not observed for the PrfA*::pPL2 strain that does not require this activation. If any change in RFP expression was observed in this PrfA*::pPL2 strain, it would be attributed to a change in the PrfA transcription or translation. Nonetheless, a possible mechanism for causing this significant reduction of PrfA activation in the WT::pPL2 strain could be a loss of the reducing environment (e.g., via degradation of GSH) caused by the culture supernatants from these inhibitory strains. Without this reducing environment present to mimic the redox potential of a mammalian cytosol, PrfA will not completely activate, thereby impacting the amount of RFP that could be induced for expression.

Taken together, these results suggested that all four of the inhibitory strains have the potential to negatively affect the virulence of *L. monocytogenes* by reducing PrfA activation. As well, a few of the culture supernatants (*B. flexus* DI3 and *B. halosaccharovorans* DP18B) also have the added potential of inhibiting the growth of *L. monocytogenes* in this reducing environment, as observed by the significant reduction in OD$_{600}$ after treatment with the supernatants. This demonstrates that the selected inhibitory strains can not only impair the growth of *L. monocytogenes* (as observed from the growth inhibition plate assay) but can also negatively impact the virulence of this pathogen.

### 3.4.7 Characterization of the anti-*Listeria* compound(s)

The preliminary characterization of the anti-*Listeria* compound(s) produced by *B. altitudinis* DS11, suggests that this anti-*Listeria* compound(s) is proteinaceous in nature, containing cleavage sites for specific proteolytic enzymes. These sites are most probably aliphatic in nature (i.e., composed of non-polar and hydrophobic amino acids), as a complete loss of activity was seen after
treatment with proteinase K – a protease that preferentially cleaves peptide bonds next to the carboxyl group of N-substituted hydrophobic, aliphatic, and aromatic amino acids. However, these sites most probably do not contain aromatic amino acids (such as F, W and Y), as full activity from this compound was present after treatment with pepsin – a protease that cleaves peptide bonds between aromatic amino acids. In addition, it is likely that this anti-Listeria compound(s) contains positively charged amino acids (such as K and R), but perhaps not near its active site as only partial activity was lost post-treatment with trypsin – a protease that cleaves peptide chains at the carboxyl side of positively charged amino acids.

These results also demonstrate how tolerant this anti-Listeria compound(s) is to denaturation or degradation, as its activity was retained post-treatment in acidic and alkaline environments, along with stability after temperature treatments up to 100°C. The heightened activity seen after exposure to alkaline pH values (e.g., pH value of 10 and 13), suggests that the antimicrobial activity of this compound(s) is more optimal under these conditions. Perhaps in this less protonated state, the compound(s) adopts a different conformation where its active site is more accessible to inhibit L. monocytogenes.

This active supernatant was also tested at different temperatures (4°C, 10°C, and RT) using the same growth inhibition plate assay. Anti-Listeria activity was observed post-incubation at 10°C (50% activity after 3 days) and RT (80% activity after day 1), but not at 4°C (even after 10 days). Perhaps at 4°C, different adaptive stress mechanisms (e.g., SOS response) may come into play, permitting L. monocytogenes to become resistance to this compound. As suggested by Cirz et al. (2005), the SOS pathway may be essential in the acquisition of bacterial mutations that confer antimicrobial resistance. However, the presence of anti-Listeria activity at 10°C suggests that this
antimicrobial compound could still be applied as a preventative measure to refrigerated foods, especially those foods that are stored in a temperature fluctuating/abused environment (e.g., busy restaurants and/or commercial kitchens).

In summary, our results suggest that the anti-
Listeria compound(s) isolated can maintain activity under a variety of different food products and storage conditions. One such example could be its use in pre-cut salads or fruits that are being served in hospitals or retirement homes. These types of produce items, which have been implicated previously in listeriosis outbreaks (CDC, 2015c; CDC, 2015d; PHAC, 2016), could be spray/mist inoculated with this anti-
Listeria compound(s) to reduce the probability of these high-risk populations from contracting listeriosis.
3.5 Conclusion

This study showed that the culturable microbiota of imported date fruits was predominantly populated by Gram-positive Firmicutes from the order Bacillales, where *Bacillus* spp. were found to represent 70 – 85% of the cultured isolates. This microbiota was additionally found to harbour bacteria that possess antagonistic properties against *L. monocytogenes*. Of the 191 isolates recovered from five different date fruits, 36 (19%) produced zones of inhibition against *L. monocytogenes* that ranged in size from 0.3 to 5.8 mm. The inhibitory strains were all identified as *Bacillus* spp. In addition, the inhibitory strains that had no association with human clinical illness (*B. flexus* DI3; *B. halosaccharovorans* DP18B; *B. velezensis/vallismortis* DS6C; and *B. altitudinis* DS11) were all found to significantly reduce the virulence of *L. monocytogenes* as measured by the inhibition of PrfA activity (p-value < 0.05). The anti-*Listeria* compound(s) produced by *B. altitudinis* DS11 were found to be a secreted compound that was proteinaceous in nature and were resistant to various treatments, including exposure to various acidic and alkaline pH values, as well as temperature treatments up to 100°C. Overall, this study showed the potential for an unconventional RTE food matrix to harbour beneficial *Bacillus* spp. producing anti-*Listeria* compound(s), either of which could be added to foods to inactivate and/or control the growth of *L. monocytogenes*. 
General conclusion

Overall, this thesis showed that the culturable microbiota from imported RTE foods, recovered using blood agar, contains a broad diversity of bacteria that possesses antagonistic properties against the foodborne pathogen *Listeria monocytogenes*. Among all the foods tested (dried apples, cumin seeds, date fruits, fennel seeds, pistachios, pollen, raisins and seaweed), the date fruit microbiota displayed the most promise for harbouring antagonistic properties against *L. monocytogenes*. This was based on the diversity of its microbiota and the ability of the associated microbiota to show inhibitory growth against this pathogen based on our in-house designed growth inhibition plate assay. As a result, date fruits were selected as a model imported RTE food to further investigate how its microbiota can control *L. monocytogenes*.

Date fruits were acquired from local retail stores from five different geographical regions that included China, Iran, Palestine, Saudi-Arabia and Tunisia. Of the 191 culturable bacterial isolates recovered from these dates, 36 (19%) were found to produce a zone of inhibition against *L. monocytogenes*. Sequencing of their 16S rRNA revealed that the anti-*Listeria* inhibitory strains all belonged to the genus *Bacillus*. In addition, the inhibitory strains that had no association with human clinical illness (*B. flexus* DI3; *B. halosaccharovorans* DP18B; *B. velezensis/vallismortis* DS6C; and *B. altitudinis* DS11) were all found to significantly reduce the virulence of *L. monocytogenes* as measured by the inhibition of this pathogen’s virulence regulator, PrfA (p-value < 0.05). In addition, the anti-*Listeria* compound(s) produced by *B. altitudinis* DS11 were found to be proteinaceous in nature, acid and alkali-tolerant and resistant to temperature treatments up to 100°C.
Taken together, these results show the potential of an unconventional RTE food matrix to harbour anti-

*Listeria* properties (i.e., beneficial *Bacillus* spp. producing anti-

*Listeria* compound(s)) that could be added to high-risk RTE foods to inactivate and/or control *L. monocytogenes*. One such example could be their use in pre-cut salads or fruits that are being served in hospitals or retirement homes. These types of produce items, which have been implicated previously in listeriosis outbreaks, could be spray/mist inoculated with a cocktail of these beneficial *Bacillus* spp. and/or their anti-

*Listeria* compound(s) to reduce the probability of high-risk populations from contracting listeriosis.

**Future Steps**

There are a number of future studies that can be done to expand and significantly increase the impact of the research done in the current thesis. The first experiments could be performed to further characterize the anti-

*Listeria* compound(s). The active supernatant could be isolated using high performance liquid chromatography and then characterized using mass spectrometry. Once identified in this manner, the suspected gene responsible for this antimicrobial could be confirmed by using a knockout mutant – if anti-

*Listeria* activity is lost, the suspected gene would be confirmed. This gene could then be cloned in an expression vector with an affinity tag (e.g., histidine tag) and then be induced for large scale production. The protein of interest could be purified using affinity, followed by size-exclusion chromatography. Once purified, the protein could be quantified and then serially-diluted to identify the minimum inhibitory concentration of the antilisterial compound.

In addition, a more detailed safety assessment of the beneficial *Bacillus* spp. and/or compound(s) could be conducted. This could be done through a series of toxicity/pathogenicity tests.
performed using a cell-culture and/or animal model. Certain immune markers (e.g., cytokines and chemokines) along with changes in phenotypes, could be tracked to assess safety.

Other future experiments could be done to test the efficacy of the beneficial *Bacillus* spp. and/or the anti-*Listeria* compound(s) directly in a food inoculated with *L. monocytogenes*. This could include produce and/or a low-moisture food, as they would most closely resemble the food matrix of the date fruits. In addition, a cocktail of the beneficial *Bacillus* spp., possibly in combination with other known antilisterial strains, could be used in order to obtain a community-based anti-*Listeria* effect. If successful, the cocktail and/or compounds could then be submitted to Health Canada for approval as a new food additive that could be used to control *L. monocytogenes* in the tested food. In addition, these candidate organisms and/or compound(s) could possibly be assessed for their effectiveness against other important foodborne pathogens.

Overall, further research in his area could lead to the discovery of either novel antimicrobial metabolites or beneficial bacteria that could be added to foods (or their respective packaging) to control foodborne pathogens in high-risk foods. These discoveries could then help reduce the prevalence of foodborne illness and thus improve the health of all Canadians.
References


111


## Appendix A – iLSM recipe

**Table A.** Incomplete *Listeria* synthetic media (iLSM) recipe (adapted by Whiteley *et al.*, 2017)

<table>
<thead>
<tr>
<th>Stock&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Stock Factor</th>
<th>Dilution</th>
<th>Ingredient</th>
<th>[Stock] in M or (µM)</th>
<th>[Final] in M or (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS (pH 7.5)</td>
<td>10</td>
<td>MOPS</td>
<td>MOPS</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>Glucose</td>
<td>Glucose</td>
<td>2.22</td>
<td>0.0555</td>
</tr>
<tr>
<td>Phosphate</td>
<td>100</td>
<td>KH₂PO₄</td>
<td>0.48</td>
<td>0.0048</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂HPO₄</td>
<td>1.15</td>
<td>0.0115</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>100</td>
<td>MgSO₄ * 7H₂O</td>
<td>0.17</td>
<td>0.0017</td>
<td></td>
</tr>
<tr>
<td>Micronutrients&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100</td>
<td>Biotin</td>
<td>(205)</td>
<td>(2.05)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Riboflavin</td>
<td>(133)</td>
<td>(1.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Para-Aminobenzoic Acid</td>
<td>(729)</td>
<td>(7.29)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lipoic Acid</td>
<td>(2.4)</td>
<td>(0.02)</td>
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<tr>
<td></td>
<td></td>
<td>Niacinamind/Cnicotinamide</td>
<td>(819)</td>
<td>(8.19)</td>
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<tr>
<td></td>
<td></td>
<td>D-pantothenic acid</td>
<td>(420)</td>
<td>(4.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyridoxal * HCl</td>
<td>(491)</td>
<td>(4.91)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thiamine * HCl</td>
<td>(296)</td>
<td>(2.96)</td>
<td></td>
</tr>
<tr>
<td>Minimum amino acids&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>L-Arginine * HCl</td>
<td>0.024</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-Histidine * HCl *H₂O</td>
<td>0.024</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DL-Isoleucine</td>
<td>0.038</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L-Leucine</td>
<td>0.038</td>
<td>0.0008</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>DL-Methionine</td>
<td>0.034</td>
<td>0.0007</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>L-Phenylalanine</td>
<td>0.030</td>
<td>0.0006</td>
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<tr>
<td></td>
<td></td>
<td>L-Tryptophan</td>
<td>0.024</td>
<td>0.0005</td>
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<tr>
<td></td>
<td></td>
<td>DL-Valine</td>
<td>0.043</td>
<td>0.0009</td>
<td></td>
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<tr>
<td>Adenine&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100</td>
<td>Adenine</td>
<td>(1850)</td>
<td>(18.5)</td>
<td></td>
</tr>
<tr>
<td>Trace metals</td>
<td>100</td>
<td>FeCl₂ * 4H₂O</td>
<td>(503)</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MnSO₄ * xH₂O</td>
<td>(5006)</td>
<td>(50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnSO₄ * 7H₂O</td>
<td>(97)</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCl₂</td>
<td>(1007)</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuSO₄ * 5H₂O</td>
<td>(8)</td>
<td>(0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CoCl₂ * 6H₂O</td>
<td>(8)</td>
<td>(0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₂BO₃</td>
<td>(9.7)</td>
<td>(0.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂MoO₄ * 2H₂O</td>
<td>(8.3)</td>
<td>(0.1)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>NaCl</td>
<td>0.856</td>
<td>0.00855</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sodium citrate</td>
<td>(10003)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>Added fresh&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10</td>
<td>L-Cysteine *HCl</td>
<td>0.8 g/L</td>
<td>0.08 g/L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>L-Glutamine</td>
<td>6 g/L</td>
<td>0.06 g/L</td>
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</tbody>
</table>

<sup>a</sup> iLSM is made by combining each of the stock solutions based on the appropriate dilution factor in the order that they are listed above. Filter-sterilize final medium and all stock solutions; store these stock solutions at 4 °C with the exception of MOPS, Glucose, and Phosphate, which are stored at room temperature. iLSM is stable at 1X for approximately 6 weeks.

<sup>b</sup> Dissolve all stock components in boiling water prior to filter sterilization.

<sup>c</sup> Dissolve these amino acids in hot 1N NaOH.

<sup>d</sup> Dissolve adenine in 0.2N HCl (8% of total volume), then dilute to final concentration in water.

<sup>e</sup> Add these ingredients fresh to iLSM; they cannot be prepared together as a stock.
Appendix B – Sample statistical analysis

The purpose of this appendix is to show a sample of the variance analysis, studentized residual plots, and coding used for one of the statistical computations run on the SAS system Proc Glimmix. This sample is of the computation run on the \textit{L. monocytogenes} WT::pPL2 fluorescence data (RFU/OD\textsubscript{600}) acquired post exposure to the supernatant treatment from select inhibitory strains (DI3, DP18B, DS6C, DS11).

\textbf{Table B1.} Mixed variance analysis of the fluorescence expression (RFU/OD\textsubscript{600}) from the \textit{L. monocytogenes} WT::pPL2 strain, post exposure to the supernatant treatments (txt) of select inhibitory strains (DI3, DP18B, DS6C, DS11)

<table>
<thead>
<tr>
<th>Random effects</th>
<th>Estimate</th>
<th>Standard error</th>
<th>Chi-square\textsuperscript{a}</th>
<th>Pr &gt; $\chi^2$</th>
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<tr>
<td>Blocks</td>
<td>2.1602 x 10\textsuperscript{9}</td>
<td>3.1784 x 10\textsuperscript{9}</td>
<td>1.32</td>
<td>0.1250</td>
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<tr>
<td>Residual</td>
<td>4.901 x 10\textsuperscript{9}</td>
<td>2.4505 x 10\textsuperscript{9}</td>
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</table>

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Numerator df</th>
<th>Denominator df</th>
<th>F Value\textsuperscript{a}</th>
<th>Pr &gt; F</th>
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<tr>
<td>Supernatant txt</td>
<td>4</td>
<td>8</td>
<td>\textbf{18.65}</td>
<td>0.0004</td>
</tr>
<tr>
<td>DI3 vs Control</td>
<td>1</td>
<td>8</td>
<td>\textbf{41.39}</td>
<td>0.0002</td>
</tr>
<tr>
<td>DP18B vs Control</td>
<td>1</td>
<td>8</td>
<td>\textbf{33.91}</td>
<td>0.0004</td>
</tr>
<tr>
<td>DS6C vs Control</td>
<td>1</td>
<td>8</td>
<td>\textbf{43.65}</td>
<td>0.0002</td>
</tr>
<tr>
<td>DS11 vs Control</td>
<td>1</td>
<td>8</td>
<td>\textbf{59.87}</td>
<td>&lt;0.0001</td>
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</table>

\textsuperscript{a}Significant effects (P<0.05) are indicated in bold.
Figure B1. Studentized residual plots of the fluorescence expression (RFU/OD$_{600}$) from the *L. monocytogenes* WT::pPL2 strain, post exposure to the supernatant treatments (txt) of select inhibitory strains (DI3, DP18B, DS6C, DS11).
SAMPLE CODING

title1 'WT::pPL2 data';
data first;
infile datalines delimiter='09'x;
id+1;
input block$ txt$ RFU OD600;
if txt='Control' then control='Control';
cards;
1       Control   544384.671  0.922
1        DI(3)   90451.01781  0.524
1       DP(18B)  128575.1458  0.743
1       DS(6C)  87650.31153  0.856
1       DS(11)  84606.99153  0.944
2       Control   492152.3349  1.035
2        DI(3)  109961.0877  0.711
2       DP(18B)  142402.027  0.888
2       DS(6C)  135324.5914  0.877
2       DS(11)  193108.119  0.78
3       Control   572468.7088  1.012
3        DI(3)  305357.1877  0.531
3       DP(18B)  339504.4522  0.861
3       DS(6C)  59225.37112  0.988
3       DS(11)  198280.6584  0.81
;
run;

title2 'RFU';
proc glimmix data=first;
class block txt;
model RFU=txt /ddfm=kr;
random block;
covtest 'block var=0' 0 ./restart;
contrast 'DI3 vs Control' txt -1 1 0 0 0;
contrast 'DP18B vs Control' txt -1 0 1 0 0;
contrast 'DS6C vs Control' txt -1 0 0 1 0;
contrast 'DS11 vs Control' txt -1 0 0 0 1;
lsmeans txt / adjust=dunnett pdiff=control;
output out=second predicted=pred
student=sresid;
run;

data second; set second; run;

title3 'residual plots for RFU';
proc sgsscatter;
plot sresid*(pred txt);
proc sgplot;
vbox sresid/ group=pred datalabel;
proc sgplot;
vbox sresid/ group=txt datalabel;
run;

title3 'sorting outliers for RFU';
proc sort;
by sresid;
proc print;

title3 'test for normal distribution for RFU';
proc univariate normal;
var sresid;
histogram sresid/normal kernel;
run;
Appendix C – Conference abstracts

C1 Ontario Food Protection Association (OFPA) Traceability Symposium

Date: April 2018 (Mississauga, ON, Canada)
Presentation type: Poster

Title: Characterizing the microbial ecology and functional diversity of bacterial communities from imported, ready-to-eat (RTE) foods

Authors: Krishna S. Gelda, Valeria R. Parreira, Jeffrey M. Farber
Affiliation: University of Guelph, CRIFS, Department of Food Science

Illnesses caused by foodborne pathogens still remain a major public health and economic concern in Canada. One approach to help control these pathogens is to better understand the interaction(s) between the microbiome of foods and the foodborne pathogens. Member(s) of the food microbiome may help inactivate pathogens and/or prevent their growth based on a number of factors, such as competitive exclusion. Thus, this research proposes to investigate the microbiome of imported, ready-to-eat (RTE) foods in the Canadian market that have not been previously studied. It is hypothesized that these foods will contain unique bacteria that show anti-bacterial properties against foodborne pathogens such as Listeria monocytogenes.

To address this research question, two complementary cultural approaches will be used. First, a culture-independent approach will be used to determine the complete microbiome of the foods. Bacterial DNA, extracted from the food’s surface, will be used to generate a library of all the bacterial signatures using 16S rRNA gene sequencing. This catalogue will then be reported as a percent-relative abundance to provide an overview of the bacterial communities found on these food types. Second, a culture-dependent approach will be used to assess whether the food’s culturable bacteria or their respective metabolites can affect the growth or virulence of L. monocytogenes. Methods to assess growth include a growth inhibition plate assay and a growth curve assessment by optical density. Methods to assess the impact upon virulence factors include fluorescence spectroscopy and qPCR by using strains of L. monocytogenes that have a fluorescent reporter linked to a virulence gene. Once determined for L. monocytogenes, these candidate organisms and respective metabolites could then be assessed for their effectiveness against other important foodborne pathogens such as Salmonella and pathogenic E. coli.

The results from this research could lead to the discovery of either novel antimicrobials or beneficial bacteria that could be added to foods to inactivate and/or control foodborne pathogen growth. These discoveries could then help reduce foodborne illness in Canada and thus improve the health of all Canadians.
Illnesses caused by foodborne pathogens still remain a major public health and economic concern in Canada. One approach to help control these pathogens is to better understand the interaction(s) between the microbiome of foods and the foodborne pathogens. Member(s) of the food microbiome can help inactivate pathogens and/or prevent their growth based on a number of factors, such as competitive exclusion. Thus, this project investigates the microbiome of imported, ready-to-eat (RTE) date fruits in the Canadian market. Date fruits were selected over other imported RTE foods we tested (such as pollen, seaweed, seeds, and pistachios) based on the microbiome diversity of the dates and the ability of the associated microbiota to show inhibitory growth against *Listeria monocytogenes*. Thus, based on our preliminary findings, it is hypothesized that date fruits contain unique bacteria that show anti-bacterial properties against the foodborne pathogen *Listeria monocytogenes*.

Dates from five different geographical regions were obtained. These regions include China, Iran, Palestine, Saudi-Arabia, and Tunisia. Bacterial strains were isolated from dates using TSA agar enriched with 5% of defibrinated sheep blood that permits both the distinction between morphological phenotypes and the growth of fastidious microorganisms. A rich diversity of bacteria was obtained from the dates, with a total of 191 isolates belonging to 91 different phenotypes being observed. The isolates were then screened using a growth inhibition plate assay against *Listeria monocytogenes*. In total, 35 isolates were found to produce a zone of inhibition against this pathogen, with zone sizes ranging from 0.5 to 5.7 mm. These isolates have all been initially identified as *Bacillus* spp. using 16S rRNA sequencing.

Characterization of the inhibition compounds will include, among other things, determining the mode of action, and the impact on the virulence of *Listeria monocytogenes*. Promising isolates will also be characterized with more scrutiny to the species/strain level. The results from this research could lead to the discovery of either novel antimicrobial metabolites or beneficial bacteria that could be added to foods to inactivate and/or control the growth of *Listeria monocytogenes*. These novel compounds will also be assessed for their potential activity against other foodborne pathogens and could eventually lead to novel probiotics and/or bio-compounds that can help reduce foodborne illness in Canada.
Illnesses caused by foodborne pathogens still remain a major public health and economic concern in Canada. One approach to help control these pathogens is to better understand the interaction(s) between the microbiome of foods and the foodborne pathogens. Member(s) of the food microbiome can help inactivate pathogens and/or prevent their growth based on a number of factors, such as competitive exclusion. Thus, this project investigates the microbiome of imported, ready-to-eat (RTE) date fruits in the Canadian market. Date fruits were selected over other imported RTE foods we tested (such as pollen, seaweed, seeds, and pistachios) based on the microbiome diversity of the dates and the ability of the associated microbiota to show inhibitory growth against *L. monocytogenes*. Thus, based on our preliminary findings, it is hypothesized that date fruits contain unique bacteria that show anti-bacterial properties against the foodborne pathogen *Listeria monocytogenes*.

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Characterization of the inhibition compounds will include, among other things, determining the mode of action, and the impact on the virulence of *L. monocytogenes*. Promising isolates will also be characterized with more scrutiny to the species/strain level. The results from this research could lead to the discovery of either novel antimicrobial metabolites or beneficial bacteria that could be added to foods to inactivate and/or control the growth of *L. monocytogenes*. This would then help to reduce the burden of foodborne illness in Canada.
C4  International Association for Food Protection (IAFP)’s European Symposium

Date: April 2019 (Nantes, France)
Presentation type: Oral (15 min)

Title: Characterizing the Culturable Bacterial Communities from Imported Date Fruits to Control *Listeria monocytogenes*

Authors: Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber
Affiliation: University of Guelph, CRIFS, Department of Food Science

Introduction: Illnesses caused by foodborne pathogens continues to remain a major health and economic concern worldwide. *Listeria monocytogenes* is one of the most important of these pathogens due to its case-fatality rate being the highest among any foodborne pathogen.

Purpose: This project investigates culturable microbiome of imported, ready-to-eat (RTE) date fruits and how well bacteria isolated from date fruits could control/inhibit the growth of *L. monocytogenes*. Date fruits were selected over other imported RTE foods tested (such as pollen, seaweed, seeds, and pistachios) based on the diversity of their microbiome and the ability of the associated microbiota to show inhibitory growth against *L. monocytogenes*.

Methods: Dates from five geographical regions were obtained that included China, Iran, Palestine, Saudi-Arabia, and Tunisia. Bacterial strains were isolated from these dates using TSA agar enriched with 5% of defibrinated sheep blood. The isolates were then screened using a growth inhibition plate assay against *L. monocytogenes* and the inhibitor strains detected were identified using 16S rRNA sequencing.

Results: A large diversity of bacteria was isolated from the dates, a total of 191 isolates belonging to 91 different phenotypes being observed. From the inhibition plate assay, 35 isolates were found to produce a zone of inhibition around *L. monocytogenes*, with zone sizes ranging from 0.5 to 5.7 mm. These inhibitor strains were identified as *Bacillus* spp. Further screening narrowed down the list of inhibitor strains to 3 – 5 to be pursued for their antimicrobial activity and whole genome sequencing.

Significance: The results from this research could lead to the discovery of either novel antimicrobial metabolites or beneficial bacteria that could be added to foods to inactivate and/or control the growth of *L. monocytogenes*. This research was conducted at CRIFS (Canadian Research Institute for Food Safety) and has been funded by CFREF (Canada First Research Excellence Fund).
Title: Characterizing the Culturable Bacterial Communities from Imported Date Fruits to Control *Listeria monocytogenes*

Authors: Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber
Affiliation: University of Guelph, CRIFS, Department of Food Science

Introduction: *Listeria monocytogenes*, a resilient and ubiquitous foodborne pathogen, remains a significant threat to public health as invasive infection from it can result in high mortality rates.

Purpose: To control/inhibit the growth and virulence of *L. monocytogenes* using culturable members of the ready-to-eat (RTE) date fruit microbiome.

Methods: Bacterial strains were isolated from imported date fruits using blood agar and then screened using a growth inhibition plate assay against *L. monocytogenes*. The inhibitor strains detected were then identified using 16S rRNA sequencing. The most promising inhibitory strains which have no association with human clinical illness were then assessed for their impact on *L. monocytogenes* virulence. This was done using a fluorescence spectroscopy model linked to this pathogen’s virulence regulator, PrfA.

Results: A large diversity of bacteria was isolated from the dates, with a total of 191 isolates belonging to 91 different phenotypes being observed. From the inhibition plate assay, 35 isolates were found to produce a zone of inhibition around *L. monocytogenes*, with zone sizes ranging from 0.5 to 5.7 mm. Sequencing revealed these inhibitor strains to all identify as *Bacillus* spp. Among those *Bacillus* spp. that were tested for their ability to inhibit PrfA, it was found that they all caused a significant reduction in PrfA expression of *L. monocytogenes* (p < 0.05).

Significance: The results from this research could lead to the discovery of either novel antimicrobial metabolites or beneficial *Bacillus* spp. that could be added to foods to inactivate and/or control *L. monocytogenes*. 
C6  Agri-Food Excellence Symposium

Date: June 2019 (Guelph, ON, Canada)
Presentation type: Poster (infographic)

Title: *Listeria monocytogenes* – a unique foodborne pathogen

Authors: Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber
Affiliation: University of Guelph, CRIFS, Department of Food Science

Our project involves isolating bacteria from imported, ready-to-eat (RTE) date fruits in order to find bacterial species that control/inhibit the growth of *Listeria monocytogenes*, a foodborne pathogen with one of the highest case-fatality rates. Date fruits were selected over other imported RTE foods tested (including pollen, seaweed, seeds, and pistachios) based on the diversity of their bacterial profile and the ability of the associated microbiota to show inhibitory growth against *L. monocytogenes*. Thus, we hypothesized that the bacterial profile of date fruits would contain bacterial species that possess antagonistic properties against the foodborne pathogen *L. monocytogenes*.

This research project is in line with Food from Thought’s micro-scale mission to promote food safety by reducing food safety risks and enhancing the health of Canadians. Specifically, the results from this project may lead to the discovery of either novel antimicrobial metabolites or beneficial bacteria that could be added to foods to inactivate and/or control *L. monocytogenes*. These novel compounds/bacteria can also be assessed for their potential activity against other foodborne pathogens. This could then lead to the production of novel probiotics and/or bio-compounds that can reduce overall foodborne illness in Canada.
C7  International Association for Food Protection (IAFP)’s Annual Meeting

Date: July 2019 (Louisville, Kentucky, USA)  
Presentation type: Poster

Title: The use of *Bacillus* spp. isolated from Ready-to-Eat (RTE) Date Fruits to control *Listeria monocytogenes*

Authors: Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber  
Affiliation: University of Guelph, CRIFS, Department of Food Science

Introduction: *Listeria monocytogenes*, an important foodborne pathogen, remains a significant threat to public health as the invasive form of infection can result in high case-fatality rates.

Purpose: To control/inhibit the growth of *L. monocytogenes* using culturable members of the ready-to-eat (RTE) date fruit microbiome.

Methods: RTE date fruits were acquired from five geographic regions: China, Iran, Palestine, Saudi-Arabia, and Tunisia. Bacterial isolates were collected by washing the surface off these dates with peptone water, then plating the wash on blood agar. Isolated strains were then individually assessed to monitor whether they can prevent growth of *L. monocytogenes* using an agar plate inhibition test (N=3). Following, bacterial strains that secreted antimicrobials were then identified using 16S rRNA Sanger sequencing.

Results: A total of 191 isolates belonging to 91 different phenotypes were observed. From this collection, 35 isolates belonging to 21 phenotypes produced a zone of inhibition against *L. monocytogenes*: Zone sizes ranged from 0.5 to 5.7 mm on the agar plate. Sequencing revealed that the inhibitory strains all belonged to the genus *Bacillus* belonging to different species. Further work was done to identify those *Bacillus* spp. which had no link to clinical illness. Those *Bacillus* spp. that were found to be safe and that produced the largest inhibition zones, are being further characterized by doing whole genome sequencing and probing the genome for potential inhibitors.

Significance: The results from this research could lead to the discovery of either novel antimicrobial metabolites or beneficial *Bacillus* spp. that could be added to foods to inactivate and/or control *L. monocytogenes*. These novel compounds can also be assessed for their potential activity against other foodborne pathogens and could eventually lead to novel probiotics and/or bio-compounds that can help to reduce foodborne illness.
Appendix D – Scientific posters

POSTER 1 – presented at:
- Food from Thought (FFT) Integration Symposium (Jan 2018)
- Food from Thought (FFT) Annual Meeting (April 2018)

POSTER 2 – presented at:
- Ontario Food Protection Association (OFPA) Traceability Symposium (April 2018)

POSTER 3 – presented at:
- Guelph Food Safety Seminars (GFSS) Symposium (Oct 2018)
- One Health Poster Day (Nov 2018)
- Plant Sciences Symposium: Agriculture with Less (Nov 2018)

POSTER 4 – presented at:
- Agri-Food Excellence Symposium (June 2019)

POSTER 5 – presented at:
- International Association for Food Protection (IAFP)’s Annual Symposium (July 2019)
Characterizing the microbial ecology and functional diversity of bacterial communities from imported, ready-to-eat (RTE) foods

Krishna S. Gelda, Valeria R. Parreira, Jeffrey M. Farber

WHAT IS THE RESEARCH ABOUT

Research Goal: Imported, RTE foods in the Canadian market will contain unique bacteria that have antibacterial properties against foodborne pathogens, like Listeria monocytogenes.

WHY?

Natural microbiome of RTE foods may be able to inactivate pathogens and/or inhibit their growth

WANTED?

Why imported?

- Probability of finding unique bacteria
- Why RTE?
  - Not further processed prior to consumption
- Why L. monocytogenes?
  - Case-fatality rate of ~20%
  - Highest of any foodborne pathogen

WHAT ARE THE RESEARCHERS DOING

Culture-dependent approach: relies on growing the microorganisms to study them

Purify isolates

1. Identify the bacteria
2. Test for growth
3. Test for the presence of virulence factor

Culture-independent approach: relies on molecular methods to study microorganisms

DNA extraction

1. Amplify the bacterial signatures
2. Deep sequencing

WHAT MAY THE RESEARCHERS FIND

A) Inhibit the growth: two-tiered test

Test 1: Co-plates inhibition screen
To assess whether a particular bacterial isolate prevents growth in a zone around L. monocytogenes.

Test 2: Growth curve test
To assess whether the metabolites from the bacteria affect the growth rate of L. monocytogenes.

B) Impact the virulence: presence/absence of fluorescence

To assess whether the metabolites from the bacteria affect the virulence of L. monocytogenes

- By using a fusion strain, where a fluorescent marker (e.g., RFP) is linked to a virulence regulator (e.g., prfA)

Bacterial isolates that inhibit growth or reduce virulence of L. monocytogenes would be further studied to determine their mode(s) of antibacterial action

WHAT DO WE NEED TO KNOW

Short Term Effects

- Potential discovery of a novel:
  - Compound (antimicrobial)
  - Beneficial bacteria
- Effective against L. monocytogenes
- Application of test methods to other foodborne pathogens
  - Salmonella
  - Pathogenic E. coli

Long Term Effects

- Addition of compounds/bacteria to foods in Canadian processing plants to control pathogen growth
- Reduce prevalence of foodborne illness
- Improve overall food safety for Canadians

To know more: Krishna Gelda, (647) 669-7795. kgeda@uoguelph.ca

*Department of Food Science, Canadian Research Institute in Food Safety (CRIFS)
University of Guelph, Guelph, Ontario, Canada, N1G 2W1

This project is a part of the Food From Thought research program at the University of Guelph. This research is undertaken in part thanks to generous funding from the Canada First Research Excellence Fund.
Characterizing the microbial ecology and functional diversity of bacterial communities from unique imported, ready-to-eat (RTE) foods

Krishna S. Gelda, Valeria R. Parreira, Jeffrey M. Farber
University of Guelph, Department of Food Science, Canadian Research Institute for Food Safety (CRIFS)

**BACKGROUND**

Hypothesis: Imported, unique RTE foods (not previously studied) in the Canadian market will contain novel bacteria that show anti-bacterial properties against foodborne pathogens such as *Listeria monocytogenes*.

**APPROACH**

**A) Inhibit the growth: plate assay & growth curve**

- To assess whether the bacteria or their respective metabolites could:
  - A) Inhibit the growth of *Listeria monocytogenes*

**B) Impact the virulence: fluorescence spectroscopy**

- To assess whether the metabolites from the bacteria affect the virulence of *L. monocytogenes*
  - By using a fusion strain, where a fluorescent marker (e.g. RFP) is linked to a virulence regulator (e.g. prfA)

**IMPACT**

**Short Term Effects**

- Potential discovery of a novel compound(s) (antimicrobial) and/or beneficial bacteria effective against *L. monocytogenes*
- Potential application of these compounds/beneficial bacteria against other foodborne pathogens

**Long Term Effects**

- Addition of compounds/bacteria to RTE foods to inactivate/control pathogen growth
- Reduce prevalence of foodborne illness
- Improve overall health for all Canadians

**Possible Solution**

Understand the microbiome of RTE foods in the market

**WHY?**

Natural microbiome of RTE foods may be able to inactivate pathogens and/or inhibit their growth

- Why imported? 🔄 Probability of finding unique bacteria
- Why RTE Foods? 🔄 They are not further processed prior to consumption
- Why *L. monocytogenes*? 🔄 Case-fatality rate of ~20-40%
  - Highest of any foodborne pathogen

**Deep sequencing**

Culture-independent approach: relies on molecular methods to study the microorganisms

**Culture-dependent approach:** relies on growing the microorganisms to study them

**To know more:** Krishna Gelda, (647) 669-7795, kgelda@uoguelph.ca

This research is undertaken in part thanks to generous funding from the Canada First Research Excellence Fund (CFREF)
Characterizing the ecology and diversity of bacterial communities from imported Date Fruits to control *Listeria monocytogenes*

Krisha S. Gelda, Valeria R. Parreira, Jeffrey M. Farber

University of Guelph, Department of Food Science, Canadian Research Institute for Food Safety (CRIFS)

**BACKGROUND**

Illnesses by foodborne pathogens... still remain a major public health & economic concern in Canada

1 IN 8 PEOPLE

OVER 11,500 HOSPITALIZATIONS

AND 240 DEATHS OCCUR EACH YEAR DUE TO FOOD-RELATED ILLNESSES

(Government of Canada, 2016)

**WHY?**

Possible solution... Understand the microbiome of foods in our market

Natural microbiome of our foods may be able to inactivate pathogens and/or inhibit their growth

**Why Date Fruits?**

- They are classified as a ready-to-eat (RTE) food
  - This can represent a high-risk product class for consumers
- They possess a rich diversity of microorganisms
  - Their associated microbiota has shown inhibitory effects against *L. monocytogenes*

**Why *L. monocytogenes***

- One of the most severe foodborne pathogens
- It displays a case-fatality rate of ~20–30%
  - The highest of any foodborne pathogen

**Hypothesis:** imported date fruits will contain unique bacteria that show bactericidal/bacteriostatic properties against the foodborne pathogen *Listeria monocytogenes.*

**APPROACH**

Date fruits→ collected from five different geographical regions
- China, Iran, Palestine, Saudi-Arabia, and Tunisia

Blood agar plates→ to screen the bacterial community from the Dates

**To assess whether these bacteria or their respective metabolites could:**

A) Impact the growth
B) Impact the virulence of *L. monocytogenes*

**RESULTS**

<table>
<thead>
<tr>
<th>Date origin</th>
<th>CFU/g</th>
<th>Isolates*</th>
<th>Inhibitors**</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>3.6 x 10⁴</td>
<td>65 (27)</td>
<td>7 (3)</td>
</tr>
<tr>
<td>Iran</td>
<td>2.0 x 10⁴</td>
<td>13 (9)</td>
<td>1</td>
</tr>
<tr>
<td>Palestine</td>
<td>9.7 x 10⁴</td>
<td>52 (24)</td>
<td>16 (10)</td>
</tr>
<tr>
<td>Saudi-Arabia</td>
<td>2.7 x 10⁴</td>
<td>33 (15)</td>
<td>10 (6)</td>
</tr>
<tr>
<td>Tunisia</td>
<td>2.5 x 10⁴</td>
<td>28 (16)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>3 – 5 logs</td>
<td>191 (91)</td>
<td>35 (21)</td>
</tr>
</tbody>
</table>

* number of bacterial isolates collected
** number of isolates that inhibit the growth of *L. monocytogenes*

**Figure 1.** Fluorescence (x/em: 550/580 nm) from strains of *L. monocytogenes* expressing RFP under the control of the Pm promoter (N=3, ± STD). Grown in *incomplete Listeria synthetic media* (ILSM) stimulated with glutathione (GSH).

**Figure 2.** Summary of inhibition zones produced by the date bacterial isolates against *L. monocytogenes* (N=3, ± STD).

**Table 1.** Summary of the bacterial community screened from the date fruits, observed phenotypes in brackets.

**Figure 3.** Fluorescence (x/em: 550/580 nm) from strains of *L. monocytogenes* expressing RFP under the control of the Pm promoter (N=3, ± STD). Grown in *incomplete Listeria synthetic media* (ILSM) stimulated with glutathione (GSH).

**Characterizing the ecology and diversity of bacterial communities from imported Date Fruits to control *Listeria monocytogenes***

**Contact:** Krishna Gelda, kgelda@uoguelph.ca

This research is undertaken in part thanks to generous funding from the Canada First Research Excellence Fund (CFREF).
Listeria monocytogenes – a unique foodborne pathogen

Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber
University of Guelph, Department of Food Science, Canadian Research Institute for Food Safety (CRIFS)

**FAST FACTS**

- Hardy & adaptive
  - pH (4.3 – 9.1)
  - Refrigeration
  - Salt (10% wt/v)

- Target population
  - O – old
  - P – pregnant
  - I – immunocompromised

- Low incidence (0.001%)
  - 1 – 10 cases per million per year

- High case-fatality rate (20–30%)
  - Highest of any foodborne pathogen

- Ready-to-eat foods
  High-risk foods include:
  - Deli-meats
  - Soft cheeses
  - Fresh-cut produce
  - Seafood (salmon)

**PATHOGENESIS – LISTERILOSIS**

**RESEARCH OVERVIEW**

Investigate the bacterial community of imported, RTE foods
- May support the growth of novel, beneficial (anti-Listeria) bacteria

- Apples (dried)
- Cumin seeds
- Date fruits
- Fennel seeds
- Pistachios
- Pollen
- Raisins
- Seaweed

Argentina
India
China, Tunisia, U.A.E
India
U.S.A
Poland
South Africa
China, Japan

**Approach**
To assess whether these bacteria/metabolites could impact growth &/or virulence of L. monocytogenes

**Application**
To add these bacteria/compounds to foods to inactivate/control L. monocytogenes
The use of *Bacillus* spp. isolated from ready-to-eat (RTE) date fruits to control *Listeria monocytogenes*

Krishna S. Gelda, Valeria R. Parreira, Gisèle LaPointe, Jeffrey M. Farber

University of Guelph, Department of Food Science, Canadian Research Institute for Food Safety (CRIFS)

**Background**
- 1 in 10 people (globally) get sick each year from contaminated food
- ~600 million cases/year
- ~420,000 estimated deaths (WHO, 2017)

**Approach**
- Date fruits → collected from five different geographical regions: China, Iran, Palestine, Saudi Arabia, and Tunisia
- Blood agar → to screen the bacterial community from the dates

**Hypothesis:**
- Imported date fruits will contain unique bacteria that show antagonistic properties against the foodborne pathogen *Listeria monocytogenes*
- This natural community may:
  - Prevent pathogen growth
  - Harbour beneficial bacteria

**Results for Impact on Growth**
- Table 1. Summary of the bacterial community screened from the date fruits (368 isolates in total)

<table>
<thead>
<tr>
<th>Date origin</th>
<th>CFU/g</th>
<th>Isolates*</th>
<th>Inhibitory strains*</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>3.6 x 10^9</td>
<td>65 (27)</td>
<td>7 (2)</td>
</tr>
<tr>
<td>Iran</td>
<td>2.0 x 10^9</td>
<td>13 (9)</td>
<td>1</td>
</tr>
<tr>
<td>Palestine</td>
<td>9.7 x 10^8</td>
<td>52 (94)</td>
<td>17 (16)</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>2.7 x 10^8</td>
<td>33 (15)</td>
<td>10 (8)</td>
</tr>
<tr>
<td>Tunisia</td>
<td>2.5 x 10^8</td>
<td>28 (16)</td>
<td>7 (5)</td>
</tr>
<tr>
<td>Total</td>
<td>10^9 - 10^10</td>
<td>181 (81)</td>
<td>54 (37)</td>
</tr>
</tbody>
</table>

* Number of bacterial isolates collected
* Number of isolates that showed growth of *L. monocytogenes*

**Results for Impact on Virulence**
- Figure 1. Summary of inhibition zones produced by the date bacterial isolates against *L. monocytogenes* (N=3, ± STD)

**Impact**
- Short-term effects:
  - Potential discovery of novel compounds and/or beneficial *Bacillus* spp. effective against *L. monocytogenes*
  - Potential application of these compounds beneficial *Bacillus* spp. against other foodborne pathogens

- Long-term effects:
  - Addition of compounds *Bacillus* spp. to foods to inactivate/control pathogen growth
  - Reduce prevalence of foodborne illness
  - Improve overall health for all consumers

**Contact:** Krishna Gelda, kgelda@uoguelph.ca; Dr. Jeff Farber, jfarber@uoguelph.ca

This research is undertaken in part thanks to funding from the Canada First Research Excellence Fund (CRIF).
Appendix E – Data management plan

Completed according to the guidelines from the Portage Network (https://portagenetwork.ca/).

Data collection

What types of data will you collect, create, link to, acquire and/or record?

- Agarose gels, bacterial isolates, photographs, and quantitative data.
- Quantitative data is from manual measurements (i.e., plate counts and inhibition zone sizes) and from spectrophotometers (i.e., optical density and fluorescent measurements).

What file formats will your data be collected in? Will these formats allow for data re-use, sharing and long-term access to the data?

- Agarose gels - collected in .scn (Image Lab) file format and .tif file format.
- Photographs - collected in .tif file format.
- Bacterial isolates - stored in freezing media at -80°C.
- Quantitative data - stored in .xlsx spreadsheet file format or .pptx powerpoint format.

What conventions and procedures will you use to structure, name and version-control your files to help you and others better understand how your data are organized?

- The organization was conducted as per the following SOP developed for our research group: DataManagement.docx (Farber LAB < Protocols). This organization is summarized below. All data collected for this thesis is stored under the Krishna folder (Farber LAB < Students) in our network Q drive.

- This folder contains subfolders for CFREF (files related to the reports made for this grant), GANTTchart (all iterations of this planning tool used by the student), IsolateLibrary (information related to the bacterial isolates collected for the project), Presentations (all powerpoint presentations used in meetings), Posters (all posters made for conferences), RawFiles (contains all raw data/MASTER files), Results (contains all compiled and up-to-date results according to the different aspects of the project), and Thesis (contains all files associated to the writing of the thesis, including drafts, chapters, figures, tables, and statistical analysis).

- Files were named by typically assigning an acronym based on the different components of the project (i.e., DropPlates, InhibitorProperties, PreliminaryResearch, SangerSequencing, ToleranceTests, and Virulence). Filenames were kept consistent, brief and specific (typically <25 characters) and were documented in the README.txt file.
Files were version controlled as follows:

- For **all files**, a separate folder was kept with the old versions of the file. For the newest version, the file was resaved corresponding to its version number.

  - EX: LmGC_Victor_v2 (corresponds to the 2\textsuperscript{nd} version of this file)

- For **Excel files**, a separate spreadsheet was kept documenting the changes made to each version of the file.

- For **Word files**, an appendix was included to document the changes made to each version of the file.

- For **PowerPoint files**, the second slide was set up to document the changes made to each version of the file.

### Documentation and metadata

**What documentation is needed for data to be read and interpreted correctly in the future?**

- **README.txt** file --> created as an annotated table of contents to document all of the work. An iteration of this file can be found in every major subfolder.

  - This file documents the following:
    - Describes the project and folder structure
    - Defines acronyms
    - Defines what files will or are found in each folder
    - Includes any additional, relevant information (units of measure, collection process, etc)

All data collected is also documented as follows directly in the file:

- Page number (in lab notebook) it corresponds to

- Date the experiment was conducted

- Purpose of the experiment

- Overview of the methodology (media, timepoints, experimental unit, sample size, etc)

- Additional notes about the data (e.g., anomalies in the data)

- Definitions to any acronyms present in the dataset
For EXCEL (.xlsx) quantitative data, this information is found in a separate tab. For POWERPOINT (.pptx) data, this information is found in the notes section of the slide.

**How will you make sure that documentation is created or captured consistently throughout your project?**

- First by following the data management SOP written for our lab group.
- Second to frequently and consistently update the README.txt annotated table of contents found in each major subfolder.

**If you are using a metadata standard and/or tools to document and describe your data, please list here.**

Data is documented and described as per the SOP written for our research group: DataManagement.docx (Farber LAB < Protocols).

This SOP outlines documentation in a README.txt file for every major subfolder. This documentation includes the following:

- Describe your project and folder structure
- Define your acronyms
- Define what files will or are found in each folder
- Include any additional, relevant information (e.g., units of measure, collection process, etc)

This SOP outlines for data collected, the following information is documented along with it:

- Page number (in lab notebook) it corresponds to
- Date the experiment was conducted
- Purpose of the experiment
- Overview of the methodology (e.g., media, timepoints, experimental unit, sample size, etc)
- Additional notes about the data (e.g., anomalies in the data)
- Definitions to any acronyms present in the dataset
Storage and backup

What are the anticipated storage requirements for your project, in terms of storage space (in megabytes, gigabytes, terabytes, etc.) And the length of time you will be storing it?

- Storage space - 15 Gb
- Storage time - up to the advisors, but I imagine the data can be stored on our network drive indefinitely.

How and where will your data be stored and backed up during your research project?

- Data is stored in hardcopy lab notebooks and on our network drive (Qdrive < Farber LAB < Students < Krishna).
- Data is additionally backed up through the University of Guelph’s iCloud service and through Time Machine on an external hard drive. iCloud backups occur retroactively, and Time Machine backups occur manually 3 - 4 times per week.

How will the research team and other collaborators access, modify, and contribute data throughout the project?

- Data can be accessed directly through either the physical lab notebooks (annotated with a list of figures and tables) or through the network drive (Qdrive < Farber LAB < Students < Krishna).
- On the network drive, data files are saved as both a master file (MF) and a working file (WF). The MF contains the raw, unprocessed data while the WF file contains the processed data. Either file can be accessed and modified.

Preservation

Where will you deposit your data for long-term preservation and access at the end of your research project?

- Data is deposited and preserved on our network drive (Qdrive: Farber LAB < Students < Krishna).
- Data is additionally saved in more accessible friendly formats (.docx; .xlsx; .pptx; .tif; .pdf).

Indicate how you will ensure your data is preservation ready. Consider preservation-friendly file formats, ensuring file integrity, anonymization and de-identification, inclusion of supporting documentation.

- At the very least, all README files are in .txt file format. As well, final copies of the thesis and manuscript are saved in .pdf file formats.
- The remaining data is stored as .tiff, .docx, .pptx, and .xlsx file formats.
Sharing and reuse

What data will you be sharing and in what form? (e.g. Raw, processed, analyzed, final).

- All forms of data are available for sharing under our network Qdrive: Farber LAB < Students < Krishna.
- Raw data are found under the **RawFiles** folder and contains subfolders for **AgaroseGels**, **Excel**, and **RawPhotos**.
- Processed and analyzed data are denoted as WF (working files) and are saved under the **IsolateLibrary** or **Results** folder in a subfolder called **OldVersions**.
- Final data are found in the same two folders, but denoted with the most up to date version of WF.

Have you considered what type of end-user license to include with your data?

- N/A

What steps will be taken to help the research community know that your data exists?

- Thesis will be published in the University of Guelph's Atrium (online repository).
- As well, the data chapters are scheduled to be published in scientific journals (either Food Control, International J of Food Microbiology, of J of Food Protection).

Ethical and legal compliance

If your research project includes sensitive data, how will you ensure that it is securely managed and accessible only to approved members of the project?

- N/A – research project does not include sensitive data

If applicable, what strategies will you undertake to address secondary uses of sensitive data?

- N/A

How will you manage legal, ethical, and intellectual property issues?

- Not handled by me.
- Handled by our research manager (Valeria Parreira) and advisor (Dr. Jeff Farber)
Responsibilities and resources

Identify who will be responsible for managing this project's data during and after the project and the major data management tasks for which they will be responsible.

- Research manager - Valeria Parreira
- Principal investigator (PI) - Dr. Jeff Farber

How will responsibilities for managing data activities be handled if substantive changes happen in the personnel overseeing the project's data, including a change of principal investigator?

- Before I leave, everything will be well documented in both the hardcopy lab notebook and our network drive to make it easier for both my manager (Valeria Parreira) and advisor (Dr. Jeff Farber) to handle the data I acquired for this project. They also both have my contact information if they experience any trouble; I would be more than happy to provide assistance.

What resources will you require to implement your data management plan? What do you estimate the overall cost for data management to be?

- Scholarship librarians from the University of Guelph along with Dr. Michelle Edwards were both consulted in the creation of the Research Data Management SOP for our research group. This SOP is the resource used in the implementation of my data management plan.
- N/A -estimate in the overall cost