Understanding the stabilisation of *Lactobacillus plantarum* by drying

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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March 2015
**Declaration**

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Sarim Khem

09 July 2015
Acknowledgements

It would not have been possible to undertake the research and write up this doctoral thesis without the help and support from generous people around me, to only some of whom it is possible to give particular mention here.

To begin with, I express my heartfelt appreciation to the Australian Government for providing me an opportunity to undertake research in Australia through the generous support of an Endeavour Postgraduate Award.

This thesis was made possible through the ongoing help, support and patience of Dr. Bee K May, who undertook to act as my principle supervisor despite her many academic and professional commitments. Her wisdom, knowledge and commitments to the highest standards inspired and motivated me throughout the entire course of my candidature. I also express my appreciation to my co-supervisor, Associate Professor Darryl M Small for his ongoing support, encouragement, and patience during my candidature, especially in polishing my English.

I express my appreciation to Dr. Meng Wai Woo and Professor Xiao Dong Chen for allowing me to use the research facilities at Monash University and for their kind advice on the single droplet drying study.

I also extend my appreciation to the Laboratory Manager, Mr Karl Lang and technical officers with the Food Science Discipline, Ms. Lillian Chuang, Ms. Yan Chen, Ms. Mary Karagiozakis, Ms. Fiona De-Mendonca for their help in offering me technical support and resources in running the project.

I thankfully acknowledge the valuable time and advice provided by Professor Robert Shanks for his timely advice and assistance provided in analysing my samples using DMA; Professor Gary Bryant for advice on the glass transition temperature concept, Mr Phil Francis and Mr Peter Rummel and their teams for advice in using the microscopy in my research, Mr. Frank Antolasic and Ms Zahra Homan for kindly provided me with access and assistance in using various facilities in Applied Chemistry. I thank Dr. Jeff Hughes and Associate Professor Anthony Bedford for their kindness and advice on the statistical aspects of my work.
Acknowledgements

Amongst my fellow postgraduate students, I acknowledge the assistance of M. Amdadul Hague for the analysis of the FTIR spectra. In addition, the effort made by Dr. Oliver Buddrick, Naksit Panyoyai, Lillian Chuang, Vilia Paramita, Yakindra Timilsena in promoting a stimulating and welcoming academic and social environment will stand as an example to those who follow us and I thank each of them for their warm friendship, help and consideration.

I especially thank my wife, Kimsan, for her personal support and great patience at all times. You have taught me a lot about sacrifice, discipline and compromise through your love, support and constant patience. My daughter, Rima and my son Sovathanak who had to go to school by themselves to allow me to focus on my project. My youngest son, Sanvarick, who had spent two years and a half without me and another year at child care to allow me to concentrate on the project. I am proud of you three and I am sorry for the time we spent apart.

Last but not least, I express my sincere thanks to my parents, brothers and sisters and my parents-in-law as well as sisters and brothers-in-law who help take care of my children and their continuing support and encouragement and as always, for which my mere expression of thanks does not suffice.
Publications and presentations

Most of the work presented in this thesis has been published and presented in the conferences.

Journal publications


Fully refereed conference proceedings papers


Other conference presentations


Abstract

There is accumulating evidence regarding the health benefits of probiotic bacteria. Accordingly, there is strong interest in the incorporation of the various species and strains of these organisms into food products. The challenges encountered include the storage of the cultures, their viability during storage, as well as the protection of the bacteria during drying prior to storage. This project has extended previous results which demonstrated the potential of two strains of *Lactobacillus plantarum* A17 and B21. The broad aim of the current research has been to investigate the drying of these bacterial strains using convective drying and to evaluate strategies to enhance viability.

A variety of techniques have been used including convective single droplet drying (SDD) and spray drying of the bacteria with a selection of protective encapsulants. Physicochemical properties of encapsulants were measured using micro differential scanning calorimetry (µ-DSC) for thermal characteristics and a tensiometer for surface properties. For the resultant capsules, thermal behaviour, structural changes during drying, surface morphology and glass transition temperature \( T_g \) were evaluated using µ-DSC, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy, modulated differential scanning calorimetry (MDSC) and dynamic mechanical analysis (DMA) respectively. In order to evaluate the interactions of bacteria and encapsulant within the matrix, electrostatic interactions was measured using zeta potential and hydrophobicity by the microbial adhesion to hydrocarbon test. Finally changes in the colour of spray dried capsules stored at different temperatures were also determined.

The first phase of the study investigated the protective effects of encapsulants using SDD during *in situ* drying of bacterial cells. This facilitated simultaneous monitoring of the kinetics of cell survival as well as the temperature and moisture contents of droplets. Among a range of the most commonly reported protective agents, whey protein isolate (WPI) and skim milk were found to provide the highest protection during the intermediate drying stage. This was attributed to the reduction in the rate of temperature increase which reduces the stress thereby preserving the bacteria. From these observations, it was proposed that the behaviour of WPI probably differs from that of other agents as it appears to form an outer layer of skin on the capsules, reducing temperature stress and preserving the bacteria.
Abstract

The influences of pH and heat on the protection provided by WPI during spray drying were investigated in the second phase. The cells survived better (~ 70%) in the matrix of native WPI at pH 7 where the protein structure was more compact and globular with 47, 7, 17 and 29% β-sheet, random coil, α-helix and β-turn structure, respectively. After exposure to acid at pH 4, the survival of A17 was reduced to ~39% corresponding to the protein having a less compact globular structure and partial unfolding as demonstrated by a significant increase in β-sheet to 53% and a complete loss of random coils as shown by the FTIR. It appears that a lower degree of protein denaturation before spray drying as characterised by µ-DSC, benefits the ability of \textit{L. plantarum} A17 to survive during spray drying. It is therefore hypothesised that a unique layer-by-layer electrostatic mechanism of different protein components based on their isoelectric points were involved during encapsulation of \textit{L. plantarum} A17 at pH 7 and this is probably responsible for the higher survival of bacteria.

In the third phase, cell survival during microencapsulation of bacteria with a selection matrix formulations was compared. Surface tension, microbial adhesion and interactions, concentration of WPI, moisture content and the morphology of spray dried microencapsulated capsules were measured. It was found that bacterial cells were effectively embedded in the whey protein layer during spray drying. The hydrophobic bacterial cells appear to be protected by attaching to hydrophobic portion of the proteins which minimises the interactions of the proteins with each other. This protective behaviour of WPI was also found to be concentration dependant with B21 strain (more hydrophobic than A17) requiring only half the amount of the encapsulant to provide a similar level of protection. The higher concentration (30%) of WPI corresponded with a thicker and larger capsules which required a longer drying time; resulting in reduced survival of the bacterial cells.

In the final phase, $T_g$ was evaluated both by MDSC and DMA and the results related to the stability of the two bacterial strains during storage at four different temperatures (4, 20, 30 and 50 °C) following drying. MDSC was not useful in the determination of $T_g$; however, it was found that the matrix has a $T_g$ of approximately 34 °C as determined by DMA. Spray dried microcapsules of both strains were stable during storage for 8 weeks at 4 and 20 °C with final cell counts of approximately 10 log CFU/g. During storage at 30 °C, which is in the vicinity of $T_g$ both strains were stable for up to two weeks followed by a 1 log reduction for B21 after 8 weeks of storage. Storing at 50 °C, which is well above the $T_g$ of the matrix...
Abstract

resulted in a ~ 2 log reduction for B21 and 4 log reduction for A17 within the first week of storage.

In conclusion, whey protein isolate, a natural dairy-based material provided adequate protection to *L. plantarum* A17 and B21 cells against spray drying conditions (110 °C inlet and ~70 °C outlet temperatures) and demonstrated promising storage stability. It is proposed that WPI offers protection to A17 and B21 via a combination of protection mechanisms namely electrostatic, hydrophobic interactions and glass transition temperature. The use of WPI as structural elements and probiotics carriers provides effective protection and viability for the strains of *L. plantarum* during storage.
# Table of contents

- Declaration: i
- Acknowledgements: ii
- Publications and presentations: iv
- Abstract: vi
- Table of contents: ix
- List of tables: xv
- List of figures: xvii
- List of abbreviations: xxii
- Explanatory notes: xxiv

## Chapter 1  Introduction

1.1 Background: 1
1.2 Research aim and objectives: 4  
  1.2.1 Aim: 4  
  1.2.2 Objectives: 4  
1.3 Thesis outline: 5

## Chapter 2  Literature review

2.1 Introduction: 6  
2.2 Lactic acid bacteria (LAB): 6  
  2.2.1 Probiotics: 7  
  2.2.2 Stability of probiotics: 9  
  2.2.3 Structure of Gram positive LAB cell: 10  
    2.2.3.1 Bacterial cell wall: 11  
    2.2.3.2 Cell wall macromolecules involving in interactions: 12  
  2.2.4 *Lactobacillus plantarum*: 13  
    2.2.4.1 *L. plantarum* in food application: 14  
    2.2.4.2 *L. plantarum* used as probiotics: 14  
    2.2.4.3 *L. plantarum* used as protective cultures: 17
Table of contents

2.3. Preservation of bacteria 18
   2.3.1 Commonly utilised protective agents 20
      2.3.1.1 Whey protein isolate (WPI) 20
      2.3.1.2 Skim milk 23
      2.3.1.3 Lactose 25
      2.3.1.4 Trehalose 26
   2.3.2 Protection hypothesis 27
      2.3.2.1 Water replacement hypothesis 27
      2.3.2.2 Glass formation hypothesis 28
   2.3.3 Encapsulation techniques 30
   2.3.4 Spray drying as a process of microencapsulation 34
   2.3.5 Selection of encapsulation materials 36
2.4 Summary of current knowledge 37
Maximizing cell survival in the preservation of lactic acid bacteria by freeze-drying 39

Chapter 3 Materials and methods 47
3.1 Introduction 47
3.2 General materials and methods 47
   3.2.1 General materials and media 47
      3.2.1.1 Whey protein isolate (WPI) 49
      3.2.1.2 de man Rogosa and Sharpe broth and agar 49
      3.2.1.3 Peptone water 49
   3.2.2 General procedure 49
   3.2.3 General media and solutions 49
   3.2.4 General microbiological methods 50
      3.2.4.1 Culture storage 50
      3.2.4.2 Cell growth 51
      3.2.4.3 Cell enumeration 52
      3.2.4.4 Bacteria cell harvesting (cell concentrating) 53
3.3 Basic principle for advanced instruments utilised 54
   3.3.1 Differential scanning calorimetry (DSC) 54
   3.3.2 Micro-differential scanning calorimetry (μ-DSC) 57
   3.3.3 Dynamic mechanical analysis (DMA) 59
Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.4</td>
<td>Scanning electron microscopy (SEM)</td>
<td>60</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
<td>62</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Zetasiser</td>
<td>65</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Surface tensiometer</td>
<td>67</td>
</tr>
<tr>
<td>3.3.8</td>
<td>Colour meter</td>
<td>67</td>
</tr>
<tr>
<td>3.3.9</td>
<td>Single droplet drying (SDD)</td>
<td>68</td>
</tr>
<tr>
<td>3.3.10</td>
<td>Spray drying</td>
<td>69</td>
</tr>
</tbody>
</table>

Chapter 4  Agent selection and protective effects during single droplet drying of bacteria

Abstract

4.1 Introduction

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Protectants

4.2.1.2 *Lactobacillus plantarum* A17

4.2.1.3 de Man Rogosa Sharpe (MRS) agar

4.2.2 Sample preparation and measurements

4.2.2.1 Preparation of protectants

4.2.2.2 Micro differential scanning calorimetry

4.2.2.3 Preparation of cells for drying experiments

4.2.2.4 Single droplet drying experiment

4.2.2.5 Isothermal heat treatment

4.2.2.6 Enumeration of bacterial cells

4.2.3 Statistical analysis

4.3 Results and discussion

4.3.1 Protein denaturation during pasteurisation

4.3.2 Protective mechanism during isothermal heat treatment

4.3.3 Skin forming protective mechanism during single droplet drying

4.3.4 Possible influence of protein denaturation and aggregation on the protective mechanism

4.3.5 Possible dual protective mechanism and future work

4.4 Conclusions
# Table of contents

## Chapter 5 Comparative influence of pH and heat on whey protein isolate in protecting *Lactobacillus plantarum* a17 during spray drying

Abstract 94

- **5.1** Introduction 95

- **5.2** Materials and methods 97
  - **5.2.1** Materials 97
    - **5.2.1.1.** *Lactobacillus plantarum* A17 97
  - **5.2.2** Sample preparation and analysis 97
    - **5.2.2.1** The growth profile of A17 97
    - **5.2.2.2** Zeta potential measurements 98
    - **5.2.2.3** Preparations of whey protein isolate solutions 98
    - **5.2.2.4** Preparation of bacterial cells for encapsulation 98
    - **5.2.2.5** Microencapsulation of A17 by spray drying 98
    - **5.2.2.6** Survival rate of bacteria during spray drying 99
    - **5.2.2.7** Analysis of moisture content 99
    - **5.2.2.8** Micro differential scanning calorimetry (DSC) 99
    - **5.2.2.9** Morphology of spray dried powder by scanning electron microscopy 100
    - **5.2.2.10** Fourier transform infrared spectroscopy (FTIR) 100
    - **5.2.2.11** Statistical analysis 101

- **5.3** Results and discussion 101
  - **5.3.1** Growth profile of *L. plantarum* A17 in varying pH conditions 101
  - **5.3.2** Effect of zeta potential in varying pH conditions on A17 cell survival 101
  - **5.3.3** Effect of heat treatment and pH of WPI on cell survival 105
  - **5.3.4** Effect of heat and acid on protein denaturation before and during spray drying 106
  - **5.3.5** Changes in protein structure 109
  - **5.3.6** Morphology of spray dried powder 111
  - **5.3.7** Protective role provided by WPI 112

- **5.4** Conclusions 113
## Chapter 6  The behaviour of whey protein isolate in protecting lactobacillus plantarum

### Abstract

6.1 Introduction

6.2 Material and methods

6.2.1 Materials

6.2.2 Lactobacillus plantarum A17 and B21

6.2.3 Methods

6.2.3.1 Preparation of bacterial cells for encapsulation

6.2.3.2 Preparation of encapsulant solutions

6.2.3.3 Microencapsulation of bacteria by spray drying

6.2.3.4 Surface tension measurement

6.2.3.5 Bacterial survival rate after spray drying

6.2.3.6 Analysis of moisture content

6.2.3.7 Morphology of spray dried powder by scanning electron microscopy (SEM)

6.2.3.8 Hydrophobicity of *L. plantarum*

6.2.3.9 Storage of spray dried microcapsules

6.2.3.10 Statistical analysis

6.3 Results and discussion

6.3.1 Effect of inlet temperature and flow rate on cell survival and final moisture content

6.3.2 Effect of protectants and concentration on the survival and moisture content of spray dried microcapsules

6.3.3 Surface tension of protectant solutions

6.3.4 Hydrophobicity of *L. plantarum*

6.3.5 Morphology of spray dried powder

6.3.6 Storage stability

6.4 Conclusions
# List of tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Some physiological, biochemical and genetic characteristics of <em>L. plantarum</em></td>
<td>13</td>
</tr>
<tr>
<td>2.2</td>
<td>Application of <em>L. plantarum</em> in foods</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Influence of <em>L. plantarum</em> on animal models, healthy volunteers and patients as assessed by <em>in vivo</em> studies</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>Amino acid composition (g AA/100 g protein) of the total protein, casein, and whey protein of bovine milk</td>
<td>20</td>
</tr>
<tr>
<td>2.5</td>
<td>Physico-chemical properties of bovine whey protein</td>
<td>21</td>
</tr>
<tr>
<td>2.6</td>
<td>Typical composition of bovine milk</td>
<td>24</td>
</tr>
<tr>
<td>2.7</td>
<td>A summary of bacteria microencapsulation techniques and the major processes involved</td>
<td>32</td>
</tr>
<tr>
<td>2.8</td>
<td>Summary of the most important characteristics of microencapsulation of methods</td>
<td>33</td>
</tr>
<tr>
<td>3.1</td>
<td>Chemicals and materials utilised in the project</td>
<td>47</td>
</tr>
<tr>
<td>3.2</td>
<td>List of other ancillary equipments utilised in this project</td>
<td>48</td>
</tr>
<tr>
<td>3.3</td>
<td>Peak assignments for secondary structure of protein of deconvoluted spectra in amide I region</td>
<td>64</td>
</tr>
<tr>
<td>4.1</td>
<td>Comparison of moisture content of droplets containing WPI with droplets containing other protectants at the start and finish of the period of rapid temperature increase</td>
<td>90</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of heat treatment and pH of WPI at 10% (w/w) on survival of A17, moisture content and protein denaturation before and after spray drying at inlet and outlet temperatures of 110 and 68 to 70 °C, respectively, and a flow rate of 6.6 mL/min</td>
<td>104</td>
</tr>
</tbody>
</table>
5.2 Percent composition of the secondary structural elements of WPI from supplier and spray dried WPI prepared at pH 7, pH 4, pH 7 plus heat at 75 °C for 1 min and pH 7 plus heat at 78 °C for 10 min

6.1 Effect of inlet temperatures and flow rates on survival and moisture content when spray drying A17 cell suspensions in WPI at pH 7 at 10% (w/w)

6.2 Effect of protective agents and their surface tensions on the survival and moisture content of spray dried microcapsules (spray drying conditions: Inlet temperature of 110 °C and outlet temperature of 69 ± 1 °C)

7.1 Moisture content and water activity of A17 and B21 microcapsules before and after storage at 4, 20, 30 and 50 °C for 8 weeks

7.2 Colour attributes of microcapsules before and after storage for 8 weeks at 4, 20, 30 and 50 °C
# List of figures

<table>
<thead>
<tr>
<th>Figure Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Cell structure of Gram positive bacteria</td>
<td>10</td>
</tr>
<tr>
<td>2.2 Cell wall structure of Gram-positive bacteria</td>
<td>11</td>
</tr>
<tr>
<td>2.3 Commonly utilised protective agents to preserve bacteria with the maximum and minimum survival rate after freeze drying (AFD)</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Schematic representation of the proposed behaviour of beta lactoglobulin during heating at temperature between 20 and 150 °C at pH &gt; 6.8</td>
<td>22</td>
</tr>
<tr>
<td>2.5 Molecular structure of lactose</td>
<td>25</td>
</tr>
<tr>
<td>2.6 Molecular structure of trehalose</td>
<td>26</td>
</tr>
<tr>
<td>2.7 Diagram showing how trehalose protects protein during drying. According to the water replacement hypothesis, as water progressively removed during dehydration, trehalose forms a protective layer by hydrogen bonding with the protein</td>
<td>28</td>
</tr>
<tr>
<td>2.8 Sugars depress the membrane phase transition temperature ($T_m$) by specifically interacting with phospholipid head groups (A1). Large polymeric sugars with rigid structures such as dextran cannot enter between the head groups (A2) whereas flexible ones such as inulin (A3) can fit into the spaces. Therefore, the latter are able to protect cells despite their relatively large size. By contrast, vitrification hypothesis suggests that the sugars can depress $T_m$ without specifically interacting with the head groups, e.g. by osmotic and volumetric effects and by vitrification of sugars between the membranes (B1). Polymeric sugars with a very high molecular weight are not capable of depressing the $T_m$ because they have fewer osmotic effects and are excluded from the intermembrane space during the removal of water (B2). This exclusion curtails the volumetric and vitrification effects of the polymeric sugars.</td>
<td>29</td>
</tr>
<tr>
<td>2.9 Microcapsules with different morphologies: A - matrix, B - simple microcapsule, C - irregular microcapsules, D - multiwall microcapsule, E - multi core microcapsule and F - aggregate of microcapsules</td>
<td>31</td>
</tr>
<tr>
<td>2.10 Cumulative number of publications related to the encapsulation of bacteria published in the last decade</td>
<td>33</td>
</tr>
</tbody>
</table>
List of figures

3.1 Typical growth characteristics of bacteria

3.2 Colonies of *L. plantarum* A17 after incubation for 24 h at 30 °C (a) and SEM examination of A17 at 3000 x magnification on a glass slide after drying in an oven at 70 °C for 15 min (b)

3.3 Sample and reference chamber for DSC (left) and schematic diagram of heat flux type DSC (right)

3.4 DSC profile showing the transitions of a material as a function of changes in temperature

3.5 An example of MDSC thermogram of a thermoplastic alloy blend of polycarbonate (PC) and polybutylene terephthalate (PBT). MDSC effectively separates the crystallisation of the PBT component into the Nonreversing heat flow, thereby allowing for accurate determination of glass transition temperature ($T_g$) of each polymer in the reversing heat flow

3.6 MDSC-Q2000 utilised in the thermal analysis in this research

3.7 Thermostatic block where the sample and reference vessel are placed during the analysis by μ-DSC

3.8 μ-DSC, Setarum VII utilised in this research

3.9 Sample preparation using material pocket

3.10 DMA 8000 (left) and the stainless pocket holding the sample (right)

3.11 Schematic comparison between light and scanning electron microscope

3.12 Environmental scanning electron microscopy (FEI Quanta 200) utilised in this research

3.13 Components of the FTIR

3.14 Perkin Elmer Spectrum 100 FTIR, which was used in this research

3.15 Optical configuration of the Zetasizer for zeta potential measurement

3.16 Zetasier Nano utilised to measure the zeta potential in this research
3.17 Opponent colour scale of L, a, b. (a) and Chroma Meter utilised in this research (b) 68

3.18 LabPlan spray dryer utilised in this research to produce microcapsules 71


4.2 Schematic figure for the experimental set up of (a) kinetics of cell survival, (b) temperature measurement and (c) mass measurement in the glass filament rig 79

4.3 The mass measurement of droplet during drying (a) a typical standard curve obtained by suspending standards bead with known mass and recording the resultant displacement of glass filament and (b) Schematic figure of the displacement of mass measuring glass filament 80

4.4 Micro differential scanning calorimetry thermogram of native and pasteurised whey protein isolate (WPI) at a concentration of 10% w/w at natural unbuffered pH of 6.6 subjected to a heating scan of 1 °C/min 81

4.5 Kinetics of cell survival during isothermal heating at 50, 60 and 90 °C of cell suspension in whey protein isolate (WPI) and lactose (Lac) 82

4.6 Droplet temperature during single droplet drying of pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture of lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C. Note that different vertical scale are used in the two graphs 85

4.7 Kinetics of cell survival during single droplet drying of cell suspension in pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture of lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C 86

4.8 Moisture content on dry basis during single droplet drying of cell suspension in pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C 88

5.1 Growth profile of A17 in MRS broth under different pH conditions 102
List of figures

5.2 Zeta potential of bacteria suspended in phosphate buffer at about $10^9$ CFU/mL and WPI at 2% (w/w) 102

5.3 Effect of pH adjustment and heat treatment on the colour of whey protein isolate solution at 10% (w/w). 103

5.4 Micro DSC thermograms of WPI solutions (a) and spray dried WPI (b) for samples obtained at an inlet temperature of 110 °C and outlet temperature of 68 to 70 °C and which have been reconstituted to the same concentration of 10% w/w (from top to bottom: pH 7 plus heat at 78 °C for 20 and 10 min, pH 7 plus 1 min heat at 75 °C, pH 7 and pH 4 respectively) 107

5.5 Absorbance (a) and second derivative spectra (b) of amide I region of WPI from supplier (black), spray dried WPI prepared at pH 7 (pink), pH 4 (red), pH 7 heat at 75 °C for 1 min (blue) and pH 7 heat at 78 °C for 10 min (green) 108

5.6 Fitted spectra of the WPI from supplier and spray dried WPI prepared at pH 4 109

5.7 Morphology of spray dried microcapsules prepared using WPI after treatment under various conditions (a. pH 7, b. pH 4 and c. pH 7 and heat treated at 78 °C for 10 min) 112

6.1 Mixture of aqueous L. plantarum A17 and B21 suspensions and hexadecane before (a) and after vortexing vigorously for 2 min followed by allowing to stand for 15 min (b). Phase contrast microscopy showing A17 (c) and B21 (d) cells attaching to the hexadecane phase with more hydrophobic cells (B21) attached better to the organic phase 125

6.2 SEM images of spray dried microcapsules prepared at temperatures of 110 (inlet) and 69 ± 1 °C (outlet). Images a-c (all at 3000× magnification): showing the increasing particle size as concentration increases. Images d-e (magnification of 10,000x for WPI at 10% and 30%): showing broken microcapsules and thickness of the capsular walls; arrow showing bacterial cell embedded in the capsule. Image f: showing a proposed morphology of a microcapsule (bacterial cells represented by white rod shapes) encapsulated in WPI matrix 126

6.3 Survival during storage at 20 °C of A17 and B21 encapsulated with WPI at pH 7 (10% w/w) by spray drying at temperatures of 110 (inlet) and 69 ± 1 °C (outlet) 128
7.1 DSC thermogram of spray dried WPI capsules prepared at pH 7 at 95% solid content at a heating rate of 2 K/min, modulation amplitude of 0.53 °C for each period of 40 s. (total heat flow (+), reversed heat flow (○) and non reversed heat flow (Δ))

7.2 Damping properties (Tan δ) of spray dried capsules prepared at pH 7 with a solid content of 95% as a function of temperature (scan rate: 2 °C/min, frequency: 1Hz.)

7.3 Survival of spray dried B21 (a) and A17 (b) encapsulated in WPI at pH 7 over a period of 8 weeks during storage at different temperatures (at 4 °C (•); 20 °C (■); 30 °C (▲) and 50 °C (●))

7.4 Linear regression model of bacterial cells survival reduction during storage at 30 °C to estimate the storage life
Abbreviations

a* Redness
AA Amino acid
APA American Psychological Association
aw water activity
ANOVA Analysis of variance
AS Australian standard
b* Yellowness
C Cytocine
CFU Colony forming unit
Da Dalton
DMA Dynamic mechanical analysis
DSC Differential scanning calorimetry
et al. And others
FTIR Fourier transform infrared spectroscopy
G Guanine
kDa kilo Dalton
L* Lightness
LAB Lactic acid bacteria
Lactose
L. plantarum Lactobacillus plantarum
MDSC Modulated differential scanning calorimetry
μ-DSC Micro differential scanning calorimetry
MRS agar de man Rogosa and Sharpe agar
MRS broth de man Rogosa and Sharpe broth
pl Isoelectric point

-xxii-
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>SDD</td>
<td>Single droplet drying</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SM</td>
<td>Skim milk</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Phase transition temperature</td>
</tr>
<tr>
<td>Tre</td>
<td>Trehalose</td>
</tr>
<tr>
<td>UATR</td>
<td>Universal attenuated total reflectance</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by weight</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>$\beta$-lg</td>
<td>Beta-lactoglobulin</td>
</tr>
<tr>
<td>$\alpha$-lac</td>
<td>alpha-lactalbumin</td>
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</tbody>
</table>
Explanatory notes

These notes are to briefly describe the approaches adopted during the preparation of this thesis. Issues including spelling and expression as well as formatting style in the reference list are consistently clarified.

1. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. For example the term colour (rather than color), words ending with –ise (rather than –ize) have been chosen throughout the whole thesis;

2. In presenting the data, SI units have been used throughout the thesis.

3. In the citation and listing of references and information sources, guideline provided by American Psychological Association (American Psychological Association, 2010) has been taken throughout. This citation style has been within the instructions to authors (Elsevier, 2015) currently recommended for manuscripts submitted to Food Chemistry, Food Hydrocolloids, and Journal of Functional Foods. These are the most highly ranked international journals in the field of food science and technology (Thomson Reuters, 2013).

4. In relation to a number of other specific formatting issues, instructions for authors currently recommended for manuscripts submitted to the Journal of Food Science (Institute of Food Technologist, 2010) is adopted. This approach was used on the basis that most of the other well recognised journals do not clearly define requirements to cover this issue. The specific formatting adopted from this source include:
   a. Space between ± and number (for example: 69 ± 1 °C);
   b. Space between measurement and number (for example: 23 μm);
   c. No space between % and number (for example: 10%);
   d. Space between degree sign and number (for example: 23 °C)
Chapter 1

Introduction

This chapter provides the background and the issues considered in developing the research project leading to the research aim and objectives. In addition, this chapter also provides an outline of different chapters of the thesis.

1.1 Background

In a previous project (Tran, 2010) some thirty retail samples of traditional Vietnamese fermented sausage (known as nem chua) were purchased from different regions of Vietnam for the isolation of lactic acid bacteria (LAB). These LAB which have been known to the locals for their health promoting and antimicrobial effects for many generations, were isolated from the products, based on their acidifying properties and their ability to produce antimicrobial compounds other than acids. From a total of more than 140 isolates, two LAB isolates, coded A17 (strong acidifying) and B21 (producing antimicrobial compound other than acids), were selected among all isolates for their strongest and broadest inhibitory spectra towards all bacterial indicators. Identification of these two LAB isolates confirmed that they are two different strains in the species of Lactobacillus plantarum. These two strains with technological and functional potential were further characterised in another project including a complete genome sequence of L. plantarum B21 (Golneshin et al., 2015). These strains which have been generally accepted as bacterial species ascribed to the general category of probiotics were further investigated in this research for their stability during processing and storage.

Interest in probiotics is at an all-time high around the world as evidenced by the world probiotics market which is expected to reach almost $29 billion in sales by 2015 (Global Industry Analyst, Inc. 2012). This interest is driven in part by the increasingly health-conscious consumers who are pursuing potential therapeutic and preventive health benefits of probiotics.
The link between the consumption of fermented foods containing live bacteria, especially dairy products, to the reduced risks of certain disease has been widely accepted. From these products as well as human derived origins, different species and strains of bacteria have been isolated and characterised as probiotics that, when administered in adequate amount, confer health benefits to the host. However, although much research does exist to show the effectiveness of some species of probiotics, a number of unsubstantiated claims by manufacturers of some products in the market have resulted in restrictions to the use of the term probiotic in some countries of the European Union (EFSA-NDA, 2011). In response to this, the International Scientific Association for Probiotics and Prebiotics has recently developed a consensus among experts to reinforce the definition and guidelines initially defined/issued by FAO/WHO some 13 years ago. This Consensus Panel (Hill et al., 2014) concurred that based on available literature there are sufficient evidence to consider certain species of *Bifidobacteria* and *Lactobacillus* including *L. plantarum* as probiotics and that the consumption of a minimum amount (10^9 CFU/g or per day) of live cells is considered to provide health benefits to the host.

This minimum threshold requirement brings challenges to the food and nutraceutical industries alike to preserve the bacteria in order to provide this high number at the time of consumption. Literature in the past ten years has seen an exponential increase in the research to preserve different strains of LAB, reporting a variety of preservation methods as well as the utilisation of different protective agents. These have led to different degrees of bacterial cell survival during processing and storage, which in many cases do not meet the minimum threshold requirement.

It has been widely observed that many microorganisms including various species and strains of LAB lose viability during storage. A considerable number of reports have sought to understand the effects of growth conditions and other factors that influence growth and the retention of viability. In order for commercial exploitation to be successful and the health benefits of the bacteria to be effectively realised, it is necessary to find ways to preserve the bacteria. This is important for transportation, as well as storage so that bacteria retain viability during transit through the various changing conditions. Although many reports in the literature have trialled numerous protectants, high retention of live probiotics depends on various factors including the species and is typically strain-specific.
Different encapsulation strategies have been developed over the years to protect probiotic bacteria during processing and storage. Among these, encapsulation by spray drying has outnumbered other strategies due to available technology, a single unit operation as well as relatively low cost of production. The existing literature on the effective drying and encapsulation of probiotics continues to grow rapidly in response to consumer demand.

Accordingly, the broad aim of the current project has been to investigate the encapsulation of *L. plantarum* and formation of microcapsules that affords protection to the bacterial cells for the purpose of retaining viability during drying and storage and was developed based on the following issues:

- Bacteria in their physiological conditions are composed of primarily water in the cell. During convective drying, water is progressively moving from the cell. Dehydrating bacterial cells encounter various stresses including thermal, osmotic, dehydration, pH and oxidation, which might lead to cellular inactivation. To overcome these stresses, protectant or encapsulation agents are added to enhance the survival of the bacteria during drying. To date different protectants including carbohydrates, proteins and biopolymers have been tested on a trial and error basis to a numbers of strains of *Lactobacilli* resulting in varying degree of survival during dehydration.

- A water replacement hypothesis has been formulated to explain the stabilisation of bacteria during drying. As drying progresses, water molecules hydrating around bacterial cell would have been replaced with protectant, thus bacterial cell wall remains undamaged. In addition, the penetration of the protective agents through bacterial cell membrane would reduce osmotic stress during drying. However, only small molecular weight compounds can transfer across the cell membrane. What happens if large molecular weight biopolymers including protein are used as protective agents? Can these protect the bacteria and if it can, how?

- Milk has been commonly used as a protectant during drying. However, there has been no report as to what constituent of milk—protein, sugar, fat—is really protecting the bacteria and the mechanism by which it is protected. There has been evidence of bacteria embedded with the whey component in cheese. However, how whey proteins
keep the bacteria alive in the dried state, especially insights into the interaction between whey proteins and bacteria are very limited.

- Disaccharides including trehalose, lactose, and sucrose have been reported to provide protection to bacterial cells due to their ability to penetrate bacterial cells thus preventing the osmotic stress during drying. However, these sugars cannot be assumed to be effective for all strains.

1.2 Research aim and objectives

1.2.1 Aim

To investigate the stabilisation of *Lactobacillus plantarum* during drying

1.2.2 Objectives

i. To evaluate five forms of protectants: whey protein isolate (WPI), trehalose, lactose, long life skim milk and a mixture of lactose and WPI in a ratio of 9.4:0.6 on the survival of *L. plantarum* A17 cells using isothermal heating and convective single droplet drying.

ii. To develop *in-situ* WPI microorganism protective mechanism for convective drying of dilute droplets at 90 °C and 110 °C.

iii. To evaluate the behaviour of WPI in protecting *L. plantarum* during spray drying.

iv. To compare the effect of denaturing WPI by pH adjustment and heat treatment on the protection of *L. plantarum* during spray drying.

v. To investigate the storage stability of *L. plantarum* encapsulated with WPI by spray drying.

Accordingly this project was also designed based on the following hypothesis:

i. The five forms of protectants including WPI, trehalose, lactose, long life skim milk and a mixture of lactose and WPI can protect the bacterial cells.

ii. Insights into the protective mechanism during convective drying of cells can be monitored using single droplet drying.
iii. WPI is able to protect *L. plantarum* cells during spray drying.

iv. Adjusting the pH of WPI close to the isoelectric point is as effective as denaturing WPI by isothermal heating at pH 7 for the protection of *L. plantarum* A17.

v. Storing bacteria encapsulated with WPI by spray drying below glass transition temperatures leads to the stability of the microencapsulated cells.

### 1.3 Thesis outline

This thesis has been written in 8 chapters including this introductory chapter. Chapter 1 provides an overview of the research project followed by background and literature review (Chapter 2) where concepts relevant to this project are reviewed. Chapter 3 describes all materials and methods utilised in the whole project including the principle of operation for some advanced instrumentation employed for analysis in this research. Chapter 4 describes results of a fundamental study of the protection mechanism of *L. plantarum* A17 during convective single droplet drying. In this, protection mechanisms afforded by commonly utilised protective agents were elucidated and form the basis for the remaining phase of the project.

Chapter 5 presents the effect of altering WPI structure by either pH or heat treatment on the protection of *L. plantarum* A17 during spray drying. Chapter 6 provides further insights into the protection mechanism of WPI on *L. plantarum* A17 and B21 during spray drying.

Chapter 7 explores the storage stability of *L. plantarum* A17 and B21 following encapsulation with WPI by spray drying. The stability of the two strains has been compared as a function of storage temperature. Finally, a general discussion and conclusions chapter (Chapter 8) discusses and summarises the key findings in this work and also presents recommendations for future research.
Chapter 2

Literature review

2.1 Introduction

The purpose of this chapter is to provide an overview of all the literature related to the study of this Ph.D. project. The review covers relevant research which has been done up to the formulation of this project including lactic acid bacteria, methods utilised to preserve them, and commonly used protective agents as carriers for bacterial cells. In addition, hypotheses, which have been formulated up to date on the protection mechanism of bacteria during desiccation, will also be reviewed. The project was initially formulated with the aim of preserving *Lactobacillus plantarum* by freeze drying. However, due to unforeseen circumstances, convective drying was used instead. Therefore, a review paper on maximising cell survival in the preservation of lactic acid bacteria (Khem, Small, & May, 2012) is attached at the end of this literature review section.

2.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of ubiquitous Gram-positive bacteria producing lactic acid as one of the major end products and have been widely used in food fermentation and probiotics. In general these bacteria are non-motile, microaerophilic, catalase, and oxidase negative and the pH of the culture during the stationary phase is less than 4. These bacteria are either Gram-positive rod or cocci, non-spore forming organisms that are devoid of cytochromes and are of non-aerobic habit but are aero-tolerant and strictly fermentative with the G+C content in the range of 33-55% (Axelsson, 1998).

These technological and beneficial microorganisms consist of different genera including *Lactobacillus, Lactococcus, Pediococcus, Leuconostoc, Carnobacterium, Enterococcus, Oenococcus, Streptococcus, Tetragenococcus, Vagococcus, Aerococcus* and *Weissella* (Bourdichon et al., 2012; Wright & Axelsson, 2011). Among these genera, *Lactobacillus, Lactococcus*, and *Pediococcus* are widely accepted as the core of the group (Ammor &
Mayo, 2007; Lucke, 2000). Some of the genera including *Lactococcus* contain species, which are recognised as animal or human pathogens (Wright & Axelsson, 2011).

*Lactobacillus* is a genus with 84 species compared to only three species each for *Pediococcus* and *Lactococcus* listed as microorganisms with “general recognition of safety” in a project jointly developed between the International Dairy Federation and the European Food and Feed Cultures Association (Bourdichon et al., 2012).

*Lactobacilli* are microaerophilic Gram-positive bacteria found in a variety of environmental niches including nutrient rich dairy systems, human mucosal surfaces and natural ecology of plants and soils (Barrangou, Lahtinen, Ibrahim, & Ouwehand, 2011). These microorganisms are presently further subdivided into three groups (Todorov & Franco, 2010):

a) Obligate homofermenters, in which there are 15 species in the group primarily classified as *Thermobacterium. L. delbrueckii* and *L. acidophilus* and *L. helveticus* are some of the examples.

b) Facultative heterofermenters, which ferments hexose sugars to lactate and other metabolites through the Embden Meyerhof Parnas (EMP) pathway. These represent some species important in food fermentation particularly *L. plantarum, L. casei, L. sakei*.

c) Strict heterofermenters in which lactic acid and acetic acid are the main final metabolites. Examples of this group are *L. brevis, L. fermentum*, and *L. reuteri*.

### 2.2.1 Probiotics

The concept of benefits of LAB was originally proven scientifically by a Russian scientist Metchnikoff Elie since the early 20th century; however, due to the pre and post world war together with the discovery of antibiotic, this concept was somehow forgotten. Indeed, very little or nothing was heard about the microbial therapy in Western country between 1908 and 1964 (Anukam & Reid, 2007). Since the late 1960s, several authors proposed different definitions of probiotics and later on, the most cited definitions was that by Fuller (1989) who described probiotics as “live microbial supplements which beneficially affects the host animal by improving its microbial balance”.

-7-
As research on probiotics grew, an expert committee was formed and sponsored by the Food and Agricultural Organisation and the World Health Organisation (FAO/WHO). This group came up with a definition of probiotics as “live microorganisms which when administered in adequate amount confer health benefits to the host” (FAO/WHO, 2001).

However, this definition of probiotics was not accepted by the European food safety authority (EFSA-NDA, 2011; McCartney, 2013) and recent rejections of health claims from consumption of probiotics by this European regulatory body has angered some prominent scientists accusing EFSA of ignoring good science (Katan, 2012). An expert panel was convened in October 2013 by the International Scientific Association for Probiotics and Prebiotics (ISAPP) to discuss the field of probiotics and the definition was reinforced as relevant and sufficiently accommodating for current and anticipated applications with only a slight grammatical change to “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014).

The expert panel also defined four categories of live micro-organisms for human use:

i. Live or active cultures from any food fermentation microbe(s). The term ‘live’ or ‘active’ do not imply probiotic activity and such fermented food may claim “Contains live and active cultures” on the label;

ii. Probiotics in food or supplement without health claims from member(s) of a safe species with proof of viability at the appropriate level. This category of food or supplement may claim “contains probiotics” on the label;

iii. Probiotics in food or supplement with a specific health claim. This category requires convincing evidence needed for specific strain(s) or strain combination in the specified health claim;

iv. Probiotic drug – this category requires appropriate trials to meet regulatory standards for drugs.

The consensus panel also concurred that certain core benefits can be ascribed to probiotics as a general class based on currently available literature, which includes well-designed clinical trials, systematic reviews and meta-analyses. In this context, the panel concurred on the strains of a number of well studied microbial species at a functional dose for use as foods or supplements in the general population. The consensus statements also accepted the following nonstrain-specific claims which could be made including *Bifidobacterium* (*adolescentis*,...
animalis, bifidum, breve and longum) and Lactobacillus (acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus and salivarius), which aligns with Italy and Canada regulatory approaches (Health Canada, 2009). The panel also set a general requirement of consuming food or supplement with at least $10^9$ CFU/g or per serving to provide health benefits. In addition, most commercial probiotic foods market consist primarily of Lactobacillus strains (Vogel et al., 2011). Some of the notable probiotic microorganisms in this genus reported in the literature include Lactobacillus rhamnosus GG, L. casei, L. casei Shirota, L. acidophilus, L. johnsonii, L. plantarum (Figueroa-González, Quijano, Ramírez, & Cruz-Guerrero, 2011).

2.2.2 Stability of probiotics

In order to provide health benefits to the host, foods or supplements should contain at least $10^9$ CFU per gram or per serving at the time of consumption (Hill et al., 2014). It is therefore, necessary for the probiotics to maintain viability that meets this minimum requirement not only during processing, but also during storage until the time of consumption. Factors affecting probiotic survival during storage including temperature, moisture content, water activity, processing method, types of protectants have been reported in the literature. Generally, temperature is inversely related to the survival of probiotic bacteria during storage and storing bacterial cells at refrigeration temperature or lower generally correlate with better survival (Corcoran, Ross, Fitzgerald, & Stanton, 2004; Peighambardoust, Golshan, & Hesari, 2011).

However, for some strains, survival is low at refrigerated conditions and cellular inactivation still occurs even at these low temperatures. For example, L. acidophilus NCIMB 701748 was report to have an activation rate of 0.011 per day when stored at 4 °C (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fist, 2013). Using alginate and chitosan as encapsulant to protect L. gasseri and B. bifidum resulted in a decrease of approximately 2 log after 28 days of storage at 4 °C (Chávarri et al., 2010). Similarly, the survival of L. plantarum microencapsulated with alginate or pectin declined from 1.95 to 6.73 log CFU/g during storage at 4 °C for 38 days in yoghurt (Brinques & Ayub, 2011).

The challenge of stability of the cultures at low temperatures is also reflected in commercial strains. Most starter and probiotic cultures available on the market as freeze dried powder are recommended to be stored frozen (-18 °C). For example, freeze dried Yogurt starters
including Viili, Vegan, Greek containing different strains of *Lactobacillus* stored in an air tight aluminium bag are recommended to be stored frozen for a shelf life of 12 months (Cultures for health, n.d). Lyofast LPRA containing freeze dried *L. rhamnosus* and *L. plantarum* and packed in an air and moisture tight container are recommended for use as protective cultures are recommended to be stored at or below -18 °C for a shelf life of 18 months (Sacco, 2009). Similarly, Lyofast SYAB 1 containing freeze dried cells of *Streptococcus thermophilus*, *L. delbrueckii* spp. *bulgaricus* added with probiotic strains of *L. acidophilus* and *B. animalis* spp. *lactis* are recommended to be stored below -20 °C (Sacco, 2011). Considering these recommended storage temperatures, understanding the stability as well as being able to produce stable dried cells at room temperature would be of great commercial interest.

### 2.2.3 Structure of Gram positive LAB cell

![Cell structure of Gram-positive bacteria](image)

*Fig. 2.1  Cell structure of Gram-positive bacteria. Adapted from (Beaudry, 2012)*
Many unique biological properties of bacteria were reflected by their unique cell structure. Due to its simplicity as compared to other higher organisms, the cell structure of bacteria has been well studied, revealing many biochemical principles. Generally, bacteria are made up of cell wall, plasma membrane, cytoplasm, ribosomes, plasmid, pili circular DNA and bacteria flagellum as illustrated in Fig. 2.1.

2.2.3.1 Bacterial cell wall

Cell wall of Gram-positive bacteria is a complex arrangement of macromolecules. It consists of a lipid layer covered by a thick layer of peptidoglycan that surrounds the cytoplasmic membrane and decorated with other glycopolymers including teichoic acids, cell wall associated polysaccharides and cell surface proteins (Fig. 2.2). Peptidoglycan is an essential polymer composed of alternating residues of β-1-4-link N-acetyl muramic acid and N-acetyl-glucosamine (Vollmer, 2008).

![Cell wall structure of Gram-positive bacteria. Adapted from (Burgain et al., 2014)](image)

There are multiple functions of bacterial cell wall including bacterial growth, maintaining bacterial cell integrity and shapes as well as resisting internal turgor pressure. The cell surface properties, which govern biointerfacial phenomena such as adhesion and cell
aggregation depends on the structure of cell wall (Dufrêne, Boonaert, van der Mei, Busscher, & Rouxhet, 2001). Polysaccharides covering the cell walls are neutral and acidic by nature, as well as cell wall protein and teichoic acid with a high acidic nature (Beveridge & Graham, 1991) that determine the bacterial interaction with the environments.

2.2.3.2 Cell wall macromolecules involved in interactions

Cell walls of LAB are quite complex consisting of a thick layer of peptidoglycan, teichoic acids, proteins and polysaccharides. Peptidoglycan is the main constituent of the cell wall of Gram positive bacteria consisting of glycan chains made of alternative $N$-acetylglucosamine and $N$-acetylmuramic acid that are linked covalently. It is mostly accepted that glycan strands run parallel to the plasma membrane, arranged perhaps as hoops or helices around the short axis of the cell resulting in a woven fabric (Tripathi et al., 2012). As summarised by Chapot-Chartier & Kulakauskas (2014), peptidoglycan of Gram positive bacteria in general, and *L. plantarum* in particular, is essential for cell growth, control of cell separation, increase carboxypeptidase activity, inhibition of major autolysin and the activation of autolysis by amidase.

Bacterial adhesion to the host or substrate depends largely on the environments (pH, temperature), the appearance of the surface roughness, the surface energy of coupled bacteria/substrate, the hydrophobic or hydrophilic character of bacteria or substrate, the surface charges, the ionic strength of the medium and the presence of specific structures on bacterial surface or substrate (An & Friedman, 1998). The adhesion of bacteria occurs in two steps including non specific and reversible interaction (such as hydrophobicity/hydrophilicity, electric charges, and the Lewis acid base) and followed by specific and non-reversible interactions involving proteaceous adhesions and complementary receptors. Other constituents of bacterial cell wall including teichoic acids, or lipoteichoic acid and polysaccharides have also been reported to be involved in adhesion phenomena. For example teichoic acids of *L. johnsonii* La1 were reported to be the major players in adhesion to human intestinal epithelial cells, possibly by hydrophobic interactions (Granato et al., 1999). Although bacterial adhesion has been mainly studied in relation to the pathogenicity; the relationship of bacterial adhesion to the formulation matrix during the preservation of bacteria is limited.
2.2.4 Lactobacillus plantarum

*L. plantarum* is a rod shape bacteria, approximately 0.9-2 × 1.0-8.0 µm in size occurring singly or grouped in short chains with cell wall consisting of ribitol and teichoic acids which are different from other *Lactobacillus* species with a usual cell wall consisting of glycerol teichoic acid in the cell envelop (Todorov & Franco, 2010). It is classified in the facultative heterofermentative species, which are able to ferment hexoses via EMP pathway resulting in the formation of D- and L-lactic acid. However, fermentation of pentose resulted in the formation of lactic and acetic acids in the presence of inductable phosphoacetolase (Garrity, Brenner, Krieg, & Staley, 2005). It is a mesophilic strain and is able to grow at temperature from 15 up to 46 °C with a pH value from 4 to 9 (Tanasupawat et al., 1992). Some of the physiological, biochemical and genetic characteristics of *L. plantarum* is presented in Table 2.1.

Table 2.1 Some physiological, biochemical and genetic characteristics of *L. plantarum* (adapted from Todorov & Franco, 2010)

<table>
<thead>
<tr>
<th>Production of lactic acid from:</th>
<th>Amygdalin, arabinose, cellobiose, fructose, galactose, glucose, gluconate, lactose, maltose, mannitol, mannose, melibiose, raffinose, ribose, salicin, sorbitol, sucrose, trehalose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid were not formed from:</td>
<td>Xylose, sorbose, inulin, inositol, starch, glycerol</td>
</tr>
<tr>
<td>Eschin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Lactic acid isomers</td>
<td>D and L</td>
</tr>
<tr>
<td>Type of teichoic acids</td>
<td>Ribitol or glycerol</td>
</tr>
<tr>
<td>Growth with 4% taurocholate</td>
<td>+</td>
</tr>
<tr>
<td>Growth in the presence of 10% NaCl</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of arginine</td>
<td>-</td>
</tr>
<tr>
<td>Production of dextran from saccharose</td>
<td>-</td>
</tr>
<tr>
<td>Mol % of Guanine and Cytosine (G and C)</td>
<td>44-46</td>
</tr>
<tr>
<td>Growth factor requirements</td>
<td>Calcium pentothenate, niacin, nicotinic acid</td>
</tr>
<tr>
<td>Antibiotic resistance to</td>
<td>Kanamycin, gentamicin, neomycin, streptomycin, polimixin B, and colistin</td>
</tr>
</tbody>
</table>
*L. plantarum* is widespread in the environment and it has been isolated from varieties of fermented foods, human vaginal as well as baby faeces. Some of the strains have been commonly utilised as starter and protective cultures as well as probiotics.

### 2.2.4.1 *L. plantarum* in food application

LAB in general and *L. plantarum* in particular are increasingly being incorporated into food formulations reflecting their potential for contributing to health and wellbeing. These microorganisms contribute not only to the development of aroma, flavour and texture, but also to the shelf life of food products. This preservative effect is due to the competition for nutrients with other microorganisms as well as the production of anti-bacterial metabolites including lactic acid and organic acids, ethanol, hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins (Belgacem, Ferchichi, Prevost, Dousset, & Manai, 2008). The strains have been isolated and applied in a range of environmental niches including dairy products, fermented plant materials, fermentation of meat, meat product and fish, bakery products, and fermented beverages (Table 2.2).

Recently, some strains of *L. plantarum* have been successfully isolated and characterised from fermented pork product in Vietnam known as *Nem Chua*. Some strains have strong acid production, which is favourable for potential starter culture. Some strains have proven inhibitory to spoilage and pathogens (Tran, 2010).

### 2.2.4.2 *L. plantarum* used as probiotics

In addition to the application of *L. plantarum* in food, some strains of these bacteria have been commercialised as probiotics. Different health effects have been reported upon the consumption of live *L. plantarum* including reducing the incidence of diarrhoea, pain and constipation of irritable bowel syndrome, bloating, flatulence and pain in irritable bowel syndrome, positive effect on immunity in HIV positive children (Parvez, Malik, Kang, & Kim, 2006). In addition, *L. plantarum* provides other benefits based on studies both *in vitro* and *in vivo* (Table 2.3). In addition to the effect shown in the *in vivo*, the *in vitro* studies have also shown other beneficial effects of *L. plantarum* including the inhibition of *Salmonella typhi* (Abdel-Daim, Hassouna, Hafez, Ashor, & Aboulwafa, 2013).
### Table 2.2 Application of *L. plantarum* in foods

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Varieties of cheese products including Pecorino, Romano, cheddar, Belgian soft cheese, Stilton and Gouda, Kopanisti and traditional Greek cheese, feta, mozzarella cheese, ovine cheeses, alberuilla cheese, Manchego cheese, Moroccan soft white cheese and danbo cheese</td>
<td>(Antonsson, Molin, &amp; Ardö, 2003; de Vries, Vaughan, Kleerebezem, &amp; de Vos, 2006)</td>
</tr>
<tr>
<td></td>
<td>Fermented milk products such as kefir, kumis, Koumiss of central Asia, Amasi, kule naoto, kajmak,</td>
<td>(Todorov &amp; Franco, 2010)</td>
</tr>
<tr>
<td>Plant</td>
<td>Olives</td>
<td>(de Vries et al., 2006; Vaughn, 1985)</td>
</tr>
<tr>
<td></td>
<td>Cocoa beans</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cassava</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cucumber</td>
<td></td>
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<tr>
<td></td>
<td>Sauerkraut</td>
<td></td>
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<tr>
<td></td>
<td>Togwa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wine</td>
<td></td>
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<tr>
<td>Meat and fish</td>
<td>Fermented dried sausage</td>
<td>(Todorov, Furtado, Saad, Tome, &amp; Franco, 2011; Toldra, 2007; Toldra, Sanz, &amp; Flores, 2001)</td>
</tr>
<tr>
<td></td>
<td>Fermented Italian sausage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fermented pork</td>
<td>(Jaichumjai, Valyasevi, Assavanig, &amp; Kurdi, 2010; Tran, 2010)</td>
</tr>
<tr>
<td></td>
<td>Fermented fish</td>
<td>(Kopermsub &amp; Yunchalard, 2010)</td>
</tr>
<tr>
<td>Bakery</td>
<td>Bread dough, sweet dough, soda cracker dough, sourdoughs</td>
<td>(Todorov &amp; Franco, 2010)</td>
</tr>
<tr>
<td>Beverage</td>
<td>Must and wine</td>
<td>(Knoll, Divol, &amp; du Toit, 2008)</td>
</tr>
<tr>
<td></td>
<td>Sorghum beer</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.3 Influence of *L. plantarum* on animal models, healthy volunteers and patients as assessed by *in vivo* studies (adopted from de Vries et al., 2006)

<table>
<thead>
<tr>
<th>Study</th>
<th>Host</th>
<th>Dose(a) (strain)</th>
<th>Subjects</th>
<th>Intake</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy people</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Johansson et al., 1993)</td>
<td>Human</td>
<td>$2.0 \times 10^{10}$ (1)</td>
<td>26</td>
<td>3 weeks</td>
<td>Increased short chain fatty acid content of faeces</td>
</tr>
<tr>
<td>(Johansson et al., 1993)</td>
<td>Human</td>
<td>$5.0 \times 10^{8}$ (1,2)</td>
<td>13</td>
<td>10 days</td>
<td>Changing microbiota in ileum and rectum Dominant recovery of <em>L. plantarum</em> (2/3 of recovered strains)</td>
</tr>
<tr>
<td>(McNaught, Woodcock, MacFie, &amp; Mitchell, 2002)</td>
<td>Human</td>
<td>$2.0 \times 10^{10}$ (1)</td>
<td>129</td>
<td>Differed</td>
<td>No effect on post-operative wound infection</td>
</tr>
<tr>
<td>(Bukowska, Pieczul-Mróz, Jastrzebska, Chelstowski, &amp; Naruszewicz, 1998)</td>
<td>Human</td>
<td>$1.0 \times 10^{10}$ (1)</td>
<td>30</td>
<td>6 weeks</td>
<td>Reduction in LDL-cholesterol (9.6%) and fibrinogen (13.5%)</td>
</tr>
<tr>
<td>(Wullt, Hagslätt, &amp; Odenholt, 2003)</td>
<td>Human</td>
<td>$5.0 \times 10^{10}$ (1)</td>
<td>20</td>
<td>38 days</td>
<td>1/3 reduction in recurrence of <em>Clostridium difficile</em>-associated diarrhoea</td>
</tr>
<tr>
<td>(Nobaek, Johansson, Molin, Ahrné, &amp; Jeppsson, 2000)</td>
<td>Human</td>
<td>$2.0 \times 10^{10}$ (1)</td>
<td>60</td>
<td>4 weeks</td>
<td>Reduction in symptoms IBS</td>
</tr>
<tr>
<td><strong>Animal models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Perdigón, Vintiñi, Alvarez, Medina, &amp; Medici, 1999)</td>
<td>Mice</td>
<td>$10^{9}$ (3)</td>
<td>20-24</td>
<td>3.5 or 7 days</td>
<td>Increase in specific and unspecific immunity</td>
</tr>
<tr>
<td>(Mangell et al., 2002)</td>
<td>Rats</td>
<td>Differed per group (1)</td>
<td>25</td>
<td>1 weeks</td>
<td>Inhibition of <em>E. Coli</em>-induced intestinal permeability</td>
</tr>
<tr>
<td>(Liu et al., 2001)</td>
<td>Rats</td>
<td>$2.0 \times 10^{9}$ (1)</td>
<td>72</td>
<td>22 days</td>
<td>Reduction in side effects of external radiation on colon anatomic healing</td>
</tr>
</tbody>
</table>

(a) CFU per day unless stated otherwise; strain (1) *L. plantarum* 299v; strain (2) *L. plantarum* 299; strain (3) *L. plantarum* CRL 936
2.2.4.3 *L. plantarum* used as protective cultures

Apart from the utilisation as starters and probiotics, *L. plantarum* has also been explored and utilised as a protective cultures. By definition protective cultures are bacteria inoculated into food with consequent *in situ* production of inhibitory molecules and/or a competitive effect against pathogen and spoilage bacteria and the use of microbial metabolites in purified form, in particular bacteriocin (Gaggia, Gioia, Baffoni, & Biavati, 2011; Gálvez, López, Abriouel, Valdivia, & Omar, 2008). Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides. These have a bactericidal or bacteriostatic effect on other closely related species; however, the producer cell exhibits specific immunity to the action of its own bacteriocin (Ammor, Tauveron, Dufour, & Chevallier, 2006).

Bacteriocins produced by LAB provide a number of desirable properties including: having generally regarded as safe status, not active and non-toxic on eukaryotic cells, they are inactivated by digestive proteases thereby minimising the effect on the desirable gut microflora, usually heat and pH tolerant, as well as having broad antimicrobial spectrum against foodborne pathogenic and spoilage bacteria. In addition to these desirable properties, using bacteriocins in food preservation offers a variety of other potential benefits such as extending shelf life, providing extra-protection during conditions of temperature abuses, decreasing the risk for transmission of foodborne pathogens, ameliorating the economic loss due to spoilage, reducing the application of chemical preservatives, permitting the application of severe heat treatment, facilitating the marketing of novel foods and satisfying industrial and consumer demand (Gálvez, Abriouel, López, & Omar, 2007). Moreover, health conscious consumers may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives (Deegan, Cotter, Hill, & Ross, 2006).

Bacteriocins can be applied in food in three different ways — by using LAB strains which produce bacteriocin in the product, addition of purified or semi-purified bacteriocin as a food preservative and the incorporation of an ingredient which has previously involved fermentation using a suitable strain so that a bacteriocin has already been produced during processing (Castro, Palavecino, Herman, Garro, & Campos, 2011). The use of live protective/probiotic culture is superior to other strategies because the microorganism is the source of the microbial peptide in addition to a wide spectrum of other useful molecules particularly organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl, the anti-
microbial action of which is well established (Vandenbergh, 1993). Nowadays, it has been accepted that an appropriate starter culture has to be chosen from an indigenous population naturally present in a food product, in order to be more competitive, well-adapted and with high metabolic capacity and to beneficially affect quality and safety (Fadda, López, & Vignolo, 2010).

Recently, the organisms involved in the fermentation of traditional Vietnamese pork products have been isolated and characterised. From this product, known as “nem chua” protective cultures have been successfully isolated and these were identified as L. plantarum. One of these strains produced bacteriocins, which showed strong anti-microbial activity against both Gram-positive and Gram-negative bacteria, as well as good pH and thermal stability (Tran, 2010). In addition, this isolate was capable of reducing E. coli by more than 4 log to less than 1 CFU/mL in vitro and less than 10 CFU/g in situ (Tran, 2010).

Whether using as starter culture, probiotics or protective cultures, bacteria strains must survive to a high number within a suitable timeframe for ease of application or exerting beneficial effect. In addition, readily available dried microbial cells would facilitate the transportation, storage and the reduction in preparation time in the applications. Recently, many reviews highlighted several challenges in producing dried microbial culture with good retention of viability over a reasonable time (Figueroa-González et al., 2011; Gaggia et al., 2011; Gupta & Abu-Ghannam, 2012).

2.3 Preservation of bacteria

Depending on preservation methods applied to the biomass, microbial cells undergo different stresses including heat, cold, dehydration, and shear or mechanical, all of which will affect the final survival of bacteria. Therefore, it has become essential that microbial cells are normally mixed with protective agents to either immobilise or encapsulate the cells against these harsh conditions (Tymczyszyn, Gerbino, Illanes, & Gómez-Zavaglia, 2011).

The term “cell immobilisation” or “encapsulation” is sometimes used interchangeably in the literature. However, they were introduced originally as two separate concepts for two different types of technologies. Encapsulation is a technology in which the bioactive components such as probiotics are completely enveloped and covered by a matrix without
any protrusion of the bioactive components whereas in immobilisation, the bioactive component is covered but not necessarily enveloped by the matrix. Nevertheless this distinction is rarely being made in the food literature (Kailasapathy, 2002; McClements, Decker, & Park, 2009).

The word protectant or encapsulation agent will be used throughout this thesis whether it is used to encapsulate or immobilise bacteria. Analysis of research findings from the literature over the last ten years on the preservation of microorganisms by freeze drying, it was found that protein and carbohydrate were the most commonly utilised protective agents to stabilise the bacteria in the dried form (Fig. 2.3) (Khem, May, & Small, 2012). As can be seen in Fig. 2.3, lactose (Lac), maltose (Mal), skim milk (SM), trehalose (Tre), and whey protein isolate (WPI) are the protective agents that provide the highest survival of bacteria following freeze drying. In this thesis, convective drying, a more economical drying process compared to freeze drying, will be investigated to stabilise different strains of L. plantarum and for this purpose protectants which have been shown to work well during freeze drying will be tested.
to provide further insights into the protection mechanism during convective drying bearing in mind that these two drying mechanisms are completely different.

2.3.1 Commonly utilised protective agents

2.3.1.1 Whey protein isolate (WPI)

Since 1830, it was reported that milk contains two types of proteins, which can be separated by acidification. Acidification to pH 4.6 resulted in an insoluble protein called casein representing approximately 78% of the total nitrogen of bovine milk and the soluble protein (approximately 20%) is called whey or serum proteins (Fox & Kelly, 2012). Ultrafiltration of whey protein resulted in whey protein concentrate (WPC) and further treatment of WPC by subsequent diafiltration or ion exchange followed by drying; whey protein isolate (WPI) is obtained (Fox & McSweeney, 2013). The main difference between WPC and WPI is the protein content in the product; in which whey with protein content of over 90% is considered to be WPI. Amino acid composition of whey protein is presented in Table 2.4.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Whey protein</th>
<th>Amino acid</th>
<th>Whey protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (G)</td>
<td>3.5</td>
<td>Serine (S)</td>
<td>5.5</td>
</tr>
<tr>
<td>Alanine (A)</td>
<td>5.5</td>
<td>Threonine (T)</td>
<td>8.5</td>
</tr>
<tr>
<td>Valine (V)</td>
<td>7.5</td>
<td>Cysteine (C)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0</td>
</tr>
<tr>
<td>Leucine (L)</td>
<td>11.8</td>
<td>Tyrosine (Y)</td>
<td>4.2</td>
</tr>
<tr>
<td>Isoleucine (I)</td>
<td>7.0</td>
<td>Asparagine (N)</td>
<td></td>
</tr>
<tr>
<td>Methionine (M)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.4</td>
<td>Glutamine (Q)</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine (F)</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan (W)</td>
<td>2.1</td>
<td>Aspartic acid (D)</td>
<td>11.0</td>
</tr>
<tr>
<td>Proline (P)</td>
<td>4.4</td>
<td>Glutamic acid (E)</td>
<td>15.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> contain sulphur

Table 2.4 Amino acid composition (g AA/100 g protein) of the total protein, casein, and whey protein of bovine milk (Belitz, Grosch, & Schieberle, 2009).
Major constituents of whey protein are β-lactoglobulin (β-lg) representing about 50% and α-lactalbumin (α-la) representing about 20% of the total whey protein with some of the physico-chemical properties listed in Table 2.5.

Table 2.5  Physico-chemical properties of bovine whey protein (Brownlow et al., 1997; Tavares, Croguennec, Carvalho, & Bouhallab, 2014)

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Concentration in milk (g/kg)</th>
<th>Concentration in whey protein (%)</th>
<th>Molecular weight (kDa)</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactoglobulin</td>
<td>3.2</td>
<td>56-60</td>
<td>18.3</td>
<td>5.2</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>1.2</td>
<td>18-24</td>
<td>14.2</td>
<td>4.2-4.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.4</td>
<td>6-12</td>
<td>66.3</td>
<td>4.7-4.9</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>0.4-0.1</td>
<td>6-12</td>
<td>146</td>
<td>5.5-8.3</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1</td>
<td>1-2</td>
<td>83</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Since β-lg is the major whey protein components, its physicochemical properties are normally reflected in whey protein. Basically, β-lg has 162 amino acids with a molecular weight of 18.3 kDa with 15% α-helix, 50% β-sheet, and 15 to 20% reverse turn (Brownlow et al., 1997). X-ray crystallography showed that β-strands are organised into two β-sheets facing each other and forming a flattened and conical barrel called a calyx which constitutes an ideal binding sites for hydrophobic ligands (Kontopidis, Holt, & Sawyer, 2002; Le Maux, Bouhallab, Giblin, Brodkorb, & Croguennec, 2014). β-lg has 5 cysteins, 4 of which forming 2 disulfide bonds. The remaining free thiol group is buried within the protein structure. The disulphide bonds contribute to the reversible denaturation of β-lg (Kitabatake, Wada, & Fujita, 2001) whereas the free thiol stabilises the native protein structure (Jayat et al., 2004).

Environmental conditions such as pH, ionic strength, stresses including heat and pressures can alter the structures and modify the functionalities of whey proteins. At pH of 5.5 to 7.5, β-lg exists as a stable covalent dimer and the structure turns to octamer when pH is adjusted close to its isoelectric point of about 5.2. The behaviour of whey protein undergoing heat treatments have been extensively studied and recently reviewed by several authors (Dufrêne et al., 2001; Ryan, Zhong, & Foegeding, 2013; Wijayanti, Bansal, & Deeth, 2014).
Like any other proteins, the denaturation of β-lg is dependent on pH, ionic strength, nature of ions, and purity of the protein, protein concentration, solvent quality and temperature. As summarised by Ryan et al. (2013) β-lg started to dissociate from dimer to monomer structures between 30 and 50 °C and unfold at higher temperatures of around 78 °C, resulting in exposure of the free thiol group and increased thiol reactivity. At low temperatures the dissociation from dimer is reversible and at higher temperatures, the denatured β-lg loses its
native tertiary and secondary structures and a previously hidden free thiol group becomes exposed leading to protein-protein interactions. Consequently, gelation or precipitation occurs due to heat induced denaturation. This summary is also consistent with the proposed behaviour of β-lg upon heat treatment at pH >6.8 as illustrated in Fig. 2.4 (de Wit, 2009).

Denatured whey proteins were reported to be stiffer, stronger and more stretchable (Kinsella & Morr, 1984). In addition, denatured protein was reported to enhance the encapsulation process as it favours adsorption at the interface resulting in the formation of a thin, gel-like layer (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). Different forms of whey proteins have been investigated in the protection of different strains of LAB resulting in different degree of survival during spray drying. For example, L. reuteri grown and spray dried in liquid whey resulted in 2 log reduction during spray drying (Jantzen, Göpel, & Beermann, 2013). Liquid whey produced by enzymatic coagulation of standard pasteurised milk was autoclaved and used as encapsulant during spray drying of Bifidobacterium Bb-12 was found to protect the cells during spray drying and acid and bile tests (De Castro-Cislaghi et al., 2012).

In addition, denatured WPI was found to provide protection to Bifidobacteria resulting in the highest survival of 25% during spray drying with an outlet temperature of 80 °C. Similarly, denatured WPI was found to provide better protection to L. plantarum both during spray drying and acidic and bile juice (Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012). Although different forms of whey proteins has been tested to encapsulated different strains of bacteria, the mechanism as to how the protein protect bacteria during drying is limited and warrant more investigations.

2.3.1.2 Skim milk

Milk is a highly complex physiochemical system containing protein, emulsified lipids as well as dissolved lactose, minerals and vitamins. It is secreted by the female mammals of approximately 4500 species to meet the complete nutritional requirements of the neotate of the species. Upon creaming by centrifugation, skim milk can be obtained and skim milk in this thesis refers to bovine milk. Milk is a very flexible material, which can be made into different foods with variety of flavours and aroma. This flexibility is mainly due to the
properties of its constituents. In addition, bovine milk, in the form of skim milk has been widely investigated in the preservation of microorganism by drying.

Table 2.6 Typical composition of bovine milk (Belitz et al. 2009)

<table>
<thead>
<tr>
<th>Composition</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.6</td>
</tr>
<tr>
<td>Fat</td>
<td>3.9</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.6</td>
</tr>
<tr>
<td>Ash</td>
<td>0.7</td>
</tr>
<tr>
<td>Casein</td>
<td>2.6</td>
</tr>
<tr>
<td>Whey protein</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Bovine milk consists mainly of disaccharide lactose, fat, casein and whey (Table 2.6) and the investigation of protection of microorganisms by milk protein is on the increase. For example, skim milk has been widely investigated to protect different strains of bacteria during spray drying resulting in survival rate ranging from 1.7 to 100% depending on outlet temperature of spray dryer and concentration of the carrier medium (Fu & Chen, 2011; Maciel, Chaves, Grosso, & Gigante, 2014). When skim milk was used as either growing and spray drying carrier, a survival of 1.7% and 95% were obtained for *L. paracasei* NFBC 338 when spray drying at outlet temperature of 95-100 and 70-75°C respectively (Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002).

Maciel et al., (2014) reported a survival of 77.7% with a cell count of 7.27 log CFU/g after spray drying *L. acidophilus* La-5 at an outlet temperature 85 to 90 °C. Sunny-Roberts & Knorr (2009) showed that spray drying with skim milk is strain specific with a survival of 75 and 55% respectively for *L. rhamnosus* GG and *L. rhamnosus* E800 when spray drying at the same outlet temperature of 65-70 °C.

Although skim milk has been studied widely using different preservation methods to protect different strains of bacteria, it is still unclear as to which constituent(s) of the milk really protect the bacterial cells (Fu & Chen, 2011). In addition, the survival appears to be strain specific and concentration dependent.
2.3.1.3 Lactose

The mild sweet taste of milk is due to a sugar called lactose, which consists of 4-6% of milk. The most stable form is alpha lactose monohydrate, C_{12}H_{22}O_{11}.H_2O. It is a reducing sugar comprising of glucose and galactose, linked by a β 1—4-O-glycosidic bond (Fig. 2.5). Lactose has a low solubility, (70 g/L) at 20 °C and crystallise in the alpha form as monohydrate. In addition, lactose, which has not been crystallised, forms an amorphous glass and is stable when the moisture is low (Fox, 2009). Due to its low sweetness, lactose has been mainly utilised in infant formulae, confectionary industry, and nutraceuticals (Paterson, 2009).

![Molecular structure of lactose](image)

Lactose has also been investigated as the protective agent in the preservation of microorganism, either used alone or in combination with other substances, especially the *Lactobacilli*. For example, lactose was investigated as matrices for the stabilisation of *L. paracasei* during storage after freeze drying in relation to the water activity and temperature. Storing the dried powder below the glass transition temperature, bacteria inactivation still occurred even though the inactivation is lower below glass transition temperature (Higl et al., 2007). Similarly, in a study to investigate the physico-chemical properties of freeze dried *L. rhamnosus*, lactose was found to provide interior protection as compared to either trehalose or mixture of trehalose and lactose in a ratio of 1:1 (Pehkonen, Roos, Miao, Ross, & Stanton, 2008). The viability of *L. acidophilus* freeze dried in the matrix of sucrose/maltodextrin or lactose/maltodextrin was found to decrease in relation to the browning and oxidation reactions (Kurtmann, Skibsted, & Carlsen, 2009). In a spray drying observation, lactose was
found to provide the least protection to *Lactococcus lactis* as compared to sodium caseinate or mixture of lactose and sodium caseinate (Ghandi, Powell, Chen, & Adhikari, 2012). In short, lactose appears to provide some protection to bacteria either during freeze or spray drying.

### 2.3.1.4 Trehalose

Trehalose is a disaccharide of glucose also known as α- D-glucopyranosyl α- D-glucopyranoside with its structure shown in Fig. 2.6. It is a non reducing sugar and is not easily hydrolysed by acid and the glycosidic bond is not cleaved by α-glycosidase.

![Molecular structure of trehalose](image)

As summarised by Ohtake & Wang (2011) trehalose has a high glass transition temperature of 110 to 120 °C, high solubility of 40.6 to 68.9 g/100 g water depending on its purity, 8 equatorial –OH, 12 hydration number at 87 °C with a concentration of 50% (w/w). Trehalose is naturally found in about 100 species including plants, algae, fungi, yeasts, bacteria, insects, and other invertebrates (Elbein, 1974). Welnicz, Grohme, Kaczmarek, Schill, & Frohme (2011) suggested that the utilisation of trehalose as a protectant to preserve microorganisms might have been based on the observation that there is an accumulation of trehalose in anydrobiotic organisms. For example, there has been a report of the accumulation of up to 25% in the cell on dry weight basis of trehalose and sucrose (Buitink & Leprince, 2004).

Trehalose was reported to enhance the survival of *L. salivarius* during freeze drying with a survival rate of 34% as compared to freeze dried cell in water where only 1% of cell survival was observed. The effect of trehalose is even more positive when protective medium was added with skim milk (Zayed & Roos, 2004). In addition, Leslie, Israeli, Lighthart, Crowe, &
Crowe (1995) reported protection of membrane due to trehalose and protein in bacteria during freeze drying.

In terms of convective drying, trehalose was found to provide different levels of protection to two strains of *L. rhamnosus* GG and E800 with survival of 75% and 55% respectively after spray drying with the medium concentration of 20% at an outlet temperature of 65 to 70°C (Sunny-Roberts & Knorr, 2009). However, trehalose did not exert positive effect on *L. plantarum* during fluidised bed drying (Linders, Wolkers, Hoekstra, & Riet, 1997).

In brief, trehalose has been utilised in a number of reports to protect microorganisms during drying with variable survival rates and it appears that the protection is strain specific.

### 2.3.2 Protection hypothesis

It is generally accepted that protective agent can depress phase transition temperature (*T_m*) from liquid crystalline to gel phase when relative water content is less than 0.25 g H₂O in a gram of dry mass (Wolfe, 1987). Two principle hypotheses including water replacement and vitrification were proposed to explain this depression effect. Due to the complexities of bacterial cell membrane, the two hypotheses were mostly formulated based on the results that had been obtained from experiments using membrane models.

#### 2.3.2.1 Water replacement hypothesis

Protectants are usually polyhydroxylated compounds whose hydroxyl groups could form hydrogen bridge bonds to the phospholipids and membrane proteins, thus avoiding the merging of lipids and the inhibition of protein denaturation. An example of the protection of protein by disaccharide trehalose following water replacement hypothesis is illustrated in Fig. 2.7. Water replacement hypothesis is the earliest commonly accepted hypothesis for membrane stabilisation by sugars (hydroxylated compounds). This protection is mainly due to the interaction between the phospholipid head group and the sugar molecules as summarised by Santivarangkna, Higl, & Foerst (2008) and shown in Fig. 2.8.
Fig. 2.7 Diagram showing how trehalose protects protein during drying. According to the water replacement hypothesis, as water progressively removed during dehydration, trehalose forms a protective layer by hydrogen bonding with the protein. Adapted from (Julca, Alaminos, González-López & Manzanera, 2012)

This hypothesis has also recently been validated in a study at atomic detail by using a model membrane and trehalose as the protectant during dehydration (Golovina, Golovin, Hoekstra, & Faller, 2009). Despite its large molecular weights, sugar polymers, polysaccharides can also get inserted and interacted with phospholipid head groups depending on the flexibility of their structures (Vereyken, Chupin, Hoekstra, Smeekens, & de Kruijff, 2003). For example, inulin which tends to form a random coil structure has a better interaction with the membrane than levan, which tends to form helix structure. Likewise, glucose based and branched chain polysaccharides dextran barely interacts with the phospholipids (Vereyken, van Kuik, Evers, Rijken, & de Kruijff, 2003).

2.3.2.2 Glass formation hypothesis

When the materials are in the amorphous metastable states similar to solid, but do not possess defined and specific molecular arrangement, it is called the glassy state. Glassy materials are very viscous and look like solid, but the molecular arrangements are like liquid without any specific order. Glassy state shows thermodynamically nonequilibrium states and their properties are temperature and time dependent (Angell, 1988). The most common parameter describing the glassy state is the glass transition temperature ($T_g$) and this occurs over a temperature range; below which materials exhibit extremely high viscosity.
Fig.2.8 Sugars depress the membrane phase transition temperature ($T_m$) by specifically interacting with phospholipid head groups (A1). Large polymeric sugars with rigid structures such as dextran cannot enter between the head groups (A2) whereas flexible ones such as inulin (A3) can fit into the spaces. Therefore, the latter are able to protect cells despite their relatively large size. By contrast, vitrification hypothesis suggests that the sugars can depress $T_m$ without specifically interacting with the head groups, e.g. by osmotic and volumetric effects and by vitrification of sugars between the membranes (B1). Polymeric sugars with a very high molecular weight are not capable of depressing the $T_m$ because they have fewer osmotic effects and are excluded from the intermembrane space during the removal of water (B2). This exclusion curtails the volumetric and vitrification effects of the polymeric sugars. The source of this figure and the caption was Santivarangkna et al., 2008.

Glass formation (vitrification) hypothesis states that sugars protect the lipid membrane by osmotic and volumetric properties and no particular sugar-lipid interaction is required (Wolfe & Bryant, 1999). It is the solidification of a liquid without crystallisation and the resulting amorphous glass not only retains the random molecular arrangement of a liquid, but also has the mechanical properties of a solid. The glassy materials are highly viscous and can arrest any chemical reactions during prolong storage (Roos, 2010).

The degree of protection provided by different sugars is believed to be due to the differences in their glass forming tendencies, which is reflected in their glass transition temperatures normally determined by differential scanning calorimetry (DSC). Normal phospholipid are hydrated and upon drying with decreasing amount of water, they may turn to gel phase or there is a gradual increase in membrane phase transition temperature ($T_m$) (Bryant, Koster, & Wolfe, 2001). The $T_m$ of *L. plantarum* shifted from 4 °C in hydrated cells to 20 °C in dried...
cells (Linders, et al., 1997). The glass transition temperature ($T_g$) of protectants should be higher than $T_m$ during drying. Trehalose which is often reported to provide better protection to different strains of bacteria might have been due to its high glass transition temperature as compared to other sugars (Green & Angell, 1989). In addition, the protection afforded by different sugars has been reported to depend on the molecular weight of the sugars or polymer sugars as well. A relatively large size polymer will not be able to fit between the surfaces in order to give volumetric effects and thus would not be able to protect the membrane lipid as schematically shown in Fig. 2.8.

Both protection mechanisms are probably supplementing each other in the preservation of microorganisms (Clegg, 2001). Although not sufficient by itself, it has been shown that vitrification is often necessary to stabilise biomolecules in the dry state (Potts, 2001). The first is related to the protection provided by the direct interaction between polyhydroxyl compounds (carbohydrates and other compatible solutes) with the membrane or water replacement. The second hypothesis relates to the role of protectants in the vitrification of cytoplasm that could preserve the biological structures by immobilisation during dehydration.

Although these two protection mechanisms have been widely accepted in the preservation of biomolecules or model membranes; questions still arises in regards to the protection of bacteria. For one thing, bacterial cell wall is a complex organisation of macromolecules consisting of peptidoglycan sacculus decorated with other glycopolymers such as teichoic acids or polysaccharides and proteins (Chapot-Chartier & Kulakauskas, 2014) embedded over the plasma membrane (Fig. 2.2). The protectant molecules might have had some kinds of association with the macromolecules in the cell wall before penetrating through peptidoglycan and interacting with the phospholipid membrane. In addition, currently most glassy states are normally referred to the protectant molecules and very limited if any studies have reported on the actual state of the bacteria cells during preservation (Santivarangkna, Aschenbrenner, Kulozik, & Petra, 2011).

### 2.3.3 Encapsulation techniques

In the food industry, encapsulation can be used in many applications including stabilising the core material, controlling the oxidative reactions, providing sustained or controlled release, masking flavours, colours or odours, extending the shelf life and protecting components
against nutritional loss. In terms of microorganisms, encapsulation improves cell viability against processing stresses, facilitates handling of cells and allows a controlled dosage (Huq, Khan, Khan, Riedl, & Lacroix, 2013). There are many encapsulation technologies that have been developed resulting in capsules with different size and shape (Fig. 2.9), but no single encapsulation technology is suitable for every application. As a result, different microcapsules can be produced depending on the encapsulating agent and the processes used. Interest in microencapsulation is growing as evidenced by an exponential growth in publications of microencapsulation by spray drying over the last decade (Fig. 2.10). The active ingredient including bacteria is usually located in the core of the microcapsules surrounded by encapsulating agent or dispersed in one matrix containing the encapsulating agent. The choice of microencapsulation method and the encapsulating agent are independent. Therefore, it is very important to understand the interaction and structural stability of bioactive ingredient including bacteria before selecting any appropriate encapsulation method and material (Corona-Hernandez et al., 2013; McClements et al., 2009). Table 2.7 summarises the commonly utilised methods to encapsulate bacteria with major steps involved in each techniques.

Fig. 2.9 Microcapsules with different morphologies: Matrix (A); simple microcapsule (B); irregular microcapsule (C); multiwall microcapsule (D); multi core microcapsule (E) and aggregate of microcapsules (F). Adapted from (Gibbs, Kermasha, Alli, & Mulligan, 1999)

There are many different encapsulation techniques to protect bacteria; however, each technique has their own benefits and drawbacks as summarised in Table 2.8. Encapsulation by spray drying has gained more interests due to low cost and available equipment in the food industry and it is also one of the oldest methods in encapsulating food ingredients since 1930s (Shahidi & Han, 1993). Furthermore, the method has been applied successfully in food industry for several decades (Gouin, 2004).
Table 2.7 A summary of bacteria microencapsulation techniques and the major processes involved (Adapted from Desai & Park 2005)

<table>
<thead>
<tr>
<th>Nº</th>
<th>Microencapsulation technique</th>
<th>Major steps in encapsulation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spray drying</td>
<td>➢ Preparation of bacteria cell in feed solution (protectant) ➢ Homogenisation of the dispersion ➢ Atomisation of the dispersion ➢ Dehydration of the atomised particles</td>
<td>Fu &amp; Chen, 2012</td>
</tr>
<tr>
<td>2</td>
<td>Freeze drying (Lyophilisation)</td>
<td>➢ Mixing of bacteria in protectant solution ➢ Freeze drying of the mixture</td>
<td>Morgan, Herman, White, &amp; Vesey, 2006</td>
</tr>
<tr>
<td>3</td>
<td>Emulsification</td>
<td>➢ Bacteria cells are dispersed in emulsion ➢ Cooling or adding cross linking agent ➢ Centrifugation or filtration to get the capsules ➢ Drying</td>
<td>Rathore, Desai, Liew, Chan, &amp; Heng, 2013</td>
</tr>
<tr>
<td>4</td>
<td>Extrusion</td>
<td>➢ Preparation of molten coating solution ➢ Dispersion of bacteria cells into molten polymer ➢ Solidification of the coating</td>
<td>Krasaekoopt, Bhandari, &amp; Deeth, 2003</td>
</tr>
<tr>
<td>5</td>
<td>Coacervation</td>
<td>➢ Formation of a three immiscible chemical phases based on electrostatic interaction between bacteria cells and coating ➢ Deposition of the coating ➢ Solidification of the coating</td>
<td>Hernández-Rodríguez, Lobato-Calleros, &amp; Pimentel-Gonálex, 2014</td>
</tr>
<tr>
<td>6</td>
<td>Fluidised bed coating</td>
<td>➢ Preparation of the coating solution ➢ Fluidisation of core particles (cells) ➢ Coating of bacterial cells</td>
<td>Schell &amp; Beermann, 2014</td>
</tr>
<tr>
<td>7</td>
<td>Spray cooling</td>
<td>➢ Preparation of the dispersion ➢ Homogenisation of the dispersion ➢ Atomisation of the dispersion into cool medium such as liquid nitrogen</td>
<td>Okuro, Junior, &amp; Favaro-Trindade, 2013</td>
</tr>
<tr>
<td>8</td>
<td>Electrospraying/electrospinning</td>
<td>➢ Bacterial cell suspension in polymer solution is sprayed using high potential electric field ➢ Using high voltage as well as high shearing force</td>
<td>Bhushani &amp; Anandharamakrishnan, 2014</td>
</tr>
</tbody>
</table>
Table 2.8 Summary of the most important characteristics of some microencapsulation methods (adapted from Gouin (2004)).

<table>
<thead>
<tr>
<th>Nature of the ingredient</th>
<th>Fluidised bed</th>
<th>Coacervation</th>
<th>Spray drying</th>
<th>Extrusion</th>
<th>Freeze drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilic</td>
<td>Straightforward</td>
<td>Challenging</td>
<td>Feasible</td>
<td>Feasible</td>
<td>Feasible</td>
</tr>
<tr>
<td>Lipophilic</td>
<td>Unfeasible</td>
<td>Challenging</td>
<td>Feasible</td>
<td>Feasible</td>
<td>Feasible</td>
</tr>
<tr>
<td>Amphiphilic</td>
<td>Challenging</td>
<td>Challenging</td>
<td>Challenging</td>
<td>Challenging</td>
<td>Feasible</td>
</tr>
<tr>
<td>Solid</td>
<td></td>
<td>Feasible</td>
<td>Unfeasible</td>
<td>Challenging</td>
<td></td>
</tr>
<tr>
<td>Liquid</td>
<td>Unfeasible</td>
<td>Feasible</td>
<td>Feasible</td>
<td>Feasible</td>
<td>Feasible</td>
</tr>
<tr>
<td>&gt;100 µm</td>
<td>Feasible</td>
<td></td>
<td>Unfeasible</td>
<td>Feasible</td>
<td>Feasible</td>
</tr>
<tr>
<td>&lt;100 µm</td>
<td>Unfeasible</td>
<td>Feasible</td>
<td>Unfeasible</td>
<td>Need grinding</td>
<td></td>
</tr>
</tbody>
</table>

| Cost in use              | Medium        | High         | Low          | High      | Very high    |
| Production capacity      | Batch wise    | 1000 kg      | 500 kg       | -         | Unfeasible   | Low         |
| Continuous               | -             | -            | 2,000 kg/h   | -         | -            |

Fig. 2.10 Cumulative number of publications related to the encapsulation of bacteria published in the last decade. (Source: [www.scopus.com](http://www.scopus.com) retrieved on 10 August 2013)
2.3.4 Spray drying as a process of microencapsulation of bacteria

The development of spray drying was primarily for the manufacture of milk powder. The drying of milk can be considered as a process of encapsulation as milk fat is the core material that is protected against oxidation by a wall material consisting of mixture of lactose and milk proteins. It is one of the cheapest methods in microencapsulation of microorganism and food ingredient. The process involves four steps including preparation of the dispersion or emulsion to be processed, homogenisation of the dispersion, and atomisation of the dispersion into the drying chamber and the dehydration of the atomised particles (Shahidi & Han, 1993).

To obtain good microencapsulation of microorganisms, some of the process parameters of the spray drying need to be optimised including the air inlet and outlet temperatures, drying time and nozzle pressures. Increasing air inlet temperature was reported to correlate with the decreasing survival of bacteria (Ghandi et al., 2012; Mauriello, Aponte, Andolfi, Moschetti, & Villani, 1999). However, Kim & Bhowmik (1990) reported that the higher inlet temperature only has a slight effect on cell survival. This is probably because the inactivation of bacteria during drying is a combined effect of temperature and time. As previously mentioned, droplet drying after atomisation happens in different stages. First, the droplet temperature corresponds to the constant drying rate period where the temperature of the droplet and the heat inactivation is limited to the wet bulb temperature. As a consequence of evaporative cooling effect during this period, the survival of bacteria is strongly related to the outlet temperature. After the constant drying rate period, particle will undergo falling rate period where particle surface becomes dry and the temperature of the product increases depending on dryer configuration (Boza, Barbin, & Scamparini, 2004). During this stage, the inactivation of bacteria depends on the drying parameters such as outlet temperature, residence time and feed rate (Santivarangkna et al., 2008).

Literature suggests that increasing outlet air temperature correlates with a lower survival of microorganisms (Lian, Hsiao, & Chou, 2002; Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). The air outlet temperature cannot be directly controlled as it depends on the drying characteristic of the materials. The outlet temperature which has been used in spray drying microorganism with varying degree of survival was reported to be 40-90°C (Fu & Chen, 2011).
The appropriate adjustment of the inlet temperature is very important because it is directly proportional to the microcapsule drying rate and the final water content. If the inlet temperature is low, the low evaporation rate causes the formation of microcapsules with high density membranes, high water content, poor fluidity, and easiness of agglomeration. However, if the inlet temperature is too high, it will inactivate the microorganism and high evaporation rate would crack the membrane. The inlet temperature is controlled by two factors—the temperature which can safely be used without damaging the product or creating operational hazards and the comparative cost of heat source (Fogler & Kleinschmidt, 1938).

It has been suggested that bacteria inactivation during spray drying happens due to both heat and dehydration inactivation. However, it is still unclear which is more damaging as both happen simultaneously.

Although heat has generally been used to inactivate harmful bacteria to ensure food safety, many studies have been focused on the more critical component so that a more effective means can be devised to inactivate bacteria. Gould (1989) presented a well-known theory which state that heat is assumed to inactivate the critical component of bacteria at the same time of destroying other less critical components. The loss of less critical component does not cause death until their numbers are reduced to very low or cell is subjected to additional stresses. The damage of critical component or the sequence of damaging is difficult to identify because heat simultaneously affects individual molecule and structure in the cell although the damage to macromolecules such as DNA or RNA from the action of heat has been identified (Abee & Wouters, 1999; Van de Guchte, Serror, Chervaux, Smokvina, & Ehrlich, 2002).

Ribosomes damage from the action of heat has been identified as the critical component leading to cell inactivation although some macromolecules such as DNA, RNA and protein and membranes have also been reported to be damaged by the action of heat (Santivarangkna, Wenning, Foerst, & Kulozik, 2007). Apart from ribosomes, other critical components including cell envelopes, DNA and RNA polymerase have also been reported as other critical components. Cell death may be due to the destruction of more than one critical component.

Since water constitutes about 70-95% of the cell mass, the removal of water imposes stresses to the cells because water molecules are connected with the stabilities of proteins, DNA,
lipids as well as providing structural order of bacteria cells (Potts, 1994). The removal of free water is believed to cause no harm to bacteria survival (Santivarangkna et al., 2007) as approximately 80% of physiological reactions of organisms mainly depend on the movement of bound water (Webb, 1960). Several cellular components are affected when drying to a certain water level. It was reported that cytoplasmic membrane is the most sensitive component in the dehydration process because there is a loss of several intracellular components when the membrane is damaged (Li, Lin, Chen, Chen, & Perce, 2006; Riveros, Ferrer, & Borquez, 2009). In addition, lipid bilayer of the membrane is a primary target of dehydration induced damage as it is thermally unstable. Apart from this, cells come in contact with large volume of air during spray drying and consequently lipid oxidation is likely to occur (Santivarangkna et al., 2007; Teixeira, Castro, & Kirby, 1995). However, the damage of cell membranes are generally reported in the membrane model rather than intact bacteria cells where the constituents are far more complicated than the lipid membrane.

2.3.5 Selection of encapsulation materials

Appropriate selection of encapsulation agent during spray drying can overcome the above mentioned inactivation of microorganisms. Since spray drying processes is carried out from aqueous feed formulation, the wall material must be soluble in water at an acceptable level. In addition, wall material should possess good properties of emulsification, film forming, and drying and the wall solutions should have low viscosity (Gouin, 2004). Many biopolymers including protein with food grade status are available in meeting the above requirements. In addition in selecting any particular proteins, polysaccharides or other components to form biopolymer particles, several factors should be considered including the ability of the components to be assembled into particles, the functional requirement for the particles, the legal status, cost, ease of use, and consistency of the ingredients and processing operation (Matalanis, Jones, & MacClements, 2011).

Using protein as an example to consider in selecting encapsulation material, the following factors are suggested by Matalanis et al., (2011). First, it requires the knowledge of physico-chemical properties of the proteins involved such as thermal denaturation temperature (for globular protein), helix-coil transition temperature (for gelatine or collagen), isoelectric points ($pI$), sensitivities to specific monovalent or multivalent ions, or susceptibility to specific enzyme or chemical cross linking or degradation reaction. Understanding these
properties enable us to establish the conditions where the protein molecules are able to associate with other protein or non-protein structure forming molecules such as environmental and solution conditions. Second, the establishment of the charge characteristic of protein molecules involved is vital as electrostatic interactions are often utilised in the structure formation. Protein is positively charged below its \( pI \), zero charge at its \( pI \) and negatively charge above the \( pI \). Although at \( pI \), the protein is supposed to be neutral (no charge), the protein still has both positive and negative region on its surface thus it can be involved in attractive and/or repulsive electrostatic interactions. Finally, it is usually important to have knowledge of the nature of the biopolymer particles that can be formed after protein association, such as their morphology, physical properties, size, charge, and stability.

2.4 Summary of current knowledge

Based on extensive literature review of the current knowledge by taking into consideration all relevant factors involved in the preservation of bacteria, it is evident that bacteria encounter various stresses which can compromise the viability no matter what preservation method is utilised. The most commonly utilised practice in the preservation of bacteria is the addition of protectant/encapsulant materials before dehydration. Although carbohydrates, proteins and milk are the most commonly utilised encapsulants in the preservation of bacteria, the retention of bacterial viability appears to vary among species and strains. In addition, a systematic study on the selection of a specific protectant for any specific microorganism is limited. Milk is a complex mixture of many components and it is also the most commonly utilised protective agent. However, it is not conclusive as to what constituent of milk is really providing protection to bacteria during spray drying.

Two hypotheses have generally been accepted to explain the protection mechanism of bacteria during dehydration including water replacement and vitrification. However, both hypotheses were based on experiments involving a model of the lipid membrane of the cells whereas the cell walls of LAB are far more complex. Apart from the lipid membrane, bacterial cell walls consist of a thick layer of peptidoglycan woven with cell wall protein and other polysaccharides. The study on the interaction between bacteria cell wall constituents and the encapsulant/protectant matrix is limited, especially the interaction between \( L.\ plantarum \) and whey protein isolate as the encapsulation material during spray drying.
Accordingly the research described in this thesis was designed to study the interaction between bacterial cell wall molecules and the encapsulation materials in order to provide further insights into the mechanism of protection of bacteria during dehydration.

It should be noted that, initially, the project was primarily focusing on the preservation of bacteria by freeze drying. Therefore, a considerable amount of time was spent on reviewing literature related to freeze drying resulting in a review paper published and presented at 18th International drying Symposium, IDS 2012 in China and this paper is presented at the end of this literature review chapter. However, due to the complication in obtaining the necessary equipment and training; the project was shifted to convective drying.
MAXIMIZING CELL SURVIVAL IN THE PRESERVATION OF LACTIC ACID BACTERIA BY FREEZE-DRYING

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Abstract: Lactic acid bacteria (LAB) are one of the most important groups of bacteria associated with the production of fermented foods and health promoting products. The significance of these bacteria has driven researchers to find ways to preserve them for food applications and the development of health promoting products. One of the most commonly utilized methods of LAB preservation is freeze drying; however, the loss of viability is inevitable for any preservation methods including freeze drying. In this article, we aim to discuss different factors including freezing temperature, protective substances and their concentration in the drying media, affecting cell survival during freeze drying and the conditions affecting storage survival.

Keywords: freeze-drying, lactic acid bacteria, protectants

INTRODUCTION

Cell survival of microorganisms is the prime concern in any drying approach and many studies have been carried out over the years. However, most scientific communications are difficult to interpret and cannot be applied without spending an extensive amount of time on the literature. One paper that does provide a comprehensive and useful summary of the different thermal drying processes used to dry different species and strains of micro-organisms, temperature and carrier (protectant) and their highest survival is that of Fu and Chen (1). However, their review was focused primarily on thermal drying.

Our aim, in this paper, is to take a further step and produce a database of information published over the last decade on the drying of lactic acid bacteria (LAB) by freeze-drying. In particular, we have focused on the freezing temperatures, protectants used, highest survival and the effect of storage on survival. LAB is of considerable interest as they are generally considered as safe (GRAS) microorganisms. In addition, most probiotic species of commercial interest belong to this group of bacteria. Freeze-drying has long been considered to be an effective method for drying temperature-sensitive materials including microorganisms. In this paper, we also discuss the results of over 30 groups of researchers on the range of protectants and the concentration used, the range of Lactobacillus species studied and the freeze drying parameters that affect the survival of lactic acid bacteria.

LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) constitute a group of Gram-positive bacteria having similar morphological, metabolic and physiological characteristics. These technological and beneficial microorganisms consist of different genera including Lactobacillus, Lactococcus, Pediococcus, Leuconostoc, Carnobacterium, Enterococcus, Oenococcus, Streptococcus, Tetracoccus, Vagococcus, Aerococcus and Weissella (2, 3). Among these genera, Lactobacillus, Lactococcus, and Pediococcus are widely accepted as the core of the group (4, 5). Some of the genera including Lactococcus contain species which are recognized as animal or human pathogens (2).

Lactobacillus is a genus with 84 species compared to only three species each for Pediococcus and Lactococcus listed as microorganisms with “general recognition of safety” in a project jointly developed between the International Dairy Federation and the European Food and Feed Cultures Association (3). In addition, most of the probiotic foods in the market consist primarily of Lactobacillus strains (6). Some of the notable probiotic microorganisms in this genus reported in the literature include Lactobacillus rhamnosus GG, L. casei, L. casei Shirota, L. acidophilus, L. johnsonii, L. plantarum (7).

Lactobacilli are microaerophilic Gram-positive bacteria found in a variety of environmental niches including nutrient rich dairy systems, human mucosal surfaces and natural ecology of plants and soil (8). These microorganisms are presently further
subdivided into three groups (9). Obligate homofermenters, in which _L. delbrueckii_ and _L. acidophilus_ and _L. helveticus_ are some of the examples. Facultative heterofermenters ferment hexose sugars to lactate and other metabolites through the EMP metabolic pathway. These represent some species important in food fermentation particularly _L. plantarum, L. casei_, _L. sakei_. The final group includes such species as _L. brevis, L. fermentum_, and _L. reuteri_ are strict heterofermentative in which lactic acid and acetic acid are the main final metabolites.

There have been extensive applications of _Lactobacilli_ in food, feed production, and commercial health-promoting probiotic products (8). To realize their full potential, strains of _Lactobacillus_ have to be readily available in a form which could facilitate the transportation, and storage along with the retention of their biological functions. Freeze drying is conventionally used to produce microbial cells which are relatively sensitive. The following sections review and discuss strategies to maintain cell survival and functionalities during freeze drying of different strains of _Lactobacillus_.

THE FREEZE-DRIYING PROCESS AND HOW IT AFFECTS CELLULAR DAMAGE

_Freeze drying equipment_

Freeze drying systems consist of a drying chamber, a condenser, a vacuum pump, and a heat source. The drying chamber in which the sample is placed and heating/cooling occurs must be vacuum-tight and with temperature controlled shelves. The condenser and vacuum pump respectively function to collect water vapour from the product and to remove non-condensable gases to achieve high vacuum levels in the chamber and condenser.

For many years, freeze drying has been applied in various industries and processes including food, pharmaceutical, plant materials, heat sensitive sample preparation, long term storage of HPLC samples as well as microbial cultures. Like any other drying technique, freeze drying is a process of removing water or other solvents from a product. The difference; however, is that the removal of the solvent from frozen products takes place through sublimation. This process of drying consists of three stages – pre-freezing, primary drying and secondary drying.

_Pre-freezing_

In the pre-freezing step, materials to be freeze dried first need to be adequately frozen because freeze drying involves the change of state from solid to gaseous phase. During this step, microbial cells are subjected to a variety of stresses including concentration and ice formation.

_Primary drying_

Primary drying involves the removal of free water and some of the bound water from the sample by sublimation, resulting in a dry, structurally intact product. Sublimation is generally capable of removing most of the free water in the matrix. This process might affect the properties of many hydrophilic macromolecules within the cells (10).

_Secondary drying_

During the desorption step, which is the removal of confined water may lead to the damage of surface proteins, cell wall structures and membranes because these water molecules have an important role in stabilizing structural and functional integrity of biological macromolecules through various types of weak bonding, including those present on the cell wall and cell membrane (11). In addition, it has been proposed that the lipid fraction of the cell membrane is the primary target area for damage during drying, where lipid peroxidation may occur and the secondary structures of RNA and DNA destabilize resulting in reduced efficacy of DNA replication, transcription and translation (11).

Freeze drying has also been demonstrated to affect some key enzymes which are important in the metabolic activity of the cells. Lactate dehydrogenase activity was reported to significantly decrease after freeze drying (12).

Removing water from the hydrogen bonding within the phospholipid bilayer increases the head group packing and forces the alkyl chains together. As a result, the lipid component may undergo a transition from lamellar to gel phase, which can be seen as a dehydrated lamellar phase in which the chains are stiff and fully extended. Moreover, certain phospholipids undergo a transition from lamellar to the hexagonal phase as water is removed (13).

FACTORS AFFECTING CELL SURVIVAL

Factors affecting cell survival of different strains of the genus _Lactobacillus_ during freeze drying have been reported by a total of 30 papers published in various journals between 2003 and 2012. These have been compiled into a database created by the authors for this study. The factors included strain, freezing temperature, protectant type and concentration. Graphs showing the interaction between different factors on survival were generated from the database.

It is important to mention that three of the published papers, Martos et al. (14), Reddy et al. (17) and Juarez Tomás et al. (38), reported cell survival rates calculated from the formula \( \frac{\log(n_1)}{\log(n_0)} \times 100\% \) (where _n_0 and _n_1 are the number of colony forming units (cfu) per mL before and after freeze drying respectively), rather than from \( \frac{n_1}{n_0} \times 100\% \). Due to the use of the inappropriate formula for calculation,
data from these studies were excluded from this analysis.

Where possible, survival rate has been calculated directly from the cfu counts reported by the various authors to ensure consistency in the method of calculation.

Different protectants have been considered and studied to aid survival during freeze drying. Protective substances also tend to minimize the stresses encountered during desiccation and storage. Sugars, alcohols, amino acids, proteins, oligosaccharides, prebiotics and anti-oxidants have been utilized in the drying medium resulting in higher survival rates compared to the drying medium without protectants (Figures 1 and 2).

The sugars most commonly utilized in drying media are the disaccharides trehalose, sucrose and lactose followed by sugar alcohols including sorbitol and glycerol, prebiotics including inulin, maltodextrin, dextran, all of which are polyhydroxy compounds as well as milk protein preparations.

The range of survival rates of different Lactobacillus after freeze drying for each of the protectants used in studies considered by this paper are presented in Figure 1.

**DISCUSSION**

**Protection of cells by sugars and protein**

In almost every Lactobacillus species, sugars and proteins provide protection against the processing conditions during freeze drying, resulting in higher cell survival rates. Sugars as cryo-protectants work in many ways to protect the cells. They provide protection against leakage resulting from membrane phase transitions, protection against cryo-injuries and vitrification in the frozen formulation and protection against thermal injury (15). Another mechanism for the protection of sugar during the process of dehydration is the direct interaction with the bacteria by establishing hydrogen bonding with the membrane proteins and/or the lipid bilayer (16). For example, the non-reducing sugar trehalose is able to form an amorphous or glassy solid which can reduce damage to the cell membrane and protein by minimizing ice formation. Moreover, it has also been shown to provide protection during desiccation by interacting with lipid bilayers and lower their transition temperature from a liquid crystalline phase to a more rigid gel phase (17). Similarly, sucrose protects cells during drying by sustaining membrane integrity, surface zeta potential, hydrophobicity and fluidity (18).

In addition to sugars, protein also provides additional protection leading to the higher survival of LAB. For instance, skim milk has been found to be capable of stabilizing the cell membrane constituents and create a porous structure in the freeze-dried product that facilitates rehydration easier (19). It also contains proteins which provide a protective coating for the cells during freeze-drying (20). Recently, Li et al., (12) measured the cell membrane fluidity and integrity and concluded that skim milk solids prevent cellular injuries by stabilizing cell membrane constituents.

Prebiotics have also been added to the drying medium in an attempt to improve the survival of probiotic bacteria. For example, in a study utilizing response surface methodology for the optimization of protective medium of Lactobacillus rhamnosus E/N cells during freeze drying, the optimal medium combination was determined to be spirulina 1.3% (w/v), lactulose 5.48% (w/v) and sucrose 13.04%
(w/v) (21). Likewise, the use of galacto-oligosaccharides as prebiotics demonstrated to be highly efficient in the preservation of *Lactobacillus bulgaricus* with higher content correlated with higher protection (22).

There are three hypotheses which have been raised to explain the protective role of polyhydroxylated compounds including sugars and polysaccharides in preservation. First, it is postulated that there are maintenance of macromolecular structures by interaction between the moieties of the protective compounds with polar residues of macromolecules in the dried state. Secondly, it is generally agreed that the compounds can result in the formation of a glassy state by raising the glass transition temperature of the dried matrix and; the final protective mechanism proposes that, in the presence of water, sugars are excluded from the surface, which may concentrate residual water molecules close to the bimolecular surface, thus preserving to a large extent its solvation and native properties. It is possible that the three mechanisms operate simultaneously during the dehydration-rehydration processes (22).

In summary, a variety of carbohydrates as well as prebiotics have been used as drying carriers resulting in enhanced cell survival after drying although there have been some reports of survival being strain dependent.

Although protectants enhance cell survival during desiccation, it should also be noted that survival following freeze drying is different for particular species and even strains within the same species. For example, the survival of *L. rhamnosus* GG freeze dried without protectants was up to 87.9% (Figure 2) (23); whereas other strains barely survived without the addition of protectants. It is important to note that this appears to be an exceptional case. This strain may be successfully produced in a more harsh environment, using the more economical method of spray drying with no difference in viability following spray and freeze drying (24). However, in another study on the same strains of *L. rhamnosus* GG, freeze dried without protectants, the survival rate was only 18% (25). This difference probably reflects the use of a complex growing medium in the latter, whereas the former involved a standard MRS broth indicating that growth medium also has an effect on survival during freeze drying. Likewise, Carvalho et al., (26) reported survival of 85.1% of *L. delbrueckii* ssp. bulgaricus in a modified growing medium compared to only 51.1% survival in a standard MRS growing medium (27) for the same strain upon freeze drying using the same parameters of freezing temperature and protectant. Similarly, different strains of *L. gasseri* show varying tolerance to freeze drying. Without protectant, *L. gasseri* CRL 1421 has a survival of 1.3% which is higher than for *L. gasseri* CRL 1412 which barely survives freeze drying (28).

**Freezing temperature**

This is another parameter affecting cell survival after freeze drying. As can be seen from Figure 3, freezing temperatures ranging from -196 to -18 ºC have been used to test the survival of different *Lactobacillus* species. Generally, most of the studies focused on the -80ºC region resulting in varying survival rates. Freezing cell suspensions at temperatures in the range of -40 to -18ºC resulted in the highest survival rates after freeze drying for some strains.

Freezing is considered to be a very important step affecting the survival of LAB, in addition to its role in determining the morphology, ice crystal size and the phase changes of the drying matrix (29). For example, approximately one third of the cells surviving the freezing step can live through the dehydration step (30). Reflecting the difficulties of determining the viability of the cells in the frozen matrix, it should be noted that cells undergo other lethal stress during thawing in addition to the cryoinjury (31).

In the cell matrix prior to freeze drying, there are two types of water — cellular water and water of hydration (32). The former is freezeable and plays an important part in sustaining the osmotic equilibrium within the bacterial cells. The freezable water is mobile depending on the freezing rate and has been reported as one of the parameters affecting cell survival (33-35).

When temperature drops to the respective freezing points, the concentration of the intracellular and extracellular solutions is increased and the cells become dehydrated which is similar that experienced during the stress encountered during drying. In this regard, Gehrke, Pralle, and Deckwer, (36) proposed two effects of cell inactivation — concentration and recrystallization. During slow freezing, extracellular ice formation leads to the increased concentration of within the cell and some precipitation of solutes due to water moving out of the cell. The higher solute concentration brings about cell damage. On the other hand, for high freezing rates, there will be small
intracellular ice crystals and these have a tendency to transform into larger crystals. These are detrimental to the cells and therefore; the freezing rate should be compromised — slow enough to prevent formation of intracellular ice crystal and fast enough to avoid water migrating from the cell leading to a concentration effect.

In this respect, it is worth mentioning that the rate of water removal from the cell depends on the surface to volume ratio of the bacterial cells as well as the structure of the cells. For example, the Gram-negative bacterium *Escherichia coli*, which has only a murein monolayer in the cell walls, has an optimal freezing rate of 10 °C/min; whereas the cells of the Gram-positive *L. plantarum*, with around 40 layers of murein has an optimal freezing rate of 1-2 °C/min as it take more time for the water to migrate out of the cells (36).

However, it was found that the optimal cooling rate of *E. coli* was 2.1°C/min and that of the Gram positive *Pseudomonas fluorescens* was very high at 233 °C/min (37). In the freeze drying of *L. paracasei*, encapsulated in a matrix of maltodextrin and trehalose, it was found that freezing the cell at -18°C resulted in a high viability of the cells (35). This appears to highlight the effect of the formulation as protective medium on the optimal cooling rate which is also reported in the *Lactobacillus coryniformis* where the best rate was shifted from 3.9 to 2.7°C/min at 2% and 20% sucrose respectively (33).

In short, it can be inferred that the cooling rate is a relative term which was defined differently by various authors without appropriate reference to sample volume and end temperatures. Consequently, cooling should be precisely described so that meaningful comparisons can be drawn.

Concentration of protectants

Concentration of the protective medium is also another parameter affecting cell survival during freeze drying. In general, higher concentrations of a protective substance are found to correspond to higher survival rate of LAB. Membrane properties of *L. casei* Zhang, measured using zeta potential, hydrophobicity, fluidity and integrity, before and after freeze drying, showed that sucrose was a better effective membrane protectant at higher concentration of 8.0% (18). In addition, the presence of 12% of either sucrose or lactose in either 6% skim milk or water resulted in a higher cell survival and enhanced functional properties during storage of some probiotic strains of *Lactobacillus* (38).

The protection by high concentrations is probably due to the effect of penetration into the cells as there have been reports indicating that protectants capable of transporting into the cell membrane result in higher survival than that of many high molecular weight polymers that cannot penetrate the membrane (35). However, concentration effect might also be strain dependent as there are also reports of very low or high concentration of protectants that can result in highest cell survival. For example, *L. delbrueckii* ssp. *bulgaricus* experienced the highest cell survival after freeze drying in the presence of galactooligosaccharides at a concentration of 38% (22). In contrast, *L. acidophilus* ATCC 4962 survived best at an optimum concentration of 0.3% of skim milk or skim milk with malt extract and monosodium glutamate (39). Again the protection by the drying medium differs between species and strains as well as the type of medium used.

Many studies have focused only on the viability of LAB following freeze drying and storage. However, the retention of viability and functionality of LAB following storage is equally important and should be examined in the evaluation of a successful drying process for any microorganism.
Survival after storage

Many factors have been reported to affect cell viability during storage including addition of protective substances, relative humidity, storage temperature, packaging materials, addition of antioxidant, final moisture content, water activity of the dried microbial powder and the mobility of the molecules.

Generally, the addition of sugars, prebiotics, protein or anti-oxidant in the drying medium enhances the stability of the dried cells during storage (26, 27, 40-42). For example, Sodium ascorbate was reported to result in significant stability of Lactobacillus acidophilus compared to samples freeze dried without ascorbate during storage (43). Likewise, with an optimum combination of cryoprotectants L. reuteri C10 stored at 4 and 30 °C for six months survived up to 96.4 and 78.3%. This is in comparison to the total viability loss of the cells without cryoprotectants of 12 and 8 weeks at 4 and 30 °C respectively (44).

Similarly, trehalose and sucrose proved to be the best protectants during storage at 35 °C of L. plantarum even though the survival declined after six months of storage (45). However, in a study on the effect of addition of glycerine, mannitol, sorbitol, inulin, dextrin and crystalline as protective carriers in the freeze drying of L. rhamnosus IMC 501 and L. casei IMC 502, glycerine was found to stabilize viability more effectively than the other compounds evaluated (46).

The retention of viability during storage appears to be controlled by water plasticization of the protectant matrix and possibly interactions of water with the dehydrated cells. Highest cell viability was obtained in glassy protective media (47).

This protective effect of sugars on biomolecules was reported only in cases of amorphous carbohydrate glasses, but not where crystalline sugars were present (48). Several components of bacterial cells that are sensitive to drying are situated in the intracellular space. This contrasts with the situation involved in drying of proteins having pharmaceutical significance. For bacteria, the protectant must be transported through the cell membrane, Thus many high molecular weight polymers, particularly those showing limited transport into cells or in penetration and interaction with the phospholipid head groups of the cell membrane, are not very effective in protecting dried probiotic (35).

Furthermore, the glassy state does not play a major protective role against major storage deteriorative reactions including lipid oxidation because the free radical reaction is not limited by diffusion processes. In addition, it has been suggested that lipid carbonyl groups, formed as secondary products from lipid oxidation, participate in browning through reaction with amino groups in Maillard-like reactions. Accordingly, browning reactions have been implicated in the loss of viability in freeze dried cells (49).

It has been widely believed that while the maintenance of the glassy state during storage stabilizes the dried matrix powder, this is only at the macroscopic level. In a study of the stability of L. bulgaricus in a freeze dried galacto-oligosaccharide matrix carried out at a molecular level, Tymczyszyn et al., (50) found a correlation between molecular mobility as measured by 1HNMR and loss of viability of the cells. The conditions correspond to short movements with local relaxation and rotational and vibrational motions in the vitreous state. These may lead to the damage of protein structures and the precipitation of salts, with negative effects on microbial survival (51).

In addition, the properties of the confined water structure in the intra-cellular of microorganisms may be relevant as it has been suggested that the interaction and conformation of the water within cells could affect the stability of the microbial cultures. It has been reported that the structure and properties of confined water play an important role in maintaining the biological functions of proteins (32).

However, the role of the glassy state has not been studied in relation to probiotic properties, although the properties are vital for probiotic products and there have been some reports about changes in the properties during storage. The amount of bacteriocin production in freeze dried vaginal Lactobacillus strains were significantly reduced after storage for 12 months, whereas the production of other antimicrobial substances (lactic acid and hydrogen peroxide) and auto aggregation were still retained (38). In addition, probiotic functionality particularly tolerance to bile and acid were moderately retained following storage of some probiotic cultures (17).

Relative humidity (RH) is another important parameter in maintaining cell survival during storage and lower RH is preferred for enhanced survival. For example, L. kefir strains significant survival occurred under conditions of RH from 0-11% (53). Higher RH resulted in a reduction in cell numbers due to the acceleration of oxidative reactions (54). There will be a removal of structural water at very low RH, which is detrimental to cells. It was found that optimally, cells should be stored at intermediate moisture contents corresponding to 7-11% RH (55). Storage at low temperature and in containers which are impermeable to oxygen appears to be beneficial to the survival of dried probiotic powders. For example, freeze dried microorganism stored at 2-4 °C in glass ampoules for 50 years contained a considerable amount of viable cells (10^6-10^7 CFU/g) that were quite sufficient for culture maintenance (56). Similarly, placing Kefir candy products in glass bottles with
deoxidant and desiccant at 4 °C, resulted in viability of up to 10^7 cfu/g after two months which is sufficient for the product to be considered as a probiotic food (57). This appears to be consistent with the previous research which indicated that storage in the presence of air, high moisture content and high temperature is detrimental to dried microorganisms (58).

As mentioned, antioxidant can assist in maintaining cellular integrity during storage; however, the antioxidant must be chosen wisely in order to avoid reaction between the antioxidant and other materials encapsulating the cells. It has been demonstrated that tocopherol alone has a positive effect whereas ascorbate alone or in combination with tocopherol has a detrimental effect on the viability of microencapsulated L. rhamnosus GG during six month storage. The beneficial effect of tocopherol is a consequence of its chemical antioxidative action. The reduced viability in sodium ascorbate containing microcapsule formulations is hypothesized to be due to acetic acid from chemical degradation reactions and the catabolism of ascorbate by LGG (59).

Last but not least, the final water content of the powder also plays an important role in maintaining viability during storage. Many reports suggest that lowering the moisture content to below 4% is preferred for a shelf stable probiotic product (60). Likewise, the optimum moisture content for storage of freeze dried L. salivarius subsp. salivarius was reported to range from 2.8 to 5.6% (61). In addition, increasing the relative humidity of the environment at which the samples were stored caused an increase in water mobility and the rate of viability loss (24).

However, lipid oxidation is enhanced at very low water contents and water therefore acts as a protectant against oxidation (49). Low moisture contents of dried cells after drying do not guarantee high stability during storage because the moisture is not constant, and desorption, adsorption and the glass transition may occur during storage depending on the specific combination of atmospheric relative humidity and storage temperature.

At ambient conditions an equilibrium may only take a few hours or days to establish (43) or it may be as long as 1 to 2 months (62). Studying the stability of freeze dried microorganisms by varying only one factor at a time will not be able to ensure the stability of the cells as there is an interaction between factors. Recently, Passot et al., (16) concluded that an integrated analysis of the relationship between water activity, glass transition temperature (T_g), water content and biological activity appear to provide a promising approach for freeze drying processes and predicting the resultant storage stability. Likewise, Tymczyszyn et al., (50) reported an integrated approach giving a strong scientific background regarding the stability of L. bulgaricus in the presence of galacto-oligosaccharides by studying the correlation between glass transition temperature and molecular mobility with the loss of viability at different water contents and storage temperature.

CONCLUSIONS

Although freeze drying has been applied in the desiccation of sensitive materials for decades, it still presents challenges especially when it is applied to live microorganisms. Several factors including intrinsic, extrinsic tolerance to the stress during processing have been reported to affect the survival of microbial cultures. Other factors which warrant investigation during the process include freezing temperature, annealing steps, primary drying kinetics and the desorption step to a final moisture content. Optimizing the freeze drying process would be useless if cultures were to lose their functional properties during storage. Therefore, it is envisaged that any studies on optimizing freeze drying survival should also be done concurrently with evaluations of the retention of functional properties during storage. To this end the structure and the conformation of confined water molecules within the cells of microorganisms should also be studied to give an enhanced understanding at the molecular level regarding the properties of confined water in relation to the cell stability during storage.

REFERENCES

12. B. Li et al., Applied Microbiology and Biotechnology 92, 609 (2011).
Chapter 3

Materials and Methods

3.1 Introduction

The purpose of this chapter is to describe the materials, instruments and methods used in this research project including other ancillary items used. This chapter only provides the general materials and procedures and the principles behind some advanced measurements, including modulated differential scanning calorimetry (MDSC), micro differential scanning calorimetry (μ-DSC), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM); surface tensiometry, colour meter and zetasizer. The specific experimental procedures are described in the relevant chapters.

3.2 General materials and methods

3.2.1 General materials and media

Chemicals and materials utilised in this project were obtained from reputable suppliers and only analytical grades were used throughout unless otherwise stated. Those chemicals and materials with the description as well as the suppliers are listed in Table 3.1. Other ancillary equipments are listed in Table 3.2.

Table 3.1 Chemicals and materials utilised in the project

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Suppliers</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein isolate instantised 894</td>
<td>Fonterra (Waikato, New Zealand)</td>
<td>COA number: 82440279-6</td>
</tr>
<tr>
<td>Lactose monohydrate</td>
<td>Sigma Aldrich, Australia</td>
<td>L3625</td>
</tr>
<tr>
<td>Trehalose dehydrate</td>
<td>Sigma Aldrich, Australia</td>
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<td>Sodium hydroxide (NaOH)</td>
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<td>Lot no. B0414382940</td>
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</tbody>
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### Materials and methods

<table>
<thead>
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<td>Lot no. 113927</td>
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<tr>
<td>MRS broth</td>
<td>Oxoid Australia</td>
<td>Lot no. 1344490</td>
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<tr>
<td>Hexadecane</td>
<td>Sigma Aldrich, Australia</td>
<td>H6703</td>
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<tr>
<td>Dipotassium phosphate, heptahydrate (K$_2$HPO$_4$,12H$_2$O)</td>
<td>Merck (Germany)</td>
<td>Lot no. 1065791000</td>
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<tr>
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<td>Merck (Germany)</td>
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</table>

<table>
<thead>
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<th>Table 3.2</th>
<th>List of other ancillary equipments utilised in this project.</th>
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</thead>
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<td><strong>Description</strong></td>
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<tr>
<td>Centrifuge (Beckman Coulter)</td>
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</tr>
<tr>
<td>Micropipettes</td>
<td>Pipet-Lite$^\text{TM}$ XLS 20 – 200 μL, 100 – 1000 μL, 500 – 5000 μL and 1 to 10 mL</td>
</tr>
<tr>
<td>Magnetic stirrer and hot plate (Ratek)</td>
<td>Serial no. CS76083V</td>
</tr>
<tr>
<td>Vortex mixer (Ratek VM1)</td>
<td>Serial no. 111126844</td>
</tr>
<tr>
<td>Water bath</td>
<td>Serial no. 108126355</td>
</tr>
<tr>
<td>Incubator</td>
<td>SEM convection incubator</td>
</tr>
<tr>
<td>Moisture analyser</td>
<td>Ohaus Moisture analyser</td>
</tr>
<tr>
<td>Water activity meter</td>
<td>Novanissa ms1</td>
</tr>
</tbody>
</table>
3.2.1.1 Whey protein isolate (WPI)

Whey protein isolate instantised 894, from Fonterra Australia Pty Ltd was utilised in this research. The composition of WPI as reported by the supplier was 90.4% protein (N × 6.38), 1.0% fat, 4.7% moisture, 0.9% total carbohydrate, 3.0% ash. pH of 5% (w/w) was 6.8, bulk density of 0.34 g/mL and aerobic plate count produced less than 10 000 CFU/g.

3.2.1.2 de man Rogosa and Sharpe broth

This was obtained from Oxoid, Australia and contained peptone (1%), meat extract (0.8%), yeast extract (0.4%), glucose (2%), sodium acetate trihydrate (0.5%), polysorbate 80 (0.1%), dipotassium hydrogen phosphate (0.2%), triammonium citrate (0.2%), magnesium sulphate heptahydrate (0.02%), manganese sulphate tetrahydrate (0.005%).

3.2.1.3 Peptone water

This was obtained from Oxoid, Australia and contained peptone (1%) and Sodium chloride (0.5%).

3.2.2 General procedure

Except otherwise stated, deionised water (Milli-Q system QGARD00R1, Millipore, Australia) was utilised in the preparation of all media and solutions in this project. All glassware, solutions and media were sterilised by autoclaving at 121 °C for 15 min, except otherwise stated. Aseptic technique was used to prepare all media and store at 4 °C until used.

3.2.3 General media and solutions

In general, the preparation of all media and agar utilised in this project followed the instruction provided by the suppliers. For example, in the preparation of 1 L of peptone water, 15.0 g of peptone powder was completely dissolved in deionised water. Likewise, in the preparation of 500 mL MRS agar, 31 g of MRS agar powder was dissolved completely in deionised water, followed by autoclaving and pour plating when it is still warm. The agar in the plate was allowed to solidify before use for plating purposes.
3.2.4 General microbiological methods

The principles behind the procedures utilised in bacterial cell storage, growth, enumeration and harvesting are described in this section.

3.2.4.1 Culture storage

For any microbiological work, the storage of culture is of utmost importance to ensure that pure viable bacteria cells are available for the whole project for further processing. It is very important to control the purity of the stock culture by limiting the potential of contamination or alteration of growth characteristic. In this project, the basic materials were frozen culture of *L. plantarum* A17 and B21, which were stored in a mixture of MRS broth and glycerol at a ratio of 1:1 at -80 °C. Basically 0.5 mL of active growing cells in MRS broth was taken and mixed with 0.5 mL of sterilised glycerol (80%), mixed thoroughly and stored at -80 °C immediately.

For the resuscitation of the cells from the frozen stock cultures at -80 °C, a loop was sterilised by the Bunsen flame till it was red hot and allowed to cool down slightly before stabbing the frozen cultures. A loopful of bacterial cells was transferred into MRS broth and incubated at 30 °C for 24 h. Once the loopful of stock culture was transferred, the stock culture cap was then closed and returned to the deep freezer while still frozen (Nagel & Kunz, 1972; Tran, 2010). A loopful of actively growing cells was then transferred from the broth and streaked onto MRS agar plates. Each time the culture was needed; one colony was taken from the plate for a further subculture to ensure purity and the purity check was also done every month using Gram staining method.

The storage period of these plates was dependent on how rapidly these organisms multiplied under the storage conditions. During growth, the organism is utilising the nutrients available in the agar. Consequently, at some point in time, the nutrients will be depleted and the organism will initially be stressed and then eventually die. The subculture must occur before cells are stressed, since stressed and non-stressed cells will respond differently to further processing such as spray drying. In this work, these working plates were kept for a maximum of two weeks at 0-4 °C and then re-subcultured into other plates as recommended by application note of Thermo Scientific Inc (2014) and Kumar, Kashyap, Singh, & Srivastava (2013).
3.2.4.2 Cell growth

For any organisms, nutrients and appropriate environment are needed for growth. Commercially available growth media for any *Lactobacilli* is de Man Rogosa and Sharpe (MRS) broth. *L. plantarum* is classified as facultative anaerobes (Todorov & Franco, 2010); thus the cells obtain their energy by converting the glucose through either anaerobic or aerobic fermentation.

Generally the growth of bacteria can be described in three separate stages with a typical growth curved presented in Fig. 3.1. First, the bacteria is in the lag stage where the microbial cells are adjusting to the new environment they have been transferred to. During this stage, the bacteria cells are biologically active, but there is no multiplication of the cells. The later stage is evidence by the rapid multiplication of the cells and is called exponential phase when the bacteria ferment sugars. When the nutrients needed for growth are depleted and the microorganism has reached the final phase, there is accumulation of metabolites, this phase is called stationary phase.

![Typical growth characteristic of bacteria](image)
Materials and methods

Bacteria multiplication is an elongation and a halving of the cell to create two equal cells. As for *L. plantarum* A17 and B21, the cells produced are round, smooth, opaque and white colonies on MRS agar with a rod shape as shown in Fig. 3.2.

![Fig. 3.2 Colonies of *L. plantarum* A17 after incubation at 30 °C for 24 h (a) and SEM examination of A17 at 3000 x magnification on a glass slide after drying in an oven at 70 °C for 15 min (b).](image)

3.2.4.3 Cell enumeration

Bacteria are so small that they cannot be seen with naked eyes and therefore cannot be enumerated directly. To overcome this problem, bacteria can be enumerated on the semi-solid media such as MRS agar for *Lactobacillus*. With appropriate incubation temperature over a certain period of time, a visible object referred to as colonies can be observed. The shape and morphology of the colony can provide preliminary identification information. Each colony represents a single cell in the broth being sampled; so by counting the colonies, the cells are being counted. For the purpose of counting, a period of at least 24 h of incubation at 30 °C was adapted throughout this research.

Another important aspect to consider when enumerating the bacterial cells is the actual cells to be enumerated. Conventionally, a count between 30 and 300 colonies on a 9 cm agar plate is taken as the estimate of the actual count number in a sample (AS 5013.1, 2004). Tenfold dilutions of the test samples was adapted throughout this project using sterile 9 mL peptone water until the count was within the acceptable range. In addition, three dilutions around the expected endpoint was also chosen to make sure that colonies in each plate is really ten fold differences in the numbers of colonies obtained on the consecutive plates after incubation. Normally, bacteria is diluted with peptone water as it is isotonic with the cells and the cells are not stressed during the dilution and plating process which is limited to approximately 30 min period to avoid cell multiplication or death. The accuracy of bacteria count can be
compromised if multiplication or death occurs during enumeration (Juste, Thomma, & Lievens, 2008).

The count was obtained by spread plating and the colonies counting after incubation was taken at least the mean of duplicate counts. The number counting of colony forming unit per mL is then calculated by multiplying the number of CFU/mL of diluted suspension by the dilution factor. For example: if 0.1 mL of a $10^{-7}$ dilution of a suspension of organisms is cultured in a spread plate and yields 35 colonies; the original suspension would contain: $35 \times 10 \times 10^7$ CFU/mL = $3.5 \times 10^9$ CFU/mL of original suspension

3.2.2.4 Bacteria cell harvesting (cell concentrating)

Bacterial cells used in this project involved mixing the cells with a protectant and subsequently encapsulated by convective drying. Therefore, bacterial cells need to be harvested and washed before mixing with a protectant or encapsulating agent. Harvesting cells at the beginning of the stationary phase has generally been accepted as cells are more resistant to subsequent processing steps (Corcoran, Ross, Fitzgerald, & Stanton, 2004; Teixeira, Castro, & Kirby, 1995; Van de Guchte, Serror, Chervaux, Smokvina, & Ehrlich, 2002, Tran, 2010).

Generally bacterial cells are concentrated and harvested by centrifugation due to the large difference in density between MRS broth and bacteria. The effectiveness of centrifugation is based on the relative centrifugal force (the value of the radius is taken from the center of the head of the radius in centimetre of the head to the outer edge of the sample container) divided by $g$ ($980\text{cm/s}^2$). The required $g$ value is dependent on various factors including particle diameter, medium density and viscosity (Karwoski, 1996).

In this work, centrifugation of the broth culture was carried out at 4000 $g$, which has been reported as suitable for the concentration of bacterial cells (Higl et al., 2007; Tymczyszyn, Sosa, Gerbino, & Hugo, 2012, Tran 2010). This centrifugal force ensures the separation of the concentrated cells and the cell free supernatant. Following centrifugation, it is necessary to wash the cell pallets with saline water (0.85%) to ensure a maximum removal of growing media.
3.3 Basic principle for advanced instruments utilised

Basic principles behind each analytical and drying methods utilised in this thesis are presented in the following sections.

3.3.1 Differential scanning calorimetry (DSC)

DSC is a widely utilised thermal analysis method to understand the material thermal transitions including from solid to solid, solid to liquid, solid to gas as well as other various transitions and reactions. Materials thermal behaviour can be understood by utilising DSC method although no visual evidence of change can be observed. Basically, two pans in the DSC are isolated from ambient environment (Fig. 3.3). One pan contains the sample to be analysed and the other pan is empty and used as a reference.

The two pans are designed to be either heated or cooled at the same constant rate \((dT/dt)\). However, the heat flow \((dH/dt)\), which is the rate at which thermal energy is supplied, is different as one pan is empty and the other contains the sample. The computer assists to ensure that the heat flows are the same and help keep track of the starting temperature, heating rate and heat flow and record the difference between the reference and the sample. A typical example of a material transition is shown in Fig. 3.4.

![Sample and reference chamber for DSC (left) and schematic diagram of heat flux type DSC (right) (TA Instrument, n.d)](image_url)
From Fig. 3.4, it can be seen that material may undergo exothermic (crystallisation) or endothermic (melting) changes as a result of changes in temperature. As materials are heated, molecules or atoms gained enough energy to break away from their rigid structure (e.g. solid) to a more unordered state (liquid).

This change of transition due to absorbing energy is called endothermic transition (melting as shown in Fig. 3.4). The energy per unit mass used in bringing this phase change is called latent heat (Lukas & LeMaire, 2009). For amorphous material, there is no latent heat and the transition associated with this material (glass transition) is referred to as second order transition.

Once the heating rate is set \((dT/dt)\), the instrument can measure the heat flow \((dH/dt)\). The heat capacity \((C_p)\) of the material can be measured by dividing the heat flow by the heating rate \(C_p = [(dH/dt)/(dT/dt)]\). Knowing the mass \((m)\) of the sample, the specific heat capacity \((C_p=C_p/m)\), as a function of temperature may be obtained, thus yielding information on the variations in the physical properties of the material. In short, the heat flow of DSC is summarised as below (Thomas, 2005):

\[
\frac{dH}{dt} = C_p \frac{dT}{dt} + f(T,t) \tag{3.1}
\]

Where \(dH/dt\) refers to total heat flow rate (can be mW which is equal to mJ/s)
Materials and methods

$C_p$ refers to sample heat capacity (specific $C_p \times$ sample mass) in J/$^\circ$C

d$T$/d$t$ refers to heating rate ($^\circ$C/min)

$f(T,t)$ refers to heat flow that is a function of temperature and time (mW)

Total heat flow = the sum of all heat flow;

Reversing heat flow = heat capacity component, $C_p \times dT/dt$; and

Non reversing heat flow = kinetic component, $f(T,t)$

Fig. 3.5 An example of MDSC thermogram of a thermoplastic alloy blend of polycarbonate (PC) and polybutylene terephthalate (PBT). MDSC effectively separates the crystallisation of the PBT component into the Nonreversing heat flow, thereby allowing for accurate determination of glass transition temperature ($T_g$) of each polymer in the reversing heat flow (TA, Instrument, n.d)

Although DSC is a useful analytical technique for the study of the properties of materials there are some limitations with respect to its ability to differentiate complex transitions into individual contributing components as shown in Fig. 3.5 as an example. Therefore, it is difficult to accurately translate the thermal events of the materials. To this end, modulated DSC can overcome some of the analytical limitations of DSC in which the samples are subjected to a more complex heating program with sinusoidal temperature modulation along with an underlying linear heating ramp (Rabel, Jona, & Maurin, 1999). Thermal transition can be both reversing (e.g. glass transition) and non-reversing (e.g. evaporation, cold
Materials and methods

In DSC, only a small amount of sample, typically in the range of 2 to 14 mg is used for the analysis of the thermal events. Small amount of sample used is desirable so as to avoid the heat loss during the analysis as the heat exchange occurs only through the bottom of the pans as shown in Fig. 3.3 (right) and significant amount of heat is lost through the wall and lids of the pan (Le Parlouer & Benoist, 2009).

In this work, modulated DSC (Q2000, TA instruments, France) equipped with refrigerated system RCS 90 (Fig. 3.6) was utilised to determine the glass transition temperature of spray dried microcapsules with solid content of approximately 95% and reported in Chapter 7.

![Fig. 3.6 MDSC-Q2000 utilised in the thermal analysis in this research](image)

### 3.3.2 Micro-differential scanning calorimetry (μ-DSC)

In contrast to DSC, μ-DSC allows the heat exchange from the sample to be totally measured by the sensor of the instrument. In addition, larger and more bulky samples can be analysed. This is ideal as food is normally a complex mixture of several components. This instrument utilises the heat flux calorimetry and its high efficiency is achieved due to the application of Calvet technological principle, in which the detection is based on a three dimensional fluxmeter sensor.

In μ-DSC, two identical calorimetric chambers (sample and reference) are placed in the thermostatic block (Fig. 3.7) at the same temperature. This block’s temperature control is carried out and checked accurately using two stages of Peltier-effect thermo-elements. This provides temperature homogeneity and stability within the block. In practice, it is common to equilibrate the temperature within the block long enough to avoid disturbance at the
Materials and methods

introduction of the container in the calorimeter and the temperature perturbation of the thermostatic block that might affect the measurement.

![Fig. 3.7 Thermostatic block where the sample and reference vessel are placed during the analysis by μ-DSC (TA Instrument, n.d)](image)

Most microcalorimeters utilise the heat flux principle (Le Parlouër & Benoist, 2009) where the heat flux difference between the sample and reference chamber at temperature $T_s$ is measured with the following equation:

$$\frac{dq}{dt} = -\frac{dh}{dt} + C_s dT_s/dt - C_r dT_r/dt$$  \hspace{1cm} (3.2)

where, $dh/dt$ is heat flux produced by the transformation of the sample or the reaction; $C_s$ is the heat capacity of the sample, including the container; $C_r$ is the heat capacity of the reference; and $T_r$ is the temperature of the reference. According to Equation 3.2, if $dh/dt$ corresponds to an endothermic transformation or reaction, the $dh/dt$ value is positive. If $dh/dt$ corresponds to an exothermic transformation or reaction, the $dh/dt$ value is negative (Le Parlouër & Benoist, 2009).

In this research, a Setaram VII μ-DSC was utilised to study the denaturation profile of whey protein solutions as it is more sensitive than MDSC in revealing thermal events in the nature of first order thermodynamic transitions and it is reported in Chapter 5 (Fig. 3.8).
3.3.3 Dynamic mechanical analysis (DMA)

In this technique, a small deformation is applied to the sample in a cyclic manner allowing the sample to respond to stress, temperature, frequency and other variables under investigation. It is one of the most suitable methods to investigate relaxation event of the sample. DMA works by applying an oscillating force to the material of known geometry and the resultant displacement of the sample is measured. However, for non rigid material like powders, flakes, films, liquids and semi solids, the Triton Technology Material Pocket (PerkinElmer Inc, 2011) has been proposed as a mechanism to support during the analysis by DMA (Fig. 3.9). From this measurement, the material stiffness can be determined and \( \tan(\delta) \) can be calculated. \( \tan(\delta) \) is the ratio of the loss component to the storage component. \( \tan(\delta) \) is plotted against temperature and the glass transition is normally observed as a peak since the material will absorb energy as it passes through the glass transition.

When analysing data from the material pocket, it is not useful to look at the modulus value as the pocket is made of stainless steel and does not display any transitions in the temperature of interest. According to PerkinElmer Inc., (2011) the \( T_g \) observed from a material in a pocket is comparable to that of the same material without the pocket present.
PerkinElmer DMA 8000 was used (Fig. 3.10) to analyse the glass transition temperature of spray dried microcapsules and reported in Chapter 7.

**3.3.4 Scanning electron microscopy (SEM)**

Like any other microscope, SEM is used to observe the structural surface morphology of materials in the range of microscale (1 micrometer = 10^{-6} m) to nanoscale (1 nanometer = 10^{-9} m). It has a power of magnifying image of approximately 10 to 300 000 times along with scale bar in each image taken. A simple analogy on how SEM looks at the surface is like a person in a dark room with a fine beamed torch to scan for objects on the wall. By scanning the torch systematically side-by-side and gradually moving down, the person can build an image of the objects with their memory. SEM uses electron beam instead of torch, an electron detector instead of eyes and a viewing screen and camera as memory.

SEM is similar to a normal light microscope fundamentally and functionally (Fig. 3.11). However, due to its complexities, SEM has better resolution (ability to distinguish two closely opposed point typically 5 nm due to short wavelength of the electron), and a higher magnification (Davis, 2005). In addition, SEM can provide better topographical detail and the ability to provide other microanalysis. One drawback of many types of SEM is the operation under vacuum as well as that the sample to be observed must be conductive. Therefore, samples are normally coated with a thin layer of metal or carbon.

Typically, SEM machine consists of three main parts. Firstly, the microscope column consists of electron gun at the top, the column, along which the electron travels and the sample...
chamber at the base. Secondly, there is a computer driving the microscope with additional bench controls. Finally, there are other ancillary equipments including the analysis of the composition of the sample.

Fig. 3.11 Schematic comparison between light and scanning electron microscope. (Australian microscopy & microanalysis research facility, n.d)

The electron column focuses and illuminates the specimen using the electron beam generated by the electron gun. As the beam is scanned over the specimen in the X- and Y- directions, secondary and backscattered electrons are produced and detected. By amplifying and modulating the brightness of the detected electron signals an image is produced.

In this research, Environmental Scanning Electron Microscopy (ESEM) (Fig. 3.12) with both functions of low and high vacuum was utilised to image the surface morphology of spray-dried powder and reported in Chapter 5 and 6.
3.3.5 Fourier transform infrared spectroscopy (FTIR)

Spectroscopy is the study of the interaction between light and matters. In principle, infrared (IR) radiation is passed through a sample where some of the infrared radiation is absorbed by the sample and some of it is transmitted through the sample. The resulting spectrum represents the molecular absorption or transmission, creating a molecular fingerprint of the sample. The unique fingerprint of each molecular component has its own distinct spectra which make the method useful in the identification analysis.

Most infrared spectra are measured and plotted from 4000 to 400 wave number (cm\(^{-1}\)) on the x axis (Smith, 2011). Wave numbers are measured in unit of wave cycle per centimetre which is abbreviated as cm\(^{-1}\). For example if a spectrum has a peak at 1500cm\(^{-1}\), it means that the sample absorbed infrared light that underwent 1500 cycles per centimetre.

Generally, FTIR analysis consists of the following components (Fig. 3.13)
Materials and methods

- The source: Infrared energy is emitted from a glowing black body source. This beam passes through an aperture which controls the amount of energy presented to the sample and the detector.

- The interferometer: the beam enters the interferometer where the spectral encoding takes place. The resulting interferogram signal then exits the interferometer.

- The sample: The beam enters the sample compartment where it is transmitted through or reflected off the surface of the sample, depending on the type of analysis being done. At this stage, specific frequencies of energy, which are uniquely characteristic of the samples, are absorbed.

- The detector: the beam finally passes to the detector for final measurement. The detectors used are specially designed to measure the special interferogram signal.

- The computer: the measured signal is digitised and sent to the computer where the Fourier transformation takes place to provide spectrum for analysis.

Fig. 3.13 Components of the FTIR (Thermo Nicolet Corporation, 2001)

The underlying reason in using FTIR to determine the protein secondary structure arises from the features associated with a characteristic hydrogen bonding pattern between amide (C=O) and N-H groups (Jackson & Mantsch, 1995). The amide I absorption contains contributions of about 80% from the C=O stretching vibration of the amide group with a minor contribution from the C-N stretching vibration; while the amide II absorption appears to be significantly less pure, arising from N-H bending and C-N stretching.
As summarised by Vedantham, Sparks, San, & Tzannis, (2000), FTIR has been reported to be the most versatile spectroscopic method for analysing protein secondary structure in various physiological environments including protein structure in solution, in aggregates, and inclusions body as well as during lyophilisation and freeze/thaw processing both qualitatively and quantitatively.

In the quantitative analysis of different features of protein secondary structure, mathematical tools such as derivatisation and Fourier self deconvolution (FSD) analyses are available to determine the unique structure features such as α-helix, β-sheets, β-turns and random coils of a protein (Yang, Griffiths, Byler, & Susi, 1985). The wave number assigned to each secondary feature is shown in Table 3.3.

<table>
<thead>
<tr>
<th>Components</th>
<th>Wavenumber (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sheet (low frequency)</td>
<td>1620-1640</td>
</tr>
<tr>
<td>Random coil</td>
<td>1641-1647</td>
</tr>
<tr>
<td>α-helix</td>
<td>1648-1660</td>
</tr>
<tr>
<td>β-turns (low frequency)</td>
<td>1661-1673</td>
</tr>
<tr>
<td>β-sheet (high frequency)</td>
<td>1674-1680</td>
</tr>
<tr>
<td>β-turns (high frequency)</td>
<td>1681-1699</td>
</tr>
</tbody>
</table>

In this thesis, Perkin Elmer Spectrum 100 FTIR spectrometer equipped with smart, plug-play-and-go universal ATR (UATR) accessory (Fig. 3.14) was utilised to analyse protein
secondary structure following spray drying and reported in Chapter 5. This system enables the analyses of samples in both liquid and solid states without further preparation. In this study, samples were measured in absorbance mode in the mid infrared region with a resolution of 16 cm$^{-1}$. The measured sample spectrum was corrected against background spectrum to ensure the elimination of extraneous artifacts.

### 3.3.6 Zetasiser

Most of the colloidal systems in aqueous media possess electrical charges which might be derived from the ionisation of surface group, preferential loss of ions from the particle surface or absorption of ions or charged molecules onto particle surface depending on the nature of the particle and its surrounding medium.

![Fig. 3.15 Optical configuration of the Zetasizer for zeta potential measurement.](Malvern Instrument, n.d)
A technique of micro-electrophoresis is commonly utilised to measure the zeta potential of colloidal dispersions where a voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attracted to the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their mobility. A typical configuration of Zetasizer to measure the zeta potential is shown in Fig. 3.15 (Malvern Instrument, nd).

Basically, a laser ① is used as a light source to illuminate the particles within the sample where it later split to incident and reference beam. The incident laser beam passes through the center of the sample cell ② and scattered light is detected at a forward angle ③. When the sample cell in contact with the electric filed, any particles moving through the measurement volume will cause the intensity of light detected to fluctuate with a frequency dependent on the particle speed. This information is later on passed through a digital signal processor ④ and PC ⑤. The software of the Zetasizer analysed the detected data to obtain the electrophoretic mobility and hence zeta potential information. It is important to note that the ratio of the intensity of the reference beam to the scattered light is carefully controlled using an attenuator ⑥ which is set around 10:1 in the Nano series.

For the purpose of measuring the zeta potential of bacteria cell surface in buffer solutions as well as the zeta potential of whey protein isolate, Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, UK) was utilised in this project (Fig. 3.16) and the result is reported in Chapter 5.

Fig. 3.16 Zetasizer Nano utilised to measure the zeta potential in this research.
### 3.3.7 Surface tensiometer

Surface activity of food colloids such as foams, emulsions and other dispersion system can be measured by the ability of an additive to readily lower the free energy state of a system and is characterised by its tendency to migrate to the boundary of two surfaces. Surface tension is defined as the amount of work (Nm) necessary to expand the surface (m²) of a liquid by one unit. Therefore, the surface tension is measured in N/m (Nm/m²).

In this work, a bubble pressure method was used to determine the surface tension of spray drying medium using SITA t60 tensiometer (SITA Messtechnik GmbH, Germany) and reported in Chapter 6. In measuring surface tension using this method, a capillary is dipped into the liquid and an air-current is directed into it. Because of the increasing gas pressure, a gas bubble forms at the lower end of the capillary, the gas bubble bursts and breaks away from the capillary. The equation (3.3) below is used to calculate the surface tension.

\[
\sigma = r_K \left( p_{\text{max}} - p_{\text{stat}} \right) = \frac{r_K}{2} \left( p_{\text{max}} - \rho g h_E \right)
\]  

Where \( \sigma \) is the surface tension; \( r_K \) refer to the capillary radius; \( p_{\text{max}} \) refers to the maximal pressure; \( p_{\text{stat}} \) refers to hydrostatic pressure; \( h_E \) refers to the depth of the capillaries’ immersion and \( \rho \) is the density of the liquid.

\( p_{\text{stat}} \) is dependent on \( h_E \), and \( \rho \) . This influence can be eliminated by means of a relative measurement of the pressure \( \Delta p \). Therefore surface tension can be written

\[
\sigma = \frac{r_K}{2} \Delta p
\]

### 3.3.8 Colour meter

Colour is one of the important visual aspects of food products. People see things and extract information through light wavelength. When communicating through colour, a language understandable for all must be followed. In this regards, colour notations can be categorised into three categories (Rhodes, 2002). These include device dependent system (for computer and other related machine display), Mathematical systems which are based on the
Materials and methods

Mathematical transformation of CIE tristimulus values such as CIELUV, CIELAB and systems based on database of aim points.

Mathematical systems are the most commonly reported within the scientific communities, which are based on the opponent colour theory (Hunterlab, 2012). Based on this theory, it is assumed that human eye receptors perceive colour as the following pairs of opposite:

- L scale: Light versus dark where low number (0-50) indicates dark and a high number (50-100) indicates light.
- a scale: Red versus green where a positive number indicates red and a negative number indicates green.
- b scale: Yellow versus blue where a positive number indicates yellow and a negative number indicates blue.

All three values are required to completely describe an object’s colour. A three dimensional representation of L, a, b colour space is shown in Fig. 3.17.

In this research, Chroma Meter (Fig. 3.17 (b)) (Model CR-400, Konica Minolta, Sensing Japan Inc) was utilised to measure the colour attribute of spray dried capsules where the bacteria was protected by whey protein isolate and reported in Chapter 7.

![Opponent colour scale of L, a, b. (a) and Chroma Meter utilised in this research (b)](image)

3.3.9 Single droplet drying (SDD)

Spray drying is a well established unit operation in the dehydration of food powder.
However, when it comes to spray drying heat sensitive materials like microorganisms, it is difficult to establish optimum drying conditions and formulations to retain the high degree of cell survival required. To this end, single droplet drying (SDD) which is able to monitor the morphological changes as well as the inactivation kinetics of microorganisms or enzymes at droplet level has been developed. By definition, SDD is the drying of a single droplet under controlled air conditions mimicking to any extent possible the spray dryer environment (Fu, Woo, & Chen, 2012). This drying method has been reported to assess inactivation kinetics of enzymes and probiotics during spray drying (Adhikari, Howes, Bhandari, & Truong, 2000; Li, Lin, Chen, Chen, & Perce, 2006; Yamamoto & Sano, 1992).

In principle, an isolated droplet is generated and placed in a conditioned air stream and the drying behaviour of the droplet is continuously monitored with a video camera. There are three different types of SDD systems, each comes with its own benefits and limitations as summarised by Fu et al., (2012). In this work, glass filament single droplet drying system was utilised to monitor the kinetics of three parameters including droplet diameter, temperature and mass as well the inactivation kinetics of microorganisms. Detailed of the determination of each parameter is reported in Chapter 4.

3.3.10 Spray drying

Spray drying is the dehydration method commonly utilised in the food industry where the feeding liquid such as a solution, an emulsion or a suspension is converted to solid particle spontaneously. The spraying droplet can be generated by different atomisers including pneumatic, pressure, spinning disk, fluid nozzle and sonic nozzle. The size of the droplet can be adjusted by either increasing or decreasing the energy provided to the atomiser. In addition the size of the formed droplet also depends on the flow rate of the feed solution as well as the viscosity of the feed solutions (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

During spray drying the inlet temperature is typically high in the range of 150-200 °C and evaporation of solvent takes place very quickly and the temperature decreases to moderate range of 50-80 °C (de Vos, Faas, Spasojevic, & Sikkema, 2010; Gharsallaoui et al., 2007). Although the high inlet temperature used in the spray drying, the time of exposition of the microparticles is very short (normally in a few seconds) and the temperature in the core particles generally does not surpass 100 °C which make the process applicable to the
encapsulation of varieties of sensitive ingredients including bacteria (Desai & Park, 2005; Peighambardoust, Golshan, & Hesari, 2011).

During the contact between hot air and liquid droplets, the balance between the temperature and vapour partial pressure are established between these phases. As a result of the temperature differences, heat transfer is carried out from air towards the products whereas water transfer is carried out in the opposite direction due to the vapour pressure difference.

According to the fundamental theory of drying, there are three steps for the drying to take place. First, hot air and droplet contact brings about the increase in droplet temperature due to heat transfer to a constant value which is considered as wet bulb temperature value (Gharsallaoui et al., 2007). After that, the evaporation of droplets water is carried out at constant temperature and water vapour partial pressure. The rate of water diffusion from the droplet core to its surface is usually considered constant and equal to the surface evaporation rate. Finally, when the droplet water content reaches a critical value, dry crust is formed at the droplet surface and the drying rate rapidly decreases with the drying front progression and becomes dependent on the water diffusion rate through this crust. Theoretically, drying is complete when the particle temperature is equal to that of the air.

In this work, LabPlant spray dryer (SD-Basic FT30MKIII spray drier, Keison products, Chelmsford, Essex, UK) was utilised to produced encapsulated capsules (Fig. 3.18). It is a bench mounted laboratory scale spray dryer with a self-priming peristaltic pump delivers the liquid sample through a small diameter jet into the main chamber and at the same time compressed air enters the outer tube of the jet which causes the liquid to emerge as a fine atomised spray into the drying chamber Jet nozzle assembly (0.5 mm standard, 1.0 and 1.5 mm jets are optional) comes with manually operated de-blocking needle for effective cleaning between runs. Other characteristics are: Product flow rate: 0-1500 mL/h; air inlet temperature: 200 °C max; heater capacity: 3 kW and drying air through flow: 70 m³/hr (fixed) (SD basic spray dryer instruction manual, 2012).

For the purpose of this research, a nozzle of 0.5 mm, and an air pressure of 392.27 kPa were used for Chapter 5, 6 and 7 to produced microcapsules. Basically before spray drying to produce microcapsules, the spray dryer was warmed up for half an hour with distilled water to make sure that the inlet temperature is stable. Similarly, the spray dryer was cleaned by
Materials and methods

pumping distilled water for another half an hour to clean the nozzle. The drying chamber, cyclone and collecting bottles were disassembled and clean between run.

Fig. 3.18 LabPlan spray dryer utilised in this research to produce microcapsules.
Chapter 4

Agent selection and protective effects during single droplet drying of bacteria

Abstract

The protective mechanisms of whey protein isolate (WPI), trehalose, lactose, and skim milk on *Lactobacillus plantarum* A17 during convective droplet drying has been explored. A single droplet drying technique was used to monitor cell survival, droplet temperature and corresponding changes in mass. WPI and skim milk provided the highest protection among the materials tested. *in situ* Analysis of the intermediate stage of drying revealed that for WPI and skim milk, crust formation reduces the rate of sudden temperature increase thereby imparting less stress on the cells. Irreversible denaturation of the WPI components might have also contributed to the protection of the cells. Skim milk, however, ‘loses’ the protective behaviour towards the latter stages of drying. This indicates that the concentration of the WPI components could be another possible factor determining the sustained protective behaviour during the later stages of drying when the moisture content is low.

Key words: *Lactobacillus plantarum*, lactose, protective mechanism, single droplet drying, trehalose, whey protein isolate,

4.1 Introduction

Lactic acid bacteria (LAB) are a group of ubiquitous Gram-positive bacteria which are generally regarded as safe in food preservation. LAB, especially those in the genus *Lactobacillus*, is most widely utilised in food and theopathic applications (Bourdichon et al., 2012; Vogel et al., 2011). In addition, LAB can enhance food safety and consumer health by preventing or reducing the incidence of pathogens (Gaggia, Gioia, Baffoni, & Biavati, 2011; Gaggia, Mattarelli, & Biavati, 2010).

The success in the utilisation of the LAB cells for such purposes often requires high cell density and retention of activity for a reasonable amount of time before incorporation into the
food formulation, in order to ensure the desired effect. A variety of different methods have been reported in the literature, including freeze drying, spray drying, vacuum drying, air drying and fluidised bed drying (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008) and these have resulted in varying degrees of cell survival.

Amongst the processing techniques for producing dried microbial cells, optimising spray drying and developing product formulations to minimise activity losses have attracted increasing research interest as it is much more cost effective than freeze drying and larger volumes can be processed (Knorr, 1998). Spray drying involves the fine atomisation of a liquid feed material and the subsequent rapid evaporation of water makes it especially suitable for heat sensitive products. Yet, the success in utilising this approach has been based on trial and error, while different research groups have reported very variable rates of cell survival ranging from 1–100% (Fu & Chen, 2011). Therefore, the objective of enhancement in cell viability warrants further investigation and an insight into the inactivation mechanisms of cells is a prerequisite (Santivarangkna, Kulozik, & Foerst, 2008).

Several factors have been reported to affect cell survival during spray drying, including the tolerance of the cells as well as processing parameters applied during the drying stages (Meng et al., 2008). It has been reported that the inactivation of microorganisms may occur due to high temperature and dehydration stress (Fu & Chen, 2011; Meng et al., 2008; Peighambardoust, Golshan, & Hesari, 2011). Damage to macromolecules particularly including DNA and RNA, as well as cell membranes and ribosomes result from the exposure of the cell to higher temperature. In addition, during dehydration, damage to cytoplasmic membranes can occur, leading to the loss of some intracellular components (Ananta, Volkert, & Knorr, 2005).

In order to overcome the stress encountered during spray drying, microbial cells are often formulated with various protectants, and research efforts have been devoted to the choice of agents providing optimal survival (Leslie, Israeli, Lighthart, Crowe, & Crowe 1995). Skim milk and carbohydrates in the form of disaccharides used either alone, or in combination, have been the most commonly used protective substances in dehydration of microorganisms in the last decade with varying effects upon cell survival. Various hypotheses have been proposed to explain the beneficial effects of sugars including the involvement of water replacement and vitrification (Santivarangkna, Higl, & Foerst, 2008). Although the protective
mechanism of skim milk has not been fully explored, it has been suggested that lactose in
skim milk interacts with the cell membrane and helps to maintain membrane integrity in a
manner similar to the protection by other sugars including trehalose (Corcoran, Ross,
Fitzgerald, & Stanton, 2004). Another major constituent of skim milk is protein and whether
or not this exerts a significant protective effect remains to be elucidated (Fu & Chen, 2011).

There has been an increasing number of reports on the application of milk protein in the
protection of probiotic cells in recent years, relating to roles in protection both whilst drying
is occurring as well as during exposure to gastrointestinal or bile fluids. This probably
reflects the desirable gelation properties of the proteins, which have been shown to be useful
for the microencapsulation of probiotics (Rathore, Desai, Liew, Chan, & Heng, 2013). There
have been a few reports specifically on the application of whey for the protection of
probiotics during spray drying. These have demonstrated good survival; however, these
studies utilised either whey protein isolates (WPI) in combination with carbohydrate or liquid
whey, which also contains lactose (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, &
Sant’Anna, 2012; Ying et al., 2011, 2013). Furthermore, none of these studies has addressed
the mechanism(s) whereby the probiotics were protected.

During pilot scale spray drying, billions of droplets are sprayed into a relatively large tower.
As a result of the varied trajectories the droplets could have quite different drying histories,
posing challenges to understand bacterial inactivation and the mechanisms of protection
afforded by various drying media. To circumvent these difficulties, a single droplet drying
device mimicking the spray drying environment was employed here to investigate different
inactivation histories of bacterial cells as the droplet was being dried under controlled
conditions. This single droplet drying device allows accurate measurements of changes in the
droplet temperature, mass and diameter as drying progresses (Fu, Woo, Lin, Zhou, & Chen,
2011). Therefore, the objective of this study was to examine the protection mechanism of
Lactobacillus plantarum A17 during convective drying using WPI, lactose, skim milk,
mixtures of lactose and WPI and the well-known non-reducing disaccharide trehalose.
4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Protectants

Five forms of protectant were used: (1) WPI 894 (Fonterra, Australia) in both pasteurised and native forms; (2) trehalose dehydrate (T9531, Sigma Aldrich, Australia); (3) lactose monohydrate (L3625, Sigma Aldrich, Australia); (4) long life skim milk (purchased from a local supermarket and used without further treatment) and (5) a mixture of lactose and WPI in a ratio of 9.4:0.6. Deionised water (Milli-Q system QGARD00R1, Millipore, Australia) was used in all experiments.

4.2.1.2 Lactobacillus plantarum A17

The test strain of L. plantarum A17 was obtained from the collection of the Laboratory of Food Microbiology, School of Applied Sciences, RMIT University, Australia. The strain was frozen at -80 °C in MRS Broth (Oxoid, Australia) with 40% (v/v) glycerol. Bacteria cells were sub-cultured and grown to the stage of peak log-phase in de Man, Rogosa and Sharpe (MRS) broth.

4.2.1.3 de Man Rogosa Sharpe (MRS) agar

This was obtained from Oxoid, Australia and contained peptone (1%), meat extract (0.8%), yeast extract (0.4%), glucose (2%), sodium acetate trihydrate (0.5%), polysorbate 80 (0.1%), dipotassium hydrogen phosphate (0.2%), triammonium citrate (0.2%), magnesium sulphate heptahydrate (0.02%), manganese sulphate tetrahydrate (0.005%) and agar (1.0%). MRS agar was used for further growth and identification of LAB colonies.

4.2.2 Sample preparation and measurements

4.2.2.1 Preparation of protectants

Protectant solutions were prepared by mixing the selected components at a concentration of 10% (w/w) in deionised water without taking into consideration the inherent moisture content.
in the original powder. WPI solution was dispersed by mixing with magnetic stirring for at least 30 min, stored at 4 °C overnight to ensure full hydration and the pH of the resulting solution was 6.6. Lactose and trehalose solutions were autoclaved at 121 °C for 15 min before use. For the WPI solution, two solutions were used: a solution pasteurised to 70 °C for 1 min and a non-pasteurised solution (native). As WPI tends to denature at temperatures beyond 65 °C, the latter solution was used as a control having minimal initial denaturation. Sterile deionised water (without protectant) was also used as a control.

4.2.2.2 Micro differential scanning calorimetry

To determine the extent of whey protein denaturation during pasteurisation, micro differential scanning calorimetry, Setaram Micro DSC VII (Setu-rau, Caluire, France) was used. Approximately 800 mg sample was filled into a DSC pan and sealed hermetically; a reference pan was also taken with deionised water of equal weight. Both pans were placed into the instrument chamber and equilibrated for 1 h at 20 °C to eliminate the effect of thermal history prior to heating to 95 °C at a programmed heating rate of 1 °C/min. Duplicate measurements were taken. During the micro differential scanning calorimetry analysis, the remaining undenatured protein in the pasteurised sample undergoes denaturation, and thus the enthalpy of denaturation is a measure of the amount of undenatured protein present in the sample after pasteurisation. The enthalpy values were calculated from the area under a line extending from 60.2 to 87.5 °C and the values were normalised to give enthalpies per gram of dry protein.

4.2.2.3 Preparation of cells for drying experiments

*L. plantarum* cells were grown in MRS broth for 17 h to reach the late growth phase. The active growing cells were homogeneously mixed and harvested by pipetting 1 mL into each of a number of Eppendorf tubes before centrifugation at 4000 g for 10 min. After decanting the supernatant, the pellet of cells in each tube was washed with sterile saline water (0.85%) and then resuspended in one of a series of different protectant dispersions (10% w/w) with a tube containing deionised water as a control. The tubes with the cell suspensions in carrier media and water were all stored at room temperature (~24 °C) for approximately 30 min before commencement of drying experiments. All tubes were placed in an ice bath throughout the drying experiments to prevent undesired cell proliferation. The viable cell concentration in the samples was checked immediately after preparation as well as after 4 h
to ensure that there was no cell proliferation. All suspensions were mixed by gently vortexing before the withdrawal of sub-samples for drying. Initial cell concentrations were $2.3 \times 10^9$ CFU/mL.

4.2.2.4 Single droplet drying experiment

A single droplet drying system in Monash University was utilised in this experiment. The principle for this unit and the procedure of droplet drying experiments were adapted from Fu et al. (2011). A schematic figure of the glass filament drier set up was adapted from Fu, Woo, Moo, & Caplice (2012) (Fig. 4.1). Briefly, an air stream with controlled temperature, velocity, and humidity was used for drying a suspended single droplet in a confined drying chamber. The single droplet was suspended at the tip of a specially made fine glass filament.

Droplets were dried for up to 240 s and cell survival was monitored every 30 s. A droplet of the cell suspension for each carrier was generated using a 5 µL gas chromatograph micro-syringe (5FX, Part# 001100, SGE Analytical Science Pty Ltd, Australia). Between drying runs, the syringe was washed with deionised, 70% ethanol, followed by sterilised deionised water to avoid cross-contamination. The initial droplet size for each run was 2 µL and the two drying temperatures (90 °C and 110 °C) with an air velocity of 0.75 m/s and moisture content of 0.0001 kg/kg were used in this study. The generated droplet was transferred and hung on the suspending glass filament inside the drying chamber with a separate transferring glass filament (Fig. 4.2a). The temperature and mass data of the droplet during drying were obtained in separate runs with identical drying conditions following the methods described by Fu et al. (2012).

For temperature measurement, the droplet was suspended by a static glass filament (Fig. 4.2b). In contrast, for mass measurement a flexible long glass filament with a bend horizontal section was used (Fig. 4.2c). Temperature data was obtained by inserting a fine-wire thermocouple (Type K, Part# CHAL-001, Omega Engineering Inc., USA) in the centre of the droplet and collecting the temperature data from the thermocouple connected to a computer via a Picometer TC-08 (Pico Technology, UK). Mass data was obtained from the changes of displacement of the mass measuring filament during drying. When there is a mass hanging at the tip, the glass filament will deflect, leading to a displacement from its original location. Throughout the drying process, this deflection will continuously reduce as moisture evaporates. The displacement history was then captured by the camcorder. From calibration, such displacement is in a linear relationship to the mass of the weight, and hence can be converted to the actual mass value by reference to a standard (Fig. 4.3). Weight standards were prepared by suspending known amounts of Vaseline-agglomerated fine glass beads (0.1-0.2 mg each) under the same drying conditions and recording the corresponding displacement of the glass filament. The actual mass of the standards was obtained using a five decimal analytical balance (Sartorius CP225D, Sartorius AG, Germany). Moisture content results were calculated from the weight measurement considering only the dissolved protectant material as the solid mass within the droplet. Values are expressed in units of kg water/kg solid matter, that is, on a dry basis (db).
Fig. 4.2 Schematic figure for the experimental set up of (a) kinetics of cell survival, (b) temperature measurement and (c) mass measurement in the glass filament rig.
Chapter 4

4.2.2.5 Isothermal heat treatment

To monitor the kinetics of cell survival during isothermal heating, cell suspensions were prepared in two separate solutions (one of WPI and the other of lactose) with the same conditions as the cell suspensions used for single droplet drying. Aliquots (1.0 mL) of the cell suspensions in either lactose or WPI (10% (w/w)) were transferred into 4 mL sterile test tubes. These tubes were then heated at 50, 60 and 90 °C for up to 180 s and the cell survival was monitored at the same time regime as used in single droplet drying. Micro-thermocouples were inserted into two additional vials containing the same amount of cell suspension in order to record the temperature history during these tests.

4.2.2.6 Enumeration of bacterial cells

For each cell suspensions, initial cell counts were taken after 30 min of mixing with carrier media and subsequently after four hours of storage in the ice bath, in order to establish whether cell proliferation was occurring. During single droplet drying experiments, drying was stopped at different time interval and the semi-dried droplet was diluted into peptone water (2 mL) without removing it from the glass filament. After appropriate dilution in peptone water, aliquots of cell suspension were plated on MRS agar followed by incubation at 30 °C for 48 h. Likewise, cell suspensions from isothermal heating, after heat treatment,
the cell suspension was serially diluted and plated on MRS agar and incubated at 30 °C for 48 h.

4.2.3. Statistical analysis

All experiments were completed in three separate runs. Statistical significance testing for the difference between treatments were analysed using an ANOVA test with Tukey Post-Hoc analysis (Minitab 16 Statistical Software). As per convention, a p value of 0.05 or below was considered statistically significant.

4.3 Results and discussion

4.3.1 Protein denaturation during pasteurisation

Fig. 4.4 Micro differential scanning calorimetry thermogram of native and pasteurised whey protein isolate (WPI) at a concentration of 10% w/w at natural unbuffered pH of 6.6 subjected to a heating scan of 1 °C/min

The extent of WPI denaturation during pasteurisation was determined by micro differential scanning calorimetry with the thermogram of native and pasteurised WPI shown in Fig. 4.4. The denaturation peak of both native and pasteurised WPI (10% w/w) is very similar, corresponding to a temperature of approximately 77 °C at pH 6.6. These values are in close agreement with those in a previous study of WPI (20% w/w) for which the denaturation
temperature was reported as 75 °C at pH of 6 (Duongthingoc, George, Katopo, Gorczyca, & Kasapis, 2013).

In the current study, enthalpy changes calculated from the thermal denaturation of native and pasteurised WPI were 0.874 and 0.748 J/g respectively. This indicates protein has been partly denatured during the pasteurisation treatment, corresponding to less than 15% of the protein has been denatured, consistent with the previous study showing that WPI only started to denature at temperatures higher than 65 °C during isothermal heating (Haque, Aldred, Chen, Barrow, & Adhikari, 2013).

4.3.2 Protective mechanism during isothermal heat treatment

![Fig. 4.5 Kinetics of cell survival during isothermal heating at 50, 60 and 90 °C of cell suspension in whey protein isolate (WPI) and lactose (Lac)](image)

The survival kinetics of cell suspension in lactose and WPI subjected to isothermal heating at 50, 60 and 90 °C are presented in Fig. 4.5. There was no significant difference \((p > 0.05)\) in survival of cells in either lactose or WPI when treated at either 50 or 60°C although cells subjected to heat treatment at 50 °C for 180 s resulted in only a slight reduction in survival. In contrast, a reduction of almost 5 log CFU/mL in viability was observed to the end of heating time when the temperature was set at 60 °C. Interestingly, there was a significant difference
(p < 0.05) in cell survival between cell suspension in lactose and WPI when subjected to water bath heating at 90 °C where almost no cells survived the first 60 s of heating for cells suspended in lactose; whereas for cells suspended in WPI, survival was slightly above 3 log CFU/mL at the end of a heating period of 180 s.

There have been numerous studies on cell death during heating as it is a commonly utilised method to eliminate or to reduce the number of microorganisms in food to ensure microbiological safety or lengthen shelf life. Apart from affecting a critical component of the cells, heat has also been assumed to influence or cause the loss of many other components, which are present in larger numbers within the cell. The loss of these less critical components does not cause cell death until the numbers are reduced to a very low number or the cell is subjected to additional stress (Gould, 1989). In addition, heat is also believed to damage some macromolecular components including DNA, RNA and proteins as well as cell membranes and ribosomes (Abee & Wouters, 1999).

Lactose at 10% (w/w) did not provide protection to strain A17 at 90 °C in comparison with WPI at the same concentration, particularly at extended heating times of 120 to 180 s. On this basis, we hypothesise that the aggregation of denatured WPI is responsible for the protection of cells during prolonged heating as it was previously reported that WPI only starts to denature at temperatures higher than 65°C during isothermal heating (Haque et al., 2013). In the current studies, although the temperature of the cell suspension reached over 65 °C only during the first 20 s of heating; we also found that less than 15% of protein becomes denatured during isothermal heating. This finding is also consistent with a previous report in which only about 30% of WPI became denatured during isothermal heating of a 10% solution at 80 °C for 60 s (Haque et al., 2013). As a consequence, we observed a statistically significant (p < 0.05) decrease in cell survival during the initial heating period. The pH of the suspension of bacterial cells in WPI used here was 6.6 and the major constituent of WPI is of β-lactoglobulin, which is a globular protein. It has been shown that upon prolonged heating at either 70 or 85 °C, particles agglomerate into large clusters and slowly precipitate (Donato, Schmitt, Bovetto, & Rouvet, 2009). King & Su (1993) also suggested that milk proteins may form a protective coating over cell wall protein, thereby protecting the cell during heating.

Another interesting trend that can be observed in Fig. 4.5 pertains to the unique strain of bacteria utilised in this research. At 50 °C, the cell survival count reduced by approximately 1
log magnitude. However, a statistically significant ($p < 0.05$) reduction occurred, by several magnitudes, at 60 and 90 °C. Therefore, it can be deduced that the apparent ‘massive destructive’ temperature threshold can be tolerated by the bacteria is in the range of $50 < T_{\text{lethal}} < 60$ °C.

The observations here indicate that whey protein reduces thermal stress as it has been shown in the isothermal heating due to denaturation of the protein, possibly forming a layer around the cell. This is consistent with the suggestion by Duongthingoc et al. (2013) that the denaturation mechanism helps protect the yeast cell by adjustment of the initial pH of the suspension close to the isoelectric point. The results in Fig. 4.5 clearly demonstrate that WPI offers a protective mechanism to the bacterium *L. plantarum* A17 strain.

### 4.3.3 Skin forming protective mechanism during single droplet drying

The temperature-time curves for the droplets containing the dissolved solids are quite distinct from those for water (Fig. 4.6) which with the latter being characterised by a long period of almost constant temperature followed by a dramatic increase to just below the drying temperature once almost all the water had evaporated. This extended period of constant temperature can be explained by evaporative cooling caused by a constant rate of moisture loss. The temperature for the droplets containing the dissolved protectants starts to increase significantly much earlier than observed for the samples of pure water. This early initiation of a temperature rise within the droplets may be caused by the polar water molecules becoming bound to the polar protectant molecules, thereby reducing the drying rate and hence the magnitude of evaporative cooling. The progressive formation of a solid outer crust will also impede the removal of moisture during the drying process. The temperature-time curve clearly shows that the temperature for the droplets containing either WPI or skim milk rose even sooner than for the droplets containing other protectants. This reflects the skin-forming surface-active behaviour of WPI enhancing the surface crust formation during convective drying. For the drying at a temperature of 110 °C, the pasteurisation of WPI resulted in enhanced skin-forming behaviour. One possible explanation for this could be that as a result of pasteurising the WPI components close to the denaturation temperature of β-lactoglobulin (78 °C) (Gulzar, Bouhallab, Jeantet, Schuck, & Croguennec, 2011), the whey proteins agglomerate and can be considered to be in a globular molten state.
Fig. 4.6  Droplet temperature during single droplet drying of pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture of lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C. Note that different vertical scale are used in the two graphs.
Fig. 4.7  Kinetics of cell survival during single droplet drying of cell suspension in pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture of lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C
Corresponding to the temperature-time trend, Fig. 4.7 shows the kinetics of cell survival with different protective substances, along with the cell suspension in water as a control, during single droplet drying at 90 and 110 °C. During the first 90 s of drying for 90 °C, there was no significant cell reduction \((p > 0.05)\) as the survival rate was approximately \(10^9\) CFU/mL for each of the protectants tested. The same trend was also observed for up to 60 s of drying at 110 °C. While the low reduction in survival rates of the other protectants during this period can be attributed to the relatively low temperature of about 30-40 °C, it was surprising that the higher temperatures, in the range of 47-60 °C for the WPI (both pasteurised and native), did not result in a significant reduction \((p > 0.05)\) in survival. This could be due to the temperature, although higher, but still being lower than the ‘massive destruction’ temperature in the range of 50-60 °C, established in the studies of isothermal heating.

Beyond the initial period characterised by relatively high cell survival, a sudden reduction in cell counts of several orders of magnitude was observed. For both drying temperatures used in this study, the sudden reduction in cell survival appeared to correspond to the sudden increase in temperature of the droplet for pure water, as well as those containing lactose, trehalose and mixture of lactose and WPI. For the water there was a slight delay of cell survival reduction due to the delay in sudden temperature increase in the absence of crust formation. This evidence supports the view that the rate of change of temperature might be the primary factor, which contributes to the death of the cell. The maximum rates of temperature increase for these droplets are approximately 1.2 °C/s (90 °C) and 1.9 °C/s (110 °C).

Throughout the time when there is a sudden reduction in cell survival (Fig. 4.7), corresponding to approximately 90-120 s (90 °C drying condition) and 60-90 s (at 110 °C), the moisture content remains relatively high, at about 0.70-2.05 kg/kg db (Fig. 4.8). This is above the critical moisture content at which WPI is reported to rapidly denature during convective drying, with values of 0.35 kg/kg db and 0.65kg/kg db during drying at 65 and 80 °C respectively (Haque et al., 2013). We suspect that this sudden reduction in cell survival may have been due to the effect of the sudden temperature rise.

On the other hand, it could also be argued that the droplet would have reached the maximum destruction temperature of approximately 50-60 °C; it is also possible that the destruction temperature may even be lower when the droplet is dehydrated to a moisture content of
Fig. 4.8  Moisture content on dry basis during single droplet drying of cell suspension in pasteurised whey protein isolate (WPI), native WPI, lactose (Lac), trehalose (Tre), skim milk (SM), mixture lactose and whey protein isolate (LacWPI) and water: (a) Drying at 90 °C, (b) Drying at 110 °C
around 50% (w/w). However, due to the rapid increase in temperature during that period, it is difficult to discern which is the dominant factor contributing to the reduction in cell viability.

Trehalose is widely reported to be effective as a protectant during spray drying of bacteria and this is particularly attributed to the replacement mechanism (Santivarangkna, et al., 2008). Surprisingly, in the current study, this disaccharide was not associated with significant protection of the bacterium. Lactose in skim milk has been suggested to interact with cell membrane and helps to maintain membrane integrity in a similar way to the non-reducing disaccharide trehalose (Corcoran et al., 2004). The latter is one of the most widely recognised osmotic protectants against both osmotic and thermal stresses (Obuchi, Iwahashi, Lepock, & Komatsu, 2000) as it exhibits a universal protective effect on anhydrobiotic cells including those of microorganisms. Water replacement, high glass transition temperature and thermo-protectant against protein denaturation have been proposed for the mechanism of protection by trehalose during dehydration (Santivarangkna et al., 2008). For example, adding 0.25 M trehalose reduces the loss of viability of *L. bulgaricus* during both heat and osmotic dehydration at 70 and 20 °C respectively (Zavaglia, Tymczyszyn, Antoni, & Anibal, 2003). Similarly, suspension of cells in 20% trehalose resulted in up to 68% of survival during spray drying for outlet temperatures in the range of 65 to 70 °C (Sunny-Roberts & Knorr, 2009). Therefore the current results indicate that, the water replacement protective mechanism might not be suitable for stresses existing under convective droplet drying conditions. This may explain why sometimes such protectants do not work for certain drying processes or strains of bacteria (Santivarangkna, Kulozik, & Foerst, 2006).

Skim milk appeared to have resulted in higher cell survival during drying than native WPI (Fig. 4.7a (120 s) and Fig. 4.7b (90 s). However, the difference was found to be not significant (*p* > 0.05). In contrast to sugar, the sudden reduction of the cell survival count for the skim milk and the WPI droplets did not correspond to the period of sudden increase in temperature of the droplet. The sudden increase in temperature occurred relatively early at approximately 45-60 s (90 °C) and 32-52 s (110 °C), whereas the sudden drop in cell survival count only began at approximately 90 s and 60 s, respectively. This could be due to the slower rate of temperature increase, approximately 0.45 °C/s (90 °C) and 0.66 °C/s (110 °C), which might have imparted less stress for the bacterial cells. This may reflect the lower rate of temperature change due to the inherent skin forming behaviour of the proteins in both the WPI and the skim milk droplets. If the slower rate of temperature increase imparts less stress
upon the cells, then what caused the sudden magnitude decrease in cell viability count at approximately the same time with the other droplet? From an examination of the temperature curves, we speculate that the subsequent decrease in cell survival count at around 120 s (90 °C) and 90 s (110 °C) was primarily due to the droplet approaching the ‘maximum destruction’ temperature. The significance of the skin forming behaviour could provide one explanation as to why the lactose-WPI mixture, which only contains a very minimal percentage of WPI, and resulting in no early crust formation behaviour, did not offer similar protection.

Although solutions used as protectants were prepared at 10% (w/w), inherent powder moisture content was not taken into consideration; thus, initial solid contents may have differed slightly from the reported values. From the moisture loss curves in Fig. 4.8, during the intermediate stage of drying, the drying rates for each of the droplets were very similar. Therefore, it is unlikely that differences in dehydration stresses will be the principal factor in the enhanced protective mechanism of the WPI and skim milk droplets. One can argue that the differences in the moisture content during the intermediate drying period might affect the cell viability count. The values of moisture content obtained at the start and end of the period of rapid temperature increase are summarised in Table 4.1. An average value is given for droplets containing protectants other than WPI and skim milk, as their temperature-time curves are quite closely superimposed.

Table 4.1 Comparison of moisture content of droplets containing WPI with droplets containing other protectants at the start and finish of the period of rapid temperature increase

<table>
<thead>
<tr>
<th>Drying temperature (°C)</th>
<th>90</th>
<th>120</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start/finish of rapid increase (s)</td>
<td>90</td>
<td>120</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>WPI</td>
<td>1.36</td>
<td>0.51</td>
<td>1.96</td>
<td>0.58</td>
</tr>
<tr>
<td>Average for lactose and trehalose</td>
<td>0.89</td>
<td>0.31</td>
<td>1.39</td>
<td>0.35</td>
</tr>
<tr>
<td>Difference</td>
<td>41%</td>
<td>48%</td>
<td>34%</td>
<td>49%</td>
</tr>
</tbody>
</table>

Droplets containing WPI had moisture contents that were 34-41% higher than those containing other protectants at the start of the period of rapid temperature increase, whilst droplets containing WPI had a content of 48-49% higher at the completion of the period of
rapid increase. It is unclear as to whether higher moisture contents help to preserve the cell viability. Nevertheless, even if it contributes to the cell counts, the higher moisture content manifests from the surface-active development of crust for WPI and skim milk; further highlighting the importance of the earlier skin formation.

Examining the survival count for extended drying periods of up to 240 s, for which the moisture content in all particles are similar, within the range of 0.1-0.2 kg/kg db, the survival count for WPI is still higher than that for the other protectants. Surprisingly, the extended drying conditions revealed that skim milk ‘loses’ the protective behaviour within the extended drying region, approaching the cell counts found for the other protectants, in contrast to WPI ($p < 0.05$). This was despite both skim milk and WPI displaying the skin formation behaviour described earlier. It is noted here that the use of the term skin forming in relation to the changes to whey proteins during drying reflects the observations subsequently made on the spray dried microcapsules and discussed in Section 6.3.5 of this thesis (page 126). The results obtained with single droplet drying indicate that a different mechanism might be responsible for the protective behaviour towards the later stages of drying. At the moment, more work is required to determine the exact protective mechanism for the latter stages of drying. One possible mechanism is the denaturation and aggregation of protein during drying (Duongthingoc et al., 2013). Previous work on various types of milk proteins (including casein) provided evidence that the aggregation of whey protein resulted in the release of sulfur-containing amino acids thereby inhibiting lipid oxidation of the bacterial membrane during spray drying (Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2013). This aspect will be discussed in the next section.

4.3.4 Possible influence of protein denaturation and aggregation on the protective mechanism

The denaturation and aggregation of WPI was previously suggested to be a dominant mechanism contributing to the protective mechanism on cell viability (Duongthingoc et al., 2013). This was deduced primarily from the conditions of the final dried product without examining in detail how this protective behaviour is developed during \textit{in situ} drying. In the current study we have attempted to unveil more on this mechanism by comparing the results for native WPI with those for the pasteurised WPI solution. Although the difference in the degree of denaturation between the two solutions was less than 15%, determined
calorimetrically, the increase in temperature of the pasteurised WPI droplet was more rapid and occurred earlier than the native one. This might indicate a higher degree of protein aggregation leading to more significant crust formation during convective drying. However, comparison of the cell survival count between both droplets for the two drying temperatures revealed no significant differences ($p > 0.05$). Therefore, the degree of denaturation of less than 15% induced by the pasteurisation step did not influence the protective mechanism observed for the WPI experiments.

We then further explored the possible influence of *in situ* development of denaturation affecting the protective mechanism on the cell viability count for the WPI and skim milk droplets. For convective drying conditions, Haque et al. (2013) reported an irreversible denaturation temperature for whey of approximately 54 °C at drying temperatures of 65 °C and 80 °C. The report also noted critical moisture of 0.35-0.56 kg/kg db below which, rapid denaturation can potentially occur due to thermal stresses. Based on this information, rapid development of denaturation could have possibly occurred between 90-120 s (for the 90 °C case) and 50-95 s (for the 110 °C case taking WPI droplet for the lower time limit). These two time durations correspond to the period of drastic reduction in cell viability count for the two drying conditions respectively. Therefore, there is a chance of *in situ* irreversible protein denaturation contributing to the enhanced protective characteristics when compared to those of the sugar protectants studied.

If the denaturation of the WPI components contributes to the protective mechanism, then why did the mixture of lactose and WPI not display such protective behaviour and why did the skim milk ‘lose’ the protective mechanism towards the latter stages of drying? In comparison with the droplet prepared with WPI, the mixture of lactose and WPI, as well as skim milk, contained only around 0.6% of whey components. Drawing an analogy to previous research, although not a direct comparison, lactose-sodium caseinate protectant mixture was shown to improve the survival rate of *Lactococcus lactis* when compared to pure lactose (Ghandi, Powell, Chen, & Adhikari, 2012). However, the proportion of sodium-caseinate to lactose used was 1:3, which was significantly much higher than for the skim milk protein and lactose used here. Study on the usage of whey permeate consisted mainly of lactose with low whey protein concentration used as a protectant in spray drying of *Lactobacillus acidophilus* resulted in only half of the survival rate when compared to reconstituted skim milk (Riveros, Ferrer, & Borquez, 2009). Therefore, the current results show strong evidence that the high
Chapter 4

proportion of lactose with a low proportion of whey protein is not sufficient to confer protection against such thermal and moisture stress, indicating that a minimal threshold amount of WPI is required to offer effective protective behaviour.

4.3.5 Possible dual protective mechanism and future work

From the discussions presented here, there appears to be two possible mechanisms in which WPI provides protection to cells during convective drying of droplets. In preventing a sudden temperature increase, the skin formation characteristic of WPI reduces the thermal stress and possibly preserves higher moisture contents for the microorganisms during the intermediate stage of drying. There are potentially many ways, which can be adopted to control the process of skin formation. An increased rate of formation might be induced by adjusting the initial pH of the cell suspension, or by using either a higher drying temperature or increased initial concentrations of protectant. The first of these, however, has to be balanced to ensure that the outlet temperature of the spray dryer is not excessively high, which would induce a different type of thermal stress due to prolonged exposure to high temperature conditions (Fu & Chen, 2011). In addition, variations in initial pH could also induce early skin formation, which could bring about cell protection and research on this is in progress. Concurrent to the skin formation mechanism, the irreversible denaturation of the WPI might also contribute to the protection. More work is required to independently assess both factors to determine which mechanism pre-dominates in contributing to this protective phenomenon.

4.4 Conclusions

A potential probiotic strain of L plantarum A17 suspended in a series of different carriers (WPI, lactose, skim milk, trehalose, as well as a mixture of lactose and WPI), were convectively dried using the single droplet drying approach to study the cell protection mechanism afforded by different substances. WPI provided the highest protection during the intermediate and latter stages of drying. During the intermediate drying stage, a reduction in the rate of temperature increase and the resultant preservation of higher moisture content, due to the skin forming behaviour, could be a major factor contributing to the protective behaviour. Concurrently, the development of irreversible denaturation might also be a further factor influencing the survival of the bacteria. It was deduced that WPI concentration might be another important factor in providing sustained protection towards the latter stage of drying.
Chapter 5

Comparative influence of pH and heat on whey protein isolate in protecting *Lactobacillus plantarum* A17 during spray drying

Abstract

*Lactobacillus plantarum* A17 was encapsulated in a series of matrices of whey protein isolate (WPI) formulated with varying degrees of denaturation induced either by acid or heat treatment prior to spray drying. The interaction window leading to complexation of WPI and the bacteria at different pH values was established by zeta potential measurements. Whey protein capsules (with moisture contents of ~ 5.5%) obtained at pH values of 7 (native) and 4 and those at pH 7 but with heat treatment at 75 °C for 1 minute and 78 °C for 10 minutes resulted in survival rates of 69.0, 39.3, 40.3 and 25.0% respectively. A significantly higher survival of cells was observed in the matrix of native WPI where the protein structure was more compact and globular with 47, 7, 17 and 29% β-sheets, random coils, α-helices and β-turns, respectively. After exposure to acid at pH 4, the survival of A17 was reduced to ~39% with the whey protein exhibiting a less compact and partial unfolding of the compact globular structure as demonstrated by a significant increase in the β-sheets to 53% and a complete loss of random coils as shown by the Fourier transform infrared spectroscopy. It appears that a lower degree of protein denaturation before spray drying as characterized by Micro differential scanning calorimetry benefits the survival of A17 during spray drying. In addition, our spray drying conditions (outlet temperature of ~70 °C) do not contribute significantly to further denaturation of the WPI matrix. A unique layer-by-layer electrostatic mechanism involved in encapsulation of A17 at pH 7 was found responsible for higher survival of these cells.

*Keywords:* spray drying, lactic acid bacteria, whey protein isolate, microencapsulation, layer-by-layer
Chapter 5

5.1 Introduction

Over the last decade, exploration to find materials and procedures suitable for the production of functional foods containing nutraceuticals or probiotics has been on a rapid rise (Heidebach, Forst, & Kulozik, 2012; Matalanis, Jones, & MacClements, 2011). One of the approaches most commonly utilised for the protection of active ingredients including bacteria is microencapsulation, a technique by which liquid droplets, solid particles, gas, or microbial cells are entrapped into a food grade microencapsulating agent (Anal & Singh, 2007; Huq, Khan, Khan, Riedl, & Lacroix, 2013). A variety of biopolymers have been reported to be effective materials for the encapsulation of probiotic bacteria (Huq et al., 2013). Among these, protein-based microparticles occupy an increasingly important position as the versatile nature of proteins which allows new design strategies to encapsulate many bio-active ingredients including microbial cells (Chen, Remondetteo, & Subirade, 2006).

Whey proteins that make up 20% of the milk proteins are of considerable interest as an encapsulant in the food industry because it is highly nutritious due to its high content of sulphur-containing amino acids (cysteine, methionine). Unlike other essential amino acids, these two are metabolised in the muscle tissues (Hayes & Cribb, 2008) and are particularly important for athletes and body builders. Although much work has been done on the important functional properties of whey proteins, application of these in the encapsulation of microorganisms is still limited. In particular, the interactions of *Lactobacillus plantarum* with respect to the influence of denatured whey proteins as an encapsulant, is still unclear. This species, which predominates the microbial communities in fermented foods because of its greater acid tolerance, (Daeschel & Fleming, 1984) is unique, so that selected strains are currently marketed as probiotics with health-promoting properties.

The composition, concentration and isoelectric points of whey proteins play a significant role in its functionality and interactions with other components. Whey proteins are made up primarily of β-lactoglobulin (70.2%) and α-lactalbumin (14.1%) along with small fractions of bovine serum albumin (8.2%), immunoglobulins (6.5%) and lactoferrin (1.0%) (Morr & Foegding, 1990; Tavares, Crouguennec, Carvalho, & Bouhallab, 2014). Most of these proteins have isoelectric points (pI) at pH values of ~5 with the exception of immunoglobulin and lactoferrin which have the pI in the pH ranges of 8.3 to 8.5 (Eigel et al., 1984; Tavares et al., 2014). The major constituents of whey proteins largely determine the overall properties of
WPI. In their native state, whey proteins are typically compact globular proteins that remain soluble at or near their isoelectric points (pH 4.2–5.0).

The stability of whey proteins during thermal processing has been comprehensively reviewed recently (Wijayanti, Bansal, & Deeth, 2014). In brief, the denaturation induced by heat results firstly in unfolding of the compact globular structure. This partial unfolding involves partial loss of helical secondary structure at approximately 65 °C (Townend & Gyuricsék, 1974) and these reversible changes are generally described as the onset of the agglomeration stage. Subsequently, further unfolding causes aggregation, primarily due to the exposure of functional groups, which were otherwise previously enclosed within the structure, leading to protein-protein interactions. These are predominantly non-polar groups as well as an abundance of sulfhydryl amino acid residues which are then able to form intermolecular disulfide bonds via the activated thiol groups (Lee, Morr, & Ha, 1992). Denatured whey proteins are stiffer, stronger and more stretchable (Kinsella & Morr, 1984). In addition, denatured protein was reported to enhance the encapsulation process as it favours adsorption at the interface resulting in the formation of a thin, gel-like layer (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). There have been some suggestions in the literature that denaturation of WPI before spray drying provided enhanced protection to microorganisms as compared to the undenatured native state. These changes were induced either by heat, in a study of *L. plantarum* (Rajam, Karthik, Parthasarathi, Joseph, & Anandharamakrishnan, 2012) or by pH changes for a yeast (Duongthingoc, George, Katopo, Gorczyca, & Kasapis, 2013). However, the encapsulation of microorganisms is highly complex where different techniques and strategies appear to be effective for particular species and these effects are also dependant on the environment (Sunny-Roberts & Knorr, 2009; Ying et al., 2010).

Processing treatments including spray drying have the potential to transform previously existing native structures into denatured or partially denatured states; or previously partially denatured proteins into aggregates. The presence of particular states may either present problems or offer opportunities. In our previous study, we reported that in comparison to other proteins and carbohydrates, whey protein isolate (WPI) in its native state provided enhanced protection to *L. plantarum* A17 during single droplet drying (Khem, Woo, Small, Chen, & May, 2015). It has been established that denatured proteins typically enhance the encapsulation of microorganisms over the use of native proteins. The mode whereby the
proteins are denatured may offer additional value to control the encapsulation process, and so the current study systematically investigates the influence of whey proteins, denatured either by acid or heat, as potential avenues for enhanced protection of *L. plantarum* A17.

### 5.2 Materials and methods

#### 5.2.1 Materials

WPI was obtained from Fonterra, Australia (WPI 894, Fonterra, Australia). According to the product specification from the typical analysis provided by the manufacturer, the bulk density was 0.34 g/mL. It had a protein content of 90.4%, fat 1.0%, moisture 4.7%, ash 3.0% and 0.9% carbohydrate. De Man Rogosa and Sharpe (MRS) agar and broth were obtained from Oxoid Australia. Phosphate buffer saline solution was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄. Other chemicals utilised in this study were of analytical grade; and also deionised water (Milli-Q system QGARD00R1, Millipore, Australia) was used in all experiments.

**5.2.1.1 Lactobacillus plantarum A17**

The test strain of *L. plantarum* A17 was obtained from the collection of the Laboratory of Food Microbiology, School of Applied Sciences, RMIT University, Australia. The strain was preserved at -80 °C in MRS Broth (Oxoid, Australia) with 40% (v/v) glycerol. The working culture was prepared by sub-culturing and growing the cells in de Man, Rogosa and Sharpe (MRS) broth and plated on MRS agar. Colonies were regularly transferred to new MRS agar every two weeks and purity check by Gram staining was done every month.

#### 5.2.2 Sample preparation and analysis

**5.2.2.1 The growth profile of A17**

To determine the growth profile of *L. plantarum* A17, 2% (v/v) of actively growing cells were sub-cultured onto MRS broth which had been adjusted to pH 2, 4, 6, 8 or 10, at the initial concentration of 7.78 log CFU/mL. After incubation at 30°C for 17 h, cells growing in the cultures were serially diluted before plating on MRS agar and incubated at 30 °C for 48 h.
5.2.2.2 Zeta potential measurements

The zeta potential of whey protein solution (2% w/w) and bacterial suspension in phosphate buffer at a cell concentration of about $10^9$ CFU/mL were determined using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, Worcestershire, UK) at different pH values, in order to gain insights into the interaction between bacterial cells and the encapsulating agent. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion. The pH of the solution was adjusted to the required values by the addition of either 0.1 M NaOH or 0.1 M HCl.

5.2.2.3 Preparations of whey protein isolate (WPI) solutions

WPI solutions were prepared by adding 10 g of powder into pre-sterilised 90 g water with the aid of a magnetic stirrer at room temperature for at least 30 min. The solutions were then kept in a refrigerator at 4 °C overnight to allow for complete protein hydration before adjusting to the desired pH by adding either 1 M NaOH or 1 M HCl. The heat treated whey proteins were obtained by heating the native whey solution (pH adjusted from 6.6 to 7.0) in shaking water bath (Ratek Instrument, Pty, Ltd., Australia) to 75 °C at a speed setting of 3 and held for 1 min, or to 78°C for either 10 or 20 min and cooled to 20°C before inoculation with the seed culture.

5.2.2.4 Preparation of bacterial cells for encapsulation

One colony of the working culture was grown in MRS broth (5 mL) for 24 h at 30°C. Cell suspension (2% v/v) was re-grown in fresh MRS broth at 30 °C for a further 17 h to reach the end of the growth phase. The actively growing cells were harvested by centrifugation at 4000 g for 10 min followed by washing with 0.85% saline water. The washed cell pellets served as the seed culture for microencapsulation.

5.2.2.5 Microencapsulation of A17 by spray drying

The seed culture was mixed with 10% (w/w) WPI solution which had been prepared as above at room temperature (~22 °C) with the aid of magnetic stirrer for 30 min. Cell suspensions in WPI contained an average cell count of approximately $3-4 \times 10^9$ CFU/mL. This was spray
dried using a LabPlant SD-Basic FT30MKIII spray drier (Keison products, Chelmsford, Essex, UK) with the help of a peristaltic pump. Controls were set at 392.26 kPa for air pressure and 6.6 mL/min for flow rate. The inlet temperature was 110 °C and this resulted in an outlet temperature of 68-70 °C. These spray drying conditions were found to provide powder with desired moisture content for storage stability. To ensure an even distribution of bacteria, the feed solution was continuously stirred using a magnetic stirrer, throughout the process of spray drying.

5.2.2.6 Survival rate of bacteria during spray drying

Bacterial cell count was enumerated by taking 1 mL of cell suspension in WPI prior to spray drying and serially diluted. Aliquots of 0.1 mL were transferred and plated onto MRS agar, followed by incubation at 30 °C for 48 h. Spray dried capsules were reconstituted with peptone water to the same initial solid content for 20 to 30 min followed by serial dilution and plating. Cell survival was calculated as:

\[
\% \text{ survival} = \frac{N}{N_o} \times 100
\]

where \(N\) and \(N_o\) are cell count after and before spray drying in CFU/mL.

5.2.2.7 Analysis of moisture content

The moisture content of spray dried powders was analysed by moisture analyser MB45 (Ohaus Corporation, USA) with a standard method of analysis. Approximately 1 g of spray dried capsules were spread on an aluminium pan and the sample was heated to 105 °C and held until the mass change of less than 1 mg for 90 s was achieved.

5.2.2.8 Micro differential scanning calorimetry (Micro DSC)

To determine the extent of whey protein denaturation after heat treatment and/or spray drying, micro DSC (Setaram Micro DSC VII, Setu-rau, Caluire, France) was used. Approximately 800 mg sample at a concentration of 10% (w/w) was placed in the sample holder, hermetically sealed and the same mass of deionised water was placed into the other chamber as a reference. Samples were scanned from 20 to 95 °C at a heating rate of 1 °C/min.
All DSC measurements were carried out in duplicate. The enthalpy of denaturation was used as a measure of the amount of undenatured protein remaining in the sample after heat treatment and/or spray drying according to the equation below (Nicorescu et al., 2009).

\[
\% \text{ denaturation} = [1 - \left( \frac{E_{\text{heat treated or spray dried}}}{E_{\text{native}}} \right)] \times 100
\]

Where \( E_{\text{heat treated or spray dried}} \) is the energy associated with the denaturation peak of heat treated or spray dried samples and \( E_{\text{native}} \) is the energy associated with the denaturation peak of the native protein (pH of the native protein was adjusted from 6.6 to 7 for consistency).

5.2.2.9 Morphology of spray dried powder by scanning electron microscopy (SEM)

Surface morphology of spray dried powders was evaluated using an FEI Quanta 200 SEM (Hillsboro, OR, USA). Powdered samples deposited on aluminium sample stubs using double-sided adhesive carbon tape were sputter coated with gold for 60 s using an SPI-Module sputter coater to avoid sample charging. Images were taken under high vacuum with an accelerating voltage of 30 kV.

5.2.2.10 Fourier transform infrared spectroscopy (FTIR)

Changes in the secondary structural elements (\( \beta \)-sheets, \( \alpha \)-helix, \( \beta \)-turns and random coils) of WPI following spray drying process were observed using a Perkin Elmer Spectrum 100 FTIR spectrometer (MA, USA). The spray dried and the control samples (WPI from supplier) were dissolved into deuterium oxide (\( \text{D}_2\text{O} \), Cambridge Isotope Laboratories, Inch, MA, USA) at 20 mg/mL. Approximately 20 \( \mu \text{L} \) from each treatment was placed on the sample diamond crystal and the spectra were recorded in the absorbance mode within the IR region of 650 to 4000 cm\(^{-1}\). For each spectrum, an average of 16 scans was recorded at 4 cm\(^{-1}\) after background subtraction to ensure elimination of extraneous artefacts. The Perkin Elmer Spectrum 100 utilised a “smart, plug-play-and-go” Universal-ATR (UATR) technology, which ensured that sample loading was performed consistently for all samples. The recorded spectra were analysed by using Perkin Elmer proprietary software (Version 10.03.06) and OPUS 6.5.92 software (Bruker Biosciences Pty Ltd., Victoria, Australia). To identify the peak position of the spectra, a 13-point second derivative analysis was done using the software. This was used to fit the Gaussian bands for the quantitative analysis and the percentage of each structural
element was estimated using the following equation (Ngarize, Herman, Adams, & Howell, 2004).

Secondary structure (%) = \( \frac{A_{\text{ind}}}{A_{\text{all}}} \times 100 \)

Where, \( A_{\text{ind}} \) refers to the sum of areas of individual secondary structural elements within amide-I band and \( A_{\text{all}} \), refers to the sum of areas of all secondary structural elements within amide-I band. The locations of the bands of WPI were assigned according to those suggested in the literature (Haque, Chen, Adldred, & Adhikari, 2015; Kong & Yu, 2007).

5.2.2.11 Statistical analysis

All experiments were carried out in duplicate from two independent experiments. Data were subjected to simple classification analysis of variance and to Turkey’s mean comparison analysis. Statistical significance between treatments of encapsulant including pH adjustment and heat treatment in comparison to the native whey protein was tested by one way ANOVA and a \( p \) value of less than 0.05 was considered as statistically significant. All statistical analyses were performed using Minitab16 software, State College, PA Inc.

5.3 Results and discussion

5.3.1 Growth profile of *L. plantarum A17* in varying pH conditions

All bacterial species have limited tolerance ranges and are individually sensitive to pH. The growth profile of *L. plantarum A17* as pH was varied from 2 to 10 is shown in Fig. 5.1. It was observed that *L. plantarum A17* can grow equally well within the broad pH range of 4 to 8.

5.3.2 Effect of zeta potential in varying pH conditions on A17 cell survival

Zeta potential measurements of *L. plantarum A17* (10⁹ CFU/mL) and WPI solution (2% w/w) at different pH values are presented in Fig. 5.2. These measurements provide the overall charge on bacterial cell surfaces and whey protein molecules as they were subjected to different pH values. A17 cells were negatively charged throughout the pH range from 2 to 10.
Fig. 5.1  Growth profile of A17 in MRS broth under different pH conditions

Fig. 5.2  Zeta potential of bacteria suspended in phosphate buffer at about $10^9$ CFU/mL and WPI at 2% (w/w)
At approximately pH 4.5, the *pI* (isoelectric point), the protein had no net charge, thereby providing maximum potential for protein-protein interactions. The WPI solution was positively charged below the *pI* and negatively charged above the *pI*. The electrostatic interactions can be explained according to the relationship between net charge of WPI, A17 and pH. At pH 3, WPI had a very high net positive charge, which means that the proteins in the solution will have high repulsive forces between them. These repulsive forces will inhibit interactions between proteins resulting in a clear solution (Fig. 5.3). As the pH was increased to 4, the net charge was decreased and the repulsive forces between proteins were also reduced resulting in an increase in turbidity (Fig. 5.3). At pH 5 (closest to the *pI*), since most of the WPI protein molecules remain in zwitterionic stage, the repulsive forces are reduced to such an extent that it leads to precipitation of WPI (Fig. 5.3). As the pH increased further from 6-10, the net charge becomes strongly negative, thereby increasing the repulsive forces between protein molecules, leading to sequential reduction in turbidity.

![Fig. 5.3](image)

**Fig. 5.3** Effect of pH adjustment and heat treatment on the colour of whey protein isolate solution at 10% (w/w)

These results, at first glance, appear to indicate that pH 4 may provide better protection to A17 cells compared to pH 7 as at pH 4 there is probably increased attachment or adhesion of cells to WPI due in part to the electrostatic interaction between the positive charge of WPI and negative charge of bacterial cell surfaces. It was also previously demonstrated that the attachment of particles to cell membrane appears to be most affected by the surface charge of
the particles (Annan, Borza, & Hansen, 2008). Variation of the charge could potentially control their binding to the cellular surfaces. However, at pH 7 of WPI (close to native state of pH 6.6), the survival rate of A17 was found to be at its highest (~ 69%) compared to pH 4 where the survival of A17 was reduced to almost half (~ 39%) (Table 5.1). It is important to note from Fig. 5.1 that pH values between 4 and 8 in a bacterial growth medium offered optimal conditions for the growth of *L. plantarum* A17 and therefore, the lower reduced survival of A17 at pH 4 cannot be assigned to this growth factor. One possible explanation for these observations is that at pH 4, all of the WPI constituents will be positively charged, that would have led to their rapid denaturation followed by precipitation. Conversely, at pH 7, while the overall charge of WPI appears to be negative from zeta potential measurements, since some of the WPI constituents, particularly immunoglobulin and lactoferrin have higher pI (~8.5), these will be positively charged, while the majority of other constituents will remain negatively charged. Notably, at pH 7, the cell surface of A17 is negatively charged.

Table 5.1 Effect of heat treatment and pH of WPI at 10% (w/w) on survival of A17, moisture content and protein denaturation before and after spray drying at inlet and outlet temperatures of 110 and 68 to 70 °C, respectively, and a flow rate of 6.6 mL/min

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Moisture (%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Protein denaturation (%)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Temp (°C)</td>
<td>Time (min)</td>
<td>Before spray drying</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>-</td>
<td>69.0 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>39.3 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.0</td>
<td>75</td>
<td>1</td>
<td>40.3 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.0</td>
<td>78</td>
<td>10</td>
<td>25.0 ± 7.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.0</td>
<td>78</td>
<td>20</td>
<td>25.0 ± 3.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Survival data was calculated from an average of at least four readings

<sup>2</sup>Calculated with respect to pH 7.0 before spray drying. Different letters in each column signify significant difference at \( p < 0.05 \)

Therefore, pH 7 offers an opportunity for WPI constituents to controllably assemble/precipitate on the surface of A17 cells through a layer-by-layer process involving electrostatic interactions. For instance, initially positively charged WPI constituents may bind to the negatively charged bacterial surface, followed by negatively charged major
constituents, further followed by positively charged proteins, and so on in a sequential electrostatic assembly fashion (Fig. 5.2). Such layer-by-layer assembly of oppositely charged macromolecules and polymers on nanoparticle surfaces has been extensively reported in the literature to create drug-delivery systems (Goethals, Shukla, Mistry, Bhargava, & Bansal, 2013; Sivakumar et al., 2009) and we believe that it is this slow layer-by-layer electrostatic assembly of WPI constituents on A17 cells, which is predominantly responsible for protecting and maintaining the active state of the bacterial cells at pH 7. This is an interesting observation as it indicates that while bacterial cells may grow equally well at a range of pH conditions; during their protection with multiple protein-based natural materials such as WPI, an active chemistry between the material constituent molecules can play a major role in dictating the appropriate pH for optimal performance of encapsulated cells.

5.3.3 Effect of heat treatment and pH of WPI on cell survival

When heat was applied at temperatures close to the denaturation of the major proteins of whey, protein aggregation and precipitation occurred readily at pH 7. The denatured proteins induced by heat at 75 °C for 1 min offered comparable protection as pH-induced denaturation, leading to 39% survival (Table 5.1). However, prolonged heating for either 10 or 20 min resulted in more aggregation of WPI proteins and survival rates were reduced to 25%. It is important to note that for various treatment groups, there was no significant difference (p > 0.05) for the final moisture content (~5%) of spray dried products using drying conditions of inlet (110 °C) and outlet (68 to 70 °C) temperature and a flow rate of 6.6 mL/min.

There have been previous reports investigating the effect of denatured whey protein on the protection of different strains of bacteria following spray drying. Denatured WPI prepared at 10% (w/v) undergoing heat treatment at 80 °C for 30 min was reported as the best encapsulant for Bifidobacterium breve and B. longum with survival rates of 25.7 and 1.4%, respectively, compared to the use of other emulsion methods followed by spray drying at an outlet temperature of 80 °C (Picot & Lacroix, 2004). Denatured WPI was reported to provide better protection to L. plantarum both during spray drying and simulated acidic and bile conditions (Rajam et al., 2012). In the context of yeast cells, it was previously reported (Duongthingoc et al., 2013) that whey protein denaturation as a result of adjusting pH to 4 (a value close to the isoelectric point) enabled whey proteins to agglomerate, thereby protecting...
yeast cells during spray drying with a survival rate of approximately 38% as compared to a survival of about 3% when at pH 7. These survival rates are far from being desirable from a functional food perspective. In contrast, the current study demonstrates that by exploiting electrostatic interactions between different WPI components, a significantly higher survival rate of 69% can be achieved for *L. plantarum* A17 cells.

Our results indicate that once denaturation is induced (whether by acid or heat), this changes the conformation of WPI causing a negative impact on cell viability. Both, either adjusting the pH value to 4.0 or heating of solutions to 75°C for 1 min at (pH 7) are accompanied by visual observations of aggregation indicating protein denaturation (Fig. 5.3). To further understand the extent of protein denaturation induced by heat and pH before and during spray drying, we employed micro DSC.

**5.3.4 Effect of heat and acid on protein denaturation before and during spray drying**

Spray drying is a mechanical and thermal process in which samples undergo shear and heat stresses which might further contribute to the denaturation of protein molecules. To determine the degree of protein denaturation, WPI solutions subjected to different heat treatments prior to spray drying and the resultant spray dried powders which were reconstituted to the same initial concentrations, were investigated using micro DSC.

The thermal events of native (before spray drying) and spray dried whey protein are presented in Fig. 5.4 for samples prepared at a concentration of 10% w/w. The WPI solution at pH 7 showed a broader peak with a lower mid-point denaturation temperature (74.6 ± 0.5 °C) reflecting the less cooperative structure of the whey protein at a pH far from the isoelectric point (pH 4.5), and stronger electrostatic repulsion between molecules due to most of the protein molecules in WPI being negatively charged at pH 7. This result corroborate well with a previous report (Ramos et al., 2012) on the denaturation temperature of 10% (w/w) mixture of β-lactoglobulin and α-lactalbumin which are the two major components of WPI. In contrast, whey protein prepared at pH 4 which is close to (slightly lower than) the *pI*, showed a significantly higher (*p < 0.05*) denaturation temperature (86.0 ± 0.4 °C) with a much narrower peak, reflecting the strongly bound structure of the protein with a slight positive
charge. These observations confirm those of Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, (2013) who also reported a significantly higher denaturation temperature for whey protein adjusted to pH values close to the pI.

Fig. 5.4  Micro DSC thermograms of WPI solutions (a) and spray dried WPI (b) for samples obtained at an inlet temperature of 110 °C and outlet temperature of 68 to 70 °C and which have been reconstituted to the same concentration of 10% w/w (from top to bottom: pH 7 plus heat at 78 °C for 20 and 10 min, pH 7 plus 1 min heat at 75 °C, pH 7 and pH 4 respectively)

A series of microcapsule preparations were reconstituted to the same concentration (10% w/w) prior to running micro DSC using the same heating program. The results indicated that the protein structure had changed only slightly at pH 7 when the denaturation temperature was increased by 2.0 °C (from 74.6 to 76.6 °C) corresponding to 6.6% denaturation. In contrast, at pH 4, the denaturation temperature decreased by 1.5 °C corresponding to 1.8% denaturation following spray drying (based on the calculated absorbed energy changes before and after spray drying, presented in Table 5.1). Whey proteins denature to a relatively small extent during spray drying at 160-190 °C inlet and 65-90 °C outlet temperatures (Guyomarch'h, Warin, Muir, & Leaver, 2000). Anandharamkrishnan, Rielly, & Stapley, (2007) also reported a relatively low denaturation while spray drying WPI at outlet temperatures of 60 or 80 °C with a feed solution concentration between 20 to 40%.

From Fig. 5.4, it is also evident that treatment of WPI at 75 °C for 1 min resulted in even broader peaks and heating at 78 °C for 10 and 20 min gave peaks that are virtually flat,
indicating almost all proteins had denatured by the heat treatment. The corresponding re-suspended spray dried powder exhibited similarly broad peaks. Comparing the energy absorbed by the native WPI at pH 7 (assuming no denatured protein was present in the sample as supplied) and the corresponding sample subjected to heat treatment at 75 °C for 1 min, resulted in 62.8% denaturation and those treated at 78 °C for 10 and 20 min resulted in denaturation of ~90% (Table 5.1).

The heat treated samples underwent only minor denaturation upon spray drying as shown in Table 5.1. WPI at 12% (w/v) was reported to fully denature with no endothermic peak observed when heat treated at 80 °C for 30 min giving a positive effect on bead stability with
pH change (Ruffin, Schmit, Lafitte, Dollat, & Chambin, 2014). It appears from our study that denaturation of whey protein induced either by heat (75 °C for 1 min) or acid (pH 4) prior to spray drying, afforded similar protection to *L. plantarum* A17.

### 5.3.5 Changes in protein structure

To investigate changes to the protein secondary structures during spray drying, samples were analysed by FTIR. Fig. 5.5 shows the absorbance and second derivative spectra of amide I region of WPI from supplier and spray dried WPI, which had undergone different treatments. The individual secondary structural feature (β-sheets, α-helix, β-turns and random coils) were quantified with band fitting software OPUS. Almost all of the spectra of amide I region were nicely fitted by producing 9-11 bands.

![Fitted spectra of the WPI from supplier and spray dried WPI prepared at pH 4](a)

![Fitted spectra of the WPI from supplier and spray dried WPI prepared at pH 4](b)

Fig. 5.6  Fitted spectra of the WPI from supplier and spray dried WPI prepared at pH 4
Two spectra of WPI from supplier and spray dried WPI prepared at pH 4 are shown in Fig. 5.6 as examples. Wave numbers from 1620 to 1640 cm\(^{-1}\) and 1674 to 1680 cm\(^{-1}\) were assigned to β-sheets, while those from 1641 to 1647 cm\(^{-1}\) were assigned to random coil and those from 1648 to 1660 were assigned to α-helix. Likewise, wave numbers appearing at and in the vicinity of 1663 cm\(^{-1}\), 1671 cm\(^{-1}\), 1683 cm\(^{-1}\), 1688 cm\(^{-1}\) and 1694 cm\(^{-1}\) were assigned to β-turns (Kong & Yu, 2007).

By fitting of the assigned bands and quantifying the magnitude of these bands, WPI from supplier was found to contain 44, 21, 26 and 9% of β-sheets, α-helix, β-turns and random coils, respectively, (Table 5.2), which corroborate well with a previous study (Ramos et al., 2013).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Secondary structures (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-sheet</td>
</tr>
<tr>
<td>WPI_Supplier</td>
<td>44(^{a})</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>47(^{ab})</td>
</tr>
<tr>
<td>pH 4.0</td>
<td>53(^{b})</td>
</tr>
<tr>
<td>pH 7.0 plus heat at 75 °C for 1 min</td>
<td>53(^{b})</td>
</tr>
<tr>
<td>pH 7.0 plus heat at 78 °C for 10 min</td>
<td>54(^{b})</td>
</tr>
</tbody>
</table>

Different letters in each column signify significant difference at p < 0.05

It can also be seen from Table 5.2 that the spray dried WPI of feed solution at pH 7 was the least affected sample as compared to the control sample. The small insignificant increase (p > 0.05) of approximately 3% each for β-sheet and β-turn structures of spray dried WPI at pH 7 was compensated by a decrease in α-helices (4%) and random coils (2%). There was a significant increase (p < 0.05) in β-sheet structures of approximately 9% each for spray dried samples prepared at pH 4, or at pH 7 after heat treatment at 75 °C for 1 min and 78 °C for 10
min. On the other hand the structural property of α-helix content decreased by about 3% for each of the treatments which were not significant ($p > 0.05$). β-sheet structure has been reported as the dominant secondary structure of WPI and the increasing proportion of β-sheet is commonly found in aggregated proteins, especially proteins that undergo thermal denaturation. The accumulation of β-sheet structures in aggregated proteins is known to derive from the formation of intermolecular antiparallel β-sheets (Lefèvre, Subirade, & Pézolet, 2005). The alterations of the other two structural elements, viz. β-turns and random coils, were quite random and did not follow any specific trend. The fluctuating behaviour of these two structural elements is in agreement with those of Tatham, Miflin, & Shewry, (1985) who suggested that the flexible structures such as β-turns play important role in folding of protein by changing the orientation of α-helices and β-sheets and therefore, these structural elements can easily alter their orientation without forming any specific structure under stresses.

### 5.3.6 Morphology of spray dried powder

Spray dried microcapsules exhibited smooth and spherical shapes as shown in Fig. 5.7. There was evidence of indentations and concavities which corroborate well with other spray-dried capsules (Anandharamkrishnan et al., 2007; Millqvist-Fureby, Elofsson, & Bergenståhl, 2001). In addition, particles were found well-separated indicating free flowing behaviour in all cases. Capsules produced at pH 7 showed smaller and more spherical shapes as compared to pH 4. Typically microcapsules undergoing denaturation before spray drying (Fig. 5.7(b) and (c)) show wrinkled and rough surfaces. It has been suggested that denatured whey protein films are more stretchable due to covalent cross-linking which could bring about a wrinkled rough surface after spray drying (Holt et al., 1998). In all cases, microcapsules were smaller than 15 μm in size and therefore, it is unlikely to affect the texture of food they are incorporated into (Champagne & Fustier, 2007).
5.3.7 Protective role provided by WPI

The survivability for the encapsulated dried cells in WPI at pH 7 (~69%) was significantly different among different treatments (Table 5.1) with the survival of A17 reduced to ~39% for WPI at pH 4. At this pH, the percentage of denatured proteins as established by Micro DSC was significantly different to that at pH 7. When the WPI was heated to 75 °C for 1 minute, both the survivability percentage and the amount of denatured proteins were non-significantly different to that when the WPI was exposed to pH 4. These results indicate that the better protection provided to the encapsulated cells when exposed to spray drying conditions was due to the degree of denaturation of the protectant before spray drying as our spray drying conditions (outlet temperature of ~70 °C) do not contribute significantly to the denaturation of whey proteins.

Native WPI at pH 7 prior to spray drying provided the best protection in this study which is in contrast to previous studies on other microbial species where denatured proteins were observed to provide better protection (Duongthingoc et al., 2013; Rajam et al., 2012). We have provided evidence that the observed behaviour of WPI in this study was not because A17 cells were sensitive to pH 4 as their optimum growth was observed across pH 4-8 (Fig. 5.1). It is also evident that the difference in survivability at different pH is unlikely to be due to electrostatic interactions between WPI and the bacterial cells alone as A17 cells were negatively charged across the whole pH range (Fig. 5.2).
In the current study, it appears that negatively charged bacteria are more effectively encapsulated with native WPI at pH 7 which will have majority of the WPI components as negatively charged while a few others appear as positively charged at this pH. Notably, in a previous study (Duongthingoc et al., 2013) while yeast cells with an overall positive charge (Stan, 2010) were found to have enhanced survival in a positively charged WPI at pH 4, a clear mechanism behind this observation was not reported. A mechanism similar to that proposed in our study involving complex layer-by-layer assembly of WPI protein constituents might be responsible for higher survivability observed at pH 4 in yeast cells. These observations support the reason for enhanced protection provided by WPI at pH 7 during spray drying in our study is unlikely to be due to electrostatic interactions alone, but is a much more complex interaction between the bacterial cell surface and the WPI matrix.

5.4 Conclusion

Native whey proteins used for encapsulating *L. plantarum* A17 by spray drying at pH 7 produced capsules with the highest survivability. This allowed us to elucidate a new layer-by-layer encapsulation mechanism that seems to be involved in protection of A17 cells at pH 7. It was also observed that the higher the degree of protein denaturation, either induced by pH changes or heat treatment as characterised by Micro DSC, FTIR and scanning electron microscopy, the lower is its protection capability during spray drying. In conclusion, native WPI with minimum denaturation could be used as structural elements and as carriers for bacterial encapsulation by spray drying.
Chapter 6

The behaviour of whey protein isolate in protecting
*Lactobacillus plantarum*

Abstract

There is increasing evidence that whey protein isolates (WPI), can be utilised to encapsulate and protect bioactive substances including lactic acid bacteria due to its physicochemical properties. However, little is known about what happens in the immediate vicinity of the cells. This study examined the protective behaviour of WPI for two strains of *Lactobacillus plantarum* A17 and B21 during spray drying. B21 was found to be more hydrophobic than A17 and required 50% of the amount of WPI to provide comparably high survival (~ 90%). We hypothesise that WPI protects the hydrophobic bacteria by initial attachment to the unfolded whey protein due to hydrophobic interactions followed by adhesion to the proteins resulting in cells being embedded within the walls of the capsules. The encapsulated strains had a moisture content of approximately 5.5% and during storage trials at 20 °C retained viability for at least eight weeks.

*Key words: Lactobacillus plantarum, hydrophobic cells, whey protein isolate, spray drying*

6.1 Introduction

Lactic acid bacteria (LAB) utilised for health promotional purposes are usually members of the genera *Lactobacillus* or *Bifidobacterium*. These bacteria are increasingly being incorporated into food formulations, reflecting their potential contributions to health and wellbeing and these benefits have been described comprehensively (de Vries, Vaughan, Kleerebezem, & de Vos, 2006; Fijan, 2014). However, to exert those benefits for the host, bacteria have to arrive at the target sites with high survival rates and this remains a challenge as reported in recent reviews (Gaggia, Gioia, Baffoni, & Biavati, 2011; Gupta & Abu-Ghannam, 2012).
The genus *Lactobacillus* includes many species and *Lactobacillus plantarum* is one which has been utilised for the purpose of food fermentation and as a health promoting agent. It is a Gram-positive bacterium in which the cell surface has an overall hydrophobic nature (García-Cayuela et al., 2014) and the wall consists primarily of peptidoglycan which is a cross-linked network of carbohydrates, peptides, teichoic acid, and polysaccharides with the proteins anchored covalently and noncovalently as part of the wall structure (Vollmer & Seligman, 2010). Some of the molecules present in the cells are involved in adhesion and interactions, and these include polysaccharides, teichoic acid, lipoteichoic acid, proteins and lipids (Busscher & Weerkamp, 1987; Burgain et al., 2014).

Although probiotic products have been on the market for over two decades (Corona-Hernandez et al., 2013), the major obstacle of protecting the bacteria is still not fully understood. Various studies (Paéz et al., 2012; Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011; Ying et al., 2010) appear to indicate that the mechanisms of protection are strain specific.

Given the claimed health benefits of probiotic bacteria, the search for suitable materials and encapsulation methods to produce functional foods containing probiotics continues to increase. The bioactive cells are usually protected by the encapsulant where the active cells are either dispersed inside the capsule or concentrated in the central region of the capsule (Kuang, Oliveira, & Crean, 2010). Biopolymers including milk proteins are the most commonly reported encapsulants/protectants used for bioactive agents including bacteria (Heidebach, Forst, & Kulozik, 2012; Tavares, Croguenec, Carvalho, & Bouhallab, 2014). This is especially the case for whey proteins as these can be designed to encapsulate in order to control targeted delivery and release of bioactive compounds due to its desirable physicochemical properties (Gunasekaran, Xiao, & Eleya, 2006; Matalanis, Jones, & MacClements, 2011; Tavares et al., 2014). In addition, the well-established nutritional values (McIntosh et al. 1998) make whey proteins a highly desirable encapsulating matrix as these are able to provide both protection of microbial cells and also enhance the health and wellbeing of consumers.

Whey protein in its native state consists of compact globular proteins with major constituents of β-lactoglobulin and α-lactalbumin. The first of these represents approximately 50% of total whey proteins and has well established primary, secondary, tertiary and quaternary
structures. Heat treatments above 65 °C in aqueous solutions (Townend & Gyuricsek, 1974) unfold the whey proteins so that part of the core, hydrophobic structure will be exposed. However, whey protein denaturation occurred at temperatures as low as 55 °C during drying above certain moisture contents (Haque, Aldred, Chen, Barrow, & Adhikari, 2013). Unlike β-lactoglobulin, α-lactalbumin, which constitutes around 20% of the total whey proteins, has about 60% of the structure that is relatively unordered and this is the most heat stable of the major constituents of whey proteins (Morr & Ha, 1993).

The combination of probiotics with whey protein isolate (WPI) will add more value to processed food products. The major disadvantage associated with this combination is the instability of the bacteria as WPI is an ideal nutritional source for microorganisms to grow and multiply if the moisture content is high. Based on these considerations, spray drying which is a well-established technology, would be a well suited choice for the preservation of bacteria for food fermentation and therapeutic applications (Peighambardoust, Golshan, & Hesari, 2011; Santivarangkna, Kulozik, & Foerst, 2007) particularly as it is more economical compared to alternative methods.

It appears that dairy products are natural carriers for LAB. In our previous study on agent selection and protective effects on bacteria using a single droplet drying approach (Khem, Woo, Small, Chen, & May, 2015), we found that WPI and skim milk provided the highest protection during the intermediate drying stage. We hypothesised that by adding hydrophobic bacteria to combine with the exposed hydrophobic parts of the whey proteins during spray drying, inactivation of the bacterial cells is prevented. In this context, the objective of the current study has been to provide further insights into the protective behaviour of WPI by relating microbial adhesion and interactions, concentration of WPI, surface tension, moisture content and morphology of microcapsules to the cell survival within the matrix when various formulations are spray dried.

6.2 Material and methods

6.2.1 Materials

WPI was obtained from Fonterra, Australia (WPI 894, Fonterra, Australia). According to the product specification from the typical analysis provided by the manufacturer, the bulk density
was 0.34 g/mL. It had a protein content of 90.4%, fat 1.0%, moisture 4.7%, ash 3.0% and 0.9% carbohydrate. Lactose monohydrate (L3625) and hexadecane (H6703) were obtained from Sigma Aldrich, Australia. Other chemicals utilised in this study were of analytical grade. Buffer solution was prepared by adding K$_2$HPO$_4$. 3H$_2$O (22.2 g), KH$_2$PO$_4$ (7.26 g), urea (1.8 g), MgSO$_4$.7H$_2$O (0.2 g) and deionised water to 1000 mL. De Man Rogosa Sharpe (MRS) agar and broth were obtained from Oxoid, Australia. Unless otherwise stated, deionised water (Milli-Q system QGARD00R1, Millipore, Australia) was used in all experiments.

6.2.2 Lactobacillus plantarum A17 and B21

The test strains of L. plantarum A17 and B21 were obtained from the collection of the Laboratory of Food Microbiology, School of Applied Sciences, RMIT University, Australia. The strains were frozen at -80 °C in MRS broth (Oxoid, Australia) with 40% (v/v) glycerol.

6.2.3 Methods

6.2.3.1 Preparation of bacterial cells for encapsulation

One colony of each of the working culture was grown in different MRS broth (5 mL) for 24 h at 30 °C. Cell suspensions (2% v/v) were re-grown in freshly prepared MRS broths at 30 °C for another 17 h to reach the end of the growth phase. The actively growing cells were harvested by centrifugation at 4000 g for 10 min followed by washing with 0.85% saline water. The washed cell pellets served as the seed culture for microencapsulation.

6.2.3.2 Preparation of encapsulant solutions

WPI solutions were prepared at concentrations of 10, 20 and 30% (w/w) by adding 10, 20 and 30 g of WPI powder into 90, 80 and 70 g of water which have been sterilised and stirred with the aid of a magnetic stirrer at room temperature for at least 30 min. The solutions were then stored in a refrigerator at 4 °C overnight to allow for complete protein hydration before standardising to pH 7 by drop-wise addition of 1M NaOH. (Note: the pH of the unbuffered “natural” solution was approximately 6.6). Similarly, a mixture of lactose and WPI (Lac:WPI) in a ratio of 9.4:0.6 was prepared by dissolving 9.4 g of lactose in 90 g of water.
and then autoclaved at 121 °C for 15 min. After cooling to room temperature, 0.6 g of WPI powder was added and mixed well before storing at 4°C overnight to allow complete protein hydration. Lactose solution was also prepared at the same concentration of 10% (w/w).

6.2.3.3 Microencapsulation of bacteria by spray drying

Each of the strains of seed culture harvested in 6.2.3.1 was mixed with encapsulant solution at room temperature (approximately 23 °C) for half an hour prior to spray drying. Cell suspensions in protectant solutions contained an average cell count of about $3-4 \times 10^9$ CFU/mL. During the optimisation of spray drying conditions, only the seed culture of A17 was mixed with 10% (w/w) WPI. Spray drying of the sample was performed using a LabPlant SD-Basic FT30MKIII spray drier (Keison products, Chelmsford, Essex, UK) fitted with an atomiser (0.5 mm) and peristaltic pump. Controls were set at 392.27 kPa for air pressure and 5 to 10 mL/min for flow rate. The inlet temperature varied between 90 and 130 °C. The outlet readings were within the range of 50 to 82 °C. In order to ensure an even distribution of bacteria throughout the drying process, the feed solution was continuously agitated using a magnetic stirrer.

Once the spray drying conditions has been chosen, encapsulation of A17 by spray drying was performed with other encapsulation solutions as described in 6.2.3.2. *L. plantarum* B21 was also spray dried in order to relate the hydrophobicity of *L. plantarum* and storage stability using WPI at only 10% (w/w) concentration.

6.2.3.4 Surface tension measurement

SITA T60 tensiometer (SITA Messtechnik, Dresden, Germany) was used to measure the surface tension of encapsulation solutions by employing the maximum bubble pressure method. Measurements were taken at room temperature (20 ± 1 °C) with a bubble pressure life time of 40 s. The instrument was calibrated using water, and a surface reading of 72.8 ± 0.5 mN/m was regarded as accurately standardised. Solutions subjected to surface tension measurement were in the same conditions as solutions used for encapsulation.
6.2.3.5 Bacterial survival rate after spray drying

Bacterial cell count was enumerated by taking 1 mL of cell suspension in feed solution prior to spray drying. After serial dilution, an aliquot of 0.1 mL was transferred and plated on MRS agar and incubated at 30 °C for 48 h. Cell count before spray drying was calculated as CFU/g of dried matter based on the initial total solids content of the feed solution before spray drying.

Similarly, 0.1000 g of spray dried powder was dissolved in peptone water for 20 to 30 min to dissolve the powder, followed by serial dilution and plating. The bacterial count was expressed as CFU/g of dried powder and cell survival calculated as:

\[
\% \text{ survival} = \left( \frac{N}{N_0} \right) \times 100
\]

where \( N \) and \( N_0 \) are number of bacteria per gram of dried matter after and before spray drying respectively.

6.2.3.6 Analysis of moisture content

The moisture content of spray dried powder was analysed by moisture analyser MB45 (Ohaus Corporation, USA). Approximately 1.000 g of spray dried powder was spread on an aluminium pan and heated at 105°C and held until mass changes of less than 1 mg for 90 s were achieved.

6.2.3.7 Morphology of spray dried powder by scanning electron microscopy (SEM)

Surface morphology of spray-dried powders was evaluated using FEI Quanta 200 SEM (Hillsboro, OR, USA). The powder samples were deposited on aluminium sample stubs using double-sided adhesive carbon tape and were sputter coated with gold for 60 s using an SPI-Module Sputter Coater. Images were taken under high vacuum with an accelerating voltage of 30 kV. Microcapsules were ground in liquid nitrogen using a mortar and pestle. The resultant powder was placed in a vacuum before depositing on the double-sided carbon tape and coated with gold as described.
6.2.3.8 Hydrophobicity of L. plantarum

Active growing cells of two different strains of L. plantarum, A17 and B21 were harvested as described in 6.2.3.1. The cell pellets were washed with buffer solution and prepared as outlined by Rosenberg, Gutnick, & Rosenberg (1980). In brief, the washed cell pellets in buffer solution (aqueous phase) were mixed with hexadecane in a ratio of 6:1 in a round bottom test tube which had been acid washed. After vigorously mixing for 2 min using a vortex mixer at high speed, to allow the hydrophobic bacteria to interact with the hydrocarbon, the mixture was allowed to stand for approximately 15 min for phase separation. A small drop of hydrocarbon from the upper layer was carefully pipetted and placed on a glass slide and observed under phase contrast microscopy (Nikon Eclipse TS 100).

6.2.3.9 Storage of spray dried microcapsules

Sub-samples (0.1000 g) of spray dried capsules of both A17 and B21, collected after spray drying were placed into series of Eppendorf tubes with rubber septa and tightly closed before storing at 20 °C for 8 weeks. Cell survival was monitored every two weeks by taking two tubes (for each strain), dissolving in peptone water for 20 to 30 min and serially diluted and plated. Cell survival was calculated as an average of four readings from two independent experiments and expressed as log CFU/g for ease of comparison.

6.2.3.10 Statistical analysis

Unless otherwise stated, all experiments were carried out in triplicate: mean values and standard deviations were calculated based on the values for the different factors studied. Two-way analysis of variance (ANOVA) was used to determine significant differences ($p < 0.05$) in temperature and flow rate. One-way ANOVA was used to determine significant differences ($p < 0.05$) in survival, surface tension and moisture content. All statistical analyses were performed using Minitab16 software, State College, PA Inc.
6.3 Results and discussion

6.3.1 Effect of inlet temperature and flow rate on cell survival and final moisture content

In the microencapsulation of bacterial cells, the selection of spray drying conditions involved a compromise between the desired moisture content and cell survival. If a high survival of cells resulted from spray drying, along with high moisture contents, it is expected that cell viability would be lost during storage (Jouppila & Roos, 1994). Accordingly, three different inlet temperatures ranging from 90 to 130 °C, along with two different flow rates of 6.6 and 9.0 mL/min were chosen in the experimental design using WPI at 10% (w/w) as spray drying encapsulant.

<table>
<thead>
<tr>
<th>Inlet</th>
<th>Outlet</th>
<th>Flow rate (mL/min)</th>
<th>Cell survival in CFU/g (\times 10^{10}) Before SD</th>
<th>Survival (%)</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>78</td>
<td>9.0</td>
<td>3.66 ± 0.91</td>
<td>34.7 ± 5.6</td>
<td>5.24 ± 0.25</td>
</tr>
<tr>
<td>130</td>
<td>82</td>
<td>6.6</td>
<td>3.05 ± 0.47</td>
<td>36.4 ± 1.4</td>
<td>4.60 ± 0.17</td>
</tr>
<tr>
<td>110</td>
<td>64</td>
<td>9.0</td>
<td>3.40 ± 0.46</td>
<td>69.1 ± 5.8</td>
<td>6.58 ± 0.15</td>
</tr>
<tr>
<td>110</td>
<td>68</td>
<td>6.6</td>
<td>3.17 ± 0.26</td>
<td>69.0 ± 1.7</td>
<td>5.57 ± 0.45</td>
</tr>
<tr>
<td>90</td>
<td>51</td>
<td>9.0</td>
<td>3.80 ± 1.41</td>
<td>71.8 ± 3.8</td>
<td>8.37 ± 0.26</td>
</tr>
<tr>
<td>90</td>
<td>55</td>
<td>6.6</td>
<td>2.98 ± 0.32</td>
<td>82.8 ± 6.6</td>
<td>6.73 ± 0.34</td>
</tr>
</tbody>
</table>

The cell survival and moisture content of spray dried WPI at three different inlet temperatures and two different flow rates are presented in Table 6.1 and the resulting cell survival ranged from 34.7 to 82.8% with corresponding moisture contents of 4.60 to 8.37%. Inlet temperature significantly \((p < 0.05)\) affected both cell survival and moisture content of the dried powder, whereas flow rate only significantly \((p < 0.05)\) influenced the moisture content of the powder. In the literature, a variety of different critical moisture contents have been described for
storage stability of dried powders. Masters (1985) reported 4% (w/w) moisture content as a good quality parameter for spray dried dairy product. However, Jouppila & Roos (1994) referred to a critical moisture content of dried milk powder of 7% for storage stability at 25 °C, based on the calculated glass transition temperature value. Zayed & Roos (2004) examined the effect of water content on the survival of bacteria and reported enhanced survival during storage for moisture contents within the range of 2.8 to 5.6%.

In this context, for subsequent experiments in the current investigation, and to avoid instability of microcapsules during storage, an inlet temperature of 110 °C and flow rate of 6.6 mL/min were selected as the spray drying conditions because these resulted in a relatively high cell survival of 69.0% and a moisture content of 5.57% and corroborated well with a previous study (Behboudi-Jobbehdar, Soukoulis, Yonekura, & Fist, 2013).

6.3.2 Effect of protectants and concentration on the survival and moisture content of spray dried microcapsules

Table 6.2 Effect of protective agents and their surface tensions on the survival and moisture content of spray dried microcapsules (spray drying conditions: Inlet temperature of 110 °C and outlet temperature of 69 ± 1 °C)

<table>
<thead>
<tr>
<th>L. plantarum</th>
<th>Protectant in 100 g solution</th>
<th>Surface tension (mN/m)</th>
<th>Survival (%)</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A17</td>
<td>Lactose (10 g)</td>
<td>73.4 ± 2.0a</td>
<td>25.8 ± 5.7d</td>
<td>4.01 ± 0.15g</td>
</tr>
<tr>
<td></td>
<td>Lac:WPI in a ratio of 9.4:0.6 (9.4 g : 0.6 g)</td>
<td>55.5 ± 0.5b</td>
<td>37.7 ± 3.1d</td>
<td>4.23 ± 0.23g,h</td>
</tr>
<tr>
<td></td>
<td>WPI at pH 7 (10 g)</td>
<td>54.7 ± 3.1bc</td>
<td>69.0 ± 1.7e</td>
<td>5.57 ± 0.45f</td>
</tr>
<tr>
<td></td>
<td>WPI at pH 7 (20 g)</td>
<td>52.9 ± 0.3c</td>
<td>91.7 ± 1.8f</td>
<td>4.90 ± 0.06b,i</td>
</tr>
<tr>
<td></td>
<td>WPI at pH 7 (30 g)</td>
<td>52.2 ± 0.4c</td>
<td>59.6 ± 8.1e</td>
<td>5.10 ± 0.24j</td>
</tr>
<tr>
<td>B21</td>
<td>WPI at pH 7 (10 g)</td>
<td>54.7 ± 3.1bc</td>
<td>88.7 ± 12f</td>
<td>5.50 ± 0.31i</td>
</tr>
</tbody>
</table>

Different letter signifies significant difference among treatments
Chapter 6

The effects of different protectants and concentrations on survival of the two strains of \textit{L. plantarum} and moisture contents after spray drying are presented in Table 6.2. A17 microcapsules prepared with WPI at a solid content of 10\% (w/w) resulted in a survival rate of 69.0\%; whereas lactose afforded the least protection with only 25.8\% cell survival. Adding a small amount of WPI (0.6\%) to the lactose solution (9.4\%) while maintaining the same solid content resulted in survival of 37.7\% during spray drying, representing a marked enhancement compared to using lactose alone. Interestingly, when the same solid content of WPI (10\%) was used to encapsulate \textit{L. plantarum} B21, there was a significant increase in survival of up to 88.7\% as compared to A17 at the same solid concentration. Although spray dried WPI microcapsules for both strains had significantly higher moisture contents ($p < 0.05$) compared to those involving lactose and the mixture of Lac:WPI, the resultant moisture content was effective in providing storage stability.

To further understand the protective behaviour of WPI on \textit{L. plantarum}, WPI at concentrations of 20 and 30\% were spray dried to encapsulate A17 at the same inlet and outlet temperatures by adjusting the flow rate so that the outlet temperature reading was in the range of $69 \pm 1$ °C. Table 6.2 shows that as the concentration of WPI was varied from 0.6\% to 20\%, the survival rate of A17 increased from 37.7\% to 91.7\%. However, at the highest protein concentration (30\%) evaluated in the current study, the survival was reduced to 59.6\%.

It appears from these results that, in comparison with the other protectants, WPI offered the highest level of protection to \textit{L. plantarum} and the protection is concentration and strain dependent. For B21 at a concentration of 10\% w/w, 50\% less WPI was required to provide similarly high survival ($\sim 90\%$) as A17 at 20\% w/w concentration. It is important to note that native WPI at 30\% w/w was still readily soluble in water at room temperature even though it has a very high protein content of 90.4\% (see Section 6.2.1). We also found that concentration affects the particle size (Fig. 6.2(a to c)) and thickness of the capsule wall (Fig. 6.2(d and e)) and this aspect is discussed in Section 6.3.5.

6.3.3 Surface tension of protectant solutions

Although the effects of whey proteins on surface tension are well known (Xu, Howes, Adhikari, & Bhandari, 2012) the surface-active behaviour of milk proteins has rarely been
reported in relation to the protection of sensitive materials particularly microorganisms. Some reports have implied that surface-active substances in liquid formulations might be useful to encapsulate and protect sensitive proteins or enzymes (Elversson & Millqvist-Fureby, 2006; Jayasundera, Adhikari, Aldred, & Ghandi, 2009). In this context, the surface tension values of protectant solutions utilised in this encapsulation study were determined and these are shown in Table 6.2. Incorporating 10% (w/w) of lactose had no significant effect upon the surface tension ($p > 0.05$) compared to the standard surface tension of water. In contrast, WPI significantly reduces the surface tension and adding as little as 0.6% of WPI to the lactose solution resulted in a significant reduction ($p < 0.05$). Higher concentrations (10 to 30% w/w) of WPI had no significant effect in further reducing the surface tension ($p > 0.05$). These results were expected as globular proteins including WPI and high molecular weight peptides diffuse slowly to the interface and only a small amount of protein is needed to saturate the interface. For encapsulation using spray drying, we found it is crucial that the encapsulation matrix contains a small amount of proteins as these have a tendency to accumulate at the surface of the powder due to their surface active behaviour and prevent adhesion to the wall of the spray dryer which is in agreement with a previous study by Wang & Langrish (2009).

6.3.4 Hydrophobicity of *L. plantarum*

Microbial adhesion to hydrocarbon, a widely used test for measuring cell surface hydrophobicity in LAB was utilised to observe the hydrophobicity of *L. plantarum* A17 and B21 and the results are shown qualitatively in Fig. 6.1. According to Ofek, Hasty, & Doyle (2003), hydrophobicity of bacterial cells is the tendency of a microorganism to adhere to a nonpolar material in preference to water. In the current study, the hydrophobicity of cell surface was measured by careful and thorough mixing of live cells with hexadecane (a non-polar solvent). Aqueous suspensions of both *L. plantarum* A17 and B21 were layered with the hydrocarbon phase as shown in Fig. 6.1a. After mixing vigorously for 2 mins, it was observed that droplets of hexadecane were dispersed into the aqueous phase and the hydrophobic cells were associated with the nonpolar material. When allowed to stand for 15 min, the hydrocarbon phase, being less dense than water, rose to the surface taking with it the cells as indicated by the creamy layer (Fig. 6.1b). Microscopic examination of the upper organic phase showed clear evidence of bacterial adherence.
Organic phase (hexadecane) on the upper part and aqueous phase on lower part

He:xdane layer became creamy and increase in thickness

Fig. 6.1 Mixture of aqueous *L. plantarum* A17 and B21 suspensions and hexadecane before (a) and after vortexing vigorously for 2 min followed by allowing to stand for 15 min (b). Phase contrast microscopy showing A17 (c) and B21 (d) cells attaching to the hexadecane phase with more hydrophobic cells (B21) attached better to the organic phase.

These results indicate that both strains of *L. plantarum* are relatively hydrophobic with B21 being more so than A17, as demonstrated in Fig. 6.1(c-d). Other researchers (García-Cayuela et al., 2014) also showed the cell wall of *L. plantarum* strains to be hydrophobic. This surface hydrophobicity is usually associated with bacterial adhesiveness, varying from species to species and strain to strains and is dependent upon environment (Sorongon, Bloodgood, & Burchard, 1991). According to Zorilla, Liang, Remondetto, & Subirade (2011), the interactions between β-lactoglobulin (the major constituent of WPI) and bioactives are primarily determined by hydrophobic bonds. Therefore, we hypothesised that the protective mechanism of WPI for *L. plantarum* was due to the hydrophobic portions of the unfolded β-lactoglobulin component of WPI during spray drying interacting hydrophobically with the particular strain followed by consolidation of the interface between the cells and WPI. In other words, during spray drying as the “native” WPI solution came into contact with heat above 65 °C, the compact globular proteins unfolded and the hydrophobic cells attached and associated with the hydrophobic portion of the whey proteins and became embedded inside or on the surface of the walls of the capsules as shown in Fig. 6.2f. To our knowledge, very limited work, if any, has been done on the interaction between *L. plantarum* and the whey protein matrix during spray drying.
6.3.5 Morphology of spray dried powder

To further evaluate our hypothesis, we employed SEM. Fig. 6.2 shows images of spray-dried microcapsules prepared with WPI at different concentrations (Fig. 6.2 a-c). We also carefully ground the microcapsules gently in liquid nitrogen to observe the location of the bacteria with respect to the WPI layer and thickness of the capsules (Fig. 6.2 d-e).

Fig. 6.2 SEM images of spray dried microcapsules prepared at temperatures of 110 (inlet) and 69 ± 1 °C (outlet). Images a-c (all at 3000× magnification): showing the increasing particle size as concentration increases. Images d-e (magnification of 10,000x for WPI at 10% and 30%): showing broken microcapsules and thickness of the capsular walls; arrow showing bacterial cell embedded in the capsule. Image f: showing a proposed morphology of a microcapsule (bacterial cells represented by white rod shapes) encapsulated in WPI matrix.
Microcapsules prepared with different concentrations (a-c) indicate spherical shapes with evidence of indentation and collapse which might be due to the rapid evaporation of moisture during drying. Microcapsules produced at a concentration of 10% show more evidence of indentations (Fig. 6.2a) compared to those prepared at higher protein concentrations of 20 and 30% (Fig. 6.2b and 6.2c). It is important to note that microcapsules produced at lower concentration (10%) resulted in smaller particles with size ranging from 1 to 7.8 µm. However, spray drying with feed solutions at a higher concentration of 20% (Fig. 6.2b) gave sizes predominantly between 2 and 14 µm and those for 30% (Fig. 6.2c) had diameters of up to 23µm.

These findings confirm the literature where higher feed solution concentrations were reported to result in larger particle sizes that require longer drying times. Consequently, microorganisms entrapped in the particles would be subjected to more heat damage leading to lower survival of bacteria (Santivarangkna et al., 2007). The results for drying of the 20% feed solution with relatively larger particle sizes, appears to provide the optimum for the protection for the cells.

When the microcapsules were gently cracked using liquid nitrogen, bacterial cells were found embedded under the surface of the WPI layer (see an example in Fig. 6.2d) and none was found inside or outside of the WPI layer despite a very careful and systematic search of many images. We consistently observed that the cells were encapsulated with a different morphology than has previously been proposed and this is illustrated diagrammatically in Fig. 6.2f.

These results support our hypothesis that spray drying with an outlet temperature of approximately 70 °C unfolds the whey proteins, thereby exposing hydrophobic portions which then interact with the hydrophobic bacterial cells. It has been well documented that in its native state, whey proteins are folded into a globular shape naturally and when heated at a temperature higher than 65 °C, they unfold exposing the hydrophobic inner sections of the protein. The unfolded forms of whey proteins tend to attach or interact with itself, walls of the equipment or other whey protein molecules and form “sticky” aggregates (Wijayanti, Bansal, & Deeth, 2014). This aggregation occurs due to two possible reasons: 1) chemical interaction involving a sulfdryl group of the β-lactoglobulin becoming accessible and 2) interaction of newly exposed hydrophobic amino acids. The aggregation of whey proteins
could be prevented by adding hydrophobic compounds to combine with the exposed hydrophobic parts of the whey proteins and this has been done commercially. However, what has never been done is to employ this physicochemical property of whey proteins to protect hydrophobic bacteria cells during spray drying.

### 6.3.6 Storage stability

Whilst minimising cell death during spray drying is important, maintaining viability during storage is necessary. Microcapsules of both strains of *L. plantarum* produced by spray drying using 10% (w/w) WPI were selected for a storage trial at 20 °C which was run over an eight week period (Fig. 6.3). Our results show that both A17 and B21 were stable with cell counts at around 10.5 log CFU/g. These storage results for A17 and B21 are promising as LAB are generally known to be stable only at refrigerated conditions (Ghandi, Powell, Broome, & Adhikari, 2013) and in the absence of oxygen (Ghandi, Powell, Howes, Chen, & Adhikari, 2012).

![Fig. 6.3](image)

**Fig. 6.3** Survival during storage at 20 °C of A17 and B21 encapsulated with WPI at pH7 (10% w/w) by spray drying at temperatures of 110 (inlet) and 69 ± 1 °C (outlet)
6.4 Conclusions

In any design of novel functional foods, it is vital to understand how each component interacts with the others. In the context of the current investigation, a good understanding of bacterial interaction with the encapsulation matrix is crucial. Our results suggest that the high survival of the bacterial strains tested could be explained by the hydrophobic interactions between the cells and the exposed hydrophobic portions of the whey proteins during spray drying and these were found to be dependent upon the concentration of protein. This finding is of great importance to the dairy and functional food industry as WPI, a highly nutritious product could be used to prevent aggregation and serve as a highly effective protectant for hydrophobic probiotic bacterial cells.
Chapter 7

Storage stability of *Lactobacillus plantarum* encapsulated with whey protein by spray drying

Abstract

Two strains of *Lactobacillus plantarum* A17 and B21 have been encapsulated by spray drying using whey protein isolate (10% w/w). The retention of viability during subsequent storage has been studied at different temperatures over a period of 8 weeks. Physical properties of the encapsulating matrix were determined calorimetrically and rheologically. Glass transition temperature ($T_g$) of the capsules was found to be approximately 34 °C by two independent methods. No significant reduction in cell viability was observed when the samples of both strains were stored at temperatures well below the $T_g$ (4 or 20 °C) with live cultures of over $1 \times 10^{10}$ CFU/g after storage. At a storage temperature in the vicinity of the $T_g$ (30 °C), both strains resulted in stable live cultures with a minimum of viable cells of $1 \times 10^9$ CFU/g for up to ~ 4 weeks for A17 and ~ 9 weeks for B21 with residual moisture contents of 7.3% and 6.5% respectively. Storage at a temperature well above the $T_g$ (50 °C) gave viable cells of $1 \times 10^9$ CFU/g for up to 5 days for B21 and up to 3 days for A17 with residual moisture contents of 5.6% and 5.1% respectively. The water activity value was < 0.2 for all storage temperatures except at 4 °C and 20 °C for B21 and A17 respectively. For temperatures above 20 °C, there was an increase of the total colour difference and a reduction of cell survival. We propose from these results that the stability of *L. plantarum* A17 and B21 protected by whey proteins and encapsulated by spray drying is unlikely to be due to the glassy state of the protectants alone.

7.1 Introduction

The species *Lactobacillus plantarum* is ascribed to the general category of probiotics and concurred by the consensus panel (Hill et al., 2014). This can reasonably be expected to impart general benefits when delivered at a functional dose for use as foods or supplements in the general population. Currently there is growing interest in probiotics among the research community. The unprecedented amount of research reflects their widespread applications
which range from fermented products to health-promoting functional foods as well as the potential for misuse or misleading marketing claims for probiotic products. The term probiotic has been defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001). This term as defined by Joint Panel of FAO/WHO Experts some 13 years ago was revisited recently and reconfirmed as relevant and sufficiently accommodating for current and anticipated applications by the International Scientific Association for Probiotics and Prebiotics (Hill et al., 2014).

For ease of commercial utilisation, bacterial cell pellets should be readily transported and also remain stable for a reasonable period of time under ambient conditions. In this context, spray drying has been utilised to encapsulate probiotic cells and achieve capsule-building as well as drying in a single step for various strains of lactic acid bacteria (LAB) (Golowczyce, Silva, Abraham, De Antoni, & Teixeira, 2010; Reddy, Madhu, & Prapulla, 2009; Simpson, Stanton, Fitzgerald, & Ross, 2005).

However, to exert the potential benefits, probiotic bacteria need to be stable and viable with adequate amounts administered. According to the Italian Ministry of Health, the use of probiotic bacteria in the food sector in the Italian market has been regulated over the past 12 years under certain conditions to include a minimum number of viable cells as $1 \times 10^9$ CFU administered per day (Ministero della Salute, Commissione unica per la nutrizione e la dietetica, 2013). Health Canada regards an adequate amount for claims regarding probiotics as being $1 \times 10^9$ CFU per serving when delivered in food for the non strain-specific claims: *Bifidobacterium* (*adolescentis, animalis, bifidum, breve and longum*) and *Lactobacillus* (*acidophilus, casei, fermentum, gasseri, johnsonii, paracasei, plantarum, rhamnosus* and *salivarius*) (Health Canada, 2009). In contrast, other researchers (Agrawal, 2005, Champagne, Gardner & Denis, 2005) have suggested $10^6$-$10^7$ CFU per gram or mL as an effective minimum.

A variety of factors have been reported to affect the survival of bacteria during storage including food ingredients and additives, water activity, storage temperature, moisture content, and the absence or presence of anti-oxidant compounds as well as the physical state of the matrix (Tripathi & Giri, 2014). For example, a moisture content ranging from 2.8 to 5.6% was reported as optimal for storage stability at -85 °C (Zayed & Roos, 2004). Weinbreck, Bodnár, & Marco, (2010) suggested that storing microencapsulated *L. rhamnosus*...
cells in conditions of elevated water activity of 0.7 was detrimental with a reduction of almost 10 log units within two weeks of storage. It was suggested that a residual water activity of 0.2 is desirable for storage stability of microbial cells in dairy matrices (Kearney et al., 2009).

From a number of studies it has been reported that temperature is inversely related to the survival of bacteria during storage and low storage temperature at 4 °C or lower maintains the stability of bacterial cells (Corcoran, Ross, Fitzgerald, & Stanton, 2004; Lapsiri, Bhandari, & Wanchaitanawong, 2012; Soukoulis, Behboudi-Jobbehdar, Yonekura, Parmenter, & Fisk, 2014). Some studies on storage stability were investigated as a function of the glass transition temperature ($T_g$) of the matrix containing the bacteria. The glass transition is the metastable state where material transforms from a stiff, highly viscous glass to a viscoelastic rubber. This transformation is associated with a primary relaxation, detectable calorimetrically by a change in heat capacity (Green, Fan, & Angell, 1994). $T_g$ was reported as the indicator for the storage stability of food, below which materials tend to be stable as deteriorative reactions are inhibited and water in the amorphous glass is immobilised and unavailable. At temperatures at and above $T_g$, the material undergoes a transition from the glass to a rubbery state which could result in significantly reduced stability of the food (Roos, 2010).

It has been recommended that amorphous pharmaceutical solids to be stored approximately 50 °C below the $T_g$ to ensure nearly complete reduction in molecular movements (Hancock, Shamblin, & Zografi, 1995). In addition, several studies report that bacteria would remain stable if the surrounding matrix is in the glassy state. This has been particularly the case when bacteria were stored in the glassy state of sugar or other carbohydrate matrices (Passot, Cenard, Douania, Tréléa, & Fonseca, 2012; Tymczyszyn, Sosa, Gerbino, & Hugo, 2012). However, there are very limited studies on the effect of the glassy state of proteins on the survival of bacterial cells. The difficulties in determining the $T_g$ of proteins, especially those having a globular structure, may account for the lack of this information (Zhou & Labuza, 2007). It has also been reported that $T_g$ was not a suitable indicator for deteriorative chemical reactions including lipid oxidation and Maillard reactions (Andersen, Fog-Petersen, Larsen, & Skibsted, 1999; Kurtmann, Carlsen, Risbo, & Skibsted, 2009).
Although whey protein isolate (WPI) has been used to encapsulate *L. plantarum* and other strains of LAB, the effect of the glassy state of the protein matrix on storage stability and cell survival remain to be elucidated. Therefore, the objective of the current study has been to explore the effect of storage temperatures with respect to the $T_g$ of the encapsulating matrix on the survival of two strains of *L. plantarum* and investigate the potential of colour measurements as an indicator of deteriorative chemical reactions.

### 7.2 Materials and methods

#### 7.2.1 Materials

WPI was obtained from Fonterra, Australia (WPI 894, Fonterra, Australia). The typical analysis in the product specification provided by the supplier, the bulk density was 0.34 g/mL, the protein content 90.4%, fat 1.0, moisture 4.7, ash 3% and there was 0.9% carbohydrate.

#### 7.2.1.1 *Lactobacillus plantarum* A17 and B21

The test strains of *L. plantarum* A17 and B21 were obtained from the collection of the Laboratory of Food Microbiology, School of Applied Sciences, RMIT University, Australia. The strain was frozen at -80 °C in MRS Broth (Oxoid, Australia) with 40% (v/v) glycerol. The working culture was prepared by sub-culturing and growing the cells in deMann, Rogosa and Sharpe (MRS) broth. All bacterial cells were used at the completion of the exponential growth phase.

#### 7.2.2 Sample preparation and methods

#### 7.2.2.1 Preparation of bacterial cells for encapsulation

One colony of the working culture on MRS agar was grown in 5 mL MRS broth for 24 h at 30 °C. A 2% (v/v) of the cell suspension was re-grown in fresh MRS broth at 30 °C for another 17 h to reach the end of the growth phase. The actively growing cells were harvested by centrifugation at 4000 g for 10 min followed by washing with 0.85% saline water. The washed cell pellets served as the seed culture for microencapsulation.
7.2.2.2 Preparation of encapsulation solutions

Solutions of WPI were prepared by adding the powder to pre-sterilised water at a concentration of 10% (w/w) with the aid of a magnetic stirrer at room temperature for at least 30 min. The solutions were then stored in the refrigerator at 4 °C overnight to allow for complete protein hydration (pH ~ 6.6) before adjusting to pH 7.0 by adding 1M NaOH drop-wise.

7.2.2.3 Microencapsulation of A17 by spray drying

Seed culture of each strain of *L. plantarum* was separately mixed with WPI solution prepared as described above. This was achieved with the aid of a magnetic stirrer for approximately half an hour before spray drying. The cell suspensions in WPI contain an average cell count of approximately 3-4 × 10⁹ CFU/mL. Spray drying of the sample was performed using a LabPlant SD-Basic FT30MKIII spray drier (Keison products, Chelmsford, Essex, UK) used in conjunction with a peristaltic pump. The air pressure was adjusted to 4.0 kgf/cm² with a flow rate of 6.6 mL/min. The inlet temperature was set at 110 °C corresponding to a reading of outlet temperature 68-70 °C. To ensure an even distribution of bacteria throughout the process, the feed solution was continuously stirred using a magnetic stirrer.

7.2.2.4 Glass transition temperature determination

*Tg* was measured as the mid-transition temperature by modulated differential scanning calorimetry (DSC Q2000, TA Instruments New Castle, DE, USA) with nitrogen purge gas (flow rate 50 mL/min). The instrument was fitted with a refrigerated cooling system to achieve subzero temperatures (as low as -90 °C). The heat signals were calibrated by a traceable indium standard (Δ*H* = 28.3 J/g) and the heat capacity response by a sapphire standard to ensure accuracy of measurements. *T*₀ pans and lids, with 3 mg of samples were used, and an empty *T*₀ pan was the reference. Ramp rates of 2, 5, 10 and 20 K/min were selected to observe the transition. All pans were scanned at those heating rates from 20 to 130 °C, followed by cooling to -90 °C and reheating to 130 °C at a modulation amplitude of 0.53 °C for each period of 40 s. *T*ᵣ values were recorded from the second heating scan.

7.2.2.5 Rheological measurements of spray dried capsules

-134-
Estimates of the $T_g$ of spray dried WPI (95% solids) were obtained using a dynamic mechanical analyser (DMA 8000, Perkin Elmer Waltham, MA, USA) with liquid nitrogen as the coolant. The spray dried sample was placed in a stainless steel pocket and subjected to single cantilever bend deformation mode within a temperature ramp of -100 to +120 °C at a scan rate of 2 K/min and frequency of 1 Hz. Results reported for thermal and rheological experiments are of individual data selected as representative of three replicates.

7.2.2.6 Storage of spray dried microcapsules

Sub-samples of spray dried capsules (0.1 g) of both A17 and B21, were placed into a series of Eppendorf tubes with rubber septa and tightly closed before storing at 4, 20, 30 and 50 °C for 8 weeks. Cell survival was monitored every two weeks by taking two tubes (for each strain), resuspending in peptone water for 20 to 30 min followed by serial dilution and plating. Cell survival was calculated as an average of at least four readings from two independent experiments and expressed as log CFU/g for ease of comparison.

7.2.2.7 Moisture content and water activity measurement

The moisture content of spray dried powders was analysed by moisture analyser MB45 (Ohaus Corporation, USA) with a standard method of analysis. Approximately 1.000 g of spray dried capsules after spray drying and capsules after storage for 8 weeks at different temperatures were spread in an aluminium pan and the sample was heated to 105 °C and held until the mass changes of less than 1 mg for 90 s were achieved.

Similarly, the water activity of the samples after spray drying and after storage at different temperature for 8 weeks were measured at room temperature ~ 23 °C using an $a_w$ meter LabMaster $a_w$ (Novassina, Precisa, Poissy, France). The powders were placed up to half of the $a_w$ cup. The $a_w$ reading was taken when equilibrium was reached.

7.2.2.8 Colour of spray dried capsules

Colour characteristics of spray dried capsules before and after storage for 8 weeks were measured in the $L^*$, $a^*$, $b^*$ space using a Chroma Meter (Model CR-400, Konica Minolta Sensing Japan Inc). Spray dried capsules were placed in the petri disc up to a thickness of
approximately 3 mm and centrally placed beneath the colour meter. The colour attributes $L^*$, $a^*$ and $b^*$ were measured using the CIELAB colour scale where $L^*$ ranges from 0 to 100, indicating colour variation from black to white; the $a^*$ axis shows the variation from red (+$a^*$) to green ($-a^*$) and $b^*$ axis indicates the variation from yellow (+$b^*$) to blue ($-b^*$). The colour difference ($\Delta E^*$) between samples before and after storage at each temperature was calculated according to the equation proposed by Francis & Clydesdale (1975).

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where $\Delta E^*$ is the lightness difference between spray dried capsules before and after storage; $\Delta a^*$ and $\Delta b^*$ are intensity of redness and yellowness respectively.

### 7.2.2.9 Statistical analysis

All experiments were carried out with a minimum of two independent trials and mean values as well as standard deviations were calculated for each of the different factors studied. Independent factors studied were bacterial strain and storage temperature while the response variables measured were survival, residual moisture, water activity and colour. One-way ANOVA with Tukey Post-Hoc analysis was used to determine significant differences ($p<0.05$) in survival, moisture content, water activity and colour. All statistical analyses were performed using Minitab16 software, State College, PA Inc.

### 7.3 Results and discussion

#### 7.3.1 Physical characterisation of spray dried capsules

Spray dried capsules (bacterial cells in WPI matrix) produced in this study with a moisture content of approximately 5% and a water activity of ~0.2 were subjected to modulated differential scanning calorimetry (MDSC) at a modulation amplitude of 0.53 °C for each period of 40 s to determine the glass transition temperature ($T_g$). Attempts were made with different heating scan rates from as low as 1 to 20 K/min. However, it was not possible to find the $T_g$, on the basis of the second heating scan in the reversing heat flow component, as no transition was evident. A typical differential component of heat flow during MDSC analysis is presented in Fig. 7.1.
Fig. 7.1  DSC thermogram of spray dried WPI capsules. Sample was prepared at pH 7 at 95% solid content at a heating rate of 2 K/min, modulation amplitude of 0.53 °C for each period of 40 s. (total heat flow (+), reversed heat flow (○) and non-reversed heat flow (Δ))

Fig. 7.2  Damping properties (Tan δ) of spray dried capsules. Sample prepared at pH 7 with a solid content of 95% as a function of temperature (scan rate: 2 °C/min, frequency: 1Hz.)
Globular protein structures have a large internal heterogeneity and the extremely broad distribution of relaxation time typically resulted in a much more gradual increase in heat capacity as compared to a sharp transition usually noted for relatively homogeneous polymers. Consequently, the $T_g$ of WPI is difficult to discern. Other authors also reported the difficulties in establishing experimental $T_g$ values for whey protein preparations (Bell & Hageman, 1996; Burin, Joupila, Roos, Kansikas, & Buera, 2000; Zhou & Labuza, 2007). Accordingly, in the currently study, spray dried microcapsules were further characterised with dynamic mechanical analysis (DMA) whereby the capsules were placed into a stainless steel pocket and subjected to small deformation mechanical spectroscopy in an effort to evaluate phenomena related to the glass transition.

The common indicator often utilised to report the glass transition of biomaterials is the peak of the tan $\delta$ trace (Menard, 2008). In the present study, the $T_g$ value was found to be approximately 34 °C, which has been derived from the peak of the tan $\delta$ curve of the WPI matrix during controlled heating (Fig. 7.2). Based on this $T_g$ value for the spray dried capsules, four different storage temperatures, two of which are well below the $T_g$ (4 and 20 °C), one in the proximity of $T_g$ (30 °C) and one well above, were chosen in the experimental design of the storage study.

### 7.3.2 Cell survival during storage at 4 and 20 °C

After spray drying, the residual moisture content of both A17 and B21 capsules was ~ 5.5% with bacterial counts of approximately 10.5 and 10.6 log CFU/g respectively. The water activity of the spray dried capsules was not significant different (p>0.05) and in the proximity of 0.17 (Table 7.1). The survival of both strains when stored at different temperatures over a period of 8 weeks is shown in Fig. 7.3. Both A17 and B21 capsules were stable when stored at 4 and 20 °C over a period of 8 weeks with final cell counts in excess of 10 log CFU/g. These storage temperatures are well below the $T_g$ of the spray-dried capsules. Therefore, it is expected that molecular mobility is restricted and thus contributing to the stability of the bacteria due to the glassy state of the matrix.

Literature on the storage of bacteria describes quite varied stability characteristics depending on strains, encapsulating materials as well as storage conditions. However, it is generally accepted that storing at refrigeration temperature is associated with better stability. For
example, *Bifidobacterium* Bb-12 encapsulated by spray drying with liquid whey produced from enzymatic coagulation of standard pasteurised milk, and having an $a_w$ of 0.23, remained stable for 12 weeks when stored at 4 °C (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). Similarly, *L. acidophilus* Ki and *L. paracasei* L26 encapsulated with whey protein concentrate with a protein content of 50%, remained high after storage for 180 days at 5 °C at a relative humidity of 0.12 (Rodrigues et al., 2011). *L. acidophilus* NCIMB 701748 spray dried with maltodextrin, whey protein concentrate and glucose in a ratio of 60:20:20 with a final moisture content of 3.8% and $a_w$ of 0.17 was also found to have a low inactivation rate of 0.011 per day when stored at 4 °C; however the inactivation rate increased four-fold when stored at 25 °C even though the $T_g$ of the powder was reported to be 59 °C (Behboudi-Jobbehda, Soukoulis, Yonekura, & Fist, 2013).

Table 7.1 Moisture content and water activity of A17 and B21 microcapsules before and after storage at 4, 20, 30 and 50 °C for 8 weeks

<table>
<thead>
<tr>
<th>Storage</th>
<th><em>L. plantarum</em> B21</th>
<th><em>L. plantarum</em> A17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (week)</td>
<td>Temp (°C)</td>
<td>Survival (logCFU/g)</td>
</tr>
<tr>
<td>0</td>
<td>10.6 ± 0.10</td>
<td>0.17 ± 0.00a</td>
</tr>
<tr>
<td>8</td>
<td>10.50 ± 0.07</td>
<td>0.21 ± 0.01a</td>
</tr>
<tr>
<td>20</td>
<td>10.35 ± 0.19</td>
<td>0.27 ± 0.01a</td>
</tr>
<tr>
<td>30</td>
<td>9.26 ± 0.41</td>
<td>0.23 ± 0.04a</td>
</tr>
<tr>
<td>50</td>
<td>4.25 ± 0.33</td>
<td>0.17 ± 0.04a</td>
</tr>
</tbody>
</table>

Different letters within a column indicates a significant difference at $p<0.05$

Other storage studies in which $T_g$ was not determined also reported that a reduction in survival continued even during storage at refrigeration temperature. For example, Oliveira et al., (2007) reported approximately 1 to 4 log reductions in survival of *L. acidophilus* and *Bifidobacterium lactis* following spray drying a complex coacervate of pectin and casein resulting in a moisture content of approximately 10% and storage at 7 °C for 120 days. The survival of *L. gasseri* and *B. bifidum* microencapsulated with alginate and chitosan by extrusion, followed by freeze drying, decreased approximately 2 log after 28 days of storage at 4 °C (Chávarri et al., 2010). Similarly, the survival of *L. plantarum* microencapsulated with alginate or pectin declined from 1.95 to 6.73 log CFU/g during storage at 4 °C for 38
days in yoghurt. In general, storing at refrigeration temperature tends to be beneficial as long as encapsulated bacteria are at a low moisture content or $a_w$ of less than 0.2.

It is interesting to note that in the current study, bacterial cells remained stable when stored at 20 °C for both strains; whereas work done by others indicated that cell inactivation occurred at a more rapid rate at this temperature. For example, Simpson et al., (2005) reported a significant reduction in survival of different strains of spray dried *Bifidobacterium* at 15 and 25 °C during storage.

### 7.3.3 Cell survival during storage at 30 °C

![Graph](image)

**Fig. 7.3** Survival of spray dried B21 (a) and A17 (b) encapsulated in WPI. Samples prepared at pH 7, stored over a period of 8 weeks at different temperatures (4 °C (●); 20 °C (■); 30 °C (▲) and 50 °C(●))
When the A17 and B21 capsules were stored at higher temperature of 30 °C, a slight decline in cell survival was observed after two weeks of storage followed by a linear decrease (Fig. 7.3). Stability of the cells at this storage temperature is of particular interest to the food industry as transportation under refrigeration is expensive. At this storage temperature which is in the vicinity of $T_g$ of the spray dried capsules, the whey protein matrix showed a gradual reduction rather than a steep drop of living cell numbers. The live cultures were stable with a minimum viable cells of $1 \times 10^9$ CFU per gram for up to ~ 4 weeks for A17 and ~ 9 weeks for B21 at residual moisture contents of 7.3% and 6.5% respectively. This is demonstrated in Fig. 7.4 where the bacterial survival declined linearly with $R^2$ values of 0.995 and 0.972 for A17 and B21 respectively. If the adequate minimum amount of viable cells to provide core benefits is taken as $10^7$ CFU/g, WPI is capable of delivering approximately 8 and 19 weeks of storage for A17 and B21 respectively. Dianawati, Mishra, & Shah, (2013) also reported a linear reduction in survival of *L. acidophilus* and *Lactococcus lactis* encapsulated by both freeze and spray drying after storage at 25 °C.

![Figure 7.4](image)

Fig. 7.4 Linear regression model of the reduction in bacterial cell survival during storage at 30 °C to estimate storage life (*L. plantarum* A17 (Δ) and B21(□))

Considering the moisture content, capsules stored at 4, 20 and 30 °C for both strains (Table 7.1) had significantly ($p<0.05$) higher moisture content after storage (6.3-7.3%). However, water activity of all stored samples were in the similar range and not significantly different ($p>0.05$). Although there is an increase in moisture content, it is expected that the $T_g$ of the
sample would not be changed significantly as Chaudhary, Small, Shanks & Kasapis (2014) reported that the \( T_g \) of spray dried WPI was 40 °C for a moisture content of 6%.

### 7.3.4 Cell survival during storage at 50 °C

With regards to capsules stored at 50 °C (well above the \( T_g \) of WPI), viable cells of \( 1 \times 10^9 \) CFU per g was achieved after storage for up to 5 days for B21 and up to 3 days for A17 with residual moisture contents of 5.6 and 5.1% respectively.

These survival results were expected because 50 °C is ~16 °C above the \( T_g \). This high storage temperature was also found to be the thermal death temperature for \( L. \) plantarum (Golneshin et al., 2015) and deteriorative reactions were expected to leading to rapid decline in viable cells. It is important to note that there is no significant difference (\( p > 0.05 \)) in terms of moisture content and water activity for both A17 and B21 microcapsules before and after storage study for samples stored at 50 °C (Table 7.1). These results confirm the protective effect of WPI during spray drying and storage.

Unlike protein or other bioactive substances, which represent a single entity, bacteria are complex assemblies of macromolecules surrounding the cytoplasm; and a particular protection mechanism during storage has been described. Pitombo, Spring, Passos, Tonato, & Vitolo, (1994) proposed that a biomaterial is most stable at or below its monolayer moisture content or \( a_w \), which differs for each particular biomaterial and is also dependent upon environmental conditions. Monolayer water contents of \( L. \) bulgaricus (Fonseca et al 2001) and encapsulated \( L. \) rhamnosus (Ying, Phoon, Sanguansri, Weerakkody, Burgar, & Augustin (2010) were found to be 10 and 3% respectively. This concept raises some questions in regard to the protection of dried cells during storage as bacterial inactivation increases with increasing temperature; however, raises in temperature were found to have minimal effects on the \( a_w \) (Santivarangkna, Aschenbrenner, Kulozik, & Petra, 2011). Some authors have suggested a strong combined effect of \( a_w \) and \( T_g \) which enhances the stability of dried bacteria during storage (Kurtmann, Carlsen, Skibsted, & Risbo, 2009).
7.3.5 Changes in colour of microcapsules during storage

Results on changes in colour of the spray-dried samples as compared to the corresponding powder immediately after spray drying are presented in Table 7.2. The decreases in lightness and redness of both A17 and B21 capsules after storage is accompanied by increases in yellowness. There is no significant difference ($p>0.05$) in terms of lightness ($L^*$) and redness ($a^*$) for samples stored at 20 °C; however, those stored at 30 and 50 °C showed significant changes ($p<0.05$) in lightness, redness and yellowness at the end of storage study. The total colour difference increased with increasing temperature of storage with values of 2.6 for samples stored at 20 °C and 9.3 for those at 50 °C (Table 7.2).

Table 7.2 Colour attributes of microcapsules before and after storage for 8 weeks at 4, 20, 30 and 50 °C

<table>
<thead>
<tr>
<th>Time (week)</th>
<th>Temp (°C)</th>
<th>$L^*$</th>
<th>$a^*$</th>
<th>$b^*$</th>
<th>$\Delta E^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>91.28 ± 1.75$^a$</td>
<td>-0.80 ± 0.32$^a$</td>
<td>5.94 ± 0.51$^a$</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>92.88 ± 0.65$^a$</td>
<td>-0.68 ± 0.04$^a$</td>
<td>7.99 ± 0.23$^b$</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>89.55 ± 0.40$^b$</td>
<td>-1.36 ± 0.01$^b$</td>
<td>9.96 ± 0.04$^c$</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>89.33 ± 0.09$^b$</td>
<td>-1.50 ± 0.02$^b$</td>
<td>13.56 ± 0.05$^d$</td>
<td>7.90</td>
</tr>
</tbody>
</table>

Different letters within a column indicates a significant difference at $p<0.05$

Total colour difference of >3 was reported to be detectable by human eyes (Martínez-Cervera, Salvador, Muguerza, Moulay, & Fiszman, 2011). Therefore it is unlikely that colour change for capsules stored at 20 °C is detectable and the encapsulated bacteria remained viable during 8 weeks of storage. However, for those capsules having detectable colour changes, inactivation of the bacteria occurred during storage. The changes in colour of stored spray dried capsules could be due to the development of Maillard reactions between protein and the reducing sugar lactose which is present in WPI. The intensity of the colour is correlated with the temperature of storage. It has previously been reported that the rates of Maillard reactions are temperature dependent. These reactions might be contributing to the instability of bacterial cells during storage which confirms the previous report on the storage stability of freeze dried $L. acidophilus$ (Kurtmann, Skibsted, & Carlsen, 2009).
7.4 Conclusion

$T_g$ which is generally accepted as an indicator for storage stability of food, was evaluated using two independent methods. Live cells embedded in WPI spray dried to approximately 95% solids, was found to exist in the glassy state at approximately 34 °C.

This natural dairy-based material provided adequate protection for $L. \text{plantarum}$ cells against harsh processing conditions (110 °C inlet temperature, ~70 °C outlet temperature and flow rate of 6.6 mL/min) and storage conditions (4 – 50 °C) over 8 weeks. Under these conditions, bacterial cells displayed resistance and survived even when the capsules were stored at or above the $T_g$ of WPI. Accordingly we propose that WPI could be used as structural elements and probiotic carriers even at ambient temperature to deliver a minimum number of viable cells of $1 \times 10^9$ CFU/g for up to 4 weeks for $L. \text{plantarum}$ A17 and up to 9 weeks for B21. These results are promising for the functional food industry.
Chapter 8

General discussion and conclusions

8.1 Introduction

The purpose of this chapter is to summarise the results obtained in this research, draw final conclusions and make recommendations for further research that would extend and develop the results presented in this thesis.

8.2 Summary of results and discussion

Initially (Chapter 4) a series of commonly utilised protective agents were screened for their abilities to protect the bacterial cells of A17 during SDD. Kinetics of bacterial survival, droplet temperature and changes in moisture content were measured during in-situ drying by this method and comparisons on the protective behaviour of each protectant were made.

Five different forms of protectants were studied: WPI, trehalose, lactose, long life skim milk, and a mixture of lactose and WPI at a concentration of 10% (w/w). These were utilised to investigate the protective behaviour by using two different drying temperatures (90 and 110 °C). It was found that skim milk and WPI provided the highest protection during the intermediate drying stage. The protection was deduced from a reduction in the rate of temperature rise for these two protectants where only the native WPI (pH ~ 6.6) appears to offer a different protective behaviour in the dry form. This finding is interesting as it appears to be in contrast with work done by Duongthingoc et al. (2013) where it was found that acid induced denaturation (pH 4) of WPI was the dominant mechanism contributing to the protection of yeast cells during spray drying.

To further elucidate the protective role provided by native WPI in the dry form, a spray dryer was used to encapsulate the bacterial cells within the WPI matrix and the results have been presented in Chapter 5. To test the hypothesis that acid or heat induced denaturation could also be a dominant mechanism for L. plantarum, the structures of whey proteins were altered either by acid or heat and the effects characterised by μ-DSC and FTIR. Zeta potential
measurements were used to study the complexation of WPI and the bacteria at different pH values.

Whey protein capsules obtained at pH 7 (native) and 4 as well as those at pH 7 but with heat treatment at 75 °C for 1 minute or 78 °C for 10 minutes showed viable cells during spray drying of 69.0, 39.3, 40.3 and 25.0% respectively. Significantly higher survival of cells was observed in the matrix of native WPI where the protein structure was more compact and globular with 47, 7, 17 and 29% β-sheets, random coils, α-helices and β-turns, respectively. After exposure to acid at pH 4, the survival of A17 was reduced to ~39% with the proteins exhibiting a less compact and partial unfolding of the compact globular structure as demonstrated by a significant increase in the β-sheets to 53% and a complete loss of random coils as shown by the Fourier transform infrared spectroscopy. It appears that a lower degree of protein denaturation before spray drying as characterized by μ-DSC benefits the survival of A17 during encapsulation. In addition, spray drying conditions (outlet temperature of ~70 °C) did not contribute significantly to further denaturation of the WPI matrix.

Surface charge of bacteria as determined by zeta potential was negative throughout the pH range of 3.0 to 10.0. It was proposed that a new layer-by-layer encapsulation mechanism may be predominantly responsible for the protection and maintenance of the active state of the bacterial cells at pH 7 based on the isoelectric points of different proteins that made up whey proteins.

To further understand the protective mechanism of WPI in the dry form (Chapter 6), a spray dryer was utilised and the survival of the cells within the matrix was related to the microbial adhesion (interaction), concentration of whey proteins, moisture content, surface tension and morphology. Two strains of *L. plantarum* (A17 and B21) were tested in this study. Measurement of the microbial adhesion showed that B21 was more hydrophobic than A17. Evidence of bacterial cells embedded in the whey protein layer was provided by SEM. It was hypothesised that hydrophobic bacteria cells appear to be protected by attaching to the hydrophobic parts of the whey proteins and prevented the proteins from attaching to each other via a different morphology than has previously been proposed as shown in Chapter 6. It was found that this protective behaviour of WPI is concentration dependant where more hydrophobic cells (B21) only required half the amount of WPI to provide similar protection.
to the less hydrophobic cells (A17). The optimum concentration of WPI to provide the best protection for *L. plantarum* A17 was found to be 20% (w/w).

In this study, spray drying produced microparticles with moisture content of approximately 5% and water activity of 0.2 are mostly spherical in shape with smooth surfaces where particle size increases with increasing protein concentration used. Microcapsules produced were in the range of 3 to approximately 23 μm. These particle sizes are unlikely to impart any grainy or mouthfeel effects when added to other foods. The highest protein concentration tested (30%) correlates with the reduced protection of bacteria due to the increase in particle size; which required longer drying time.

To exert any core benefits of probiotics, bacteria need to be stable and viable with adequate amounts administered. Therefore the final study in this thesis (Chapter 7) was focused on evaluating the effect of storage temperatures with respect to the *Tg* of the encapsulating matrix on the survival of two strains of *L. plantarum* and use colour measurements as an indicator of deteriorative chemical reactions.

The *Tg* of spray dried microcapsules produced in this research with solid content of about 95%, was investigated by two independent methods. Modulated differential scanning calorimetry was not useful in determining the *Tg* of the capsules; however, measurements of the *Tg* using dynamic mechanical analysis gave values of approximately 34 °C. Based on this *Tg*, four different storage temperatures of 4, 20, 30 and 50 °C were chosen to study the storage stability of both A17 and B21 microcapsules for a period of 8 weeks.

It was found that bacterial cells of both strains were stable with the final count of approximately 10\(10^9\) CFU/g when capsules were stored at 4 and 20 °C, which were below the *Tg* of the spray dried capsules. At storage temperature in the proximity of the *Tg* (30 °C), both strains resulted in stable live cultures with a minimum of viable cells of 1×10\(^9\) CFU/g for up to ~ 4 weeks for A17 and ~ 9 weeks for B21 at residual moisture content of 7.3% and 6.5% respectively. As expected, storing at 50 °C resulted in a rapid reduction of bacterial cell counts with an approximately 2 log reduction for B21 and 4 log reduction for A17 within the first week of storage corresponding to viable cell count of 1×10\(^9\) CFU/g for up to 5 days for B21 and up to 3 days for A17 with residual moisture content of 5.6% and 5.1% respectively. The water activity of microcapsules at the end of the storage study was found to be less than
0.2. Storing spray-dried microcapsules at higher temperature resulted in an increase in total colour difference and changes in the colour were attributed to the Maillard like reactions between protein and reducing sugar (lactose).

The result from the storage study is promising and could be of interest to the food industry as storing capsules slightly above room temperature (30 °C) resulted in a stable bacterial cell count of up to $10^9$ CFU/g for ~4 and 9 weeks respectively for A17 and B21. This is a general minimum threshold to provide health effects to the host as recently agreed among the International scientific association for probiotics and prebiotics. Products stable at room temperature would provide a lot of advantages as this can reduce the cost of storage and transportation. Currently most if not all commercial probiotics are stored either refrigerated (2 – 8 °C) or frozen (-18 °C). These findings are useful to the dairy and functional food industry as protein-based microparticles occupy an increasingly important position as the versatile nature of proteins allows new design strategies to encapsulate many food ingredients including microbial cells.

The above studies have confirmed the effective encapsulation of *L. plantarum* and formation of microcapsules that protects the bacterial cells for the purpose of retaining viability during drying and storage. This thesis provides a strong basis for understanding the mechanism of protection of WPI and the interaction between whey proteins and bacterial cells using a combination of advanced instrumental techniques including differential scanning calorimetry, Fourier transform infra-red spectroscopy, zeta potential analysis and scanning electron microscopy. These have been used to compare the changes in protein at the level of the secondary structure as a key to understanding the mechanism of the interactions between the proteins and the bacteria. The applications of Zeta potential measurement as well as the bacteria adhesion to hydrocarbon test have facilitated the development of a clear picture of how the interaction between different whey protein components as well as the interaction between hydrophobic bacteria and protein protect bacterial cells during drying and storage. Evidence of cells embedded in the whey protein layer was confirmed by SEM via a different morphology than has previously been proposed in literature. The effectiveness of WPI in stabilising *L. plantarum* during storage at ambient temperature (30 °C) demonstrated commercial potential of this approach. This research work is therefore unique in combining a series of measurements that evaluate the molecular and denaturation properties of whey protein and using that to develop a new model to explain the protective effects observed.
8.3 Major conclusions

From different studies conducted in this research project, the following final conclusions have been drawn:

i. Among the commonly utilised protective agent, WPI and skim milk provided the best protection during intermediate drying stage during SDD. This protection was deduced from the reduction in the rate of temperature rise during SDD at two different temperatures of 90 and 110 °C, which reduces thermal stress, thus preserving the bacteria. In addition, WPI provided effective protective behaviour in the dry form;

ii. Whey protein capsules obtained at pH 7 (native) and 4 as well as those at pH 7 but with heat treatment at 75 °C for 1 minute or 78 °C for 10 minutes showed viability during spray drying of 69.0, 39.3, 40.3 and 25.0% respectively. Significantly higher survival of cells was observed in the matrix of native WPI where the protein structure was more compact and globular with 47, 7, 17 and 29% β-sheets, random coils, α-helices and β-turns, respectively. After exposure to acid at pH 4, the survival of A17 was reduced to ~39% with the proteins exhibiting a less compact and partial unfolding of the compact globular structure as demonstrated by a significant increase in the β-sheets to 53% and a complete loss of random coils. The protection to bacterial cells at pH 7 was attributed to the layer-by-layer electrostatic interactions between different components of WPI which differed in their isoelectric point;

iii. Measurement of the microbial adhesion showed that B21 was more hydrophobic than A17. Evidence of bacterial cells embedded in the whey protein layer was provided by SEM. It was hypothesised that hydrophobic bacteria cells appear to be protected by attaching to the hydrophobic parts of the whey proteins and prevented the proteins from attaching to each other via a different morphology than has previously been proposed

iv. The protection of L. plantarum by WPI during spray drying is concentration dependent, where 20% provided the optimum protection. Using highest
concentration in this study (30% of WPI) resulted in reduced protection during spray drying as higher concentration resulted in larger particle size; thus requiring longer drying times which ultimately induced thermal stress to the bacterial cells;

v. Storing microcapsules containing both A17 and B21 at 4 and 20 °C, which are well below the $T_g$ of the matrix, resulted in the stability of bacterial cells. Product-keeping quality of the microcapsules was also evident for up to two weeks of storage at room temperature (30 °C), before a gradual decline with a final viable cell count of $10^7$ and $10^9$ CFU/g respectively after 8 weeks of storage;

vi. Capsules stored at 50 °C (close to the thermal death temperature of A17) gave viable cells of $1\times10^9$ CFU/g for up to 5 days for B21 and up to 3 days for A17 with residual moisture content of 5.6% and 5.1% respectively. Storage at this high temperature was accompanied by changes in total colour difference of the microcapsules.

**8.4 Possible area for further research**

The investigations reported in this thesis demonstrated that the two strains of *L. plantarum* could be successfully encapsulated with WPI by spray drying and the resultant capsules with a moisture content of approximately 5% were stable when stored at 4 and 20 °C. A further recommendation from the current studies is to evaluate the effectiveness of the strong acid producer (A17) and bacteriocin producer (B21) microcapsules produced as described in this thesis for its effectiveness in acid production, and inhibition against certain pathogen as well as the production of anti-microbial compounds.

In addition, it is recommended that the capsules developed in this study, be trialled as a starter culture for fermentation of various food substrates including meat and dairy products.

Another related area is to extend the study to the release of these bacterial cells from the microcapsules into the various changing conditions of the digestive tract including the resistance against acid or bile test.
Finally, it is the hope of the author that the work described in this thesis provides a strong basis for ongoing research in this important area. Furthermore, may the current studies contribute to the enhanced health and well-being of the consumers around the world.
References


-155-


References


References


References


References


Stan, I. (2010). *Electrical charge of a single living cell measured by optical trap technique* (Master), ICFO- Institut de Ciències Fotòniques, Optical Tweezers Group, Spain


-181-
