

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**The biotherapeutic potential of *Lactobacillus reuteri* DPC16  
and bovine lactoferrin in controlling some pathogens,  
genotoxicity and inflammation in the gut**

**Hong Tian**

2013

**The biotherapeutic potential of *Lactobacillus reuteri* DPC16  
and bovine lactoferrin in controlling some pathogens,  
genotoxicity and inflammation in the gut**

A thesis presented in partial fulfilment of the requirements for the degree of  
**Doctor of Philosophy in Engineering and Advanced Technology**  
at Massey University, Auckland, New Zealand

**Hong Tian**

2013

## **Abstract**

This study investigated the effects of the probiotic bacterium, *L. reuteri* DPC16, alone and in combination with bovine lactoferrin, on intestinal pathogens, intestinal inflammation and carcinogenesis. Human and animal cellular model systems were designed and applied to this evaluation.

The identity of the *L. reuteri* DPC16 strain was confirmed using 16S rRNA analysis and its ability to produce the antibacterial compound, reuterin. It was able to tolerate pH 2 and physiological concentrations of bile salts in a simulated gastrointestinal tract environment in the presence of protective nutrients. It was able to adhere to a Caco-2 human epithelial monolayer (modelling the human GI tract) and it did not degrade mucin.

Both bovine lactoferrin and *L. reuteri* DPC16 inhibited the growth of the intestinal pathogens *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* O157:H7, with much less effect on tested probiotic bacteria. Together, *L. reuteri* DPC16 and bovine lactoferrin showed synergistic inhibitory effects.

*L. reuteri* DPC16 was also able to remove indole from faecal water. Using human/animal cellular model systems, combined with the use of *E. coli* endotoxin and genotoxic factors present in faecal water, bovine lactoferrin was shown to down-regulate inflammation by affecting the signalling pathway on immune receptors that recognize the endotoxin, while both bovine lactoferrin and strain DPC16 were shown to have the potential to prevent epithelial cell DNA damage.

The study has demonstrated several significant properties of *L. reuteri* DPC16 and bovine lactoferrin, including antibacterial, antigenotoxic, and anti-inflammatory activities, and possible mechanisms for these activities have been proposed. Based on the information obtained from this work, a combination of the probiotic *L. reuteri* DPC16 and bovine lactoferrin could possibly be developed as a novel probiotic formula

for human consumption, to maintain beneficial bacteria while controlling harmful bacteria in the GI tract. However, advanced *in vitro* model systems and *in vivo* studies are suggested to confirm these findings in order to consider the feasibility of commercialisation.

## **Acknowledgement**

I would like to take this opportunity to express my sincere gratitude to all my supervisors, Professor Ian Maddox, Dr. Quan Shu, Professor Margot Skinner, Dr. Jeffrey Greenwood and Professor Lynnette Ferguson, from Massey University, Bioactive Research New Zealand, the New Zealand Institute for Plant & Food Research and the University of Auckland. This thesis would not have been possible without their advice, support and encouragement.

In particular, I wish to thank my chief supervisor, Professor Ian Maddox for his expert supervision, professional guidance, and continuous encouragement during my study. I am very thankful to him for giving me the opportunity to study at the School of Engineering and Advanced Technology, Massey University, and providing me the travel grant to attend national and international conferences. His generosity, optimism and enthusiasm were always very inspiring throughout the tenure of my study.

I am sincerely grateful to Dr. Quan Shu for his leadership in the probiotics research programme and for offering me the TIF scholarship to complete the PhD project. I would like to thank him for organising and providing the research facility and funding, and for his innovative thoughts, strong motivation and guidance.

I would like to thank Principle scientist Margot Skinner and Dr. Jeffrey Greenwood for including me within their Food & Wellness research team at Plant & Food Research. Their expertise in the New Zealand functional food research field provided guidance to me through developing my research interests, skills and techniques in this field. Particularly I would like to thank the valuable advice in the areas of experimental design and statistical analysis from Dr. Jeffrey Greenwood.

I wish to express my sincere and appreciative acknowledgment to Graham Fletcher, Senior scientist and seafood team leader, Plant & Food Research, for his kind support and for hosting me in his microbiology laboratory and office facilities. Valuable

suggestions and advice in the areas of microbiology and molecular microbiology were received from Dr. Douglas Rosendale, Dr. Cristina Durante and Dr. Muktifar Ahmed, moral support was provided by Dr. Christelle Andre, Dr. Guangjian Lu, and assistance in the laboratory and assay development were provided by Sean Chen and Swapna Gannabathula. Thanks to Graeme Summers, Jill Chantarachoti and Jessicah Riley for the laboratory management. Without their kind support and help, this thesis would not have been completed. In addition, I would like to acknowledge Dr. Denise Hunter in the Food & Wellness research group, Plant & Food Research, for proof-reading the thesis.

I am also grateful to my friends and colleagues, Michael Bian, Sunny Cui, Ping Gao, Sravani Gupta and Edward Walker for their friendship and science discussion.

Finally, I would like to thank my family, my husband Irwin Xu, my sons Tian and Kyle, my sister, and my parents in China. Words cannot describe my heartiest gratitude towards all of them for their endless love, understanding, and continuous support and encouragement throughout this thesis.

## **Abbreviations**

|        |   |
|--------|---|
| AC     | Aberrant crypt  |
| ACF    | Aberrant crypt foci   |
| AAD    | Antibiotic-associated diarrhea  |
| AICR   | The American Institute for Cancer Research                            |
| ANOVA  | Analysis of variance  |
| AOM    | Azoxymethane  |
| ATCC   | American Type Culture Collection                                      |
| AFB1   | Aflatoxin B1  |
| B(a)P  | Benzo(a) pyrene   |
| BCA    | Bicinchoninic acid  |
| BLAST  | The Basic Local Alignment Search Tool                                 |
| BLf    | Bovine lactoferrin  |
| BHI    | Brain heart infusion broth  |
| BM     | Basal medium  |
| BSA    | Bovine serum albumin  |
| CD     | Crohn's disease   |
| CFU/mL | Colony Forming Units per milliliter                                   |
| CRC    | Colorectal cancer   |
| DC     | Dendritic cells   |
| DCA    | Deoxycholic acid  |
| Dg     | Supernatant of DPC16 3h cell suspension in 250 mM glycerol PBS buffer |
| DMH    | 1, 2-dimethylhydrazine  |
| DNA    | Deoxyribonucleic acid   |
| DMSO   | Dimethyl sulfoxide  |
| DS     | DPC16 culture supernatant in MRS medium                               |
| DSg    | DPC16 culture supernatant in 250 mM glycerol-containing MRS medium    |
| CPS    | Capsular polysaccharides  |
| EaggEC | Enteroaggregative <i>E.coli</i>                                       |
| EDTA   | Ethylenediamine tetra-acetic acid                                     |



|               |   |
|---------------|---|
| e.g.          | <i>exempli gratia</i> , means "for example"       |
| EHEC          | Enterohaemorrhagic <i>E. coli</i>                 |
| ELISA         | Enzyme-linked immunosorbent assay                 |
| EPEC          | Enteropathogenic <i>E. coli</i>                   |
| EPS           | Extracellular polysaccharide                      |
| ETEC          | Enterotoxigenic <i>E. coli</i>                    |
| FAO           | Food and Agriculture Organization                 |
| FDA           | The U.S. Food and Drug Administration             |
| GI            | Gastrointestinal                                  |
| GIT           | Gastrointestinal tract                            |
| HCAs          | Heterocyclic amines                               |
| HMGB1         | High-mobility group protein B1                    |
| HIV           | Human immunodeficiency virus                      |
| HK cells      | Heat-killed cells                                 |
| 3-HPA         | 3-hydroxypropionaldehyde                          |
| HFA           | Human flora associated                            |
| IARC          | International Agency for Research into Cancer     |
| IBD           | Inflammatory bowel diseases                       |
| IBS           | Irritable bowel syndrome                          |
| i. e.         | Abbreviation for id est. Latin meaning "that is," |
| IFN- $\gamma$ | Interferon- $\gamma$                              |
| IgA           | Immunoglobulin A                                  |
| IgG           | Immunoglobulin G                                  |
| IL            | Interleukin                                       |
| iNOS          | Inducible nitric oxide synthase                   |
| IQ            | 2- amino-3methylimidazo[4,5- <i>f</i> ]-quinoline |
| LAB           | Lactic acid bacteria                              |
| LCA           | Lithocholic acid                                  |
| LDH           | Lactate dehydrogenase                             |
| LOS           | Lipooligosaccharide                               |
| LPS           | Lipopolysaccharide                                |
| MAM           | Methylazoxymethanol                               |

|                  |  |
|------------------|--|
| MAP              | Modified atmosphere packaging                          |
| MIC              | Minimum inhibitory concentration                       |
| MICA/MICB        | MHC class I chain-related genes                        |
| MLCK             | Myosin light chain kinase                              |
| MW               | Molecular weight                                       |
| NAD <sup>+</sup> | Nicotinamide adenine dinucleotide                      |
| NADH             | Reduced form of NAD <sup>+</sup>                       |
| NADP             | Nicotine adenine dinucleotide phosphate                |
| NK cells         | Natural killer cells                                   |
| NO               | Nitric Oxide   |
| NOD2             | Nucleotide-binding oligomerization domain containing 2 |
| NPD              | 4-nitro-o-phenylenediamine                             |
| NSP              | Non-starch polysaccharides                             |
| NZMP             | New Zealand milk products                              |
| OD               | Optical density  |
| PAMPs            | pathogen-associated molecular patterns                 |
| PBS              | Phosphate buffered saline                              |
| PCR              | Polymerase chain reaction                              |
| PAD              | Photodiode Array Detector                              |
| pH               | -.Log <sub>10</sub> (aH <sup>+</sup> )                 |
| PGM              | Porcine gastric mucin                                  |
| PMA              | Phorbol myristate acetate                              |
| PRRs             | Pattern-recognition receptors                          |
| r DNA            | r RNA gene   |
| RNA              | Ribonucleic acid                                       |
| RNS              | Reactive nitrogen species                              |
| ROS              | Reactive oxygen species                                |
| r RNA            | Ribosomal RNA  |
| SCGE             | Single cell gel electrophoresis                        |
| SCFAs            | Short chain fatty acids                                |
| SD               | Standard deviation                                     |
| SDS              | Sodium dodecyl sulphate                                |

|               |                                       |
|---------------|---------------------------------------|
| SE            | Standard error                        |
| sIgA          | Secretory Immunoglobulin A            |
| TEER          | Transepithelial electrical resistance |
| TNF- $\alpha$ | Tumour necrosis factor-alpha          |
| TLRs          | Toll-like receptors                   |
| UC            | Ulcerative colitis                    |
| UV            | Ultraviolet light                     |
| VLBW          | Very low birth weight                 |
| WCRF          | World Cancer Research Fund            |
| WHO           | World Health Organization             |

## **Table of Contents**

|  |            |
|--|------------|
| <b>Abstract</b> .....  | <b>i</b>   |
| <b>Acknowledgement</b> .....   | <b>iii</b> |
| <b>Abbreviations</b> .....   | <b>v</b>   |
| <b>Table of Contents</b> .....   | <b>ix</b>  |
| <b>List of Figures</b> .....   | <b>xiv</b> |
| <b>List of Tables</b> .....  | <b>xix</b> |
| <b>Chapter 1 General Introduction</b> .....                                  | <b>1</b>   |
| Overview .....   | 1          |
| Literature review .....  | 2          |
| 1.1 Gastrointestinal microflora .....  | 2          |
| 1.1.1 The development of gastrointestinal microflora .....                   | 2          |
| 1.1.2 Factors influencing the composition of the intestinal microflora ..... | 5          |
| 1.1.3 Imbalanced intestinal microflora and disease incidence .....           | 9          |
| 1.1.4 Summary .....  | 25         |
| 1.2 Probiotics .....   | 25         |
| 1.2.1 Probiotics characteristics and selection criteria .....                | 26         |
| 1.2.2 Probiotic research and the existing gaps .....                         | 28         |
| 1.2.3 <i>Lactobacillus reuteri</i> .....                                     | 30         |
| 1.3 Milk beneficial factors .....  | 35         |
| 1.3.1 Lactoferrin.....   | 36         |
| 1.3.2 Structure .....  | 38         |
| 1.3.3 Functions .....  | 38         |
| 1.4 Probiotics and lactoferrin .....   | 41         |
| 1.5 Aim of this study .....  | 42         |

|                  |  |           |
|------------------|--|-----------|
| <b>Chapter 2</b> | <b>Characterisation of the probiotic properties of <i>Lactobacillus reuteri</i> DPC16.....</b>                             | <b>44</b> |
| 2.1              | Introduction.....  | 44        |
| 2.1.1            | The background of the <i>Lactobacillus reuteri</i> DPC16 strain.....   | 44        |
| 2.1.2            | Aims of this chapter.....  | 45        |
| 2.2              | Materials and Methods.....   | 46        |
| 2.2.1            | Chemicals.....   | 46        |
| 2.2.2            | Bacterial strains and culture media.....   | 46        |
| 2.2.3            | 16S rRNA analysis.....   | 46        |
| 2.2.4            | Preparation of <i>L. reuteri</i> DPC16 culture supernatants.....   | 49        |
| 2.2.5            | Agar diffusion assay.....  | 50        |
| 2.2.6            | Spectrophotometric turbidity bioassay.....   | 50        |
| 2.2.7            | Analysis of reuterin and short chain fatty acids in <i>L. reuteri</i> DPC16 supernatants.....                              | 51        |
| 2.2.8            | <i>L. reuteri</i> DPC16 environmental tolerance assays.....  | 52        |
| 2.2.9            | Tolerance to low pH values and high bile salts concentrations in nutrient-deficient conditions.....                        | 54        |
| 2.2.10           | Human intestinal epithelium (Caco-2) adhesion assays.....  | 55        |
| 2.2.11           | Mucin degradation studies.....   | 56        |
| 2.2.12           | Statistical analysis.....  | 57        |
| 2.3              | Results.....   | 59        |
| 2.3.1            | <i>L. reuteri</i> DPC16 genotype confirmation.....   | 59        |
| 2.3.2            | Antibacterial activities of <i>L. reuteri</i> DPC16 against pathogens.....   | 60        |
| 2.3.3            | Identification of the antibacterial substances in <i>L. reuteri</i> DPC16 fermentation products.....                       | 64        |
| 2.3.4            | The effect of DSg on the growth kinetics of some pathogens and probiotics.....   | 66        |
| 2.3.5            | Tolerance of <i>L. reuteri</i> DPC16 to pH and bile salts under nutrient-sufficient and nutrient-deficient conditions..... | 69        |
| 2.3.6            | Adhesion of <i>L. reuteri</i> DPC16 to an intestinal epithelial Caco-2 monolayer.....                                      | 71        |
| 2.3.7            | Mucin degradation assessment of <i>L. reuteri</i> DPC16 and other bacteria.....  | 72        |

|  |     |
|--|-----|
| 2.4 Discussion .....   | 73  |
| <b>Chapter 3 Effects of bovine lactoferrin on the growth of bacteria and the possible mechanisms.....79</b>  |     |
| Introduction .....   | 79  |
| 3.1.1 Lactoferrin.....   | 79  |
| 3.1.2 Aim of this chapter.....   | 80  |
| 3.2 Materials and Methods .....  | 81  |
| 3.2.1 Bovine Lactoferrin .....   | 81  |
| 3.2.2 Bacterial strains and culture media .....  | 81  |
| 3.2.3 Skim milk medium.....  | 81  |
| 3.2.4 Spectrophotometric turbidity bioassay and bacterial drop plate count<br>technique .....  | 81  |
| 3.2.5 Statistical analysis .....   | 81  |
| 3.3 Results .....  | 82  |
| 3.3.1 The effects of bovine lactoferrin on the growth of some pathogenic and<br>probiotic bacteria.....  | 82  |
| 3.3.2 The effect of polymyxin B on the growth of selected pathogens and<br>probiotics.....   | 86  |
| 3.3.3 The effect of bovine lactoferrin in combination with a probiotic<br>supernatant on the growth of selected pathogens and probiotics.....            | 88  |
| 3.4 Discussion .....   | 90  |
| <b>Chapter 4 Protective effects of <i>L. reuteri</i> DPC16 and bovine lactoferrin on faecal<br/>genotoxin-induced epithelial cell DNA damage..... 95</b> |     |
| 4.1 Introduction .....   | 95  |
| 4.1.1 DNA damage and carcinoma development in the colon.....   | 96  |
| 4.1.2 Genotoxicity of human faecal water and probiotic interventions .....   | 97  |
| 4.1.3 Aim of this chapter.....   | 99  |
| 4.2 Materials and methods .....  | 100 |
| 4.2.1 Bacterial strains and growth conditions .....  | 100 |
| 4.2.2 Bacterial supernatant and cells preparation.....   | 100 |
| 4.2.3 Human faecal water and faecal flora preparation .....  | 100 |

|  |   |            |
|--|---|------------|
| 4.2.4  | Cell lines and growth conditions.....   | 101        |
| 4.2.5  | Cytotoxicity determination (MTT assay).....   | 101        |
| 4.2.6  | Colon carcinogenesis model and genotoxicity measurement .....   | 102        |
| 4.2.7  | Indole determination .....  | 105        |
| 4.2.8  | Statistical analysis .....  | 106        |
| 4.3  | Results.....  | 107        |
| 4.3.1  | Cytotoxicity of human faecal water .....  | 107        |
| 4.3.2  | Genotoxicity of human faecal water and the effects of potential protectants<br>.....                          | 108        |
| 4.3.3  | Antigenotoxic effects of bovine lactoferrin on faecal water-induced colon<br>epithelial cell DNA damage ..... | 111        |
| 4.3.4  | Indole determination .....  | 113        |
| 4.4  | Discussion .....  | 115        |
| <br><b>Chapter 5 The effects of <i>L. reuteri</i> DPC16 and bovine lactoferrin on models of<br/>endotoxin- induced intestinal inflammation .....</b> |   | <b>120</b> |
| 5.1  | Introduction.....   | 120        |
| 5.1.1  | Endotoxin –lipopolysaccharide (LPS) .....   | 120        |
| 5.1.2  | Lipopolysaccharide-induced inflammatory response .....  | 122        |
| 5.1.3  | Aim of this chapter.....  | 123        |
| 5.2  | Materials and Methods.....  | 124        |
| 5.2.1  | Chemicals.....  | 124        |
| 5.2.2  | Bacterial supernatants and cell preparations .....  | 124        |
| 5.2.3  | Human and murine immune cell lines and culture conditions.....  | 125        |
| 5.2.4  | Human colon adenocarcinoma Caco-2 cell line and culture conditions  | 125        |
| 5.2.5  | Inflammatory cellular models .....  | 125        |
| 5.2.6  | Intracellular cytokine detection (Flow Cytometry).....  | 125        |
| 5.2.7  | Nitric oxide determination (Griess reaction assay).....   | 126        |
| 5.2.8  | Cell proliferation assessment (MTT assay).....  | 127        |
| 5.2.9  | TNF- $\alpha$ analysis (ELISA).....   | 127        |
| 5.2.10   | Intestinal epithelium/immune co-culture model (Caco-2/THP-1) .....  | 127        |
| 5.2.11   | Cell death determination (LDH assay).....   | 130        |

|                  |  |            |
|------------------|--|------------|
| 5.2.12           | Statistical analysis .....   | 130        |
| 5.3              | Results .....  | 130        |
| 5.3.1            | Establishment of the RAW 264.7 cell model system .....                                     | 130        |
| 5.3.2            | Establishment of the THP-1 cell model system .....   | 139        |
| 5.3.3            | Effects of bovine lactoferrin on LPS-induced TNF- $\alpha$ production in THP-1 cells ..... | 141        |
| 5.3.4            | Establishment of the Caco2/THP-1 co-culture model system .....                             | 143        |
| 5.4              | Discussion .....   | 149        |
| <b>Chapter 6</b> | <b>General Discussion .....</b>  | <b>152</b> |
| 6.1              | Discussion .....   | 152        |
| 6.1.1            | The applications of 16S rRNA gene extraction and identification .....                      | 153        |
| 6.1.2            | The probiotic properties of <i>L. reuteri</i> DPC16 .....                                  | 154        |
| 6.1.3            | Lactoferrin and <i>L. reuteri</i> DPC16 .....  | 157        |
| 6.2              | Future work and challenges .....   | 158        |
| 6.2.1            | Extended work based on the present study .....   | 158        |
| 6.2.2            | Probiotic formulae in human GI intervention .....  | 159        |
| 6.3              | Concluding remarks .....   | 161        |
|                  | <b>Bibliography .....</b>  | <b>163</b> |
|                  | <b>Publications.....</b>   | <b>184</b> |
|                  | <b>Appendix (I to III).....</b>  | <b>CD</b>  |



## **List of Figures**

|   |    |
|---|----|
| Figure 1.1 The distribution of microbes in the gastrointestinal tract of the human adult<br>(adapted from Roccarina et al. 2010) .....                            | 4  |
| Figure 1.2 Faecal flora from 4-28 days of age in breast-fed babies and formula-fed<br>babies (adapted from Balmer and Wharton, 1989) .....                        | 6  |
| Figure 1.3 Faecal pH from 4-28 days of age in breast-fed babies and formula-fed babies<br>(adapted from Balmer and Wharton, 1989).....                            | 6  |
| Figure 1.4 Changes in the faecal flora with increasing age in human (adapted from<br>Mitsuoka 1996) .....   | 8  |
| Figure 1.5 Functions of intestinal microbes in humans (modified from Gibson and<br>Roberfroid 1995) .....   | 9  |
| Figure 1.6 A model for the pathogenesis of inflammatory bowel disease .....   | 17 |
| Figure 1.7 Summary of cancer risk factors on cellular DNA and consequences of DNA<br>damage (Friedberg 1995) .....  | 18 |
| Figure 1.8 Structure of heterocyclic amines and polycyclic aromatic hydrocarbons<br>(modified from Sugimura, 1997) .....  | 22 |
| Figure 1.9 The major toxic metabolites produced by intestinal bacteria and their<br>proposed negative health effects .....  | 24 |
| Figure 1.10 Intestinal microflora and disease incidence (from Benno, 2004) .....  | 25 |
| Figure 1.11 Beneficial effects of probiotics on human health (adapted from Parvez <i>et al.</i> ,<br>2006).....   | 29 |
| Figure 1.12 A proposed glucose (pink outline) and glycerol (blue outline) metabolic<br>pathway of <i>L. reuteri</i> JCM112T (from Morita <i>et al.</i> 2008)..... | 31 |
| Figure 1.13 Three-dimensional structure of bovine lactoferrin (adapted from Baker<br>and Baker, 2009) .....   | 38 |

|  |    |
|--|----|
| Figure 2.1 Other derivatives of Reuterin (3-hydroxypropionaldehyde, 3-HPA) produced from glycerol (modified from Bauer <i>et al.</i> 2010a).....               | 51 |
| Figure 2.2 A 10-fold serial dilution of a bacterial suspension conducted in a 96-well plate .....  | 54 |
| Figure 2.3 4x4 drop plate count method: 4 samples displayed vertically with 4 dilutions displayed horizontally on an agar plate.....                           | 54 |
| Figure 2.4 A sigmoidal concentration-response (variable slope, four-parameter logistic equation) model.....  | 58 |
| Figure 2.5 Agarose gel electrophoresis of 16S rRNA gene PCR product from <i>L. reuteri</i>   | 59 |
| Figure 2.6 Antibacterial activities of <i>L. reuteri</i> DPC16 against growth of the indicator pathogen <i>E.coli</i> O157:H7 in an agar diffusion assay ..... | 61 |
| Figure 2.7 Antibacterial concentration responses of three <i>L. reuteri</i> DPC16 culture supernatants .....   | 62 |
| Figure 2.8 Comparison of the antibacterial effects of original and pH-neutralized <i>L. reuteri</i> DPC16 supernatants .....                                   | 63 |
| Figure 2.9 Comparison of the inhibitory effects of glycerol and Dg .....   | 64 |
| Figure 2.10 An acrolein standard curve for determination of reuterin using the colorimetric method (Circle <i>et al.</i> 1945).....                            | 65 |
| Figure 2.11 A time course study of <i>L. reuteri</i> DPC16 supernatant (DSg) effects on the growth of pathogens .....  | 67 |
| Figure 2.12 A time course study of <i>L. reuteri</i> DPC16 supernatant (DSg) effects on the growth of probiotics .....   | 68 |
| Figure 2.13 Survival of <i>L. reuteri</i> DPC16 and other probiotics challenged with low pH and high bile salts under conditions of nutrient sufficiency.....  | 69 |
| Figure 2.14 Survival of probiotics challenged with low pH or high bile salts under conditions of nutrient deficiency.....                                      | 70 |
| Figure 2.15 Correlation of added <i>L. reuteri</i> DPC16 numbers and those adhered to the  |    |

|  |     |
|--|-----|
| Caco-2 monolayer .....   | 71  |
| Figure 2.16 The epithelial adhesion of <i>L. reuteri</i> DPC16 and other probiotic strains ....  | 72  |
| Figure 2.17 Agarose plate mucinolytic assay .....  | 73  |
| Figure 3.1 The effects of bovine lactoferrin on the growth of pathogens and probiotics   | 83  |
| Figure 3.2 The effects of bovine serum albumin on the growth of pathogens and<br>probiotics .....  | 84  |
| Figure 3.3 The effects of bovine lactoferrin and bovine serum albumin on the growth of<br><i>E. coli</i> (EC) in BHI medium tested using the standard drop plate count<br>method ..... | 85  |
| Figure 3.4 The effects of bovine lactoferrin, at 20 mg/mL, on the growth of selected<br>bacteria in skim milk medium .....   | 86  |
| Figure 3.5 The effects of polymyxin B on the growth of selected bacteria .....   | 87  |
| Figure 3.6 The effects of penicillin and streptomycin on growth of selected bacteria ...   | 88  |
| Figure 3.7 Effects of a combination of BLf and DSg on the growth of pathogens and<br>probiotics .....  | 89  |
| Figure 3.8 Mechanisms of action of antibacterial peptides (from Jenssen <i>et al.</i> 2006)..  | 91  |
| Figure 3.9 A proposed model for the interaction of lactoferrin with LPS in Gram-<br>negative bacterial outer membranes .....   | 94  |
| Figure 4.1 The metabolism of tryptophan to indole by tryptophanase .....   | 96  |
| Figure 4.2 Carcinoma development in the colon (adapted from Gill and Rowland 2002)<br>97   | 97  |
| Figure 4.3 Molecular structure of MTT and its corresponding colorimetric reaction<br>product in the presence of mitochondrial reductase enzyme activity .....                          | 102 |
| Figure 4.4 Comet image showing original cell DNA (A) and damaged DNA (B) spread<br>out by electrophoresis and highlighted using a fluorescent stain .....                              | 103 |
| Figure 4.5 Diagram of typical comet and DNA tail moment analysis .....   | 105 |
| Figure 4.6 Cytotoxicity of faecal water measured using the MTT assay.....  | 108 |

|   |     |
|---|-----|
| Figure 4.7 Genotoxicity of three faecal water samples.....  | 109 |
| Figure 4.8 The antigenotoxic effects of tested probiotics and pathogens .....   | 110 |
| Figure 4.9 The antigenotoxic effects of <i>L. reuteri</i> DPC16 heat-killed cells and cell-free culture supernatant .....   | 111 |
| Figure 4.10 Antigenotoxic effects of bovine lactoferrin and bovine serum albumin....  | 112 |
| Figure 4.11 Comet images showing the effects of bovine lactoferrin and bovine serum albumin on faecal water-induced HT29 cell DNA damage .....  | 112 |
| Figure 4.12 Biochemical test and HPLC analysis for indole production.....   | 113 |
| Figure 4.13 Indole standard curve obtained using HPLC analysis .....  | 114 |
| Figure 4.14 The effect of <i>L. reuteri</i> DPC16 and <i>E.coli</i> on indole concentration in faecal water .....   | 114 |
| Figure 4.15 A proposal for mechanisms by which <i>L. reuteri</i> may counteract <i>E.coli</i> in the large intestine .....  | 118 |
| Figure 5.1 CD14/TLR4/MD2 receptor complex.....  | 121 |
| Figure 5.2 Caco-2/THP-1 co-culture system in separate compartments of a transwell culture.....  | 128 |
| Figure 5.3 Caco-2/THP1 co-culture system work flow chart .....  | 128 |
| Figure 5.4 EndOhm-6 chamber for measurement of transepithelial electrical resistance  | 129 |
| Figure 5.5 Flow cytometry dot plot graphs .....   | 132 |
| Figure 5.6 Flow cytometry cytokine histogram .....  | 133 |
| Figure 5.7 The effect of <i>L. reuteri</i> DPC16 cells and cell-free supernatants on nitric oxide production by RAW 264.7 cells .....   | 134 |
| Figure 5.8 The effect of BLf on nitric oxide production by RAW 264.7 cells .....  | 135 |
| Figure 5.9 Microscope images showing the effects of 24 h treatment with LPS (1 µg/mL) alone and LPS plus lactoferrin (1.25 mg/mL) on the morphology of murine macrophage RAW 264.7 cells..... | 136 |

|  |     |
|--|-----|
| Figure 5.10 Effects of bovine lactoferrin (BLf) on LPS-induced nitric oxide production and cell proliferation in RAW 264.7 cells .....                 | 137 |
| Figure 5.11 Effects of <i>L. reuteri</i> DPC16 supernatant and heat-killed cells on LPS-induced nitric oxide production by RAW 264.7 cells .....       | 138 |
| Figure 5.12 PMA differentiated THP-1 cells .....   | 140 |
| Figure 5.13 Anti-inflammatory effects of bovine lactoferrin .....  | 142 |
| Figure 5.14 The effect of bovine lactoferrin treatments on TNF- $\alpha$ production by THP-1 cells.....  | 143 |
| Figure 5.15 Changes in epithelial barrier integrity of the Caco-2 monolayer .....  | 145 |
| Figure 5.16 Lactate dehydrogenase (LDH) determination on the Caco-2 monolayer under the treatment conditions .....                                     | 146 |
| Figure 5.17 The TEER reduction of a Caco2 monolayer during co-cultivation with 200 nM PMA-differentiated THP-1 cells at an increased cell density..... | 147 |
| Figure 5.18 The TEER reduction of a Caco-2 monolayer THP-1 coculture following treatment with DPC16 supernatant and bovine lactoferrin .....           | 148 |
| Figure 5.19 The negative charges on lipopolysaccharide (LPS) that may be a binding site for cationic lactoferrin .....                                 | 151 |
| Figure 6.1 A hypothetical model of DPC16 + lactoferrin on intestinal disease prevention  | 159 |

## **List of Tables**

|  |     |
|--|-----|
| Table 1.1 Commonly used probiotics.....  | 28  |
| Table 2.1 pH values, bile salts concentrations and food retention times in the human<br>intestinal tract .....                 | 53  |
| Table 2.2 Relative MIC50 of different <i>L. reuteri</i> DPC16 supernatants (DS, DSg and Dg)<br>against pathogens .....         | 63  |
| Table 2.3 pH and SCFA analysis of MRS growth medium and <i>L. reuteri</i> DPC16 culture<br>supernatants (DS and DSg) .....     | 65  |
| Table 3.1 Combination Index of the antibacterial activities of BLf (10 mg/mL) and DSg<br>[12.5 % (v/v)] against pathogens..... | 90  |
| Table 4.1 Summary of dietary studies assessing genotoxicity in human faecal samples  | 98  |
| Table 4.2 Treatment preparation for antigenotoxicity assays.....   | 104 |