Understanding the pathobiology of *Brachyspira pilosicoli* in order to develop novel intervention strategies against avian intestinal spirochaetosis

A thesis submitted by

Luke John Mappley

For the degree of

Doctor of Philosophy

2012

Department of Bacteriology
Animal Health and Veterinary Laboratories Agency
&
School of Biological Science
Royal Holloway, University of London

“Remember to look up at the stars and not down at your feet”

- *Prof. Stephen Hawking*

I dedicate this thesis to

My parents, John and Nicola Mappley

Throughout all my endeavours, you believe in me
Your support, encouragement and generosity are endless

In loving memory of my grandmother, Hilda Leah ‘May’ Bull

Always with me in spirit, if not in body
You continue to inspire me

Declaration of authorship

I Luke John Mappley hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed:

Date:

Abstract

The anaerobic spirochaete *Brachyspira pilosicoli* causes enteric disease in avian, porcine and human hosts, amongst others. Avian intestinal spirochaetosis (AIS), the resulting disease from colonisation of the caeca and colon of poultry by *Brachyspira* leads to production losses, with an estimated annual cost of circa £18 million to the commercial layer industry in the United Kingdom. A lack of knowledge of the metabolic capabilities and little genomic information for *Brachyspira* has resulted in a limited understanding of the pathobiology of this genus. In addition, an emergence of antibiotic resistance in *Brachyspira*, together with bans on the prophylactic use of antimicrobials in animal feed, drive an urgent requirement for alternative treatment strategies for diseases such as AIS.

In the first intra-species genome comparison within the genus *Brachyspira*, these studies report the whole genome sequence of an avian strain of *B. pilosicoli,* B2904, and the incomplete genome sequence of a human strain of *B. pilosicoli,* WesB. Comparisons are made between the *de novo* sequenced strains and those of *B. pilosicoli* 95/1000, a pig strain and other available *Brachyspira* genome sequences from public databases. Furthermore, this study reports the first application of the high-throughput Biolog phenotype screening tool to *Brachyspira* for detailed phenotypic analysis and confirmation of metabolic deductions made from the genotypic data.

Probiotics have been reported as protecting against infection with common enteric pathogens in livestock and in this study investigations into which aspects of the biology of *Brachyspira* they antagonise were undertaken. Lactobacilli reduced the growth and motility of *B. pilosicoli* and its ability to adhere and invade epithelial cells *in vitro*. Following these encouraging results, an *in vivo* intervention study was performed using a *B. pilosicoli* challenge model in poultry to elucidate the potential for probiotic intervention against AIS. This study demonstrated that when administered in drinking water, *L. reuteri* LM1, isolated from a healthy chicken, reduced all aspects of the clinical presentation of AIS.

Acknowledgments

The past four years of work that have contributed to this thesis would not have been possible without the support and contribution of many people.

I have been very fortunate to have worked towards this thesis under excellent supervision. To Prof. Roberto La Ragione, I am extremely thankful for your continuous and unconditional scientific, financial and moral support and for giving me the flexibility to develop into an independent scientist. To Prof. Martin Woodward, I am grateful to have shared in your enormous appetite for research and I have a great deal of respect for your true feedback, though at times frustrating, it was always fruitful. My thanks extend to Prof. Peter Bramley.

During my studies, I was the privileged recipient of three travel grants from the Society of General Microbiology, two of which funded my attendance to international conferences that facilitated my first meeting with Prof. David Hampson and the third permitted a research visit to his laboratories at Murdoch University, Perth. To Prof. David Hampson and your family, I owe an enormous debt of gratitude for the hospitality, kindness and generosity you showed during my visit and also for some of the most meaningful and invaluable supervision and guidance that I received. At Murdoch, I owe great thanks to the people at the Centre for Comparative Genomics and the School of Veterinary and Biomedical Science for making me most welcome and notably to Prof. Matthew Bellgard, Dr. Nyree Phillips and Dr. Tom La, for your assistance and for permitting the use of additional data that contributed to the genome comparisons. I must especially thank Dr. Michael Black, without the wealth of time and expertise you dedicated and your willingness to provide help whenever asked, the comparative genomic research would have failed to be so prosperous.

I am delighted to acknowledge Dr. Alistair Darby for your help with genome sequencing and assembly and for accommodating me on my numerous visits to the University of Liverpool. I acknowledge Prof. Julian Parkhill and Dr. Keith Turner for providing genome sequence data and Dr. Barry Bochner, for your generosity in providing additional Biolog resources and also for lending your knowledge and advice. My thanks extend to Dr. Alejandro Núñez and Bill Cooley for the help you both have

given with histopathological and electron microscopical analysis and interpretation. I thank Andrew Steventon and David Welchman for your help and teaching me the basics of the isolation and culture of *Brachyspira*.

I greatly acknowledge the receipt of funding from the British Egg Marketing Board Research and Education Trust and particularly Alan and Anne Beckett, for your moral and financial support.

A special thank you to all my colleagues in the Department of Bacteriology at the Animal Health and Veterinary Laboratories Agency. It has been a wonderfully fulfilling and rewarding experience to work and study in this group and with some of my closest friends. To Christine Boinett, from day one your assistance and support has been greatly appreciated and I have been lucky to have found a friend in you from so early on in my studies. To Monika Tchórzewska, your assistance with *in vitro* and *in vivo* studies was endless, when it comes to helping others, you are selfless and in you, is someone who I can always count on. To Dr. Laura Searle, your guidance and assistance were second to none and I could not have asked for a better first lab partner and friend. To Manal AbuOun, I am eternally grateful for all your help and supervision, it is hard to think of an aspect of this work where your contribution was not key and I could not have wished to have work with a better postdoctorate. My experience would not have been so much fun without all your help, laughter and jokes.

My deepest and most sincere thanks to my parents, John and Nicola, my sisters, Claire and Joanna and my late grandmother, May. Your tireless support, encouragement and enthusiasm has always been appreciated. Without your love and guidance, I would not have become the person I am today. You inspire me. I am forever in your debt and will love you always.

Finally, this thesis would not have been accomplished without the faith, love and companionship of my best friend, soul mate and partner, Whitney. Thank you for your patience, your support, for being my strength and for sharing this achievement with me. You encourage me to aim for the stars and I cannot wait for our future.

Contribution of others

David Welchman organised sample collection from affected flocks and Andrew Steventon supported in isolation in the of *Brachyspira* strains at the Animal Health and Veterinary Laboratories Agency, Winchester.

Dr. Alistair Darby performed the genome sequencing for *Brachyspira pilosicoli* B2904 and provided assistance during its assembly and annotation.

Prof. Julian Parkhill and Dr. Keith Turner provided the genome sequence for *Brachyspira pilosicoli* WesB.

Prof. David Hampson, Dr. Nyree Phillips and Dr. Tom La provided the draft genome scaffolds for *B. aalborgi* 513^T, *B. alvinipulli* $C1^T$ and *B. intermedia* HB60.

Dr. Michael Black provided guidance and technical assistance to the comparative genomic analyses.

The Cell and Tissue Culture Section at the Animal Health and Veterinary Laboratories Agency prepared HT29-16E monolayers for *in vitro* studies.

The Animal Service Unit at the Animal Health and Veterinary Laboratories Agency assisted in day to day husbandry and veterinary input for the *in vivo* studies.

Dr. Alejandro Núñez at the Animal Health and Veterinary Laboratories Agency assisted with *post-mortem* examinations and histopathological examination of stained tissues.

Bill Cooley at the Animal Health and Veterinary Laboratories Agency processed cells and tissues from *in vitro* studies and undertook the electron microscopical examinations.

Table of contents

Title page 1
Declaration 4
Abstract 5
Acknowledgements 6
Contribution of others 8
Table of contents 9
List of figures 17
List of tables 20
List of abbreviations 22
List of publications 24
Chapter 1. Introduction 25
1.1 Avian intestinal spirochaetosis 25
1.1.1 Definition of avian intestinal spirochaetosis 25
1.1.2 History of avian intestinal spirochaetosis 26
1.1.3 Significance of avian intestinal spirochaetosis 27
1.1.3.1 Economic 27
1.1.3.2 Public Health 27
1.1.4 Aetiology of avian intestinal spirochaetosis 27
1.1.5 Epidemiology of avian intestinal spirochaetosis 28
1.1.5.1 Host range 28
1.1.5.2 Transmission 28
1.1.5.3 Incidence and prevalence 29
1.1.5.4 Incubation period and persistence 30
1.1.5.5 Influencing factors 31
1.1.6 Pathology and clinical symptoms of avian intestinal spirochaetosis 32
1.1.6.1 Subclinical colonisation 32
1.1.6.2 Mild to moderate clinical disease 33
1.1.6.3 Severe disease 35
1.1.6.4 Anatomical location 36
1.1.6.5 Immunity 36
1.1.7 Diagnosis of avian intestinal spirochaetosis 37
1.1.7.1 Isolation and visualisation of spirochaetes 37
1.1.7.2 Identification of spirochaetes by serological properties 37

1.1.7.3 Identification of spirochaetes by biochemical properties 37
1.1.7.4 Identification of spirochaetes by genotypic properties 38
1.1.8 Intervention and treatment of avian intestinal spirochaetosis 39
1.1.8.1 Biosecurity and disinfectants 39
1.1.8.2 Antimicrobial control 39
1.1.8.3 Pre- and probiotic control 40
1.1.8.4 Vaccination 41
1.1.9 Animal models for avian intestinal spirochaetosis 41
1.2 *Brachyspira* 43
1.2.1 Phylogeny and taxonomy of *Brachyspira* 43
1.2.1.1 Phylum *Spirochaetes* 43
1.2.1.2 Genus *Brachyspira* 45
1.2.2 Phenotypic characteristics of *Brachyspira* 47
1.2.2.1 Morphology 47
1.2.2.2 Culture 48
1.2.2.3 Colony morphology 49
1.2.2.4 Biochemical properties 49
1.2.2.5 Metabolic properties 50
1.2.3 Genotypic characteristics of *Brachyspira* 51
1.2.4 Host range of *Brachyspira* 52
1.2.5 Virulence of *Brachyspira* 54
1.2.5.1 Motility and chemotaxis 54
1.2.5.2 Adherence and invasion 54
1.2.5.3 Haemolysis 55
1.2.5.4 NADH oxidase activity 55
1.2.5.5 Lipooligosaccharides 55
1.2.5.6 Iron import system 55
1.3 Probiotics 56
1.3.1 Definitions of probiotic, prebiotic and synbiotic 56
1.3.2 History of probiotics 56
1.3.2.1 History of probiotic use in humans 57
1.3.2.2 History of probiotic use in livestock 57
1.3.3 Design and selection of probiotics 58
1.3.4 The use of probiotics in poultry 61
1.3.5 The use of *Lactobacillus* species as probiotics in poultry 62

1.3.6 Dose and administration of probiotics to poultry 63
1.3.7 Mechanisms of action of probiotics 63
1.3.7.1 Competitive exclusion 63
1.3.7.2 Antagonistic activity 65
1.3.7.3 Digestion and gastrointestinal health 66
1.3.7.4 Immunomodulation 66
1.3.8 Other food supplements and their effect on reducing infection in poultry 67
1.4 Aims and objective 68
Chapter 2. Materials and methods 69
2.1 Bacteriological methodology 69
2.1.1 Bacterial strains and culture conditions 69
2.1.2 Isolation of *Brachyspira* from chicken faeces 72
2.1.3 Isolation of *Lactobacillus* from chicken faeces 72
2.1.4 Gram stain 73
2.1.5 *Brachyspira* identification and characterisation 73
2.1.5.1 Indole test 73
2.1.5.2 Hippurate test 73
2.1.5.3 α-Glucosidase, β-glucosidase and α-galactosidase activity tests 73
2.1.5.4 Slide agglutination test 74
2.1.5.5 Indirect fluorescent antibody test 74
2.1.5.6 Antimicrobial susceptibility testing of *Brachyspira* strains 75
2.1.5.7 Scanning electron microscopy of *B. pilosicoli* 75
2.1.6 Identification and characterisation of *Lactobacillus* 76
2.1.6.1 API 50 CH identification system 76
2.1.6.2 Catalase test 76
2.1.6.3 Hydrogen peroxide production assay 76
2.1.6.4 Antimicrobial susceptibility testing of *Lactobacillus* strains 76
2.1.6.5 Gastric acid tolerance assay 77
2.1.6.6 Bile tolerance assay 78
2.1.7 Growth and inhibition assay of *B. pilosicoli* 78
2.1.8 Agar motility and haemolysis inhibition assay of *B. pilosicoli* 79
2.1.9 Biolog Phenotype MicroArray™ 79
2.2 Molecular biology methodology 81
2.2.1 Extraction of genomic DNA 81

2.2.2 Extraction of bacterial DNA from faeces 81
2.2.3 Polymerase chain reaction 82
2.2.4 Long-range PCR 83
2.2.5 Purification of PCR products 83
2.2.6 Gel electrophoresis 83
2.2.7 Identification of *Brachyspira* using PCR 84
2.2.8 Identification and characterisation of *Lactobacillus* 85
2.2.8.1 Multiplex PCR 85
2.2.8.2 16S rRNA gene sequencing 85
2.2.8.3 Gram-positive antimicrobial resistance microarray 86
2.2.9 Genome sequencing and comparative genomics of *B. pilosicoli* 87
2.2.9.1 Genomic DNA preparation, library construction and sequencing 87
2.2.9.2 Sequence analysis and annotation 88
2.2.9.3 Multilocus sequence typing 89
2.2.9.4 Protein cluster analysis 89
2.3 *In vitro* methodology 90
2.3.1 Culture of mucus-secreting colonic, HT29-16E cells 90
2.3.2 Adhesion and invasion assays using HT29-16E cell models 90
2.3.3 Avian caecal *in vitro* organ culture association assay 91
2.3.4 Electron microscopy 92
2.4 *In vivo* methodology 94
2.4.1 Experimental birds 94
2.4.1.1 Experimental challenge study 94
2.4.1.2 Intervention study 94
2.4.2 Preparation of bacterial strains for administration to birds 94
2.4.2.1 Experimental challenge study 94
2.4.2.2 Intervention study 95
2.4.3 Experimental challenge 95
2.4.3.1 Experimental challenge study 95
2.4.3.2 Intervention study 95
2.4.4 Experimental monitoring during *in vivo* studies 95
2.4.5 *Post-mortem* examinations 96
2.4.5.1 Experimental challenge study 96
2.4.5.2 Intervention study 97
2.4.6 Bacteriological enumeration at *post-mortem* examination 97

2.4.7 Haematoxylin and eosin stain 98
2.5 Statistical analyses 99
Chapter 3. Characterisation of *Brachyspira* and development of a novel experimental challenge model for avian intestinal spirochaetosis 100
3.1 Introduction 100
3.2 Results 102
3.2.1 Speciation and characterisation of *Brachyspira* 102
3.2.1.1 Phenotypic characterisation 102
3.2.1.2 Molecular characterisation 102
3.2.1.3 Antimicrobial susceptibility 105
3.2.1.4 Cell shape and structure 107
3.2.2 Establishing a novel experimental challenge model for avian intestinal spirochaetosis using *B. pilosicoli* 108
3.2.2.1 Colonisation of poultry by *B. pilosicoli* 108
3.2.2.2 Bird weights 108
3.2.2.3 Faecal moisture content 109
3.2.2.4 Egg production 109
3.2.2.5 *Post-mortem* examination of five days post final challenge 111
3.2.2.6 *Post-mortem* examination at eighteen days post final challenge 112
3.3 Discussion 115
Chapter 4. Comparative genomics of three strains of *Brachyspira pilosicoli* and correlation with phenotypic diversity 119
4.1 Introduction 119
4.2 Results 122
4.2.1 Genetic relatedness of *Brachyspira* genomes 122
4.2.2 Comparison of general genome features 122
4.2.3 Genome sequence alignments 125
4.2.4 Global genome feature comparisons 128
4.2.4.1 Functional prediction comparisons 128
4.2.4.2 Genome feature comparisons between *B. pilosicoli* genomes 129
4.2.4.3 Genome feature comparisons between *B. pilosicoli* and other *Brachyspira* species genomes 129
4.2.5 Screening for potential virulence features 130
4.2.6 Comparison of central metabolism phenotypes 133
4.2.6.1 Comparison of carbon source utilisation by *Brachyspira* 133

4.2.6.2 Correlation of differences in *B. pilosicoli* phenotype with genotype 136
4.3 Discussion 138
4.3.1 Comparison of general genome features 138
4.3.2 *B. pilosicoli* genome architecture 139
4.3.3 Global feature comparisons between the strains of *B. pilosicoli* 140
4.3.4 Global feature comparisons between the *B. pilosicoli* genomes and other *Brachyspira* genomes 140
4.3.4.1 Features unique to the *B. pilosicoli* species 141
4.3.4.2 Unique features shared by two strains of *B. pilosicoli* 142
4.3.4.3 *B. pilosicoli* strain-unique features 142
4.3.5 Comparison of potential virulence features 143
4.3.5.1 Lipooligosaccharides 143
4.3.5.2 Motility and chemotaxis 144
4.3.5.3 Adhesion and membrane proteins 144
4.3.5.4 Host tissue degradation 145
4.3.5.5 Oxidative stress 146
4.3.5.6 Ankrin-like protein 146
4.3.5.7 Phage and other mobile genetic elements 146
4.3.6 Central metabolism and correlation with phenotype 148
4.3.6.1 Carbohydrate metabolism 148
4.3.6.2 Amino acid metabolism 149
4.3.6.3 Nucleotide metabolism 150
4.3.6.4 Lipid metabolism 150
4.3.6.5 Differences in carbon source utilisation by *Brachyspira* 150
4.3.7 Concluding remarks 152
Chapter 5. The characterisation and selection of *Lactobacillus* species as prospective probiotics for the control of avian intestinal spirochaetosis 153
5.1 Introduction 153
5.2 Results 155
5.2.1 Speciation and characterisation of poultry isolates of *Lactobacillus* 155
5.2.1.1 Phenotypic characterisation 155
5.2.1.2 Molecular characterisation 155
5.2.2 Selection of *Lactobacillus* strains as prospective probiotics 159
5.2.2.1 Screening for an inhibitory effect of *Lactobacillus* cell-free supernatant on the growth of *B. pilosicoli* 159

5.2.2.2 Screening for an inhibitory effect of *Lactobacillus* on the motility of *B. pilosicoli* 159
5.2.3 Further characterisation of *L. reuteri* LM1 and *L. salivarius* LM2 162
5.2.3.1 Antimicrobial susceptibility 162
5.2.3.2 Tolerance to avian gastric acid 163
5.2.3.3 Tolerance to avian and bovine bile salts and avian bile extract 164
5.3 Discussion 167
Chapter 6. Investigating the ability of lactobacilli to antagonise *Brachyspira pilosicoli in vitro*: a potential intervention against avian intestinal spirochaetosis 173
6.1 Introduction 173
6.2 Results 175
6.2.1 pH-dependent inhibition of *B. pilosicoli* growth by *Lactobacillus* cell-free supernatant 175
6.2.2 Inhibitory effect of *Lactobacillus* whole cells on *B. pilosicoli* motility 175
6.2.3 Adhesion and invasion assays using HT29-16E cell culture models 177
6.2.3.1 Comparison of the monolayer and three-dimensional models 177
6.2.3.2 Inhibitory effect of lactobacilli on the adherence and invasion of *B. pilosicoli* to HT29-16E cells 178
6.2.3.3 Electron microscopical analysis of HT29-16E monolayer and three-dimensional cells following adhesion and invasion assays 181
6.2.4 Association assays using an avian caecal *in vitro* organ culture models 184
6.2.4.1 Establishment of the avian caecal *in vitro* organ culture model 184
6.2.4.2 Inhibitory effect of lactobacilli on the association of *B. pilosicoli* to avian caecal *in vitro* organ culture tissues 184
6.2.4.3 Electron microscopical analysis of avian caecal *in vitro* organ culture tissues following association assays 185
6.3 Discussion 188
Chapter 7. Investigating the ability of *Lactobacillus reuteri* LM1 to reduce *Brachyspira pilosicoli*-induced pathology in experimentally challenged chickens 193
7.1 Introduction 193
7.2 Results 195
7.2.1 Colonisation of 18 week-old chickens by *B. pilosicoli* and *L. reuteri* 195
7.2.2 Bird weights 196

7.2.3 Faecal moisture content 196
7.2.4 Egg production 198
7.2.5 Bacteriological findings at *post-mortem* examination 200
7.2.6 Histopathological findings at *post-mortem* examination 202
7.3 Discussion 204
Chapter 8. General discussion **209**
References **222**
Appendices **268**
Appendix I 269
Appendix II 273

List of figures

Figure 1.1 Histopathological observations in the caeca of a laying hen naturally infected with *B. pilosicoli*. 35

Figure 1.2 Transmission electron micrograph of *B. aalborgi* end-on attached to the colonic mucosa forming a "false brush border" in a human. 36

Figure 1.3 Phase-contrast photomicrograph of wet mount preparation of *B. alvinipulli* $C1^T$. 47

Figure 2.1 The anatomy of the gastrointestinal tract and the female reproductive tract of poultry. 97

Figure 3.1 Examination of *B. pilosicoli* B2904 from broth culture by scanning electron microscopy. 107

Figure 3.2 Experimental monitoring of birds throughout the experimental challenge study showing the percentage of swabs positive for *B. pilosicoli* by culture and PCR, the average bird weight and the faecal moisture content. 110

Figure 3.3 Egg production and average egg weights each day of the experimental challenge study for the point of the first egg lay. 111

Figure 3.4 Colonisation of *B. pilosicoli* in different tissues sampled at *post-mortem*, at five days post final challenge and 18 days post final challenge. 113

Figure 3.5 Histopathological findings in haematoxylin and eosin-stained caecal tonsil, liver and spleen tissues of birds experimentally challenged with *B. pilosicoli* B2904 and CPSp1. 114

Figure 4.1 A dendrogram illustrating the relationships among nine strains of *Brachyspira*, representing six of the seven known species. 122

Figure 4.2 Circos circular representation of the complete 2.77 Mb *B. pilosicoli* B2904 genome with annotated genes. 124

Figure 4.3 Dot matrix plots comparing the genomes of *B. pilosicoli* B2904, 95/1000 and WesB. 126

Figure 4.4 Pairwise genome alignments of *B. pilosicoli* strains 95/1000, B2904 and WesB. 127

Figure 4.5 Venn diagram of genes unique to and shared between *B. pilosicoli* 95/1000, B2904 and WesB. 130

Figure 4.6 Comparison of the organisation of the bacteriophages in the three *B. pilosicoli* genomes and *B. murdochii* 56-150^{T} genome. 148

Figure 5.1 Growth of *B. pilosicoli* B2904 in broth culture supplemented with 10% cell-free supernatant (pH 3.8) of *Lactobacillus* strains that gave significant inhibition of the growth of the spirochaete. 160

Figure 5.2 Tolerance of *L. reuteri* LM1 and *L. salivarius* LM2 to bile salts and bile collected from the gall bladder of specific pathogen-free chickens. 165

Figure 6.1 Growth of *B. pilosicoli* B2904 in broth culture supplemented with 10% cell-free supernatant of *L. reuteri* LM1 and *L. salivarius* LM2 adjusted to pH 3.8, 4.5 and 7.2 and heat-inactivated lactobacilli. 176

Figure 6.2 Examination of human, mucus-secreting colonic cell, HT29-16E, monolayer and three-dimensional cell uninfected control samples by scanning and transmission electron microscopy. 177

Figure 6.3 Effect of *Lactobacillus* treatment on *B. pilosicoli* B2904 adherence to and invasion of HT29-16E monolayers. 179

Figure 6.4 Effect of *Lactobacillus* treatment on *B. pilosicoli* B2904 adherence to and invasion of HT29-16E three-dimensional cells. 180

Figure 6.5 Examination of HT29-16E monolayers infected with *B. pilosicoli* B2904 and following pre-treatment with *L. reuteri* LM1 and *L. salivarius* LM2 by scanning and transmission electron microscopy. 182

Figure 6.6 Examination of HT29-16E three-dimensional cells infected with *B. pilosicoli* B2904 and following co-incubation with *L. reuteri* LM1

and *L. salivarius* LM2 by scanning and transmission electron microscopy. 183

Figure 6.7 Examination of uninfected control avian caecal *in vitro* organ culture tissue samples by scanning electron microscopy. 184

Figure 6.8 Effect of *Lactobacillus* treatment on association of *B. pilosicoli* B2904 with avian caecal *in vitro* organ culture tissues. 186

Figure 6.9 Examination of avian caecal *in vitro* organ culture tissues infected with *B. pilosicoli* B2904 and co-incubation with viable *L. reuteri* LM1 and *L. salivarius* LM2 by scanning electron microscopy. 187

Figure 7.1 Experimental monitoring of birds throughout the intervention study demonstrating the percentage of cloacal swabs positive *for B. pilosicoli* by culture and PCR, the mean bird weight and the mean faecal moisture content. 197

Figure 7.2 Monitoring of egg production; the number of eggs laid and average egg weights were recorded alongside average scores for the faecal staining of eggshells on a daily basis throughout the intervention study. 199

Figure 7.3 Colonisation of *B. pilosicoli* in different tissues sampled at *post-mortem*, at five days and 21 days post final challenge. 201

Figure 7.4 Haematoxylin and eosin-stained tissues demonstrating histopathological changes in birds experimentally challenged with *B. pilosicoli* B2904 from the untreated and *L. reuteri* LM1-treated groups. 203

List of tables

Table 1.1 Experimental challenge studies in chickens with strains of validated *Brachyspira* species. 42

Table 1.2 Proposed taxonomic outline of phylum *Spirochaetes*. 43

Table 1.3 Validated and proposed *Brachyspira* species and their known host range. 45

Table 1.4 Phenotypic characteristics and major hosts of the seven validated *Brachyspira* species. 48

Table 1.5 Grouping of *Brachyspira* species based on biochemical properties. 50

Table 1.6 General genome feature comparison of the available *Brachyspira* genome sequences. 52

Table 1.7 Commercially available veterinary probiotics deemed safe by the Scientific Committee for Animal Nutrition in the European Union. 59

Table 2.1 *Brachyspira* strains used in the studies presented in this thesis. 70

Table 2.2 *Lactobacillus* strains used in the studies presented in this thesis. 71

Table 2.3 Primers for *Brachyspira* genus- and species-specific PCRs. 84

Table 2.4 Primers for the *Lactobacillus* multiplex PCR. 85

Table 2.5 Experimental strategies for HT29-16E monolayer and three-dimensional cell and avian caecal *in vitro* organ culture studies. 93

Table 2.6 Summary of *p* value categorisation. 99

Table 3.1 Biochemical properties of *Brachyspira* strains used in further studies that aided in the phenotypic speciation of these strains. 103

Table 3.2 Molecular speciation of *Brachyspira* strains by PCR. 104

Table 3.3 Minimum inhibitory concentrations of the antimicrobials tiamulin, tylosin and gentamicin against the *Brachyspira* strains used in the studies presented in this thesis. 106

Table 4.1 General genome feature comparison for strains of *B. pilosicoli* of different host origin. 123

Table 4.2 Distribution of Cluster of Orthologous Group categories in *B. pilosicoli* 95/1000, B2904 and WesB. 128

Table 4.3 Protein blastmatrix analysis of nine *Brachyspira* genomes. 131

Table 4.4 Conserved and shared protein clusters between the six genome-sequenced strains of *Brachyspira*. 132

Table 4.5 The number of genes with potential roles in pathogenesis and virulence in the three *B. pilosicoli* genomes. 133

Table 4.6 Differences in the utilisation of unique carbon sources by six species of *Brachyspira* and two proposed species tested analysed using the Biolog Phenotype MicroArray™ system. 134

Table 4.7 Correlation between differences in carbon source utilisation and genotype of *B. pilosicoli* 95/1000, B2904 and WesB. 137

Table 5.1 Phenotypic and molecular speciation and characterisation *Lactobacillus* strains used in the studies presented in this thesis. 157

Table 5.2 The effect of viable or heat-inactivated *Lactobacillus* strains on the motility of *B. pilosicoli* B2904 following 4 and 24 hour contact times. 161

Table 5.3 Minimum inhibitory concentration of nine antimicrobials against *L. reuteri* LM1 and *L. salivarius* LM2. 163

Table 5.4 Survival of *L. reuteri* LM1 and *L. salivarius* LM2 following 3 hours incubation in poultry gastric juice adjusted to pH 2.0, 2.5 and 3.0. 164

Table 5.5 Tolerance measured in lag time of *L. reuteri* LM1 and *L. salivarius* LM2 to poultry bile salts, sodium taurochenodeoxycholate and taurocholic acid and oxgall, containing bovine bile salts at 0.3% and their tolerance to bile collected from specific pathogen-free chickens at 0.3%, 0.6% and 0.9%. 166

List of abbreviations

˚C	Degree Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
AIS	Avian intestinal spirochaetosis
ABC	ATP-binding cassette
ACT	Artemis comparison tool
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BEB	*Brachyspira* enrichment broth
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CDAD	*C. difficile*-associated disease
CDS	Coding DNA sequence
CE	Competitive exclusion
CFS	Cell-free supernatant
CFU	Colony forming unit
COG	Cluster of orthologous groups
CRISPR	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyltrimethylammonium bromide
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dUTP	Deoxyuridine triphosphate
DTT	Dithiothreitol
EC	European commission
EDTA	Ethylene-diamine tetra-acetic acid
EFSA	European food safety authority
ELISA	Enzyme-linked immunosorbent assays
EM	Electron microscopy
EPS	Exopolysaccharide
EU	European union
FABA	Fastidious anaerobe blood agar
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
g	Gram
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
GRAS	Generally regarded as safe
GTA	Gene transfer agent
GTR	General time reversible
HBSS	Hank's balanced salt solution
HE	Haematoxylin and eosin
HGT	Horizontal gene transfer
HIB	Heart infusion broth
HIS	Human intestinal spirochaetosis
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
iCORN	Iterative correction of reference nucleotides
(I)FAT	(Indirect) fluorescent antibody test
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IMAGE	Iterative mapping and assembly for gap elimination
ISE	Insertion sequence element
IVOC	*In vitro* organ culture
JCICSB	Judicial commission of the international committee on systematics of bacteria
KAAS	KEGG automatic annotation server
Kb	Kilobase pair
KEGG	Kyoto encyclopaedia of genes and genomes
KO	KEGG orthology
L	Litre
LAB	Lactic acid bacteria
LB(-G)	Lysogeny broth (without glucose)
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide

LSM	LAB susceptibility medium
M	Molar
MALDI	Matrix-assisted laser desorption/ionisation
MAT	Micro-agglutination test
Mb	Megabase pair
MCL	Markov clustering algorithm
mg	Milligram
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
ml	Millilitre
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mm	Millimetre
mM	Millimolar
MRS	de Man-Rogosa-Sharpe
NADH	Nicotinamide adenine dinucleotide
NCBI	National centre for biotechnology information
ng	Nanogram
nm	Nanometre
nM	Nanomolar
NMR	Nuclear magnetic resonance
nr	Non-redundant
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PID	Percentage identity
PIS	Porcine intestinal spirochaetosis
PM	Phenotype Microarray™
pmol	picomole
PTS	Phosphotransferase system
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
rRNA	Ribosomal RNA
RWV	Rotating wall vessel
SAT	Slide agglutination test
SCAN	Scientific committee for animal nutrition
SCFA	Short-chain fatty acid
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SNP	Single nucleotide polymorphism
SOAP	Short oligonucleotide alignment program
SPF	Specific pathogen-free
STCDC	Sodium taurochenodeoxycholate
TA	Taurocholic acid
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
TEM	Transmission electron microscopy
TMB	Tetramethylbenzidine
tmRNA	Transfer-messenger RNA
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TTSS	Type three secretion system
UV	Ultraviolet
V	Volts
v/v	volume/volume
VSH	Virus of *S. hyodysenteriae*
w/v	weight/volume
ZnB	Zinc bacitracin
3D	Three-dimensional

List of publications

Mappley, L. J., M. A. Tchórzewska, W. A. Cooley, M. J. Woodward, R. M. La Ragione. (2011). Lactobacilli antagonise growth, motility and adherence of *Brachyspira pilosicoli*: a potential intervention against avian intestinal spirochaetosis. *Appl Environ Microbiol*. 77: 5402–5411.

Mappley, L. J., M. L. Black, M. AbuOun, A. C. Darby, M. J. Woodward, J. Parkhill, A. K. Turner, M. I. Bellgard, T. La, N. D. Phillips, R. M. La Ragione, D. J. Hampson. (2012). Comparative genomics of *Brachyspira pilosicoli* strains: genome rearrangements, reductions and correlation of genetic compliment with phenotypic diversity. *BMC Genomics*. 13: 454.

Mappley, L. J., M. A. Tchórzewska, A. Nunez, M. J. Woodward, R. M. La Ragione. (2012). Evidence for systemic spread of the potentially zoonotic intestinal spirochaete *Brachyspira pilosicoli* in experimentally challenged laying chickens. *J Med Micro*.

Mappley, L. J., M. A. Tchórzewska, A. Nunez, M. J. Woodward, P. M. Bramley, R. M. La Ragione. (2012). Oral treatment of chickens with *Lactobacillus reuteri* LM1 reduces *Brachyspira pilosicoli*-induced pathology. *J Med Micro*.

Chapter 1.

Introduction

1.1 Avian intestinal spirochaetosis

1.1.1 Definition of avian intestinal spirochaetosis

Avian intestinal spirochaetosis (AIS) refers to the disease resulting from the colonisation of the caeca and/or colo-rectum of poultry with spirochaetes. The disease presents in a number of ways but generally occurs at the onset of lay and in adult hens and is associated with diarrhoea, a reduction in growth rate, reductions in both egg production and egg weights, faecal staining of eggs and there may be increased mortality rates within infected flocks The two most commonly reported species in AIS are *B. pilosicoli* and *B. intermedia* (McLaren *et al*., 1997; Stephens and Hampson, 1999, 2001) although *B. alvinipulli* is also considered a cause of disease in chickens (Stanton *et al*., 1998) and geese (Nemes *et al*., 2006). The term has also been associated with *B. hyodysenteriae* infection causing severe typhlitis in rheas.

The term "intestinal spirochaetosis" is not limited to poultry and has been used to describe the colonisation of humans and swine with pathogenic *Brachyspira* species. Human intestinal spirochaetosis (HIS) describes the colonisation of humans with *B. pilosicoli* or *B. aalborgi* (Mikosza and Hampson, 2001). Porcine intestinal spirochaetosis (PIS) describes the colonisation of swine with *B. pilosicoli* (Hampson and Duhamel, 2006). *B. hyodysenteriae* is pathogenic in swine, but colonisation by this species and the resulting disease are described by the term "swine dysentery" (Hampson *et al*., 2006a).

1.1.2 History of avian intestinal spirochaetosis

Initial accounts of the isolation of spirochaetes from avian species were of *Borrelia anserina* infecting chickens, ducks and geese, causing septicaemia and increased mortality rates (Marchoux and Salimbeni, 1903; Sakharoff, 1891). Spirochaetal colonisation of the avian gastrointestinal (GI) tract was first reported in the early twentieth century in the United Kingdom (Fantham, 1910). Helical bacteria which were 16.5 – 32.5 μm in length with 4 – 7 waves with pointed-to-tapered cell ends were isolated from the caeca and rectum of young and adult grouse, and were named "*Spirochaeta lovati*". In 1930, spirochaete-like bacteria were visualised in the caecal droppings of 25% of randomly selected hens (Harris, 1930). The caecal droppings from many of the birds were described as yellowish-brown, semi-solid to pasty and exhibited a strong odour. Three morphologically distinct spirochaetal organisms were described; however no intestinal spirochaetes were identified in chicks or immature chickens. Moreover, some chickens received an oral inoculation of faeces containing spirochaete-like bacteria, which did not induce clinical disease or intestinal lesions.

In 1955, spirochaete-like bacteria associated with large caseous nodules in the caecal walls of turkeys, pheasants and chickens were isolated and propagated in chicken embryos (Mathey and Zander, 1955). When orally inoculated into turkeys, these organisms produced caecal nodules and the spirochaete-like bacteria were re-isolated. Interestingly, intravenous inoculation of day-old chicks failed to produce clinical disease or intestinal pathology. Although none of the organisms described in these early reports are available for examination, the descriptions of their size, morphology and mode of motility are consistent with spirochaetes.

Not until the 1980s, were associations made between intestinal spirochaetes and enteric disease in poultry. Enteric disease syndromes associated with intestinal spirochaetes were reported in commercial laying hens and pullets in the Netherlands (Davelaar *et al*., 1986), United Kingdom (Griffiths *et al*., 1987) and United States of America (Swayne *et al*., 1992). Chickens exhibiting clinical diarrhoea without increased mortality rates were reported, but the species of spirochaete associated with the disease was unknown. However, subsequent studies in Australia (McLaren *et al*., 1996; Phillips *et al*., 2005; Stephens *et al*., 2005), Europe (Bano *et al*., 2008; Burch *et al*., 2006) and the United States of America (Trampel *et al*., 1994), which confirmed and extended this work, have identified and named the *Brachyspira* species causing AIS. Since 1990, AIS has been correlated with severe intestinal necrotic lesions and increased mortality in captive-bred greater or common rheas (Buckles *et al*., 1994; Sagartz *et al*., 1992). More

recently, AIS has been reported in geese (Nemes *et al*., 2006), partridges (Jansson *et al*., 2001), pheasants (Webb *et al*., 1997) and turkeys (Shivaprasad and Duhamel, 2005).

1.1.3 Significance of avian intestinal spirochaetosis

1.1.3.1 Economic

The cost of the disease to the United Kingdom commercial laying industry was estimated at £14 million (Burch *et al*., 2006) and more recently, £18 million, based on a national laying flock of 30 million hens, with 30 eggs lost per hen and an egg price of 5 pence (Burch, D. J. S., 2009 personal communication). Losses similar to those in the United Kingdom are likely to occur elsewhere, since epidemiological surveys have reported a prevalence of AIS amongst laying hen flocks in Australia (McLaren *et al*., 1996) and Italy (Bano *et al*., 2008), as common as it is across the United Kingdom.

1.1.3.2 Public health

Of the pathogenic avian intestinal spirochaetes, *B. pilosicoli* is considered a pathogen in humans, causing HIS. Some avian *B. pilosicoli* strains are closely related to strains of human origin (and from other animals) and it is likely that there is no barrier to cross-species transmission of *B. pilosicoli* (Hampson *et al*., 2006b). *B. pilosicoli* strains isolated from humans have successfully colonised day-old chicks (Dwars *et al*., 1992a; Trott *et al*., 1995) and adult laying hens (Jamshidi and Hampson, 2003), however the potential for transmission from birds to humans remains to be explored. *B. pilosicoli* colonisation in humans is common in developing countries, but is mainly confined to immunocompromised, homosexual males in developed countries (Trivett-Moore *et al*., 1998; Trott *et al*., 1997b). The occurrence of *B. pilosicoli* colonisation in humans has been linked with crowded and unhygienic living conditions, contaminated drinking water (Margawani *et al*., 2004) and has a high prevalence in cholera patients (Nelson *et al*., 2009). *B. pilosicoli* has been recovered from dam water inhabited by affected ducks, highlighting a potential source for zoonosis (Oxberry *et al*., 1998).

1.1.4 Aetiology of avian intestinal spirochaetosis

The genus *Brachyspira* is described in greater detail in section 1.2, but of seven documented and several proposed species, three are considered pathogenic to poultry; *B. intermedia*, *B. pilosicoli* and to a lesser extent *B. alvinipulli*. These three species are capable of inducing clinical disease in experimentally challenged adult chickens

(Hampson and McLaren, 1999; Stephens and Hampson, 2002a). Despite having been isolated from avian species including laying hens (Feberwee *et al*., 2008), *B. hyodysenteriae* is considered non-pathogenic to poultry, although it is the aetiological agent of swine dysentery and causes necrotising typhilitis in rheas and ducks (Glavits *et al*., 2011; Sagartz *et al*., 1992). "*B. pulli"* is widespread and potentially pathogenic in poultry (Jordan and Hampson, 2007). Other *Brachyspira* species isolated from avian hosts are generally considered commensals. *B. innocens* has been associated with reduced egg production in free-range flocks (Burch *et al*., 2009), however, this study employed biochemical testing as the sole means of identification. *B. aalborgi* and "*B. canis*" are the only species that have not been recovered from avian hosts to date. It was postulated that avian species may have been the original hosts of an ancestral *Brachyspira*-like spirochaete when it first colonised the GI tract, explaining the wide diversity of species found in avian hosts (Hampson and Swayne, 2008).

1.1.5 Epidemiology of avian intestinal spirochaetosis

1.1.5.1 Host range

Intestinal spirochaetes can colonise the caeca and colo-rectum of a variety avian species. Of domestic poultry, laying hens (Davelaar *et al*., 1986; Dwars *et al*., 1989; Griffiths *et al*., 1987; Swayne *et al*., 1992), broiler hens (Dwars *et al*., 1990), broiler breeder hens (Stephens and Hampson, 2002a), turkeys (Mathey and Zander, 1955), common rheas (Sagartz *et al*., 1992), corvid birds (Jansson *et al*., 2008), flamingos (Trott *et al*., 1996c), grouse (Fantham, 1910), laying geese (Nemes *et al*., 2006), mallards (Rasback *et al*., 2007a), ostriches (Stoutenburg and Swayne, 1992), pheasants (Webb *et al*., 1997), swans (Trott *et al*., 1996c), water birds (Oxberry *et al*., 1998) and even a snowy sheathbill from Antarctica (Jansson *et al*., 2009b).

1.1.5.2 Transmission

Avian intestinal spirochaetes colonise poultry from approximately 15 weeks of age and therefore hatchery and rearing flocks are an uncommon source of infection (Jordan and Hampson, 2007). *Brachyspira* can survive for a limited time in poultry faeces and are susceptible to a wide range of common disinfectants, thus making the local environment an unlikely source of infection in newly-stocked sheds provided it has been cleaned and rested (Phillips *et al*., 2003). A probable means of infection is via feral birds, rodents and domestic animals such as dogs, which can harbour intestinal

spirochaetes. Wild waterbirds can shed *Brachyspira* in their faeces and these may survive in ponds or dams providing drinking water (Jansson *et al*., 2001; Jansson *et al*., 2004). *Brachyspira* can remain viable for up to 66 days in lake water at 6˚C (Oxberry *et al*., 1998). Contact between swine and poultry may provide a route of infection, since porcine intestinal spirochaetes can infect hens (Trott *et al*., 1995). The movement of staff and equipment between sheds is a likely route for transmission. Transmission can occur quickly via faeces and aerosols and hence, the prevalence within a flock increases with age, with up to a 100% colonisation rate in older flocks (Bano *et al*., 2008).

1.1.5.3 Incidence and prevalence

AIS has been reported across Europe, North America and Australasia and is believed to be endemic worldwide. Some studies have suggested that outdoor free-range flocks are more commonly infected than caged or housed flocks (Burch *et al*., 2009; Wagenaar *et al*., 2003). Few epidemiological surveys of AIS have been performed and of those that have, the methodology for the detection of the spirochaetes has varied greatly, hindering correlation between studies. The incidence of the disease in North America has not been investigated whereas surveys in Europe and Australasia have shown AIS to be particularly common in laying and broiler breeder flocks.

In 1989 in the Netherlands, use of a fluorescent antibody test (FAT) demonstrated spirochaetes in the caeca of birds from 37 of 134 (27.6%) flocks with enteritis and only two of 45 (4.4%) flocks without intestinal disorders (Dwars *et al*., 1989). In 1996 in Western Australia, selective culture was employed to reveal intestinal spirochaetes in the faeces of 16 of 30 (53.3%) randomly selected laying flocks and 13 of 37 (35.1%) broiler breeder flocks (McLaren *et al*., 1996). Spirochaetes were isolated from 64.0% of flocks exhibiting diarrhoea or poor production and 28.0% of flocks with no disease symptoms. The greater sensitivity of selective culture compared to FAT may account for the greater reported prevalence of infection observed in Western Australia.

More recent surveys have used selective culture alongside polymerase chain reaction (PCR) for the identification of the spirochaete species present. Such studies found a greater prevalence of the infection in Eastern Australia, where spirochaetes were recovered from 12 of 28 (42.9%) randomly selected broiler breeder flocks, 15 of 22 (68.2%) laying flocks and none of 19 broiler flocks included in the study (Stephens and Hampson, 1999). Colonisation was significantly correlated with wet litter, whereby infected flocks exhibited 14.0% greater faecal moisture content than uninfected flocks. In 2008 in Northern Italy, selective culture was used to demonstrate the colonisation of

21 of 29 (72.4%) laying flocks with intestinal spirochaetes (Bano *et al*., 2008). Colonisation was associated with reduced egg production but no difference in faecal moisture content. In these studies in Australia and Italy, prevalence increased with age and was significantly greater in flocks over 40 weeks of age than in younger flocks.

In 2007, a survey in the United Kingdom suggested 70.0% of flocks with poor performance were infected with intestinal spirochaetes (Burch, 2007). In 2009, selective culture was used alongside biochemical testing to reveal colonisation by intestinal spirochaetes in 99 of 108 (91.7%) free-range and organic laying flocks, 57 of 74 (77.0%) caged laying flocks, 2 of 8 (25.0%) breeder and none of the 24 in-rear pullet flocks tested (Burch *et al*., 2009). Colonisation of free-range flocks was significantly correlated with poor performance and incidence increased with age.

In the studies described above, approximately 70.0% and 50.0% of laying and breeding flocks, respectively, were colonised by intestinal spirochaetes. Approximately two-thirds of flocks were colonised by pathogenic species, of which *B. intermedia* accounts for about two-thirds and *B. pilosicoli* for the remainder (Bano *et al*., 2008; Stephens *et al*., 2005). In some cases, flocks were colonised by both of these pathogenic species (Phillips *et al*., 2005). *B. alvinipulli* has been isolated from laying hens in the United States of America (Swayne *et al*., 1992) and the Netherlands (Feberwee *et al*., 2008), geese in Hungary (Nemes *et al*., 2006) and mallards in Sweden (Jansson *et al*., 2011). High rates of faecal carriage, particularly of *B. pilosicoli* have been noted in waterbirds, which are a probable reservoir of infection for chickens (Oxberry *et al*., 1998; Stoutenburg *et al*., 1995). *B. hyodysenteriae* has been isolated from laying chickens in the United Kingdom (Thomson *et al*., 2007), the Netherlands (Feberwee *et al*., 2008) and is widespread in rheas in the United States of America (Buckles *et al*., 1997) and some feral and farmed mallards in Sweden (Jansson *et al*., 2004).

1.1.5.4 Incubation period and persistence

The incubation period of AIS is variable since dose, species and environment can have a profound influence (Hampson and Swayne, 2008). Clinical symptoms can manifest as early as 5 days following experimental challenge (Swayne *et al*., 1995). However, significant levels of colonisation and disease symptoms often appear several weeks after experimental challenge (Hampson *et al*., 2002a; Hampson *et al*., 2002b).

Intestinal spirochaetes can colonise the caeca persistently (Davelaar *et al*., 1986; Dwars *et al*., 1990, 1992b). In experimentally challenged chickens, a *B. intermedia* strain was detected in the faeces from challenge until the end of the study, 23 weeks

later (Dwars *et al*., 1993; McLaren *et al*., 1997) and in another study this strain was present in the faeces of the chickens at 9 months after challenge (Dwars *et al*., 1990). Persistent colonisation, extending over 4 – 7 weeks, has been noted following experimental challenge of hens with *B. pilosicoli* (Jamshidi and Hampson, 2002, 2003).

1.1.5.5 Influencing factors

The different clinical outcomes in infected hens arise from different influences on intestinal colonisation, which may explain the heterogeneity in clinical signs and pathology in flocks with AIS. Housing birds in close proximity, facilitating transmission between birds, enhances the probability of high infection rates. However, a survey in the United Kingdom revealed greater colonisation of *Brachyspira* amongst free-range flocks than caged flocks and that free-range birds exhibit poorer performance (Burch *et al*., 2009). It was postulated that contact of free-range birds with one another and with feral birds and mammals carrying intestinal spirochaetes facilitated initial infection. Stress periods may predispose colonisation with spirochaetes since infection increases at the onset of lay and times of moulting (Jordan and Hampson, 2007).

The pathogenicity of avian intestinal spirochaetes is greatest when delivered by oral gavage to day-old chicks (Swayne, 1994; Swayne *et al*., 1995), however natural intestinal spirochaete colonisation has not been noted in young birds. Older flocks are more commonly infected, with flocks over 40 weeks of age significantly more likely to be infected with intestinal spirochaetes and infection less common in birds under 15 weeks of age (Bano *et al*., 2008; Myers *et al*., 2009; Phillips *et al*., 2005). Above 15 weeks of age, infection increases gradually and the average prevalence in sheds containing birds aged 10 – 39 weeks was 40.5%, 40 – 69 weeks was 44.9% and 70 – 100 weeks was 81.1% (Stephens and Hampson, 1999). Correlation been pathogenicity and incidence with age may reflect increasing exposure or changes in gut microbiota rather than age susceptibility, but this concept is yet to be challenged experimentally.

Intestinal spirochaetes must reach the lower GI tract and establish interactions with the local niche environment for successful colonisation. Survival through the upper GI tract may be facilitated by the spirochaetes residing within boluses of food or faeces. Spirochaetes may interact with other anaerobic bacteria in the caeca and colon of pigs, including *Clostridium* and the species act in synergy to facilitate colonisation, inflammation and lesion formation (Harris *et al*., 1978; Joens *et al*., 1981; Whipp *et al*., 1979). Such species were co-isolated with spirochaetes in hens (Feberwee *et al*., 2008).

The influence of diet on colonisation has been demonstrated in experimentally challenged hens. Wheat-based diets and particularly wheat varieties with high levels of non-starch polysaccharides promote colonisation by *B. intermedia* compared to barley and sorghum diets (Phillips *et al*., 2004a, b). The addition of dietary enzyme designed to hydrolyse non-starch polysaccharides in wheat reduced colonisation by *B. intermedia* in hens (Hampson *et al*., 2002b). Zinc bacitracin (ZnB), a supplemental growth promoter, reduced colonisation by *B. intermedia* but enhanced colonisation by *B. pilosicoli* in experimentally challenged hens (Hampson *et al*., 2002a; Jamshidi and Hampson, 2002; Stephens and Hampson, 2002a). Since ZnB primarily acts on Gram-positive bacteria, the conflicting results indicate that there may be complex interactions between different components of the intestinal microbiota and different species of spirochaetes.

1.1.6 Pathology and clinical symptoms of avian intestinal spirochaetosis

Understanding of the pathology and clinical symptoms of AIS has come from three sources; experimental challenge of day-old chicks, experimental challenge of adult chickens and observations of natural cases of the disease. Data from day-old chicks should be treated with caution as although they provide insight into the pathogenic potential, the associated disease is not representative of natural infection in adult birds. Experimental challenge of adult hens is more representative of natural disease, but unlike many commercial, caged flocks where AIS is apparent, the birds are usually individually caged, appropriately fed and relatively stress-free and birds often display only mild symptoms with an absence of histopathological changes (Hampson and McLaren, 1999). Moreover, the studies are often restricted to using one or few spirochaete strains, standard dietary and other conditions and there are no co-infections, which is common in commercial flocks. Observations from natural cases of AIS are of direct relevance to industry, however the studies are limited by the potential of other co-infections that may go unrecognised or make it difficult to attribute aspects of pathology or clinical symptoms and early AIS case reports failed to speciate the aetiological agent (Davelaar *et al*., 1986; Griffiths *et al*., 1987). The resulting disease and colonisation of birds with intestinal spirochaetes has been classified into subclinical colonisation, mild to moderate clinical disease or severe clinical disease.

1.1.6.1 Subclinical colonisation

Colonisation with intestinal spirochaetes in the absence of clinical symptoms of disease is most common in wild birds and waterbirds, where they are considered to be

commensals. Subclinical colonisation in chickens has been associated with non-pathogenic species such as *B. murdochii* (McLaren *et al*., 1996). In wild birds, subclinical colonisation may be by pathogenic and/or non-pathogenic species without clinical symptoms of the enteric disease (Jansson *et al*., 2004; Oxberry *et al*., 1998). Inoculation of non-pathogenic spirochaete strains from wild birds into day-old chicks caused mild diarrhoea, foamy caecal contents and reduced growth rates with the spirochaetes attaching to the surface epithelium, inducing inflammatory cell infiltration into the lamina propria (Prapasarakul *et al*., 2011; Swayne *et al*., 1993).

1.1.6.2 Mild to moderate clinical disease

Mild to moderate clinical disease is associated with colonisation by *B. alvinipulli*, *B. intermedia* and/or *B. pilosicoli*, especially in laying and broiler breeder hens. Clinical symptoms are not pathognomic, but indicate enteric disease (Stephens and Hampson, 2001). Colonisation in laying hens was initially associated with prolonged diarrhoea and reduced egg production (Davelaar *et al*., 1986). Naturally infected birds exhibited mild typhlitis with increased numbers of goblet cells and focal lesions in the caecal epithelium containing spirochaetes with leukocytic infiltration. Subsequently, retarded growth rates and delayed onsets of lay were associated with spirochaetes colonising the caeca in adult pullets, inducing distended crypts and epithelial sloughing (Griffiths *et al*., 1987). In broiler breeder hens, AIS causes increased feed consumption, production of eggs too light for hatching and weak broiler chicks with retarded growth and poor feed conversion hatched from eggs of infected hens (Smit *et al*., 1998).

To improve understanding of infection with *B. alvinipulli*, challenge of day-old chicks and adult hens resulted in foamy contents in dilated caeca and wet, yellow faeces in both age groups (Swayne *et al*., 1995). Infected birds displayed severe lymphoplastic typhlitis, caecal villous epithelial cell hyperplasia and submucosal lymphocytic follicles. Spirochaetes formed dense layers over the epithelial surface of the villi and crypts, often penetrating between caecal epithelial cells and in the crypt lumina. Challenge of day-old ducklings with *B. alvinipulli* resulted in a weight reduction but no gross pathology (Thuma *et al*., 2011). In naturally infected flocks, *B. alvinipulli* caused diarrhoea, faeces smeared around the vent (pasty vent), faecal staining on eggshells (Swayne *et al*., 1992) and in hens that presented typhlitis, focal necrosis and necrotic material containing spirochaetes in the lumen were noted (Feberwee *et al*., 2008).

B. intermedia challenge of broiler chicks and laying hens resulted in reduced growth rates, wet droppings with increased lipid content, increased serum protein, lipid,

carotenoid and biliruben content and reduced egg production in adult hens (Dwars *et al*., 1992a, 1993; Dwars *et al*., 1990, 1992b). The spirochaetes penetrated the caecal mucosa and accumulated under the epithelium, causing erosion but no signs of inflammation. Eggs from infected hens had lower weights, paler yolks and low carotenoid content. Despite an absence of spirochaetes, broiler chicks hatched from the eggs of infected hens exhibited pale, wet faeces, reduced weights and developed rickets. Other challenge studies with *B. intermedia* report reduced growth, increased faecal moisture content and decreased egg production (Hampson and McLaren, 1999; Phillips *et al*., 2004a).

In experimental challenge studies with *B. pilosicoli* in chicks, diarrhoea and depressed growth rates were noted (Trott *et al*., 1995). In a similar study, clinical symptoms were absent, but vacuolation in the cytoplasm of enterocytes and crypt elongation were recorded (Dwars *et al*., 1992a). Challenge of adult hens with *B. pilosicoli* elicited increased faecal moisture content, faecal staining of eggshells, reduced egg production and foamy caecal contents but mild pathology was recorded if at all (Jamshidi and Hampson, 2003; Stephens and Hampson, 2002a). Natural infection of flocks with *B. pilosicoli* is associated with reduced egg production, diarrhoea, foamy caecal contents, pasty vent, typhlitis, non-productive ovaries and lethargy with dense layers of spirochaetes covering the apical surface of caecal enterocytes (Feberwee *et al*., 2008; Trampel *et al*., 1994). The dense "false brush border", characteristic of *B. pilosicoli* colonisation, can damage microvilli and terminal web microfilaments (Muniappa *et al*., 1996; Prapasarakul *et al*., 2011). Intestinal spirochaetes may act as copathogens with indigenous bacilli to cause caecal lesions (Swayne and McLaren, 1997). Reactive and mild inflammatory changes can occur in the caeca alongside crypt hyperplasia, epithelial erosion and increased numbers of goblet cells (Figure 1.1A) with the spirochaetes forming dense fringes (Figure 1.1B). *B. pilosicoli* colonisation was associated with increased mortality rates in turkeys (Shivaprasad and Duhamel, 2005) and typhlocolitis, renal degeneration and hepatic/splenic amyloidosis in ducks (Glavits *et al*., 2011). Similar histopathology was noted in the turkeys and ducks as in the hens.

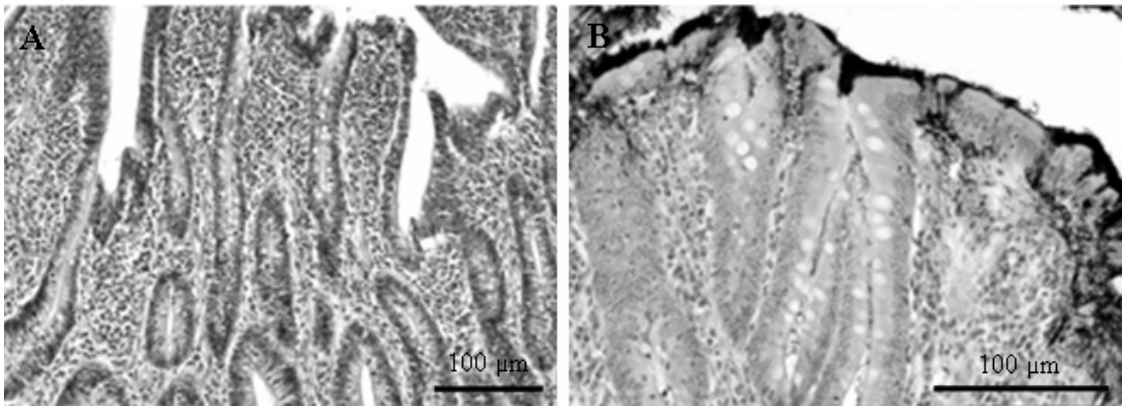

Figure 1.1 Histopathological observations in the caeca of a laying hen naturally infected with *B. pilosicoli* (Feberwee *et al*., 2008). The caecal wall displays crypt hyperplasia and infiltration of heterophils in the lamina propria (A). Spirochaetes form a dense layer on the surface of and penetrate between enterocytes (B).

1.1.6.3 Severe disease

Severe disease is associated with typhlitis observed in rheas naturally colonised by *B. hyodysenteriae*, where mortality rates can reach as high as 80% (Buckles *et al*., 1994; Sagartz *et al*., 1992). Typically, infected rheas are over 6 months of age and adult cases coincide with stress. Fatalities often occur without clinical symptoms (Sagartz *et al*., 1992), however 1 – 2 days prior to death the rheas may present depression, reduced weights and wet faeces with caseous cores (Swayne, 1994). The caeca can be dilated, with thickened walls and ulcerations, severe mucosal necrosis, crypt elongation and goblet cell hyperplasia and the caecal lumina may be colonised by spirochaetes with fibrinonecrotic debris. Similar histopathology was noted in day-old chicks, turkeys and rheas challenged with *B. hyodysenteriae* isolated from rheas with severe AIS (Jensen *et al*., 1996). Challenge of day-old chicks with porcine strains of *B. hyodysenteriae* produced reduced weight, caecal atrophy with epithelial and goblet cell hyperplasia and crypt elongation (Adachi *et al*., 1985; Sueyoshi and Adachi, 1990; Trott and Hampson, 1998). Severe disease was reported in geese colonised by *B. alvinipulli* presenting mortality rates ranging 18 – 28% (Nemes *et al*., 2006). The infection was linked with haemorrhagic and necrotic inflammation in the colo-rectal region and fibrinonecrotic typhlitis. Additionally, swollen kidneys displaying degeneration of tubular epithelial cells were reported and lymphohistiocytic inflammation was noted in the liver.

1.1.6.4 Anatomical location

Avian intestinal spirochaetes are anaerobic chemoheterotrophs making the lower GI tract (colo-rectum and/or caeca) of animals and humans ideal for their colonisation. The spirochaetes situate in close physical proximity to the mucosal epithelium. Intestinal mucus secreted by goblet cells is likely to be important as a physical matrix and a chemical substrate. Mucin has been implicated as a chemo-attractant to *B. hyodysenteriae* and *B. pilosicoli* (Milner and Sellwood, 1994; Naresh and Hampson, 2010). *B. pilosicoli* and *B. aalborgi* can colonise intestinal mucosal surfaces by attaching to enterocytes via one cell end to form densely packed parallel arrays of spirochaetes, often referred as a "false brush border" (Figure 1.2). *B. pilosicoli* forms such attachments in humans and pigs and chickens (Jensen *et al*., 2000; Muniappa *et al*., 1996; Trott *et al*., 1996a), however they can colonise without attachment (Jamshidi and Hampson, 2003). Occurrences of spirochaetaemia have been reported in humans but no animals or avian species (Bait-Merabet *et al*., 2008; Prim *et al*., 2011); further investigation into the potential for blood infection of *Brachyspira* is required.

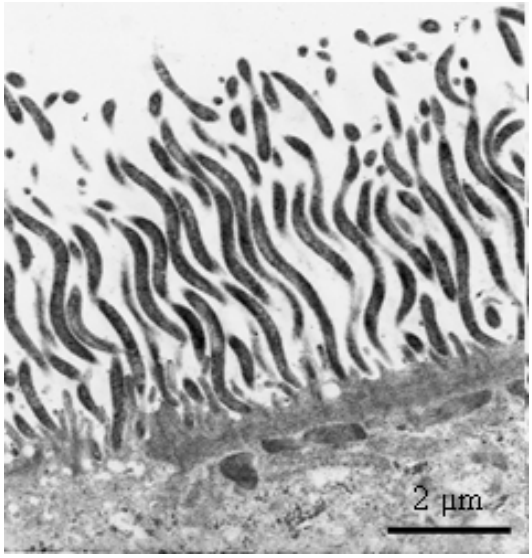

Figure 1.2 Transmission electron micrograph of *B. aalborgi* end-on attached to the colonic mucosa forming a "false brush border" in a human (Kraaz *et al*., 2000).

1.1.6.5 Immunity

The immunological response to infection with intestinal spirochaetes can be prolonged as shown in experimentally challenged birds (Dwars *et al*., 1990), but remains to be explored fully. Humoral antibody responses to intestinal spirochaetes appear to be non-specific since they may or may not be evident in birds from which spirochaetes have or have not been isolated (Mantle *et al*., 1989a; Stoutenburg *et al*., 1995).

1.1.7 Diagnosis of avian intestinal spirochaetosis

Clinical signs of AIS are indicative of the disease, but cannot be used for diagnosis and the presence of micro- or macro-pathological lesions alone is rarely sufficient to provide certain diagnosis of AIS. Thus, diagnosis of AIS is confirmed by microbiological and molecular identification of the aetiological agent.

1.1.7.1 Isolation and visualisation of spirochaetes

Isolation of spirochaetes by selective culture (as discussed in section 1.2.2.2) is important for further characterisation and speciation of the causative spirochaetes. The sensitivity of culture for detection depends on the number of organisms, type and condition of the sample. Fresh faeces or caecal mucosa are preferable samples for culture and may be chilled at 4˚C for a week (Hampson and Swayne, 2008). The presence of spirochaetes in faeces may be demonstrated visually by examining a wet smear under light, phase-contrast or dark-field microscopy and observing their characteristic morphology and movement (discussed in section 1.2.2.1). Their presence may be clarified by electron microscopy (EM), although this can be expensive and time-consuming. The periplasmic flagella can be enumerated, which varies between species.

1.1.7.2 Identification of spirochaetes by serological properties

The presence of spirochaete antigens may be confirmed by direct or indirect (I)FAT (Davelaar *et al*., 1986; Dwars *et al*., 1989) and immunohistochemcial (IHC) methods using polyclonal antibodies (Fisher *et al*., 1997; Webb *et al*., 1997). Neither morphology, IFAT or IHC methods are sufficient to distinguish between all species. Antisera specific for the detection of *B. hyodysenteriae* by IFAT (Lemcke and Burrows, 1981; Lysons and Lemcke, 1983) and monoclonal antibodies to envelope proteins of *B. pilosicoli*, which may be used to develop IFAT (Lee and Hampson, 1995; Tenaya *et al*., 1998), have been reported. Serological tests including enzyme-linked immunosorbent assays (ELISA) (La *et al*., 2009; Song *et al*., 2012), slide and micro-agglutination (S/MAT) tests (Hampson, 1991; Lee and Hampson, 1996) can detect spirochaetes but with low specificity sensitivity (La and Hampson, 2001).

1.1.7.3 Identification of spirochaetes by biochemical properties

The difference in haemolytic patterns on blood agar and biochemical reactivity (discussed in section 1.2.2.4) can be used to speciate and group intestinal spirochaetes. Culture and biochemical testing can be time-consuming due to the fastidious nature of

Brachyspira (Phillips *et al*., 2006), although the sensitivity of such tests for identification is highly regarded (Rasback *et al*., 2006). Culture is useful for testing antimicrobial efficacy against intestinal spirochaetes (Brooke *et al*., 2003a).

1.1.7.4 Identification of spirochaetes by genotypic properties

The development of molecular-based tools for the identification and detection of spirochaetes was encouraged due to the inconsistencies of phenotyping and mixed species infections. PCR assays were developed to detect intestinal spirochaetes isolated by selective culture (Atyeo *et al*., 1998; Leser *et al*., 1997). The most reliable PCRs for *B. pilosicoli* were designed on the 16S ribosomal ribonucleic acid (rRNA) gene and for *B. intermedia* were designed on the nicotinamide adenine dinucleotide (NADH) oxidase (*nox*) gene (Phillips *et al*., 2005, 2006). PCRs based on the *nox* gene and a haemolysin (*tly*) gene have been used for *B. hyodysenteriae* (Fellstrom *et al*., 2001). Two duplex PCRs have been developed, one to detect *B. intermedia* and *B. pilosicoli* (Phillips *et al*., 2006) and the other to detect *B. hyodysenteriae* and *B. pilosicoli* (La *et al*., 2003) from deoxyribonucleic acid (DNA) extracted from faeces. A *Brachyspira* genus-specific PCR may be performed prior to a species-specific duplex PCR, in a two-step nested duplex PCR to increase the detection limit. Two multiplex PCRs have been developed, one to simultaneously detect *B. hyodysenteriae*, *B. pilosicoli* and *Lawsonia intracellularis* (Nathues *et al*., 2007; Reiner *et al*., 2011) and another to detect the species considered pathogenic to poultry, *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* (Abdelrahman *et al*., 2009). Sequencing of the 16S rRNA and *nox* genes aid in identification although this is not a preferred method due to sequence similarity between species (Jansson *et al*., 2011). Poultry faeces contain PCR inhibitors such as uric acid, reducing the detection limit of PCR on faecal DNA, but this may be overcome using washed faecal samples.

Other molecular methodology for identification include restriction fragment length polymorphism (RFLP) involving restriction digestion of specific PCR products to give species-specific banding patterns upon gel electrophoresis (Barcellos *et al*., 2000; Rohde and Habighorst-Blome, 2012). Fluorescent *in situ* hybridisation (FISH), using fluorescent oligonucleotide probes specific for *Brachyspira* can visualise spirochaetes associated with tissues (Boye *et al*., 1998; Jensen *et al*., 2000), which may be captured by laser microdissection and subjected to 16S rRNA gene PCR and sequence analysis to allow simultaneous identification and localisation of spirochaetes (Klitgaard *et al*., 2005). Multilocus enzyme electrophoresis (MLEE) was pivotal in the differentiation of spirochaetes (Lee *et al*., 1993; McLaren *et al*., 1997), but has been replaced by pulsed

field gel electrophoresis (PFGE), which provides better discrimination of species (Atyeo *et al*., 1996). Recently, randomly amplified polymorphic DNA (RAPD) (Backhans *et al*., 2011; Fellstrom *et al*., 2008) and multilocus sequence typing (MLST) (Osorio *et al*., 2012; Rasback *et al*., 2007b) have been developed to type intestinal spirochaetes.

1.1.8 Intervention and treatment of avian intestinal spirochaetosis

1.1.8.1 Biosecurity and disinfectants

Cleaning and disinfection is required to prevent transmission of AIS between flocks in addition to strict biosecurity to prevent the introduction of spirochaetes. Effective rodent, insect and wild bird control with consistently high levels of shed hygiene is essential in preventing the disease and its spread (Hampson and Swayne, 2008). Physical containment of the birds should include security fencing and bird-proof netting around openings in the containment facilities. Entry of personnel should be restricted, preferably with shower-in and shower-out facilities and disinfectant boot-dips. A clean water source and measures applied to minimise contact of birds with potentially infected faeces are important. Feed ingredients, such as wheat that can enhance colonisation, could be avoided. It is best to avoid raising rheas on swine farms and to separate flocks into age groups, implementing strict biosecurity measures to avoid transmission of spirochaetes from asymptomatic adult birds to susceptible chicks.

Disinfectants including quaternary ammonium compounds, iodine, chlorine, and hydrogen peroxide can immediately inactivate *Brachyspira* in organic matter (Phillips *et al*., 2003). Several studies have revealed reduced effectiveness of disinfectants, such as Virkon S against porcine and human enteric organisms, including *B. pilosicoli* over time and have suggested an emerging resistance of spirochaetes to some disinfectants (Corona-Barrera *et al*., 2004; de la Puente Redondo *et al*., 1998; Isenberg *et al*., 1988).

1.1.8.2 Antimicrobial control

Spirochaetes are susceptible to a range of antimicrobials, however a limited number are effective in treating the clinical disease. It was postulated that compounds used to treat swine dysentery would be applicable to treat AIS (Swayne, 1997). The first *in vitro* antimicrobial susceptibility testing of avian intestinal spirochaetes demonstrated high susceptibilities of *B. alvinipulli*, *B. hyodysenteriae* and *B. pilosicoli* strains from chickens and rheas to antimicrobials used to treat swine dysentery, such as carbadox, lincomycin and tiamulin (Trampel *et al*., 1999). However, a resistance to streptomycin

was revealed in addition to variable results for bacitracin, chlortetracycline, eryhtromycin, neomycin, oxytetracycline and penicillin. Subsequent studies demostrated susceptibilites to lincomycin, metronidazole, tetracycline and tiamulin in *B. intermedia* and *B. pilosicoli* strains (Hampson *et al*., 2006c), although elevated minimum inhibitory concentration (MIC) values for ampicillin and tylosin were noted. Decreased susceptibilities to ampicillin, doxycycline, tiamulin, tylosin and valnemulin were later found in a range of *Brachyspira* strains from laying hens and wild mallards (Jansson and Pringle, 2011). Acquired resistance to doxycycline, tilmicosin, tylosin and lincomycin and low-level resistance to tiamulin and valnemulin has been shown in *B. intermedia* strains from laying hens (Verlinden *et al*., 2011). Resistance to macrolides and lincosamides, such as tylosin and erythromycin is conferred by a mutation in the peptidyl transferase region of the 23S rRNA gene (Karlsson *et al*., 1999).

Although no antimicrobials have been registered for the specific treatment of AIS, several studies have investigated the use of antimicrobials to treat AIS. In-feed dimetridazole has been successful in treating laying hens with AIS (Griffiths *et al*., 1987). In-water 5-nitroimidazole was effective in treating broiler hens with AIS when administered before the onset of lay (Smit *et al*., 1998). The condition of broiler breeder hens with AIS was improved using either in-water lincospectin or tiamulin although infection did reappear following both treatments and in-water oxytetracycline was then used to remove or reduce the reoccuring infection (Stephens and Hampson, 1999), highlighting a requirement for regular treatment with courses of antimicrobials to prevent reinfection. In-water tiamulin successfully treated laying hens infected with *B. pilosicoli*, improving production and reducing mortality (Burch *et al*., 2006). Tiamulin and lincomycin have been effective in removing *B. intermedia* and *B. pilosicoli* from challenged birds (Stephens and Hampson, 2002b). Dimetridazole, erythromycin and lincomycin have been successful in treating severe AIS in rheas (Hanley *et al*., 1994).

Antimicrobial treatment in laying hens can be problematic due to the withdrawl times required to avoid the presence of residues in eggs. Moreoever, compounds such as nitroimidazoles are unsuituable for use in food-producing animals due to their genotoxicity (Franklin *et al*., 2006) and the use of tiamulin must be carefully considered as it cannot be used with ionophores due to toxicity (Weisman *et al*., 1983).

1.1.8.3 Pre- and probiotic control

Antimicrobial treatment in laying hens can be problematic (as discussed in section 1.1.7.2) and in addition to emerging resistances to current antimicrobials and bans on

subtherapeutic antimicrobial usage, there is a drive to develop alternative treatment strategies for AIS. Few studies exist on investigating the potential of pre- and probitoic treatment of *Brachyspira* infection. In 2008, a patent application was published for the use of *L. johnsonii* as a probiotic, based on its ability to inhibit *B. pilosicoli* and *B. hyodysenteriae* growth, through the production of hydrogen peroxide and a proteineous antimicrobial (Se *et al*., 2008). *L. rhamnosus* and *L. farciminis* strains have been implicated in inhibiting *Brachyspira* motility via co-aggregation and eliciting a stress response in the *Brachyspira* (Bernardeau *et al*., 2009). Interestingly, the cell-free supernatant (CFS) of *L. salivarius* but not *L. reuteri* had a pH-dependant inhibitory effect on *B. pilosicoli* growth (Klose *et al*., 2010). Moreover, supplementing the diet of pigs experimentally challenged with *B. hyodysenteriae* with the prebiotic inulin protected the pigs against swine dysentery (Hansen *et al*., 2010), potentially through modifying the intestinal microbiota and microbial fermentation (Hansen *et al*., 2011).

1.1.8.4 Vaccination

A number of vaccines have been developed against *Brachyspira* to treat swine dysentery, although the protection provided has been ineffective and inconsistent (Hampson *et al*., 1993) whilst some vaccines have increased and accelerated swine dysentery caused by *B. hyodysenteriae* (Olson *et al*., 1994). There are currently no commercially available *Brachyspira* vaccines for use in poultry (Hampson and Swayne, 2008). A bacterin vaccine was investigated to control *B. intermedia* in laying hens and although it gave a humoral response, antibody levels declined significantly following challenge with *B. intermedia* (Amin *et al*., 2009). Reverse vaccinology and the use of recombinant proteins has shown promise in reducing *B. pilosicoli* colonisation in a mouse model (Movahedi and Hampson, 2009). Experimental vaccines using live attenuated strains (Hyatt *et al*., 1994), recombinant proteins (La *et al*., 2004) or DNA (Davis *et al*., 2005) have been developed but not yet reached commercial production.

1.1.9 Animal models for avian intestinal spirochaetosis

Pathogenicity, colonisation, host range and immune responses have been investigated in pig, guinea pig, mice and chicken models for intestinal spirochaetosis. Many different *Brachyspira* species and strains have been used to experimentally challenge chicks and adult hens (Table 1.1) and only a minority of strains (155-5, B256^T, 155-20, 27042-94B, and 513A^T) were unable to colonise the GI tract of chickens with a minority (CPSi1, SP16, 16242-94, Rosie 2299) also unable to produce symptoms

or lesions. Moreover, few challenge studies have been performed in other bird species including geese (Ivanics *et al*., 2007), common rheas (Swayne, 1994) and mallards (Jansson *et al*., 2009a). Swine dysentery is commonly investigated by experimental challenge of pigs and mice (Hutto and Wannemuehler, 1999; Jacobson *et al*., 2004).

Table 1.1 Experimental challenge studies in chickens with strains of validated *Brachyspira* species (Jansson, 2009).

Species	Strain(s)	Origin	Reference
B. aalborgi	513A^T	Monkey	(Trott and Hampson, 1998)
B. alvinipulli	C1	Chicken	(Swayne *et al*., 1995)
B. hyodysenteriae	R1	Rhea	(Swayne, 1994)
	B78^T, B204R, WA15, SA3	Pig	(Adachi *et al*., 1985; Sueyoshi and Adachi, 1990; Sueyoshi *et al*., 1987; Sueyoshi *et al*., 1986; Trott and Hampson, 1998; Trott *et al*., 1995)
B. innocens	CPSi1	Chicken	(Stephens and Hampson, 2002a)
	B256^T, 155-5	Pig	(Muniappa *et al*., 1997; Trott *et al*., 1995)
	27042-94B	Dog	(Muniappa *et al*., 1996)
B. intermedia	1380, HB60	Chicken	(Dwars *et al*., 1993; Dwars *et al*., 1990, 1992b; Hampson and McLaren, 1999; Hampson *et al*., 2002b; Phillips *et al*., 2004a, b)
	889	Pig	(Trott and Hampson, 1998)
B. murdochii	155-20	Pig	(Trott and Hampson, 1998)
B. pilosicoli	CPSp1	Chicken	(Jamshidi and Hampson, 2002; Stephens and Hampson, 2002a, b)
	1648, 3295, UNL-3, UNL-5, UNL-8, D9201243A, T9300098, T9301604B	Pig	(Muniappa *et al*., 1997; Trott and Hampson, 1998; Trott *et al*., 1995)
	S76	Widgeon	(Swayne *et al*., 1993)
	K9-12, 16242-94	Dog	(Muniappa *et al*., 1996)
	SP16, WesB, Kar, GAP 401, Rosie 2299, HIV3AB2	Human	(Jamshidi and Hampson, 2003; Muniappa *et al*., 1996; Muniappa *et al*., 1998; Trott and Hampson, 1998; Trott *et al*., 1995)
	MMU27669, MMU26986, MMU26717	Monkey	(Muniappa *et al*., 1998)

1.2 *Brachyspira*

1.2.1 Phylogeny and taxonomy of *Brachyspira*

1.2.1.1 Phylum *Spirochaetes*

The existence of helical-shaped, motile microorganisms in human faeces and the oral cavity has been noted shortly after the development of microscopy in the 17th century (Dobell, 1932) and the first named spirochaete (etymology Gr. speira "coil" and chaite "hair") was *Spirochaeta plicatilis* (Ehrenberg, 1835). Until the 1960 – 1970s, spirochaetes were confused with protists, and their bacterial nature was eventually proven based on ultrastructural features (Holt, 1978; Ryter and Pillot, 1965).

The domain *Bacteria* is currently subdivided in 24 phyla, including *Spirochaetes*, which contains all spirochaete species shown in Table 1.2 and this represents a monophyletic lineage and a major branch in eubacterial evolution with deeply branching subclusters within the clade, corresponding to different families and genera (Ludwig *et al*., 2008; Woese, 1987). There are over 200 proposed species and phylotypes of spirochaete, of which over half remain to be cultured *in vitro* (Paster and Dewhirst, 2000). Spirochaetes are considered to be evolutionary ancient organisms (Canale-Parola, 1977) supported by phylogenetic studies (Brown *et al*., 2001; Daubin *et al*., 2002), their presence in ancient ecosystems such as phototrophic bacterial mat communities (Margulis *et al*., 1993) and their symbiotic relationship with living fossils and termites in 15 – 20 million year-old amber (Pernice *et al*., 2007; Wier *et al*., 2002).

Table 1.2 Proposed taxonomic outline (families, genera) of phylum *Spirochaetes* (Ludwig *et al*., 2008).

Phylum *Spirochaetes*				
Family I	**Family II**	**Family III**	**Family IV**	**Family V**
Spirochaetaceae	***Brachyspiraceae***	***Brevinemataceae***	***Leptospiraceae***	***Incertae sedis*[a]**
Spirochaeta	*Brachyspira*	*Brevinema*	*Leptospira*	*Clevelandina*
Borrelia			*Leptonema*	*Diplocalyx*
Cristispira			*Turneriella*	*Hollandina*
Treponema				*Pillotina*

[a] Uncertain placement of genera because strains and DNA sequences are unavailable.

Spirochaetes are metabolically diverse chemoorganoheterotrophs with complex and different demands for nutrients and oxygen. They inhabit a diverse range of

environments, with both mesophilic and extremophilic (thermophile, alkaliphile and halophile) spirochaetes noted (Charon and Goldstein, 2002; Euzeby, 2008; Margulis *et al*., 1993). Many species are free-living in marine environments, hot springs, oil fields, soil and microbial mat communities. Some spirochaetes exist as commensals in eukaryotic hosts including insects, molluscs and vertebrates, with the genera *Brachyspira* and some *Treponema* capable of colonising the GI tract. Spirochaetes can exist in the hindgut of wood-eating termites participating in bio-recycling and nitrogen fixation (Warnecke and Hugenholtz, 2007) and in the digestive and excretory organs of molluscs as commensals (Pernice *et al*., 2007). Spirochaetes cause a range of diseases of vertebrate hosts with the epidemiology, host range, tissue tropism and invasiveness varying greatly between species. *Treponema* species are the aetiological agent of syphilis and yaws in humans, periodontitis in humans and dogs and digital dermatitis in cattle; *Borrelia* species cause Lyme disease in humans, dogs and horses and avian spirochaetosis in a range of poultry; *Leptospira* species cause leptospirosis in humans, dogs, cattle, pigs, sheep and horses and *Brachyspira* species can cause swine dysentery in pigs and intestinal spirochaetosis in humans, pigs and poultry.

Spirochaetes divide by binary fission and are typically helical-shaped, but a flat wave and coccoid shape have been recorded (Charon *et al*., 2009; Droge *et al*., 2006). Spirochaetes in the genera *Treponema*, *Borrelia*, *Leptospira*, and *Brachyspira* can form spherical bodies, perhaps in response to adverse conditions (de Ciccio *et al*., 1999; Wood *et al*., 2006). Cell size range from 0.1 – 3.0 μm in diameter and 2.0 – 180.0 μm in length depending on the species (Charon and Goldstein, 2002; Hovind-Hougen *et al*., 1982). The cell wall consists of a thin peptidoglycan layer on the cytoplasmic membrane and an outer membrane bilayer. Periplasmic flagella are subterminally attached at each cell end and reside in the periplasmic space forming bundles that overlap in the midsection of the cell (Charon *et al*., 2009). Their numbers vary from 2 – 100s per cell, depending on the species. They contribute to morphology and motility by asymmetrical rotation allowing cells to penetrate and move efficiently in viscous media that would otherwise cause immobilisation, although immotile species have been noted (Droge *et al*., 2006). Spirochaetes typically possess circular chromosomes, however *Borrelia burgodorferi* has a linear chromosome with linear and circular plasmids (Kobryn and Chaconas, 2002). Currently, genome sequences are available for species of the genera *Borrelia*, *Brachyspira*, *Leptonema*, *Leptospira*, *Spirochaeta*, *Treponema* and *Turneriella* (GenBank, National Centre for Biotechnology Information, NCBI).

1.2.1.2 Genus *Brachyspira*

Brachyspira (etymology Gr. brachy "short" and speira "coil") are oxygen-tolerant, anaerobic spirochaetes, which have been isolated from the mammalian and avian GI tract, faecal-contaminated habitats and human blood. The genus currently consists of seven species that have standing in nomenclature and several proposed species detailed in Table 1.3.

Table 1.3 Validated and proposed *Brachyspira* species and their known host range.[a]

Species[a]	Published host range	Reference to species description or proposition
B. aalborgi	Human, non-human primates	(Hovind-Hougen *et al*., 1982)
B. alvinipulli	Chicken, domestic goose, Red breasted, merganser (Mergus serrator), dog	(Stanton *et al*., 1998)
B. hyodysenteriae	Pig, rat, mouse, common rhea, mallard, chicken, goose	(Harris *et al*., 1972a; Taylor and Alexander, 1971)
B. innocens	Pig, dog, horse, chicken	(Kinyon and Harris, 1979; Stanton, 1992)
B. intermedia	Pig, chicken	(Stanton *et al*., 1997)
B. murdochii	Pig, rat, chicken	(Stanton *et al*., 1997)
B. pilosicoli	Pig, dog, horse, non-human primates, human, chicken, pheasant, grey partridge, feral water birds, common rhea	(Trott *et al*., 1996d)
"*B. canis*"	Dog	(Duhamel *et al*., 1998b)
"*B. christiani*"	Human	(Jensen *et al*., 2001)
"*B. corvi*"	Jackdaw, hooded crow, rook	(Jansson *et al*., 2008)
"*B. hampsonii*"	Pig	(Mantle *et al*., 1989b)
"*B. ibaraki*"	Human	(Tachibana *et al*., 2003)
"*B. pulli*"	Chicken, dog	(Stephens and Hampson, 1999)
"*B. suanatina*"	Pig, mallard	(Rasback *et al*., 2007a)

[a] Species within quotation marks are proposed (unvalidated).

[b] Additional references to host ranges: (Duhamel, 2001; Duhamel *et al*., 1997; Feberwee *et al*., 2008; Hampson *et al*., 2006d; Jansson *et al*., 2008; Jansson *et al*., 2004; Jansson *et al*., 2011; Jensen *et al*., 1996; Joens and Kinyon, 1982; Johansson *et al*., 2004; McLaren *et al*., 1997; Munshi *et al*., 2003; Nemes *et al*., 2006; Oxberry *et al*., 1998; Thomson *et al*., 2007; Trivett-Moore *et al*., 1998; Trott *et al*., 1996c; Trott *et al*., 1996d; Webb *et al*., 1997).

Prior to the genus *Brachyspira*, spirochaetes that now come under this genus underwent several taxonomic changes. *B. hyodysenteriae* was first described as a vibrio-

like microorganism (*Vibrio coli*) (Vimal *et al*., 2000) and was renamed *Treponema hyodysenteriae* a decade later having been identified as a spirochaete and that fulfilled Koch's postulates (Harris *et al*., 1972a; Taylor and Alexander, 1971). All intestinal spirochaetes isolated from swine regardless of phenotype and pathogenicity were designated to this species (Harris *et al*., 1972a). It was later shown that pathogenic and non-pathogenic strains shared only 28% sequence homology (Miao *et al*., 1978) and weakly haemolytic, presumably non-pathogenic species isolated from swine faeces were classified as a new species, *T. innocens* (Kinyon and Harris, 1979). *T. hyodysenteriae* and *T. innocens* were distantly related to genus *Treponema* based on 16S rRNA sequencing, DNA-DNA relative reassociation, protein electrophoretic profiles and genomic DNA restriction enzyme analysis and thus, were reclassified to a new genus, *Serpula* (Paster *et al*., 1991; Stanton *et al*., 1991), later changed to *Serpulina* (Stanton, 1992). A spirochaete proposed as *Anguillina coli* (Lee *et al*., 1993) was added to this genus as *S. pilosicoli* (Trott *et al*., 1996d), followed by *S. intermedia* and *S. murdochii* (Stanton *et al*., 1997). *S. hyodysenteriae, S. innocens* and *S. pilosicoli* were unified with *B. aalborgi*, isolated from humans in the genus *Brachyspira* (Ochiai *et al*., 1997). This genus was added as a footnote to the descriptions of *S. alvinipulli* and *S. intermedia* (Stanton *et al*., 1998) and *S. murdochii* was last to be unified (Hampson and La, 2006).

Brachyspira is the sole genus in the family *Brachyspiraceae* within the order *Spirochaetales*, which contains four other families including *Leptospiraceae* and *Spirochaetaceae*. The order, *Spirochaetales*, belongs to the class *Spirochaetes*, which is a monophyletic lineage of the phylum Spirochaetes. *Brachyspira* share some general characteristics of spirochaetes, including a similar helical shape and ultrastructure, 16S rRNA gene sequences with signature sequences and natural resistance to rifampicin (Paster and Dewhirst, 2000). The unique ultrastructure and 16S rRNA gene sequences can be used to distinguish *Brachyspira* from other spirochaetes (Paster *et al*., 1991).

To identify, differentiate and determine phylogenetic relationships of *Brachyspira*, MLEE has been a key molecular tool, permitting the prediction of new species and assembly of strains into MLEE groups (Duhamel *et al*., 1998b; Selander *et al*., 1986). *Brachyspira* represent a distinct line in spirochaete evolution based on comparative analysis of 16S rRNA gene (*rrs*) sequences (Paster *et al*., 1991; Stanton *et al*., 1996). Due to the similarity of 16S rRNA gene (*rrs*) sequences among *Brachyspira*, new species should be designated only after their phylogenetic relationships with known *Brachyspira* species are confirmed by MLEE and DNA sequence homology analysis by DNA-DNA relative reassociation (Stanton, 2006). Novel, uncharacterised *Brachyspira*

species have been proposed, based on 16S rRNA gene sequencing (Pettersson *et al*., 2000). Phylogenetic analyses of 23S rRNA gene (*rrl*) sequences of *Brachyspira* are consistent with MLEE and 16S rRNA gene (*rrs*) phylogenies and 23S rRNA gene-targeted PCR and PCR-RFLP can differentiate species despite high sequence similarity (Barcellos *et al*., 2000; Leser *et al*., 1997). In the future, it is likely that whole genome sequencing will be involved in determining phylogenetic relationships of *Brachyspira*.

1.2.2 Phenotypic characteristics of *Brachyspira*

1.2.2.1 Morphology

Brachyspira are long, slender, helical-shaped, motile, Gram-negative bacteria as depicted in Figure 1.3 (Stanton *et al*., 1998). Cell lengths and widths ranges 2.0 – 14.0 μm and 0.19 – 0.40 μm, respectively, with amplitudes ranging 0.45 – 0.79 μm and wave lengths ranging 2.7 – 3.7 μm (Harris *et al*., 1972a; Stanton *et al*., 1997; Stanton *et al*., 1991; Stanton *et al*., 1998; Trott *et al*., 1996d). The cell end can be blunt, pointed or tapered, depending on the species (Hovind-Hougen *et al*., 1982; Ochiai *et al*., 1997; Stanton *et al*., 1997; Stanton *et al*., 1998; Trott *et al*., 1996b). The characteristics of the seven validated *Brachyspira* species are detailed in Table 1.4.

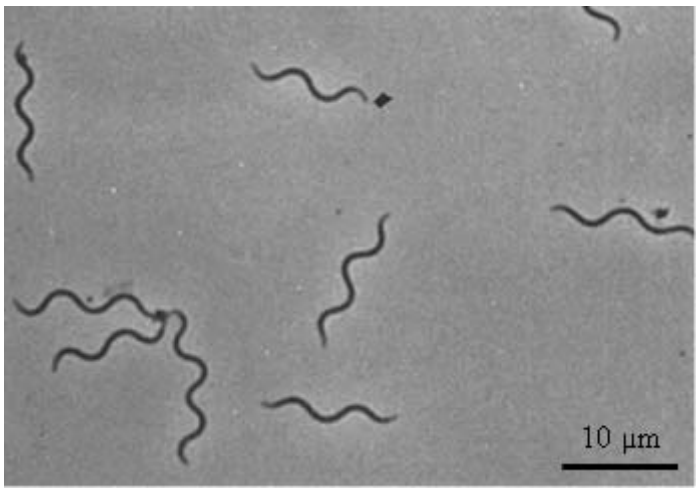

Figure 1.3 Phase-contrast photomicrograph of wet mount preparation of *B. alvinipulli* $C1^T$ (Stanton *et al*., 1998).

Typical of spirochaetes, *Brachyspira* have equal sets of flagella originating at either pole of the central protoplasmic cylinder, overlapping with each other midway along the cell (Canale-Parola, 1984). Rotation of periplasmic flagella between the outer sheath and the inner protoplasmic cylinder drives the characteristic sinuous spirochaetal movement that permit the spirochaetes to transverse viscous mucus, which would otherwise immobilise externally flagellated bacteria (Berg, 1976; Nakamura *et al*.,

2006). The number of periplasmic flagella at each end of the cell varies between species (Table 1.4) and can be an important consideration when distinguishing strains using EM (Sellwood and Bland, 1997). Avian intestinal spirochaetes typically have end:middle:end flagella number ratios of 8:16:8 or 5:10:5 (Stoutenburg *et al*., 1995). When cells divide by binary fission, the new flagella appear prior to the division. The morphology of the spirochaete cell end can aid in strain characterisation (Table 1.4).

Table 1.4 Phenotypic characteristics and major hosts of the seven validated *Brachyspira* species.[a]

Species	Length (µm)	Diameter (µm)	Flagella per cell	Cell pole	Major host
B. aalborgi	2.0 – 6.0	0.20	8	Tapered	Human
B. alvinipulli	8.0 – 11.0	0.20 – 0.35	22 – 30	Blunt	Chicken
B. hyodysenteriae	7.0 – 9.0	0.30 – 0.40	22 – 28	Blunt	Pig
B. innocens	7.0 – 9.0	0.30 – 0.40	20 – 26	Blunt	Pig, chicken
B. intermedia	8.0 – 10.0	0.35 – 0.45	24 – 28	Blunt	Pig, chicken
B. murdochii	5.0 – 7.0	0.23 – 0.30	22 – 26	Blunt	Pig, chicken
B. pilosicoli	5.0 – 2.0	0.20 – 0.40	8 – 12	One or both ends pointed	Pig, chickens, rodent, human

[a] References to information: (de Smet *et al*., 1998; Duhamel *et al*., 1998a; Fellstrom and Gunnarsson, 1995; Fellstrom *et al*., 1999; Fellstrom *et al*., 1997; Harris *et al*., 1972b; Hovind-Hougen *et al*., 1982; Kinyon and Harris, 1979; Kraaz *et al*., 2000; McLaren *et al*., 1997; Sellwood and Bland, 1997; Stanton *et al*., 1997; Stanton *et al*., 1991; Stanton *et al*., 1998; Stanton *et al*., 1996; Trott *et al*., 1996a; Trott *et al*., 1996c; Trott *et al*., 1997a).

1.2.2.2 Culture

Brachyspira are anaerobic but aerotolerant and tolerate transient exposure to air due to high activity of NADH oxidase (Harris *et al*., 1972a; Stanton and Lebo, 1988). Their growth is limited by strict anaerobic conditions and is optimal at atmosphere containing approximately 1% oxygen (Stanton and Cornell, 1987). Some species such as *B. pilosicoli* are able to grow in a concentration of up to 7% oxygen (Stephens and Hampson, 2001). *Brachyspira* are cultivable on solid or liquid media, supplemented with blood and/or serum. On solid blood medium, colonies are weakly or strongly β-haemolytic depending on the species. Primary isolation of *Brachyspira* can be accomplished using blood agar base medium, such as Trypticase Soy agar with 5 – 10% (v/v) sheep blood and one to five selective antimicrobials, including spectinomycin, rifampin, spiramycin, vancomycin, polymixin and/or colistin (Swayne and McLaren,

1997). Generally, *Brachyspira* selective agar contains 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Selective media is important to inhibit the growth of non-spirochaetal bacteria that would otherwise outgrow the slow-growing, fastidious spirochaetes. Typically, the growth of *Brachyspira* is viable after anaerobic incubation (10% hydrogen and 10% carbon dioxide in nitrogen) at 37 – 42˚C for 2 – 5 days (Brooke *et al*., 2003b). Mucin can be added to improve the growth, whilst citrated blood can increase haemolytic variation (Stephens and Hampson, 2001). *Brachyspira* can be propagated in broth culture, growing between 10^8 – 10^9 colony-forming units (CFU)/ml within 2 – 3 days. Typically, broth media includes pre-reduced anaerobic Trypticase Soy broth, containing 2% (v/v) foetal calf serum (FCS) and a 1% (v/v) cholesterol solution (Kunkle *et al*., 1986; Kunkle and Kinyon, 1988). *Brachyspira* utilise soluble sugars as carbon sources, such as glucose, from which they produce acetate, butyrate, hydrogen and carbon dioxide (Stanton, 2006). Cholesterol and phospholipids, of which erythrocytes are a source, are considered essential for *Brachyspira* growth (Lemcke and Burrows, 1980).

1.2.2.3 Colony morphology

On agar medium, avian intestinal spirochaetes grow as a dull, flat sheet that spreads over the surface and particularly strongly β-haemolytic spirochaetes may penetrate the agar. They form a confluent, cloudy film with defined edges surrounded by a zone of haemolysis. Most species are weakly β-haemolytic, although *B. hyodysenteriae* is typically strongly β-haemolytic. Occasionally, *B. intermedia* and other unidentified avian strains can cause intermediate to strong β-haemolysis (Jansson *et al*., 2001; McLaren *et al*., 1997). Examining a wet smear under dark field or phase contrast microscopy is routinely used to confirm spirochaetal growth and visualise their characteristic morphology and sinuous movement (Stephens and Hampson, 2001).

1.2.2.4 Biochemical properties

Brachyspira produce an array of specific enzymes and can be differentiated based on their biochemical properties, including testing for indole production and hippurate hydrolysis (Fellstrom *et al*., 1999) and commercial kits such as API-ZYM can be used for characterisation (Hunter and Wood, 1979). *Brachyspira* strains are differentiated biochemically by their intensity of haemolysis on blood agar, indole production, hippurate hydrolysis and activities of α-galactosidase, α-glucosidase, and β-glucosidase (Fellstrom *et al*., 1997; Fellstrom *et al*., 1995). *Brachyspira* species are assigned to a

group based on biochemical test results (Table 1.5) (Fellstrom *et al*., 1999). Tests for carbohydrate utilisation are uncommon in diagnostics for *Brachyspira* and have been tested for few strains (Stanton *et al*., 1997; Trott *et al*., 1996b). Due to phenotypic variations and a lack of understanding of the metabolic capabilities of *Brachyspira* species, molecular techniques have replaced biochemical testing for identification.

Table 1.5 Grouping of *Brachyspira* species based on biochemical properties (Fellstrom *et al*., 1999).

Group	Haemolysis	Indole production[a]	Hippurate hydrolysis[b]	α-gal[c]	α-glu[d]	β-glu[e]	Species indicated
I	strong	±	–	–	±	+	*B. hyodysenteriae*
II	weak	+	–	–	+	+	*B. intermedia*
IIIa	weak	–	–	–	–	+	*B. murdochii*
IIIb	weak	–	–	±	–	+	*B. innocens*
IIIc	weak	–	–	±	+	+	*B. innocens*
IV	weak	–	+	±	±	–	*B. pilosicoli*
ND	weak	–	+	–	–	+	*B. alvinipulli*

[a] Test for ability to cleave indole from tryptophan; +, positive; −, negative; ±, positive/negative.

[b] Test for ability to hydrolyse hippurate; +, positive; −, negative, ±, positive/negative.

[c] Test for α-galactosidase activity; +, positive; −, negative; ±, positive/negative.

[d] Test for α-glucosidase activity; +, positive; −, negative; ±, positive/negative.

[e] Test for β-glucosidase activity; +, positive; −, negative; ±, positive/negative.

1.2.2.5 Metabolic properties

The central metabolic pathways present within *Brachyspira*, which have been supported by recent genome sequencing, have revealed adaptations to life in the lower GI tract. The central metabolic pathways for energy production include a glycolysis, gluconeogenesis and the non-oxidative pentose phosphate pathway, from which many metabolic pathways originate (Bellgard *et al*., 2009; Hafstrom *et al*., 2011; Pati *et al*., 2010; Wanchanthuek *et al*., 2010). The end products of glucose metabolism in *Brachyspira* are acetate, butyrate, hydrogen and carbon dioxide, showing the species have similar catabolic routes (Stanton, 1989; Stanton *et al*., 1997; Stanton and Lebo, 1988; Stanton *et al*., 1998; Trott *et al*., 1996b). Following glycolysis, pyruvate is catabolised to acetyl-CoA, hydrogen and carbon dioxide. Acetyl-CoA is further converted to acetate or butyrate via a branched fermentation pathway. Moreover, the complete repertoire of genes for nucleotide metabolism, lipopolysaccharides (LPS) biosynthesis and a respiratory electron transport chain have been identified, alongside

an incomplete tricarboxylic acid (TCA) cycle. Hence, adenosine triphosphate (ATP) is yielded via sugar fermentation and ATP-yielding mechanisms are substrate level phosphorylations mediated by phosphoglycerate kinase, pyruvate kinase and acetate kinase, genes for which were detected in the available *Brachyspira* genomes. Despite producing stoichiometricically less ATP than oxidative phosphorylation, substrate level phosphorylations form ATP more rapidly, which may be necessary to fuel the high motility of *Brachyspira*. Aerobic pyruvate dehydrogenase and anaerobic pyruvate formate lyase are present in *Brachyspira* and associated with mixed-acid fermentation. The incomplete TCA cycle may function in carbon assimilation and the generation of precursors for biosynthesis (Romano and Conway, 1996). The respiratory transport chain may enable the generation of ATP oxidative phosphorylation and interestingly, *B. hyodysenteriae* grow optimally in an atmosphere containing 1% oxygen and consume oxygen during growth (Stanton and Cornell, 1987; Stanton and Lebo, 1988).

1.2.3 Genotypic characteristics of *Brachyspira*

Brachyspira possess a circular chromosome with low G+C content (24.2 – 27.9%) (Liolios *et al*., 2008; Ochiai *et al*., 1997; Stanton *et al*., 1997; Stanton *et al*., 1998; Trott *et al*., 1996d; Wanchanthuek *et al*., 2010) and 16S rRNA gene sequences are highly conserved amongst *Brachyspira* (Stanton, 2006). Whole genome sequences are now available for *B. hyodysenteriae* WA1 (Bellgard *et al*., 2009), *B. intermedia* PWS/A^{T} (Hafstrom *et al*., 2011), *B. murdochii* 56-150^{T} (Pati *et al*., 2010) and *B. pilosicoli* 95/1000 (Wanchanthuek *et al*., 2010) and genome projects are currently being undertaken for other species and strains (GenBank, NCBI). Of the available genomes, *B. pilosicoli* 95/1000 is the only strain not to harbour a plasmid. The general features of the four available *Brachyspira* genomes are compared in Table 1.6, based on a previous comparative genomic study (Hafstrom *et al*., 2011). As expected, the total number of predicted open reading frames (ORF) increased with genome size.

Few tools are available for the genetic manipulation of *Brachyspira*, which has hindered the understanding of the pathobiology of this organism. *Brachyspira* strains with specific mutations in genes, such as those coding for haemolysins (ter Huurne *et al*., 1992), flagellar proteins (Rosey *et al*., 1995) and NADH oxidase (Stanton *et al*., 1999) have been created by cloning the gene into *Escherichia coli*, inactivating the gene by insertion of a kanamycin resistance gene and then introducing the construct into *Brachyspira* cells by electroporation. Mutant *Brachyspira* cells, in which the knockout mutation had undergone allelic exchange with the wild type gene, can be selected by

plating on media containing kanamycin. Bacteriophage-like particles, smaller than but morphologically similar to the lambda (λ) phage of *E. coli* were initially described in *B. hyodysenteriae* and *B. innocens* (Humphrey *et al*., 1995) then in *B. pilosicoli* and *B. intermedia* (Motro *et al*., 2009; Stanton *et al*., 2003) and has been found in all available *Brachyspira* genomes alongside unique bacteriophages. This phage, named virus of *S. hyodysenteriae* (VSH)-1 can package random, 7.5 Kb linear DNA fragments and act an avirulent, gene transfer agent (GTA). VSH-1 can transfer of chloramphenicol resistance between *B. hyodysenteriae* strains (Humphrey *et al*., 1997). The VSH-1 genome is divided into clusters of 16.3 and 3.6 Kb and contains at least six genes that do not function in its production (Stanton *et al*., 2009). The use of phages for gene manipulation to improve understanding of *Brachyspira* remains to be explored.

Table 1.6 General genome feature comparison of the available *Brachyspira* genome sequences including the number of open reading frames (ORFs) predicted from the comparative genomic study (Hafstrom *et al*., 2011).

Feature	***B. hyodysenteriae* WA1[b]**	***B. intermedia* PWS/A^{T}[a]**	***B. murdochii* 56-150^{T}[c]**	***B. pilosicoli* 95/1000**
Size (bp)	3,000,694	3304788	3241804	2586443
Coding region (%)	86.7	85.0	85.9	88.6
G+C content (%)	27.1	27.2	27.6	27.9
Total predicted ORFs	2613	2870	2809	2299
with function prediction	1755	1854	1993	1615
conserved/hypothetical	858	1016	816	684
ribosomal (r)RNA genes	3	3	3	3
transfer (t)RNA genes	34	33	34	33

[a] *B. hyodysenteriae* WA1 has a 35940 bp plasmid; coding region 91.2%; G+C content, 22.4%; 29 ORFs with predicted function and 2 conserved/hypothetical ORFs.

[b] *B. intermedia* PWS/A^{T} has a 3260 bp plasmid; coding region, 53.0%; G+C content, 21.0%; 3 conserved/hypothetical ORFs.

[c] *B. murdochii* 56-150^{T} was also reported to have a 3.2 Kb plasmid with approximately 96% identity at the nucleotide sequence level to the *B. intermedia* PWS/A^{T} plasmid.

1.2.4 Host range of *Brachyspira*

The presence of spirochaetes in the human GI tract has been known since the 1900's, although they were originally regarded as harmless commensals (Rotterdam, 1997). Following the identification of large numbers of spirochaetes in the stools of human patients with dysentery-like diseases, it became apparent that some intestinal

spirochaetes were pathogenic (Parr, 1923). Human colonic and rectal mucosae can be colonised by dense arrays of spirochaetes, producing HIS (Barrett, 1997; Harland and Lee, 1967; Jensen *et al*., 2001). *B. aalborgi*, *B. pilosicoli* and as yet uncharacterised species have been associated with HIS (Brooke *et al*., 2003b; Hovind-Hougen *et al*., 1982). HIS is more prevalent among humans living in poor conditions and immunocompromised patients and has been associated with intestinal disorders but also observed in healthy humans (Brooke *et al*., 2006; Mikosza and Hampson, 2001).

Other than causing disease in humans, intestinal spirochaetosis poses significant problems to the porcine and poultry industries. Koch's postulates have been fulfilled for *B. pilosicoli* using gnotobiotic (Neef *et al*., 1994) and conventional swine (Duhamel, 1996; Taylor *et al*., 1980; Trott *et al*., 1996a). The swine challenged with porcine or human *B. pilosicoli* exhibited diarrhoea, growth reduction and spirochaetes attached to apical surfaces of colonic enterocytes. Experimental challenge of hens with pathogenic *Brachyspira* species have also resulted in clinical disease (Dwars *et al*., 1993; Hampson and McLaren, 1999; Jamshidi and Hampson, 2003). Additionally, the inoculation of chicks with porcine and human *B. pilosicoli* led to the attachment of the spirochaetes to the caecal epithelium and invasion of the caecal wall (Dwars *et al*., 1992a; Muniappa *et al*., 1996; Trott *et al*., 1995). Alongside others, these studies fulfilled Koch's postulates, confirming the pathogenicity of certain *Brachyspira* species to poultry.

B. hyodysenteriae and *B. pilosicoli* are the agents of the disease in swine; *B. hyodysenteriae* infection is associated with swine dysentery and *B. pilosicoli* infection results in PIS (Hampson and Duhamel, 2006; Hampson *et al*., 2006a). Swine dysentery is a severe mucohaemorrhagic diarrhoeal disease that affects growing and finishing pigs in all major pig producing countries, caused by the colonisation of the caeca and colo-recta by *B. hyodysenteriae*. Clinical symptoms include mucoid diarrhoea containing blood, reduced growth, poor feed conversion, and increased mortality. PIS often affects pigs a few weeks after weaning and causes mucoid diarrhoea often containing blood, unthriftiness, poor feed conversion and reduced growth rates (Hampson and Duhamel, 2006). *B. innocens* and *B. murdochii* are considered as non-pathogenic in swine, however the enteropathogenic potential of *B. intermedia* is undetermined (Hampson *et al*., 2006a). The agents of AIS are *B. intermedia*, *B. pilosicoli* and less commonly, *B. alvinipulli* (Stephens and Hampson, 2001; Swayne, 1997) (as discussed in section 1.1).

The potential for zoonotic transmission of *Brachyspira* has been investigated and described in a number of studies (Hampson *et al*., 2006b). Some species, including *B. pilosicoli* may infect across species barriers, since a *B. pilosicoli* strain recovered from a

human immunodeficiency virus (HIV)-infected patient with diarrhoea could infect adult hens, causing chronic diarrhoea (Jamshidi and Hampson, 2003). Moreover, *Brachyspira* have been isolated from dogs (Trott *et al*., 1997b), non-human primates (Munshi *et al*., 2003), rodents including rats and mice (McLaren *et al*., 1997; Smith, 2005; Trott *et al*., 1996c) and species detailed in Table 1.3. Feral birds, rodents and domestic animals can harbour *Brachyspira* and hence, are potential sources for transmission.

1.2.5 Virulence of *Brachyspira*

Brachyspira exert their pathogenic effect within close proximity of mucus in the lumen and crypts of the caeca and/or colon, but virulence mechanisms are poorly understood. *Brachyspira*-associated diseases are multifactorial and the aetiological agent is influenced by factors such as host immunity, diet and the intestinal microbiota.

1.2.5.1 Motility and chemotaxis

Chemotaxis and motility are important virulence factors for *Brachyspira*, facilitating penetration of the mucus and association to the intestinal mucosa (Kennedy *et al*., 1988). *B. hyodysenteriae* are highly motile in viscous media and exhibit chemoattraction towards mucosal glycoproteins (Kennedy and Yancey, 1996). The chemical components of mucin, fucose and L-serine, are potent chemo-attractants for *Brachyspira* (Milner and Sellwood, 1994; Naresh and Hampson, 2010). A homologue of the *mglB* gene, which mediates chemotaxis towards mucin was identified in *B. pilosicoli* (Zhang *et al*., 2000). Flagella enable high motility within mucus (Kennedy *et al*., 1988) and disruption of flagella genes reduces colonisation and virulence of *Brachyspira* in challenged pigs and mice (Kennedy *et al*., 1997; Rosey *et al*., 1996).

1.2.5.2 Adherence and invasion

Pathogenesis of AIS and PIS caused by *B. pilosicoli* is poorly understood, but in these infections, the spirochaetes attach to the apical cell membrane of enterocytes in large numbers forming a “false brush border” and also invade crypts, epithelium and the lamina propria (Hampson and Duhamel, 2006). Only *B. aalborgi* and *B. pilosicoli* adhere to healthy epithelial cells, whereas other species adhere and enter the cytoplasm of disrupted epithelial cells (Kennedy and Strafuss, 1976; Teige *et al*., 1981). In natural and experimental *B. pilosicoli* infections in pigs, chicken and mice, one cell end of the spirochaetes may invaginate into mature columnar cells (Jensen *et al*., 2000; Sacco *et al*., 1997; Trott *et al*., 1995). Upon end-on attachment, the membrane remains intact, but

the attachment disrupts microvilli, interfering with fluid and nutrient absorption (Gad *et al*., 1977; Muniappa *et al*., 1998). Attachment and invasion determinants of *Yersinia*, *Shigella* and *E. coli* were not identified in *B. pilosicoli* (Hartland *et al*., 1998) and genes encoding known adhesins or toxins were not found in *Brachyspira* genomes.

1.2.5.3 Haemolysis

Haemolysin production is considered a virulence factor particularly in the strongly β-haemolytic *B. hyodysenteriae*. Other *Brachyspira* species are weakly β-haemolytic and the haemolysin is cytotoxic to a number of cell lines and is likely to contribute to lesion formation (Lysons *et al*., 1991; Muir *et al*., 1992). Cholesterol and phospholipids are essential growth factors and haemolysins are employed to obtain these and other nutrients from host cells. Extracted haemolysins caused lysis of erythrocytes, cytotoxic effects in eukaryotic cells and epithelial lesions in a murine model (Hutto and Wannemuehler, 1999). The β-haemolysin gene *hlyA* was identified in *B. hyodysenteriae*, but also in *B. pilosicoli* (Hsu *et al*., 2001; Zuerner and Stanton, 1994).

1.2.5.4 NADH oxidase activity

As anaerobes, the ability of *Brachyspira* to colonise the colonic mucosa amongst respiring tissues is enhanced by NADH oxidase activity, protecting from oxygen toxicity (Stanton *et al*., 1999). All species contain NADH oxidase activity (Stanton *et al*., 1995). Mutation of the *nox* gene reduces virulence (Stanton and Jensen, 1993).

1.2.5.5 Lipooligosaccharides

Lipooligosaccharide (LOS) in the cell wall is associated with virulence. *B. hyodysenteriae* possesses rough LOS, which unlike smooth LOS, lacks *O*-side chains (Greer and Wannemuehler, 1989). Non-pathogenic *B. innocens* lacks a high molecular weight LOS (Joens, 1997). LOS is likely to be responsible for mucosal inflammation, as demonstrated in a mouse model (Halter and Joens, 1988; Nuessen *et al*., 1983).

1.2.5.6 Iron import system

B. hyodysenteriae possess an iron import system, to sequester iron from the host (Dugourd *et al*., 1999). This active transport system for iron import is common amongst Gram-negative bacteria and pathogens such as *Neisseria* (Adhikari *et al*., 1996). Genes for components for a secretory (Sec) pathway are present in *Brachyspira*, although no genes for toxin injection have been detected (Bellgard *et al*., 2009).

1.3 Probiotics

1.3.1 Definitions of probiotic, prebiotic and synbiotic

The term ‘probiotic’ (etymology Gr. pro “for” and biotic “life”) was coined by in 1965 to describe secreted compounds from one microorganism that are able to stimulate the growth of another (Lilly and Stillwell, 1965). The term was also applied to the description of tissue extracts that promote microbial growth (Sperti, 1971). In 1974, the term was applied in the sense to which it is currently understood, defining it as “organisms and substances which contribute to intestinal microbial balance” (Parker, 1974). The definition was amended in 1989 to specify that a probiotic is “a live microbial feed supplement, which beneficially affects the host animal by improving its microbial balance” (Fuller, 1989). This is arguably the most widely accepted and cited definition to date. More recently, the definition was updated to “a preparation of viable microorganisms, which is consumed by humans or other animals with the aim of inducing beneficial effects by qualitatively or quantitatively influencing their gut microbiota and/or modifying their immune status” (Fuller, 2004).

The term ‘prebiotic’ (etymology Gr. pre “before” and biotic “life”) was introduced in 1995 as “non-digestible food ingredients that beneficially affect the host by selectively promoting growth and/or activity of one or a limited number of health-promoting bacteria indigenous to the GI tract” (Gibson and Roberfroid, 1995). The term ‘synbiotic’ (etymology Gr. pre “together with” and biotic “life”) was also proposed for the simultaneous application of prebiotics and probiotics to humans or other animals, whereby the former selectively stimulates the latter (Gibson and Roberfroid, 1995).

1.3.2 History of probiotics

The concept of probiotics and cultured dairy products that presumably contained live microorganisms for consumption dates back to a Persian version of the Old Testament (Genesis 18:8) that states “Abraham owed his longevity to the consumption of sour milk” (Schrezenmeir and de Vrese, 2001). Additionally in 76 BC, the Roman historian Plinius advocated the administration of fermented milk products to treat symptoms of gastroenteritis, including diarrhoea (Bottazzi, 1983). Interestingly, the therapeutic use of these products existed prior to the first description of bacteria (Shortt, 1999). A fundamental breakthrough placing probiotics in the context of their current understanding, were observations published in 1907 by Eli Metchnikoff, who is widely accepted as the developer of the concept of probiotics. The seminal publication

attributed the regular consumption of fermented yoghurt containing *Lactobacillus* species to the extended life span of Bulgarian peasants (Metchnikoff, 1907). Shortly prior to Metchnikoff's publication, Tissier demonstrated that the gut flora of breast fed infants consisted predominantly of *Bifidobacterium* species and proposed a need for specific bacteria in maintaining health (Tissier, 1900) and suggested administering bifidobacteria to infants suffering from diarrhoea, claiming that they supersede the putrefactive bacteria (Tissier, 1906). In 1916, Nissle demonstrated the use of non-lactic acid bacteria, including *E. coli*, as agents to "fight" against pathogens (Nissle, 1916).

Encouraged by the work of Metchnikoff and subsequent research on probiotics, in 1919, Carasso established the Spanish company Danone™ and promoted the beneficial effects of yoghurts (Shortt, 1999). In the 1930's, Shirota founded the Japanese company, Yakult™, producing a fermented yoghurt containing *L. casei* strain Shirota, capable of surviving transit through the human GI tract. By the 1950's, the use of live microbial food supplements was established in food and medical industries and interest focused on their use to re-establish natural intestinal microbiota following antimicrobial treatment (Cheplin and Rettger, 1922; Rettger *et al*., 1936; Salminen *et al*., 1998).

1.3.2.1 History of probiotic use in humans

In humans, probiotics have been used clinically to treat diseases such as *C. difficile*-associated disease (CDAD), inflammatory bowel disease (IBD) and inflammatory bowel syndrome (IBS) (Nomoto, 2005). Moreover, probiotics have been administered as a functional foods based on the notion that their consumption may reduce constipation, gastritis, hypertension, lactose intolerance and serum cholesterol (Andersson *et al*., 2001; Harish and Varghese, 2006). The health benefits conferred by probiotics have been extensively reviewed (Andersson *et al*., 2001; Floch and Montrose, 2005; Harish and Varghese, 2006; Isolauri, 2001; Montrose and Floch, 2005), however the reviews unanimously conclude that the majority of clinical trials for probiotics were poorly designed with too few subjects to draw significant conclusions.

1.3.2.2 History of probiotic use in livestock

The application of probiotics in livestock is based on the principle that healthy intestinal microbiota confers resistance to disease (Fuller, 1992). In intensive farming, it is common practice to remove offspring into isolated, clean environments, which limits the contact with the mother and limits the acquisition of passive immunity and the development of a normal healthy intestinal microbiota, rendering the animals more

susceptible to infections. Moreover, the offspring are subjected to a multitude of stresses such as transport, nutrition and temperature fluctuations, which may induce microbiota imbalance (Burkholder *et al*., 2008; Tannock and Savage, 1974). Nutritional stresses, such as a change in feed, can increase susceptibility to disease. Probiotics are used to restore and beneficially alter the microbiota present in young, stressed or antimicrobial-treated animals. Natural microbiota plays a vital role in resistance to enteric disease with health benefits including improved digestion, feed conversion and growth, resistance to infection, reduced mortality, increased egg production and quality (in poultry) and increased milk yield (in cattle) (Nurmi and Rantala, 1973). Although effective in removing target microorganisms causing infection, antimicrobials are associated with side effects due to alterations of the natural microbiota, such as antimicrobial-associated diarrhoea. The use of probiotics with antimicrobials is effective in preventing infection whilst restoring the microbiota (Surawicz, 2008).

The range of bacteria selected for use as probiotics in livestock varies greatly and they are typically developed for use in monogastric animals, including poultry. Their use in ruminants is more complicated and probiotics are selected and targeted to alleviate acidosis, alter the feed conversion ratio and/or reduce the incidence of disease (Krehbiel *et al*., 2003). In addition to bacterial probiotics, yeasts and fungi have demonstrated particular efficacy in ruminants (Wallace, 1994). The commercially available veterinary probiotics, detailed in Table 1.7, are deemed safe for use in the target animals by the Scientific Committee for Animal Nutrition (SCAN). SCAN recognises that these probiotics pose no risk to the environment or to individuals handling or consuming products derived from animals that consumed the probiotics.

Recently, interest in probiotics in the veterinary, medical and food industry has been enhanced due to the requirement for alternative therapies to antimicrobials, following the ban on subtherapeutic antimicrobial usage in Europe, the potential ban in the United States and the emerging resistance to current antimicrobials.

1.3.3 Design and selection of probiotics

Criteria for the selection of probiotics can be divided into four categories (Klaenhammer and Kullen, 1999). Appropriateness criteria ensure the bacteria are generally regarded as safe (GRAS) and of host origin. Technological suitability criteria determine the practicalities of production and storage. Competitiveness criteria consider survival within the host and the ability to withstand environmental stresses. Performance and functionality criteria evaluate the beneficial effects on the host.

Table 1.7 Commercially available veterinary probiotics deemed safe by the Scientific Committee for Animal Nutrition (SCAN) in the European Union (EU) (SCAN, 2003b).

Product name	Probiotic organism	Culture collection	Target organism
Adjulact 2000®	*Streptococcus infantarius* *Lactobacillus plantarum*	CNCM I-841 CNCM I-840	Calves
Bactocell®	*Pediococcus acidilactici*	CNCM MA 18/5	Broilers
Biacton®	*Lactobacillus farciminis*	CNCM MA 67/4	Piglets
Bioplus 2B®	*Bacillus licheniformis* *Bacillus subtilis*	DSM 5749 DSM 5750	Piglets / pigs for fattening Broilers, calves and piglets / pigs for fattening
Biosprint®	*Saccharomyces cerevisiae*	BCCM / MUCL 39885	Beef cattle and piglets / pigs for fattening
Bonvital®	*Enterococcus faecium* *Lactobacillus rhamnosus*	DSM 7134 DSM 7133	Calves and pigs for fattening
Biosaf SC 47®	*Saccharomyces cerevisiae*	NCYC Sc 47	Beef / dairy cattle and piglets / pigs
Cylactin LBC®	*Enterococcus faecium*	NCIMB 10415	Broilers and calves and piglets / pigs for fattening
Fecinor plus®	*Enterococcus faecium*	CECT 4515	Calves / beef cattle and piglets / pigs for fattening
Gardion®	*Lactobacillus casei* *Enterococcus faecium*	NCIMB 30096 NCIMB 30098	Calves
Kluyten®	*Kluyveromyces marxiamus*	MUCL 39434	Dairy cattle
Lactiferm®	*Enterococcus faecium*	NCIMB 11181	Calves and piglets
L. acidophilus D2/CSL®	*Lactobacillus acidophilus*	CECT 4529	Broilers / laying hens
Levucell SB20®	*Saccharomyces cerevisiae*	CNCM I-1079	Piglets / pigs
Levucell SC20®	*Saccharomyces cerevisiae*	CNCM I-1077	Beef / dairy cattle
Microferm®	*Enterococcus faecium*	DSM 5464	Broilers, calves and piglets
Mirimil-Biomin®	*Enterococcus faecium*	DSM 3520	Calves
Oralin®	*Enterococcus faecium*	NCIMB 10415	Broilers, calves and pigs for fattening
Primver Pro®	*Enterococcus mundtii*	CNCM MA 27/4	Lambs
Probios PDFM Granular®	*Enterococcus faecium* *Enterococcus faecium*	DSM 4788 DSM 4789	Broilers
Yea-Sacc®	*Saccharomyces cerevisiae*	CBS 493 94	Calves / beef / dairy cattle

In correlation with guidelines proposed by SCAN, the European Union (EU) devised regulations for the use of probiotics as animal feed supplements (von Wright, 2005). Regulation 1831/2003 of the parliament and council of the EU regulates the use of animal feed additives, whilst council directive 87/153/EEC, which stipulates the assessment guidelines for the safety of feed additives. This directive states that probiotics must not produce toxins, virulence factors and antimicrobial substances of clinical significance or carry transmissible antimicrobial resistance determinants.

There is increasing evidence for the transferability of antimicrobial resistance genes between resistant and sensitive species of probiotic species, such as enterococci and lactobacilli within the GI tract of humans and animals (Ashraf and Shah, 2011; Egervarn *et al*., 2010; Lester *et al*., 2006). Resistance can occur naturally within bacteria, for example, some lactobacilli are intrinsically resistant to vancomycin by possessing a different termini on peptidoglycan precursors, to which vancomycin cannot bind and prevent cross-linking of peptidoglycan in the cell wall (Roper *et al*., 2000). Acquired resistance is mediated by mobile genetic elements (MGE), such as plasmids and transposons and there is concern over the risk of such transfer from probiotic bacteria to pathogens. *Lactobacillus* species may be an environmental reservoir for antimicrobial resistance genes in broilers (Cauwerts *et al*., 2006; Danielsen, 2002), driving a requirement to monitor the carriage of antimicrobial resistance genes. When selecting probiotic candidates, it is useful to refer to the recommended MICs of clinically important antimicrobials in potential probiotic species (SCAN, 2003a). Bacteria that express mobile or acquired resistance genes are not suitable as probiotics.

Competitiveness criteria tend to focus on the ability of the probiotic candidate to withstand environmental stresses, typically acid and bile, but also heat and desiccation to reflect manufacture and storage. Assays to determine the tolerance of probiotic candidates to gastric acid and biles are commonly performed (Barbosa *et al*., 2005; Hyronimus *et al*., 2000; Jin *et al*., 1998; Wang *et al*., 2012). *In vitro* models to simulate porcine gastric fluid have proved effective in selection (de Angelis *et al*., 2006).

It is unclear as to whether fulfilling performance and functionality criteria will result in the selection of an efficacious probiotic since the mechanisms underlying the functional roles of probiotics remain to be elucidated. Beneficial probiotic effects may include the exclusion of pathogens, immunomodulation, reduction of the severity of diarrhoea, maintenance of mucosal integrity, improvement of host serum cholesterol and blood pressure, prevention of vaginitis and reduction of mutagenic activity (Klaenhammer and Kullen, 1999). Screening for *in vitro* adherence of probiotic

candidates to epithelial cells has been used as a selection criterion (Duary *et al*., 2011; Haeri *et al*., 2012), but whether such screening provides adequate evidence for the probiotic candidate to persist in the GI tract *in vivo* is unknown. The ability of probiotic candidates to exhibit antimicrobial activity or antagonism against pathogens is often screened (de Keersmaecker *et al*., 2006; Dunne *et al*., 2001). As the understanding of the modes of action of probiotics improves, the selection criteria for competitiveness, performance and functionality will become more appropriate and definitive.

1.3.4 The use of probiotics in poultry

The first probiotic preparation developed for poultry used crude caecal extracts from adult birds and was administered directly into the crop of chicks by oral gavage, conferring increased resistance to *Salmonella* infection (Nurmi and Rantala, 1973; Rantala and Nurmi, 1973). The studies introduced of the concept of competitive exclusion (CE) to describe the exclusion of pathogens from an ecological niche by out-competition by probiotics. To date, studies have demonstrated CE by probiotics to protect hosts against pathogens including *E. coli*, *Campylobacter*, *Salmonella* and *Yersinia* (Soerjadi-Liem *et al*., 1984; Soerjadi *et al*., 1982a; Weinack *et al*., 1982).

Poultry probiotics can be separated into defined and undefined products. The microorganisms that comprise the product have been identified in defined products, such as Protexin Pro-soluble®. However, undefined products such as Aviguard® and BROILACT®, which consist of bacterial cultures that are partially or completely undefined have demonstrated the most effective CE of pathogens, such as *Salmonella*, to date (Nakamura *et al*., 2002; Nuotio *et al*., 1992; Schneitz *et al*., 1992). Many of the probiotic products were derived from caecal contents, although single strain probiotics, particularly of the *Lactobacillus* genera can control pathogens such as *E. coli* and *Salmonella* (Higgins *et al*., 2007; La Ragione *et al*., 2004; Vicente *et al*., 2008). Bacteriocin-producing strains also reduced the numbers of viable pathogens in *in vivo* studies (Corr *et al*., 2007). Lactobacilli and bifidobacteria are the most extensively used probiotics in humans, whereas bacilli, enterococci and yeast are widely used in livestock (Simon *et al*., 2001). Recently, research on the use of *Lactobacillus* as probiotics in poultry has increased (Jin *et al*., 2000; Kalavathy *et al*., 2003; La Ragione *et al*., 2004).

Studies of the use of probiotics in poultry have focused on CE against zoonoses to which poultry are a major reservoir including *Campylobacter*, *Clostridium*, *E. coli* and *Salmonella*. *L. johnsonii* can mitigate colonisation by *Clostridium* and *E. coli* in specific pathogen-free (SPF) chicks (La Ragione *et al*., 2004). *Bacillus* spores are effective in

the CE of *Clostridium* from day-old chicks, however a delayed effect suggests a need for the spores to germinate in the GI tract or immunomodulation to occur (Cartman *et al*., 2008). The protective effect of undefined CE preparations and caecal contents against *Campylobacter*, *E. coli* and *Salmonella* in chicks and chickens is well documented (Stavric *et al*., 1993; Weinack *et al*., 1981, 1982). However, the caecal contents required to protect against *Campylobacter* differs from that required against *Salmonella* (Fuller, 1992; Zhang *et al*., 2007b) and studies now focus on defining caecal preparations active against *Campylobacter* and *Salmonella* (Bhaskaran *et al*., 2011). Defined probiotic mixtures containing *Citrobacter*, *Klebsiella* and *E. coli* are effective in the CE of *Campylobacter* in chicks (Schoeni and Wong, 1994; Timmerman *et al*., 2004). Treatment with undefined probiotics presents concerns over the potential of containing human or animal pathogens and regulatory bodies are becoming stricter with their policy and requirement for defined probiotics. The future of probiotics in poultry requires research of defined probiotics that exclude, preferably multiple, pathogens.

1.3.5 The use of *Lactobacillus* species as probiotics in poultry

The *Lactobacillus* (etymology Gr. lac "milk" and bacillum "small rod") genus is in the family *Lactobacillaceae*, order *Lactobacillales* and class *Bacilli*, which belong to the phylum Firmicutes (Hammes and Hertel, 2009). Lactobacilli are Gram-positive, usually non-motile, fermentative, facultative anaerobes with varying morphology from long, slender, sometimes bent rods to short, often coryneform coccobacilli.

In poultry, lactobacilli exist as commensals, commonly colonising the GI tract and lower reproductive tract. Dietary supplementation with lactobacilli, including *L. reuteri* and *L. salivarius*, in poultry is associated with CE of pathogens including *Campylobacter* (Ghareeb *et al*., 2012; Stern *et al*., 2001), *Clostridium* (Decroos *et al*., 2004; Kizerwetter-Swida and Binek, 2009), *E. coli* (Edens *et al*., 1997) and *Salmonella* (Higgins *et al*., 2008; Pascual *et al*., 1999). Lactobacilli have been implicated in immunomodulation, whereby oral treatment with lactobacilli modulate systemic antibody- and cell-mediated immune responses, induce cytokine production and enhance phagocytic activity of cells in the caecum and ileum to protect against enteric pathogens (Ben Salah *et al*., 2012; Dalloul *et al*., 2003; Koenen *et al*., 2004). In poultry, gut-associated lymphoid tissue (GALT) plays an important role in protecting against viral, bacterial and toxic matter due to an absence of peripheral lymph nodes. The use of lactobacilli in poultry can improve body weight gain and feed conversion ratios and confer a hypolipidaemic effect (Capcarova *et al*., 2010; Kalavathy *et al*., 2003; Zulkifli

et al., 2000) and can enrich beneficial lactobacilli, suppress non-beneficial bacterial groups and maintain a natural stability of indigenous microbiota (Lan *et al*., 2004; Nakphaichit *et al*., 2011). The growth-promoting effects of certain probiotics have been compared to antimicrobial therapies (Cavazzoni *et al*., 1998; Mountzouris *et al*., 2007).

1.3.6 Dose and administration of probiotics to poultry

The dose of probiotics varies between commercial products based on the strength of probiotic action and industrial production limitations (Carita, 1992). Recommended doses usually range 10^8 – 10^{10} CFU/kg feed. Incubator hatched chicks are particularly amenable to probiotic intervention since they are deprived of protective microbiota that they would naturally acquire from the hen and environment. Probiotics are likely to be most effective when administered to newly hatched chicks, before the caecal microbiota stabilises at approximately 4 – 6 weeks of age (Mead, 1989).

The first probiotic preparation for poultry was administered by oral gavage (Nurmi and Rantala, 1973), which, although useful in trials when precise control of the dose is required, is impractical for poultry farmers. Subsequently, administration via drinking water was introduced (Rantala, 1974), which has been as effective as oral gavage (Seuna *et al*., 1978; Wierup *et al*., 1988), although chicks may refuse to drink water containing the probiotic and the viability of anaerobes rapidly declines (Seuna *et al*., 1978). Aerosol (Pivnick and Nurmi, 1982) and spray applications (Goren *et al*., 1984) have been developed and, particularly alongside administration in drinking water, have been effective in the CE of *Salmonella* and *E. coli* (Schneitz *et al*., 1990; Wolfenden *et al*., 2007). Probiotics rarely produce optimal results when administered in pelleted feed, which may be due to the partial or total destruction of the bacteria by the pelleting process. *Bacillus* species that sporulate can survive high temperatures and are ideal candidates for administration in feed (Cartman *et al*., 2008; La Ragione *et al*., 2001). Other methods for administration to livestock include capsules, paste and powder (Fuller, 1992). The method of administration may depend on the aim of the use of the probiotic, the frequency of administration and the animal to which it is administered.

1.3.7 Mechanisms of action of probiotics

1.3.7.1 Competitive exclusion

CE implies the prevention of entry of an entity into a certain environment by occupying the available space. This may occur by the competing entity being better

suited to establish and maintain itself in the environment or producing a product toxic to its competitor. The beneficial effects of consuming fermented yoghurt were first attributed to the colonisation of the GI tract by *L. acidophilus* in fermented yoghurt (Rettger and Chaplin, 1921). It was later demonstrated that natural resistance to infection in chickens develops with the establishment of mature intestinal microbiota (Milner and Shaffer, 1952) and that poultry caecal cultures inhibit the growth of pathogens *in vitro* (Royal and Mutimer, 1972), producing a similar effect to antimicrobials (Tortuero, 1973). The term "competitive exclusion" was introduced following studies that orally inoculated chicks with intestinal content from adult birds to increase resistance to *Salmonella* infection (Nurmi and Rantala, 1973). CE against a number of pathogens including *Campylobacter*, *Clostridium*, *E. coli* and *Salmonella* was subsequently demonstrated. Undefined preparations reduced the prevalence of *Salmonella* in experimentally challenged chicks (Goren *et al*., 1984), however results are variable under field conditions (Stavric and d'Aoust, 1993). Defined cultures have had less significant results under experimental conditions, with potency decreasing during storage and repeated laboratory manipulation (Mead *et al*., 1989; Stavric *et al*., 1991). Defined cultures can act as CE agents in poultry, adhering to intestinal tissues and mitigating the colonisation of pathogens (Fuller, 1977; Jin *et al*., 1996b). Lactobacilli compete with pathogens for receptor sites of adherence on the intestinal surface (Sissons, 1989). The attachment of probiotic bacteria to host epithelial cells is well documented and their close association may affect intracellular activity and cell metabolism, suggesting a potential for cross-talk between the intestinal microbiota and epithelial cells (Hooper *et al*., 2002; Xu and Gordon, 2003). Adherence via the action of proteins or glycoproteins termed lectins, is essential for proliferation and reducing the rate of removal of the probiotic from the GI tract due to the peristaltic movement of digesta. In chicks, maximal colonisation by intestinal microbiota occurs at 48 – 72 hours after treatment (Soerjadi *et al*., 1982b). Early colonisation by a dense mat of microbiota is important to protect against infection since *Salmonella* colonise the GI tract of chickens in the absence of microbiota, supporting the notion of CE by direct competition for receptor binding sites (Conway *et al*., 1987; Stavric, 1987).

CE is considered to result also from competition for nutrients leading to the out-competition of pathogens by probiotics and indigenous microbiota. For example, the consumption of monosaccharides by probiotics may reduce the growth of clostridia, which rely on monosaccharides (Wilson and Perini, 1988). The complexity of nutrient utilisation in the GI tract makes it difficult to define the specifics of this mechanism.

1.3.7.2 Antagonistic activity

Probiotics can antagonise pathogens through the production of antimicrobial substances. Both homo- and heterofermentative lactobacilli produce organic acids, which exert an antimicrobial effect on pathogens. Specifically, formate, proprionate and butyrate can modulate bacterial pathogenicity (Gantois *et al*., 2006; van Immerseel *et al*., 2006) and also mediate the pH of the GI tract, which further exerts a strong antimicrobial effect (Skrivanova and Marounek, 2007). The pH affects the amount of undissociated acid available to diffuse across the cell membrane and dissociate, creating a high proton concentration and depleting the proton motive force and intracellular ATP, which causes membrane permeabilisation and sensitises the cell to bacteriocins, detergents and bile (Coconnier-Polter *et al*., 2005; de Keersmaecker *et al*., 2006). Much of the antimicrobial activity of probiotics is attributed to the production of lactic acid, which acts as a membrane permeabiliser by lowering the internal pH of susceptible bacteria, causing sublethal damage (Fayol-Messaoudi *et al*., 2005; Makras *et al*., 2006).

Bacteriocins have a biologically active protein moiety with bactericidal action (Tagg *et al*., 1976). Lactobacilli produce numerous antagonistic substances, including many characterized bacteriocins (Joerger and Klaenhammer, 1986; Upreti and Hinsdill, 1975). One class of bacteriocins is lantibiotics, which are bactericidal and form pores in the cell membrane, causing non-specific leakage or inhibit peptidoglycan biosynthesis and cell wall formation (Brotz and Sahl, 2000). Another class of bacteriocins are the bacteriolysins, which hydrolyse the cell wall of sensitive bacteria, often via glycylglycine endopeptidase activity, which cleaves pentaglycine cross-bridges (Wu *et al*., 2003).

Reuterin or β-hydroxypropionaldehyde is a broad spectrum antimicrobial produced by some *L. reuteri* strains active against a range of pathogens and across a wide pH range (Rodriguez *et al*., 2003). It is produced by the activity of coenzyme B12-dependant glycerol dehydratase in the presence of glycerol and anaerobic conditions. Moreover, reutericyclin is a tetrameric acid produced by *L. reuteri* that exhibits antimicrobial activity by dissipating the proton motive force (Ganzle, 2004).

Some lactobacilli produce hydrogen peroxide in presence of oxygen (Martin *et al*., 2008a; Strus *et al*., 2004). Hydrogen peroxide-producing lactobacilli have shown strong antimicrobial activity against a range of pathogens, including *Salmonella* (Eschenbach *et al*., 1989). The antimicrobial effect is likely to result from the formation of a highly reactive oxygen species (hydroxyl group) from its dissociation (Halliwell, 1978).

1.3.7.3 Digestion and gastrointestinal health

Supplementing the diet with probiotics may aid in the metabolism of dietary nutrients. The addition lactobacilli in maize, barely and/or soybean diets of poultry can stimulate appetite and increase fat and mineral retention (Nahanshon *et al*., 1994, 1996). Lactobacilli produce digestive enzymes that may enrich the concentration of intestinal enzymes and specifically, lactobacilli from the GI tract of chickens have exhibited amylase, protease, and lipase activity (Jin *et al*., 1996a; Szylit *et al*., 1980). A probiotic mixture containing *Lactobacillus* species significantly increased carbohydrase activity in the mucosal tissue of pigs (Collington *et al*., 1990). In chickens fed *L. acidophilus* or yoghurt, the enzymic activities of nitroreductase, azoreductase and β-glucuronidase decreased (Cole *et al*., 1984; Coloe *et al*., 1984; Goldin and Gorbach, 1984).

L. acidophilus and *B. subtilis* reduce the concentration of ammonia in the litter of poultry and improve the condition and growth of animals as ammonia damages the cell surface and increases susceptibility to infection (Chiang and Hsiem, 1995). Probiotics, including lactobacilli act as potent inducers of mucin secretion, which is vital in clearing the contents of the intestinal lumen and preventing the translocation of bacteria across the intestinal epithelium (Mack *et al*., 2003). Secreted mucins act as barriers to enteropathogens, although some such as *Salmonella* bind and utilise mucins (Vimal *et al*., 2000) and thus, the stimulation of mucins may not be ideal for their antagonisation. Probiotics can produce substances, perhaps secondary metabolites, that neutralise enterotoxins, such as a metabolite produced by *L. bulgaricus* that neutralises enterotoxins released from coli-forms (Schwab *et al*., 1980; Stuart *et al*., 1978).

1.3.7.4 Immunomodulation

Immunity resulting from exposure to a variety of antigens, including pathogens, is essential in the defence of young animals against enteric infections (Perdigon *et al*., 1995). The administration of probiotics manipulates the gut microbiota and influences the immune response, but the mechanisms that facilitate immunomodulation are unclear (McCracken and Gaskins, 1999). Probiotics stimulate different immune cells to produce cytokines to regulate the immune response (Christensen *et al*., 2002; Lammers *et al*., 2003). Lactobacilli can induce cytokines including γ-interferon, interleukins (IL) and tumour necrosis factor (TNF)-α (Blum *et al*., 2002; Rakoff-Nahoum *et al*., 2004; Schultz *et al*., 2003). The induction of IL-4 and IL-10 aids in the development of B cells and immunoglobulin (Ig) isotype switching required for the antibody production.

The effect of probiotics on immunomodulation in poultry has been investigated extensively. Lactobacilli can modulate the systemic antibody response to antigens in chickens (Apata, 2008; Huang *et al*., 2004; Kabir *et al*., 2004). Birds treated with *L. reuteri* exhibit longer ileal villi and deeper crypts in a response associated with enhanced T cell function and increased production of anti-*Salmonella* IgM antibodies (Dunham *et al*., 1993). Supplementation of chicken diets with lactobacilli can increase the cellularity of Peyer's patches in the ileum and stimulate the mucosal immune system (Nahanshon *et al*., 1994) and production of antibodies, enhancing serum and intestinal natural antibody levels (Haghighi *et al*., 2005). Lactobacilli administered to poultry infected with *Eimeria* provided protection via immunomodulation and stimulation of early immune responses to improve defence against coccidiosis (Dalloul *et al*., 2003).

1.3.8 Other food supplements and their effect on reducing infection in poultry

In contrast to probiotics, where live microorganisms are ingested, prebiotics are administered to stimulate the growth and/or activity of the resident microbiota, which may suppress deleterious bacteria and have other health benefits on the host (Parracho *et al*., 2007; Sako *et al*., 1999). Prebiotics are typically oligosaccharides, found naturally in breast milk (galactooligosaccharide), chicory (fructooligosaccharide), garlic and onions (fructans) (Delzenne *et al*., 2003; Mikkelsen *et al*., 2003). They are non-digestible in the GI tract and may bind to pathogens directly and be utilised by probiotic bacteria such as lactobacilli to produce metabolites such as bacteriocins in the lower GI tract (Collins and Gibson, 1999). Prebiotics can modify the composition and metabolic activity of the microbiota in the GI tract of poultry due to the fermentation of these oligosaccharides forming short-chain fatty acids (SCFA) and lactate (Corrigan *et al*., 2011; Kim *et al*., 2011). Prebiotics can reduce intestinal colonisation by pathogens including *Salmonella* and *Clostridium* in poultry (Agunos *et al*., 2007; Collins *et al*., 2009; Spring *et al*., 2000) and enhance the growth of intestinal microbiota and quantities of fermentation products, such as SCFAs (Depeint *et al*., 2008; Macfarlane *et al*., 2008; Rabiu *et al*., 2001). SCFAs can aid in mineral absorption, proliferation of enterocytes, suppression of inflammation, inhibition of pathogens and stimulation of mucus production (Ito *et al*., 1997; Scheppach, 1994; Tzortzis *et al*., 2005). Additionally, prebiotics can increase serum antibody levels and induce pro-inflammatory cytokine and mucosal IgA responses, which may aid in bacterial clearance (Janardhana *et al*., 2009; Scholtens *et al*., 2008; Vos *et al*., 2007).

1.4 Aims and objectives

The aforegoing introduction provides evidence that *Brachyspira* is an important zoonotic pathogen that is a major cause of enteric disease in laying, broiler and meat-breeder hens. The resulting disease, AIS, poses significant problems to farmers worldwide with significant economic impact. Alternative intervention strategies are urgently required against this disease that is reported to be increasing, at least partially attributed to the EU ban on the use of antimicrobials as growth promoters in livestock. A limited understanding of the pathobiology of the aetiological agent has hindered the development of effective intervention strategies for *Brachyspira*-related diseases, such as AIS. The aim of this study was towards gaining a better understanding of *B. pilosicoli*, a species known to cause AIS, through genotyping and phenotyping and to investigate potential probiotic intervention strategies against AIS. Thus, the testable hypothesis of this project is:

Improving our understanding of the pathobiology of B. pilosicoli will aid the development of novel intervention strategies for related diseases, such as AIS

To fulfil this testable hypothesis, the specific aims and objectives of this project are:

- To develop an improved experimental challenge model for AIS in laying hens.
- To improve understanding of the pathobiology of *B. pilosicoli* by sequencing the whole genome of a strain that has fulfilled Koch's postulates, performing comparative genomics on available *B. pilosicoli* genome sequences and using phenotypic screening methods to validate genotypic differences.
- To select *Lactobacillus* strains as probiotic candidates to be used in further studies to intervene in AIS.
- To develop novel avian *in vitro* systems to model *Brachyspira* infection to evaluate probiotic intervention candidates prior to *in vivo* studies, using experimentally challenged laying hens.

Chapter 2.

Materials and methods

2.1 Bacteriological methodology

2.1.1 Bacterial strains and culture conditions

A total of 29 *Brachyspira* strains were used in the studies presented in this thesis, representing six of the seven known species in addition to two proposed species. The host and source of each of the *Brachyspira* strains is detailed in Table 2.1. *Brachyspira* stock cultures were maintained in FCS (Sigma-Aldrich) + 30% (v/v) *Brachyspira* enrichment broth (BEB) (Rasback *et al*., 2005), at -80˚C. *Brachyspira* were cultured on fastidious anaerobe blood agar (FABA) or *Brachyspira* selective agar (Rasback *et al*., 2005), which contains the selective ingredients spectinomycin dihydrochloride, vancomycin and colistin sulphamethane, in an anaerobic cabinet (10% hydrogen and 10% carbon dioxide in nitrogen) (Don Whitley Scientific) at 37˚C for 3 – 5 days. Where *Brachyspira* broth cultures were required, under anaerobic conditions, bacterial cells were aseptically picked from the agar surface with a sterile cotton swab, suspended in BEB and incubated under the same conditions.

Table 2.1 *Brachyspira* strains used in the studies presented in this thesis. Details of the provenance of each strain are provided.

Species	Strain	Host	Source[a]
B. alvinipulli	AN1263/2/04	Chicken	SVA
B. alvinipulli	AN3382/2/03	Chicken	SVA
B. alvinipulli	C1^T	Chicken	Murdoch University
B. alvinipulli	C5	Chicken	Murdoch University
"B. canis"	DogB	Chicken	Murdoch University
"B. canis"	D24	Chicken	Murdoch University
B. hyodysenteriae	B78^T	Pig	Murdoch University
B. hyodysenteriae	P18A	Pig	Murdoch University
B. hyodysenteriae	Q9348.6	Pig	Murdoch University
B. hyodysenteriae	WA1	Pig	Murdoch University
B. innocens	AN3165/2/03	Chicken	SVA
B. innocens	AN4113/03	Chicken	SVA
B. innocens	AN4341/03	Chicken	SVA
B. innocens	B2960	Chicken	AHVLA
B. innocens	Q97.3289.5.5	Chicken	Murdoch University
B. intermedia	AN3370/03	Chicken	SVA
B. intermedia	P280-1	Pig	Murdoch University
B. intermedia	Q98.0446.2	Chicken	Murdoch University
B. intermedia	UNL-2	Pig	Murdoch University
B. murdochii	155/20	Pig	Murdoch University
B. murdochii	56-150^T	Pig	Murdoch University
B. murdochii	AN181/1/04	Chicken	SVA
B. murdochii	AN3549/1/03	Chicken	SVA
B. pilosicoli	95/1000	Pig	Murdoch University
B. pilosicoli	B2904	Chicken	AHVLA
B. pilosicoli	WesB	Human	Murdoch University
B. pilosicoli	CPSp1	Chicken	Murdoch University
"B. pulli"	B37ii	Chicken	Murdoch University
"B. pulli"	Bp605	Chicken	Murdoch University

[a] Source: Murdoch University, Perth, Australia (received from David J. Hampson); SVA, National Veterinary Institute, Uppsala, Sweden (received from Désirée S. Jansson); AHVLA, Animal Health and Veterinary Laboratories Agency, Winchester, United Kingdom (isolated from faeces of chickens).

Eighteen *Lactobacillus* strains were used in the studies presented in this thesis and are detailed in Table 2.2. All strains were obtained from the culture collection at the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, United Kingdom). *Lactobacillus* stock cultures were maintained in heart infusion broth (HIB) + 30% (v/v) glycerol (Oxoid) at -80˚C. Lactobacilli were cultured on de Man-Rogosa-Sharpe (MRS) agar and in MRS broth (de Man *et al*., 1960), microaerophilically in an anaerobic jar using a GasPak plus system (BBL) (94% hydrogen and 6% carbon dioxide) at 37˚C for 16 hours. Heat-inactivated lactobacilli were prepared by resuspending the lactobacilli in 0.1 M sterile phosphate buffered saline (PBS) at the desired cell density and heating aliquots of the viable bacterial suspensions at 80˚C for 20 minutes in a thermal cycler (Alpha Labs). Lactobacilli spent growth medium was obtained by centrifuging the lactobacilli broth cultures (10^9 CFU/ml) (2500 × g) for 10 minutes at ambient temperature and filtering the supernatant through a 0.2 μm filter (Sartorius Stedim) to yield the CFS. The pH value of the CFS was adjusted accordingly for each assay using 10 M sodium hydroxide (Sigma-Aldrich).

Table 2.2 *Lactobacillus* strains used in the studies presented in this thesis. Details of the provenance of each strain are provided.

Species	Strain	Origin
L. acidophilus	B2990	Human
L. acidophilus	B2993	Human
L. buchneri	B2997	Vegetable
L. bulgaricus	B2991	Dairy
L. bulgaricus	B2999	Dairy
L. casei	B2986	Human
L. casei	B2995	Dairy
L. fermentum	B2992	Vegetable
L. plantarum	B2989	Vegetable
L. plantarum	B2994	Vegetable
L. plantarum	B2996	Vegetable
L. plantarum	JC1 (B2028)	Swine
L. reuteri	B2026	Swine
L. reuteri	LM1	Poultry
L. rhamnosus	B2987	Human
L. rhamnosus	B2988	Human
L. rhamnosus	B2998	Dairy
L. salivarius	LM2	Poultry

E. coli K12 was obtained from Gibco. *E. faecalis* ATCC 29212 and *E. faecium* SF11770 were obtained from the culture collection at the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, United Kingdom). *E. coli* and *E. faecalis* stock cultures were maintained in HIB + 30% (v/v) glycerol at -80˚C. Both species were cultured aerobically on lysogeny broth (LB) agar without glucose (LB-G) (Bertani, 1951, 2004) at 37˚C for 16 hours and in LB-G broth under the same conditions, with gentle agitation (225 rpm).

2.1.2 Isolation of *Brachyspira* from chicken faeces

The two *Brachyspira* strains obtained from the Animal Health and Veterinary Laboratories Agency (AHVLA, Winchester, United Kingdom) (Table 2.1), were isolated in the United Kingdom from chicken faeces. *B. pilosicoli* B2904 was originally isolated from the faeces of a chicken exhibiting clinical signs of AIS and *B. innocens* B2960 was isolated from the faeces of a healthy chicken.

For the primary culture, a sterile swab was used to aseptically sample from the core of the faecal sample. The swab was then inoculated onto *Brachyspira* selective agar, which was incubated anaerobically at 37˚C for 3 – 8 days. When haemolytic growth was observed on the surface of the agar, the presence of *Brachyspira* was confirmed using a wet smear examined under dark field microscopy (Olympus CX21, ×1000). Following confirmation of the presence of *Brachyspira* from uncontaminated primary culture, a small haemolytic block of *Brachyspira* selective agar was homogenised in 0.1 M sterile PBS and inoculated onto FABA agar. In cases where other contaminating bacterial species were evident, contaminants were removed from the surface of the agar using a sterile swab and small haemolytic blocks of *Brachyspira* selective agar were placed in the centre of a 0.2 µm filter disc (Whatman) on FABA agar and incubated anaerobically at 37˚C for 2 – 5 days to achieve pure *Brachyspira* culture.

2.1.3 Isolation of *Lactobacillus* from chicken faeces

L. reuteri LM1 and *L. salivarius* LM2 were isolated from the faeces of healthy conventional, commercial laying hens that were proven free from *Brachyspira* by culture and PCR (Phillips *et al*., 2005). Serial dilutions of the faeces in 0.1 M sterile PBS were inoculated onto MRS agar, which facilitated lactobacilli selection. Single colonies of different morphology were selected and stored in HIB with 30% (v/v) glycerol (Oxoid) at -80˚C for further characterisation.

2.1.4 Gram stain

A sterile swab was used to smear surface growth of *Brachyspira* on FABA agar or *Lactobacillus* on MRS agar onto a glass slide, which was then heat-fixed and 1% (w/v) crystal violet (Sigma-Aldrich) added at ambient temperature for 60 seconds. After washing with sterile water, the slide was treated with Lugol's iodine solution (Sigma-Aldrich) for 60 seconds, then washed in acetone (Sigma-Aldrich) and the counter stain, dilute carbol fuchsin (Sigma-Aldrich) applied for 60 seconds. The slide was then washed with sterile water, air-dried and examined under oil immersion light microscopy (Olympus CX21, ×1000). The morphology and Gram stain of the bacteria present was recorded.

2.1.5 *Brachyspira* identification and characterisation

2.1.5.1 Indole test

A heavy suspension of *Brachyspira* in BEB (≥ McFarland 4.0) was prepared by transferring surface growth from FABA agar using a sterile swab. The inoculated BEB broth was incubated anaerobically, at 37˚C for 24 hours, after which 150 µl API JAMES (Kovac's) reagent (BioMérieux) was added. Following a 10 minute incubation at ambient temperature, results were recorded whereby the formation of a pink-red pellicle was regarded as positive and a yellow pellicle as negative for the ability to cleave indole from tryptophan.

2.1.5.2 Hippurate test

A cloudy suspension of *Brachyspira* in 1% (w/v) sodium hippurate solution (Sigma-Aldrich) (≥ McFarland 2.0) was prepared by transferring surface growth from FABA agar using a sterile swab. The suspensions were incubated anaerobically at 37˚C for 24 hours, after which 150 µl API NIN (ninhydrin) reagent (BioMérieux) was added. Following 10 minute incubation at ambient temperature, results were recorded whereby a blue-purple colour change was regarded as positive and a clear-orange colour change as negative for the ability to hydrolyse sodium hippurate to glycine and sodium benzoate.

2.1.5.3 α-Glucosidase, β-glucosidase and α-galactosidase activity tests

A heavy suspension of *Brachyspira* in 0.1 M sterile PBS (≥ McFarland 4.0) was prepared by transferring surface growth from FABA agar using a sterile swab. Three

aliquots of the suspension were transferred into separate universal tubes and an α-glucosidase (p-nitrophenyl-α-D-glucopyranoside), β-glucosidase (p-nitrophenyl-β-D-glucopyranoside) or α-galactosidase (p-nitrophenyl-α-D-galactopyranoside) diatab (Rosco Diagnostics) was added to each. The suspensions were incubated at anaerobically at 37˚C for 16 hours. Results were recorded whereby a yellow colour change was regarded as positive and no colour change as negative for the respective enzyme activity.

2.1.5.4 Slide agglutination test

Where necessary, the speciation of *B. hyodysenteriae* was confirmed using the SAT (Burrows and Lemcke, 1981; Hampson, 1991). A drop of *B. hyodysenteriae* antiserum was applied onto a glass slide. Surface growth of pure *Brachyspira* culture was transferred from FABA agar using a sterile swab and mixed with the antiserum to produce a milky suspension. After gentle agitation at ambient temperature for 30 seconds, results for agglutination were recorded as positive or negative.

2.1.5.5 Indirect fluorescent antibody test

Where necessary, the speciation of *B. hyodysenteriae* was confirmed using the IFAT (Lemcke and Burrows, 1981; Lysons and Lemcke, 1983). A weak suspension of *Brachyspira* in sterile water (≥ McFarland 0.5) was prepared by transferring surface growth from FABA agar using a sterile swab. A wet smear was prepared on a glass slide and examined under dark field microscopy (×1000) to ensure the presence of approximately 20 – 30 *Brachyspira* cells per field. The slide was air-dried, fixed in acetone at ambient temperature for 10 minutes, air-dried again and a drop of *B. hyodysenteriae* antiserum was applied at 37˚C for 45 minutes. The slide was then washed in 0.1 M sterile PBS for 15 minutes, air-dried and a drop of anti-rabbit-fluorescein isothiocyanate (FITC) secondary antibody (Sigma-Aldrich) was applied at 37˚C for 45 minutes. After air-drying, the slide was mounted in Vectashield mountant (Vector Laboratories) and examined using confocal laser scanning microscopy (Leica TCS SP2 AOBS system attached to a Leica DM IRE2 microscope equipped with Ar-Kr, 488 nm, He-Ne, 546 nm and diode, 405 nm laser excitation, Leica Microsystems) under oil-immersion, using ×40 and ×63 objectives. Results for fluorescence were recorded as positive or negative.

2.1.5.6 Antimicrobial susceptibility testing of *Brachyspira* strains

Antimicrobial susceptibility testing in *Brachyspira* was performed by the agar dilution method as previously described (Brooke *et al*., 2003a). The antimicrobials tested were gentamicin, tiamulin and tylosin, which were obtained from Sigma-Aldrich. The antimicrobial doubling series for both gentamicin and tylosin ranged 0.25 – 512 µg/ml and for tiamulin the doubling series ranged 0.016 – 0.5 µg/ml. Antimicrobial stock solutions were prepared as according to the manufacturer's instructions, diluted in sterile water to double the highest tested concentration and filter-sterilised using a 0.2 µm filter. Sterile antimicrobial solutions were further diluted into 10 ml volumes of sterile water containing defibrinated ovine blood and diluted over the required MIC range. The diluted antimicrobial solutions were poured into 10 ml molten double strength agar, melted at 54˚C in a water bath, gently agitated and immediately poured into a sterile Petri dish (Sterilin). The agar was then cooled to set and dried. The agar plates were prepared immediately prior to inoculation.

Surface growth from a pure *Brachyspira* culture was transferred from FABA agar to BEB using a sterile swab and incubated anaerobically at 37˚C for 24 hours. The organisms were then dispersed by gentle agitation and suspensions prepared in 0.1 M sterile PBS (≥ McFarland 1.0), which were inoculated on the antimicrobial-containing agar plates using a multipoint inoculator. After 3 – 5 days of anaerobic incubation at 37˚C, MICs were recorded as the minimum concentration to inhibit growth of the test organism by 90% of its normal growth. A negative control plate containing no antimicrobials was prepared and *B. hyodysenteriae* P18A was used as a control strain (Burch, 2008).

2.1.5.7 Scanning electron microscopy of *B. pilosicoli*

Surface growth from a pure *Brachyspira* B2904 culture was transferred from FABA agar to BEB using a sterile swab and incubated anaerobically at 37˚C for 3 days. The culture (5×10^7 CFU/ml) was washed in 0.1 M sterile PBS and fixed in 3% (v/v) glutaraldehyde (Sigma-Aldrich). Fixed cells were washed with 0.1 M sterile PBS, post-fixed in 1% (w/v) osmium tetroxide (Agar Scientific), dehydrated in ethanol (Sigma-Aldrich), of increasing concentrations to 100% (v/v), and subsequently treated with hexamethyldisilazane (Sigma-Aldrich) for 5 minutes. Air-dried cells were settled on poly-L-lysine glass cover slips (Sigma-Aldrich), attached to aluminium stubs, sputter-coated with gold and examined under scanning (S)EM (Stereoscan S-250 MK3 SEM, Cambridge Instruments).

2.1.6 Identification and characterisation of *Lactobacillus*

2.1.6.1 API 50 CH identification system

The API 50 CH identification system (BioMérieux) was used to identify and speciate lactobacilli, according to the manufacturer's instructions. Briefly, suspensions of *Lactobacillus* cells (≥ McFarland 2.0) were obtained by transferring colonies from MRS agar culture into suspension medium (BioMérieux). Six drops of this suspension was transferred into the API 50 CHL medium (BioMérieux), which was used to inoculate the tubes of the API strip. Mineral oil was added on top of each of the tubes of the strips, which were incubated aerobically at 37˚C for 48 hours. Results were recorded visually at 24 and 48 hours post-inoculation and analysed using an online database (apiweb^{TM}, BioMérieux).

2.1.6.2 Catalase test

Surface growth of *Lactobacillus* was transferred from MRS agar and smeared onto a glass slide. A drop of 3% (w/v) hydrogen peroxide solution (Sigma-Aldrich) was applied to the slide. Results were recorded immediately with the production of gaseous bubbles regarded as catalase positive and the absence of gaseous bubbles as catalase negative. Catalase-positive *E. coli* K12 was used as a positive control.

2.1.6.3 Hydrogen peroxide production assay

The ability of lactobacilli to produce hydrogen peroxide was tested using an established method (Martin *et al*., 2008a). Briefly, MRS agar plates containing 100 μM 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) and 10 μg/ml horseradish peroxidase (HRP) (Sigma-Aldrich) was prepared. After air-drying, the lactobacilli were cultured on the agar plates and incubated micro-aerophilically at 37˚C for 48 hours. Following a 30 minute exposure to atmospheric air, results were recorded whereby the development of a pale or intense blue colour was regarded as positive for weak or strong hydrogen peroxide production, respectively and no colour change was regarded as negative.

2.1.6.4 Antimicrobial susceptibility testing of *Lactobacillus* strains

Antimicrobial susceptibility testing in *Lactobacillus* was performed by the broth microdilution method as recommended in the guidelines for testing for antimicrobial resistance in potential probiotic candidates (EFSA, 2008). All antimicrobials were

obtained from Sigma-Aldrich. The antimicrobial doubling series for ampicillin, chloramphenicol, clindamycin, erythromycin and gentamicin ranged 0.06 – 128 µg/ml and for kanamycin, streptomycin, tetracycline and vancomycin ranged 1 – 256 µg/ml. Antimicrobial stock solutions were prepared as according to the manufacturer's instructions, diluted in sterile water to double the highest tested concentration and filter-sterilised using a 0.2 µm filter. The double strength concentration of antimicrobial was added to the first well and double diluted into Lactic acid bacteria (LAB) susceptibility medium (LSM), which supports *Lactobacillus* growth and has no effect on the potency of antimicrobials tested (Klare *et al*., 2005).

E. faecalis ATCC 29212 was used as a control strain. Suspensions of *Lactobacillus* and *E. faecalis* in 0.1 M sterile PBS (≥ McFarland 1.0) were prepared by transferring surface growth of pure culture MRS or LB-G agar respectively, using a sterile swab. The cell suspension (10 µl) was added to the wells of a 24-well plate (Nunc) containing the test antimicrobial dilutions (1 ml) and incubated aerobically at 37˚C for 24 hours. MICs were recorded as the lowest antimicrobial concentration to inhibit growth of the test organism.

2.1.6.5 Gastric acid tolerance assay

Gastric juice was retrieved and prepared from SPF chickens as described previously (Lin *et al*., 2007). Briefly, SPF chickens were euthanased by cervical dislocation and the gizzard aseptically removed. Digesta was collected from the gizzard, mixed with double the volume of sterile water and centrifuged (3000 × g) for 30 minutes. The supernatants were collected, pH measured and adjusted where necessary to pH 2.0, 2.5 and 3.0. Prior to use, the gastric juice was filtered through a 0.45 µm sterile filter (Sartorius Stedim).

To test the tolerance of *L. reuteri* LM1 and *L. salivarius* LM2 to the avian gastric acid, *Lactobacillus* MRS broth culture (10^9 CFU/ml) was centrifuged (5000 × g) for 10 minutes and the pellet was resuspended in an equal volume of sterile gastric juice, at pH 2.0, 2.5 or 3.0 and 0.1 M PBS (pH 7.2) as a control. The lactobacilli were incubated in these conditions micro-aerophilically at 37˚C for 3 hours. Viable bacterial counts were determined at 0 and 3 hours, by plating serial dilutions in 0.1 M sterile PBS onto MRS agar and incubating micro-aerophilically at 37˚C for 48 hours. Assays were performed in triplicate on three separate occasions.

2.1.6.6 Bile tolerance assay

The tolerance of the *L. reuteri* LM1 and *L. salivarius* LM2 to oxgall (Sigma-Aldrich), containing bovine bile salts, the two major poultry bile salts, taurocholic acid and sodium taurochenodeoxycholate (Sigma-Aldrich) and bile collected from the gall bladder of SPF chickens was determined by modification of previously described methods (Tsai *et al*., 2005; Yu and Tsen, 1993). *Lactobacillus* MRS broth culture (10^9 CFU/ml) was inoculated at 0.1% (v/v) into MRS broth with and without oxgall, taurocholic acid, sodium taurochenodeoxycholate at 0.3% (w/v) and poultry bile at 0.3%, 0.6% and 0.9% (v/v). For each condition, 100 µl of each broth mixture was transferred to a 96-well microtitre plate (Iwaki) and incubated in the FLUOstar OPTIMA (BMG Labtech) at 37˚C in triplicate. MRS broth mixtures without lactobacilli were added to standardise the optical density (OD) readings, which were taken at an absorbance of 600 nm, every 15 minutes for 30 hours. Assays were performed in triplicate on three separate occasions.

Data were analysed using a previously proposed method (Chateau *et al*., 1994), whereby the length of time it took growth to reach a 0.3 unit difference in absorbance at 600 nm was used to determine the delay of growth, or lag time, between the control and test conditions. The lag time was used to categorise strains as resistant, where the delay was equal to or less than 15 minutes; tolerant, where the delay was between 15 and 40 minutes; weakly tolerant, where the delay was between 40 and 60 minutes and sensitive, where the delay was over 60 minutes.

2.1.7 Growth and inhibition assay of *B. pilosicoli*

In preliminary growth and inhibition assays, *Lactobacillus* CFS (pH 3.8) at 10% (v/v) was added to BEB inoculated with *B. pilosicoli* B2904 (10^6 CFU/ml) and incubated anaerobically at 37˚C. Control broths were prepared containing MRS (pH 5.8) at 10% (v/v). *Brachyspira* were enumerated at 24 hour intervals over a 120 hour period using a Helber counting chamber (Hawksley) under dark field microscopy (×1000). Assays were performed in triplicate on three separate occasions.

In subsequent assays, heat-inactivated lactobacilli (10^6 CFU/ml) and their CFS, at 10% (v/v), at original (3.8) or adjusted (4.5 and 7.2) pH value, was added to BEB inoculated with either *B. pilosicoli* B2904 or *B. innocens* B2960 (10^6 CFU/ml) and incubated anaerobically at 37˚C. Control broths were prepared containing MRS, at 10% (v/v), at pH 3.8, 4.5 and 7.2. *Brachyspira* were enumerated at 24 hour intervals over a 120 hour period using a Helber counting chamber under dark field microscopy (×1000).

Additionally, 100 µl of each broth mixture was transferred to a 96-well microtitre plate and incubated in the FLUOstar OPTIMA under anaerobic atmosphere, at 37˚C. BEB broth mixtures without *Brachyspira* were added to standardise the OD readings, which were taken at an absorbance of 600 nm, every 2.77 hours for 125 hours. Assays were performed in triplicate on three separate occasions.

2.1.8 Agar motility and haemolysis inhibition assay of *B. pilosicoli*

Agar motility inhibition assays were performed using the 'spot test' as previously described (Bernardeau *et al*., 2009). Each *Lactobacillus* strain, viable or heat-inactivated, resuspended in 0.1 M sterile PBS (10^9 CFU/ml) was pre-incubated with either *B. pilosicoli* B2904 or *B. innocens* B2960 cell suspensions in 0.1 M PBS (10^9 CFU/ml) (1/1, v/v) in a microcentrifuge tube (Eppendorf), anaerobically, at 37˚C for 4 and 24 hours. Following pre-incubation, 5 µl of each mixed suspension was spotted in triplicate onto *Brachyspira* selective agar (Rasback *et al*., 2005) and incubated anaerobically at 37˚C for 8 days. The extent of motility and hemolysis were examined visually at 24 hour intervals and compared to the growth of *B. pilosicoli* B2904 or *B. innocens* B2960 cell suspension controls, respectively. Following the monitoring period, growth from each assay was subcultured onto FABA to assess viability. Assays were performed in triplicate on three separate occasions.

2.1.9 Biolog Phenotype MicroArray™

All *Brachyspira* strains (Table 2.1) excluding *B. innocens* B2960 were analysed using the Biolog Phenotype Microarray™ (PM) technology (Bochner *et al*., 2001; Borglin *et al*., 2009) for high throughput substrate utilisation screening, which included 191 unique carbon sources (PM1 and PM2). Biolog PM™ panels and reagents were supplied by Biolog and used according to the manufacturer's instructions. Briefly, under anaerobic conditions, bacterial cells were aseptically picked from the FABA agar surface with a sterile cotton swab and suspended in 10 ml of Biolog inoculating fluid (IF-0) until a cell density of 40% transmittance was reached on a Biolog turbidimeter. Prior to addition to 96-well Biolog PM™ microtitre plates, bacterial suspensions were further diluted into 12 ml of IF-0 (per plate) in sterile water. Biolog PM™ microtitre plates were pre-incubated with two AGELESS® oxygen absorbers (Mitsubishi) 48 hours prior to inoculation, at ambient temperature. The resuspended bacterial cells were pipetted into the 96-well plates at a volume of 100 µl/well. Prior to removal from the anaerobic chamber, one AGELESS® oxygen absorber and one CO_2GEN compact sachet

(Oxoid) were attached per Biolog PM™ panel, which were then placed into 4 oz Whirl-Pak® Long-Term Sample Retention Bags (Nasco) with the open end heat-sealed.

Substrate utilisation was measured via the reduction of a tetrazolium dye (clear yellow) to formazan (purple), indicative of cellular respiration at 37˚C. Experiments were also run at 42˚C, using bacteria cultured at this temperature. Formazan formation was monitored at 15 minute intervals for 120 hours in OmniLog apparatus. Kinetic data were analysed with OmniLog-PM software. Each experiment was performed on at least two separate occasions for each strain. It was noted that although tetrazolium dye reduction is indicative of cellular respiration, it can occur independent of cell growth (Bochner, 2009; Bochner *et al*., 2001).

Blank PM1 and PM2 controls were run, whereby IF-0 was added in place of the bacterial cell suspension, to assess for abiotic reactions that occur in the anaerobic atmosphere across the 120 hour monitoring period. The following compounds were omitted from analysis due to the nature of the abiotic reactions that occurred in wells containing these compounds, under the conditions of the study: D-arabinose and L-arabinose, dihydroxyacetone, D-glucosamine, 5-keto-D-gluconate, L-lyxose, palatinose, D-ribose, 2-deoxy-D-ribose, sorbate, D-tagatose and D-xylose.

2.2 Molecular biology methodology

2.2.1 Extraction of genomic DNA

The cetyltrimethylammonium bromide (CTAB) DNA extraction method was used to purify high molecular weight genomic DNA from pure bacterial culture (Wilson, 1990). A heavy suspension of *Brachyspira* or *Lactobacillus* in 0.1 M sterile PBS (≥ McFarland 4.0) was prepared by transferring surface growth from FABA or MRS agar, respectively using a sterile swab. Cell suspensions were centrifuged (2500 × g) for 10 minutes and the supernatant discarded. The pellet was resuspended in 400 µl Tris-ethylene-diamine tetra-acetic acid (EDTA) (TE) buffer (1×) (Sigma-Aldrich) and the cells lysed by the addition of 10% (w/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich) and proteinase K solution (20 mg/ml) (Sigma-Aldrich) at 55˚C for 10 minutes. For lactobacilli, prior to the addition of SDS and proteinase K solution, the pellet was resuspended in TE buffer (1×) containing lysozyme (20 mg/ml) at 37˚C for 60 minutes. RNase (10 mg/ml) (Sigma-Aldrich) was added and incubated at 37˚C for 30 minutes followed by 100 µl 5 M sodium chloride (Sigma-Aldrich) and 80 µl CTAB (Sigma-Aldrich) at 55˚C for 10 minutes. Subsequently, 750 µl chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich) was added and the suspension centrifuged (16000 × g) for 5 minutes. The aqueous phase was transferred to a phase lock tube (Eppendorf) and the chloroform/isoamyl alcohol wash was repeated twice. The aqueous phase was then transferred to a microcentrifuge tube and 400 µl isopropanol (Sigma-Aldrich) was added at -20˚C for 30 minutes and then centrifuged (16000 × g) for 10 minutes. The pellet was washed in 70% (v/v) ethanol, centrifuged briefly and the supernatant carefully removed using a pipette. After air-drying, the pellet was resuspended in 50 µl nuclease-free water (Ambion). The NanoDrop™ spectrophotometer (Thermo Scientific) was used to determine the concentration of DNA in the extract, measured by absorbance at 260 nm and the purity of the DNA was determined from the ratio of absorbance at 260 nm to absorbance at 280 nm, of which pure DNA has a ratio of 1.7 – 1.9. The DNA extract was stored at -20˚C.

2.2.2 Extraction of bacterial DNA from faeces

The QIAamp DNA stool mini kit (Qiagen) was employed for faecal DNA extraction from poultry faecal samples according to the manufacturer's instructions. Briefly, a sample (180 – 220 mg) was taken from the core of the faeces and placed in a microcentrifuge tube on ice. The faecal sample was lysed by the addition of buffer ASL,

vortexed to homogenise the sample and incubated at 70˚C for 5 minutes. The sample was then vortexed for 15 seconds and centrifuged (16000 × g) at ambient temperature for 60 seconds. The supernatant was transferred to a new microcentrifuge tube and an InhibitEX tablet was added and vortexed to suspend the tablet and remove DNA-degrading substances and PCR inhibitors present in the stool sample, which adsorb to the InhibitEX matrix. The sample was centrifuged (16000 × g) for 3 minutes and the supernatant transferred to a microcentrifuge tube and centrifuged (16000 × g) for 3 minutes. The supernatant was transferred to a microcentrifuge tube with proteinase K and the cell lysis buffer AL was added, vortexed and incubated at 70˚C for 10 minutes. Subsequently, 100% (v/v) ethanol was added to the lysate and this was transferred to the QIAamp spin column placed in a collection tube. The spin column was centrifuged (16000 × g) for 60 seconds and then placed in a new collection tube. The DNA bound to the QIAamp silica membrane in the spin column was washed with buffer AW1 and then AW2, centrifuging (16000 × g) for 3 minutes after the application of each buffer, to ensure removal of residual impurities. The spin column was transferred to a microcentrifuge tube and 50 µl elution buffer AE was applied directly to the QIAamp membrane and centrifuged (16000 × g) for 60 seconds to elute the DNA. The concentration and purity of the genomic DNA was assessed using the NanoDrop™ spectrophotometer. The DNA extract was stored at -20˚C.

2.2.3 Polymerase chain reaction

PCR was use to amplify target DNA sequence up to 1500 bp (Saiki *et al*., 1988). Briefly, a 20 µl reaction mixture was prepared, consisting of HotStarTaq® DNA polymerase MasterMix (Qiagen), forward and reverse primers designed for the amplification of the target sequence (20 pmol each) (Sigma-Aldrich), template DNA (20 – 50 ng/µl) and sterile water. The volume of each of the constituents was scaled accordingly for PCR reactions of greater volumes.

PCR amplifications were performed using a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems) as follows; 95˚C for 15 minutes, followed by 30 cycles of denaturation at 95˚C for 60 seconds, annealing at the required temperature (45 – 65˚C) for 60 seconds and extension at 72˚C for the required extension time (45 – 60 seconds per Kb), and a final extension at 72˚C for 7 minutes before cooling to 4˚C.

2.2.4 Long-range PCR

Where the amplification of target DNA sequence >1500 bp was necessary, long-range PCR was performed using Elongase® enzyme mix (Invitrogen). The 50 µl reaction consisted of 200 µM deoxyribonucleotide triphosphate (dNTP) mix, 200 nM each of the forward and reverse primers, genomic DNA template (100 ng), 60 mM tris-sulphate, 18 mM ammonium sulphate, 1.6 mM magnesium sulphate, Elongase® enzyme mix and sterile water.

PCR amplifications were performed on a GeneAmp® PCR system 9700 thermal cycler as follows; 94˚C for 30 seconds, followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at the required temperature (55 – 65˚C) for 60 seconds and extension at 68˚C for the required time (45 – 65 seconds per Kb), then temperature cooled to 4˚C.

2.2.5 Purification of PCR products

The QIAquick® PCR purification kit (Qiagen) was used for the purification of PCR products (100 bp – 10 Kb) by the removal of unwanted primers and impurities according to the manufacturer's instructions. Briefly, five volumes of the binding buffer PB was added to one volume of the PCR product and the mixture was transferred to a QIAquick spin column in a collection tube, which was centrifuged (16000 × g) for 60 seconds. The flow-through was discarded and the ethanol-containing buffer PE was applied to the spin column to remove salts. After centrifuging (16000 × g) for 60 seconds, the flow-through was discarded and an additional centrifugation step performed to remove any residual buffer. The spin column was placed in a microcentrifuge tube and 30 µl elution buffer EB applied to the centre of the QIAquick silica membrane. The PCR product was eluted by centrifugation (16000 × g) for 60 seconds and the eluent was stored at -20˚C.

2.2.6 Gel electrophoresis

Agarose (Promega) was melted in Tris-Acetate-EDTA (TAE) buffer (1×) (Sigma-Aldrich) at 0.8% (w/v) and set in a gel casting tray using a comb to form loading wells. The gel was placed in a Sub-Cell tank (Bio-Rad), submerged in TAE buffer (1×) and 1 µl DNA loading buffer (6×) (Promega) was added to each 5 – 10 µl DNA or PCR sample prior to loading. A 100 bp or 1 Kb DNA molecular marker ladder (Promega) was run alongside DNA samples. Once all samples were loaded, the gel was electrophoresed at 70 volts (V) for approximately 60 minutes. Subsequently, the gel was

stained in 1 µg/ml ethidium bromide solution (Sigma-Aldrich) for 30 minutes, de-stained in distilled water for 10 minutes and then visualised under ultra-violet (UV) light using a GeneGenius gel imaging system (Syngene).

2.2.7 Identification of *Brachyspira* using PCR

Established *Brachyspira* genus- and species-specific PCRs were employed for the identification of *Brachyspira* strains and the detection of *Brachyspira* in the *in vitro* and *in vivo* studies presented in this thesis; primer sequences and product size for each PCR are detailed in Table 2.3. Two-genus specific PCRs based on the 16S rRNA gene were used for the initial identification of all strains and also to confirm the presence or absence of *Brachyspira* in chicken faeces (Johansson *et al*., 2004; Phillips *et al*., 2005). Species-specific PCRs based on the NADH oxidase (*nox*) and 16S rRNA genes were used for the identification of *B. hyodysenteriae*, *B. intermedia* and *B. pilosicoli* strains (La *et al*., 2003; Phillips *et al*., 2006). A further *B. pilosicoli*-specific PCR based on the 16S rRNA gene was used for the detection of this species in chicken faeces (Mikosza *et al*., 2001a). The standard method was followed for each PCR (section 2.2.3).

Table 2.3 Primers for *Brachyspira* genus- and species-specific PCRs. Details are provided of the target gene, primer sequence and product size for each primer pair.

Target species	Target gene	Primer name	Primer sequence (5' – 3')	Size (bp)	Reference
Brachy[a]	16S rRNA	Br16S-F Br16S-R	TGAGTAACACGTAGGTAATC GCTAACGACTTCAGGTAAAAC	1309	(Phillips *et al*., 2005)
Brachy[a]	16S rRNA	kag007F kag009R	GTTTGATYCTGGCTCAGARCKAACG CTTCCGGTACGGMTGCCTTGTTACG	1509	(Johansson *et al*., 2004)
Bhy[b]	*nox*	H1-F H2-R	ACTAAAGATCCTGATGTATTTG CTAATAAACGTCTGCTGC	354	(La *et al*., 2003)
Bim[c]	*nox*	Int1-F Int2-R	AGAGTTTGATGATAATTATGAC ATAAACATCAGGATCTTTGC	567	(Phillips *et al*., 2006)
Bpi[d]	16S rRNA	P1-F P2-R	AGAGGAAAGTTTTTTCGCTTC GCACCTATGTTAAACGTCCTTG	823	(La *et al*., 2003)
Bpi[d]	16S rRNA	Acoli-F Acoli-R	AGAGGAAAGTTTTTTCGCTTC CCCCTACAATATCCAAGACT	439	(Mikosza *et al*., 2001a)

[a] *Brachyspira* genus-specific PCR.

[b] *B. hyodysenteriae* species-specific PCR.

[c] *B. intermedia* species-specific PCR.

[d] *B. pilosicoli* species-specific PCR.

2.2.8 Identification and characterisation of *Lactobacillus*

2.2.8.1 Multiplex PCR

An established multiplex PCR was employed for the identification of *Lactobacillus* species and also individual primer sets were used for the detection of *Lactobacillus* species from poultry faeces (Kwon *et al*., 2004). The species-specific primers were based on unique regions extending from the 16S rRNA to the 23S rRNA gene and are detailed in Table 2.4. The 50 µl reaction mixture consisted of HotStarTaq® DNA polymerase MasterMix, primers (20 pmol each), *Lactobacillus* genomic DNA template (20 – 50 ng/µl) and sterile water.

PCR amplifications were performed on a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems) as follows: 95˚C for 15 minutes, followed by 35 cycles consisting of denaturation at 95˚C for 30 seconds, annealing at 56˚C for 30 seconds and extension at 72˚C for 30 seconds, and a final 7 minute extension step at 72˚C before cooling to 4˚C.

Table 2.4 Primers for the *Lactobacillus* multiplex PCR (Kwon *et al*., 2004). Details are provided of the primer sequence and product size for each primer pair.

Target species	Primer name	Primer sequence (5' – 3')	Size (bp)[a]
All *Lactobacillus*	IDL03R	CCACCTTCCTCCGGTTTGTCA	–
All *Lactobacillus*	IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	–
L. casei group[b]	IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	727
L. acidophilus	IDL22R	AACTATCGCTTACGCTACCACTTTGC	606
L. delbrueckii	IDL31F	CTGTGCTACACCTAGAGATAGGTGG	184
L. gasseri	IDL42R	ATTTCAAGTTGAGTCTCTCTCTC	272
L. reuteri	IDL52F	ACCTGATTGACGATGGATCACCAGT	1105
L. plantarum	IDL62R	CTAGTGGTAACAGTTGATTAAAACTGC	428
L. rhamnosus	IDL73R	GCCAACAAGCTATGTGTTCGCTTGC	448

[a] Approximated length of each PCR product derived from primer pairs composed of species-specific primer and *Lactobacillus* conserved primer (IDL03R or IDL04F).

[b] *L. casei* group includes all *L. casei*-related species, including *L. casei* and *L. rhamnosus*.

2.2.8.2 16S rRNA gene sequencing

The 16S rRNA gene was amplified by PCR from a *Lactobacillus* genomic DNA template using primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al*., 1998). The 1324 bp PCR product was confirmed by gel electrophoresis and purified prior to sequencing. ABI sequencing

reactions were performed at the Central Sequencing Unit (AHVLA, Weybridge, United Kingdom). The BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used for sequencing according to the manufacturer's instructions and run on an ABI sequencer. The 10 µl reaction consisted of sequencing buffer, primer (1 pmol), DNA template (1 – 2 ng DNA per 100 bp) BigDye®. ABI cycle sequencing was carried out on 3130*xl* Genetic Analyzer (Applied Biosystems) with a 50 cm capillary using the following PCR parameters: 25 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 2 minutes.

Sequence traces were analysed and edited in SeqMan (DNAstar, Lasergene) and consensus DNA sequences were saved in EditSeq (DNAstar, Lasergene). Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) to compare the sequences to an online database (GenBank, NCBI).

2.2.8.3 Gram-positive antimicrobial resistance microarray

The Identibac AMR+ve™ microarray was employed to detect antimicrobial resistance genes in *L. reuteri* LM2 and *L. salivarius* LM2, using the *E. faecium* SF11770 control strain, according to the manufacturer's instructions (Perreten *et al*., 2005). Genomic DNA was labelled by a randomly primed polymerisation reaction using Sequenase (USB Corporation) as previously described (Bohlander *et al*., 1992). Genomic DNA (10 – 100 ng) was denatured at 94˚C for 2 minutes with primer A (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3') (40 pmol) with Sequenase buffer and whilst cooling to 10˚C for 5 minutes, Sequenase polymerase was added with dNTPs, bovine serum albumin (BSA) and dithiothreitol (DTT). In a thermal cycler, the reaction mixture was subjected to temperature ramping from 10˚C to 37˚C across 8 minutes, 37˚C for 8 minutes, 94˚C for 2 minutes, 10˚C whilst adding diluted Sequenase buffer for 5 minutes, ramping from 10˚C to 37˚C across 8 mintes and finally 37˚C for 8 minutes. The product was added with sterile water to a master mix containing dNTPs, biotin-16-deoxyuridine triphosphate (dUTP), Taq polymerase and primer B (5'-GTTTCCCAGTCACGATC-3') (100 pmol). In a thermal cycler, the reaction mixture was subjected to 35 cycles of 94˚C for 30 seconds, 40˚C for 30 seconds, 50˚C for 30 seconds and 72˚C for 2 minutes before cooling to 4˚C. The PCR product was subjected to gel electrophoresis to check for a smear (0.5 – 1 Kb), confirming the labelling amplification reaction as successful.

Microarray tubes were washed with sterile water and then hybridisation buffer was added at ambient temperature for 5 minutes. The PCR product, diluted in hybridisation buffer, was incubated at 95˚C for 5 minutes and cooled on ice prior to adding to the microarray tube at 60˚C for 60 minutes (550 rpm). Microarray tubes were subsequently washed three times with washing buffer at weakening concentrations at 30˚C for 5 minutes (550 rpm). The microarrays were blocked with a solution containing 2% (w/v) milk powder at 30˚C for 15 minutes (550 rpm). Poly-HRP Streptavidin (Clondiag) was added and incubated at 30 for 15 minutes (550 rpm). Microarrays were again washed three times with washing buffer at weakening concentrations at 30˚C for 5 minutes (550 rpm). Hybridised probes were enhanced by adding a 3,3',5,5'-TMB analog (Seramun Grün) at 25˚C for 15 minutes. Microarray images were generated by ATR-01 Reader and the data were analysed using Iconoclust software (Clondiag). Hybridisation analyses were performed in duplicate.

2.2.9 Genome sequencing and comparative genomics of *B. pilosicoli*

2.2.9.1 Genomic DNA preparation, library construction and sequencing

CTAB extraction was used to purify high molecular weight genomic DNA (Wilson, 1990) (as described in section 2.2.1). The *B. pilosicoli* B2904 and WesB genomes were sequenced on a Roche 454 FLX platform (454 Life Sciences), using a standard preparation for a 3 Kb and 8 Kb library, respectively.

For the B2904 genome, a *de novo* assembly of the sequence reads into contiguous sequences was generated using Newbler assembler software (454 Life Sciences). The reads were assembled into one scaffold of 173 contigs with an average coverage of ×20. Remaining gaps were closed by PCR walking between unlinked, contiguous sequences (Wilson, 1990), followed by Sanger sequencing. In total, 170 Sanger reads were incorporated into the assembly.

For the WesB genome, sequence data were initially assembled with Short Oligonucleotide Alignment Program (SOAP) (Li *et al*., 2008) and subsequently Newbler assembler software was used to create a combined assembly with Illumina reads. Iterative Mapping and Assembly for Gap Elimination (IMAGE) (Tsai *et al*., 2010) improved genome assemblies by targeted re-assembly of Illumina reads to span gaps within scaffolds. To check for indels (insertion/deletions) and single nucleotide polymorphisms (SNP), Iterative Correction of Reference Nucleotides (iCORN) (Otto *et al*., 2010) was applied to the genome and appropriate corrections were made. All repeats

over 100 bp were checked to ensure that they were confirmed by at least two spanning read pairs. The incomplete WesB genome was sequenced within one scaffold, with an average coverage of ×34.

2.2.9.2 Sequence analysis and annotation

The complete nucleotide sequence and annotation of *B. pilosicoli* B2904 (accession number: CP003490 Project ID: 80999) and partial nucleotide sequence and annotation of *B. pilosicoli* WesB B2904 (accession number HE793032; Project ID: 89437) have been deposited in GenBank. Scaffold sequences for unpublished genomes *B. alvinipulli* $C1^T$ and *B. intermedia* HB60 can be accessed from the Centre for Comparative Genomics, Murdoch University via e-mail request (ccg.murdoch.edu.au/). The draft genome scaffolds for *B. aalborgii* are available at the MetaHit website (www.sanger.ac.uk/resources/downloads/bacteria/metahit/).

Sequence and protein analysis and annotation (including rRNA and tRNA prediction) for the complete *B. pilosicoli* B2904 and partial *B. pilosicoli* WesB genomes was as previously described for *B. hyodysenteriae* WA1 (Bellgard *et al*., 2009) and *B. pilosicoli* 95/1000 (Wanchanthuek *et al*., 2010) unless otherwise stated.

Gene prediction and gene and protein sequence extraction was achieved using prodigal 2.50 (compbio.ornl.gov/prodigal/) (Hyatt *et al*., 2010). Initial coding DNA sequence (CDS) annotation was completed with an in-house updated compilation of the Automatic Functional Annotation and Classification Tool (AutoFACT) 3.4 (Koski *et al*., 2005), which uses BLAST to compare the CDS sequences to online databases; UniRef90 and UniRef100 (Suzek *et al*., 2007), Cluster of Orthologous Groups (COG) (Tatusov *et al*., 1997), Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000), Pfam (Sonnhammer *et al*., 1997), Simple Modular Architecture Research Tool (SMART) (Schultz *et al*., 1998), EST others (Boguski *et al*., 1993), Large SubUnit (LSU) rRNA (de Rijk *et al*., 2000), Small SubUnit (SSU) rRNA (van de Peer *et al*., 2000) and the non-redundant (nr) database (NCBI). When there is a significant match based on length and percentage identity (PID), to one of the rRNA databases, the sequence is classified as rRNA. Non-rRNA CDS are compared against the remaining databases based on a threshold e-value of 1e-05. Resulting annotations were manually checked and edited where appropriate to be consistent with previous *Brachyspira* genome annotation methodologies for comparative purposes (Bellgard *et al*., 2009; Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010). Final annotations were

assessed with the NCBI Microbial Genome Submission Tool (preview.ncbi.nlm.nih.gov/genomes/frameshifts/).

Circular maps of the chromosome were produced using Circos 0.48 (mkweb.bcgsc.ca/circus/) (Krzywinski *et al*., 2009). The chromosomes were orientated from the *oriC*, which were identified using the Ori-Finder program (tubic.tju.edu.cn/Ori-Finder/) (Gao and Zhang, 2008). All genes were colour-coded according to COG functional categories. The open source utility 'Freckle' was used for sequence dot plotting (code.google.com/p/freckle/), which was an in-house development of the Dotter tool (Sonnhammer and Durbin, 1995). Pairwise genome alignments were performed with the Artemis Comparison Tool (ACT) (Carver *et al*., 2005).

2.2.9.3 Multilocus sequence typing

The MLST dendrogram of six *Brachyspira* strains that have undergone genome sequencing, and three that are currently within unpublished genome sequencing projects being undertaken by the authors was calculated and constructed from the concatenation of seven gene nucleotide sequences (*adh*, *pgm*, *est*, *glp*, *gdh*, *thi*, *alp*) (Rasback *et al*., 2007b). These concatenated sequences were aligned by ClustalW (Larkin *et al*., 2007) and the maximum likelihood dendrogram was generated via molecular evolutionary genetics analysis (MEGA) 5 (Tamura *et al*., 2011). The condensed bootstrap maximum likelihood dendrogram was constructed from the General Time Reversible (GTR) model with a Gamma of 2.83 (+G) and an assumption that a fraction of sites (0.27) are evolutionarily invariable (+*I*).

2.2.9.4 Protein cluster analysis

Protein reciprocal blast similarity searches with a threshold maximum expected value 1e-20 were conducted with BlastlineMCL, which is an implementation of the Markov clustering algorithm (MCL) for graphs (www.micans.org/mcl/). The granularity of the output cluster was set with an inflation value of 2.5.

2.3 *In vitro* methodology

2.3.1 Culture of mucus-secreting colonic, HT29-16E cells

HT29-16E bead stock cultures were stored in liquid nitrogen (-196˚C). Cells were thawed at 37˚C in a water bath and were then reconstituted in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids (100×) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and gentamicin (50 μg/ml), in a 250 ml tissue culture flask and incubated in the presence of 5% carbon dioxide, at 37˚C, until a confluent monolayer was present. For monolayer growth, trypsinised cells were suspended in the supplemented DMEM medium, sown at 2×10^5 cells per well in 6-well plates (Nunc) and grown to confluency for 120 hours prior to use (Searle *et al*., 2009).

HT29-16E cells were cultured in a three-dimensional (3D) cell model as previously described (Honer zu Bentrup *et al*., 2006). Trypsinised cells were resuspended in GTSF-2 medium (Lelkes *et al*., 1997), to yield 2×10^5 cells/ml and combined with 5 mg/ml Cytodex^{TM} microcarrier beads (Cytodex 3, 133 – 215 μm) (Sigma-Aldrich) and 500 μl sterile glucose solution (1 g/L), then dispensed into a 50 ml rotating wall vessel (RWV) (Synthecon). The RWV was incubated in the presence of 5% carbon dioxide at 37˚C for 21 – 23 days to allow cell adherence to the beads. The culture medium was not changed for the first 48 hours, but subsequently, 90% of the culture medium was changed every 24 hours. Over the 21 – 23 days, the rotation speed was gradually increased from 13.0 to 30.0 rpm, to ensure cell-bead aggregates remained in suspension. Following 21 – 23 days of incubation, cell aggregates were removed from the RWV, resuspended to yield 5×10^5 cells/ml and seeded into 1.5 ml microcentrifuge tubes immediately prior to use.

2.3.2 Adhesion and invasion assays using HT29-16E cell models

Human, mucus-secreting colonic cells, HT29-16E, were selected for use in adhesion and invasion assays since this cell line is well-established in studies of the interaction of enteroinvasive bacteria, such as *Salmonella* and *E. coli*, with the intestinal epithelium (Kerneis *et al*., 1994; Mellor *et al*., 2009), and have demonstrated an ability to differentiate (Cohen *et al*., 1999; Jessup *et al*., 2000). Moreover, preliminary studies (not presented) confirmed that *B. pilosicoli* B2904 adhered and invaded this cell line.

Adhesion and invasion assays were performed essentially, as described previously (Dibb-Fuller *et al*., 1999; Searle *et al*., 2009; Searle *et al*., 2010). Briefly, *B. pilosicoli*

and lactobacilli inocula were prepared by centrifugation (2447 × g, 10 minutes) of a 5 day and 24 hour broth culture, respectively, after which, the pellet was resuspended in tissue culture media to yield 5×10^7 CFU/ml.

The HT29-16E monolayer and 3D cells were inoculated under different experimental strategies detailed in Table 2.5. A total of 4 ml bacterial inoculum was added to the cells seeded in the 6-well plate in the monolayer assays, whereas 1 ml bacterial inoculum was added to the cell aggregates in microcentrifuge tubes in 3D cell assays and incubated at 37˚C in an anaerobic cabinet with gentle agitation.

To enumerate the *B. pilosicoli* associated with the HT29-16E cells following the respective incubation, the cells were washed three times with Hank's balanced salt solution (HBSS) (Sigma-Aldrich) and a homogenous cell suspension was achieved by gentle pipetting and by the use of a cell scraper (Corning) in monolayer assays. To differentiate the number of intracellular (invaded) *B. pilosicoli*, 100 µg/ml gentamicin solution (Sigma-Aldrich) was added to incomplete DMEM and delivered to each well, in monolayer assays or microcentrifuge tube, in 3D cell assays, and incubated for a further 2 hours. Cells were subsequently washed three times with HBSS and a homogenous cell suspension prepared as described above. Cell suspensions were diluted serially ($10^0 – 10^{-7}$), plated onto *Brachyspira* selective agar and incubated anaerobically at 37˚C for 3 – 5 days. The number of adherent *Brachyspira* was determined by subtracting the number of invaded from the number of associated *Brachyspira*. All HT29-16E cell experiments were conducted in triplicate on three separate occasions.

2.3.3 Avian caecal *in vitro* organ culture association assay

Immediately prior to *in vitro* organ culture (IVOC) studies, thirty-six commercial 20 week-old ISA brown laying hens (confirmed free of *Brachyspira* by pooled faeces culture and PCR) were euthanased by cervical dislocation and, at *post-mortem* examination, the caeca were aseptically sampled and stored in pre-cooled complete Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% (v/v) FCS, 0.25% (w/v) lactalbumin hydrosylate, 75 mM mercaptoethanol, 0.2 µg/ml hydrocortisone (chloroform/ethanol 1:1), 0.1 µg/ml insulin and 2 mM L-glutamine and L-aspartate (Girard *et al*., 2005); all constituents were obtained from Sigma-Aldrich. Tissues were prepared as described previously (Collins *et al*., 2010). Briefly, tissues were washed in complete medium and trimmed aseptically to remove excess mesenteric adipose tissue. Tissue sections were immobilised in CellCrowns™ (Scaffdex) so that the mucosal side was immobilised between the CellCrown™ and its base, providing a

polarised IVOC system with fixed surface area and where the bacterial inoculum was limited to the mucosal side of the explant tissue. Immobilised tissues were placed into a 24-well plate (Nunc) with the mucosal side face-upwards and submerged in 500 µl complete medium.

For IVOC association assays, *B. pilosicoli* and both viable and heat-inactivated lactobacilli inocula were prepared by centrifugation (2447 × g, 10 minutes) of a 5 day and 24 hour broth culture, respectively, after which, the pellet was resuspended in complete medium to yield 10^8 CFU/ml. Tissues were inoculated under different experimental strategies detailed in Table 2.5. A total of 1 ml bacterial inoculum was added to the tissues in the 24-well plate and incubated anaerobically at 37˚C. Uninfected control tissues were used to confirm the absence of pre-existing *Brachyspira* infection. Following infection, tissues were washed thoroughly using HBSS, homogenised in 0.1 M PBS and serially diluted to facilitate enumeration. Dilutions (10^0 – 10^{-7}) were plated onto *Brachyspira* selective agar and incubated anaerobically at 37˚C for 3 – 5 days; to determine numbers of associated *Brachyspira*. All IVOC experiments enumerating viable bacteria were repeated in quadruplicate on two separate occasions.

2.3.4 Electron microscopy

Samples from 3D cell and IVOC studies were fixed in 3% (v/v) glutaraldehyde (Sigma-Aldrich) for at least 24 hours prior to processing by the Electron Microscopy Unit (AHVLA, Weybridge). SEM and transmission (T)EM were carried out as previously described (La Ragione *et al*., 2002). Duplicate samples of each condition were examined blind. For SEM, fixed specimens were washed in 0.1 M sterile PBS, post-fixed in 1% (w/v) osmium tetroxide, rinsed with 0.1 M sterile PBS, dehydrated in ethanol and placed in hexamethyldisizane (Sigma-Aldrich) for 5 minutes. Specimens were critical point dried with liquid carbon dioxide, fixed to aluminium stubs with silver conductive paint, sputter-coated with gold and examined under SEM (Stereoscan S-250 mk 3 SEM, Cambridge Instruments).

For TEM, fixed specimens were cut to 1 – 2 mm thickness, washed in 0.1 M sterile PBS, post-fixed in 1% (w/v) osmium tetroxide, dehydrated in ethanol and placed in propylene oxide prior to embedding in araldite resin (Sigma-Aldrich). The resin was polymerised at 60˚C for 48 hours and ultrathin sections at 70 – 90 nm thickness were prepared on copper grids and stained with uranyl acetate and lead citrate (Sigma-Aldrich). Sections were examined under TEM (CM-10 TEM, Philips).

Table 2.5 Experimental strategies for HT29-16E monolayer and three-dimensional (3D) cell and avian caecal *in vitro* organ culture (IVOC) studies.

Assay	Experimental strategy or step	Bacterial inoculation (CFU/ml) (incubation time)	
		HT29-16E monolayer / 3D cells	**Avian caecal IVOC[c]**
Protection[a]	*L. reuteri* or *L. salivarius* pre-incubation.	5×10^7 (30 minutes)	10^8 (30 minutes)
	B. pilosicoli inoculation.	5×10^7 (5 hours)	10^8 (2 hours)
Competition	Simultaneous inoculation of *L. reuteri* or *L. salivarius* and *B. pilosicoli*.	5×10^7 (5 hours)[d]	10^8 (2 hours)[d]
Displacement[a]	*B. pilosicoli* inoculation.	5×10^7 (5 hours)	10^8 (2 hours)
	L. reuteri or *L. salivarius* post-incubation.	5×10^7 (30 minutes)	10^8 (30 minutes)
CFS	*B. pilosicoli* inoculation with 10% (v/v) *L. reuteri* or *L. salivarius* CFS.	5×10^7 (5 hours)	10^8 (2 hours)
B. pilosicoli B2904[b]	*B. pilosicoli* inoculation with medium in place of *Lactobacillus* inoculation (containing 10% (v/v) MRS for CFS assay control).	5×10^7 (5 hours)	10^8 (2 hours)
L. reuteri LM1/ *L. salivarius* LM2[b]	*L. reuteri* or *L. salivarius* inoculation with medium in place of *B. pilosicoli* inoculation.	5×10^7 (30 minutes in protection and displacement and 5 hours in competition assays)	10^8 (30 minutes in protection and displacement and 2 hours in competition assays)
Uninfected[b]	Complete media added only (supplemented with 10% (v/v) MRS, pH 5.8 and 3.8, for CFS assay control).	No bacterial inoculum added	No bacterial inoculum added

[a] Between delivery of different bacterial inocula, HT29-16E cells or IVOC tissues were washed twice with HBSS.

[b] Control conditions.

[c] Both viable and heat-inactivated lactobacilli were administered in separate conditions for avian caecal IVOC assays.

[d] The values refer to the concentrations and incubation times of both *B. pilosicoli* and the *Lactobacillus* spp.

2.4 *In vivo* methodology

2.4.1 Experimental birds

2.4.1.1 Experimental challenge study

Thirty ISA Warren laying pullets were purchased from a commercial breeder at approximately 16 weeks of age. Upon arrival (day 1), the birds were randomly divided into three groups of ten birds each. The groups of birds were housed in separate, negative pressure rooms within a biosecure containment facility with 12 hours of artificial light per 24 hours. Commercial un-medicated pelleted feed and drinking water were provided *ad libitum*.

2.4.1.2 Intervention study

Thirty ISA Warren laying pullets were purchased from a commercial breeder at approximately 17 weeks of age. Upon arrival (day 1), the birds were randomly divided into two groups of fifteen birds each. The groups of birds were housed in separate, negative pressure rooms within a biosecure containment facility with 12 hours of artificial light per 24 hours. Commercial un-medicated pelleted feed and drinking water were provided *ad libitum*. One group received distilled water and the other, probiotic-treated group, received distilled water containing 2.5×10^8 CFU/ml *L. reuteri* LM1 throughout the study, from day 1 with replacements provided daily. *L. reuteri* LM1 was shown to survive in distilled water for 2.5 hours without significant reduction in viability and assuming chicken drink approximately 200 ml per day (20.8 ml in 2.5 hours), the chicken should consume circa 5×10^9 CFU viable lactobacilli. Furthermore, non-viable lactobacilli were shown to also inhibit *B. pilosicoli* motility.

2.4.2 Preparation of bacterial strains for administration to birds

2.4.2.1 Experimental challenge study

B. pilosicoli strains CPSp1, isolated from a chicken in Australia (Stephens and Hampson, 2002b) and B2904, isolated from a chicken in the United Kingdom were harvested by centrifugation of a 5 day BEB broth culture (2447 × g, 10 minutes) and the pellet was resuspended in 0.1 M sterile PBS to yield 5×10^8 CFU/ml.

2.4.2.2 Intervention study

B. pilosicoli B2904, isolated from a chicken in the United Kingdom was harvested by centrifugation of a 5 day BEB broth culture (2447 × g, 10 minutes) and the pellet was resuspended in 0.1 M sterile PBS to yield 5×10^8 CFU/ml. *L. reuteri* LM1, isolated from healthy conventional laying hen faeces, was harvested by centrifugation of a MRS broth culture (2447 × g, 10 minutes). The pellets were resuspended in 10 L distilled water, in which they have been shown to remain viable without significant depletion in numbers for 2.5 hours, yielding 2.5×10^8 CFU/ml.

2.4.3 Experimental challenge

2.4.3.1 Experimental challenge study

Following a week of acclimatisation, at 17 weeks of age, ten birds in the three groups were challenged with *B. pilosicoli* or sham-challenged with 0.1 M sterile PBS by oral gavage on three alternate days (days 6, 8 and 10). Each of the birds in all three groups was first dosed with 2 ml 10% (w/v) sodium bicarbonate solution (Sigma-Aldrich) by oral gavage to neutralise the crop acid, as used previously (Carroll *et al*., 2004; Randall *et al*., 2006). Following 20 minutes after the dose of sodium bicarbonate, all birds in one group were challenged by oral gavage, with 1 ml *B. pilosicoli* B2904 suspension (5×10^8 CFU/ml), another with *B. pilosicoli* CPSp1 suspension and a negative control group was sham-challenged with 1 ml 0.1 M sterile PBS.

2.4.3.2 Intervention study

For the intervention study, after a week of acclimatisation, at 18 weeks of age, fifteen birds in the two groups were challenged with *B. pilosicoli* by oral gavage on three alternate days (days 8, 10 and 12). Each of the birds in both groups was first dosed with 2 ml 10% (w/v) sodium bicarbonate solution by oral gavage to neutralise the crop acid. Following 20 minutes after the dose of sodium bicarbonate, all birds were challenged by oral gavage, with 1 ml *B. pilosicoli* B2904 suspension (5×10^8 CFU/ml).

2.4.4 Experimental monitoring during *in vivo* studies

The chickens were weighed and cloacally swabbed upon arrival and throughout both the experimental challenge and intervention studies. Cloacal swabs were plated onto *Brachyspira* selective agar, which were incubated anaerobically at 37˚C for 8 days; the plates were examined at 24 hour intervals for spirochaetal growth. When growth

was detected visually, cells were picked from the plate using a sterile swab and subjected to PCR to verify genus (Phillips *et al*., 2005) and species (Mikosza *et al*., 2001b).

Eggs were collected daily and weighed. Furthermore, in the intervention study, eggs were scored on the degree of faecal eggshell staining without knowledge of which group they belonged; scores ranged from 0 for a clean eggshell to 5 for a heavily stained eggshell, as previously described (Stephens and Hampson, 2002b).

A representative sample of fresh faeces were taken from the floor of each of the rooms on a daily basis for the duration of the study and mixed portions (1 g) were weighed and dried to constant weight to determine faecal moisture content. In the intervention study, three faecal DNA extracts were prepared from the mixed faecal samples and subjected to PCR, using previously described primers to test for the presence of the *Brachyspira* genus (Phillips *et al*., 2005), *B. pilosicoli* (Mikosza *et al*., 2001b) and *L. reuteri* (Kwon *et al*., 2004).

2.4.5 *Post-mortem* examinations

2.4.5.1 Experimental challenge study

Three birds per group were euthanased by cervical dislocation and subjected to *post-mortem* examination at 5 and 18 days after the final challenge with *B. pilosicoli*. At both *post-mortem* examinations, the caeca were aseptically removed and weighed and sections (approximately 1 g) of each of the duodenum, jejunum, ileum, caeca, colon, liver, spleen were aseptically sampled and placed in 0.1 M sterile PBS for bacterial enumeration and 10% (v/v) neutral buffered formalin (Sigma-Aldrich) for histopathological examination by haematoxylin and eosin (HE) staining. The anatomical locations of tissues sampled from the chicken GI tract is outlined in Figure 2.1A. In the first *post-mortem* examination, 5 days after the final dose, the oviduct was aseptically removed and fixed for histopathological examination. In the final *post-mortem* examination, 18 days after the final dose, sections (approximately 1 g) from the ovary, infundibulum, magnum, isthmus and the uterus/vagina region were sampled for bacteriology and histopathology. The anatomical locations of tissues sampled from the chicken oviduct is outlined in Figure 2.1B.

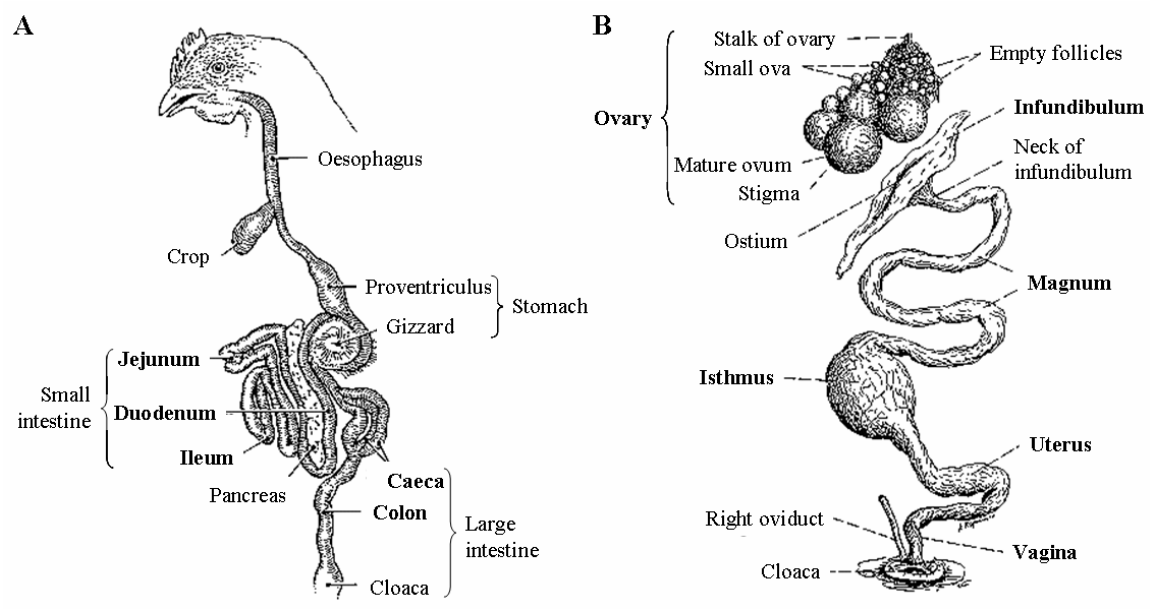

Figure 2.1 The anatomy of the gastrointestinal (GI) tract and the female reproductive tract of poultry (IACUC, 2007). Tissues sampled at *post-mortem* examination in the experimental challenge and intervention studies are emboldened.

2.4.5.2 Intervention study

Three birds per group were euthanased by cervical dislocation and subjected to *post-mortem* examination at 5 and 21 days after the final challenge with *B. pilosicoli*. At both *post-mortem* examinations, the caeca were aseptically removed and weighed and sections (approximately 1 g) of each of the duodenum, jejunum, ileum, caeca, colon, liver, spleen, ovary, infundibulum, magnum, isthmus, uterus and vagina (Figure 2.1) were aseptically sampled and placed in 0.1 M sterile PBS for bacterial enumeration. Sections of each of the caecal tonsil, caecum, colon and liver were fixed in 10% (v/v) neutral buffered formalin for histopathological examination by HE staining.

2.4.6 Bacteriological enumeration at *post-mortem* examination

For bacteriology, tissues were homogenised in 9 ml 0.1 M sterile PBS, diluted serially (10^0 – 10^{-7}) and 20 µl samples plated onto *Brachyspira* selective agar and incubated anaerobically at 37˚C for 3 – 5 days. This process was performed swiftly to minimise exposure to oxygen. *B. pilosicoli* colonies were subsequently enumerated and colonies confirmed as *B. pilosicoli* by PCR (Mikosza *et al*., 2001b; Phillips *et al*., 2005). Similarly, PCR tests were performed on the tissue homogenates to confirm the presence/absence of *Brachyspira* in tissues that were negative by culture. In the absence of distinguishing features of the *B. pilosicoli*, it was assumed that these were the strains

used for challenge, since the poultry were *B. pilosicoli*-free prior to challenge and were kept in biosecure containment.

2.4.7 Haematoxylin and eosin stain

Trimmed tissues fixed in 10% (v/v) neutral buffered formalin were processed in paraffin wax and 4 μm sections were HE stained. The samples were examined and photographed using a light microscope (Leica DM4000B microscope attached to a Leica DFC480 digital camera, Leica Microsystems, ×100, ×200, ×400).

2.5 Statistical analyses

All experiments presented in this thesis excluding the *in vivo* intervention study, consisted of three or more experimental groups, including control groups. Therefore, equivalent data from each group were compared using the one-way analysis of variance (ANOVA) and the Bonferroni post test with a 95% confidence interval. This statistical analysis compares all groups of data as a whole and indicates whether the results of at least one group differ significantly from those of another. The data were presented in graphs and tables as the mean and standard deviation of the mean and significance (*p* value) between the groups of data were calculated

The *in vivo* intervention study consisted of two experimental groups, the data of which were compared using an unpaired Student's t-test with a 95% confidence interval. The data were presented in graphs and tables as the mean and standard deviation of the mean and the significance (*p* value) between the groups of data were calculated.

For the analysis of bacterial counts from *in vitro* and *in vivo* studies, the bacterial counts were transformed to their logarithms to base ten ($\log_{10}$) for statistical analysis.

Statistical analysis of the data presented in this thesis was performed and the exact *p* values calculated using commercially available software (GraphPad Prism). Where the *p* value was below 0.05 on comparing groups of data, the difference was considered significant. Furthermore, categorisation of the *p* values was applied on graphs using an asterisk (*) grading system (Table 2.6).

Table 2. 6 Summary of *p* value categorisation. In the graphs and tables presented in this thesis, *p* values are associated with an asterisk representing the degree of significance between groups of data.

***p* value**	**Degree of significance**	**Asterisk grade**
≤0.001	Extremely significant	***
>0.001 to ≤0.01	Very significant	**
>0.01 to ≤0.05	Significant	*
>0.05	Not significant	ns

Chapter 3.

Characterisation of *Brachyspira* and development of a novel experimental challenge model for avian intestinal spirochaetosis

3.1 Introduction

AIS is an enteric disease of poultry that arises from the colonisation of the caeca and colon by the anaerobic spirochaete, *Brachyspira* (Stephens and Hampson, 2001; Swayne and McLaren, 1997), as introduced in Chapter 1 (specifically in section 1.1). Currently, three species of this genus are considered avian pathogens; *B. alvinipulli* (Stanton *et al*., 1998), *B. intermedia* (Hampson and McLaren, 1999) and *B. pilosicoli* (Stephens and Hampson, 2002a). *B. pilosicoli* has a wide host range, also causing PIS in pigs (PIS) (Trott *et al*., 1996d) and HIS in humans (Tsinganou and Gebbers, 2010), with a potential for zoonosis (Hampson *et al*., 2006b).

AIS is common in adult layer and broiler breeder flocks (Stephens and Hampson, 1999), and has been associated with a delayed onset of lay, reduced egg weights, diarrhoea, faecal staining of eggshells, reduced growth rates, increased feed consumption and non-productive ovaries (Davelaar *et al*., 1986; Feberwee *et al*., 2008; Griffiths *et al*., 1987; Swayne *et al*., 1992). A characteristic of colonisation of birds by *B. pilosicoli* is the ability to form end-on attachments to the intestinal epithelial surface and invade (Feberwee *et al*., 2008; Jensen *et al*., 2001; Jensen *et al*., 2000). Moreover, *B. pilosicoli* has been found to form a dense fringe penetrating between enterocytes that is associated with reactive and mild inflammatory responses, crypt hyperplasia and increased numbers of goblet cells (Feberwee *et al*., 2008). Similar observations have been recorded in experimental challenge studies that used *B. pilosicoli* but the disease

induced is often reported as milder than that observed in the field (Jamshidi and Hampson, 2002, 2003; Stephens and Hampson, 2002a, b). To date, no experimental challenge studies have produced a quantitative output for the colonisation of poultry by *Brachyspira*.

Until recently, the identification of *Brachyspira* species has been highly reliant on phenotypic characteristics involving biochemical testing, such as tests for indole production and hippurate hydrolysis, and microscopy to differentiate species by cell length, morphology and the number of periplasmic flagella. However, in recent years a number of molecular methods for the detection and speciation of *Brachyspira* have been developed (La *et al*., 2003; Phillips *et al*., 2006). For the studies presented in this chapter, this array of phenotypic and genotypic tests were used to accurately identify the *Brachyspira* species. Accurate identification was imperative to future studies relating to AIS given that only *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* are considered pathogenic to poultry (Hampson and McLaren, 1999; Stanton *et al*., 1998; Stephens and Hampson, 2002a) and an additional objective was set, namely to confirm Koch's postulates by poultry challenge studies using a newly isolated *B. pilosicoli* strain. As part of these *in vivo* studies, the aim was to improve on recent variable AIS models by the development of a novel *in vivo* model for AIS that produced novel clinical observations and a quantitative output of colonisation.

3.2 Results

3.2.1 Speciation and characterisation of *Brachyspira*

3.2.1.1 Phenotypic characterisation

Brachyspira strains were isolated from the faeces of poultry with suspected AIS using *Brachyspira* selective agar and subsequent subculture onto FABA agar to ensure purity. The role of the anaerobic bacteriology group at the Animal Health and Veterinary Laboratories Agency (AHVLA, Winchester, United Kingdom) who aided in this process is acknowledged. In addition, well-characterised strains were obtained from Prof. David Hampson at Murdoch University (Perth, Australia) and Dr. Désirée Jansson at the National Veterinary Institute (SVA, Uppsala, Sweden) and these were successfully cultured on FABA and *Brachyspira* selective agar.

Gram staining performed on each of the 29 *Brachyspira* strains, followed by examination under light microscopy revealed Gram-negative, helical spirochaetes. Examination of wet smears under dark field microscopy confirmed all strains as highly motile, helical spirochaetes. The 29 *Brachyspira* strains were used in biochemical tests (Fellstrom and Gunnarsson, 1995; Hommez *et al*., 1998) to identify or confirm their speciation (Table 3.1). All strains were grown on FABA agar and typical of *B. hyodysenteriae*, all strains of this species were strongly β-haemolytic and those of the other species were weakly β-haemolytic. Where speciation as *B. hyodysenteriae* was suspected, this was confirmed serologically via the SAT and/or IFAT tests, which involved the application of *B. hyodysenteriae* antiserum (Burrows and Lemcke, 1981).

3.2.1.2 Molecular characterisation

In order to perform molecular characterisation tests on the *Brachyspira* strains, genomic DNA was extracted, using the CTAB genomic DNA extraction method for use in two different *Brachyspira* genus-specific PCRs; both were based on the amplification of the 16S rRNA gene but one specifically of a 1309 bp fragment (Phillips *et al*., 2005) and the other of a 1509 bp fragment (Johansson *et al*., 2004). Species-specific PCR tests for *B. hyodysenteriae*, targeting a 354 bp region of the NADH oxidase (*nox*) gene, for *B. intermedia*, targeting a 557 bp region also of the *nox* gene and for *B. pilosicoli*, targeting a 823 bp region of the 16S rRNA gene, were also performed on DNA extracted from all strains (La *et al*., 2003; Phillips *et al*., 2006). Gel electrophoresis of the PCR products was performed and the results for each of the PCR tests are summarised in Table 3.2.

Table 3.1 Biochemical properties of *Brachyspira* strains used in further studies that aided in the phenotypic speciation of these strains. Where possible, the group to which each strain/species is assigned is given (Fellstrom and Gunnarsson, 1995; Hommez *et al*., 1998).

Species	Strain	Group	Ind[a]	Hipp[b]	α-gal[c]	α-glu[d]	β-glu[e]
B. alvinipulli	AN1263/2/04		−	+	−	−	+
B. alvinipulli	AN3382/2/03		−	+	−	−	+
B. alvinipulli	C1^T		−	+	−	−	+
B. alvinipulli	C5		−	+	−	−	+
"*B. canis*"	DogB	III	−	−	−	−	+
"*B. canis*"	D24	III	−	−	−	−	+
B. hyodysenteriae	B78^T	I	+	−	−	+	+
B. hyodysenteriae	P18A	I	+	−	−	+	+
B. hyodysenteriae	Q9348.6	I	+	−	−	+	+
B. hyodysenteriae	WA1	I	+	−	−	+	+
B. innocens	AN3165/2/03	IIIb	−	−	+	−	+
B. innocens	AN4113/03	IIIb	−	−	+	−	+
B. innocens	AN4341/03	IIIb	−	−	+	−	+
B. innocens	B2960	IIIc	−	−	+	+	+
B. innocens	Q97.3289.5.5	IIIc	−	−	−	+	+
B. intermedia	AN3370/03	II	+	−	−	+	+
B. intermedia	P280-1	II	+	−	−	+	+
B. intermedia	Q98.0446.2	II	+	−	−	+	+
B. intermedia	UNL-2	II	+	−	−	+	+
B. murdochii	155/20	III	−	−	−	−	+
B. murdochii	56-150^T	III	−	−	−	−	+
B. murdochii	AN181/1/04	III	+	+	+	+	+
B. murdochii	AN3549/1/03	III	+	+	+	+	+
B. pilosicoli	95/1000	IV	−	+	+	−	−
B. pilosicoli	B2904	IV	−	+	+	−	−
B. pilosicoli	WesB	IV	−	+	+	−	−
B. pilosicoli	CPSp1	IV	−	+	+	−	−
"*B. pulli*"	B37ii		−	−	+	−	+
"*B. pulli*"	Bp605		−	−	+	−	+

[a] Test for ability to cleave indole from tryptophan; +, positive; −, negative.

[b] Test for ability to hydrolyse hippurate to glycine and benzoate; +, positive; −, negative.

[c] Test for α-galactosidase activity; +, positive; −, negative.

[d] Test for α-glucosidase activity; +, positive; −, negative.

[e] Test for β-glucosidase activity; +, positive; −, negative.

Table 3.2 Molecular speciation of *Brachyspira* strains by PCR. Results of the two *Brachyspira* genus-specific and the three species-specific PCR tests are displayed.

Species	**Strain**	**PCR**[a]			
		Brachy[b]	***Bhy***[c]	***Bim***[d]	***Bpi***[e]
B. alvinipulli	AN1263/2/04	+	−	−	−
B. alvinipulli	AN3382/2/03	+	−	−	−
B. alvinipulli	C1^T	+	−	−	−
B. alvinipulli	C5	+	−	−	−
"*B. canis*"	DogB	+	−	−	−
"*B. canis*"	D24	+	−	−	−
B. hyodysenteriae	B78^T	+	+	−	−
B. hyodysenteriae	P18A	+	+	−	−
B. hyodysenteriae	Q9348.6	+	+	−	−
B. hyodysenteriae	WA1	+	+	−	−
B. innocens	AN3165/2/03	+	−	−	−
B. innocens	AN4113/03	+	−	−	−
B. innocens	AN4341/03	+	−	−	−
B. innocens	B2960	+	−	−	−
B. innocens	Q97.3289.5.5	+	−	−	−
B. intermedia	AN3370/03	+	−	+	−
B. intermedia	P280-1	+	−	+	−
B. intermedia	Q98.0446.2	+	−	+	−
B. intermedia	UNL-2	+	−	+	−
B. murdochii	155/20	+	−	−	−
B. murdochii	56-150^T	+	−	−	−
B. murdochii	AN181/1/04	+	−	−	−
B. murdochii	AN3549/1/03	+	−	−	−
B. pilosicoli	95/1000	+	−	−	+
B. pilosicoli	B2904	+	−	−	+
B. pilosicoli	WesB	+	−	−	+
B. pilosicoli	CPSp1	+	−	−	+
"*B. pulli*"	B37ii	+	−	−	−
"*B. pulli*"	Bp605	+	−	−	−

[a] PCR result for DNA band when subjected to gel electrophoresis; +, positive; −, negative.

[b] Two *Brachyspira* genus-specific PCR. (Johansson *et al*., 2004; Phillips *et al*., 2005).

[c] *B. hyodysenteriae* species-specific PCR (La *et al*., 2003).

[d] *B. intermedia* species-specific PCR (Phillips *et al*., 2006).

[e] *B. pilosicoli* species-specific PCR (Phillips *et al*., 2006).

3.2.1.3 Antimicrobial susceptibility

As part of the characterisation, antimicrobial susceptibility testing was performed on all *Brachyspira* strains used in the studies presented in this thesis. The MIC of the antimicrobials gentamicin, tiamulin and tylosin were determined. Tiamulin and tylosin were selected as these antimicrobials have been reported as effective against *Brachyspira* but with an emerging resistance against them (Karlsson *et al*., 1999; Pringle *et al*., 2006). It was important to determine the MIC of gentamicin against *Brachyspira* for its use in tissue culture adhesion and invasion/association studies (as discussed in Chapter 6), where this aided the determination of the number of invading *B. pilosicoli*. *B. hyodysenteriae* P18A also acted as a control for MIC testing (Burch, 2008). The MICs of the three antimicrobials against each of the *Brachyspira* strains are displayed in Table 3.3.

Table 3.3 Minimum inhibitory concentrations (MIC) of the antimicrobials tiamulin, tylosin and gentamicin against the *Brachyspira* strains used in the studies presented in this thesis.

Species	Strain	MIC (µg/ml)		
		Tiamulin[a]	Tylosin[b]	Gentamicin[b]
B. alvinipulli	AN1263/2/04	0.016	2	2
B. alvinipulli	AN3382/2/03	0.016	2	1
B. alvinipulli	C1^T	0.016	1	4
B. alvinipulli	C5	0.016	2	4
"*B. canis*"	DogB	0.016	2	2
"*B. canis*"	D24	0.016	2	2
B. hyodysenteriae	B78^T	0.25	4	2
B. hyodysenteriae	P18A	0.062	256	1
B. hyodysenteriae	Q9348.6	0.062	4	8
B. hyodysenteriae	WA1	0.062	16	8
B. innocens	AN3165/2/03	0.016	4	8
B. innocens	AN4113/03	0.125	1	8
B. innocens	AN4341/03	0.016	4	8
B. innocens	B2960	0.03	128	1
B. innocens	Q97.3289.5.5	0.125	128	8
B. intermedia	AN3370/03	0.016	16	2
B. intermedia	P280-1	0.016	2	1
B. intermedia	Q98.0446.2	0.062	128	4
B. intermedia	UNL-2	0.062	16	4
B. murdochii	155/20	0.016	2	1
B. murdochii	56-150^T	0.016	256	2
B. murdochii	AN181/1/04	0.016	4	8
B. murdochii	AN3549/1/03	0.016	1	4
B. pilosicoli	95/1000	0.016	256	2
B. pilosicoli	B2904	0.125	256	1
B. pilosicoli	WesB	0.016	2	1
B. pilosicoli	CPSp1	0.016	1	4
"*B. pulli*"	B37ii	0.016	2	1
"*B. pulli*"	Bp605	0.016	2	1

[a] Antimicrobial tested doubling series ranged 0.016 – 0.5 µg/ml.

[b] Antimicrobial tested doubling series ranged 0.25 – 512 µg/ml.

3.2.1.4 Cell shape and structure

Each *Brachyspira* was examined morphologically by SEM and an example of the outputs for one strain, *B. pilosicoli* B2904, which was the strain used in all studies and was also selected for whole genome sequencing, is displayed in Figure 3.1. SEM revealed spirochaete cells of approximately 12.0 µm in length and 0.25 µm in width. Both cell ends of this strain were pointed, with five periplasmic flagella originating at either end (Figure 3.1C). Midway along the cells where the periplasmic flagella from each pole of the cell overlap, ten periplasmic flagella were observed wrapped around the cell (Figure 3.1D). This resulted in a periplasmic flagella end:middle:end conformation of 5:10:5.

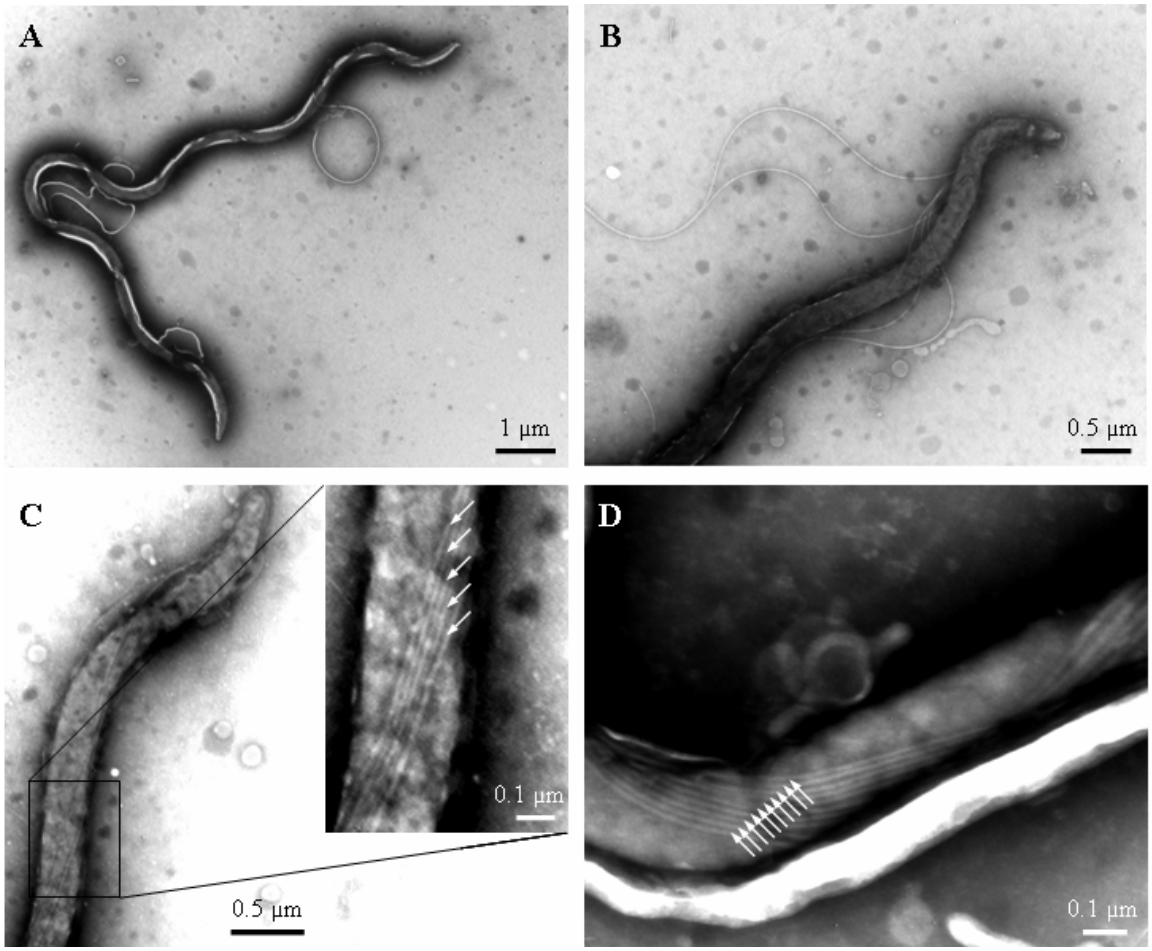

Figure 3.1 Examination of *B. pilosicoli* B2904 from broth culture (BEB) by scanning electron microscopy (SEM). The entire cell showing helical morphology (A) and once pointed cell end of *B. pilosicoli* B2904 with the flagella exposed due to disruption of outer sheath (B), are shown. Periplasmic flagella (arrows) originating at both cell ends, wrap around the spirochaete (C) and midway along the *B. pilosicoli* axis, the periplasmic flagella from either end overlap (D).

3.2.2 Establishing a novel experimental challenge model for avian intestinal spirochaetosis using *B. pilosicoli*

Due to its isolation from the faeces of a chicken exhibiting clinical symptoms of AIS and ability to grow well *in vitro*, *B. pilosicoli* B2904 was selected for use in an experimental challenge study to demonstrate its pathogenicity in chickens and fulfil Koch's postulates. *B. pilosicoli* CPSp1 was used as a positive control, to challenge a group of ISA Warren laying pullets alongside the B2904-challenged group, as experimental challenge with the CPSp1 strain was shown previously to elicit clinical symptoms comparable to that of AIS (Jamshidi and Hampson, 2002; Stephens and Hampson, 2002b). In order to improve on previous experimental challenge models for AIS, birds were pre-dosed with 10% sodium bicarbonate to neutralise the crop acid, as used previously in chickens experimentally challenged with *Salmonella* (Carroll *et al*., 2004; Randall *et al*., 2006). Furthermore, as will be demonstrated this is the first study to produce a quantitative output on *Brachyspira* colonisation at *post-mortem* examination.

3.2.2.1 Colonisation of poultry by *B. pilosicoli*

The birds in all groups were cloacally swabbed upon arrival and throughout the experiment (on days indicated in Figure 3.2A) and swiftly plated onto *Brachyspira* selective agar and incubated anaerobically at 37˚C for 8 days, to minimise exposure to oxygen. The plates were examined at 24 hour intervals for spirochaetal growth and growth was detected visually, cells were picked from the plate and subjected to PCR to verify genus (Phillips *et al*., 2005) and species (Mikosza *et al*., 2001b). All birds were free from *Brachyspira* as tested by culture and PCR prior to challenge. Following challenge, cloacal swabs from 80.0 – 85.7% of birds were positive for *B. pilosicoli* by culture with species confirmation by PCR (Figure 3.2). However, the sensitivity of this method for the detection of *B. pilosicoli* is unknown.

3.2.2.2 Bird weights

The chickens were weighed upon arrival and throughout the study (on days indicated in Figure 3.2B). Weight gain was reduced in the challenged groups with the final mean weight significantly lower than the negative control in both the B2904- ($p<0.05$) and CPSp1-challenged ($p<0.01$) groups (Figure 3.2B).

3.2.2.3 Faecal moisture content

A representative sample of fresh faeces were taken from the floor of each of the rooms for the duration of the study (on days indicated in Figure 3.2C) and portions (1 g) were weighed and dried to constant weight to determine faecal moisture content. Following challenge with CPSp1, faecal moisture content increased by 0.5 – 5.3%, but by the end of the study there was no significant difference compared to the control group (Figure 3.2C). However, challenge with B2904 increased faecal moisture by 4.1 – 7.6% compared to the control group, which was a significant difference that remained until the end of study ($p<0.01$).

3.2.2.4 Egg production

For the *in vivo* model, the age of inoculation with *B. pilosicoli* was selected as 17 weeks of age, as the birds come into lay. The objective was to assess the impact of the deliberate inoculations on the timing of coming into to lay and on egg production. Eggs were collected daily and weighed. Control birds came into lay at 18 weeks of age (day 15) whereas a delay was noted in both challenged groups (onset of lay day 22/23) (Figure 3.3). CPSp1-challenged birds laid considerably fewer eggs (n = 7) compared with B2904-challenged birds (n = 25) that were productively comparable to the control birds (n = 26). However, egg weight for the control group (54.05 g ±5.92) was significantly greater than the B2904- (48.55 g ±3.49, $p<0.001$) and CPSp1-challenged (46.65 g ±3.12, $p<0.01$) groups. Faecal staining was detected only on the eggs of the challenged groups only.

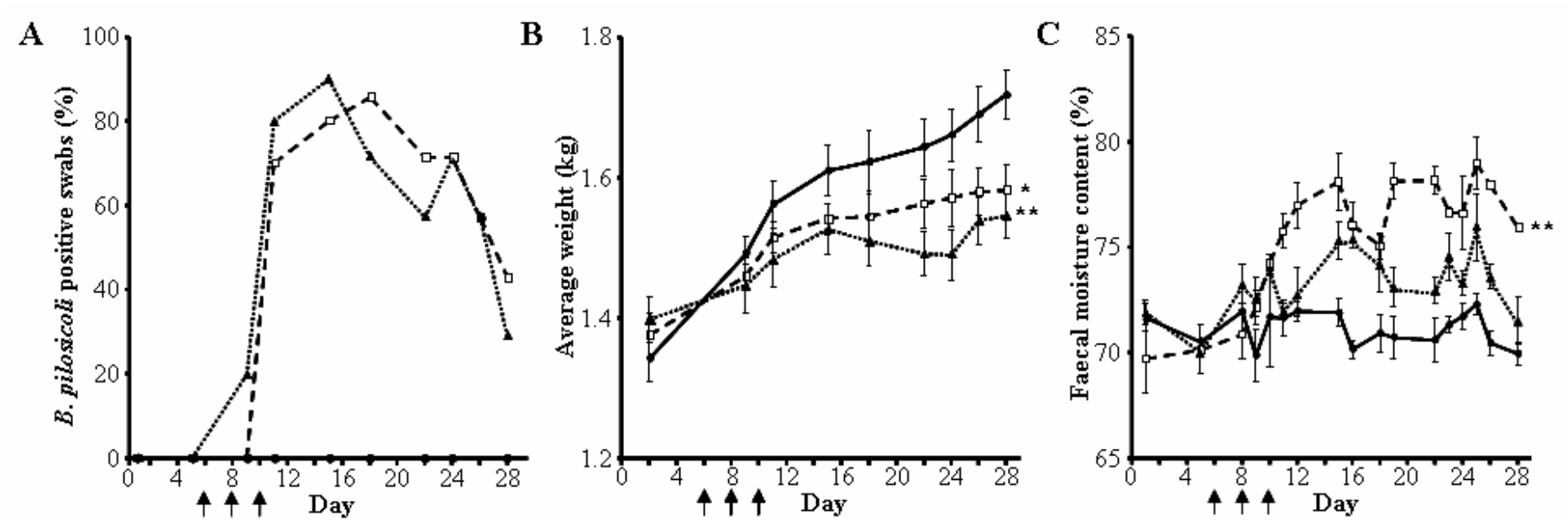

Figure 3.2 Experimental monitoring of birds throughout the experimental challenge study showing the percentage of swabs positive for *B. pilosicoli* by culture and PCR (A), the average bird weight (B) and the faecal moisture content (C). The average was based on ten birds until day 15 and then seven birds until the study end, in each of the negative control (closed circle, solid line), B2904-challenged (open square, dashed line) and CPSp1-challenged (closed triangle, dotted line) groups. The arrows on the x-axis indicate the days of challenge. Significance is shown in cases where the final data points of the challenged groups and the negative control group differed significantly; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

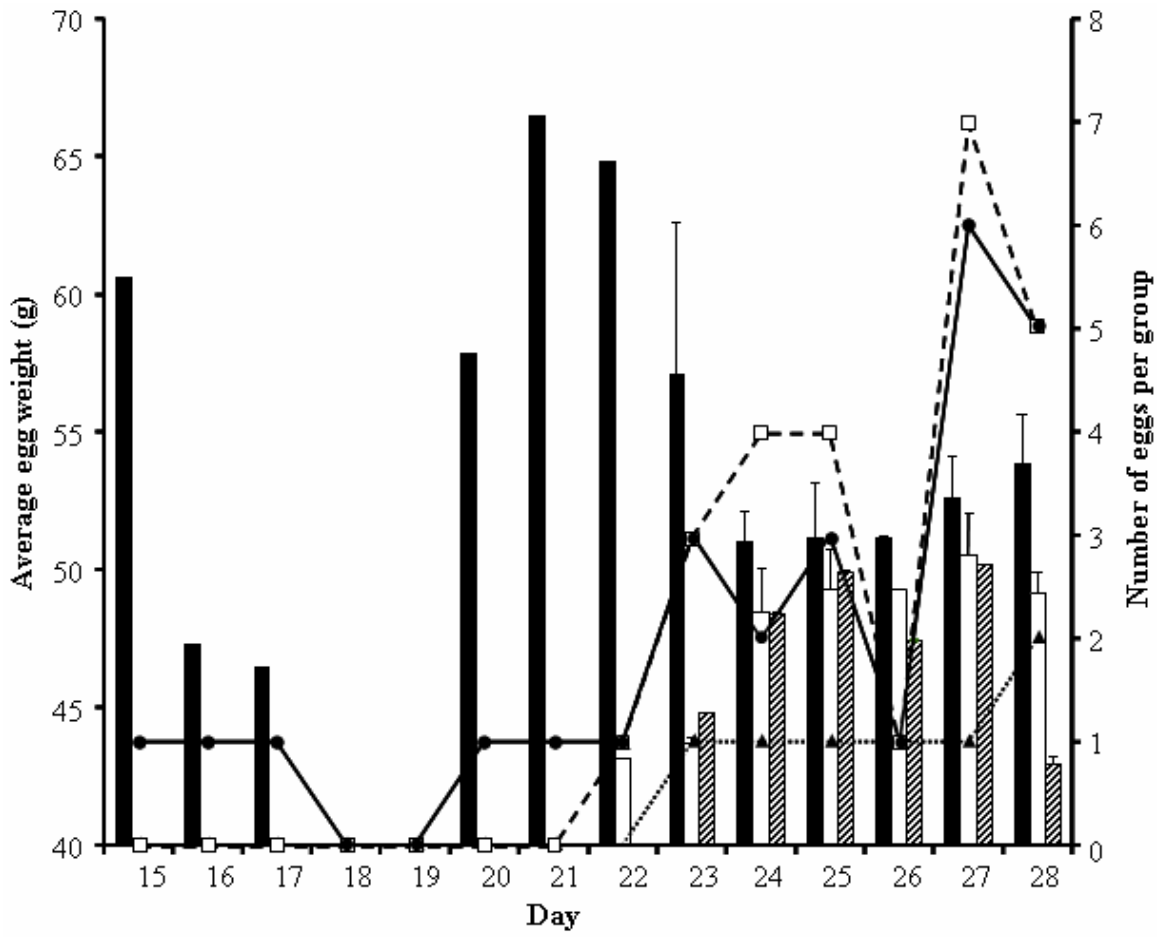

Figure 3.3 Egg production (lines) and average egg weights (bars) each day of the experimental challenge study for the point of the first egg lay. Seven birds were present from day 15 in each of the negative control (closed bar/closed circle, solid line), B2904-challenged (open bar/open square, dashed line) and CPSp1-challenged (hatched bar/closed triangle, dotted line) groups.

3.2.2.5 *Post-mortem* examination at five days post final challenge

At five days after the final challenge (day 15) three birds from each group were euthanased by cervical dislocation and subjected to *post-mortem* examination. *B. pilosicoli* were isolated at approximately 10-fold higher numbers from the caeca of the B2904- than CPSp1-challenged birds (Figure 3.4A). Caecal weight of the B2904-challenged (13.25 g ±1.48, $p<0.05$) but not the CPSp1-challenged (14.22 g ±0.80) birds was significantly lower than the control group (16.00 g ±0.24). The presence of lymphoplasmacytic cells in the caeca and colon was variable in the control and challenged birds without evidence of typhlitis. Other than the caeca and colon, *B. pilosicoli* were recovered in low numbers from the ileum, liver and spleen of two B2904-challenged birds. Pathology in the ileum of these birds included lymphoid hyperplasia, crypt abscesses and intraepithelial trafficking, mainly lymphocytic in villi

tips and crypts. In the spleen, lymphoid hyperplasia with proliferation of ellipsoid macrophages and germinal centres were recorded with increased pyknotic and karyorrhectic debris. *B. pilosicoli* was isolated from the liver of two B2904-challenged birds with moderate to severe hepatic lipidosis noted in all three birds. Petchia were detected in the duodenum of CPSp1-challenged birds, but no spirochaetes were isolated from this tissue.

3.2.2.6 *Post-mortem* examination at eighteen days post final challenge

At 18 days after the final challenge (day 28) three birds from each group were euthanased by cervical dislocation and subjected to *post-mortem* examination. Contrary to the first *post-mortem*, by the end of the study, the number of CPSp1 associated with caecal tissue was significantly greater than B2904 ($p<0.05$) (Figure 3.4B). The number of *B. pilosicoli* associated with caecal tissue was significantly greater at the end of the study compared to at five days post final challenge ($p<0.05$). Moreover, B2904 was not isolated from the ileum, liver and spleen whereas CPSp1 was isolated from the liver and spleen of one of the three birds examined.

Non-specific histological changes were noted in B2904-challenged birds, such as haemorrhages in lymphoid tissue, distended crypts and secondary follicle proliferation in the caeca of B2904-challenged birds (Figure 3.5A). By the end of the study, prominent changes were recorded in tissues of CPSp1-challenged birds with larger, more numerous crypt abscesses and dilated crypts containing cellular debris and inflammatory cells in the caecal tonsil (Figure 3.5B), indicating bacterial colonisation. The epithelium surrounding crypts displayed attenuation, degradation and necrosis alongside crypt hyperplasia, which was previously associated with *Brachyspira* infection (Feberwee *et al*., 2008). Caecal haemorrhages were common in CPSp1-challenged birds and they had more lymphocytic aggregates in the liver (Figure 2.5C) with moderate granulocytic hepatitis in one bird, perhaps associated to the isolation of *B. pilosicoli* from the liver. Prominent sheathed capillaries and secondary follicle proliferation were also noted in the spleen (Figure 3.5D), correlating with the isolation of *B. pilosicoli* from these tissues. The ileum of two CPSp1-challenged birds was distended with no spirochaetes isolated from this tissue. Regions of the oviduct were sampled at this point and both *B. pilosicoli* strains were isolated from the uterus/vagina region of one bird of each group (Figure 3.4B). Aside from a focal area of lymphoplasmacytic cells accumulated in the magnum of a B2904-challenged bird, no other significant histopathological changes were noted.

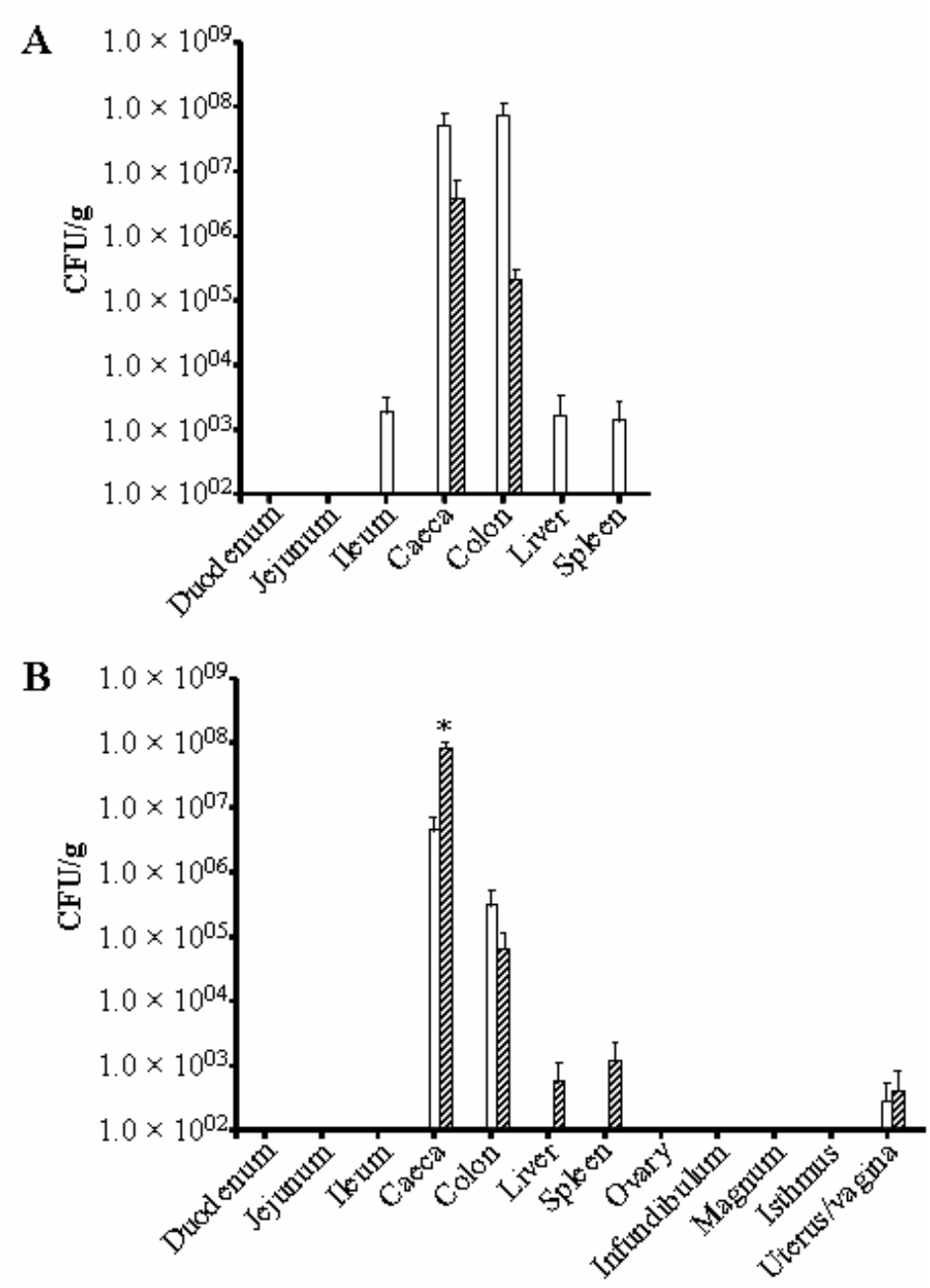

Figure 3.4 Colonisation of *B. pilosicoli* in different tissues sampled at *post-mortem*, at five days post final challenge (A) and 18 days post final challenge (B). The average was taken from three birds examined at the two time-points, from birds of the negative control (closed bar), B2904-challenged (open bar) and CPSp1-challenged (hatched bar) groups. Significance is shown in cases where colonisation of the two *B. pilosicoli* strains differed significantly; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

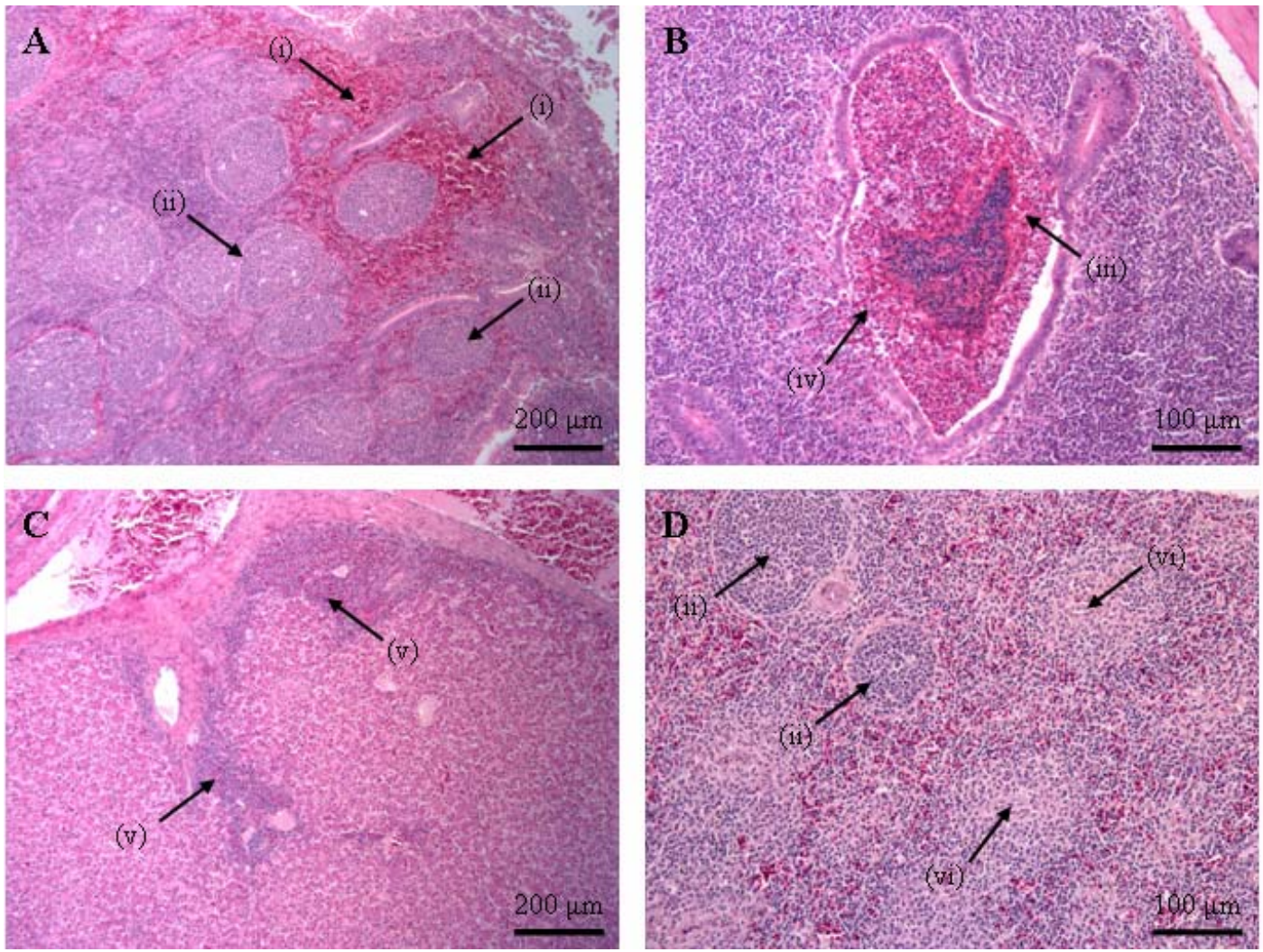

Figure 3.5 Histopathological findings in haematoxylin and eosin (HE)-stained caecal tonsil (A and B), liver (C) and spleen (D) tissues of birds experimentally challenged with *B. pilosicoli* B2904 (A) and CPSp1 (B-D). Pathology included haemorrhages in lymphoid tissue (i) and secondary follicle proliferation in caecal tonsils and spleen (ii). In the caecal tonsils, distended crypts containing a mixture of hyaline material, slough epithelial cells and inflammatory cells (crypt abscess) (iii) with epithelial attenuation and heterophilic infiltration (iv) were noted. Acute hepatitis was noted in the liver with heterophilic infiltration in periportal region and sinusoids (v). Prominent sheathed capillaries (vi) were observed in the spleen.

3.3 Discussion

In the studies presented in this thesis, two strains of each of "*B. canis*" and "*B. pulli*", four strains of each of *B. alvinipulli*, *B. hyodysenteriae*, *B. intermedia*, *B. murdochii* and *B. pilosicoli* and five strains of *B. innocens* were used. This included two newly isolated strains, *B. innocens* B2960 and *B. pilosicoli* B2904. All strains were cultured on FABA and *Brachyspira* selective agar, the latter of which contains the selective ingredients, spectinomycin, vancomycin and colistin, demonstrating all strains exhibited the intrinsic resistance characteristic to *Brachyspira* (Jenkinson and Wingar, 1981; Songer *et al*., 1976). Gram staining and examination of wet smears under dark filed microscopy revealed the *Brachyspira* strains to be Gram-negative and possess the characteristic helical morphology and sinuous movement of *Brachyspira*.

Phenotypic speciation of *Brachyspira* strains was by means of biochemical testing and a grouping system that has been described previously (Fellstrom and Gunnarsson, 1995; Hommez *et al*., 1998) (Table 3.1). In conjunction with molecular speciation by application of genus and species-specific PCRs (Table 3.2), two strains isolated from the faeces of poultry at the Animal Health and Veterinary Laboratories Agency (AHVLA, Winchester, United Kingdom) were speciated and the species of the strains received from Murdoch University (Perth, Australia) and the National Veterinary Institute (SVA, Uppsala, Sweden) was confirmed.

Antimicrobial MIC testing was performed on all *Brachyspira* strains as part of their characterisation. Tiamulin and tylosin were tested as these are the antimicrobials most frequently used to treat *Brachyspira* infection, whereas the MIC of gentamicin was important for use as the antimicrobial in tissues culture experiments described in Chapter 6. Clinical breakpoint values previously reported for tiamulin and tylosin against intestinal spirochaetes, interpreted strains with an MIC >4 μg/ml as resistant, >1 to ≤4 μg/ml as intermediate and ≤1 μg/ml as sensitive (Ronne and Szancer, 1990). Moreover, clinical breakpoint values reported for gentamicin against intestinal spirochaetes, interpreted strains with an MIC ≥10 μg/ml as resistant, 5 μg/ml as intermediate and ≤1 μg/ml as sensitive (Duhamel *et al*., 1998a). In spite of reports of emerging tiamulin resistance amongst *Brachyspira* (Clothier *et al*., 2011; Fossi *et al*., 1999; Pringle *et al*., 2006), all strains used in the studies presented in this thesis were susceptible to tiamulin (Table 3.3). This was an interesting observation because the strains were isolated from many farms with AIS where tiamulin had been used for control purposes on several of those premises (Burch, D. J. S., 2009 personal

communication). The antimicrobial effect of tiamulin and also tylosin is through their ability to inhibit protein synthesis by binding to the 23S ribosomal subunit at or close to the peptidyl transferase region. Point mutations in domain V of the 23S rRNA gene and/or the ribosomal protein L3 gene, particularly G→A mutations at position 2032, have been associated with resistance to tiamulin and other pleuromutilin antimicrobials in *Brachyspira* (Hidalgo *et al*., 2011; Pringle *et al*., 2004) and such resistance has been demonstrated i*n vitro* through exposure to the antimicrobial (Karlsson *et al*., 2001). Of the 29 *Brachyspira* test strains, only four were susceptible to tylosin, with 15 strains classed as intermediate and 10 as resistant. Tylosin resistance is more widespread amongst *Brachyspira*, particularly *B. hyodysenteriae*, which is not surprising in view of selective pressure due to the wide use of tylosin as a therapeutic and growth promoter in swine production (Karlsson *et al*., 2003; Kitai *et al*., 1987; Molnar, 1996). Resistance to tylosin and other macrolide antimicrobials in *Brachyspira* has been associated with an A→T or A→G mutation in the nucleotide position homologous with position 2058 of the *E. coli* 23S rRNA gene and susceptible strains have become resistant *in vitro* through exposure to the antimicrobial (Karlsson *et al*., 1999). No strains were considered resistant to gentamicin and nine were considered sensitive with an MIC of 1 µg/ml, including both *B. pilosicoli* B2904 and *B. innocens* B2960. This was fortuitous and as will be described later in the thesis, this permitted tissue culture studies to be undertaken where gentamicin was applied in order to allow determination of the numbers of bacteria that had invaded and are consequently protected from the action of gentamicin as this antimicrobial does not penetrate eukaryotic cells. The range of MIC values for gentamicin against the *Brachyspira* strains were similar to those previously noted (Clothier *et al*., 2011; Duhamel *et al*., 1998a).

For morphological characterisation, SEM was performed on a broth culture of the *Brachyspira* strains, as displayed for *B. pilosicoli* B2904 (Figure 3.1). This analysis revealed the typical helical morphology of *Brachyspira* species with periplasmic flagella winding around the protoplasmic cylinder, enclosed in the outer sheath. The cell dimensions were consistent with previous analysis for each species and *B. pilosicoli* B2904 had an approximate width and length of 0.25 µm and 12.0 µm, respectively, which is typical of this species (Sellwood and Bland, 1997). Moreover, this strain had pointed cell ends with five periplasmic flagella inserted at each pole, giving a periplasmic flagella end:middle:end ratio of 5:10:5 (Figure 3.1C and D), which is characteristic of this species (Sellwood and Bland, 1997) and more widely of avian intestinal spirochaetes (Stoutenburg *et al*., 1995).

In order to fulfil Koch's postulates, the newly isolated *B. pilosicoli* B2904 was selected for use in a novel experimental challenge model. This strain was selected due to it being isolated from the faeces of a chicken exhibiting clinical symptoms of AIS, displaying an ability to grow well *in vitro* and also demonstrating susceptibility to gentamicin (Table 3.3), which was important for *in vitro* tissue culture assays discussed in Chapter 6. In the experimental challenge study, *B. pilosicoli* CPSp1 was used as a positive control, due its ability to induce clinical symptoms comparable to that of AIS in experimentally challenged hens, as described previously (Jamshidi and Hampson, 2002; Stephens and Hampson, 2002b).

The experimental challenge model developed and described here appears to have produced more severe clinical symptoms, consistent with AIS, than previously described models. The colonisation rates, assessed by culture from cloacal swabs, reached 80.0 – 85.7% (Figure 3.2A), which is greater than previously described models that also used the CPSp1 strain in which colonisation rates were as low as 10.0% in some groups (Jamshidi and Hampson, 2002; Stephens and Hampson, 2002b). Furthermore, weight gain was significantly reduced in both challenged groups ($p<0.05$) (Figure 3.2B), whereas the CPSp1 strain had little effect on hen weight in previous experimental challenge studies (Jamshidi and Hampson, 2003; Stephens and Hampson, 2002a, b). This suggests that neutralising the crop prior to infection challenge may improve the survival of *B. pilosicoli* transversing the crop acid barrier facilitating enhanced colonisation of the distal intestinal tract and causing disease.

At *post-mortem*, the pathology recorded in the caeca, particularly of the B2904-challenged birds at five days post final challenge, was consistent with lymphoid hyperplasia, previously described in chickens naturally infected with *B. pilosicoli* (Feberwee *et al*., 2008) and an increased frequency of crypt abscesses colonised by *B. pilosicoli* was noted. Aside from the caeca and colon, *B. pilosicoli* were recovered in low numbers from the ileum, liver and spleen of the challenged birds. In humans, *B. pilosicoli*-like spirochaetes have been isolated from extra-intestinal tissues, including the liver (Kostman *et al*., 1995) and the bloodstream (Trott *et al*., 1997a). In duck flocks, *Brachyspira* infection was associated with hepatic and splenic amyloidosis (Glavits *et al*., 2011). The findings presented in this chapter report the first isolation of *B. pilosicoli* from the liver and spleen of chickens.

Despite a reduction in the severity of clinical symptoms by the final *post-mortem* examination, colonisation by *B. pilosicoli* persisted in both groups. *B. pilosicoli* were isolated from the caeca and colon of birds from both the B2904- and CPSp1-challenged

groups, despite their detection from cloacal swabs diminishing toward the study end (Figure 3.2A). At this stage, B2904 was not isolated from the ileum, liver and spleen, however CPSp1 was. This finding suggests B2904 may have produced pathology in the birds at an early stage of infection, relating to the normalisation of egg production and bird weight by the end of the study (Figures 3.2B and 3.3). The lack of pathology and absence of colonisation of the upper oviduct of both groups does not provide explanation for the delayed and reduced egg production. However, it is possible that *B. pilosicoli* produced pathology in the upper oviduct at an earlier stage of infection.

This experimental challenge model for AIS revealed more pathological changes previously unreported in the literature of *Brachyspira* infection in chickens, such as in the liver and spleen. Direct visualisation of the aetiological agent will be necessary to determine the specificity of the changes. The differences in the clinical symptoms and pathology between the B2904- and CPSp1-challenged birds may arise from differences in the pathogenicity of the two strains, requiring further investigation that may be aided by the increasing availability of *Brachyspira* genome sequence, although they may arise from other factors such as dose. The experiment was controlled and thus, the deep tissue colonisation is believed to be a true phenomenon and not the result of an extraneous factor, such as co-infection. Overall, the novel model of *Brachyspira* infection has improved rates of colonisation and induced hitherto unreported pathology. Furthermore, subsequent studies using the B2904-challenge model to evaluate AIS intervention strategies have shown findings consistent with this study (as discussed in Chapter 7).

In conclusion, this chapter has described the identification and characterisation of 29 *Brachyspira* strains, which will be used in the studies presented in the subsequent chapters of this thesis. *B. pilosicoli* B2904 was selected for use in the development of a novel experimental challenge model for AIS. The establishment of this experimental challenge model was vital for *in vivo* studies to evaluate AIS intervention strategies (as discussed in Chapter 7). In turn, this study demonstrated the pathogenicity of *B. pilosicoli* B2904 in chickens, providing a *B. pilosicoli* strain for which Koch's postulates have been fulfilled. As will be discussed in Chapter 4, *B. pilosicoli* B2904 was also selected for whole genome sequencing.

Chapter 4.

Comparative genomics of three strains of *Brachyspira pilosicoli* and correlation with phenotypic diversity

4.1 Introduction

Spirochaetes represent a monophyletic lineage and a major branch in eubacterial evolution; *Brachyspira* is the sole genus of the family *Brachyspiraceae* within the order Spirochaetales, which belongs to the spirochaete phylum (Ludwig *et al*., 2008). *Brachyspira* are Gram-negative, loosely coiled, aerotolerant anaerobes that colonise the lower GI tract of mammals and birds, but vary in pathogenicity. There are seven species of *Brachyspira* that are currently officially recognised: *B. aalborgi*, a potential human pathogen (Hovind-Hougen *et al*., 1982); the porcine pathogen, *B. hyodysenteriae* (Harris *et al*., 1972a); the avian pathogens, *B. alvinipulli* (Stanton *et al*., 1998) and *B. intermedia* (Stanton *et al*., 1997); the avian, porcine and human pathogen, *B. pilosicoli* (Trott *et al*., 1996d); non-pathogenic *B. innocens* (Kinyon and Harris, 1979) and *B. murdochii*, which is of uncertain pathogenic potential (Stanton *et al*., 1997). In addition, there are a number of proposed species including "*B. canis*" (Duhamel *et al*., 1998b), "*B. pulli*" (Stephens and Hampson, 1999) and "*B. suanatina*" (Rasback *et al*., 2007a) amongst others. The classification of the genus is still immature and the often used descriptors of certain *Brachyspira* as pathogenic, intermediate pathogenic or non-pathogenic is subject to debate.

B. pilosicoli is an aetiological agent of colitis and occasional spirochaetaemia in humans (Tsinganou and Gebbers, 2010), and a cause of PIS and AIS (Smith, 2005). It

may also cause disease in other species (Duhamel *et al*., 1998b) and has demonstrated potential for zoonotic transmission (Hampson *et al*., 2006b). *B. pilosicoli* is commonly found in humans living in densely populated areas with poor hygienic conditions (Lee and Hampson, 1992; Margawani *et al*., 2004; Munshi *et al*., 2004; Trott *et al*., 1997b), and in homosexual males (Trivett-Moore *et al*., 1998). *B. pilosicoli* infections are highly prevalent in intensively farmed swine and poultry, inducing inflammation in the colon and caeca, diarrhoea and reducing growth and productivity (Smith, 2005). Motility and chemotaxis are deemed important virulence factors, and, as with *B. hyodysenteriae*, *B. pilosicoli* has a chemoattraction to mucin that facilitates penetration of the mucus and association with the underlying intestinal epithelial surface (Milner and Sellwood, 1994; Naresh and Hampson, 2010). The intimate contact with the epithelia induces an outpouring of mucus and epithelial sloughing (Kennedy *et al*., 1988). An unusual feature of *B. pilosicoli* infection, which is shared only by *B. aalborgi*, is the ability to insert one cell end into the luminal surface of enterocytes in the large intestine, forming a pit-like structure, with arrays of such attached spirochaetes giving the appearance of a "false brush-border" (Harland and Lee, 1967; Trott *et al*., 1996a). This unusual form of attachment of *B. pilosicoli* also occurs in Caco-2 cells *in vitro*, resulting in apoptosis, actin rearrangement and elevated interleukin expression (Naresh *et al*., 2009).

The paucity of genomic information and absence of tools for genetic manipulation are responsible, at least partly, for the lack of knowledge regarding the adaptations that *Brachyspira* have undergone to colonise the lower GI tract of animals and humans, and for the pathogenic species to induce disease. Hence, this has also hindered the development of novel intervention strategies for *Brachyspira*-related diseases. *Brachyspira* whole genome sequences have only recently been made available for the following species: *B. hyodysenteriae* (Bellgard *et al*., 2009), *B. intermedia* (Hafstrom *et al*., 2011), *B. murdochii* (Pati *et al*., 2010) and *B. pilosicoli* (Wanchanthuek *et al*., 2010). Analysis of the four published sequences showed substantial genetic diversity, and their availability has facilitated research on the corresponding species. However, the availability of only one genome sequence per species has limited the conclusions that can be drawn from the genome as a representation for the species as a whole, and does not allow analysis of intra-species genomic variation. In this chapter, the whole genome sequence of *B. pilosicoli* B2904 is reported; this strain was isolated from a chicken that exhibited clinical symptoms of AIS in the United Kingdom and shown to fulfil Koch's postulates by inducing disease with symptoms akin to AIS in chickens (as discussed in Chapter 3). The genome of *B. pilosicoli* WesB, isolated from an Australian Aboriginal

child with diarrhoea, was also partially sequenced. Experimentally, the latter strain has been shown to colonise and cause disease in pigs (Trott *et al*., 1996a). Although the strains were isolated originally from different host species, it is unlikely that the differences that were found between them were related to their host species of origin (Hampson *et al*., 2006b). The genomes are presented alongside the whole genome sequence of *B. pilosicoli* 95/1000, isolated from a pig with PIS in Australia, and which has been confirmed to be virulent in experimental infection studies in pigs (Trott *et al*., 1996a). In order to correlate genotype with phenotype, Biolog PM™ technology was employed (Bochner, 2009; Bochner *et al*., 2001) to assess carbon source utilisation in the three strains of *B. pilosicoli*. These studies facilitated the validation of genotypic variation observed in the genome sequences and permitted detailed correlation between genotype and phenotype.

4.2 Results

4.2.1 Genetic relatedness of *Brachyspira* genomes

A dendrogram was produced based on MLST data derived from genome sequence data for six strains of *Brachyspira* that have been genome sequenced, and three that are currently within unpublished genome sequencing projects (Figure 4.1). MLST highlighted the close relationship between the *B. pilosicoli* 95/1000, B2904 and WesB, with *B. aalborgi* being distinct, but most closely related to *B. pilosicoli* and distantly related to *B. hyodysenteriae*. The two strains of *B. intermedia* included appeared less closely related than might be expected.

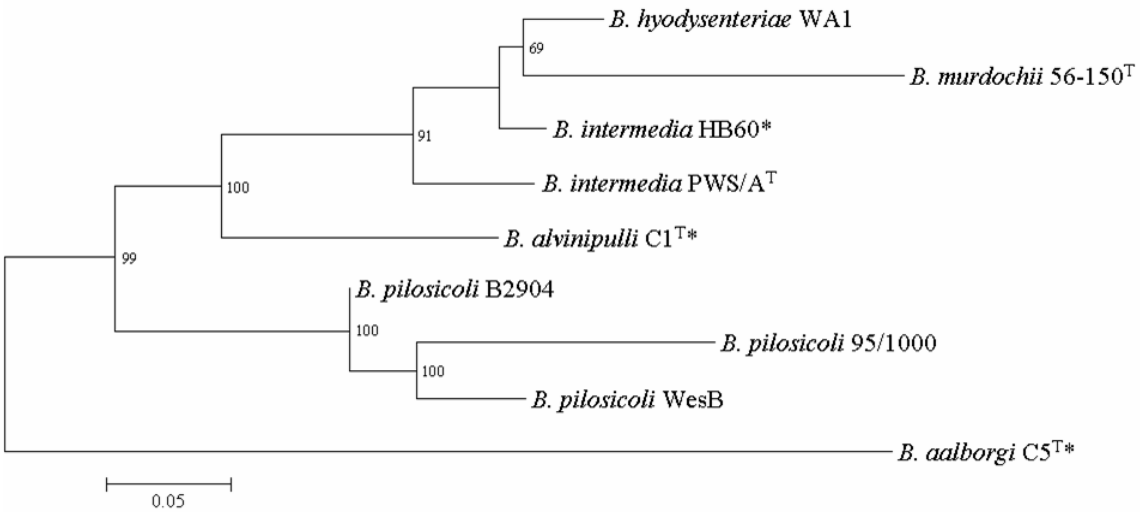

Figure 4.1 A dendrogram illustrating the relationships among nine strains of *Brachyspira*, representing six of the seven known species. Analysis was based on concatenated DNA sequences of seven multilocus sequence typing (MLST) loci (Rasback *et al*., 2007b). The genome sequences of the strains used in the analysis have either been completed or are currently within a genome sequencing project (*). The tree was constructed using the maximum likelihood method. Bootstrap values (%) are shown for stable nodes. The length of the scale bar is equivalent.

4.2.2 Comparison of general genome features

The general genome features of the three sequenced *B. pilosicoli* genomes are compared in Table 4.1. The G+C content of the *B. pilosicoli* genomes were very similar to each other (27.44% to 27.9%), and to that of other chromosomes in the genus, which range from 27.1% to 27.9% (Hafstrom *et al*., 2011). The complete genome sequence of *B. pilosicoli* B2904 consisted of a 2,765,477 bp circular chromosome (Figure 4.2),

whereas the incomplete WesB genome was larger, at 2,889,522 bp. The 2,586,443 bp genome of strain 95/1000 was the smallest of the three genomes. Not only did the *B. pilosicoli* genomes show size variability, but they were smaller than the genomes of the other sequenced species, apart from *B. aalborgi* 513^T which preliminary findings suggest is ~2.5 Mb.

Table 4.1 General genome feature comparison for strains of *B. pilosicoli* of different host origin. The comparison includes strains 95/1000 (porcine), B2904 (avian) and WesB (human).

Genome features	**95/1000**	**B2904**	**WesB**[a]
Genome size (bp)	2586443	2765477	2889522
G+C content	27.90%	27.79%	27.45%
Total predicted ORFs	2339	2696	2690
Non-significant PID and coverage ORFs	3	23	101
Significant PID and/or coverage ORFs	2336	2673	2589
rRNA genes	3	3	3
tRNA genes	34	34	34
tmRNA genes	1	1	1
hypothetical/conserved hypothetical proteins	657	590	545
genes with function prediction	1641	2045	2006
Genes assigned to COG[b]	1201	1196	1276
Genes assigned a KO number[bc]	1048	1082	1128
Genes assigned E.C. numbers[b]	523	567	563
Genes with signal peptide	244	322	316
Genes with transmembrane helices	48	61	68
Suspected truncated proteins	55	130	64
Suspected protein frameshift/deletions	4	223	50

[a] The incomplete WesB strain genome was within one scaffold.

[b] Those genes with significant PID and/or query/target coverage hits; significance equals blastx/blastp PID of at least 25% and/or 75% query or target coverage.

[c] Assigned to KO via KEGG Automatic Annotation Server (KAAS).

The disparity between the number of ORFs and genome size between the B2904 and WesB strains and the high number of non-significant PID and coverage ORFs in the WesB genome may be an artefact of the incomplete nature of this genome, which is the largest of the three strains. In 95/1000, 44.8% of ORFs were assigned a KEGG Orthology (KO), whereas only 40.5% and 43.6% of ORFs were assigned in B2904 and

WesB, respectively. A lower proportion of ORFs were matched in the COG database for B2904 and WesB compared to 95/1000.

All three strains of *B. pilosicoli* harboured the same number of tRNA, rRNA and transfer-messenger (tm)RNA genes (Table 4.1). The tRNA genes represented all 20 amino acids and there were single copies of the 5S, 16S and 23S rRNA genes. The *rrf* (5S) and *rrl* (23S) genes were co-located in all three *B. pilosicoli* genomes, with the *rrs* (16S) gene located approximately 645 Kb, 679 Kb and 773 Kb from the other rRNA genes in the 95/1000, B2904 and WesB genomes, respectively.

The origin of replication of the *B. pilosicoli* genomes was set according to the position of the *oriC* and GC-skew pattern, as previously suggested (Hafstrom *et al.*, 2011); this was supported by the Ori-Finder program (Gao and Zhang, 2008).

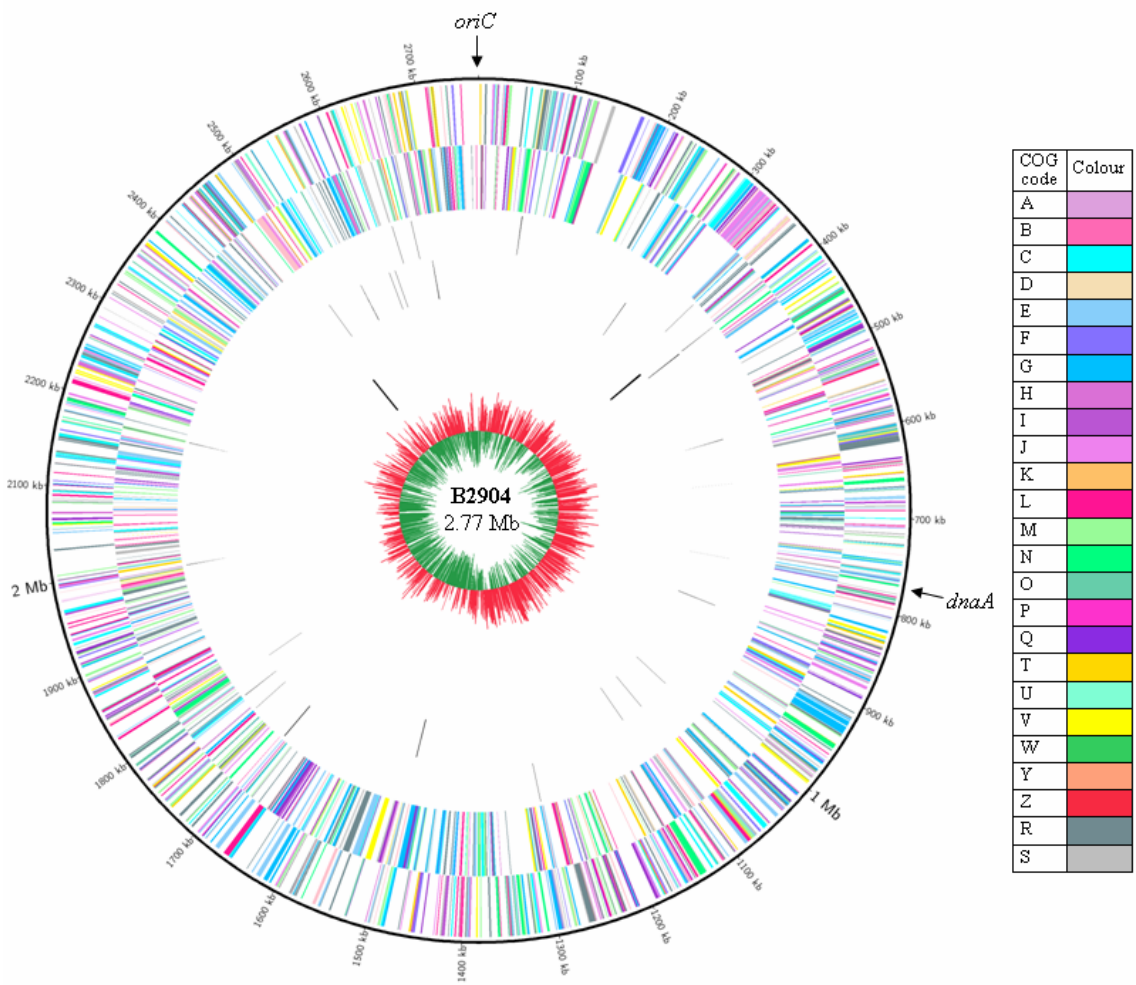

Figure 4.2 Circos circular representation of the complete 2.77 Mb *B. pilosicoli* B2904 genome with annotated genes. The genome is orientated from the *oriC* and also displays the location of *dnaA*. Circles range from 1 (outer circle) to 7 (inner circle). Circle 1, Cluster of Orthologous Group (COG)-coded forward strand genes; circle 2, COG-coded reverse strand genes; circle 3, forward strand tRNA; circle 4, reverse (cont'd p. 125)

Figure 4.2 (cont'd) strand tRNA; circle 5, forward strand rRNA; circle 6, reverse strand rRNA; circle 7, GC skew ((G-C)/(G+C); red indicates values >0; green indicates values <0). All genes are colour-coded according to COG functions shown in the key table; A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control, cell division and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; M, cell wall, membrane and envelope biogenesis; N, cell motility and secretion; O, post-translational modification, protein turnover and chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport and catabolism; T, signal transduction mechanisms; U, intracellular trafficking, secretion and vesicular transport; V, defence mechanisms; W, extracellular structures; Y, nuclear structure wheat for cell division and chromosome partitioning; Z, cytoskeleton; R, general function prediction only; S, function unknown.

4.2.3 Genome sequence alignments

Dot plot comparisons of the three *B. pilosicoli* genomes revealed that there have been chromosomal rearrangements that are arranged symmetrically around the origin or terminus of replication, highlighted by the X-patterns in the alignments (Figure 4.3). The genome rearrangements were confirmed by PCR.

Whole genome comparisons of the strains of *B. pilosicoli* were performed using ACT (Carver *et al*., 2005). On comparing B2904 with 95/1000, four major genome rearrangement events appeared to have occurred, whereas two profound rearrangements were evident when comparing WesB to 95/1000 (Figure 4.4). Mobile genetic elements (MGE) were found adjacent to or within close proximity of the sites where recombination events appear to have occurred in the B2904 and WesB genomes. Fifty-seven and 30 MGEs, including insertion sequence elements (ISE), recombinases, transposases and integrases were identified in the B2904 and WesB genomes, respectively, compared to just four in the 95/1000 genome. The proportion of these features therefore seems to correlate with the extent of rearrangement within the genome. Furthermore, multiple copies of a transposase/integrase gene that was absent from the 95/1000 genome was identified in the genomes of B2904 (n = 43) and WesB

(*n* = 7) (Appendix I). The lower number of copies in WesB may be an artefact of the genes not assembling in the incomplete genome.

The fewest suspected pseudogenes (gene truncation or frameshift) were found in 95/1000 and the most in B2904 (Table 4.1), a finding that correlates to the number of MGEs and degree of genome rearrangements in these strains. Of the total number of pseudogenes in each strain, 91.5%, 84.5% and 81.3% were in a cluster with orthologs in the other two strains in 95/1000, B2904 and WesB, respectively. Most strikingly, all strains shared clusters included either multiple B2904 and/or WesB pseudogenes with a complete 95/1000 gene.

Despite extensive chromosomal rearrangements, the genome alignments (Figure 4.3 and 4.4), show that the majority of the genome sequence is shared between the three strains, with B2904 and WesB possessing the greatest proportion of unique sequences. Furthermore, a 26 Kb region, likely to have involvement in horizontal gene transfer (HGT), and that is partially conserved in all previously reported *Brachyspira* genomes as well as *E. faecalis* and *E. coli* (Motro *et al*., 2008), was identified in the *B. pilosicoli* B2904 and WesB genomes.

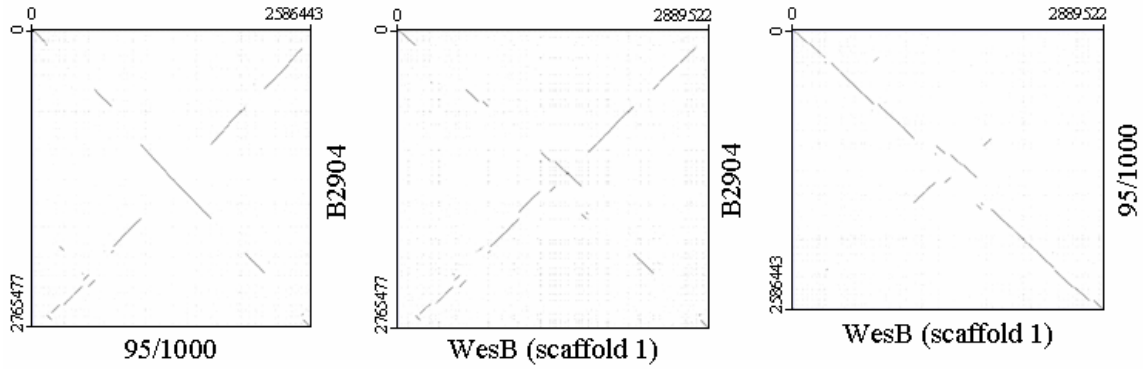

Figure 4.3 Dot matrix plots comparing the genomes of *B. pilosicoli* B2904, 95/1000 and WesB. The dot matrix plots of the three genome sequences linearised at the *oriC* were generated using Freckle. The incomplete WesB strain genome was within one scaffold. The output displays a two-dimensional plot, whereby the dots represent matched regions between the three genomes. The minimum size of matched sequences was set to 20 bp.

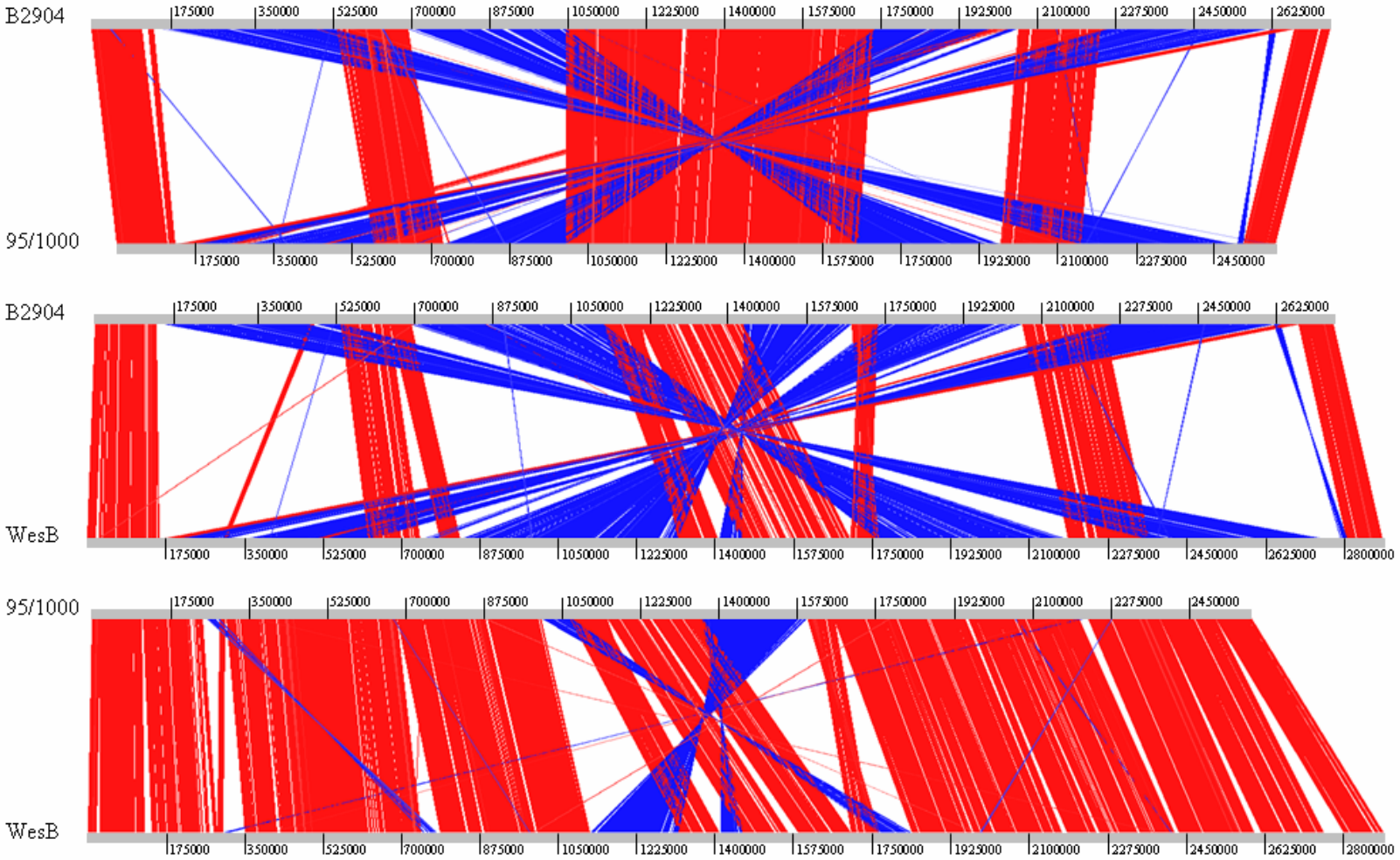

Figure 4.4 Pairwise genome alignments of *B. pilosicoli* strains 95/1000, B2904 and WesB. The Artemis Comparison Tool (ACT) was used to compare the three genome sequences against each other. The genome sequences were aligned from the predicted *oriC* and visualised in ACT with a cut-off set to blast scores >500. Red and blue bars indicate regions of similarity in the same orientation (red) and inverted (blue).

4.2.4 Global genome feature comparisons

4.2.4.1 Functional prediction comparisons

Functional classifications were assigned to each of the protein-coding genes of the three strains of *B. pilosicoli* using the COG database. The analysis revealed that the general distribution of features into categories was similar for the three strains (Table 4.2), and this highlighted their close relationship.

Table 4.2 Distribution of Cluster of Orthologous Group (COG) categories in *B. pilosicoli* 95/1000, B2904 and WesB. The number and percentage of total genes within each of the genomes, assigned to each functional group are shown (cont'd p 129).[a]

Function (COG category)	95/1000	%	B2904	%	WesB[b]	%
Cellular Processes						
Translation, ribosomal structure and biogenesis (J)	122	5.22	119	4.45	125	4.83
Transcription (K)	51	2.18	49	1.83	61	2.36
Replication, recombination and repair (L)	51	2.18	56	2.10	61	2.36
Cellular Processes and Signalling						
Cell cycle control, cell division and chromosome partitioning (D)	10	0.43	8	0.30	9	0.35
Defence mechanisms (V)	35	1.50	33	1.23	35	1.35
Signal transduction mechanisms (T)	16	0.68	15	0.56	15	0.58
Cell wall, membrane and envelope biogenesis (M)	74	3.17	72	2.69	79	3.05
Cell motility (N)	40	1.71	39	1.46	40	1.54
Intracellular trafficking, secretion and vesicular transport (U)	11	0.47	7	0.26	9	0.35
Post-translational modification, protein turnover and chaperones (O)	40	1.71	36	1.35	39	1.51
Metabolism						
Energy production and conservation (C)	89	3.81	84	3.14	84	3.24
Carbohydrate transport and metabolism (G)	101	4.32	123	4.60	139	5.37
Amino acid transport and metabolism (E)	141	6.03	138	5.16	149	5.76
Nucleotide transport and metabolism (F)	49	2.10	54	2.02	57	2.20
Coenzyme transport and metabolism (H)	47	2.01	44	1.65	48	1.85
Lipid transport and metabolism (I)	41	1.75	33	1.23	34	1.31
Inorganic ion transport and metabolism (P)	53	2.27	53	1.98	49	1.89

Table 4.2 (cont'd).

Function (COG category)	95/1000	%	B2904	%	WesB[b]	%
Secondary metabolites biosynthesis, transport and catabolism (Q)	9	0.38	10	0.37	9	0.35
Poorly characterised						
General function prediction only (R)	149	6.37	147	5.50	157	6.06
Function unknown (S)	72	3.08	76	2.84	77	2.97
Unassigned						
Not in COG (X)	1137	48.63	1477	55.26	1313	50.71
TOTAL	2338	100	2673	100	2589	100

[a] Those genes with significant PID and/or query/target coverage hits; significance equals blastx/blastp PID of at least 25% and/or 75% query or target coverage.

[b] The incomplete WesB strain genome was within one scaffold.

4.2.4.2 Genome feature comparisons between *B. pilosicoli* genomes

The three strains of *B. pilosicoli* contained 2,132 conserved genes, and these contribute to defining the *B. pilosicoli* pan-genome (Figure 4.5); this related to 92.6%, 80.2% and 80.4% of the total genes of the 95/1000, B2904 and WesB genomes, respectively. *B. pilosicoli* WesB harboured the greatest number of unique genes, with 10.0% of its genes being absent from the other genomes; B2904 had a similar proportion (9.5%), whereas 95/1000 had considerably fewer (4.9%). *B. pilosicoli* B2904 and WesB shared the greatest proportion of genes (~8.9%) while B2904 shared a greater percentage of its genes with 95/1000 (1.4%) than with WesB (0.7%).

4.2.4.3 Genome feature comparisons between *B. pilosicoli* and other *Brachyspira* species genomes

Complete genome sequences of *B. hyodysenteriae* WA1, *B. intermedia* PWS/A^T, *B. pilosicoli* 95/1000 and *B. murdochii* 56-150^T have previously undergone comparative analysis (Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010). Genome sequences of *B. pilosicoli* B2904 and WesB can now be added to these comparisons, giving the first opportunity for a *Brachyspira* intra-species genome comparison. A protein blastmatrix comparison was performed on the four previously sequenced genomes, the two newly-sequenced *B. pilosicoli* genomes and the unpublished, draft genome scaffolds of *B. aalborgi* 513^T, *B. alvinipulli* C1^T and *B. intermedia* HB60 (Table 4.3).

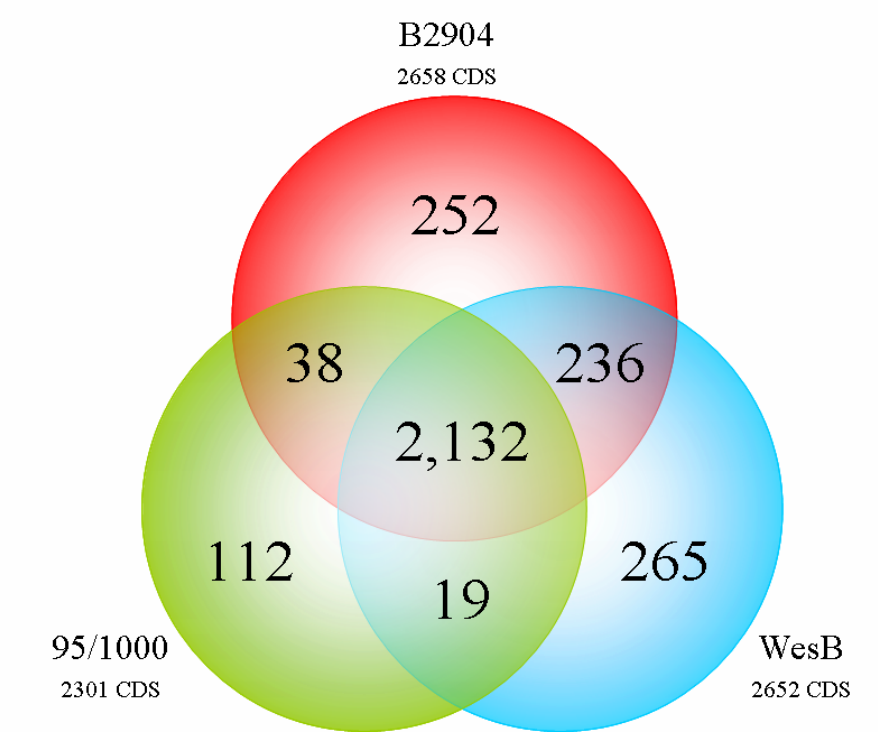

Figure 4.5 Venn diagram of genes unique to and shared between *B. pilosicoli* 95/1000, B2904 and WesB. The Venn diagram was resolved via BLASTlineMCL protein clustering. Each circle represents the total number of protein-coding genes in the genome, whereby overlapping regions indicate the number of genes shared between the respective genomes.

A protein Markov clustering analysis of the six published *Brachyspira* genomes identified 1,647 protein clusters shared by all six strains (Table 4.4), the encoding genes of which may be used to define a *Brachyspira* species pan-genome.

4.2.5 Screening for potential virulence features

Virulence factor screening in *Brachyspira* genomes was performed as described previously (Bellgard *et al*., 2009; Wanchanthuek *et al*., 2010), but with the analysis encompassing a greater array of genes, particularly in categories relating to adhesion and/or surface proteins and MGEs (Table 4.5). The greatest number of potential virulence features was in B2904, however additional features may be identified in the WesB genome once it is completed.

Table 4.3 Protein blastmatrix analysis of nine *Brachyspira* genomes. The percentage of the total coding DNA sequences (CDS) that were identified in other genomes (green) and the proportion of protein repeats within the genome (red) is shown. A cut-off e-value of 1e-05 was used.

	B. aalborgi 513[Ta] 2257 CDS	*B. alvinipulli* C1[Ta] 3228 CDS	*B. hyodysenteriae* WA1 2613 CDS	*B. intermedia* PWS/A[T] 2890 CDS	*B. intermedia* HB60[a] 3392 CDS	*B. murdochii* 56-150[T] 2809 CDS	*B. pilosicoli* B2904 2658 CDS	*B. pilosicoli* 95/1000 2301 CDS	*B. pilosicoli* WesB[b] 2652 CDS
WesB[b]	21.36%	21.65%	22.73%	24.95%	19.84%	25.95%	68.43%	65.32%	1.73%
95/1000	17.94%	18.56%	20.78%	20.90%	16.77%	22.11%	54.93%	0.74%	
B2904	20.47%	21.25%	21.74%	23.60%	19.25%	25.35%	2.71%		
56-150[T]	22.37%	31.51%	33.68%	36.40%	29.33%	5.30%			
HB60[a]	20.65%	31.23%	46.77%	57.65%	1.77%				
PWS/A[T]	21.05%	32.03%	50.33%	1.56%					
WA1	17.72%	27.48%	1.11%						
C1[Ta]	23.48%	2.54%							
513[Ta]	1.11%								

[a] Incomplete genome currently within a genome sequencing project.

[b] The incomplete WesB strain genome was within one scaffold.

Table 4.4 Conserved and shared protein clusters between the six genome-sequenced strains of *Brachyspira*. *B. hyodysenteriae* WA1 (H), *B. intermedia* PWS/A^T (I), *B. murdochii* 56-150^T (M) and *B. pilosicoli* 95/1000 (Pa), B2904 (Pb) and WesB (Pc) strains were included in the protein cluster analysis. A cut-off e-value of 1e-20 was used.

Combination of species/strains	Number of clusters
H	186
I	277
M	223
Pa	85
Pb	138
Pc	185
H I	61
H M	14
H Pa	3
H Pb	2
H Pc	3
I M	30
I Pa	4
I Pb	3
I Pc	36
M Pa	2
M Pb	7
M Pc	16
Pa Pb	12
Pa Pc	6
Pb Pc	47
H I M	173
H I Pa	4
H I Pb	2
H I Pc	7
H M Pa	1
H M Pb	5
H M Pc	2
H Pa Pb	1
H Pa Pc	1
H Pb Pc	7
I M Pa	2

Combination of species/strains	Number of clusters
I M Pb	1
I M Pc	7
I Pa Pb	5
I Pa Pc	0
I Pb Pc	2
M Pa Pb	9
M Pa Pc	0
M Pb Pc	29
Pa Pb Pc	110
H I M Pa	10
H I M Pb	8
H I M Pc	1
H I Pa Pb	3
H I Pa Pc	0
H I Pb Pc	3
H M Pa Pb	0
H M Pa Pc	0
H M Pb Pc	2
H Pa Pb Pc	4
I M Pa Pb	4
I M Pa Pc	1
I M Pb Pc	9
I Pa Pb Pc	14
M Pa Pb Pc	58
H I M Pa Pb	3
H I M Pa Pc	6
H I M Pb Pc	16
H I Pa Pb Pc	45
H M Pa Pb Pc	8
I M Pa Pb Pc	34
H I M Pa Pb Pc	1,647

Table 4.5 The number of genes with potential roles in pathogenesis and virulence in the three *B. pilosicoli* genomes. The analysis categorised the genes from the genomes of *B. pilosicoli* 95/1000, B2904 and WesB.

Role of putative gene	95/1000	B2904	WesB[a]
Core genes involved in LPS biosynthesis[b]	27	30	32
Chemotaxis			
putative methyl-accepting chemotaxis protein	7	7	10
methyl-accepting chemotaxis protein A (*mcpA*)	2	0	2
methyl-accepting chemotaxis protein B (*mcpB*)	8	11	11
chemotaxis protein	15	15	15
Flagella	42	42	42
Adhesion and membrane protein			
lipoprotein	21	31	29
variable surface protein	3	4	4
integral membrane protein	1	1	1
outer membrane protein	25	25	23
periplasmic protein	25	25	28
inner membrane protein	75	83	83
Host tissue degradation			
haemolysis	12	12	12
phospholipase	2	3	2
peptidase	44	48	48
protease	19	19	17
Oxidative stress	7	7	7
Ankyrin-like protein	31	34	35
Phage and other MGEs	46	109	100
Total	412	506	501

[a] The incomplete WesB strain genome was within one scaffold.

[b] Core LOS biosynthesis genes.

4.2.6 Comparison of central metabolism phenotypes

4.2.6.1 Comparison of carbon source utilisation by *Brachyspira*

Analysis of the genomes of *B. hyodysenteriae* and *B. pilosicoli* has revealed that these species share many metabolic capabilities (Bellgard *et al*., 2009; Wanchanthuek *et al*., 2010). In these studies, the analysis of central metabolic pathway detection in *Brachyspira* genomes was extended by application of Biolog PM^{TM} technology for phenotypic determination of carbon source utilisation of four strains each of *B. alvinipulli*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B.*

pilosicoli and two strains each of "*B. canis*" and "*B. pulli*". The utilisation of 178 unique carbon compounds by the strains was screened (Appendix II).

All strains of *Brachyspira* tested were capable of utilising the following compounds as a carbon source; N-acetyl-D-glucosamine, D-galactose, D-alanine, D-glucose-6-phosphate, D-fructose, α-D-glucose, maltose, D-melibiose, α-ketoglutaric acid, α-ketobutyric acid, α-D-lactose, lactulose, D-glucose-1-phosphate, D-fructose-6-phosphate, maltotriose, D-threonine, glyoxylic acid, L-serine, L-threonine, L-alanine and arbutin. When analysing the results at a species level, differences in the utilisation of 71 compounds between species were noted (Table 4.6); this included differences that were based only on consistent results of strains within each species.

Table 4.6 Differences in the utilisation of unique carbon sources by six species of *Brachyspira* and two proposed species tested analysed using the Biolog Phenotype MicroArray™ (PM) system. This summary table is based on the results of two repeats for four strains each of *B. alvinipulli*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. pilosicoli* and two strains each of "*B. canis*" and "*B. pulli*" (Appendix II). Results are shown only for compounds where differences in its utilisation were observed for all tested strains of a species (cont'd p. 135).

	Ability to utilise carbon source[a]							
	Bal[b]	***Bca***[c]	***Bhy***[d]	***Bin***[e]	***Bim***[f]	***Bmu***[g]	***Bpi***[h]	***Bpu***[i]
L-Aspartic acid	+	±	±	+	±	±	−	+
L-Proline	±	+	+	+	+	±	−	+
D-Trehalose	+	+	+	±	±	±	−	±
D-Serine	+	+	+	+	+	+	−	+
Glycerol	−	−	−	−	−	−	+	−
D-Gluconic acid	±	+	+	±	±	+	−	−
Formic acid	±	−	−	−	−	−	−	+
L-Glutamic acid	+	+	+	+	+	+	−	+
D,L-Malic acid	±	−	+	−	±	−	+	±
Tween 20	+	−	+	+	±	±	−	±
L-Rhamnose	−	−	+	−	+	±	+	±
Thymidine	−	−	±	±	±	±	+	−
L-Asparagine	−	−	+	±	±	±	−	−
D-Aspartic acid	±	+	+	±	±	+	−	+
Tween 40	±	−	±	+	±	±	−	±
α-Methyl-D-galactoside	−	−	+	+	−	±	−	+
Uridine	−	±	+	+	+	±	+	+

Table 4.6 (cont'd p. 136).

	Ability to utilise carbon source[a]							
	Bal[b]	***Bca***[c]	***Bhy***[d]	***Bin***[e]	***Bim***[f]	***Bmu***[g]	***Bpi***[h]	***Bpu***[i]
L-Glutamine	±	+	±	+	±	+	−	−
m-Tartaric acid	±	−	+	±	±	±	−	±
Tween 80	±	−	−	+	±	±	−	±
β-Methyl-D-glucoside	+	−	+	+	±	+	−	+
Adonitol	±	−	−	+	−	−	−	+
2`-Deoxyadenosine	+	−	+	−	+	+	+	−
Adenosine	±	+	+	−	+	−	+	−
Glycyl-L-aspartic acid	+	+	+	+	+	+	−	+
m-Inositol	+	+	+	+	±	±	−	+
Propionic acid	+	−	+	+	+	+	+	+
Mucic acid	+	−	+	+	±	+	−	−
Glycolic acid	+	+	±	+	±	+	−	−
Inosine	+	±	+	−	+	±	+	±
Glycyl-L-glutamic acid	±	±	+	+	+	±	−	±
L-Alanyl-glycine	+	+	±	+	−	±	−	+
Acetoacetic acid	+	+	+	+	+	−	+	−
N-Acetyl-β-D-mannosamine	+	−	+	+	+	±	+	+
Methyl pyruvate	+	±	−	+	+	±	−	±
D-Malic acid	±	−	−	+	−	±	−	±
Glycyl-L-proline	+	+	+	+	±	+	−	+
p-Hydroxyphenylacetic acid	±	−	±	+	±	±	−	−
m-Hydroxyphenylacetic acid	±	−	±	+	±	±	−	−
Tyramine	+	+	±	+	+	+	−	+
Glucuronamide	−	+	±	−	±	±	±	−
β-Phenylethylamine	±	+	±	+	±	+	−	+
Ethanolamine	±	+	±	+	±	+	−	+
α-Cyclodextrin	−	−	−	+	+	+	−	−
Dextrin	−	−	−	−	+	−	+	−
Inulin	±	−	+	−	−	−	−	−
Pectin	±	−	+	+	+	±	−	+
N-Acetyl-D-galactosamine	±	−	+	−	−	±	+	−
N-Acetylneuraminic acid	±	−	+	−	+	−	+	−
β-D-Allose	±	±	+	−	±	−	±	±
3-*O*-β-D-Galactopyranosyl-D-arabinose	±	−	±	±	−	−	+	±
Gentiobiose	−	±	+	+	−	±	+	±

Table 4.6 (cont'd).

	Ability to utilise carbon source[a]							
	Bal[b]	***Bca***[c]	***Bhy***[d]	***Bin***[e]	***Bim***[f]	***Bmu***[g]	***Bpi***[h]	***Bpu***[i]
L-Glucose	−	±	+	±	−	±	+	±
D-Lactitol	±	−	+	+	±	−	−	±
β-Methyl-D-galactoside	±	−	+	±	+	−	+	−
3-*O*-Methyl-glucose	+	±	+	−	−	−	+	±
D-Raffinose	−	−	±	±	+	±	−	±
Salicin	+	−	+	−	+	−	+	−
Turanose	±	±	±	−	−	±	+	+
N-Acetyl-D-glucosaminitol	+	±	+	+	±	+	+	−
D,L-Citramalic acid	+	−	−	−	−	±	−	−
β-Hydroxybutyric acid	±	−	+	±	±	±	−	−
γ-Hydroxybutyric acid	+	−	+	±	−	−	−	−
α-Ketovaleric acid	±	±	+	±	−	±	−	±
Oxalomalic acid	−	−	+	±	−	−	−	±
D-Tartaric acid	±	−	±	−	−	−	+	−
L-Tartaric acid	−	−	+	−	−	−	+	−
Glycine	−	−	+	−	+	−	+	−
L-Histidine	±	−	+	−	+	−	−	−
L-Leucine	−	−	+	−	+	−	−	−
D,L-Carnitine	±	−	+	−	−	−	−	−

[a] Utilisation of compound as a carbon source; +, all strains of species able of utilisation; −, all strains of the species unable of utilisation; ±, some but not all strains of the species able of utilisation.

[b] *B. alvinipulli*.

[c] "*B. canis*".

[d] *B. hyodysenteriae*.

[e] *B. innocens*.

[f] *B. intermedia*.

[g] *B. murdochii*.

[h] *B. pilosicoli*.

[i] "*B. pulli*".

4.2.6.2 Correlation of differences in *B. pilosicoli* phenotype with genotype

The metabolic capabilities of the strains of *B. pilosicoli* tested were highly conserved (Appendix II). Differences were found in the utilisation of just seven carbon sources, which were directly correlated with genotypic variations (Table 4.7).

Table 4.7 Correlation between differences in carbon source utilisation and genotype of *B. pilosicoli* 95/1000, B2904 and WesB. Possible explanations for the differences in phenotype relate to differences in genomic features.

Unique carbon source compound tested	95/1000	B2904	WesB	Possible explanation for difference in phenotype based on genotype
D-Mannose	−	−	+	WesB is the only strain with the mannose/sorbose-specific PTS system IIABCD components (wesB_1269, wesB_1270, wesB_1271 and wesB_1272) for uptake and phosphorylation of D-mannose.
D-Glucuronic acid	−	+	+	95/1000 lacks the pfkB carbohydrate kinase, 2-dehydro-3-deoxygluconate kinase, which links D-glucuronic acid metabolism to glycolysis. This enzyme is found in both B2904 (B2904_orf899 and B2904_orf900) and WesB (wesB_1781).
D-Mannitol	−	+	−	B2904 is the only strain with the D-mannitol PTS system IIABC components (B2904_orf2447) and also a mannitol-1-phosphate 5-dehydrogenase (B2904_orf2446) for D-mannitol, uptake, phosphorylation and catabolism.
Glucuronamide	−	+	+	95/1000 lacks the pfkB carbohydrate kinase, 2-dehydro-3-deoxygluconate kinase, which links D-glucuronic acid and related compound metabolism to glycolysis. This enzyme is found in both B2904 (B2904_orf899 and B2904_orf899 and B2904_orf900) and WesB (wesB_1781).
β-D-Allose	−	−	+	WesB is the only strains with D-allose ABC transporter components (wesB_1171, wesB_1172 and wesB_1175) and D-allose kinase (wesB_0259 and wesB_1174) for uptake and metabolism of D-allose.
β-Methyl-D-glucuronic acid	−	+	+	95/1000 lacks the pfkB carbohydrate kinase, 2-dehydro-3-deoxygluconate kinase, which links D-glucuronic acid and related compound metabolism to glycolysis. This enzyme is found in both B2904 (B2904_orf899 and B2904_orf900) and WesB (wesB_1781).
L-Sorbose	−	−	+	WesB is the only strain with the mannose/sorbose-specific PTS system IIABCD (wesB_1269, wesB_1270, wesB_1271, wesB_1272) components for uptake and phosphorylation of L-sorbose.

4.3 Discussion

4.3.1 Comparison of general genome features

The dendrogram based on the MLST data for the nine strains of *Brachyspira* demonstrated relatedness between the species that was concordant with previous findings (Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010). A close relationship was noted between the three strains of *B. pilosicoli* however the two strains of *B. intermedia* were less closely related, which supports reports of extensive diversity in this species based on results of PFGE (Fellstrom *et al*., 2008), and a previous MLST study which indicated that these two strains belong to distinct groups (Phillips *et al*., 2010). It has been suggested that not all isolates with the *B. intermedia* phenotype should be assigned to this species (Hafstrom *et al*., 2011).

The relatively small size of the *B. pilosicoli* genomes is most likely due to them being members of a more specialised species that has undergone a high degree of reductive genome evolution. Its has been suggested that if this is the case, then *B. pilosicoli* is likely to be an older pathogen than other *Brachyspira* species such as *B. hyodysenteriae* (Hafstrom *et al*., 2011). Such reductive genome evolution may have allowed improved energy efficiency and enhanced pathogenic potential. Reductive genome evolution is particularly evident in obligate, intracellular bacterial pathogens (Andersson and Kurland, 1998) and consistent with this, of the *Brachyspira* species, only *B. pilosicoli* and *B. aalborgi* show long-term intimate associations with the surface of enterocytes, into which they interdigitate one of their cell ends. In addition to their small genomes, the sequenced strains of *B. pilosicoli* lacked plasmids, whereas the genomes of the other fully sequenced *Brachyspira* species have included plasmids (Hafstrom *et al*., 2011).

The rRNA gene organisation observed in the strains of *B. pilosicoli* has been considered a distinguishing feature of *Brachyspira* (Zuerner and Stanton, 1994), since other spirochaetes typically have differing copy numbers and organisations (Fukunaga *et al*., 1992b; Fukunaga *et al*., 1992c). However, similar arrangements to *Brachyspira* have been detected in the spirochaete *Borrelia burgdorferi* (Fukunaga *et al*., 1992a). Situated between the *rrs* gene and *rrf-rrl* cluster, which are either side of the *oriC*, was the tmRNA (*ssrA*, 10Sa RNA) gene and nine of the total 34 tRNAs that were dispersed throughout the genome (Figure 4.2).

The origin of replication was originally considered to be adjacent to the *dnaA* gene (Bellgard *et al*., 2009; Wanchanthuek *et al*., 2010), however there was no association

between the *oriC* and *dnaA* genes in the *B. pilosicoli* B2904 genome (Figure 4.2), as found in other *Brachyspira* genomes (Hafstrom *et al*., 2011). The arrangement of genes surrounding the *dnaA* gene was consistent between the strains of *B. pilosicoli*, as with the other sequenced *Brachyspira* genomes (Wanchanthuek *et al*., 2010). The genes at the *oriC*, although consistent between the strains of *B. pilosicoli* analysed, appear to vary extensively between the species.

4.3.2 *B. pilosicoli* genome architecture

MGEs were found adjacent to or within close proximity of the sites where recombination events appear to have occurred in the B2904 and WesB genomes (Figure 4.3 and 4.4). The greater number of MGEs, including ISEs, recombinases, transposases and integrases identified in the B2904 and WesB genomes compared to the 95/1000 genome seems to correlate with the extent of genome rearrangement (Appendix I). MGEs have been implicated in chromosomal rearrangements, gene disruptions resulting in pseudogenes, and eventual loss of genes, which may contribute to reductive genome evolution (Moran and Plague, 2004). Species and strains that are undergoing or have recently undergone reductive genome evolution, and hence become more specialised pathogens, typically harbour large numbers of MGEs (Plague *et al*., 2008; Schmitz-Esser *et al*., 2011; Song *et al*., 2010). Coincidentally, fewest suspected pseudogenes were noted in 95/1000 and most in B2904 (Table 4.1).

Differences in the number of MGEs in the three *B. pilosicoli* genomes may relate to their different stages of reductive genome evolution. Strain 95/1000, which had the smallest genome, also had the fewest MGEs and this could be interpreted as indicating that the MGEs that induced the genome reduction in this strain have become lost. Alternatively, MGE expansion may not have occurred in 95/1000 to the same degree as in B2904 and WesB, as MGEs are generally lost in a fragmentary manner by pseudogenisation. This would mean that 95/1000 has not undergone niche specialisation relative to the other two strains, and hence its genome is unlikely to have been reduced in this way. On the other hand, the greater number of pseudogenes in the larger B2904 and WesB genomes does suggest that they may be undergoing genome reduction. A possible explanation would be that these strains are in the initial stages of genome reduction, at the point at which MGE expansion occurs (Moran and Plague, 2004; Song *et al*., 2010). Genome reduction and MGE expansion is often associated with niche specialisation or host restriction (Parkhill *et al*., 2003; Parkhill *et al*., 2001). However, *B. pilosicoli* are not considered host-restricted, and WesB, of human origin, has been

shown also to have the capacity to infect chickens and pigs (Trott *et al*., 1996a; Trott *et al*., 1995). *B. pilosicoli* is a highly recombinant species (Trott *et al*., 1998), and despite differences in genome arrangement and the number of pseudogenes, part of the variation in the genome sizes simply reflects the carriage of different subsets of the pan-genome.

The dot plot comparison of the three *B. pilosicoli* genomes revealed that the rearrangements were symmetrical around the origin or terminus of replication (Figure 4.3). It has been postulated that symmetrical rearrangements occur because recombination events are determined by the replication forks that are approximately equal distance from the o*riC* during bidirectional replication (Tillier and Collins, 2000). It has also been argued that non-symmetrical rearrangements can be disadvantageous, and so genome rearrangements such as those found in the strains of *B. pilosicoli* are a product of selection (Mackiewicz *et al*., 2001).

4.3.3 Global feature comparisons between the strains of *B. pilosicoli*

The general distribution of features into COG categories was similar for the three strains (Table 4.2), highlighting their close relationship. Despite having the smallest genome, *B. pilosicoli* 95/1000 possessed the greatest number of features in six categories. B2904 contained the most features in eight categories, and WesB in one category. A striking difference between the strains was in the carbohydrate (G), amino acid (E) and nucleotide (F) transport and metabolism categories, with the larger WesB genome containing considerably more features than the B2904 and 95/1000 genomes. In addition, compared to other *Brachyspira* species the strains of *B. pilosicoli* had a reduced number of features associated with inorganic ion transport and metabolism (P) (Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010).

From the three *B. pilosicoli* genomes available, the pan-genome could be defined by 2,132 conserved genes (Figure 4.5). As expected, there was a greater number of core genes between the strains of *B. pilosicoli* than between strains of different species; substantially fewer core genes (1,087) were identified for *B. hyodysenteriae* WA1, *B. pilosicoli* 95/1000 and *B. murdochii* 56-150^{T} (Wanchanthuek *et al*., 2010).

4.3.4 Global feature comparisons between the *B. pilosicoli* genomes and other *Brachyspira* genomes

The protein blastmatrix comparison performed on the nine available *Brachyspira* genomes (published and unpublished), revealed that the strains of *B. pilosicoli* shared

the greatest proportion of proteins (54.9 – 68.4%) (Table 4.3). Of the strains of *B. pilosicoli*, B2904 harboured the greatest proportion of protein repeats relating to paralogs (2.7%), despite not possessing the largest genome. Overall, the non-pathogenic *B. murdochii* had the greatest proportion of protein repeats (5.3%), perhaps relating to its large genome. High proportions of shared proteins highlighted the close relationships of *B. hyodysenteriae* with *B. intermedia* (>46.7%) and *B. murdochii* (33.7%) (Figure 4.1). *B. aalborgi* shared the lowest percentage of proteins with other *Brachyspira* species, consistent with evidence that this is the most divergent species (Figure 4.1).

The protein Markov clustering analysis of the six published *Brachyspira* genomes revealed *B. intermedia* PWS/A^T harboured the greatest number of clusters not found in the other sequenced *Brachyspira* genomes (n = 277) and it has the largest genome (Table 4.4). The greatest number of clusters shared only between two strains was with *B. intermedia* PWS/A^T and *B. hyodysenteriae* WA1 (n = 61), consistent with the close relationship of these species (Figure 4.1). Of the strains of *B. pilosicoli*, B2904 and WesB shared the most unique protein clusters (n = 47), and WesB also shared the greatest number of clusters with a non-*B. pilosicoli* strain, having 36 clusters in common with *B. intermedia* and 16 with *B. murdochii*. The strains of *B. pilosicoli* collectively shared the most clusters with *B. murdochii* 56-150^T (n = 58), and fewest with *B. hyodysenteriae* WA1 (n = 4), as noted previously (Hafstrom *et al*., 2011). Non-*B. pilosicoli* strains shared 173 clusters, whereas the strains of *B. pilosicoli* shared 110 clusters, reflecting gene loss and genome reduction.

4.3.4.1 Features unique to the *B. pilosicoli* species

Of 110 protein clusters present only in the *B. pilosicoli* genomes (Table 4.4), 54.6% were hypothetical or unclassified. The majority of protein clusters were metabolic features, including an α-galactosidase (BP951000_0276; B2904_orf1586; wesB_1069), the activity of which is a distinguishing feature of the species (Fellstrom and Gunnarsson, 1995; Fellstrom *et al*., 1997). Although it was suggested that *B. pilosicoli* had lost many transport-related genes during reductive evolution (Hafstrom *et al*., 2011), 13 clusters were found to be for transport proteins. Sialidase family-like protein genes unique to *B. pilosicoli* 95/1000 (BP951000_0858, BP951000_0859 and BP951000_0861) (Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010) were also present in B2904 (B2904_orf1812, B2904_orf1813 and B2904_orf1814) and WesB (wesB_0922, wesB_0923 and wesB_0924); the products of such genes may play a role in adherence to host cells, forming binding sites through glycosaminoglycans

modification (Piotrowski *et al*., 1991). Clusters for an α-1,2-fucosyl transferase (BP951000_1232; B2904_orf14; wesB_0014), two membrane proteins (BP951000_1751; B2904_orf2268; wesB_0587) (BP951000_1752; B2904_orf2267; wesB_0586) and two glycosyltransferases (BP951000_0003; B2904_orf1276; wesB_1428) (BP951000_2338; B2904_orf1277 and B2904_orf1282; wesB_1429) were unique to *B. pilosicoli* and may contribute to host cell adherence. Other *B. pilosicoli*-specific clusters were for an ankyrin repeat protein (BP951000_0080; B2904_orf1369; wesB_1511), a β-lactamase (BP95100_1338; B2904_orf2576; wesB_0148), two peptidases (BP951000_1129; B2904_orf205; wesB_2479) (BP951000_1260; B2904_orf40; wesB_0047) and phage proteins (BP951000_1211; B2904_orf2686; wesB_2642) (BP951000_1258; B2904_orf39; wesB_0046).

4.3.4.2 Unique features shared by two strains of *B. pilosicoli*

Of the strains of *B. pilosicoli*, B2904 and WesB shared most unique clusters (Table 4.4). Fewer clusters were shared with 95/1000, but of twelve clusters unique to 95/1000 and B2904, all but N-acetyl mannosamine-6-phosphate 2-epimerase (BP951000_2135; B2904_orf1689) were hypothetical. Six clusters were unique to 95/1000 and WesB, all lacking a specified function. Of 47 clusters unique to B2904 and WesB, 51.1% were hypothetical; notable clusters shared between these strains were for a further sialidase-like protein (B2904_orf1811; wesB_0925) and a peptidase (B2904_orf863; wesB_1557). The glycine reductase complex locus of 95/1000 (BP951000_1852 – BP951000_1860) and *B. murdochii* 56-150^{T} (Bmur_2720 – Bmur_2728) (Wanchanthuek *et al*., 2010) was identified in B2904 (B2904_orf665 – B2904_orf673) and WesB (wesB_0746 – wesB_0754), but with an additional ATP-binding cassette (ABC)-type glycine betaine transport component in a separate locus (B2904_orf1065; wesB_1632). Moreover, a cluster for a transposase unique to B2904 (n = 47) and WesB (n = 7) was detected. Genes that were shared only by the larger B2904 and WesB genomes and were absent from 95/1000, without apparent detriment, presumably have some specialised function that is not essential for survival. These features may have been lost from 95/1000, as they are not essential, or acquired in B2904 and WesB, perhaps by HGT.

4.3.4.3 *B. pilosicoli* strain-unique features

B. pilosicoli 95/1000 harboured the fewest and WesB the most unique features (Table 4.4), correlating with their genome size. As discussed above, the 95/1000 strain

may have become more specialised, having lost non-essential features through reductive evolution (Andersson and Kurland, 1998); alternatively, the absence of orthologs in other strains or species may suggest that these features have been acquired via HGT. Of the strain-unique clusters, 77.7%, 65.9% and 68.1% were for hypothetical proteins in 95/1000, B2904 and WesB, respectively. In 95/1000, unique clusters included a sodium/ pantothenate symporter and an outer membrane lipoprotein (BP951000_0731) with a potential role in host cell adherence (BP951000_0634). In B2904, unique clusters included putative phage proteins (B2904_orf136, B2904_orf143 and B2904_orf816), additional glycine reductase complex proteins (B2904_orf2051 and B2904_orf2052) and proteins involved in ascorbate metabolism (B2904_orf1019, B2904_orf1020 and B2904_orf1024) and mannitol metabolism (B2904_orf2446 and B2904_orf2447). In WesB, unique features included mannose/sorbose-specific phosphotransferase system (PTS) components (wesB_1270, wesB_1271 and wesB_1272), fructose-specific PTS components (wesB_2317 and wesB_2318) and a D-allose kinase (wesB_1174). Six unique phage-related features and an integrase were identified at two loci in the WesB genome (wesB_0297, wesB_0298, wesB_2528, wesB_2540, wesB_2545, wesB_2550 and wesB_2567). Interestingly, each of the strains harboured unique genes for ankyrin proteins (BP951000_0037; B2904_orf892 and B2904_orf1944; wesB_0903).

4.3.5 Comparison of potential virulence features

4.3.5.1 Lipooligosaccharides

A *rfbBADC* cluster, encoding proteins for nucleotide sugar biosynthesis and with a suggested role in *O*-antigen assimilation (Whitfield, 1995; Wildschutte *et al*., 2004), was identified on the *B. hyodysenteriae* WA1 plasmid (Bellgard *et al*., 2009). Although lacking this cluster, the three strains of *B. pilosicoli* possessed *rfbA* (BP951000_1687; B2904_orf2229; wesB_0523) and *rfbB* (BP951000_1148; B2904_orf2569; wesB_2572), but *rfbC* was noted only in B2904 (n = 1) and WesB (n = 2) (B2904_orf117; wesB_0130 and wesB_0131). Genes involved in the biosynthesis of 3,5-dideoxyhexose, an *O*-antigen component of LPS (Kessler *et al*., 1993), were found located adjacent to the *rfbC* gene(s) in B2904 and WesB; both strains contained *rfbF* (B2904_orf115; wesB_0127) and *rfbG* (B2904_orf116; wesB_0128), but *rfbH* was present only in WesB (wesB_0129). The absence of such genes in the pathogenic strain 95/1000 suggests that they may have a limited impact on virulence.

4.3.5.2 Motility and chemotaxis

As with 95/1000, the two other strains of *B. pilosicoli* possessed fewer chemotaxis genes than *B. hyodysenteriae* and *B. murdochii* (Table 4.5) (Wanchanthuek *et al*., 2010). No *mcpC* genes were found in the strains of *B. pilosicoli*, despite their detection in the genomes of the other fully sequenced *Brachyspira* species. The inter-species differences in the number and complement of chemotaxis-related genes may account for differences in their attraction to mucins and affinity to local host niches (Naresh and Hampson, 2010). No *mcpA* genes were identified in B2904, but two copies were found in the other strains of *B. pilosicoli*. The same complement of chemosensory transducer genes was identified in all three strains, as was the previously described cluster of seven such genes (Wanchanthuek *et al*., 2010). Differences in the number of chemotaxis-related genes between the three strains may translate from differences in genome size. This may denote a redundancy of features that can be lost without apparent detriment to long-term survival. The same flagella genes were shared by all three strains of *B. pilosicoli*.

4.3.5.3 Adhesion and membrane proteins

End-on attachment of the spirochaete to the luminal epithelial surface of the lower intestinal tract is characteristic of colonisation by *B. pilosicoli* and *B. aalborgi* (Hovind-Hougen *et al*., 1982; McLaren *et al*., 1997). Thus, surface-associated proteins or lipoproteins are potential candidates for virulence. All lipoprotein genes in 95/1000 were found in both B2904 and WesB strains. However, these strains also had a predicted secreted lipoprotein (B2904_orf1676; wesB_1576) and a lipoprotein carrier protein, LolA (B2904_orf608; wesB_0637), which anchors lipoproteins to the outer membrane (Takeda *et al*., 2003). The same complement of genes encoding variable surface proteins found in 95/1000 (Wanchanthuek *et al*., 2010) and the putative integral membrane virulence factor, MviN (B2904_orf469; wesB_2218) were noted in B2904 and WesB. Genes for outer membrane proteins with a potential role in virulence were identified, including BspA antigens, which may bind fibronectin and initiate a serological response (Sharma *et al*., 1998), OmpA proteins, similar to proteins implicated in *Leptospira* virulence (Ristow *et al*., 2007), and Tia invasion determinants. Genes encoding TolC were identified in all three strains of *B. pilosicoli*, and this protein has been implicated in host invasion, virulence gene expression, and as an outer membrane component of efflux pumps (Ferhat *et al*., 2009; Minato *et al*., 2011; Zgurskaya *et al*., 2011). The periplasmic proteins identified were predicted to be primarily associated with other membrane proteins, and constitute ABC transporters

with putative roles in virulence (Davidson *et al*., 2008). Gene duplications were largely responsible for the greater number of inner membrane virulence factors in B2904 and WesB, but since they were absent from 95/1000, it is unlikely that they have significant impact on virulence. WesB harboured two additional genes encoding OppA, which has suggested involvement in spirochaete-host interactions in *Treponema denticola* (Fenno *et al*., 2000). Genes encoding P-type ATPase components, such as *cadA* and *zntA*, were noted in the three strains and these have been implicated in the ability of pathogens to sense and adapt to intracellular environments through heavy metal ion regulation (Francis and Thomas, 1997; Silver and Walderhaug, 1992), in addition to Trk potassium transport components, required for invasion and intracellular growth of *Salmonella* (Su *et al*., 2009). Genes encoding outer, periplasmic and inner membrane proteins that constitute transport systems implicated in bacterial virulence mechanisms were detected, such as polyamine ABC-type transport, which is important for *Streptococcus pneumoniae* pathogenesis (Shah *et al*., 2008), TonB-dependant iron transport, which is related to *Shigella dysenteriae* virulence (Reeves *et al*., 2000), and PTS systems implicated in the virulence of *Mycobacterium tuberculosis* and *E. coli* (Lamarche *et al*., 2005; Peirs *et al*., 2005). Genes were found encoding components of the AcrAB-TolC complex, which confers antimicrobial resistance and survival in the GI tract (Perez *et al*., 2012), a ferrous iron transporter, *feoB*, for iron acquisition, gut colonisation and intracellular survival of multiple enteropathogens (Naikare *et al*., 2006; Velayudhan *et al*., 2000), and a glutamine transporter gene, *glnQ*, which has been implicated in *Streptococcus* adherence and virulence (Tamura *et al*., 2002). In the strains of *B. pilosicoli*, an *mgl* operon similar to one with a proposed role in virulence expression in *Treponema pallidum* (Porcella *et al*., 1996) was noted. Multidrug efflux features were found in all three strains, which aside from drug resistance, are attributed with a range of roles in pathogenesis (Piddock, 2006a). Genes for the Sec pathway described in 95/1000 (Wanchanthuek *et al*., 2010), with no needle-associated genes were also noted in B2904 and WesB, with an additional *secA*-like gene in WesB (wesB_0869).

4.3.5.4 Host tissue degradation

The complement of haemolysis-related genes was identical between the three strains. Compared to previous analysis, other genes were detected including a haemolysin, previously undetected in 95/1000 (BP951000_1925) and three putative streptolysin genes, *sagB* (BP951000_0919; B2904_orf445; wesB_2241), *sagC* (BP951000_0918; B2904_orf446; wesB_2240) and *sagD* (BP951000_0917;

B2904_orf447; wesB_2239), involved in β-haemolysis and virulence in streptococci (Betschel *et al*., 1998; Sierig *et al*., 2003). A putative phospholipase/carboxylesterase (B2904_orf1218) was found in B2904. The three strains contained similar numbers of peptidases and proteases, which may participate in local degradation of host tissues, however 95/1000 lacked peptidase E, which had no effect on protein degradation in *Salmonella* (Carter and Miller, 1984), and hence, this non-essential enzyme may have been lost through reductive evolution.

4.3.5.5 Oxidative stress

Genes related to oxidative stress were shared by the three strains. A partial *BatI* (*Bacteroides* aerotolerance) operon (Tang *et al*., 1999) was noted in all strains, in close proximity to one of the *nox* genes and consisted of *batB* (BP951000_0196; B2904_orf1493; wesB_1155), *batC* (BP951000_0195; B2904_orf1492; wesB_1156), *batD* (BP951000_0194; B2904_orf1491; wesB_1157) and *batE* (BP951000_0193; B2904_orf1490; wesB_1158). The *batA* gene was in a distinct locus in each of the three strains (BP951000_1387; B2904_orf2546; wesB_0200).

4.3.5.6 Ankyrin-like protein

There was little difference in the number of genes encoding ankyrin-like proteins between the strains of *B. pilosicoli*, which may be involved in host cell interactions through their ability to bind host chromatin (Cho *et al*., 2005). *B. pilosicoli* harboured consistently fewer of these genes than *B. hyodysenteriae* (Wanchanthuek *et al*., 2010).

4.3.5.7 Phage and other mobile genetic elements

Outside of bacteriophage regions, four, 54 and 28 MGEs were identified in 95/1000, B2904 and WesB, respectively, correlating with the extent of genomic rearrangements. The types and copy number of all MGEs in the *B. pilosicoli* genomes are detailed in Appendix I. The region encoding genes related to the VSH-1 prophage-like GTA in 95/1000 (Wanchanthuek *et al*., 2010), was identified in B2904 (B2904_orf2669 – B2904_orf2692) and WesB (wesB_2625 – wesB_2648). This region was ~15 Kb in 95/1000 compared to ~21 Kb in B2904 and WesB due to an insertion between genes encoding OrfE and Hvp53, containing genes for a monosaccharide-transporting ATPase (B2904_orf2671; wesB_2628), an ABC transporter-related protein (B2904_orf2672; wesB_2629), a ROK family protein (B2904_orf2674; wesB_2631), an integrase in B2904 only (B2904_orf2675), and a periplasmic binding protein/LacI

transcriptional regulator (B2904_orf2673; wesB_2627 and wesB_2630). Generally, these features had high homology with those in *Clostridium carboxidivorans* (e-value < 1e-74), consistent with the finding that *Brachyspira* share a high degree of gene similarity with *Clostridium* (Bellgard *et al*., 2009), and supporting the notion that the bacteriophages exchange genetic material between species (Hafstrom *et al*., 2011). In WesB, an additional cluster of VSH-1-associated genes, flanked by a phage terminase, was detected (wesB_2527 – wesB_2553); the different genes in this region shared highest homology with *C. carboxidivorans*, *B. hyodysenteriae*, *B. intermedia*, *B. pilosicoli* and *B. murdochii*, suggesting that the GTA had involvement in intra- and inter-species gene transfer. The bacteriophage that was identified in *B. pilosicoli* 95/1000 (pP1), and in *B. murdochii* 56-150^T (pM1, pM2 and pM3) (Hafstrom *et al*., 2011; Wanchanthuek *et al*., 2010), was also found in B2904 (pP2; B2904_orf1942 – B2904_orf1970) and WesB (pP3; wesB_0739 – wesB_0708) (Figure 4.6). In *B. pilosicoli*, the bacteriophage size was proportional to genome size. Hypothetical proteins encoded in this region were shared between 95/1000 and B2904, however WesB contained four unique hypothetical genes. The B2904 pP2 bacteriophage possessed a unique ankyrin repeat protein (B2904_orf1943). An adenine-specific DNA methyltransferase gene was present only in the WesB pP3 bacteriophage (wesB_0711), adjacent to the DNA methylase gene found in bacteriophages of *B. pilosicoli* (BP951000_1480; B2904_orf1968; wesB_0710), but absent from those of *B. murdochii* 56-150^T. Two separate novel bacteriophages regions were found in B2904 (pP4) and WesB (pP5). The ~29 Kb pP4 bacteriophage contained seven phage proteins (B2904_orf133 – B2904_orf180), six predicted proteins with homology to sequences of other *Brachyspira* species, and 35 unique hypothetical genes. The ~28 Kb pP5 bacteriophage (wesB_0301 – wesB_0341) shared all the components of the pI1 bacteriophage of *B. intermedia* PWS/A^T, suggesting transfer of the bacteriophage in an inter-species HGT event. Interestingly, pP5 was flanked by VSH-1 components (wesB_0297, wesB_0298 and wesB_0343), and hence the VSH-1 GTA may be responsible for mediating the HGT event. Two nuclease genes (wesB_0306 and wesB_0308) and a number of unique hypothetical genes in pP5 were not identified in pI1. Clustered regularly interspaced short palindromic repeats (CRISPR), which provide bacteria with acquired resistance to bacteriophages (Sorek *et al*., 2008), were only identified in the non-pathogenic *B. murdochii* 56-150^T, which suggests a role for bacteriophages in the pathogenicity of *Brachyspira*. *B. pilosicoli* B2904 and *B. intermedia* PWS/A^T did however possess a bacteriophage resistance protein

(B2904_orf2624; Bint_2390) which has been implicated in protecting against bacteriophages (Fineran *et al*., 2009).

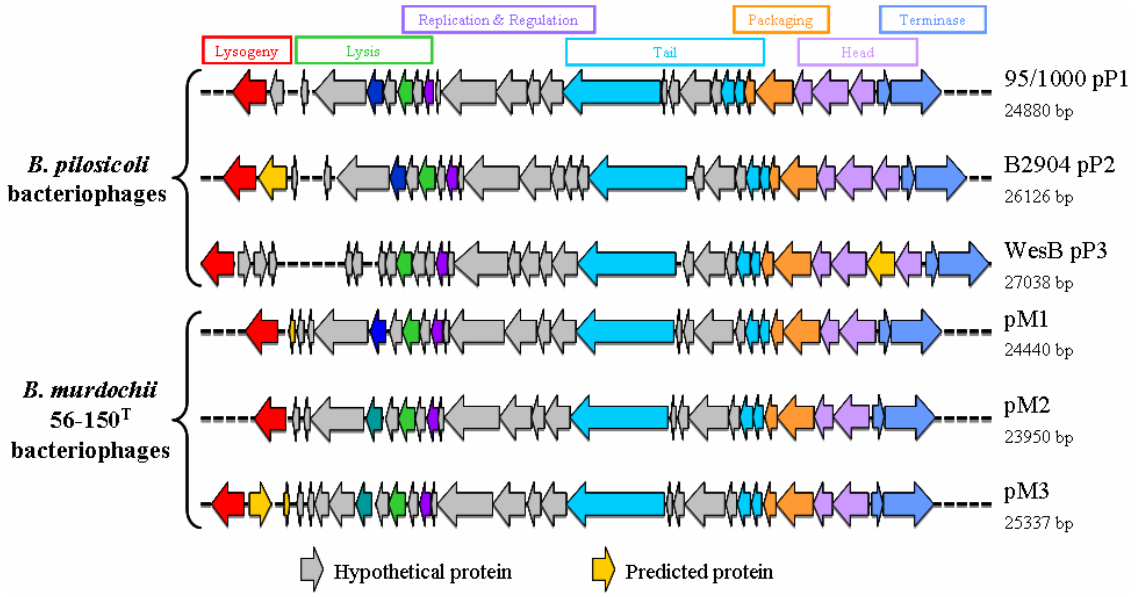

Figure 4.6 Comparison of the organisation of the bacteriophages in the three *B. pilosicoli* genomes and *B. murdochii* 56-150^{T} genome. A comparison of bacteriophages pP1 in 95/1000, pP2 in B2904 and pP3 in WesB and also the three bacteriophages found in *B. murdochii* 56-150^{T}; pM1, pM2 and pM3. Genes encoding hypothetical proteins (grey) and genes with predicted protein function (yellow) are indicated.

4.3.6 Central metabolism and correlation with phenotype

4.3.6.1 Carbohydrate metabolism

High proportions (4.32 – 5.37%) of the *B. pilosicoli* genomes were associated with carbohydrate transport and metabolism (Table 4.2), and from metabolic pathway reconstructions it is evident that glycolysis constitutes a major backbone of energy production (Wanchanthuek *et al*., 2010). Collectively the strains of *B. pilosicoli* utilised 51.9% of carbohydrate compounds tested, and more specifically 69.4% of hexose sugars (Appendix II). Genes for enzymes involved in converting glucose-6-phosphate to ribulose-5-phosphate that were identified in *B. hyodysenteriae* WA1 (Bellgard *et al*., 2009), were found in the *B. pilosicoli* genomes. These features are likely to direct carbohydrate oxidation towards the non-oxidative pentose phosphate pathway, to generate reducing power required for biosynthetic pathways. *B. pilosicoli* is characterised by an absence of β-glucosidase activity (Fellstrom and Gunnarsson, 1995), however a novel system for metabolising β-glucosides found in 95/1000

(Wanchanthuek *et al*., 2010) was also present in B2904 and WesB, which, alongside specific PTS systems, is likely to be involved in the utilisation of D-cellobiose and arbutin as carbon sources. Despite lacking β-glucosidase, metabolism of β-glucosides may be important to the virulence of *B. pilosicoli* as this phenotype is associated with bacterial growth, adhesion and colonisation (Kilic *et al*., 2004). Of the disaccharides tested, 64.3% were utilised by the strains of *B. pilosicoli*, whereas, of the oligosaccharides only dextrin was utilised, which is likely to be attributed to α-glucosidase activity (BP951000_1130; B2904_orf204; wesB_2480).

4.3.6.2 Amino acid metabolism

Of the COG categories related to metabolism, the greatest proportion of the genome was related to amino acid transport and metabolism (Table 4.2). Phenotypic studies revealed that despite the high number of genes for amino acid/oligopeptide transporters found in the genomes, only five of the tested amino acids were able to support *B. pilosicoli* as a sole carbon source (Appendix II). Genes encoding enzymes to direct these amino acids towards pyruvate metabolism and hence energy production were identified, including alanine dehydrogenase (BP951000_0036; B2904_orf1321; wesB_1465), threonine aldolase (BP951000_1568; B2904_orf2409; wesB_0396), glycine hydroxymethyltransferase (BP951000_1528; B2904_orf2450; wesB_0361) and L-serine dehydratase (BP951000_0452 and BP951000_0453; B2904_orf939 and B2904_orf940; wesB_1746 and wesB_1747). Interestingly, *B. pilosicoli* was the only species that was unable to utilise the amino acids D-serine and L-glutamic acid (Table 4.6), consistent with the absence of genes required for their catabolism in the *B. pilosicoli* genomes. Moreover, a glycine reductase complex found in the strains of *B. pilosicoli*, which catalyses the reductive deamination of glycine, forming ATP, would be involved in the utilisation of glycine. A high proportion of amino acid metabolic features in *B. pilosicoli* were related to biosynthesis and potentially maintaining intermediates of the partial TCA cycle identified in this species (Wanchanthuek *et al*., 2010), rather than catabolism to produce energy. L-glutamic acid and L-glutamine were insufficient to sustain *B. pilosicoli* as a sole carbon source; these amino acids are primary products of ammonia assimilation used in peptidoglycan, LOS and outer membrane protein biosynthesis (Merrick and Edwards, 1995), hence their metabolism is redirected to energy yielding pathways. The strains of *B. pilosicoli* possessed genes for glutamate dehydrogenase (BP951000_1312; B2904_orf93; wesB_0103), which catalyses the reversible synthesis of glutamic acid from α-ketoglutaric acid and

ammonium. Since α-ketoglutaric acid was able to sustain *B. pilosicoli*, the presence of a transporter for α-ketoglutaric acid and not glutamic acid may explain this phenotype. The ability to utilise amino acids as an energy source may have become redundant in *Brachyspira*, which typically occupy the nutrient-rich lower GI tract, and hence associated features may have been lost through reductive evolution.

4.3.6.3 Nucleotide metabolism

The strains of *B. pilosicoli* were able to utilise three purine and two pyrimidine nucleosides tested as a sole carbon source (Appendix II). The enzymes suggested to complete a metabolic link between nucleoside and central metabolism in *B. hyodysenteriae* WA1 (Bellgard *et al*., 2009) were identified in the strains of *B. pilosicoli*.

4.3.6.4 Lipid metabolism

Despite the presence of enzymes involved in the β-oxidation of fatty acids, including a long chain fatty acid-CoA ligase (BP951000_0887; B2904_orf479; wesB_2210), no long chain fatty acids tested were utilised by *B. pilosicoli* as a carbon source; however, the short chain fatty acids, butyric acid and propionic acid, were utilised (Appendix II). Uniquely to *B. pilosicoli* (Table 4.6), glycerol was utilised as a carbon source, and genes for its metabolism were detected including those for a glycerol uptake facilitator (BP951000_0799; B2904_orf2190; wesB_2118), glycerol kinase (BP951000_0800; B2904_orf2191; wesB_2119) and glycerol-3-phosphate dehydrogenase (BP951000_1696; B2904_orf2220; wesB_0532). Glycerol is an important carbon and energy source for pathogens; glycerol-catabolising enzymes have been considered crucial for intracellular growth of *Listeria* (Joseph *et al*., 2006) and the ability to utilise this compound has been considered a key factor in allowing the reductive evolution of *Mycoplasma* (Halbedel *et al*., 2004). The gene set required for fatty acid biosynthesis was incomplete in B2904 and WesB, as it was in 95/1000 (Wanchanthuek *et al*., 2010).

4.3.6.5 Differences in carbon source utilisation by *Brachyspira*

Previous genomic analysis *B. hyodysenteriae* and *B. pilosicoli* revealed that these species share many metabolic capabilities (Bellgard *et al*., 2009; Wanchanthuek *et al*., 2010). By application of Biolog PM^{TM} technology for phenotypic determination of carbon source utilisation of four strains each of *B. alvinipulli*, *B. hyodysenteriae*, *B.*

innocens, *B. intermedia*, *B. murdochii* and *B. pilosicoli* and two strains each of "*B. canis*" and "*B. pulli*" (Appendix II), differences in carbon source utilisation between species were determined. *B. alvinipulli*, *B. hyodysenteriae*, *B innocens* and *B. murdochii* had the most intra-species differences in the utilisation of carbon sources, with intra-species differences in the utilisation of 42 to 47 carbon sources each (Appendix II). "*B. canis*", *B. intermedia* and "*B. pulli*" had between 19 and 26 intra-species differences in carbon source utilisation each (Appendix II), with *B. pilosicoli* having fewest, with just 7, all of which were accounted for genotypically (Table 4.7). As more *Brachyspira* genome sequences become available for intra-species comparisons, particularly in the species *B. alvinipulli*, *B. hyodysenteriae*, *B innocens* and *B. murdochii*, considerable differences in the complement of metabolic features in the strains of each species may be expected. The analysis, however was based only on four strains of each of the known species and two strains of both proposed species, and therefore, may have underestimated the extent of the intra-species differences.

At a species level, differences in the utilisation of 71 carbon sources were noted (Table 4.6). Amongst the other species tested, *B. pilosicoli* demonstrated a unique ability to utilise glycerol and a unique inability to utilise L-glutamic acid, glycyl-L-aspartate and D-serine. *B. pilosicoli* was the only species in which all tested strains were able to utilise 3-*O*-β-D-galactopyranosyl-D-arabinose, D-tartaric acid and thymidine, and unable to utilise D-aspartic acid, L-aspartic acid, ethanolamine, glycyl-L-glutamic acid, glycyl-L-proline, *m*-inositol, β-phenylethylamine, L-proline, D-trehalose and tyramine. These phenotypic differences have potential to improve current biochemical identification testing, however a wider strain set for each of the species would be required to confirm these unique phenotypes amongst the species. Moreover, further work is required in order to correlate phenotypic and genotypic differences between the strains and species, for which genome sequences are available.

Preliminary work to associate phenotypic differences with genotype in other *Brachyspira* species did find some correlations. For example, *B. alvinipulli* was the only species where all the strains tested were unable to utilise uridine (Table 4.6) and coincidentally the gene encoding uridine phosphorylase for the first step in uridine catabolism, was found in all available *Brachyspira* genomes (in two or more copies) except that of *B. alvinipulli* $C1^T$. Moreover, *B. alvinipulli* was the only species in which strains, including $C1^T$, showed the ability to utilise the dicarboxylates, oxalic acid, bromosuccinic acid, sebacic acid and citraconcic acid (Appendix II), and this may be explained by the presence of the *dctA* gene, encoding a dicarboxylate transport protein,

found only in the *B. alvinipulli* C1^T genome. Similarly, the ability of only strains of *B. hyodysenteriae* to utilise the tricarboxylate, citric acid (Appendix II), may be associated to the presence of the genes *tctA*, *tctB* and *tctC*, which encode components of a tricarboxylate transport protein and *citD*, *citE* and *citF*, which encode subunits of the enzyme citrate lyase, only in the *B. hyodysenteriae* WA1 genome.

4.3.7 Concluding remarks

In this chapter, the genome of *B. pilosicoli* strain B2904 and the near complete genome of strain WesB were reported. Together with the previously reported 95/1000 genome, this allowed the first intra-species genome comparison within the genus *Brachyspira*. The feature-based analysis revealed a high level of similarity between the three strains and identified genes that different strains of the spirochaete may have lost in a process of reductive genome evolution. Sequence-based comparisons showed the majority of sequence was shared between the strains, with few unique regions; however, genome rearrangements were observed around the *oriC*. MGEs were found associated to areas of rearrangements, and these features may be a factor that has driven or is driving reductive evolution. Novel bacteriophages were identified in the newly-sequenced genomes, which displayed evidence of intra- and inter-species HGT, and these may have key practical applications for use in genetic manipulation. This is the first analysis of the spirochaete in a high-throughput phenotype screening tool, allowing correlation between genotype and phenotype. Future work may focus on the application of this technology to a wider range of *Brachyspira* species to validate genome differences, potentially providing a means by which these phenotypes can be used for rapid screening to infer genotypes and improve current diagnostic methods. With the increasing availability of *Brachyspira* genome sequences, such technology should facilitate the validation of metabolic models based on genome sequence.

Chapter 5.

The characterisation and selection of *Lactobacillus* species as prospective probiotics for the control of avian intestinal spirochaetosis

5.1 Introduction

An approach to the control of AIS has been the use of antimicrobials such as tiamulin. However, given the drive in animal husbandry to reduce antimicrobial usage, alternative control measures are required. Probiotics are viable microorganisms used as feed supplements, which lead to beneficial effects in the host (Fuller, 1989) and were introduced in Chapter 1 (specifically in section 1.3). The bacterial genera commonly used as probiotics include *Bacillus*, *Bifidobacteium*, *Enterococcus*, *Lactobacillus* and *Streptococcus*. Probiotics are thought to exert beneficial health benefits to the host by immunomodulation of the gut mucosa, enhancing epithelia barrier function, modulation of the gut microbiota and by CE of pathogenic microbes, via competition for nutrients and receptor sites and production of antimicrobial compounds (Collins *et al*., 2009; Gerritsen *et al*., 2011; Klaenhammer and Kullen, 1999). One testable hypothesis of this thesis is that lactic acid bacteria such as lactobacilli from poultry may have properties suitable for the control of AIS, specifically by interference with aspects of the biology of *Brachyspira* species.

Since May 2003, the responsibility over the risk assessment of animal feed additives for use in Europe was taken over by the European Food Safety Authority (EFSA) from the SCAN (Anadon *et al*., 2006; von Wright, 2005). The approval and risk management of a probiotic product is the responsibility of the European Commission

(EC) and its constituent member states, to which, the EFSA provide scientific advice. Accurate and consistent characterisation and identification of probiotic lactobacilli strains was fundamental to the studies presented in this thesis, as probiotic candidates must meet the EU guidelines proposed in council directive 87/153/EEC, which stipulate the assessment guidelines for the safe use of probiotic feed additives (von Wright, 2005). To fulfil current EU directives on animal feed additives, probiotic products must be clearly identified and characterised to the species level, supported in claims on their efficacy, tolerated by the target animal species, safe for the operator have no risk to the safety of the end-consumer (SCAN, 2001). Moreover, novel probiotic products must not harbour acquired antimicrobial resistance determinants, which have the potential to be transferred to other bacteria (EFSA, 2005, 2008; SCAN, 2001, 2003b). Poor quality control has been reported previously of probiotic bacteria in the commercial sector (Coeuret *et al*., 2004; Yeung *et al*., 2002); to avoid this, it is important to accurately and consistently identify the species in use from an early stage in the selection process.

Selection criteria for probiotics can be divided into four categories (Klaenhammer and Kullen, 1999). Appropriateness criteria ensure the bacteria are GRAS and of host origin. Technological suitability criteria determine practical aspects of production and storage. Competitiveness criteria consider bacterial survival within the host and tolerance to environmental stresses, such as gastric acid and bile. Performance and functionality criteria relate to the beneficial effect of the probiotic to the host.

This chapter describes the phenotypic and molecular characterisation of *Lactobacillus* strains, which were used as probiotic candidates in the studies discussed in Chapters 6 and 7. Furthermore, the initial screening for properties of the lactobacilli indicative of them acting as potential probiotics and the further characterisation of the selected strains, including acid and bile tolerance assays and screening for antimicrobial resistance is described in this chapter. This further characterisation was conducted in order to comply with current EU regulations on animal feed additives, hereby highlighting essential future work required for the commercial use of such potential probiotic strains.

5.2 Results

5.2.1 Speciation and characterisation of poultry isolates of *Lactobacillus*

5.2.1.1 Phenotypic characterisation

Of the eighteen *Lactobacillus* strains utilised in the studies presented in this thesis, sixteen were obtained from the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, United Kingdom) culture collection. A further two strains, designated LM1 and LM2, were isolated as part of this study from the faeces of healthy conventional, commercial laying hens by the inoculation of serial dilutions of faeces (in 0.1 M sterile PBS) onto MRS agar, which facilitated the selection of lactobacilli. Single colonies of the presumptive *Lactobacillus* strains were picked and expanded by further subculture on MRS agar prior to preparation of a stock culture.

Gram staining was performed on all of the 16 AHVLA *Lactobacillus* strains and the two presumptive *Lactobacillus* strains, LM1 and LM2, followed by examination under light microscopy. All strains were Gram-positive, rod-shaped bacteria. Biochemical speciation of all *Lactobacillus* strains was performed using the API 50 CH system. API result profiles of all of the strains were recorded and analysed using the online database of API fermentation profiles (apiweb^{TM}, BioMérieux), which returned PID of the speciation of each of the strains (Table 5.1). All of the AHVLA derived strains were correctly identified by this method with the notable exception for strains classified by AHVLA as *L. reuteri* were identified as *L. fermentum*. Strains LM1 and LM2 were given presumptive identities of *L. fermentum* and *L. salivarius* respectively.

The catalase test revealed all *Lactobacillus* strains to be catalase-negative (Table 5.1). Furthermore, the ability of the strains to produce hydrogen peroxide, a potential antimicrobial substance, was assayed using an established method (Martin *et al*., 2008a; Rabe and Hillier, 2003). Five of the strains were positive for hydrogen peroxide production including the LM1 strain.

5.2.1.2 Molecular characterisation

A multiplex PCR, which amplified unique regions of sequence between the 16S and 23S rRNA genes (Kwon *et al*., 2004), was employed for molecular speciation of the eighteen *Lactobacillus* strains. The multiplex PCR is capable of the detection of *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *L. reuteri* and *L. rhamnosus*, amplifying products of specific length for each; 606, 727, 184, 272, 428,

1105 and 448 bp, respectively. Following the multiplex PCR, the products were subjected to gel electrophoresis in order to determine the amplicon length, if any, and provide a speciation based on the multiplex PCR (Table 5.1). Three of the strains, including the LM2 strain, were not speciated by the multiplex PCR employed and gave inconclusive results with either no amplicons or amplicons of multiple lengths. However, speciation of the LM1 strain was indicated as *L. reuteri*, a result at variance with the API data.

Further to the multiplex PCR, 16S rRNA gene sequencing was employed to confirm and in some cases clarify speciation. Universal 16S rRNA gene primers were employed for amplification of the gene (Marchesi *et al*., 1998), and the resultant amplicons were sequenced by ABI sequencing. Following alignment of the forward and reverse sequences, the 16S rRNA gene sequence of each of the strains was compared to an online database (GenBank, NCBI), using BLAST. The species and GenBank accession number with highest PID match to the 16S rRNA gene sequence of each strain in GenBank database is shown in Table 5.1. The LM1 strain was confirmed as *L. reuteri* and the LM2 strain as *L. salivarius*.

Table 5.1 Phenotypic and molecular speciation and characterisation *Lactobacillus* strains used in the studies presented in this thesis. Results of the API 50 CH system, multiplex PCR and 16S rRNA gene sequencing were used for speciation of the *Lactobacillus* strains. Results of the API 50 CH system were analysed using the online database of API fermentation profiles (apiweb™, BioMérieux) and 16S rRNA gene sequences were compared to an online database (GenBank, NCBI), using BLAST. As part of characterisation, the lactobacilli were tested for the activity of catalase and their ability to produce hydrogen peroxide (cont'd p 158).

Species	**Strain**	**API 50 CH ID**		**Multiplex PCR**[a]	**16S rRNA sequencing**[b]			**Catalase**[c]	**Hydrogen peroxide**[d]
		Species	**PID**		**Match species**	**Genbank accession**	**PID**		
L. acidophilus	B2990	*L. acidophilus*	47.00%	*L. acidophilus*	*L. acidophilus*	EU878007.1	99.00%	−	+
L. acidophilus	B2993	*L. acidophilus*	76.40%	*L. acidophilus*	*L. acidophilus*	JQ350808.1	100.00%	−	+
L. buchneri	B2997	*L. buchneri*	45.50%	−	*L. buchneri*	AB425940.1	100.00%	−	−
L. bulgaricus	B2991	*L. delbrueckii*	57.80%	*L. delbrueckii*	*L. bulgaricus*	FJ749381.1	98.00%	−	+
L. bulgaricus	B2999	*L. delbrueckii*	88.60%	*L. delbrueckii*	*L. bulgaricus*	EU547306.1	100.00%	−	+
L. casei	B2986	*L. paracasei*	80.00%	*L. casei*	*L. casei*	JN560879.1	99.00%	−	−
L. casei	B2995	*L. paracasei*	80.00%	*L. casei*	*L. casei*	JN560917.1	100.00%	−	−
L. fermentum	B2992	*L. fermentum*	59.80%	−	*L. fermentum*	EU626018.1	99.00%	−	−
L. plantarum	B2989	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	GU451062.1	98.00%	−	−
L. plantarum	B2994	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	GU451062.1	98.00%	−	−
L. plantarum	B2996	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	AM279764.2	100.00%	−	−
L. plantarum	JC1 (B2028)	*L. plantarum*	99.90%	*L. plantarum*	*L. plantarum*	JQ278711.1	100.00%	−	−
L. reuteri	B2026	*L. fermentum*	99.30%	*L. reuteri*	*L. reuteri*	JN981867.1	98.00%	−	−
L. reuteri	LM1	*L. fermentum*	99.30%	*L. reuteri*	*L. reuteri*	JF927766.1	100.00%	−	+
L. rhamnosus	B2987	*L. rhamnosus*	96.50%	*L. rhamnosus*	*L. rhamnosus*	HQ293051.1	97.00%	−	−

Table 5.2 (cont'd).

Species	Strain	API 50 CH ID		Multiplex PCR[a]	16S rRNA sequencing[b]			Catalase[c]	Hydrogen peroxide[d]
		Species	PID		Match species	Genbank accession	PID		
L. rhamnosus	B2988	*L. rhamnosus*	99.70%	*L. rhamnosus*	*L. rhamnosus*	JQ621982.1	98.00%	−	−
L. rhamnosus	B2998	*L. rhamnosus*	99.90%	*L. rhamnosus*	*L. rhamnosus*	HQ293051.1	100.00%	−	−
L. salivarius	LM2	*L. salivarius*	94.80%	−	*L. salivarius*	HQ293056.1	99.00%	−	−

[a] Multiplex PCR (Kwon *et al*., 2004) detects *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *L. reuteri* and *L. rhamnosus*; −, inconclusive result.

[b] Species and accession number with highest PID match to the 16S rRNA gene sequence in GenBank database.

[c] Test for catalase activity; +, positive; −, negative.

[d] Test for ability to produce hydrogen peroxide; +, positive; −, negative.

5.2.2 Selection of *Lactobacillus* strains as prospective probiotics

5.2.2.1 Screening for an inhibitory effect of *Lactobacillus* cell-free supernatant on the growth of *B. pilosicoli*

The test panel of *Lactobacillus* strains (Table 5.1) were screened for their inhibitory activity against the growth and motility of *B. pilosicoli*. Whilst a relatively trivial test, the technicality of preparing and testing multiple *Brachyspira* isolates was logistically not possible. Thus, all initial screening was limited to the use of strain B2904. The CFS of each *Lactobacillus* strain was obtained from spent MRS, of which the median pH was pH 3.8. The CFS was added to BEB at 10% (v/v), using a control whereby 10% (v/v) MRS (pH 5.8) was added. The effect of the *Lactobacillus* CFS on the growth of *B. pilosicoli* B2904 was monitored at 24 hours intervals over a 120 hour period using a Helber counting chamber for bacterial enumeration. Of the CFS from eighteen *Lactobacillus* strains screened, seven gave significant inhibition of the growth compared to the MRS control ($p<0.05$) (Figure 5.1). These strains were *L. acidophilus* B2990, *L. bulgaricus* B2991, *L. plantarum* B2994, *L. reuteri* B2026, *L. reuteri* LM1, *L. rhamnosus* B2988 and *L. salivarius* LM2.

5.2.2.2 Screening for an inhibitory effect of *Lactobacillus* on the motility of *B. pilosicoli*

Agar motility and haemolysis inhibition assays were performed as described previously (Bernardeau *et al*., 2009), as motility is considered an essential virulence factor and inhibition of this feature may be key in the intervention of *Brachyspira*. The ability of the panel of *Lactobacillus* strains (Table 5.1), both viable and heat-inactivated, to inhibit the motility of the motile *B. pilosicoli* B2904 strain following 4 and 24 hour contact times, was tested. Heat-inactivated lactobacilli cells were used to eliminate the potential effect of competition for nutrients and the synthesis of antimicrobial substances. A 5 µl spot of the suspension containing *B. pilosicoli* and lactobacilli (1:1) was inoculated on FABA agar after the respective contact time and incubated anaerobically for 8 days. The extent of motility and haemolysis was examined visually each day. With the exception of *L. casei* B2986, *L. casei* B2995 and *L. fermentum* B2992, all *Lactobacillus* strains tested had a supressive effect on the motility or haemolysis of *B. pilosicoli*. The inhibition of motility and haemolysis for the *Lactobacillus* strains that also significantly inhibited the growth of *B. pilosicoli* is

shown in Table 5.2. *B. pilosicoli* were recovered by subculture from all assays that displayed motility and hemolytic growth of the spirochaete, indicating viability.

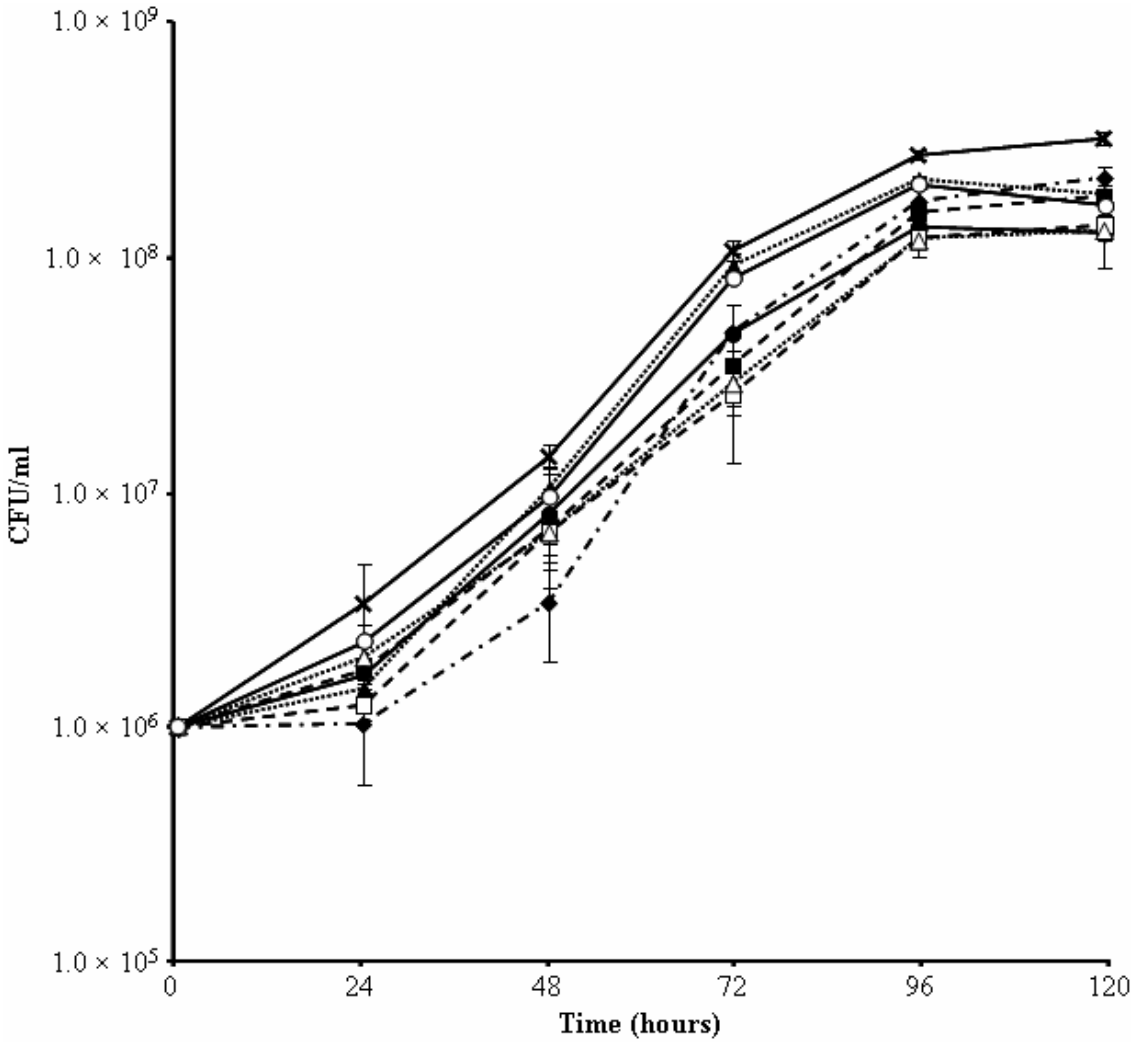

Figure 5.1 Growth of *B. pilosicoli* B2904 in broth culture (BEB) supplemented with 10% (v/v) cell-free supernatant (CFS) (pH 3.8) of *Lactobacillus* strains that gave significant inhibition of the growth of the spirochaete ($p<0.05$); *L. acidophilus* B2990 (closed squares, dashed line), *L. bulgaricus* B2991 (closed triangles, dotted line), *L. plantarum* B2994 (closed circles, solid line), *L. reuteri* B2026 (open circles, solid line), *L. reuteri* LM1 (closed diamonds, dash-dotted line), *L. rhamnosus* B2988 (open squares, dashed line) and *L. salivarius* LM2 (open triangles, dotted line). Controls with MRS (pH 5.8) supplemented at 10% (v/v) to the *B. pilosicoli* culture (x's) are shown. *B. pilosicoli* were enumerated using a Helber counting chamber at 25 hour intervals over the 120 hour period. Values presented are means with standard deviation of 9 repeats. Significance was determined between the final growth point readings of the *B. pilosicoli* with lactobacilli CFS compared to the MRS (pH 5.8) control.

Table 5.3 The effect of viable or heat-inactivated *Lactobacillus* strains on the motility of *B. pilosicoli* B2904 following 4 and 24 hour contact times. A *B. pilosicoli* B2904 only suspension in PBS was used as a control. *Lactobacillus* strains, of which the cell-free supernatant (CFS) significantly inhibited *B. pilosicoli* B2904 growth, are shown.

Condition		Contact time (hr)	Haemolysis[a]	Motility[b]
PBS control		4	+	+
		24	+	+
L. acidophilus B2990	Viable	4	+	–
		24	+	–
	Heat-inactivated	4	+	+
		24	+	–
L. bulgaricus B2991	Viable	4	+	+
		24	–	–
	Heat-inactivated	4	+	+
		24	–	–
L. plantarum B2994	Viable	4	+	–
		24	–	–
	Heat-inactivated	4	+	+
		24	+	–
L. reuteri B2026	Viable	4	+	+
		24	+	–
	Heat-inactivated	4	+	+
		24	+	–
L. reuteri LM1	Viable	4	–	–
		24	–	–
	Heat-inactivated	4	+	–
		24	+	–
L. rhamnosus B2988	Viable	4	–	–
		24	–	–
	Heat-inactivated	4	+	+
		24	–	–
L. salivarius LM2	Viable	4	+	+
		24	–	–
	Heat-inactivated	4	+	+
		24	+	–

[a] Haemolytic ability of *B. pilosicoli* B2904 following the incubation with the *Lactobacillus* strain; +, positive; −, negative.

[b] Motile ability of *B. pilosicoli* B2904 following the incubation with the *Lactobacillus* strain; +, positive; −, negative.

5.2.3 Further characterisation of *L. reuteri* LM1 and *L. salivarius* LM2

From the initial studies performed on the panel on *Lactobacillus* strains (discussed in sections 5.2.1 and 5.2.2), *L. reuteri* LM1 and *L. salivarius* LM2 were selected as potential probiotic candidates for further investigation of their ability to intervene against AIS. These strains were selected as their origin was the same as the host for the intended treatment, which is an important selection criterion for probiotics (Klaenhammer and Kullen, 1999) and also indicates an ability to colonise the intended host. Furthermore, selection criteria stipulate that probiotic candidates should exhibit antimicrobial activity against target pathogens or some degree of antagonism, which both of these strains have demonstrated in growth and motility inhibition assays. For the purpose of intellectual property protection and potential commercial exploitation, the use of novel strains, such as LM1 and LM2, is ideal.

5.2.3.1 Antimicrobial susceptibility

It is essential to demonstrate that novel probiotic products do not harbour any acquired antimicrobial resistance determinants, which may be transferable to other bacteria, in order to comply with current EU regulations for animal feed additives (EFSA, 2008). The MICs of ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin were determined for *L. reuteri* LM1 and *L. salivarius* LM2, which were the probiotic candidate strains selected for further investigation in *in vitro* studies and *L. reuteri* LM2 in *in vivo* studies (Table 5.3). This array of antimicrobials was selected in order to maximise the identification of resistance genotypes to the most common antimicrobials used by assessing the resistance phenotypes. It is important to note that some *Lactobacillus* species are innately resistant to ampicillin and vancomycin, which does not pose the risk of transmissible antimicrobial resistance genes.

Preliminary studies that used the Identibac AMR+ve^{TM} microarray for the detection of 90 antimicrobial resistance genes in Gram-positive bacteria (Perreten *et al*., 2005), indicated the presence of tetracycline resistance genes in both *L. reuteri* LM1 (*tetM*) and *L. salivarius* LM2 (*tetW* and *tetZ*). Moreover, the microarray indicated the presence of the *cat-86* gene, which mediates chloramphenicol resistance in *L. salivarius* LM2. However, no studies have been performed to confirm the presence of such genes in the *Lactobacillus* strains.

Table 5.4 Minimum inhibitory concentration (MIC) of nine antimicrobials against *L. reuteri* LM1 and *L. salivarius* LM2. The MIC was determined for nine of the ten stipulated antimicrobials in the guidelines for antimicrobial resistance screening in probiotic candidates (EFSA, 2008).

Antimicrobial	**MIC (µg/ml)**	
	***L. reuteri* LM1**	***L. salivarius* LM2**
Ampicillin[a,c]	1	0.5
Chloramphenicol[a]	4	8[d]
Clindamycin[a]	0.25	0.125
Erythromycin[a]	0.5	0.5
Gentamicin[a]	1	32[d]
Kanamycin[b]	32	128[d]
Streptomycin[b]	32	128[d]
Tetracycline[b]	256[d]	128[d]
Vancomycin[b,c]	256[d]	>256[d]

[a] Antimicrobial tested doubling series ranged 0.06 – 128 µg/ml.

[b] Antimicrobial tested doubling series ranged 1 – 256 µg/ml.

[c] Some *Lactobacillus* species and strains are inherently resistant.

[d] Strain considered resistant as the MIC is greater than the proposed breakpoint (EFSA, 2008).

5.2.3.2 Tolerance to avian gastric acid

L. reuteri LM1 and *L. salivarius* LM2, which were selected as probiotic candidates for further *in vitro* and *in vivo* testing, were assessed for their ability to tolerate gastric acid retrieved and prepared from SPF chickens. The lactobacilli were resuspended at 10^9 CFU/ml in sterile gastric juice prepared from SPF poultry gizzard digesta and adjusted to pH 2.0, 2.5 and 3.0. Both strains were also resuspended in 0.1 M PBS (pH 7.2). A significant reduction in the numbers of *L. salivarius* LM2 was observed after 3 hours incubation in gastric juice at all three pH levels tested, whereas a significant reduction in numbers of *L. reuteri* LM1 was evident only at the lowest tested pH level (Table 5.4). No significant difference in the numbers of either *Lactobacillus* strain was observed when incubated for 3 hours in 0.1 M PBS (pH 7.2). A 3 hour incubation was used as it is unlikely that the bacteria would be exposed to the low pH for any longer amount of time, due to the shorter GI tract of poultry.

Table 5.5 Survival of *L. reuteri* LM1 and *L. salivarius* LM2 following 3 hours incubation in poultry gastric juice adjusted to pH 2.0, 2.5 and 3.0. Values presented are means with standard deviation of 9 repeats. Significance is shown where *Lactobacillus* cell numbers differed significantly from 0 hours. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

	***L. reuteri* LM1 (log_{10} CFU/ml)**		***L. salivarius* LM2 (log_{10} CFU/ml)**	
	0 hr	**3 hr**	**0 hr**	**3 hr**
pH 3.0	9.19 ± 0.04	9.18 ± 0.07	9.25 ± 0.04	8.93 ± 0.10*
pH 2.5	9.27 ± 0.02	9.18 ± 0.02	9.14 ± 0.05	8.02 ± 0.15***
pH 2.0	9.16 ± 0.01	8.79 ± 0.10***	9.35 ± 0.01	8.14 ± 0.10***

5.2.3.3 Tolerance to avian and bovine bile salts and avian bile extract

The tolerance of *L. reuteri* LM1 and *L. salivarius* LM2 to two major poultry bile salts, taurocholic acid and sodium taurochenodeoxycholate (Yeh and Hwang, 2001) and oxgall, containing bovine bile salts, was determined by measuring OD_{600} to monitor growth of the lactobacilli in the presence of 0.3% (w/v) of each of the bile salts tested over a 30 hour period (Figure 5.2A and B). Furthermore, the tolerance of the lactobacilli to three concentrations (0.3%, 0.6% and 0.9%, v/v) of bile collected from the gall bladder of SPF chicken was also determined (Figure 5.2C and D). The delay in growth of lactobacilli was associated with the addition of bile products (Table 5.5) was deduced by the application of a previously described method (Chateau *et al*., 1994).

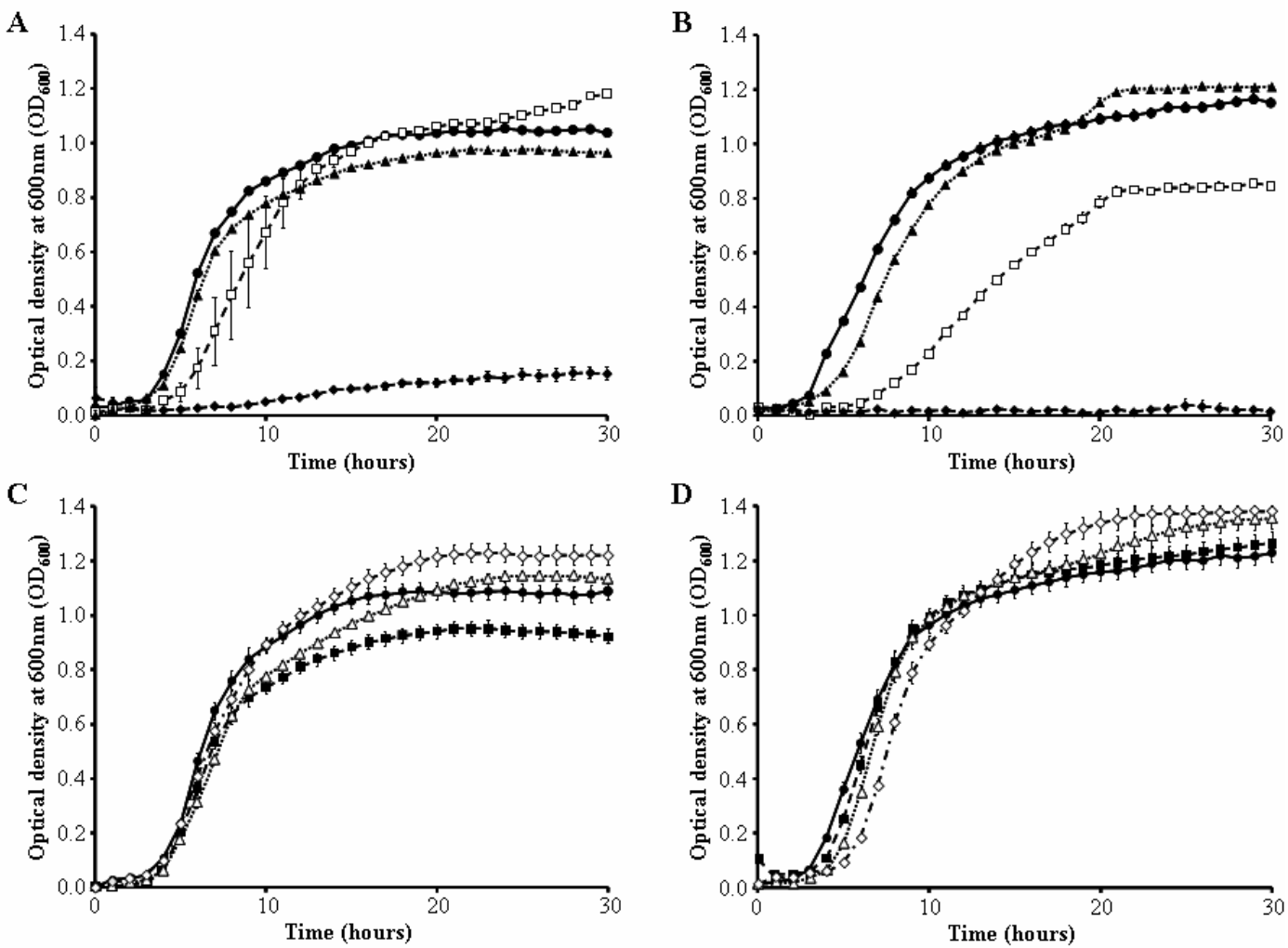

Figure 5.2 Tolerance of *L. reuteri* LM1 (A and C) and *L. salivarius* LM2 (B and D) to bile salts and bile collected from the gall bladder of specific pathogen-free (SPF) chickens. Poultry bile salts, taurocholic acid (closed triangles, dotted line) and sodium taurochenodeoxy-cholate (open squares, dashed line) and the bovine bile salt, oxgall (closed diamonds, dash-dotted line), were tested at 0.3% (w/v) in MRS (A and B). Poultry bile extracts were tested at 0.3% (closed squares, dashed line), 0.6% (open triangles, dotted line) and 0.9% (v/v) (open diamonds, dash-dotted line) in MRS (C and D). MRS only controls were used (closed circles, solid line). Growth was measured using OD_{600} as a reporter. Values presented are means with standard deviation of 9 repeats.

Table 5.6 Tolerance measured in lag time of *L. reuteri* LM1 and *L. salivarius* LM2 to poultry bile salts, sodium taurochenodeoxycholate (STCDC) and taurocholic acid (TA) and oxgall, containing bovine bile salts at 0.3% (w/v) and their tolerance to bile collected from specific pathogen-free (SPF) chickens at 0.3%, 0.6% and 0.9% (v/v). Values presented are means with standard deviation of 9 repeats.

Bile product added	**Time to reach 0.3 unit difference in OD_{600} (hr)**					
	***L. reuteri* LM1**			***L. salivarius* LM2**		
	No bile	**With bile**	**Lag time[a]**	**No bile**	**With bile**	**Lag time[a]**
0.3% STCDC[b]	5.00 ± 0.10	7.37 ± 1.70	2.37 ± 1.63	4.57 ± 0.15	10.9 ± 0.17	6.33 ± 0.32
0.3% TA[c]	5.00 ± 0.10	5.43 ± 0.25	0.43 ± 0.32	4.57 ± 0.15	6.13 ± 0.06	1.57 ± 0.21
0.3% Oxgall	5.00 ± 0.10	–	–	4.57 ± 0.15	–	–
0.3% Bile[d]	5.27 ± 0.15	5.77 ± 0.32	0.50 ± 0.17	4.63 ± 0.21	5.27 ± 0.15	0.63 ± 0.06
0.6% Bile[d]	5.27 ± 0.15	5.93 ± 0.29	0.67 ± 0.15	4.63 ± 0.21	5.73 ± 0.12	1.10 ± 0.10
0.9% Bile[d]	5.27 ± 0.15	5.50 ± 0.17	0.23 ± 0.06	4.63 ± 0.21	6.70 ± 0.10	1.07 ± 0.12

[a] Lag time between the no bile and with bile conditions; **–,** 0.3 unit difference not reached.

[b] Sodium taurochenodeoxycholate, poultry bile salt.

[c] Taurocholic acid, poultry bile salt.

[d] Bile extracts collected from the gall bladder of SPF chickens.

5.3 Discussion

Eighteen candidate probiotics, of the genus *Lactobacillus*, were screened for their antagonistic properties against *B. pilosicoli*; two strains of *L. acidophilus*, one *L. buchneri*, two *L. delbrueckii* subsp. *bulgaricus*, two *L. casei*, one *L. fermentum*, four *L. plantarum*, two *L. reuteri*, three *L. rhamnosus* and one *L. salivarius*. All *Lactobacillus* strains were cultured on MRS agar, which is widely used for the culture of lactobacilli and aids in the selection of this bacterial species (de Man *et al*., 1960). *L. reuteri* LM1 and *L. salivarius* LM2 were isolated from the faeces of healthy conventional laying hens using MRS agar, and they conformed to the basic biochemical and physiological parameters of *Lactobacillus* strains (Kandler and Weiss, 1986). All strains were confirmed as Gram-positive, rod-shape bacteria and were catalase negative, which aside from a minor proportion of strains, is typical of the *Lactobacillus* genus (Dacre and Sharpe, 1956).

The API 50 CH system was used as a preliminary tool to speciate the strains isolated from poultry faeces and confirm the species of strains obtained from the Animal Health and Veterinary Laboratories Agency (AHVLA, Weybridge, United Kingdom) culture collection (Table 5.1). The majority of the *Lactobacillus* strains were correctly identified by this biochemical method of speciation with high PID, but with some as low as 45.5%, perhaps due to strain variation in fermentation profiles or incorrect interpretation of indistinguishable results. The two *L. bulgaricus* strains were identified as *L. delbrueckii* as the former is a subspecies of the latter, *L. delbrueckii* subsp. *bulgaricus*. The two *L. casei* strains were identified as *L. paracasei*, resulting from the close relationship and the phenotypic similarity of these species, which, until recently, were under dispute of the Judicial Commission of the International Committee on Systematics of Bacteria (JCICSB) in the field of taxonomy (Felis *et al*., 2009; JCICSB, 2008). Both *L. reuteri* strains, one of which was isolated from the poultry faeces, were incorrectly identified as *L. fermentum* using the API 50 CH system; the API 50 CH system has previously incorrectly identified *L. reuteri* as *L. fermentum* with high identification (>92.5%) (Kwon *et al*., 2004). Both *L. reuteri* strains had identical fermentation profiles to an *L. reuteri* strain previously tested, fermenting only L-arabinose, ribose, galactose, glucose, maltose lactose melibiose, saccharose, raffinose and gluconate in API 50 CH (Rosander *et al*., 2008). It was not unreasonably concluded that reliance upon API identification alone would likely be unreliable. Thus, additional classification approaches were used.

Molecular methods were used to further validate the speciation of each strain in the form of multiplex PCR and 16S rRNA sequencing (Table 5.1). The multiplex PCR employed was able to detect the species, *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. gasseri, L. plantarum*, *L. reuteri* and *L. rhamnosus*, using primers based on the species-specific sequences of 16S rRNA or 16S-23S rRNA intergenic spacer region (Kwon *et al*., 2004). For species that were detectable by the multiplex PCR, bands of expected sizes were noted when the PCR product was subjected to gel electrophoresis. As with the API 50 CH speciation, *L. bulgaricus* was identified as *L. delbrueckii*, as the former is a subspecies of the latter. The results of the multiplex PCR were inconclusive for the strains of *L. buchneri*, *L. fermentum* and *L. salivarius*, having either no band or multiple weak bands. Final clarification of the species of each strain was achieved by sequencing the product of a PCR performed using universal primer to amplify the 16S rRNA gene (Marchesi *et al*., 1998). The 16S rRNA sequence of each strain had a high PID match to the sequence of this gene in strains of the expected species (≥97.0%), found in the GenBank database (Table 5.1). As anticipated from the debates in the taxonomy literature, multiple approaches were required to establish presumptive identifications within the existing classification for the lactobacilli.

Initial studies to select *Lactobacillus* strains as prospective probiotics focused on screening for an inhibitory effect of the CFS of the *Lactobacillus* strains on the growth of *B. pilosicoli* B2904. These growth inhibition assays revealed the CFS, at 10% (v/v), of seven of the eighteen strains screened gave a significant inhibition of *B. pilosicoli* growth, including that of *L. reuteri* LM1 and *L. salivarius* LM2 (Figure 5.1). In these initial studies, the pH of the CFS was not adjusted (as described in Chapter 6), and was approximately pH 3.8 for all strains. Furthermore, the composition of the acids in the CFS that generated the pH drop to pH 3.8 was not investigated; the relative ratios of acid depend on whether the strains were hetero- or homofermentative. Not all CFS had a significant effect on the growth of *B. pilosicoli* and, hence, the effect observed for the CFS of the seven significantly inhibiting strains is unlikely to be wholly pH-dependant. For example, acetic acid is more inhibitory than lactic acid and it may be that the relative abundance of acetic acid at pH 3.8 is of significance in these CFS tests. Also, the production of antimicrobial compounds, such as bacteriocins or reuterin, by these strains may explain their inhibitory effect on the growth of *B. pilosicoli* (Klose *et al*., 2010). *L. acidophilus* B2990, *L. bulgaricus* B2991 and *L. reuteri* LM1 are capable of hydrogen peroxide production (Table 5.1), which may be responsible for the inhibitory effect by these strains, since this GRAS antimicrobial agent can be lethally oxidising to

other microorganisms. Other strains that did not inhibit the growth of *B. pilosicoli* significantly were, however, capable of the production of hydrogen peroxide; quantification of the production levels would be necessary to assess the potential contribution of this compound. A metabonomic approach to assess the type and abundance of the products of the metabolism of the lactobacilli may be a useful research area in the future.

Motility and haemolysis are properties of *Brachyspira* that are considered to contribute to their pathogenicity; motility to gain access to the site of colonisation and haemolysis demonstrates the ability to damage eukaryotic cells. Assays for these two properties were performed and to test the inhibitory effect of live and heat-inactivated cells of the *Lactobacillus* strains on these properties of *B. pilosicoli* B2904, after 4 and 24 hour contact. Aside from the *L. casei* and *L. fermentum* strains, all strains had an inhibitory effect on the motility and/or haemolysis of *B. pilosicoli*, which included the seven strains of which the CFS significantly inhibited the growth of *B. pilosicoli* (Table 5.2). Inhibition of motility and haemolysis occurred most commonly with viable cells and after a 24 hour contact time. Inhibition of motility often occurred in the absence of an inhibition of haemolysis, indicating that that *B. pilosicoli* were viable and they were subsequently subcultured on *Brachyspira* selective agar. Furthermore, where inhibition of motility and/or haemolysis was observed with the viable *Lactobacillus* strain, the heat-inactivated bacterial cells also elicited inhibition, suggesting the effect was not due to competition for nutrients between the two bacterial species or synthesis of antimicrobial substances by the lactobacilli. It is likely that the prevention of the motility of *B. pilosicoli* is a consequence of co-aggregation between the lactobacilli and the spirochaetes, as previously suggested (Bernardeau *et al*., 2009). However, it is also possible that metabolites from the lactobacilli are still present in heat-inactivated cells and these act *in trans*.

L. reuteri LM1 and *L. salivarius* LM2 were deemed suitable candidates for further investigation of their inhibitory effect on *Brachyspira* as a potential treatment strategy for AIS and related diseases. Ideally, the origin of the probiotic strains should be the same as the host species for the intended treatment, which supports the selection of *L. reuteri* LM1 and *L. salivarius* LM2, both of poultry origin, for further study (Collins *et al*., 2009; Klaenhammer and Kullen, 1999). This also indicates that the probiotic is able to colonise the intended host, although this does need testing and confirmation. Selection criteria stipulate that probiotic candidates should exhibit antimicrobial activity against target pathogens or some degree of antagonism, which both of these strains have

demonstrated in the growth and motility inhibition assays. *L. reuteri* LM1 has also been shown to produce the antimicrobial compound, hydrogen peroxide. However, it is important to note and that the abundance of metabolites and the degree of inhibition seen in these *in vitro* tests may have less significant impact on *B. pilosicoli in vivo*. Further work should consider the site in the gut where the *Lactobacillus* probiotic candidate strains will colonise and their metabolites potentially have greatest impact.

It is generally accepted that lactobacilli are GRAS and do not cause disease in humans or food producing animals, however, with the increasing use of this species in probiotic food products, concerns over the safety of its use include deleterious metabolic activation, excessive immune stimulation and gene transfer of virulence and antimicrobial resistance genes amongst microorganisms (Agostoni *et al*., 2004; Marteau, 2001). Such health considerations have led to the publication of guidelines for the selection of probiotics proposed in council directive 87/153/EEC (von Wright, 2005). The guidelines state that probiotic strains must not produce toxins, virulence factors or antimicrobial substances of clinical or veterinary significance and they must not carry transmissible antimicrobial resistance determinants. Since both *L. reuteri* LM1 and *L. salivarius* LM2 were isolated from healthy chickens, they are unlikely to possess any virulence factors that would pose significant risk, however, screening for virulence determinants and antimicrobial substance production in these two strains would be required.

Guidelines for screening for antimicrobial resistance stipulate that probiotic candidates must be examined to establish their susceptibility to a relevant range of antimicrobials of human or veterinary importance (EFSA, 2008). As a basic requirement the MIC of nine of the ten stipulated antimicrobials, excluding quinupristin/dalfopristin was determined for both strains (Table 5.3). These antimicrobials were selected to maximise the identification of resistance genotypes to the most common antimicrobials by assessing phenotype. Aside from antimicrobials that lactobacilli are considered intrinsically resistant to, *L. reuteri* LM1 demonstrated resistance to tetracycline and *L. salivarius* LM2 to chloramphenicol, gentamicin, kanamycin, streptomycin and tetracycline. The genetic basis of this resistance requires further investigation, however preliminary results of the Identibac AMR+ve^{TM} microarray indicated the presence of tetracycline resistance genes in *L. reuteri* LM1 (*tetM*) and *L. salivarius* LM2 (*tetW* and *tetZ*), with a chloramphenicol resistance (*cat-86*) gene also noted in the latter. Antimicrobial resistance genes, including *tetM* have been associated with MGEs in lactobacilli and can be transferred to other bacterial

strains and species (Gevers *et al*., 2003; Teuber *et al*., 1999). The *tetM* and *tetW* genes encode ribosomal protection proteins that intervene in the binding of tetracycline to the ribosome and prevent its inhibitory effect on translation, whereas the *tetZ* gene encodes a efflux system to remove tetracycline from the cytoplasm. The *cat-86* gene encodes a chloramphenicol acetyltransferase, which binds and acetylates the antimicrobial and in turn, prevents it binding ribosomes and inhibiting protein synthesis. Curative strategies may be applied to probiotic strains to remove antimicrobial resistance genes or plasmids carrying such genes (Huys *et al*., 2006); such as the removal of two plasmids, one carrying a *tetW* gene, from the commercial probiotic strain *L. reuteri* ATCC 55730, deriving the daughter strain DSM 17938, without losing probiotic characteristics (Rosander *et al*., 2008).

As part of competitiveness criteria, probiotic candidates should demonstrate an ability to resist environmental stresses, such as bile and acids (Klaenhammer and Kullen, 1999). Tests were performed at pH 2.0, 2.5 and 3.0 covering the range of poultry gastric juice that ranges from pH 2.0 to 3.0 depending on the feeding state and growth stage of the birds (Lin *et al*., 2007; Yu and Tsen, 1993). The 3 hour incubation length was selected as it is unlikely that the bacteria would be exposed to the low pH for any longer than 3 hours, due to the shorter GI tract of poultry, for which entire passage has been estimated at 2 – 5 hours (Duke, 1977). Gastric acid tolerance assays demonstrated that *L. reuteri* LM1 tolerated avian gastric juice to a greater extent at pH 3.0 and 2.5 than *L. salivarius* LM2, which after 3 hours had significantly reduced viable counts ($p<0.001$) (Table 5.4). The normal pH of gastric juice in chickens can be as low as pH 2.0 (Ehrmann *et al*., 2002), and after incubation at pH 2.0 for 3 hours, the numbers of surviving *L. reuteri* LM1 and *L. salivarius* LM2, were significantly decreased from the 0 hour control ($p<0.001$). Despite this, a proportion of the initial inocula for both strains did survive this low pH and *in vivo* it might be anticipated that a higher proportion may survive due to protection from acids by other materials in the chyme and hence, the surviving bacteria may reach and populate the lower GI tract, exerting their probiotic effect.

Bile tolerance assays illustrated the sensitivity of both *L. reuteri* LM1 and *L. salivarius* LM2 to 0.3% (w/v) oxgall, a product containing bovine bile salts (Figure 5.1A and B). Enhanced growth was observed in the presence of the poultry bile salts, sodium taurochenodeoxycholate and taurocholic acid, at 0.3% (w/v) (Figure 5.1A and B). Strains were categorised as resistant, where the lag time was equal to or less than 15 minutes; tolerant, where the lag time was between 15 and 40 minutes; weakly tolerant,

where the lag time was between 40 and 60 minutes and sensitive, where the lag time was over 60 minutes (Chateau *et al*., 1994). Both *L. reuteri* LM1 and *L. salivarius* LM2 were deemed sensitive to 0.3% (w/v) sodium taurochenodeoxy-cholate and, although *L. salivarius* LM2 was sensitive to 0.3% (w/v) taurocholic acid, *L. reuteri* LM1 was classed as tolerant (Table 5.5). Furthermore, both strains grew well in the presence of the poultry bile extract and in some cases to greater extents than the control (Figure 5.1C and D), suggesting a potential capability in utilising components of the poultry bile. From the lag time analysis, *L. reuteri* LM1 was resistant to weakly tolerant to the three tested concentrations of poultry bile, but *L. salivarius* LM2 was tolerant at 0.3% (v/v), but sensitive at 0.6% (v/v) and 0.9% (v/v) (Table 5.5). Despite any sensitivity to the bile salts or extract, in most cases, bacterial growth was observed (Figure 5.1), hence, when viable bacteria reach the lower GI tract, they may populate these areas and exert their probiotic effect.

L. reuteri LM1 and *L. salivarius*, LM2, isolated from the faeces of healthy convention laying hens, were selected as probiotic candidates for use in further experiments to evaluate their potential in the intervention of AIS. The novel strains were selected since they are of host origin for the intended host and they displayed an ability to significantly inhibit the growth of *B. pilosicoli* B2904 and also the motility of this strain. *L. reuteri* LM1 has been shown to be resistant to fewer antimicrobials and demonstrated better tolerance to acids and biles, making this strain more ideal for use as a probiotic.

Chapter 6.

Investigating the ability of lactobacilli to antagonise *Brachyspira pilosicoli in vitro*: a potential intervention against avian intestinal spirochaetosis

6.1 Introduction

AIS is an enteric disease that affects layer and broiler breeder chickens leading to clinical enteritis and reduced performance, resulting from the colonisation of the caeca and colo-rectum by the spirochaete, *Brachyspira* (Stephens and Hampson, 2001). Clinical symptoms of AIS include reduced egg production with delayed onset of lay, chronic diarrhoea with faecal staining of eggs, weight loss and increased flock morbidity rates (Burch *et al*., 2006; Smit *et al*., 1998). *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* are considered pathogenic to poultry (Hampson and McLaren, 1999; Stanton *et al*., 1998; Stephens and Hampson, 2002a). Although the mechanisms of pathogenesis are unclear, colonisation of poultry, swine and humans by *B. pilosicoli* is characterised by its ability to form end-on attachments to and invade the intestinal surface epithelium (Feberwee *et al*., 2008; Jensen *et al*., 2001; Jensen *et al*., 2000).

In the United Kingdom, the incidence of *Brachyspira* in commercial and free-range flocks has been estimated at 74% and 90%, respectively (Burch, 2010). AIS disease associated with *Brachyspira* infection is reported to be increasing, which may be at least partially attributed to the 2006 EU ban on the use of antimicrobials as growth promoters in livestock (Castanon, 2007). In 2006, the annual cost of the disease to the United Kingdom laying industry was estimated at £14 million and this figure continues to rise (Burch *et al*., 2006). In addition, antimicrobial resistance appears to be increasing amongst *Brachyspira*, including an emerging resistance to the most commonly-used

antimicrobial for AIS treatment, tiamulin; resistance has been reported in porcine strains of *Brachyspira* (Karlsson *et al*., 2003; Lobova *et al*., 2004; Pringle *et al*., 2006), and elevated MICs have been demonstrated in strains of poultry origin (Hampson *et al*., 2006c) and were also demonstrated in strains used in the studies presented in this thesis (as discussed in Chapter 3). Moreover, antimicrobial resistance genes have been reported in the genomes of *Brachyspira* (Jansson and Pringle, 2011; Karlsson *et al*., 1999; Karlsson *et al*., 2004; Mortimer-Jones *et al*., 2008) and the *B. pilosicoli* genomes reported in this thesis (as discussed in Chapter 4).

The rise of endemic diseases since the ban and antimicrobial resistance has renewed interest in developing alternative intervention strategies; one such alternative therapy which is being extensively researched is the use of probiotics (Collins *et al*., 2009). Probiotics that include genera such as bifidobacteria and lactobacilli (Collins *et al*., 2009) are described as live microorganisms that confer health benefits on the host when administered in adequate quantities (FAO/WHO, 2001). Multiple mechanisms have been proposed for the protective effect that probiotics confer against pathogenic microorganisms, including secretion of antimicrobial compounds, competition for essential nutrients, competition for host cell binding receptors and immunomodulation of the gut mucosa (Vanderpool *et al*., 2008). Probiotics have demonstrated promise, *in vitro* and *in vivo*, as CE agents against *E. coli*, *Salmonella*, *Clostridia* and *Campylobacter* infection in poultry (La Ragione *et al*., 2004; La Ragione and Woodward, 2003; Schoeni and Wong, 1994; Stern *et al*., 2006; Vicente *et al*., 2008) and can colonise the caeca (Pascual *et al*., 1999), the host niche of many pathogens, such as *Brachyspira*. Recently, a patent application was published (Se *et al*., 2008) describing the use of *L. johnsonii* D115 as a probiotic against *Brachyspira*. Additionally, *L. rhamnosus* and *L. farciminis* strains have been implicated in inhibiting the motility of *Brachyspira* by co-aggregation and eliciting a stress response (Bernardeau *et al*., 2009).

To date, no studies have investigated the adherence and invasion dynamics of avian *B. pilosicoli* to epithelial cells in relation to treatment with probiotics. Hence, in this chapter, the development and use of a human, colonic epithelial 3D cell and avian caecal IVOC model to aid such investigations is reported. From preliminary probiotic screening studies described in Chapter 5, *L. reuteri* LM1 and *L. salivarius* LM2 were selected for further investigation of their potential use in the intervention against *B. pilosicoli*. The studies presented in this chapter employed motility, growth inhibition and adhesion and invasion assays to investigate *in vitro* the antagonistic effect of *L. reuteri* LM1 and *L. salivarius* LM2 on *B. pilosicoli* B2904.

6.2 Results

6.2.1 pH-dependent inhibition of *B. pilosicoli* growth by *Lactobacillus* cell-free supernatant

The CFS obtained from spent MRS after culture of *Lactobacillus* was pH 3.8 whereas MRS was pH 5.8 prior to growth. The effect of a 10% (v/v) dilution of the CFS of *L. reuteri* LM1 and *L. salivarius* LM2 and adjusted to pH 3.8, 4.5 and 7.2 on the growth of *B. pilosicoli* B2904 in BEB was monitored using FLUOstar OPTIMA to measure OD (Figure 6.1) and bacterial cell counts using a Helber counting chamber. In comparison with MRS controls at the respective pH value, significant inhibition of growth of *B. pilosicoli* was observed with CFS from both *L. reuteri* ($p<0.05$) and *L. salivarius* ($p<0.001$) at pH 3.8 and with the CFS of *L. reuteri* only at pH 4.5 ($p<0.001$). At pH 7.2 neither CFS had an effect on the growth of *B. pilosicoli*. Neither of the two heat-inactivated lactobacilli strains had a significant impact on the growth of *B. pilosicoli*. Furthermore, the effect of the CFS on the growth of non-pathogenic *B. innocens* was also tested in order to understand if the effect was strain or species-dependant. The growth of *B. innocens* in each of the conditions was similar to that of *B. pilosicoli*.

6.2.2 Inhibitory effect of *Lactobacillus* whole cells on *B. pilosicoli* motility

As discussed in Chapter 5, agar motility inhibition assays were performed to investigate the ability of viable and heat-inactivated lactobacilli to inhibit the motility of *B. pilosicoli* B2904. The motility of *B. pilosicoli* was inhibited by both viable and heat-inactivated *L. reuteri* LM1 and *L. salivarius* LM2 following a 4 hour and a 24 hour contact time, respectively (Table 5.3). Viable lactobacilli inhibited hemolysis by *B. pilosicoli* in these tests. *B. pilosicoli* recovered by subculture from all assays displayed motility and hemolytic growth, indicating viability. The effect of the lactobacilli on the motility and haemolysis of non-pathogenic *B. innocens* was also tested in order to understand if the effect was strain or species-dependant. The effect of each of the conditions on motility was similar for both *B. pilosicoli* and *B. innocens*.

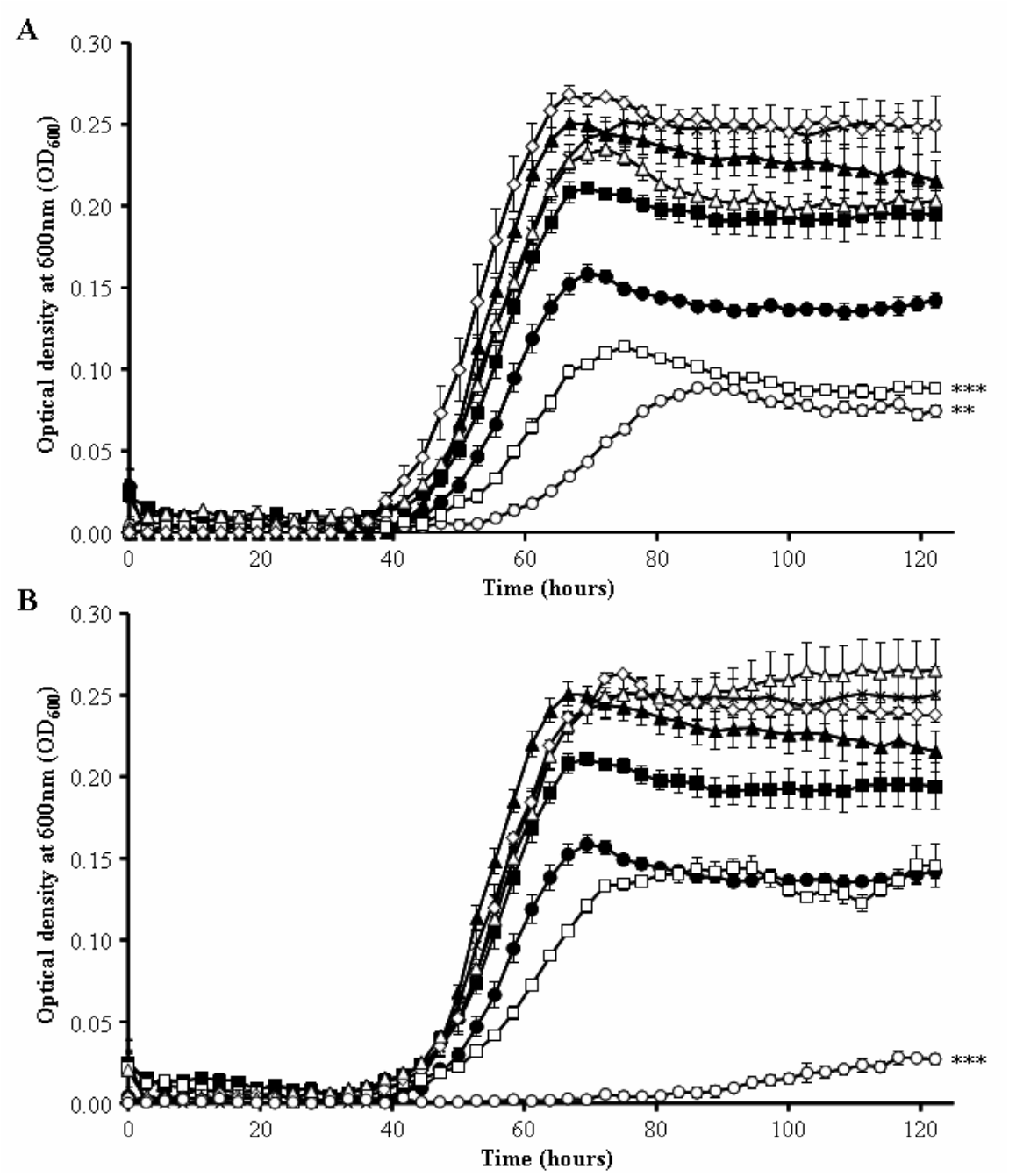

Figure 6.1 Growth of *B. pilosicoli* B2904 in broth culture (BEB) supplemented with 10% (v/v) cell-free supernatant (CFS) of *L. reuteri* LM1 (A) and *L. salivarius* LM2 (B) (open shapes) adjusted to pH 3.8 (circles), 4.5 (squares) and 7.2 (triangles) and heat-inactivated lactobacilli (diamonds), measured using OD_{600} as a reporter of growth. Controls with pH-adjusted MRS (closed shapes) and with no additive to *B. pilosicoli* culture (x's) are shown. *B. pilosicoli* cells were also enumerated using a Helber counting chamber; an OD_{600} of 0.05 represents ~10^7 CFU/ml and an OD_{600} of 0.25 represents ~3.5×10^8 CFU/ml. Means with standard deviation of 9 repeats are presented. Significance is shown for differences between the final growth point readings of *B. pilosicoli* with the CFS of *Lactobacillus* and those for the respective control (+ 10% (v/v) MRS); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.2.3 Adhesion and invasion assays using HT29-16E cell culture models

6.2.3.1 Comparison of the monolayer and three-dimensional models

Human, mucus-secreting colonic cells, HT29-16E, were selected for use in adhesion and invasion assays as this is a well-established cell line for studying the interactions of enteric pathogens such as *Salmonella* and *E. coli* with the intestinal epithelium (Kerneis *et al*., 1994; Mellor *et al*., 2009), and capable of differentiation (Cohen *et al*., 1999; Jessup *et al*., 2000). Preliminary studies illustrated an ability of *B. pilosicoli* B2904 to adhere and invade the cell line, which may be attributed to a chemoattraction towards mucin secreted by this cell line (Naresh and Hampson, 2010).

HT29-16E 3D cells displayed differentiated brush borders, confluent across the cell surface and tight junctions between cells (Figure 6.2B and D), that were superior in comparison to monolayers (Figure 6.2A and C), as previously noted (Honer zu Bentrup *et al*., 2006). HT29-16E 3D cells grew predominantly as multilayered cell aggregates, measuring up to 350 µm in diameter, similar to previous findings (Searle *et al*., 2010).

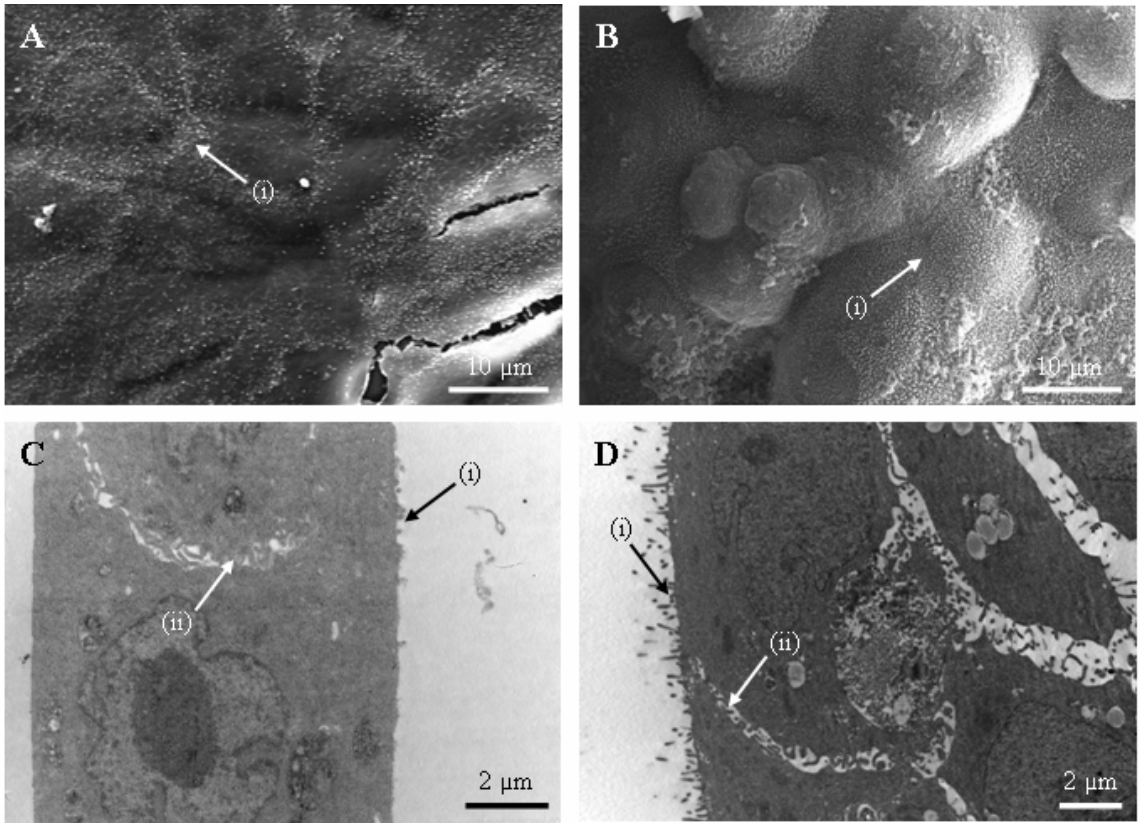

Figure 6.2 Examination of human, mucus-secreting colonic cell, HT29-16E, monolayer (A, C) and three-dimensional (3D) cell (B, D) uninfected control samples by scanning and transmission electron microscopy (S/TEM). The three-dimensional (3D) cells have more prominent brush borders (i) and superiorly differentiated tight junctions (ii).

6.2.3.2 Inhibitory effect of lactobacilli on the adherence and invasion of *B. pilosicoli* to HT29-16E cells

To determine whether *L. reuteri* LM1 or *L. salivarius* LM2 were able to reduce the adherence and invasion of *B. pilosicoli* to HT29-16E cells, protection, competition and displacement assays (as described in Chapter 2 and specifically section 2.3.2) were performed using monolayer and 3D cell models (Table 2.5). Generally, results were similar between the two models. However, on comparing the numbers of adhering and invading *B. pilosicoli* in control assays, adhesion was significantly greater and invasion was significantly lower in the 3D cell model ($p<0.001$). Adherence and invasion of *B. pilosicoli* was significantly reduced by *L. reuteri* and *L. salivarius*, in protection and competition assays in the monolayer (Figure 6.3A and B) and 3D cell (Figure 6.4A and B) models ($p<0.01$). Both strains gave greater reduction in the adherence and invasion of *B. pilosicoli* in competition assays than protection assays. In the monolayer model, *L. reuteri* and *L. salivarius* reduced the invasion of *B. pilosicoli* to a significantly greater degree in competition as opposed to protection assays ($p<0.05$) and in the 3D cell model, *L. reuteri* reduced invasion by *B. pilosicoli* 13.6-fold in protection assays and 30.0-fold in competition assays. In displacement assays, little effect was observed on the adherence and invasion of *B. pilosicoli* (Figure 6.3C and 6.4C), with a reduction in adherence noted only with *L. reuteri* in the monolayer model ($p<0.05$). In both models, *L. reuteri* was associated with a significantly greater reduction of adherence and invasion by *B. pilosicoli* than *L. salivarius* ($p<0.05$).

The assays described above were also performed with CFS rather than lactobacilli (Table 2.5) to determine whether the inhibitory effect on the adherence and invasion of *B. pilosicoli* was a result of compounds secreted by the lactobacilli or the bacteria themselves. No significant reduction of the adherence of *B. pilosicoli* was observed with lactobacilli CFS (Figure 6.3D and 6.4D). However, the invasion of *B. pilosicoli* was significantly reduced in the presence of 10% (v/v) *L. reuteri* CFS ($p<0.001$), but only in the monolayer model.

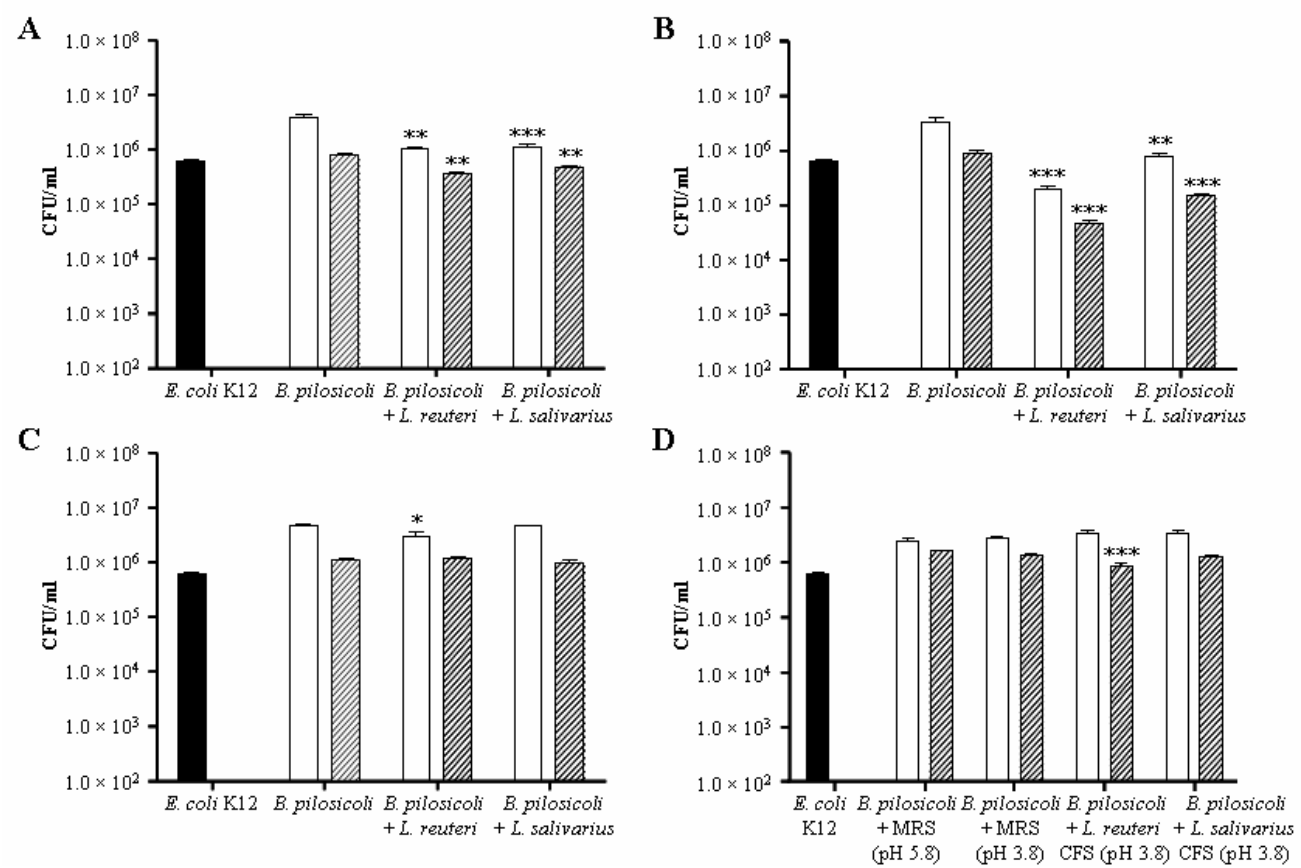

Figure 6.3 Effect of *Lactobacillus* treatment on *B. pilosicoli* B2904 adherence to (white bars) and invasion of (hatched bars) HT29-16E monolayers. *L. reuteri* LM1 and *L. salivarius* LM2 were used in protection (A), competition (B) and displacement (C) assays and their cell-free supernatant (CFS) were used at 10% (v/v) (D). *B. pilosicoli* only controls are shown, where tissue culture medium was added in place of lactobacilli and 10% (v/v) MRS (pH 5.8 and 3.8) was added as a control in CFS assays. *E. coli* K12 acted as a negative control for invasion (black bars). Values presented are means with standard deviation of 9 repeats. Significance is shown where adhered or invaded *B. pilosicoli* cell numbers differed significantly between *Lactobacillus* treatment and no-*Lactobacillus* control or where CFS treatment differed significantly from the MRS (pH 3.8) control. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

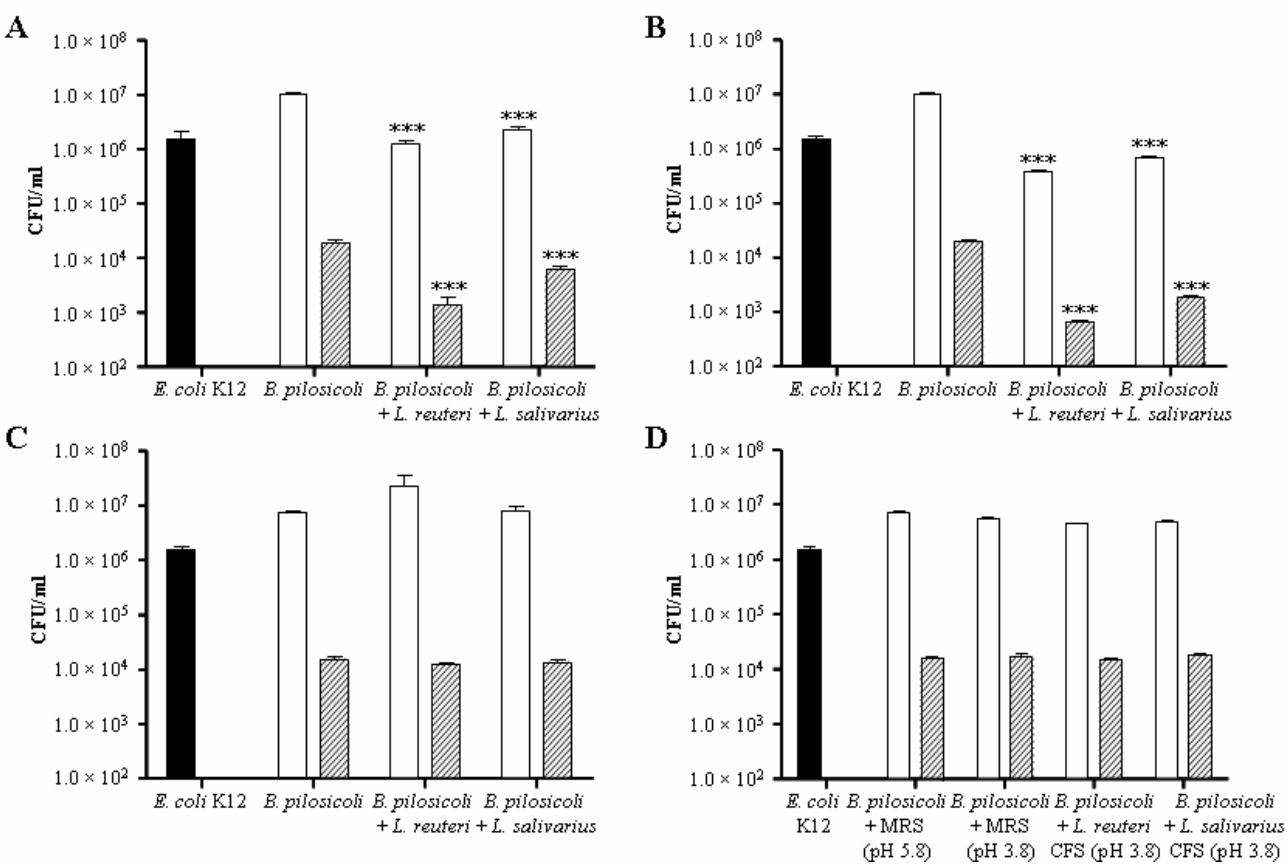

Figure 6.4 Effect of *Lactobacillus* treatment on *B. pilosicoli* B2904 adherence to (white bars) and invasion of (hatched bars) HT29-16E three-dimensional (3D) cells. *L. reuteri* LM1 and *L. salivarius* LM2 were used in protection (A), competition (B) and displacement (C) assays and their cell-free supernatant (CFS) were used at 10% (v/v) (D). *B. pilosicoli* only controls are shown, where tissue culture medium was added in place of lactobacilli and 10% (v/v) MRS (pH 5.8 and 3.8) was added as a control in CFS assays. *E. coli* K12 acted as a negative control for invasion (black bars). Values presented are means with standard deviation of 9 repeats. Significance is shown where adhered or invaded *B. pilosicoli* cell numbers differed significantly between *Lactobacillus* treatment and no-*Lactobacillus* control or where CFS treatment differed significantly from the MRS (pH 3.8) control. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

6.2.3.3 Electron microscopical analysis of HT29-16E monolayer and three-dimensional cells following adhesion and invasion assays

From the monolayer assays, samples from protection assays, in which significant reductions in the adherence and invasion of *B. pilosicoli* were noted, were examined by SEM and TEM (Figure 6.5). In the absence of lactobacilli, high numbers of *B. pilosicoli* were noted adhering by end-on attachment (Figure 6.5A) and invading the cells (Figure 6.5B). Fewer *B. pilosicoli* were observed adhering to and invading the monolayers following pre-treatment with *L. reuteri* LM1 (Figure 6.5C and D) or *L. salivarius* LM2 (Figure 6.5E and F), supporting the bacteriological count data. Direct interactions and co-aggregation were noted between *B. pilosicoli* and the lactobacilli (Figures 6.5C-F).

From the 3D cell assays, samples from competition assays, in which the greatest effect was observed on the adherence and invasion of *B. pilosicoli*, were examined by SEM and TEM (Figure 6.6). Qualitatively, greater numbers of adherent *B. pilosicoli* were observed in the absence of lactobacilli, with dense populations invading at tight junctions (Figure 6.6A), supporting the data derived from bacteriological counts. Of particular note were direct interactions between *B. pilosicoli* and both *L. reuteri* LM1 (Figure 6.6D and E) and *L. salivarius* LM2 (Figure 6.6F). EM of cell aggregates that were infected with *Brachyspira* alone indicated apoptosis (blebbing, loss of microvilli, disintegrated cytoplasm with vacuolation, chromatin condensation and fragmentation and cell sloughing) (Figure 6.6B and C). With co-administration of *L. reuteri* LM1 or *L. salivarius* LM2 (Figure 6.6D-F), end-on attachment of *B. pilosicoli* was observed less frequently, with apparent interactions between the two bacterial species and co-localisation at the cell surface. Minimal pathology was apparent in the presence of lactobacilli and the integrity of the brush border was maintained (Figure 6.6D-F).

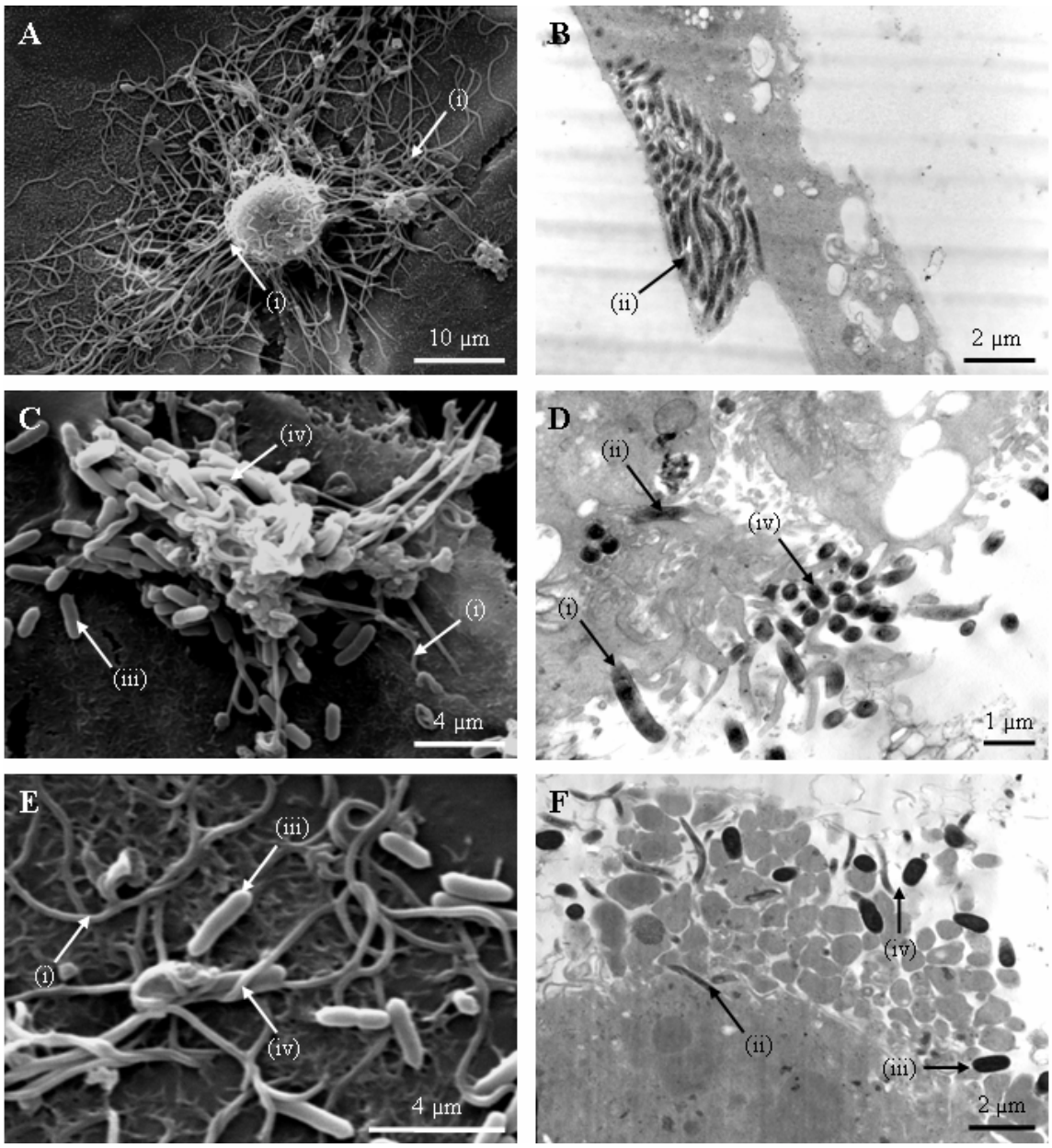

Figure 6.5 Examination of HT29-16E monolayers infected with *B. pilosicoli* B2904 (A, B) and following pre-treatment with *L. reuteri* LM1 (C, D) and *L. salivarius* LM2 (E, F) by scanning and transmission electron microscopy (S/TEM). *B. pilosicoli* were observed adhering to the cells via end-on attachment (i) and invading the cells (ii). Following pre-treatment with lactobacilli, adherent lactobacilli were observed (iii) in addition to their interactions and co-aggregation with the *B. pilosicoli* (iv).

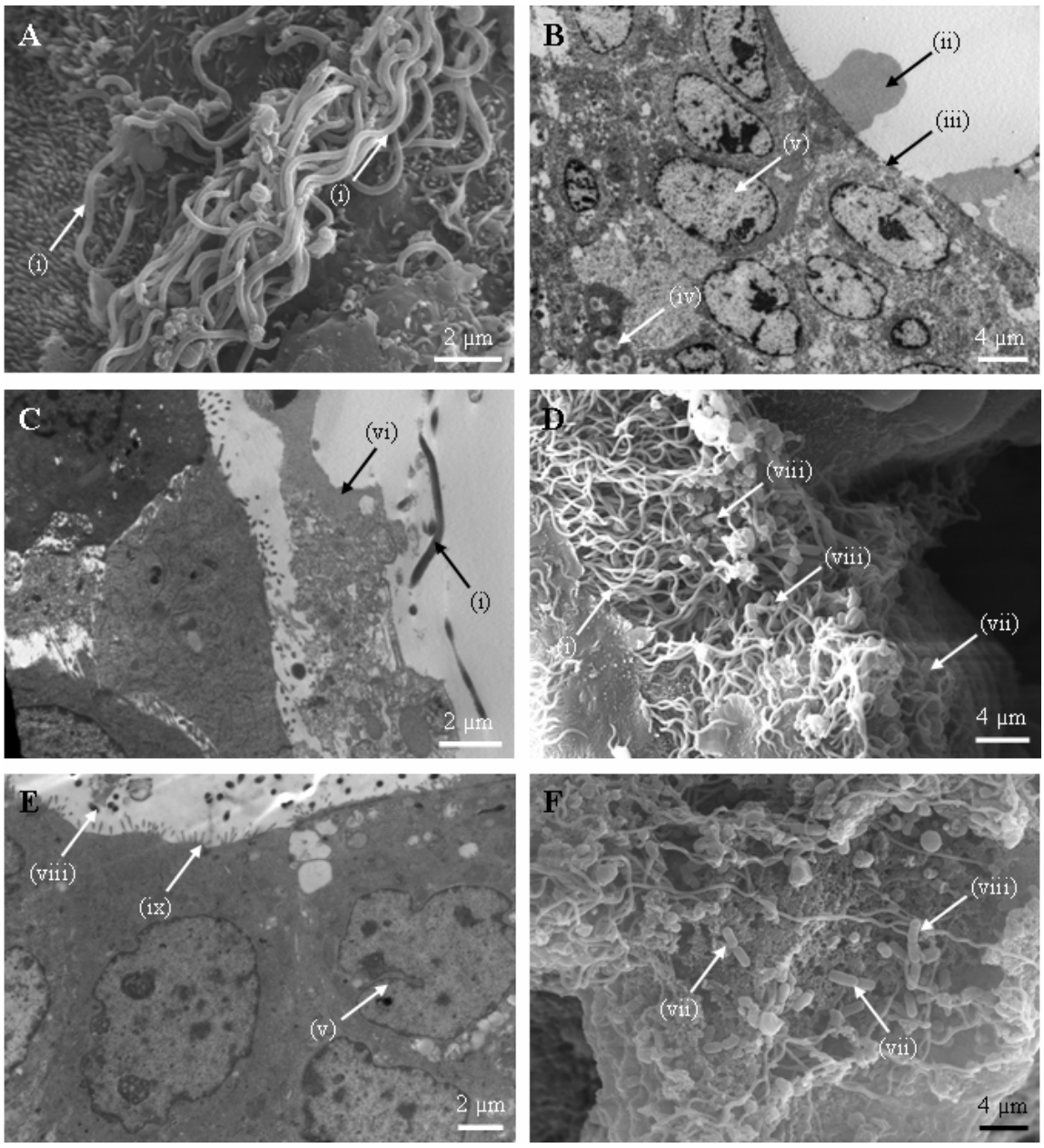

Figure 6.6 Examination of HT29-16E three-dimensional (3D) cells infected with *B. pilosicoli* B2904 (A-C) and following co-incubation with *L. reuteri* LM1 (D, E) and *L. salivarius* LM2 (F) by scanning and transmission electron microscopy (S/TEM). *B. pilosicoli* were observed adhering at the epithelial cell surface (i) and cells treated with *B. pilosicoli* only, exhibited signs of blebbing (ii), loss of microvilli (iii), disintegrated cytoplasm with vacuolation (iv), chromatin condensation and fragmentation (v) and cell sloughing (vi). Where lactobacilli were co-administered, adherent lactobacilli were observed (vii) in addition to their interactions and co-aggregation with the *B. pilosicoli* (viii). Overall minimal pathology was apparent in the presence of lactobacilli and specifically the integrity of the brush border was maintained (ix).

6.2.4 Association assays using an avian caecal *in vitro* organ culture models

6.2.4.1 Establishment of the avian caecal *in vitro* organ culture model

CellCrown™ technology was employed to create a polarised IVOC system; avian caecal tissue explants were immobilised within the CellCrown™, in a modification of a previously described method (Collins *et al*., 2010). This model allowed the separation of the apical, mucosal surface and basolateral side of the tissue and the subsequent inoculation of a defined concentration of *B. pilosicoli*, lactobacilli or a combination of the two (Table 2.5), onto a fixed area of the mucosal surface of the caecal explants. Hence, the model generated a physiologically relevant platform from which reproducible bacterial association values could be obtained. SEM showed the IVOC tissues remained well-preserved throughout the study (Figure 6.7), however a limitation of the model was the inability to remove the entire natural microflora associated with the surface of the tissue.

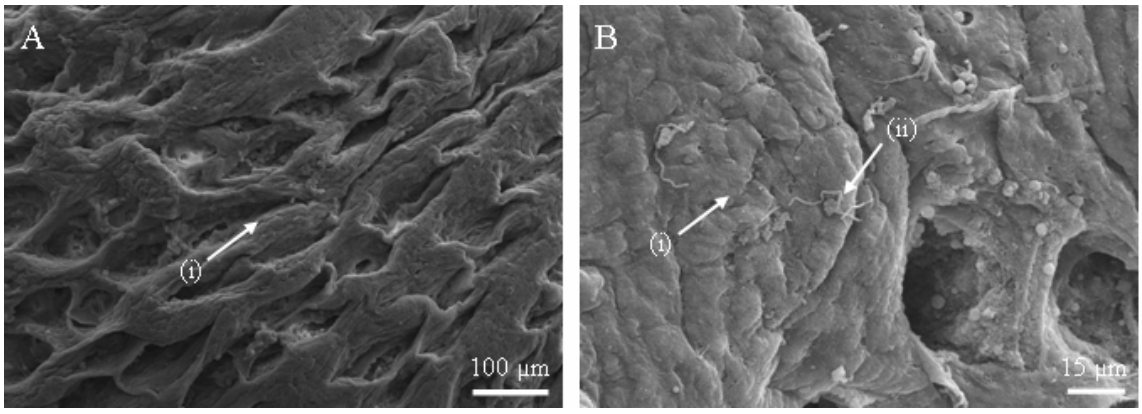

Figure 6.7 Examination of uninfected control avian caecal *in vitro* organ culture (IVOC) tissue samples by scanning electron microscopy (SEM). Tissues maintained an intact surface epithelium (i) with few resident microflora associated with the mucosal surface (ii).

6.2.4.2 Inhibitory effect of lactobacilli on the association of *B. pilosicoli* to avian caecal *in vitro* organ culture tissues

Avian caecal IVOC association assays were performed to determine whether *L. reuteri* LM1 or *L. salivarius* LM2, viable or heat-inactivated, were able to reduce the association of *B. pilosicoli* (as described in Chapter 2 and specifically section 2.3.3). This model generated reproducible bacterial association values and was used alongside the data generated in monolayer and 3D cell assays to compare and validate the findings

of each method. As found in the HT29-16E cell assays, viable *L. reuteri* and *L. salivarius* significantly reduced the association of *B. pilosicoli* to caecal IVOC tissue in protection and competition assays (Figure 6.8A and B) ($p<0.001$). Heat-inactivated lactobacilli had a similar effect ($p<0.01$), with the level of reduction in association observed in protection and competition assays decreased by up to 6.5-fold. A greater reduction of the association of *B. pilosicoli* resulted when viable or heat-inactivated lactobacilli were administered in competition, as previously noted in the HT29-16E cell assays and *L. reuteri* reduced the association of *B. pilosicoli* to a greater degree than *L. salivarius*. In displacement assays neither of the *Lactobacillus* strains displayed any significant ability to reduce *B. pilosicoli* association (Figure 6.8C).

CFS assays were also performed in the IVOC model (Table 2.5), to assess the effect of secreted compounds from the lactobacilli on *B. pilosicoli* association. The *L. salivarius* CFS exerted no effect on *B. pilosicoli* association. However, association was decreased significantly in the presence of the CFS from *L. reuteri* ($p<0.001$) (Figure 6.8D).

6.2.4.3 Electron microscopical analysis of avian caecal *in vitro* organ culture tissues following association assays

Samples from competition assays were processed by SEM as the most significant reduction in *B. pilosicoli* association was noted in this condition (Figure 6.9). From qualitative analysis, adherent *B. pilosicoli* were observed in greater abundance in the absence of lactobacilli co-administration in competition assays (Figure 6.9A and B), confirming bacteriological counts. In the presence of viable *L. reuteri* LM1 (Figure 6.9C and D) and *L. salivarius* LM2 (Figure 6.9E and F), direct interactions between the two bacterial species were apparent.

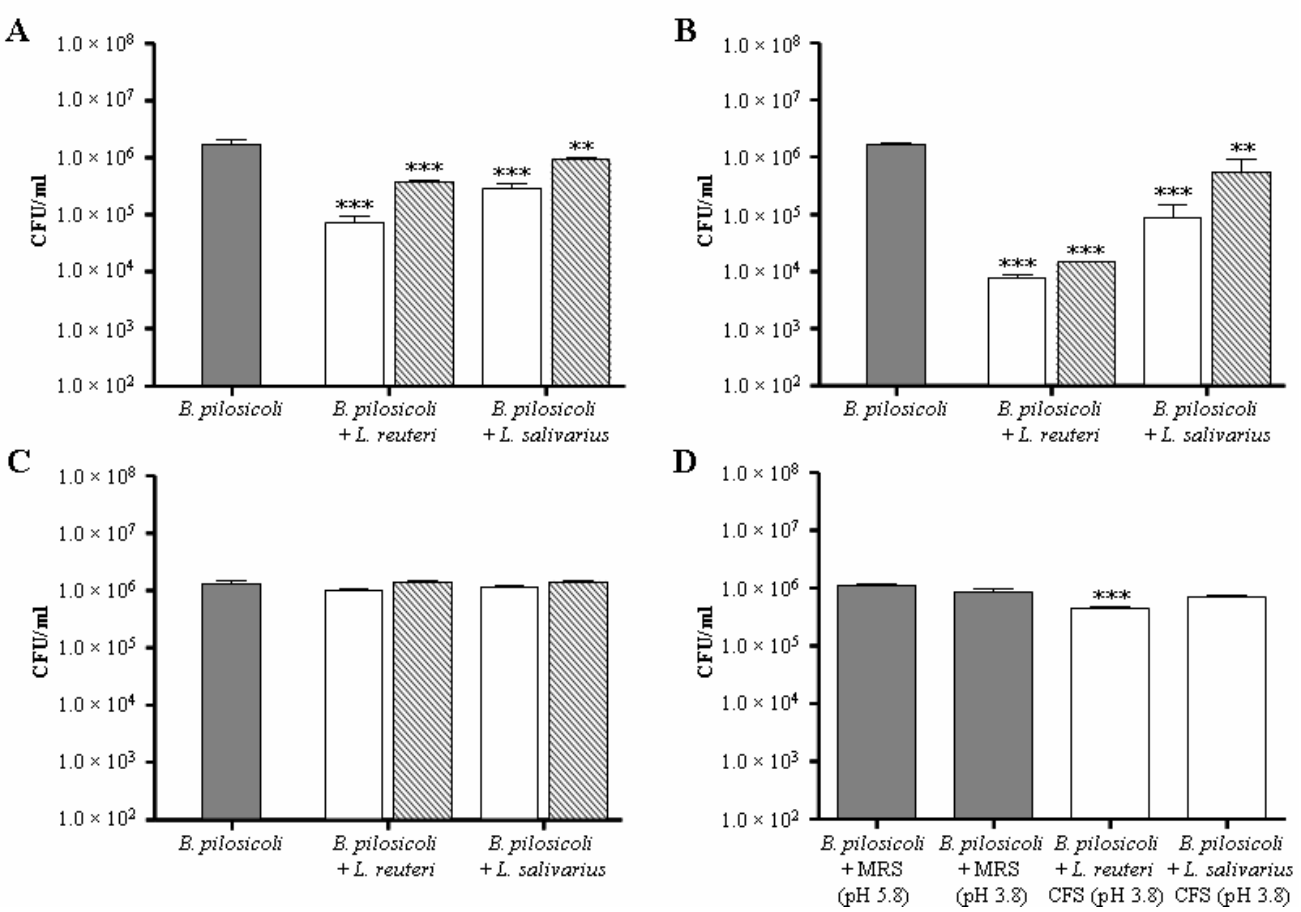

Figure 6.8 Effect of *Lactobacillus* treatment on association of *B. pilosicoli* B2904 with avian caecal *in vitro* organ culture (IVOC) tissues. Viable (white bars) and heat-inactivated (hatched bars) *L. reuteri* LM1 and *L. salivarius* LM2 were used in protection (A), competition (B), and displacement (C) assays and their cell-free supernatant (CFS) were used at 10% (v/v) in CFS studies (D). *B. pilosicoli* only controls (gray bars) are shown, where tissue culture medium was added in place of lactobacilli and 10% (v/v) MRS (pH 5.8 and 3.8) was added as a control in CFS assays. Values presented are means with standard deviation of 8 repeats. Significance is shown where associated *B. pilosicoli* cell numbers differed significantly between *Lactobacillus* treatment and no-*Lactobacillus* control or where CFS treatment differed significantly from the MRS (pH 3.8) control. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

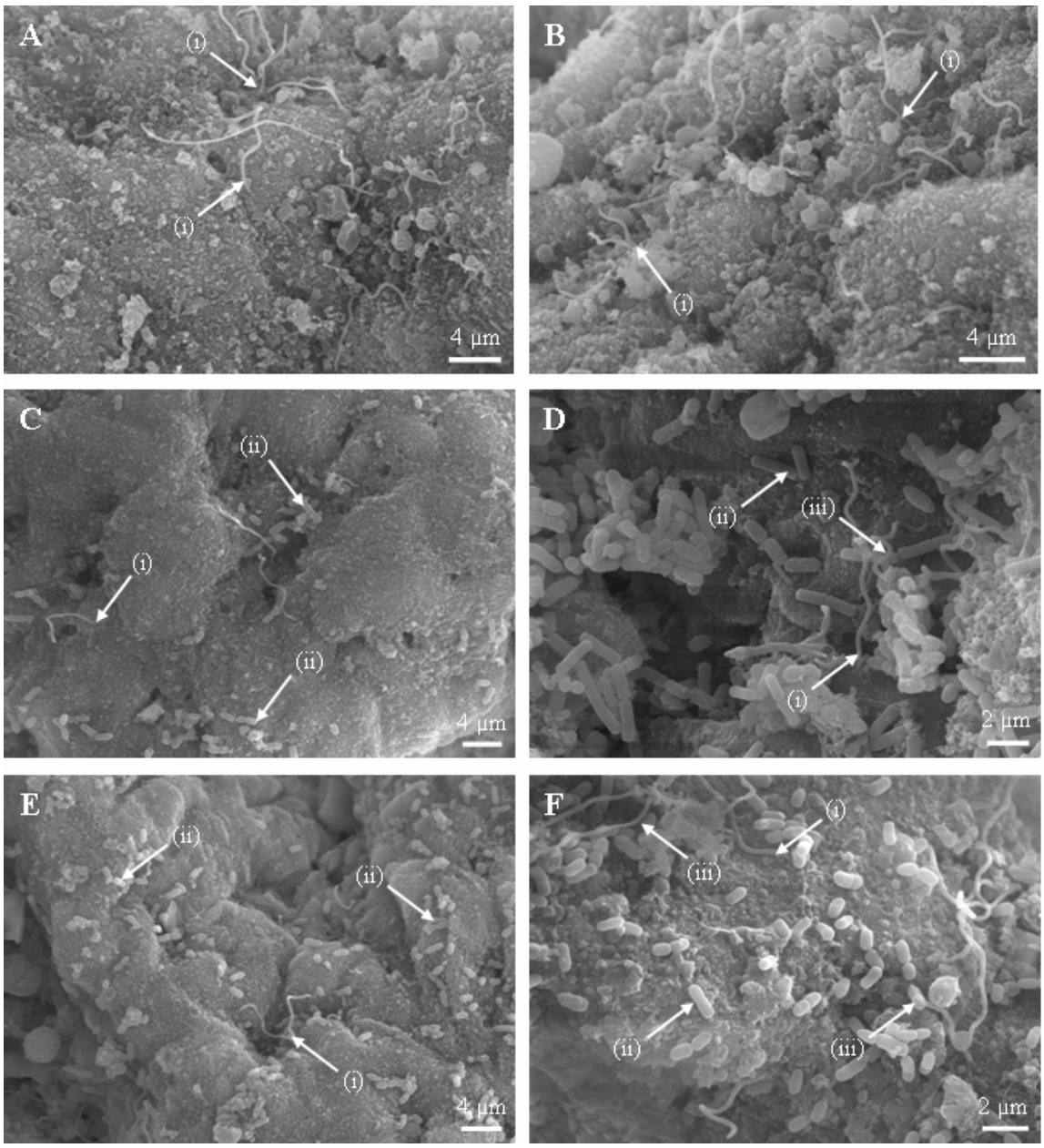

Figure 6.9 Examination of avian caecal *in vitro* organ culture (IVOC) tissues infected with *B. pilosicoli* B2904 (A, B) and co-incubation with viable *L. reuteri* LM1 (C, D) and *L. salivarius* LM2 (E, F) by scanning electron microscopy (SEM). *B. pilosicoli* were observed adhering to the mucosal surface of the tissue (i) and where lactobacilli were co-administered, adherent lactobacilli were also observed (ii) in addition to their interactions with *B. pilosicoli* (iii).

6.3 Discussion

Growth inhibition studies indicated that the CFS of *L. reuteri* LM1 and *L. salivarius* LM2 suppressed the growth of *B. pilosicoli* B2904 in a pH-dependent manner. *L. salivarius* CFS elicited a greater suppressive effect on the growth of *B. pilosicoli* at pH 3.8 than the CFS of *L. reuteri* although the CFS of *L. reuteri* also induced significant suppression of *B. pilosicoli* growth at pH 4.5 (Figure 6.1). The suppression induced by CFS at pH 3.8 was significantly greater than that by MRS at the same pH value suggesting that the suppressive effect is not attributed solely to acidity. A possible explanation may be the strain-dependent production of pH-dependent active compound(s) such as hydrogen peroxide and/or other antimicrobial compounds such as reuterin or bacteriocins (Klose *et al*., 2010). Lactobacilli CFS has been shown to induce a stress response in *Brachyspira*, often with lethal effect, attributable to lactic acid (Bernardeau *et al*., 2009). Heat-inactivated lactobacilli had no effect on the growth of *B. pilosicoli*, supporting the role of secreted compounds in inhibiting the growth. Since heat-inactivated lactobacilli and CFS had a similar effect on both *B. innocens* and *B. pilosicoli* growth, it appears the CFS has a universal effect on both pathogenic and non-pathogenic *Brachyspira* growth.

Incubation of *B. pilosicoli* B2904 or *B. innocens* B2960 with both lactobacilli strains, whether viable or heat-inactivated, resulted in a loss of motility (Table 3.5). The similarity of results for the viable and heat-inactivated assays suggests this was a passive process and not physiological. Since *B. pilosicoli* and *B. innocens* motility was inhibited in similar conditions, the factor which results in this inhibition most likely does not relate to the pathogenicity of the *Brachyspira*. The EM observations showed co-aggregation between the two bacterial species (Figure 6.5C-F, 6.6C-F and 6.9C-F) supporting similar observations described previously (Bernardeau *et al*., 2009). Co-aggregation may prove detrimental to *Brachyspira* by rendering them incapable of escaping the eliminating effect of mucus for which motility and chemotaxis are considered key virulence features of spirochaetes (Lux *et al*., 2000; Nakamura *et al*., 2006). Interestingly, the motility of *B. pilosicoli* and *B. innocens* was inhibited after 4 hour incubation with *L. reuteri* but, only after 24 hour incubation with *L. salivarius*. The biochemical basis of adherence and the avidity of binding (Aslim *et al*., 2007; Ruas-Madiedo *et al*., 2006; Schachtsiek *et al*., 2004) is worthy of further investigation as this may identify the lectins for further development and exploitation.

Prior to adhesion and invasion studies, preliminary studies confirmed the adhesive and invasive properties of *B. pilosicoli* B2904 to HT29-16E cells; this tropism may be attributed to the mucus-secreting properties of this cell line, since *B. pilosicoli* have previously exhibited chemoattraction to mucin (Naresh and Hampson, 2010). Further to the monolayer studies, a 3D cell model which maintains a differentiated 3D architecture of the parental tissue (Figure 6.2) that creates a more physiologically relevant platform was adopted for adhesion and invasion assays. Results from the assays in the 3D cell model reflected those in the monolayer model; however the number *B. pilosicoli* cells that adhered was significantly greater and those that invaded was significantly lower in the 3D cell model. This tropism was noted previously for *E. coli*, *C. difficile* and *S.* Typhimurium (Eveillard *et al*., 1993; Honer zu Bentrup *et al*., 2006; Kerneis *et al*., 1994; Searle *et al*., 2010) and results from cell differentiation, which affects pathogen infection, up-regulating adherence and down-regulating invasion (Gabastou *et al*., 1995). Findings from HT19-16E cell assays encouraged the continuation of studies using *in vitro*-cultured caecal tissue explants from laying hens; a novel and possibly more physiologically relevant *in vitro* platform on which to study the potential use of probiotics to protect against AIS. In IVOC studies, tissues were maintained physiologically active with their natural architecture and mucin layers; SEM showed the tissues remained well-preserved throughout the study (Figure 6.7). However, the nature of the IVOC study did not allow separate enumeration of adherent and invaded *B. pilosicoli*, thus total association was assessed.

L. reuteri LM1 and *L. salivarius* LM2 reduced significantly the adherence and invasion of *B. pilosicoli* to HT29-16E cells and the mucosal surface of avian caeca IVOC tissues when administered in protection and competition assays (Figure 6.3, 6.4 and 6.8). This may be most probably explained by co-aggregation between the lactobacilli and *B. pilosicoli* (Figure 6.5C-F, 6.6C-F and 6.9C-F). High levels of exopolysaccharide (EPS) production have been associated with the co-aggregative properties of lactobacilli with enteric pathogens such as *E. coli* (Aslim *et al*., 2007); EPSs are produced by other probiotic members of the normal gut microflora including bifidobacteria (Ruas-Madiedo *et al*., 2006) and thus, may provide additional protection against *Brachyspira* infection. Surface proteins, such as co-aggregation promoting factor (Cpf), have also been implicated in the co-aggregative phenotype of lactobacilli with pathogens (Schachtsiek *et al*., 2004). Whether there was any physiological, pH-dependent impact on adherence and invasion is unclear. However, this is less likely to have occurred than passive co-aggregation because there was no evidence of pH-

associated cellular tissue damage of the HT29-16E cells and the mucosal surface of avian caeca IVOC tissues.

When co-administered in protection assays, the viable lactobacilli were evenly distributed across the cell surface of the HT29-16E monolayers (Figure 6.5C-F), 3D cells (Figure 6.6C-F) and the mucosal surface of the avian caecal IVOC tissue (Figure 6.9C-F), potentially occupying specific receptor sites, limiting the number of adherent *Brachyspira* by niche competition. Greater numbers of lactobacilli would have been present in competition assays, as compared with protection assays, where the washes following the 30 minute pre-treatment would have removed non-adhered lactobacilli; hence, this may explain the greater reduction of the adherence and invasion of *B. pilosicoli* in competition assays, as more lactobacilli were available to interact with the *Brachyspira*. *L. reuteri* induced a greater reduction of *B. pilosicoli* adherence and invasion than *L. salivarius*, regardless of delivery. This trait may be attributed to an ability of *L. reuteri* to compete for a wider range of receptor binding sites, produce additional antimicrobial compounds or co-aggregate more efficiently.

The inability of either lactobacilli strain to reduce the adherence or invasion of *B. pilosicoli* in displacement assays (Figure 6.3C, 6.4C and 6.8C) may be due to the absence of lactobacilli to interact with the *B. pilosicoli* during the 5 hour incubation and the inability of the lactobacilli post-treatment to reverse adherence and invasion. These results suggest the lactobacilli must be present prior to or with the *Brachyspira* in order to interact with the spirochaete and prevent association with the epithelial cells. This data suggests that *Lactobacillus* treatment may have little effect in birds that are already colonised with *B. pilosicoli*. However, this approach may aid recovery after antimicrobial treatment when re-infection from the environment is possible. In this study the opportunity to assess other health benefits of the lactobacilli, other than to control AIS was not possible due to the lack of time and animal studies. It would be valuable to assess a combined antimicrobial-probiotic approach to not only control disease but also speed recovery.

The inability of lactobacilli CFS to reduce the adherence and invasion of *B. pilosicoli* in the 3D cell model (Figure 6.4D) further supports the notion that direct interactions with lactobacilli are crucial in reducing *B. pilosicoli* association. In spite of this, CFS studies in the monolayer and IVOC models revealed a small, but statistically significant ability of the CFS of *L. reuteri,* but not that of *L. salivarius,* to reduce the association of *B. pilosicoli*. The effect of *L. reuteri* CFS on the association of *B. pilosicoli* may be due to the production and release of one or more metabolic by-

product(s) capable of limiting *B. pilosicoli* association or bioactive component(s) that may block adhesion molecules on the *B. pilosicoli* or host cells. Interestingly, the CFS of *L. delbrueckii* spp. bulgaricus inhibits the cytotoxic effects of *C. difficile* and its adhesion to Caco-2 cells attributable to the production of bioactive compounds that inhibit the toxin, its receptors or bacterial adhesion molecules (Banerjee *et al*., 2009).

Further supporting the concept of passive co-aggregation between the lactobacilli and *Brachyspira* was the ability of heat-inactivated lactobacilli to elicit a significant reduction in *Brachyspira* association in protection and competition assays in the IVOC model (Figure 6.8A and B). However, heat-inactivated lactobacilli reduced *B. pilosicoli* association to a lesser degree than viable lactobacilli; this may be due to the increasing numbers of the viable lactobacilli throughout the assays, or perhaps the active production and secretion of inhibitory compounds. A probable mechanism by which the lactobacilli induce a significant reduction in *B. pilosicoli* association when delivered prior to or with the *Brachyspira*, result in the passive co-aggregation between the different species which inhibits *Brachyspira* motility, hence trapping the spirochaete and mitigating its ability to adhere and invade host cells.

In 3D cell assays, HT29-16E cells that had been infected with *B. pilosicoli* showed signs of membrane blebbing (Figure 6.6B and C), indicative of apoptosis due to physical or chemical stresses (Cunningham, 1995; Fackler and Grosse, 2008). *B. pilosicoli* may induce bleb formation via a type three secretion system (TTSS)-dependent invasion mechanism, as has been observed with *Pseudomonas aeruginosa* (Angus *et al*., 2008), or they may adopt a similar mechanism to *Bacteroides fragilis*, which produce an enterotoxin that acts on the cytoskeleton (Donelli *et al*., 1996). Genes encoding components of a TTSS and putative cytotoxin genes have been reported in *Brachyspira* (Bellgard *et al*., 2009; Wanchanthuek *et al*., 2010). Effacement of microvilli was observed in addition to shrunken cytoplasm, intracellular vacuolation and cell sloughing, indicative of apoptosis; these findings are consistent with histopathological studies of tissues from infected birds (Jansson *et al*., 2009a; Shivaprasad and Duhamel, 2005). Moreover, chromatin condensation was noted, which has been observed in infected avian tissues and human colo-rectal cell (Caco-2) monolayers (Naresh *et al*., 2009); genes encoding ankyrin proteins which bind host cell chromatin, have been identified in *Brachyspira* (Bellgard *et al*., 2009). Further supporting the ability of lactobacilli to intervene in *Brachyspira* infection *in vitro* was the apparent protection against this cellular pathology that they conferred in competition assays (Figure 6.6D-F).

The effect of lactobacilli on the growth, motility and association of *B. pilosicoli* to host cells encourages *in vivo* studies to assess the efficacy of these strains to protect against AIS. The rapid growth and robust nature of lactobacilli as compared with the slow-growing, fastidious *Brachyspira* make these species ideal probiotic candidates for intervention against *Brachyspira* infection by niche competition. The results indicate that acidification will inhibit the *B. pilosicoli*, however this may be detrimental to the host and therefore a key effector in control may be the passive co-aggregation that was observed. Supplementing the diet of poultry with co-aggregative lactobacilli may therefore be a useful control strategy for AIS.

Chapter 7.

Investigating the ability of *Lactobacillus reuteri* LM1 to reduce *Brachyspira pilosicoli*-induced pathology in experimentally challenged chickens

7.1 Introduction

AIS is a disease of poultry that arises from the colonisation of the caeca and colon of birds by the anaerobic spirochaete, *Brachyspira* (Stephens and Hampson, 2001; Swayne and McLaren, 1997). Of the seven documented *Brachyspira* species, three are considered pathogenic in poultry as demonstrated by *in vivo* experimental challenge with *B. alvinipulli* (Stanton *et al*., 1998), *B. intermedia* (Hampson and McLaren, 1999) and *B. pilosicoli* (Stephens and Hampson, 2002a). *B. pilosicoli* has a wide host range, also causing PIS in pigs (Trott *et al*., 1996) and HIS in humans (Tsinganou and Gebbers, 2010), with potential for zoonosis (Hampson *et al*., 2006b). AIS in layer and broiler breeder flocks has been associated with a delayed onset of lay, reduced egg weights, diarrhoea, faecal staining of eggshells, reduced growth rates, increased feed consumption and non-productive ovaries (Davelaar *et al*., 1986; Feberwee *et al*., 2008; Griffiths *et al*., 1987; Swayne *et al*., 1992). Colonisation by *B. pilosicoli* has been characterised by an ability of the organism to form end-on attachments to and invade the intestinal epithelia, forming dense fringes penetrating between enterocytes associated with inflammatory responses (Feberwee *et al*., 2008; Jensen *et al*., 2001; Jensen *et al*., 2000).

In the United Kingdom, the incidence of *Brachyspira* in commercial and free-range flocks has been estimated at 74% and 90%, respectively (Burch, 2010), with AIS

reported to be increasing at least partially attributed to the 2006 EU ban on the use of antibiotics as growth promoters in livestock (Castanon, 2007). With increasing antimicrobial resistance amongst *Brachyspira,* including to tiamulin which is currently the preferred therapeutic for AIS (Hampson *et al*., 2006c; Pringle *et al*., 2006), there is increased interest in developing alternative intervention strategies (Collins *et al*., 2009).

Probiotics have been described as live microorganisms that confer health benefits on the host when administered in adequate quantities (FAO/WHO, 2001) and include many enteric commensal species including bifidobacteria and lactobacilli. With regard to the CE of pathogens by probiotics, several mechanisms have been suggested that include the secretion of antimicrobial compounds, competition for essential nutrients and host cell binding receptors and immunomodulation of the gut mucosa (Vanderpool *et al*., 2008). Probiotics have been demonstrated to reduce the colonisation of the lower GI tract by various pathogens in poultry including *E. coli*, *Salmonella*, *Clostridia* and *Campylobacter* (La Ragione *et al*., 2004; La Ragione and Woodward, 2003; Pascual *et al*., 1999; Schoeni and Wong, 1994; Stern *et al*., 2006; Vicente *et al*., 2008). In this chapter, the aim was to determine whether probiotics may reduce the colonisation of the GI tract by *Brachyspira* especially as this pathogen has been shown to colonise the same niche as many of these pathogens.

Lactobacillus strains have been shown to inhibit various aspects of the biology of *Brachyspira in vitro* in Chapter 6 and in other reported studies. For example, *L. johnsonii* exhibited antimicrobial activity against *B. pilosicoli* and *B. hyodysenteriae* through the production of hydrogen peroxide and a proteineous antimicrobial compound (Se *et al*., 2008). *L. rhamnosus* and *L. farciminis* strains have also been implicated in inhibiting the motility of *Brachyspira* by co-aggregation with the spirochaetes and eliciting a bacterial stress response (Bernardeau *et al*., 2009). In Chapter 6, *L. reuteri* LM1 and *L. salivarius* LM2 were demonstrated to inhibit the motility, growth and adherence to host cells of *B. pilosicoli* B2904 *in vitro*. This chapter describes *in vivo* studies using the novel experimental challenge model described in Chapter 3 to test the hypothesis that *L. reuteri* LM1 will compete against and reduce the pathogenic impact of *B. pilosicoli* in the chicken.

7.2 Results

7.2.1 Colonisation of 18 week-old chickens by *B. pilosicoli* and *L. reuteri*

The birds in all groups were cloacally swabbed upon arrival and throughout the experiment (on days indicated in Figure 7.1A). Swabs were plated swiftly onto *Brachyspira* selective agar and incubated anaerobically at 37˚C for 8 days to minimise exposure to oxygen. The plates were examined visually at 24 hour intervals for spirochaetal growth and to verify the genus and species of any putative spirochaetes, cells were picked from the plate and subjected to PCR (Mikosza *et al*., 2001b; Phillips *et al*., 2005). All cultures from cloacal swabs from both groups of birds prior to challenge with *B. pilosicoli* B2904 were negative for spirochaetal growth. Moreover, representative random faecal samples taken from the floor of both of the rooms of birds were negative by PCR for the *Brachyspira* genus and *B. pilosicoli* species.

Following the three challenges with *B. pilosicoli* B2904, the percentage of birds that tested positive by culture from the cloacal swab increased from zero to 83.3% and 75.0% in the untreated control and *L. reuteri* LM1-treated groups, respectively (Figure 7.1A). In the untreated group, ≥75.0% remained culture positive until 12 days after final challenge (day 24), whereas, in the *L. reuteri* LM1-treated group by this point, the percentage of positive birds decreased to 50.0%. By 19 days after final challenge (day 33), the percentage of culture positive birds decreased to 50.0% in the untreated and 16.7% in the *L. reuteri* LM1-treated group.

Faecal DNA isolated from representative random faeces taken from the floor of each of the rooms of birds tested positive for the *Brachyspira* genus and *B. pilosicoli* species by PCR from the day of the final challenge (day 12) and remained positive throughout the study in the untreated group. In the *L. reuteri* LM1-treated group, however, the faecal samples tested positive until 16 days post final challenge (day 28) and then negative until the end of the study. The detection of *B. pilosicoli* by culture was mostly synonymous with direct PCR on faecal DNA. However *B. pilosicoli* was detected by PCR only on five days from untreated group faeces (days 12, 14, 24, 30, 32) and two days from *L. reuteri* LM1-treated group faeces (days 13 and 27).

L. reuteri LM1 was shown to survive in distilled water for 2.5 hours without significant reduction in viability and hence, assuming each chicken consumed approximately 200 ml per day (20.8 ml in 2.5 hours) (Grashorn and Simonovic, 2009), each chicken should have consumed circa 5×10^9 CFU viable lactobacilli. Furthermore, non-viable lactobacilli have been shown to inhibit the motility of *B. pilosicoli* (as

discussed in Chapter 6) and hence any non-viable cells ingested may aid in the intervention of *B. pilosicoli*. To detect the *L. reuteri* species in faeces, faecal DNA extracts were prepared from a mixed pool of faecal samples and subjected to PCR to specifically detect *L. reuteri* (Kwon *et al*., 2004). Representative faecal samples from the *L. reuteri* LM1-treated group tested positive for *L. reuteri* by PCR from day 1 and on each day throughout the study. Faecal samples from the untreated group tested positive from day 1 throughout the study excluding days 11, 13 to 18 inclusive, 23 and 24, which tested negative for *L. reuteri*.

7.2.2 Bird weights

The chickens were weighed upon arrival and throughout the study (on days indicated in Figure 7.1B). The average weight of the birds in both groups increased throughout the course of the experiment, although the rate of weight gain was greater in the *L. reuteri* LM1-treated group (Figure 7.1B). By the end of the study (day 31), the mean weight of the birds in the *L. reuteri* LM1-treated group was approximately 0.09 kg higher than that of the untreated group ($p<0.01$).

7.2.3 Faecal moisture content

Representative samples of fresh faeces were taken from the floor of each of the rooms for the duration of the study (on days indicated in Figure 7.1C) and portions (1 g) were weighed and dried to constant weight to determine faecal moisture content. The average faecal moisture content of the two groups was similar until the day following the final *Brachyspira* challenge (day 13), where the faecal moisture content of the untreated group increased significantly compared to the *L. reuteri* LM1-treated group ($p<0.05$) (Figure 7.1C). The faecal moisture content of the untreated group continued to increase until eight days post final *Brachyspira* challenge (day 20), where it was significantly greater (approx. 6.8%) than that of the *L. reuteri* LM1-treated group ($p<0.05$). The faecal moisture content of both groups decreased after day 21 to the end of the study (day 33). There was no significant difference in faecal moisture content of the *L. reuteri* LM1-treated group between the first and final days of the study. However there were significant differences between these time points for the untreated group ($p<0.05$). On the final day of the study, the faecal moisture content of the *L. reuteri* LM1-treated group was significantly lower than that of the untreated group ($p<0.05$).

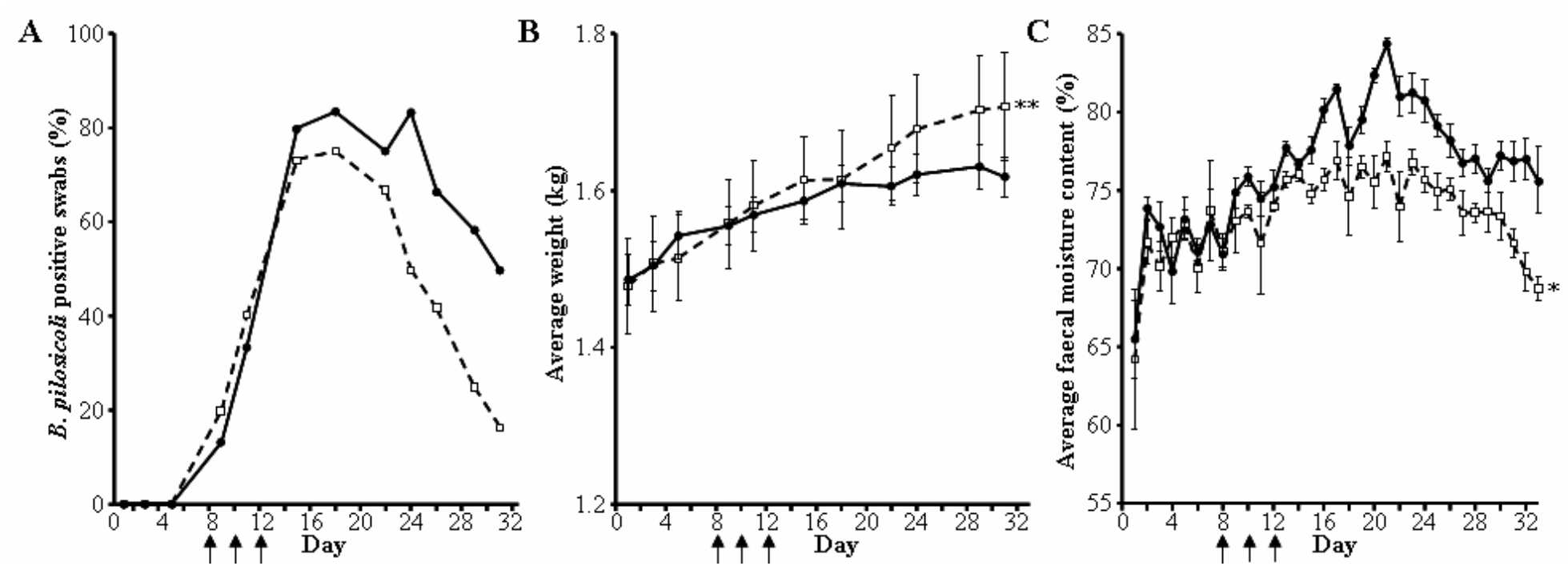

Figure 7.1 Experimental monitoring of birds throughout the intervention study demonstrating the percentage of cloacal swabs positive for *B. pilosicoli* by culture and PCR (A), the mean bird weight (B) and the mean faecal moisture content (C). The mean was based on fifteen birds until day 17 and then twelve birds until the study end in both the untreated (closed circle, solid line) and *L. reuteri* LM1-treated (open square, dashed line) groups. The arrows on the x-axis indicate the days of challenge. Significance is shown in cases where the final data points of the challenged groups and the negative control group differed significantly; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

7.2.4 Egg production

Eggs were collected daily and weighed and scored for the extent of faecal eggshell staining without knowledge of which group they belonged to. Scoring was performed blind with 0 denoting a clean eggshell and 5 for a heavily stained eggshell, as previously described (Stephens and Hampson, 2002b). Egg production in both groups of birds commenced at the start of the study (day 1). The number of eggs produced per day in both groups increased at similar rates until the challenge period, following which, the number of eggs laid by the *L. reuteri* LM1-treated group continued to rise, peaking at 14 eggs laid by the 15 birds on day 16, whereas the untreated group also peaked at this time but laying two fewer eggs (Figure 7.2A). From six days post final challenge (day 18), the number of eggs laid per day appeared to stabilise with the birds laying between eight and eleven eggs per day.

The average egg weight between the two groups was similar up to and during the challenge period. However, on all days following the final challenge except days 15, 17 and 32, the average egg weight of the *L. reuteri* LM1-treated group was greater than that of the untreated group (Figure 7.2A). The average egg weights of the *L. reuteri* LM1-treated group were significantly greater than that of the untreated group on day 18, days 25 – 31 and day 33 ($p<0.05$). The average weight of eggs from across the whole study was significantly greater for the *L. reuteri* LM1-treated (54.81 g ±8.23) than the untreated (52.27 g ±5.58) group ($p<0.001$).

Following the final challenge (day 12), although the average score for the faecal staining of the eggs did increase in the *L. reuteri* LM1-treated group, the average score was greater in the untreated group on most days for the duration of the experiment (Figure 7.2B); specifically, greater average scores were noted on nine of the 21 days of the experiment after the final challenge ($p<0.05$).

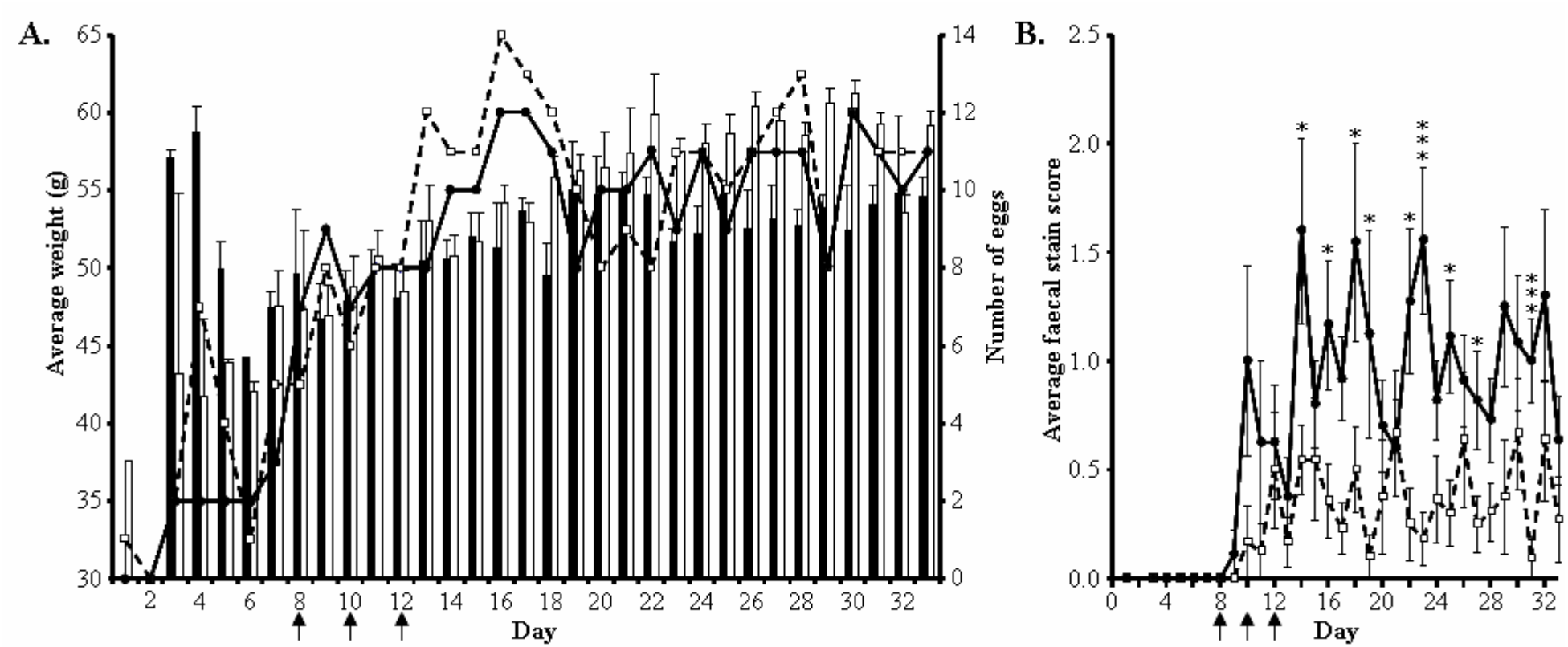

Figure 7.2 Monitoring of egg production; the number of eggs laid (lines) and average egg weights (bars) were recorded (A) alongside average scores for the faecal staining of eggshells (B) on a daily basis throughout the intervention study. Fifteen birds were present in the study until day 17 and then twelve birds present until the study end in both the untreated (closed bar/closed circle, solid line) and *L. reuteri* LM1-treated (open bar/open square, dashed line) groups. The arrows on the x-axis indicate the days of challenge. Significance is shown in cases where the mean score for the faecal staining differed significantly between the two groups; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

7.2.5 Bacteriological findings at *post-mortem* examination

At five and 21 days after the final challenge with *B. pilosicoli*, three birds per group were euthanased by cervical dislocation and subjected to *post-mortem* examination. At five days post final challenge, *post-mortem* examination revealed few macroscopic pathological findings however, petchia were detected in the duodenum and upper jejunum of two birds in both groups. However, no *Brachyspira* were isolated from these tissues. Interestingly, the caeca from the untreated group were smaller, with the average caecal weight of the untreated group (12.6 g ±2.4) lower, without significance, than that of the *L. reuteri* LM1-treated group (13.8 g ±1.2). *B. pilosicoli* were recovered from the caeca and colon of all three birds in both groups, but at significantly greater numbers in the caeca ($p<0.05$) and colon ($p<0.001$) of birds from the untreated group (Figure 7.3A). The average numbers of *B. pilosicoli* recovered from the caeca and colon of the untreated group were nine- and 50-fold greater, respectively, than the numbers recovered from these tissues of the birds from the *L. reuteri* LM1-treated group. *B. pilosicoli* were also isolated from the ileum, spleen and isthmus of one bird from the untreated group and two birds from the *L. reuteri* LM1-treated group, the vagina of two birds from the untreated group and one bird from the *L. reuteri* LM1-treated group, the liver of one bird from each group and the uterus of just one bird from the untreated group.

Post-mortem examination at 21 days after the final challenge revealed the caeca were again smaller in the birds of the untreated group with their average caecal weight (18.1 g ±3.7) 1.6 g lower, without significance, than the average caecal weight of the *L. reuteri* LM1-treated group birds (19.7 g ±4.4). *B. pilosicoli* were recovered from the caeca and colon of all three birds of the untreated group, but from the caeca of only two birds and colon of one bird from the *L. reuteri* LM1-treated group. The average number of *B. pilosicoli* isolated from the caeca of birds from the untreated group was approximately 33-fold greater than that of the *L. reuteri* LM1-treated group ($p<0.05$) (Figure 7.3B). Moreover, the average number isolated from the colon of birds from the untreated group was approximately 24-fold greater than that of the *L. reuteri* LM1-treated group ($p<0.01$). Interestingly, *Brachyspira* were recovered from the ileum, liver and uterus of one bird from the untreated group, the isthmus of one bird of the untreated and two birds of the *L. reuteri* LM1-treated group and the vagina of two birds from the untreated and one bird from the *L. reuteri* LM1-treated group.

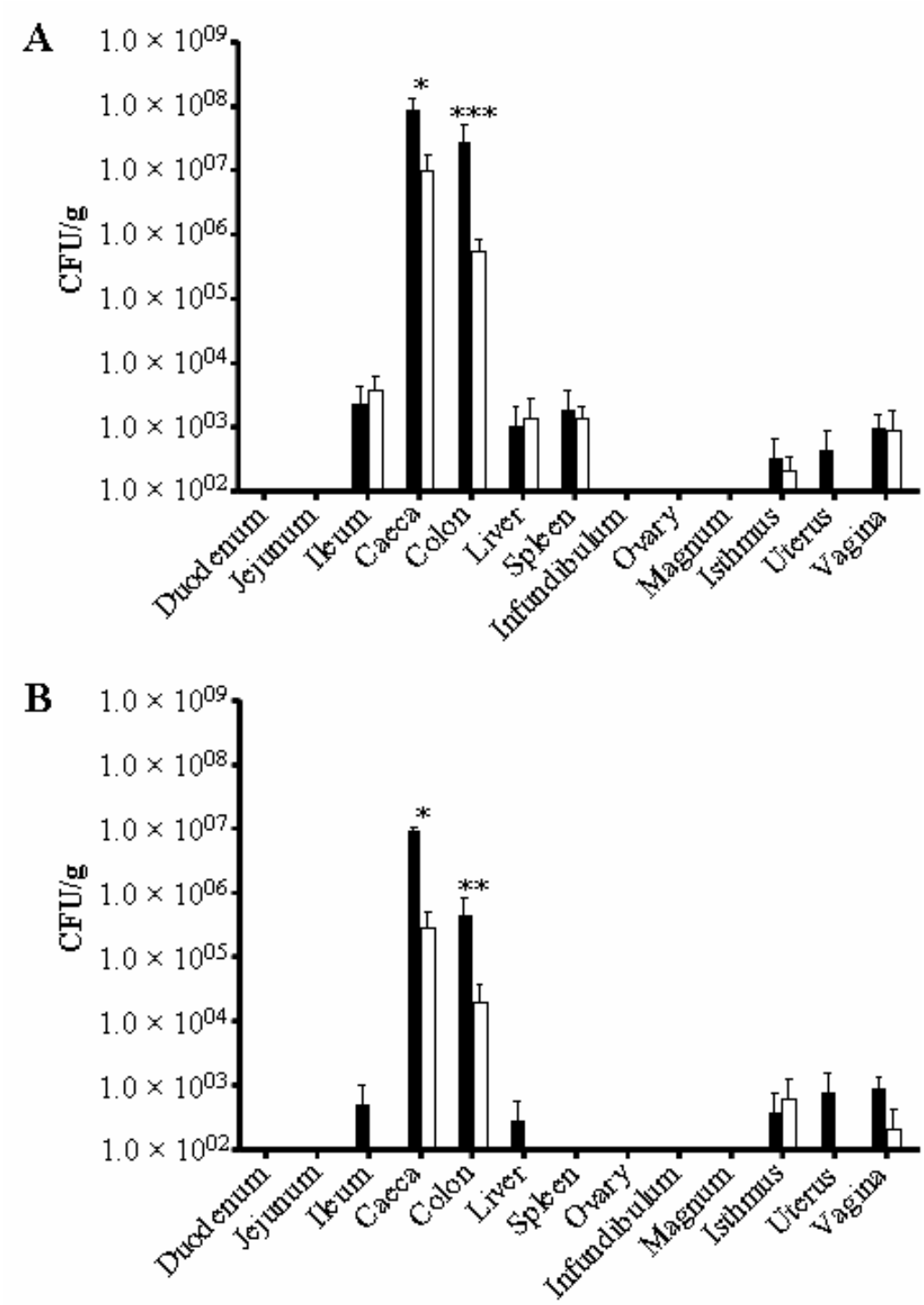

Figure 7.3 Colonisation of *B. pilosicoli* in different tissues sampled at *post-mortem*, at five days (A) and 21 days post final challenge (B). The average was taken from three birds examined at the two time-points from birds of the untreated (closed bar) and *L. reuteri* LM1-treated (open bar) groups. Significance is shown in cases where colonisation by *B. pilosicoli* in the same tissue samples of the two groups differed significantly; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

7.2.6 Histopathological findings at *post-mortem* examination

Microscopical examination of the tissues sampled at five days post final *Brachyspira* challenge, frequent multifocal lymphohistiocytic infiltration was observed in the periportal spaces in the liver of two birds examined at *post-mortem* from the untreated group (Figure 7.4A). This pathology was not observed in the birds examined from the *L. reuteri* LM1-treated group (Figure 7.4B). However, small crypt abscesses, a paucity of secondary follicles and abundant diffuse GALT were noted in the caecal tonsil of one bird of this group.

At 21 days post final *Brachyspira* challenge, less evident pathology was noted in the liver, with only a few areas of lymphocytic infiltration in one of the three birds examined at *post-mortem* from the untreated group. In the three birds examined from the untreated group, lymphoid hyperplasia and secondary follicle proliferation (Figure 7.4C) were noted in addition to crypt abscesses, distended crypts containing bacterial colonies (and rare protozoa) and subepithelial haemorrhages (Figure 7.4C and E). In birds from the *L. reuteri* LM1-treated group, active secondary follicles were noted in the caecal tonsil of one bird and crypt abscesses in the caecal tonsil of another. Generally the tissues examined from the *L. reuteri* LM1-treated exhibited less pathology than that those from the untreated group (Figure 7.4D and F).

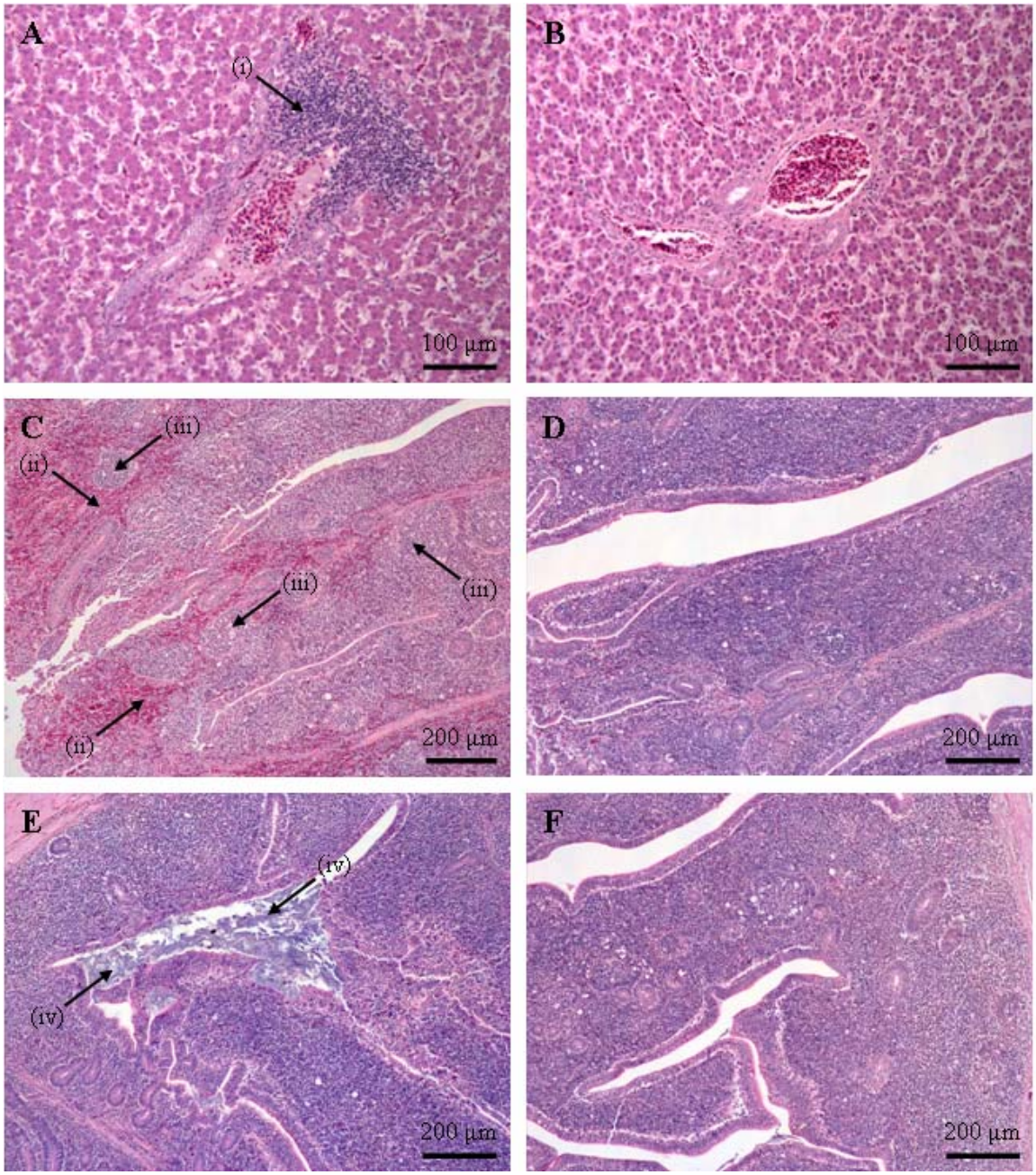

Figure 7.4 Haematoxylin and eosin (HE)-stained tissues demonstrating histopathological changes in birds experimentally challenged with *B. pilosicoli* B2904 from the untreated (A, C and E) and *L. reuteri* LM1-treated groups (B, D and F). Pathology was noted in the liver at five days post *Brachyspira* challenge (A and B) and the caecal tonsil (C-F) at 21 days post final *Brachyspira* challenge. In tissues examined from birds in the untreated group, periportal lymphocytic infiltration was observed in the in liver (i). Haemorrhages (ii), abundant secondary follicles (iii) and distended crypts containing bacterial colonies (iv) were observed in caecal tonsil tissues. Only mild pathology was noted in the tissues examined from the *L. reuteri* LM1-treated group.

7.3 Discussion

Lactobacillus strains have effectively excluded various enteric pathogens from poultry including *Campylobacter* (Zhang *et al*., 2007a), *Clostridium* (La Ragione *et al*., 2004), *E. coli* (Edens *et al*., 1997) and *Salmonella* (Higgins *et al*., 2008; Pascual *et al*., 1999; Vicente *et al*., 2008; Zhang *et al*., 2007b). In Chapter 6, a variety of *in vitro* assays were employed to demonstrate that *Lactobacillus* strains including *L. reuteri* LM1 mitigate against the pathobiology induced by *Brachyspira* by significantly inhibiting growth, motility and adherence. Thus, the scope of the studies described in this chapter focused upon the potential CE effect of *L. reuteri* LM1 against AIS in chickens experimentally challenged with *B. pilosicoli* B2904 using the novel experimental challenge model described in Chapter 3. The studies compared the clinical symptoms and colonisation of *Brachyspira* in *L. reuteri* LM1-treated birds with untreated birds. Given the weight of data indicating a reduction in clinical symptoms and the reduction in colonisation, it can be argued that *L. reuteri* LM1 does indeed confer health benefits *in vivo* and can be truly described as a probiotic.

Following *B. pilosicoli* challenge, the spirochaetes were readily re-isolated from cloacal swabs of both groups. However, fewer birds were positive for *B. pilosicoli* by culture in the *L. reuteri* LM1-treated group (75.0%) than in the untreated group (83.3%) (Figure 7.1A), perhaps attributed to the protective effect of *L. reuteri* LM1 provided in the drinking water from a week prior to challenge and throughout the study. The number of *B. pilosicoli*-positive birds decreased throughout the study but at a greater rate in the *L. reuteri* LM1-treated group. The mechanism of this effect is unclear and may be due to CE by *L. reuteri* LM1 and/or via a potential effect on the modulation of the intestinal microbiota. Given the *in vitro* study data (discussed in Chapter 6), it is not unreasonable to argue that the negative impact on growth, motility and cellular invasion mediated by *L. reuteri* LM1 contributed to the protective effects observed against *B. pilosicoli*. It would be interesting in further *in vivo* work to interrogate these mechanisms. Interestingly, the sensitivity of culture for the detection of *B. pilosicoli* from representative random samples of fresh faeces taken from the floor of each of the rooms was lower than that of PCR on faecal DNA with *B. pilosicoli* detected only by PCR and not culture on five days for the untreated group and two days for the *L. reuteri* LM1-treated group. *B. pilosicoli* were detected by PCR on all days of the study for the untreated group and only until 16 days post final challenge (day 28) for the *L. reuteri*

LM1-treated group, perhaps reflecting an extensive reduction in the number of *B. pilosicoli*-positive birds.

The average bird weight increased at a similar rate for both groups up to *Brachyspira* challenge period, where the rate of weight gain in the *L. reuteri* LM1-treated-group was unaffected, whereas in the untreated group, the rate decreased (Figure 7.1B). By the end of the study, the average weight of the *L. reuteri* LM1-treated birds was significantly greater than that of the untreated birds ($p<0.01$). Interestingly, the administration of *Lactobacillus* probiotics has previously demonstrated a positive effect on weight gain in chickens (Lan *et al*., 2003; Timmerman *et al*., 2006).

Following the challenges with *B. pilosicoli*, the faecal moisture content of the untreated group increased significantly compared to the *L. reuteri* LM1-treated group ($p<0.05$) (Figure 7.1C). Rather than a direct effect of the lactobacilli on reducing faecal moisture content, it is probable that this effect resulted from the ability of lactobacilli to mitigate against the infection and colonisation of *B. pilosicoli* (as discussed in Chapter 6), hence, alleviating clinical symptoms such as the increase in faecal moisture content (Jamshidi and Hampson, 2003).

Egg production in both groups was similar up to *Brachyspira* challenge, after which there was an eight day period where the *L. reuteri* LM1-treated group generally laid slightly greater numbers of eggs per day than the untreated group (Figure 7.2A). Previous studies have related probiotic supplementation to improved egg production (Kurtoglu *et al*., 2004; Tortuero and Fernandez, 1995); however others have noted no effect on egg production (Balevi *et al*., 2001). Moreover, the average egg weight for the *L. reuteri* LM1-treated group was significantly greater than the untreated group ($p<0.001$) (Figure 7.2A). Significant increases in egg weight were previously associated with probiotic supplementation and it was postulated that this resulted from increased digestion of nutrients by probiotic bacteria (Nahanshon *et al*., 1992; Nahanshon *et al*., 1994; Tortuero and Fernandez, 1995). In this study, the greater egg weights of the *L. reuteri* LM1-treated group may simply reflect the mitigation of *B. pilosicoli* infection aided by administration of *L. reuteri* LM1. Eggs were scored on the extent of faecal staining without knowledge to which group they belonged. The average score for the faecal staining of eggs from the *L. reuteri* LM1-treated group were generally lower than the untreated group after the challenge with *B. pilosicoli* (Figure 7.2B); on nine of the 21 days after the final challenge, there was significant difference between the average scores of the two groups ($p<0.05$).

At five days after final challenge, *B. pilosicoli* were recovered at significantly greater numbers from the caeca and colon of the untreated group ($p<0.05$) (Figure 7.3A). This suggests that *L. reuteri* LM1 may have inhibited the growth, motility and/or adherence of *B. pilosicoli*, an effect that was observed *in vitro*, as discussed in Chapter 6. This greater colonisation in the untreated birds may relate to the reduced average weight of the caeca. Interestingly, no pathology was noted in the colon and caecal tissues in the untreated birds examined at *post-mortem*. However, crypt abscesses, secondary follicles and abundant diffuse GALT were identified in the caecal tonsils of one of the three birds examined from the *L. reuteri* LM1-treated group. *B. pilosicoli* were also isolated from the ileum, liver, spleen, isthmus and vagina of birds from both groups and the uterus of a bird from the untreated group. Upon histopathological examination at 21 days after challenge, the liver of two birds from the untreated group showed multifocal lymphohistiocytic infiltration in periportal spaces (Figure 7.4A), whilst in the livers of the three birds from the *L. reuteri* LM1-treated group no pathology was noted (Figure 7.4B). *B. pilosicoli* have previously been isolated from the liver (Kostman *et al*., 1995) and bloodstream (Bait-Merabet *et al*., 2008; Prim *et al*., 2011) in humans and *Brachyspira* infection was associated with hepatic and splenic amyloidosis in duck flocks (Glavits *et al*., 2011). However, this is the first report of the isolation of *Brachyspira* from the liver of chickens.

At 21 days post final challenge, *B. pilosicoli* were isolated at significantly lower numbers from the caeca and colon of the birds examined at *post-mortem* from both groups, compared to the *post-mortem* at five days after challenge (Figure 7.3B). The greatest numbers of *B. pilosicoli* were isolated from the caeca and colon, but at significantly lower numbers in birds from the *L. reuteri* LM1-treated group ($p<0.05$). These findings correlated with the histopathological findings, whereby more severe pathology was noted in birds from the untreated group including secondary follicle proliferation, lymphoid hyperplasia (Figure 7.4C), crypt abscesses, distended crypts and subepithelial haemorrhages (Figure 7.4E), which have been described previously in poultry colonised by *B. pilosicoli* (Feberwee *et al*., 2008). The smaller caeca from birds of the untreated group at this time-point may again be explained by the greater extent of colonisation by *B. pilosicoli* in untreated birds. The spirochaete was also isolated from the isthmus and vagina of birds from both groups and the ileum, liver and uterus of birds from one birds of the untreated group. The liver of the bird from the untreated group, from which *B. pilosicoli* was isolated displayed few areas of lymphocytic infiltration, perhaps induced by *B. pilosicoli* infection. The reduction in the colonisation

of *B. pilosicoli* and the severity of the pathology observed in *L. reuteri* LM1-treated birds highlights a potential protective function of *L. reuteri* LM1 against AIS.

The data strongly support the hypothesis that *L. reuteri* LM1 ameliorated the effects of the pathogenicity of *B. pilosicoli*. However, faecal samples from the *L. reuteri* LM1-treated group tested positive for *L. reuteri* by PCR at all time points throughout the study, whereas those from the untreated group were variably positive. Specifically, the birds of both groups were *L. reuteri*-positive at day 1. However, since the birds of the untreated group were negative for *L. reuteri* on nine days of the study, the level of colonisation of this species was likely to have been lower in this group due to its absence from the drinking water. The PCR test used was not specific for *L. reuteri* LM1 and therefore the PCR will have detected both LM1 and other strains native to that bird population. Thus, *L. reuteri* species are considered to have colonised the birds of both groups prior to the study. As this species is a common commensal of the GI tract (Abbas Hilmi *et al*., 2007) and the birds were 17 weeks of age on arrival, this is not unreasonable. It may be argued however, that as groups were kept in biosecure containment, the effects seen were due to the additional *L. reuteri* LM1 treatment. Without distinguishing features to enable specific detection of *L. reuteri* LM1, detection of *L. reuteri* primarily in the treated group strongly supported the argument that it was indeed *L. reuteri* LM1, a strain of chicken origin that colonised the chickens to which it was administered in the drinking water. In summary, the data indicate elevated numbers of *L. reuteri* in the treated group, which were probably strain LM1 plus any native strains already present. The control group contained *L. reuteri* but in lower numbers and, therefore, it is not unreasonable to argue that the biological impact on *B. pilosicoli* infection may be due to the elevated numbers of all *L. reuteri* strains or LM1 specifically. Furthermore, nutritional modification by prebiotics for example may enhance *L. reuteri* numbers to induce the same effect as supplementation of water with strain LM1. Clearly further work is required to assess this.

Further to the *in vitro* studies presented in Chapter 6, the promise of *L. reuteri* LM1 as a probiotic that affords some protection against AIS has been demonstrated by *in vivo* experimentation, since the administration of *L. reuteri* LM1 in the drinking water reduced the severity of clinical symptoms associated with *B. pilosicoli* infection. Whilst some differences between the two groups may be related to a direct effect from the probiotic supplementation, such as weight gain and increase in egg weight, it is possible that the antagonistic effect *L. reuteri* LM1 exerts on *B. pilosicoli* is largely responsible. The rapid growth and robust nature of lactobacilli as compared with the

slow-growing, fastidious *Brachyspira* make these species ideal probiotic candidates for intervention against AIS, possibly by niche competition, however further work would be required to study the mode of action. *L. reuteri* LM1 may have reduced colonisation of *B. pilosicoli*, alleviating the associated clinical symptoms by acidification, which has been shown to inhibit *B. pilosicoli* and/or passive co-aggregation (as discussed in Chapter 6). Supplementing the diet of poultry with co-aggregative lactobacilli may therefore be a useful control strategy for AIS. This study warrants further investigation into the development of *L. reuteri* LM1 as a prophylactic probiotic to protect against enteric infections such as *Brachyspira* and potentially as a therapeutic treatment for these infections.

Chapter 8.

General discussion

AIS is a worldwide-recognised enteric disease that affects laying and broiler breeder chickens and hence, poses significant economic complications to poultry farmers. The aetiological agent of the disease is the spirochaete, *Brachyspira*, which colonises the caeca and/or colo-rectum of infected poultry. The limited understanding of the pathobiology of aetiological agent has hindered the development of novel intervention strategies for AIS and other *Brachyspira*-related diseases, such as swine dysentery. Recently, whole genome sequencing of *Brachyspira* has assisted in improving the understanding of this pathogen and particularly, comparative genome studies are important for elucidating the genotypic explanations for differences in pathogenicity between species (Bellgard *et al*., 2009; Hafstrom *et al*., 2011; Pati *et al*., 2010; Wanchanthuek *et al*., 2010). However, genome sequence information is still not available for all *Brachyspira* species, and genome sequence information is restricted to one strain for those that have been genome sequenced, limiting the conclusions that can be drawn from such analysis. Indeed, it can be justifiably argued that the need is for not only comprehensive coverage of pathogenic, intermediate and non-pathogenic strains, but also geospatially distinct populations of each species in order to draw firmer conclusions. This thesis has contributed to this research by sequencing and analysing one novel *B. pilosicoli* genome.

One aim of the studies presented in this thesis was to gain a better understanding of the pathobiology of *B. pilosicoli*, one of the *Brachyspira* species considered

pathogenic to poultry, in order to develop novel intervention strategies against AIS. *B. pilosicoli* is currently the only species that is acknowledged as a pathogen in poultry, swine and humans and hence, a strain of this species was selected for use in further studies, including whole genome sequencing, due to the potential of the results to have wider implications. All *Brachyspira* strains used in the studies presented in this thesis were characterised thoroughly (as discussed in Chapter 3 and specifically section 3.2.1), and *B. pilosicoli* B2904 was selected for use in the further studies. This strain was isolated from the faeces of a chicken that exhibited clinical symptoms of AIS in the United Kingdom and was selected due to its ability to grow well *in vitro*, susceptibility to gentamicin, which was important for tissue culture assays (as discussed in Chapter 6) and its isolation from a case of AIS. It seems unlikely that these selection criteria for this specific strain introduced any undue bias. Given there was no genome sequence available for *B. pilosicoli* of poultry origin, and the strain selected was associated with pathology in the chicken, it can be fairly argued that that this new data is a valuable building block for further studies and is likely to be genuinely representative of *B. pilosicoli*, at least of those in circulation in the United Kingdom.

In order to fulfil the aim of sequencing the whole genome of a *B. pilosicoli* strain for which Koch's postulates had been proven in a chicken, *B. pilosicoli* B2904 was used in an experimental challenge model. This aim coincided with the aim to develop a novel and improved experimental challenge model for AIS for which the efficacy of future potential interventions could be evaluated (such as that discussed in Chapter 7). In order to make this novel experimental infection model comparable with those described previously, *B. pilosicoli* CPSp1 was used to challenge one of the groups of birds, as this strain was used in previous experimental challenge studies to induce AIS-like clinical symptoms (Jamshidi and Hampson, 2002; Stephens and Hampson, 2002a, b) and hence, also served as a positive control in the studies reported here.

The experimental challenge model developed and described in this thesis produced improved rates of colonisation and induced hitherto more severe clinical symptoms consistent with AIS and unreported pathology, compared to previously described models (Amin *et al*., 2009; Hampson and McLaren, 1999; Hampson *et al*., 2002a; Hampson *et al*., 2002b; Jamshidi and Hampson, 2002, 2003; Jamshidian *et al*., 2004; Stephens and Hampson, 2002a). The model for AIS that has been described in this thesis used sodium bicarbonate to neutralise the crop acid barrier prior to challenge, which may have improved the survival of *B. pilosicoli* transversing the crop, facilitating enhanced colonisation of the distal GI tract and thus, resulting in more severe clinical

symptoms. In addition to more severe clinical symptoms that are akin to those observed with AIS in the field (Dwars *et al*., 1990; Feberwee *et al*., 2008; Smit *et al*., 1998), this was the first study to provide a quantitative output on *Brachyspira* colonisation at *post-mortem*. *In vivo* models for AIS have been important tools to evaluate the efficacy of novel intervention strategies, such as vaccines (Amin *et al*., 2009), antimicrobials (Hampson *et al*., 2002a; Stephens and Hampson, 2002a, b) and dietary supplements (Hampson *et al*., 2002b). Thus, this novel *in vivo* model for AIS would be useful in testing such intervention strategies and would allow investigators to determine whether the treatment elicited a reduction in colonisation in specific tissues. This model was applied to test the efficacy of probiotic intervention in AIS (as discussed in Chapter 7) and future studies to test other novel intervention strategies or the pathogenicity of *Brachyspira* strains in poultry, may also benefit from using sodium bicarbonate to neutralise the crop acid prior to challenge and the quantitative approach to evaluate *Brachyspira* colonisation at *post-mortem*. Whilst the data from the *in vivo* studies reported in this thesis give considerable encouragement for the wider application of the modified inoculation approach, care must be taken in that the *in vivo* studies were modest in terms of numbers of animals used and that no larger scale study was performed to repeat the findings. However, confidence in the findings arises from the comparability with CPSp1 as both a control and a reference to prior infection studies. It seems highly unlikely, given the containment and general health status of the animals used, that the pathological outcome of the infections were in any way artefactual or associated with adventitious infectious agents co-infecting during the study.

In the experimental challenge model described in this thesis, novel pathology was associated with *B. pilosicoli* colonisation in the liver and spleen. Previously, *Brachyspira* have been isolated from extra-intestinal tissues in humans, including the liver (Kostman *et al*., 1995) and the bloodstream (Trott *et al*., 1997a) and have also been associated with hepatic and splenic amyloidosis in duck flocks (Glavits *et al*., 2011). However, this was the first report of the recovery of *Brachyspira* from such tissues in chickens. Future work should focus on the direct visualisation of the aetiological agent in order to determine the specificity of the pathological changes noted in these tissues. *In situ* visualisation of *Brachyspira* has previously been performed via IHC (Feberwee *et al*., 2008; Ivanics *et al*., 2007; Thuma *et al*., 2011), FISH (Herzog *et al*., 2005; Jensen *et al*., 2001) or staining methods, such as Warthin-Starry (Jansson *et al*., 2009a; Jensen *et al*., 2000; Trott and Hampson, 1998). Such intricate analysis of samples may reveal insight into the different colonisation patterns and pathology caused by different strains,

as noted for B2904 and CPSp1 and may aid in understanding the delayed and reduced egg production. Differences in the disease caused by the two strains may arise from differences in their pathogenicity. Future work may involve sequencing the whole genome of the CPSp1 strain for a comparative analysis with the B2904 genome in order to improve the understanding of such differences. The experiment was carefully controlled and thus, the deep tissue colonisation was considered to be a true phenomenon and not the result of an extraneous factor, such as co-infection. Furthermore, subsequent studies using the B2904-challenge model to evaluate AIS intervention strategies have shown findings consistent with this study (as discussed in Chapter 7). It may be of interest, however, for further work to be performed to provide additional validation of the novel observations from these studies.

B. pilosicoli B2904 was isolated from a chicken that exhibited clinical symptoms of AIS and having proven the pathogenicity of this strain in experimentally challenged chickens, the whole genome of this was sequenced. Alongside the incomplete genome sequence of *B. pilosicoli* WesB, of human origin, and the previously reported whole genome sequence of *B. pilosicoli* 95/1000 (Wanchanthuek *et al*., 2010), of porcine origin, this permitted the first intra-species genome comparison within the *Brachyspira* genus (as discussed in Chapter 4). Comparing the *B. pilosicoli* genome sequences and features with each other and also with available genome sequences for other *Brachyspira* species may reveal insight into the unique pathogenicity of this species, since this is the only species that is considered capable of inducing disease in poultry, swine and humans. Alongside *B. aalborgi*, these species are also unique in their ability to form intimate end-on attachments to the intestinal epithelium of the host. Of other *Brachyspira* species, whole genome sequences have been published for *B. hyodysenteriae* WA1 (Bellgard *et al*., 2009), *B. intermedia* PWS/A^{T} (Hafstrom *et al*., 2011) and *B. murdochii* 56-150^{T} (Pati *et al*., 2010) and the unpublished, draft genome scaffolds of *B. aalborgi* 513^{T}, *B. alvinipulli* C1^{T} and *B. intermedia* HB60 were also available for limited analysis.

MLST analysis revealed the three *B. pilosicoli* strains to be closely related; however future work could validate such findings and the relationships between the other *Brachyspira* strains, by use of other molecular typing methods such as MLEE, PFGE, RAPD and 16S rRNA gene sequence analysis, as previously performed for *Brachyspira* (Fellstrom *et al*., 2008; Fossi *et al*., 2004; Hidalgo *et al*., 2009; Rasback *et al*., 2007b). The relatively small size of the *B. pilosicoli* genomes may suggest that they

are members of a more specialised species that has undergone a high degree of reductive genome evolution. This would suggest that *B. pilosicoli* is likely to be an older pathogen than other *Brachyspira* species (Hafstrom *et al*., 2011) and is likely to have allowed improved energy efficiency and enhanced pathogenic potential. Reductive genome evolution is particularly evident in obligate, intracellular bacterial pathogens (Andersson and Kurland, 1998) and consistent with this, *B. pilosicoli* and *B. aalborgi*, which appear to have the smallest genomes, develop long-term intimate associations with the surface of enterocytes, into which they interdigitate one of their cell ends.

Genome rearrangements oriented around the *oriC* were observed in the *B. pilosicoli* genomes, particularly of the B2904 and WesB strains, which had larger genomes. The rearrangements correlated largely with the positions of MGEs, of which greater numbers were noted in the B2904 and WesB genomes and were likely to have driven chromosomal rearrangements, gene disruptions and eventual loss of genes (Moran and Plague, 2004; Plague *et al*., 2008; Schmitz-Esser *et al*., 2011; Song *et al*., 2010). Strain 95/1000 had the fewest MGEs and this may be interpreted as indicating that the MGEs that induced the genome reduction in this strain have become lost. Alternatively, the B2904 and WesB genomes may be in the initial stages of genome reduction at which point MGE expansion occurs (Moran and Plague, 2004; Song *et al*., 2010). The MGEs are then generally lost in a fragmentary manner by pseudogenisation, which correlates with the greater number of pseudogenes recorded in the B2904 and WesB genomes. Genome reduction and MGE expansion is often associated with niche specialisation or host restriction (Parkhill *et al*., 2003; Parkhill *et al*., 2001), although *B. pilosicoli* are not considered host-restricted and the WesB strain, of human origin, has been shown to have the capacity to infect chickens and pigs (Trott *et al*., 1996a; Trott *et al*., 1995). MGEs driving genome rearrangements and reduction may be a key factor in the pathogenicity of *B. pilosicoli*. With an increasing availability of genome sequences for *Brachyspira* species, it is likely that further intra-species genome comparisons will be performed in the future, and it would be interesting to investigate the relationships of genome rearrangements and MGEs in other species.

B. pilosicoli is a highly recombinant species (Trott *et al*., 1998), and despite differences in genome arrangement and the number of pseudogenes, part of the variation in the genome sizes simply reflects the carriage of different subsets of the pan-genome. Feature-based analysis revealed a high level of similarity between the three *B. pilosicoli* strains and allowed the identification of genes that may have been lost in a process of reductive genome evolution due to their absence from some strains, such as a

peptidase, glycine reductase complex components and transposases that were absent from the 95/1000 strain. Virulence factor screening was also performed in the three *B. pilosicoli* strains, highlighting the presence of genes for LOS biosynthesis, chemotaxis and motility, adhesion, host tissue degradation, oxidative stress, ankyrin repeat proteins and phage, all of which may contribute to the pathogenicity of the strains. Novel bacteriophages were detected in the newly-sequenced *B. pilosicoli* genomes, which appeared to have involvement in intra- and inter-species HGT and hence, may have had involvement in the pathogenicity of these strains. Alongside other MGEs, these may have also played a role in the acquisition of antimicrobial resistance genes noted in the *B. pilosicoli* genomes

To complement the genomic comparisons, this study was also the first to apply a high-throughput phenotype screening tool to correlate genotype with phenotype in *Brachyspira*. This allowed validation of phenotypic differences predicted from genome analysis, such as the lack of genes for glucuronate catabolism in 95/1000. The phenotypic data for the 28 *Brachyspira* strains of different known and proposed species presented in this thesis has potential for thorough interrogation to elucidate metabolic differences between different species of varying pathogenicity. Future work may focus on linking this phenotypic data to genotypic findings in species for which genome sequences are available or may become available. It would be interesting to perform MLST on all 28 strains used to compare the relatedness of these strains based on molecular as well as phenotypic methods. Such analysis of genotype and phenotype alongside one another, could play an important role in the validation of proposed species such as "*B. canis*", "*B. pulli*" and the recently reported "*B. hampsonii*" (Chander *et al*., 2012). Application of this technology to other genome-sequenced *Brachyspira* will be greatly beneficial to understanding the differences in pathogenicity within this genus. This technology will allow comparison on the metabolic profiles of different strains of different species and also has potential to be an important application in the validation of metabolic models, as has previously been performed for *E. coli* (Feist *et al*., 2007) and *Salmonella* (Abuoun *et al*., 2009). With the increasing availability of *Brachyspira* genome sequences, such technology should aid in improving the understanding of *Brachyspira* metabolic competence that is likely to relate to or even contribute directly to pathogenicity. Genes for motility, LOS synthesis, iron metabolism and others associated with pathogenesis were identified but the contribution to the pathogenic process of other genes such as those associated with central or peripheral (e.g. import and use of monosaccharides) metabolism are worthy of further

investigation. Certain metabolic competences may lead to host preference, niche colonisation or energetically favourable catabolism of mucins for example. The need is for the ability to rapidly generate knock-out and knock-down mutants to start this type of interrogation.

Both genomic and phenotypic analysis revealed interesting differences between the three *B. pilosicoli* strains, worthy of further investigation. These strains were isolated from different hosts and geographical locations. However, with just three strains the conclusions that could be drawn were limited. Research is in this field would greatly benefit from sequencing the genomes of wider strain sets from more and each of the hosts and geographical locations.

Antimicrobials such as tiamulin are usually employed for the treatment of *Brachyspira*-related diseases including AIS. However, novel intervention strategies are required for such diseases due to the ban of the subtherapeutic use of antimicrobials in livestock and an emergence of resistance within enteric pathogens including *Brachyspira* (Clothier *et al*., 2011; Duhamel *et al*., 1998a; Hampson *et al*., 2006c; Karlsson *et al*., 2003; Karlsson *et al*., 2004; Pringle *et al*., 2006; Pringle *et al*., 2004). Antimicrobial resistance genes were demonstrated in the three *B. pilosicoli* strains for which the genomes have been sequenced including multidrug efflux pumps (as discussed in Chapter 4) which can confer resistance to multiple antimicrobials (Piddock, 2006b; Poole, 2001; Webber and Piddock, 2003). The use of lactobacilli as probiotics for the CE of *Brachyspira* was considered to be an ideal potential alternative intervention as the sensitivity of *Brachyspira* to organic acids, such as those produced by lactobacilli, has been reported (Corona-Barrera *et al*., 2004). Moreover, adherence to the host intestinal epithelium is considered an important virulence trait in *B. pilosicoli* (Jensen *et al*., 2000) and lactobacilli have been suggested to compete for receptor binding sites, to prevent binding of the pathogen. Genes that are potentially associated with adherence were identified in all three *B. pilosicoli* genomes sequenced (as discussed in Chapter 4 and specifically section 4.3.5.3). Furthermore, the use of lactobacilli as probiotics in poultry is well established and such treatment has been reported as effective against other enteric disease in poultry including *Campylobacter*, *Clostridium*, *E. coli* and *Salmonella* (Collins *et al*., 2009; Patterson and Burkholder, 2003; Schneitz, 2005).

Recently, some studies have investigated the potential of probiotics to intervene in *Brachyspira* infection. Inhibitory effects of probiotics, including lactobacilli, have been

reported on *Brachyspira* growth and attributed to the production and release of organic acids, particularly lactic acid, hydrogen peroxide and other antimicrobial compounds (Klose *et al*., 2010; Klose *et al*., 2009; Se *et al*., 2008). Furthermore, *Lactobacillus* probiotics have been implicated in inhibiting *Brachyspira* motility via co-aggregation with the spirochaetes and eliciting a stress response in the *Brachyspira* (Bernardeau *et al*., 2009). Motility is regarded as an important virulence feature of *Brachyspira* and is considered vital for inhabiting the GI tract and inducing enteric disease. Hence, inhibition of such features would be detrimental to the survival of *Brachyspira* in the host environment.

Of eighteen probiotic candidates of the genus *Lactobacillus* that were screened for their ability to inhibit *B. pilosicoli* growth, motility and haemolysis, *L. reuteri* LM1 and *L. salivarius* LM2 were selected (as discussed in Chapter 5). These strains were isolated from the faeces of healthy conventional, commercial laying hens, fulfilling the probiotic selection criterion of being of host origin, whilst also indicating that the strains are able to colonise the intended host. Selection criteria stipulate that probiotic candidates should exhibit antimicrobial activity against target pathogens or some degree of antagonism, which both of these strains demonstrated against the growth, motility and haemolysis of *B. pilosicoli*. It is important to note and that the abundance of metabolites and the degree of inhibition observed in these *in vitro* tests may be of less significant impact on *B. pilosicoli in vivo*. Further work should consider this and also the site in the gut where the *Lactobacillus* probiotic candidate strains colonise and hence, where their metabolites potentially have greatest impact.

In concordance with guidelines for the selection of probiotics (von Wright, 2005), *L. reuteri* LM1 and *L. salivarius* LM2 were screened for antimicrobial resistance, which suggested these strains may carry resistance genes to chloramphenicol and tetracycline. Curative strategies have been applied to probiotic strains to remove antimicrobial resistance genes or plasmids carrying such genes previously (Huys *et al*., 2006). For the commercial use of these strains as probiotics, further work to remove any such antimicrobial resistance genes is essential. The ability of *L. reuteri* LM1 and *L. salivarius* LM2 to resist gastric acid and bile was investigated to fulfil some selection criteria that ensure probiotics candidates are tolerant to environmental stresses. Both strains demonstrated sufficient tolerance to both poultry gastric acid and bile and it can be argued that these properties would enable passage of viable probiotic organisms to the lower GI tract. Again, it is assumed that the impact of the probiotic is mediated in the lower GI tract and most likely in the caeca and colon, however evidence for this

needs to be gained. Future work could investigate a multitude of other selection criteria that have been suggested and focus on an array of properties, such as amenability to mass production and storage, genetic stability, resistance to bacteriocins, acids and other antimicrobials produced by resident microflora and immunostimulatory capacity (Klaenhammer and Kullen, 1999).

L. reuteri LM1 and *L. salivarius* LM2 were used in studies to further investigate their ability to inhibit the growth, motility and adherence of *B. pilosicoli* and elucidate some of the potential mechanisms giving rise to these effects (as discussed in Chapter 6). The suppression of the growth of *B. pilosicoli* B2904 was determined to be of a pH-dependent manner and the findings suggested that the suppressive effect was not attributed solely to acidity. This would indicate that there may have been a strain-dependent production of pH-dependent active compound(s) such as hydrogen peroxide and/or other antimicrobial compounds such as reuterin or bacteriocins, as suggested previously to have been involved in the inhibition of *Brachyspira* growth (Klose *et al*., 2010). *L. reuteri* LM1 has been shown to produce hydrogen peroxide (as discussed in Chapter 5), which may have contributed to its inhibitory effect. However, further investigation is required to evaluate the composition of the acids in CFS that generated the pH drop to pH 3.8 and the relative ratios of acid, which may depend on whether the strains were hetero- or homofermentative. Metabolomic and metabonomic approaches to assess the type and abundance of the products of the metabolism of the lactobacilli and the metabolic response of the host to probiotic treatment would also be a useful research area in the future. Metabolomic approaches using chromatographic methods, such as high-performance liquid chromatography (HPLC) to separate the constituents of the CFS of probiotic strains and proteomic methods, usually mass spectrometry, such as matrix-assisted laser desorption/ionisation (MALDI) to identify active components of the CFS (Kim *et al*., 2008; Laughton *et al*., 2006; Svetoch *et al*., 2011). Metabonomic approaches, often using nuclear magnetic resonance (NMR) have been used to investigate probiotic modulation of symbiotic gut microbial-host metabolic interactions in mouse models (Hong *et al*., 2010; Martin *et al*., 2008b) and in human samples (Hong *et al*., 2011).

EM examination revealed that the two probiotic candidates inhibited the motility of *B. pilosicoli* by co-aggregation, which would render the spirochaetes incapable of escaping the eliminating effect of mucus for which motility and chemotaxis are considered key virulence features (Lux *et al*., 2000; Nakamura *et al*., 2006). This aspect

of inhibition was also considered to play a key role in the reduction of *B. pilosicoli* adherence and invasion in *in vitro* protection and competition assays (as discussed in Chapter 6). Genes encoding membrane proteins that could be involved in such interactions were identified in the three genome-sequenced *B. pilosicoli* strains (as discussed in Chapter 4 and specifically in section 4.3.5.3). Surface proteins, such as co-aggregation-promoting factor (Cpf) and EPSs have been implicated in the co-aggregation of lactobacilli with enteric pathogens (Aslim *et al*., 2007; Schachtsiek *et al*., 2004). However, the biochemical basis of adherence and the avidity of binding in both *Brachyspira* and the *Lactobacillus* strains is worthy of further investigation as this may identify the lectins for further development and exploitation. It is also probable that the lactobacilli competed with the *B. pilosicoli* for specific receptor binding sites limiting the number of adherent *Brachyspira* by niche competition. The potential ability of the lactobacilli to produce antimicrobial compounds, such as hydrogen peroxide production in *L. reuteri* LM1, or bioactive components, which may block adhesion molecules on the *B. pilosicoli* or host cells, may have further contributed to this effect. This is supported by the small reduction in the adherence and invasion of *B. pilosicoli* in CFS assays. Further supporting the ability of *L. reuteri* LM1 and *L. salivarius* LM2 to intervene in *Brachyspira* infection *in vitro* was the apparent protection against this cellular pathology that they conferred in competition assays using 3D cells. If intimate association is one of the mechanisms of inhibition, it becomes very reasonable to argue that other factors that act *in trans*, such as hydrogen peroxide and organic acids, will be involved in the overall inhibition process because of proximity and reduced opportunity for dilution or metabolism of products by other organisms. A key question arises as to which of these speculated mechanisms of inhibition plays the greater, sole or any role *in vivo*.

The rapid growth and robust nature of lactobacilli as compared with the slow-growing, fastidious *Brachyspira* make these species ideal probiotic candidates for intervention against *Brachyspira* infection by niche competition. The results indicated that acidification inhibited the *B. pilosicoli*, however this may be detrimental to the host and therefore a key effector in control may be the passive co-aggregation that was observed. Supplementing the diet of poultry with co-aggregative lactobacilli may therefore be a useful control strategy for AIS. The effect of *L. reuteri* LM1 and *L. salivarius* LM2 on the growth, motility and association of *B. pilosicoli* to host cells encouraged *in vivo* studies to assess the efficacy of these strains to protect against AIS. *L. reuteri* LM1 was selected for use in an *in vivo* intervention study against *B. pilosicoli*

B2904 that was used to experimentally challenge laying hens in the novel model that was developed (as discussed in Chapter 3). *L. reuteri* LM1 was selected since it had a more significant impact in the inhibition of the growth, motility, adherence and invasion of *B. pilosicoli* in the *in vitro* assays.

The scope of the *in vivo* intervention study focused upon the potential CE effect of *L. reuteri* LM1 against AIS in chickens experimentally challenged with *B. pilosicoli* B2904 by comparing the clinical symptoms and colonisation of *Brachyspira* in *L. reuteri* LM1-treated birds with untreated birds (as discussed in Chapter 7). Given the weight of data indicating a reduction in clinical symptoms and the reduction in colonisation, there is reason to strongly argue that *L. reuteri* LM1 does indeed confer health benefits *in vivo* and can be truly described as a probiotic. Of interest, is the apparent controlling effects of the *L. reuteri* LM1 *in vivo* and the question arises as to whether those features of inhibition observed *in vitro* (as discussed in Chapter 6), are those that impact *in vivo*. It could be argued that the addition of *L. reuteri* provided in the drinking water is a minority in the gut population and any inhibitory metabolites produced may be utilised by other gut commensals or even be diluted to become ineffectual. The fact that *Lactobacillus* did afford protection clearly indicates that the probiotic was effective but the mechanism which could be assumed to be those demonstrated in the *in vitro* studies could be challenged.

Although the mechanism of the effect of *L. reuteri* LM1 against *B. pilosicoli in vivo* is unclear, it may be due to CE by *L. reuteri* LM1 and/or via a potential effect on the modulation of the intestinal microbiota. Given the *in vitro* study data (as discussed in Chapter 6) it is not unreasonable to argue that the negative impact on growth, motility and cellular invasion mediated by *L. reuteri* LM1 contributed to the protective effects observed against *B. pilosicoli*. It would be interesting in further *in vivo* work to interrogate these mechanisms. This study was focused on the CE effect of the probiotics and their effect on clinical symptoms, however in the future, it would be interesting to investigate the other aspects by which this strain by exert beneficial effects to the health of chickens, such as increasing feed intake and digestion, reducing ammonia production, modulating the gut microbiota, modulating the secretion of intestinal mucins and immunomodulation.

A limitation of the *in vivo* intervention study was the absence of distinguishing features of *L. reuteri* LM1 and hence, inability to specifically and quantitatively detect this strain. The detection of *L. reuteri* primarily in the treated group strongly supported

the argument that it was indeed the LM1 strain, which was of chicken origin and hence, was likely to have colonised the chickens to which it was administered. It would be of interest to develop a method for the specific detection of *L. reuteri* LM1 and to further investigate the colonisation patterns of this strain in poultry. A method to screen the expression of putative adhesin genes involved in the binding mechanism in lactobacilli has been previously described and could be employed (Turpin *et al*., 2012). This would help determine whether this probiotic strain was capable of colonising the caecal and colo-rectal regions and exclude *B. pilosicoli* by niche competition. Although the chickens were already colonised by *L. reuteri*, it was not necessarily identical to the probiotic added in the drinking water, and it can be argued that the inhibitory effects were enhanced by an accumulative effect. Perhaps the increased numbers of *L. reuteri* overcame any diluting effect that was discussed earlier. Also, if the position is taken that increased numbers are important in inhibition, then co-localisation of the *L. reuteri* and the *Brachyspira* should have a similar controlling effect. Thus, it becomes imperative to study sites of colonisation of the gut of both pathogen and probiotic in order to shed further light on the true mechanisms of inhibition.

This *in vivo* study was conducted in a controlled environment and future work should investigate the ability of *L. reuteri* LM1 to intervene in AIS in the field and perhaps in chickens already infected with *Brachyspira*. Moreover, *B. pilosicoli* was focused upon as an aetiological agent of AIS and it would be of interest to this research to investigate the effect of *L. reuteri* LM1 treatment in chickens infected with other species known to cause AIS, namely *B. alvinipulli* and *B. intermedia*. Since *B. pilosicoli* is the aetiological agent of HIS and PIS, the use of *L. reuteri* LM1 to intervene in *Brachyspira*-related diseases in other host species may be worthy of further investigation. In order to develop this probiotic for commercial use, future work on this strain will need to focus on the production and storage practicalities, since in these preliminary studies the lactobacilli were harvested from fresh broth culture, which would be an impractical application in the commercial poultry industry.

The novel research studies presented in this thesis have focused on gaining a better understanding of the pathobiology of *B. pilosicoli* in order to develop novel intervention strategies against AIS. *B. pilosicoli* are unique in their pathogenicity in poultry, swine and human host and despite this wide host range, comparative genomic analysis has demonstrated the similarity of strains of different origins. Therefore, it is likely that some of the findings presented in this thesis regarding probiotic intervention

against AIS, will be applicable to other diseases caused by this pathogen in different hosts, such as PIS and HIS. *In vitro* and *in vivo* studies have indicated that the use of *L. reuteri* LM1 as a probiotic intervention against AIS will be an effective treatment. Future work will need to focus on the efficacy of this intervention in treating AIS in the field and caused by other strains and species that cause the disease. Moreover, research should focus on developing practical and safe methods for the use of *L. reuteri* LM1 as a commercial probiotic in livestock.

References

Abbas Hilmi, H. T., A. Surakka, J. Apajalahti, and P. E. Saris. (2007). Identification of the most abundant *Lactobacillus* species in the crop of 1- and 5-week-old broiler chickens. *Appl Environ Microbiol*. 73: 7867-7873.

Abdelrahman, W. H. A., M. AbuOun, K. D. C. Stark, B. Wieland, and R. M. La Ragione. (2009). A rapid molecular tool for the simultaneous detection of avian *Brachyspira* species in chicken faeces. *In*: Proceedings of the 5th International Conference on Colonic Spirochaetal Infections in Animals and Humans. Leon, Spain.

AbuOun, M., P. F. Suthers, G. I. Jones, B. R. Carter, M. P. Saunders, C. D. Maranas, M. J. Woodward, and M. F. Anjum. (2009). Genome scale reconstruction of a *Salmonella* metabolic model: comparison of similarity and differences with a commensal *Escherichia coli* strain. *J Biol Chem*. 284: 29480-29488.

Adachi, Y., M. Sueyoshi, E. Miyagawa, H. Minato, and S. Shoya. (1985). Experimental infection of young broiler chicks with *Treponema hyodysenteriae*. *Microbiol Immunol*. 29: 683-688.

Adhikari, P., S. A. Berish, A. J. Nowalk, K. L. Veraldi, S. A. Morse, and T. A. Mietzner. (1996). The *fbpABC* locus of *Neisseria gonorrhoeae* functions in the periplasm-to-cytosol transport of iron. *J Bacteriol*. 178: 2145-2149.

Agostoni, C., I. Axelsson, C. Braegger, O. Goulet, B. Koletzko, K. F. Michaelsen, J. Rigo, R. Shamir, H. Szajewska, D. Turck, L. T. Weaver, and E. C. o. Nutrition. (2004). Probiotic bacteria in dietetic products for infants: a commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. 38: 365-374.

Agunos, A., M. Ibuki, F. Yokomizo, and Y. Mine. (2007). Effect of dietary beta1-4 mannobiose in the prevention of *Salmonella enteritidis* infection in broilers. *Br Poult Sci*. 48: 331-341.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. (1990). Basic local alignment search tool. *J Mol Biol*. 215: 403-410.

Amin, M. M., N. D. Phillips, T. La, and D. J. Hampson. (2009). Vaccination with an autogenous bacterin fails to prevent colonization by *Brachyspira intermedia* in experimentally infected laying chickens. *Vet Microbiol*. 133: 372-376.

Anadon, A., M. R. Martinez-Larranaga, and M. Aranzazu Martinez. (2006). Probiotics for animal nutrition in the European Union: regulation and safety assessment. *Regul Toxicol Pharmacol*. 45: 91-95.

Andersson, S. G., and C. G. Kurland. (1998). Reductive evolution of resident genomes. *Trends Microbiol*. 6: 263-268.

Andersson, H., N.-G. Asp, A. Bruce, S. Roos, T. Wadstrom, and A. E. Wold. (2001). Health effects of probiotics and prebiotics: A literature review on human studies. *Scand J Nutr*. 45: 58-75.

Angus, A. A., A. A. Lee, D. K. Augustin, E. J. Lee, D. J. Evans, and S. M. Fleiszig. (2008). *Pseudomonas aeruginosa* induces membrane blebs in epithelial cells, which are utilized as a niche for intracellular replication and motility. *Infect Immun*. 76: 1992-2001.

Apata, D. F. (2008). Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of *Lactobacillus bulgaricus*. *J Sci Food Agric*. 88: 1253-1258.

Ashraf, R., and N. P. Shah. (2011). Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. *Int Food Res J*. 18: 837-853.

Aslim, B., D. Onal, and Y. Beyatli. (2007). Factors influencing autoaggregation and aggregation of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from handmade yogurt. *J Food Prot*. 70: 223-227.

Atyeo, R. F., S. L. Oxberry, and D. J. Hampson. (1996). Pulsed-field gel electrophoresis for sub-specific differentiation of *Serpulina pilosicoli* (formerly "*Anguillina coli*"). *FEMS Microbiol Lett*. 141: 77-81.

Atyeo, R. F., S. L. Oxberry, B. G. Combs, and D. J. Hampson. (1998). Development and evaluation of polymerase chain reaction tests as an aid to diagnosis of swine dysentery and intestinal spirochaetosis. *Lett Appl Microbiol*. 26: 126-130.

Backhans, A., D. S. Jansson, A. Aspan, and C. Fellstrom. (2011). Typing of *Brachyspira* spp. from rodents, pigs and chickens on Swedish farms. *Vet Microbiol*. 153: 156-162.

Bait-Merabet, L., A. Thille, P. Legrand, C. Brun-Buisson, and V. Cattoir. (2008). *Brachyspira pilosicoli* bloodstream infections: case report and review of the literature. *Ann Clin Microbiol Antimicrob*. 7: 19.

Balevi, T., U. S. Ucan, B. Coskun, V. Kurtoglu, and I. S. Cetingul. (2001). Effect of dietary probiotic on performance and humoral immune response in layer hens. *Br Poult Sci*. 42: 456-461.

Banerjee, P., G. J. Merkel, and A. K. Bhunia. (2009). *Lactobacillus delbrueckii* ssp. *bulgaricus* B-30892 can inhibit cytotoxic effects and adhesion of pathogenic *Clostridium difficile* to Caco-2 cells. *Gut Pathog*. 1: 8.

Bano, L., G. Merialdi, P. Bonilauri, G. Dall'Anese, K. Capello, D. Comin, G. Cattoli, V. Sanguinetti, D. J. Hampson, and F. Agnoletti. (2008). Prevalence, disease associations and risk factors for colonization with intestinal spirochaetes (*Brachyspira* spp.) in flocks of laying hens in north-eastern Italy. *Avian Pathol*. 37: 281-286.

Barbosa, T. M., C. R. Serra, R. M. La Ragione, M. J. Woodward, and A. O. Henriques. (2005). Screening for *Bacillus* isolates in the broiler gastrointestinal tract. *Appl Environ Microbiol*. 71: 968-978.

Barcellos, D. E., M. de Uzeda, N. Ikuta, V. R. Lunge, A. S. Fonseca, Kader, II, and G. E. Duhamel. (2000). Identification of porcine intestinal spirochetes by PCR-restriction fragment length polymorphism analysis of ribosomal DNA encoding 23S rRNA. *Vet Microbiol*. 75: 189-198.

Barrett, S. P. (1997). Human intestinal spirichaetosis, pp. 243-265. *In*: D. J. Hampson and T. B. Stanton (eds.), Intestinal Spirochaetes of Domestic Animals and Humans. CAB International, Oxon.

Bellgard, M. I., P. Wanchanthuek, T. La, K. Ryan, P. Moolhuijzen, Z. Albertyn, B. Shaban, Y. Motro, D. S. Dunn, D. Schibeci, A. Hunter, R. Barrero, N. D. Phillips, and D. J. Hampson. (2009). Genome sequence of the pathogenic intestinal spirochete *Brachyspira hyodysenteriae* reveals adaptations to its lifestyle in the porcine large intestine. *PLoS ONE*. 4: e4641.

Ben Salah, R., I. Trabelsi, R. Ben Mansour, S. Lassoued, H. Chouayekh, and S. Bejar. (2012). A new *Lactobacillus plantarum* strain, TN8, from the gastrointestinal tract of poultry induces high cytokine production. *Anaerobe*. 18: 436-444.

Berg, H. C. (1976). How spirochetes may swim. *J Theor Biol*. 56: 269–273.

Bernardeau, M., M. Gueguen, D. G. Smith, E. Corona-Barrera, and J. P. Vernoux. (2009). *In vitro* antagonistic activities of *Lactobacillus* spp. against *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*. *Vet Microbiol*. 138: 184-190.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol*. 62: 293-300.

Bertani, G. (2004). Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *J Bacteriol*. 186: 595-600.

Betschel, S. D., S. M. Borgia, N. L. Barg, D. E. Low, and J. C. de Azavedo. (1998). Reduced virulence of group A streptococcal Tn*916* mutants that do not produce streptolysin S. *Infect Immun*. 66: 1671-1679.

Bhaskaran, H. P., A. M. Donoghue, K. Arsi, A. Wooming, I. Reyes-Herrera, L. R. Bielke, G. Tellez, J. A. Byrd, P. J. Blore, B. M. Hargis, and D. J. Donoghue. (2011). *In vitro* selection of enteric microflora for potential use as a competitive exclusion culture against *Campylobacter* in poultry. *Int J Poult Sci*. 10: 940-945.

Blum, S., D. Haller, A. Pfeifer, and E. J. Schiffrin. (2002). Probiotics and immune response. *Clin Rev Allergy Immunol*. 22: 287-309.

Bochner, B. R. (2009). Global phenotypic characterization of bacteria. *FEMS Microbiol Rev*. 33: 191-205.

Bochner, B. R., P. Gadzinski, and E. Panomitros. (2001). Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res*. 11: 1246-1255.

Boguski, M. S., T. M. Lowe, and C. M. Tolstoshev. (1993). dbEST--database for "expressed sequence tags". *Nat Genet*. 4: 332-333.

Bohlander, S. K., R. Espinosa, 3rd, M. M. Le Beau, J. D. Rowley, and M. O. Diaz. (1992). A method for the rapid sequence-independent amplification of microdissected chromosomal material. *Genomics*. 13: 1322-1324.

Borglin, S., D. Joyner, J. Jacobsen, A. Mukhopadhyay, and T. C. Hazen. (2009). Overcoming the anaerobic hurdle in phenotypic microarrays: generation and visualization of growth curve data for *Desulfovibrio vulgaris* Hildenborough. *J Microbiol Methods*. 76: 159-168.

Bottazzi, V. (1983). Other fermented dairy products, pp. 315-364. *In*: G. Reed (ed.), Biotechnology, vol. 5, Food and Feed Production with Microorganisms. Verlag Chemie, Weinheim.

Boye, M., T. K. Jensen, K. Moller, T. D. Leser, and S. E. Jorsal. (1998). Specific detection of the genus *Serpulina*, *S. hyodysenteriae* and *S. pilosicoli* in porcine intestines by fluorescent rRNA *in situ* hybridization. *Mol Cell Probes*. 12: 323-330.

Brooke, C. J., D. J. Hampson, and T. V. Riley. (2003a). *In vitro* antimicrobial susceptibility of *Brachyspira pilosicoli* isolates from humans. *Antimicrob Agents Chemother*. 47: 2354-2357.

Brooke, C. J., T. V. Riley, and D. J. Hampson. (2003b). Evaluation of selective media for the isolation of *Brachyspira aalborgi* from human faeces. *J Med Microbiol*. 52: 509-513.

Brooke, C. J., T. V. Riley, and D. J. Hampson. (2006). Comparison of prevalence and risk factors for faecal carriage of the intestinal spirochaetes *Brachyspira aalborgi* and *Brachyspira pilosicoli* in four Australian populations. *Epidemiol Infect*. 134: 627-634.

Brotz, H., and H. G. Sahl. (2000). New insights into the mechanism of action of lantibiotics--diverse biological effects by binding to the same molecular target. *J Antimicrob Chemother*. 46: 1-6.

Brown, J. R., C. J. Douady, M. J. Italia, W. E. Marshall, and M. J. Stanhope. (2001). Universal trees based on large combined protein sequence data sets. *Nat Genet*. 28: 281-285.

Buckles, E., D. Swayne, and K. Eaton. (1994). Cases of necrotising typhlitis associated with caecal spirochaete in common rheas (*Rhea americana*). *Vet Pathol*. 31: 612.

Buckles, E. L., K. A. Eaton, and D. E. Swayne. (1997). Cases of spirochete-associated necrotizing typhlitis in captive common rheas (*Rhea americana*). *Avian Dis*. 41: 144-148.

Burch, D. G. S. (2007). Avian intestinal spirochaetosis in layers - spirochaete survival, www.thepoultrysite.com/articles/828/avian-intestinal-spirochaetosis-in-layers-spirochaete-survival/ 18/08/09.

Burch, D. G. S. (2008). Tiamulin activity against *Brachyspira hyodysenteriae*. *Vet Rec*. 163: 698.

Burch, D. G. S. (2010). Egg production drops from *Brachyspira*. *World Poult*. 26: 24-25.

Burch, D. G. S, C. Harding, R. Alvarez, and M. Valks. (2006). Treatment of a field case of avian intestinal spirochaetosis caused by *Brachyspira pilosicoli* with tiamulin. *Avian Pathol*. 35: 211-216.

Burch, D. G. S., B. W. Strugnell, A. Steventon, E. N. Watson, and C. Harding. (2009). Survey of 222 flocks in Great Britain for the presence of *Brachyspira* species and their effect on production. *In*: Proceedings of the 5th International Conference on Colonic Spirochaetal Infections in Animals and Humans. Leon, Spain.

Burkholder, K. M., K. L. Thompson, M. E. Einstein, T. J. Applegate, and J. A. Patterson. (2008). Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella* Enteritidis colonization in broilers. *Poult Sci*. 87: 1734-1741.

Burrows, M. R., and R. M. Lemcke. (1981). Identification of *Treponema hyodysenteriae* by a rapid slide agglutination test. *Vet Rec*. 108: 187-189.

Canale-Parola, E. (1977). Physiology and evolution of spirochaetes. *Bacteriol Rev*. 41: 181-204.

Canale-Parola, E. (1984). The spirochaetes. *In*: N. R. Krieg and J. G. Holt (eds.), Bergey's Manual of Systematic Bacteriology, vol. 1. Williams & Wilkins, Baltimore.

Capcarova, M., J. Weiss, C. Hrncar, A. Kolesarova, and G. Pal. (2010). Effect of *Lactobacillus fermentum* and *Enterococcus faecium* strains on internal milieu, antioxidant status and body weight of broiler chickens. *J Anim Physiol Anim Nutr*. 94: e215-224.

Carita, S. (1992). Research note: automated droplet application of competitive exclusion preparation. *Poult Sci*. 71: 2125-2128.

Carroll, P., R. M. La Ragione, A. R. Sayers, and M. J. Woodward. (2004). The O-antigen of *Salmonella enterica* serotype Enteritidis PT4: a significant factor in gastrointestinal colonisation of young but not newly hatched chicks. *Vet Microbiol*. 102: 73-85.

Carter, T. H., and C. G. Miller. (1984). Aspartate-specific peptidases in *Salmonella typhimurium*: mutants deficient in peptidase E. *J Bacteriol*. 159: 453-459.

Cartman, S. T., R. M. La Ragione, and M. J. Woodward. (2008). *Bacillus subtilis* spores germinate in the chicken gastrointestinal tract. *Appl Environ Microbiol*. 74: 5254-5258.

Carver, T. J., K. M. Rutherford, M. Berriman, M. A. Rajandream, B. G. Barrell, and J. Parkhill. (2005). ACT: the Artemis comparison tool. *Bioinformatics*. 21: 3422-3423.

Castanon, J. I. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci*. 86: 2466-2471.

Cauwerts, K., F. Pasmans, L. A. Devriese, F. Haesebrouck, and A. Decostere. (2006). Cloacal *Lactobacillus* isolates from broilers often display resistance toward tetracycline antibiotics. *Microb Drug Resist*. 12: 284-288.

Cavazzoni, V., A. Adami, and C. Castrovilli. (1998). Performance of broiler chickens supplemented with *Bacillus coagulans* as probiotic. *Br Poult Sci*. 39: 526-529.

Chander, Y., A. Primus, S. Oliveira, and C. J. Gebhart. (2012). Phenotypic and molecular characterization of a novel strongly hemolytic *Brachyspira* species, provisionally designated "*Brachyspira hampsonii*". *J Vet Diagn Invest*. 24: 903-910.

Charon, N. W., and S. F. Goldstein. (2002). Genetics of motility and chemotaxis of a fascinating group of bacteria: the spirochetes. *Annu Rev Genet*. 36: 47-73.

Charon, N. W., S. F. Goldstein, M. Marko, C. Hsieh, L. L. Gebhardt, M. A. Motaleb, C. W. Wolgemuth, R. J. Limberger, and N. Rowe. (2009). The flat-ribbon configuration of the periplasmic flagella of *Borrelia burgdorferi* and its relationship to motility and morphology. *J Bacteriol*. 191: 600-607.

Chateau, N., A. M. Deschamps, and A. Hadj Sassi. (1994). Heterogeneity of bile salts resistance in the *Lactobacillus* isolates of a probiotic consortium. *Lett Appl Microbiol*. 18: 42-44.

Cheplin, H., and L. Rettger. (1922). The therapeutic application of *Lactobacillus acidophilus*. *Abstr Bacteriol*. 6: 24.

Chiang, S. H., and W. M. Hsiem. (1995). Effect of direct feed microorganisms on broiler growth performance and litter ammonia level. *Asian-Aust J Anim Sci*. 8: 159-162.

Cho, N. H., J. M. Kim, E. K. Kwon, S. Y. Kim, S. H. Han, H. Chu, J. H. Lee, M. S. Choi, and I. S. Kim. (2005). Molecular characterization of a group of proteins containing ankyrin repeats in *Orientia tsutsugamushi*. *Ann N Y Acad Sci*. 1063: 100-101.

Christensen, H. R., H. Frokiaer, and J. J. Pestka. (2002). Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J Immunol*. 168: 171-178.

Clothier, K. A., J. M. Kinyon, T. S. Frana, N. Naberhaus, L. Bower, E. L. Strait, and K. Schwartz. (2011). Species characterization and minimum inhibitory concentration patterns of *Brachyspira* species isolates from swine with clinical disease. *J Vet Diagn Invest*. 23: 1140-1145.

Coconnier-Polter, M. H., V. Lievin-Le Moal, and A. L. Servin. (2005). A *Lactobacillus acidophilus* strain of human gastrointestinal microbiota origin elicits killing of enterovirulent *Salmonella enterica* serovar Typhimurium by triggering lethal bacterial membrane damage. *Appl Environ Microbiol*. 71: 6115-6120.

Coeuret, V., M. Gueguen, and J. P. Vernoux. (2004). Numbers and strains of lactobacilli in some probiotic products. *Int J Food Microbiol*. 97: 147-156.

Cohen, E., I. Ophir, and Y. B. Shaul. (1999). Induced differentiation in HT29, a human colon adenocarcinoma cell line. *J Cell Sci*. 112: 2657-2666.

Cole, C. B., P. H. Anderson, S. M. Philips, R. Fuller, and D. Hewitt. (1984). The effect of yogurt on the growth, lactose-utilizing gut organisms and β-glucuronidase activity of caecal contents of a lactose-fed, lactose-deficient animal. *Food Microbiol*. 1: 217-222.

Collington, G. K., D. S. Parker, and D. G. Armstrong. (1990). The influence of inclusion of either an antibiotic or a probiotic in the diet on the development of digestive enzyme activity in the pig. *B J Nutr*. 64: 59-70.

Collins, M. D., and G. R. Gibson. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr*. 69: 1052S-1057S.

Collins, J. W., R. M. La Ragione, M. J. Woodward, and L. E. J. Searle. (2009). Application of prebiotics and probiotics in livestock, pp. 1123-1192. *In*: D. Charalampopoulos and R. A. Rastall (eds.), Prebiotics and Probiotics Science and Technology. Springer, New York.

Collins, J. W., N. G. Coldham, F. J. Salguero, W. A. Cooley, W. R. Newell, R. A. Rastall, G. R. Gibson, M. J. Woodward, and R. M. La Ragione. (2010). Response of porcine intestinal *in vitro* organ culture tissues following exposure to *Lactobacillus plantarum* JC1 and *Salmonella enterica* serovar Typhimurium SL1344. *Appl Environ Microbiol*. 76: 6645-6657.

Coloe, P. J., T. J. Bagust, and L. Ireland. (1984). Development of the normal gastrointestinal microflora of specific pathogen-free chickens. *J Hyg (Lond)*. 92: 79-87.

Conway, P. L., S. L. Gorbach, and B. R. Goldin. (1987). Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci*. 70: 1-12.

Corona-Barrera, E., D. G. Smith, B. Murray, and J. R. Thomson. (2004). Efficacy of seven disinfectant sanitisers on field isolates of *Brachyspira pilosicoli*. *Vet Rec*. 154: 473-474.

Corr, S. C., Y. Li, C. U. Riedel, P. W. O'Toole, C. Hill, and C. G. Gahan. (2007). Bacteriocin production as a mechanism for the anti-infective activity of *Lactobacillus salivarius* UCC118. *Proc Natl Acad Sci U S A*. 104: 7617-7621.

Corrigan, A., K. Horgan, N. Clipson, and R. A. Murphy. (2011). Effect of dietary supplementation with a *Saccharomyces cerevisiae* mannan oligosaccharide on the bacterial community structure of broiler cecal contents. *Appl Environ Microbiol*. 77: 6653-6662.

Cunningham, C. C. (1995). Actin polymerization and intracellular solvent flow in cell surface blebbing. *J Cell Biol*. 129: 1589-1599.

Dacre, J. C., and M. E. Sharpe. (1956). Catalase production by lactobacilli. *Nature*. 178: 700.

Dalloul, R. A., H. S. Lillehoj, T. A. Shellem, and J. A. Doerr. (2003). Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. *Poult Sci*. 82: 62-66.

Danielsen, M. (2002). Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. *Plasmid*. 48: 98-103.

Daubin, V., M. Gouy, and G. Perriere. (2002). A phylogenomic approach to bacterial phylogeny: evidence of a core of genes sharing a common history. *Genome Res*. 12: 1080-1090.

Davelaar, F. G., H. F. Smit, K. Hovind-Hougen, R. M. Dwars, and P. C. Vandervalk. (1986). Infectious typhlitis in chickens caused by spirochetes. *Avian Pathol*. 15: 247-258.

Davidson, A. L., E. Dassa, C. Orelle, and J. Chen. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev*. 72: 317-364.

Davis, A. J., S. C. Smith, and R. J. Moore. (2005). The *Brachyspira hyodysenteriae ftnA* gene: DNA vaccination and real-time PCR quantification of bacteria in a mouse model of disease. *Curr Microbiol*. 50: 285-291.

de Angelis, M., S. Siragusa, M. Berloco, L. Caputo, L. Settanni, G. Alfonsi, M. Amerio, A. Grandi, A. Ragni, and M. Gobbetti. (2006). Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. *Res Microbiol*. 157: 792-801.

de Ciccio, A., R. McLaughlin, and E. C. Chan. (1999). Factors affecting the formation of spherical bodies in the spirochete *Treponema denticola*. *Oral Microbiol Immunol*. 14: 384-386.

de Keersmaecker, S. C., T. L. Verhoeven, J. Desair, K. Marchal, J. Vanderleyden, and I. Nagy. (2006). Strong antimicrobial activity of *Lactobacillus rhamnosus* GG against *Salmonella typhimurium* is due to accumulation of lactic acid. *FEMS Microbiol Lett*. 259: 89-96.

de la Puente Redondo, V. A., C. B. Gutierrez Martin, N. G. Del Blanco, and E. F. Rodriguez Ferri. (1998). The effect of N-duopropenide (a new disinfectant with quaternary ammonium iodides) and formaldehyde on survival of organisms of sanitary interest in pig slurry. *Zentralbl Veterinarmed B*. 45: 481-493.

de Man, J. C., M. Rogosa, and M. E. Sharpe. (1960). A medium for the cultivation of lactobacilli. *J Appl Microbiol*. 23: 130-135.

de Rijk, P., J. Wuyts, Y. van de Peer, T. Winkelmans, and R. de Wachter. (2000). The European large subunit ribosomal RNA database. *Nucleic Acids Res*. 28: 177-178.

de Smet, K. A., D. E. Worth, and S. P. Barrett. (1998). Variation amongst human isolates of *Brachyspira* (*Serpulina*) *pilosicoli* based on biochemical characterization and 16S rRNA gene sequencing. *Int J Syst Bacteriol*. 48: 1257-1263.

Decroos, K., T. Vercauteren, G. Werquin, and W. Verstraete. (2004). Repression of *Clostridium* population in young broiler chickens after administration of a probiotic mixture. *Commun Agric Appl Biol Sci*. 69: 5-13.

Delzenne, N., C. Cherbut, and A. Neyrinck. (2003). Prebiotics: actual and potential effects in inflammatory and malignant colonic diseases. *Curr Opin Clin Nutr Metab Care*. 6: 581-586.

Depeint, F., G. Tzortzis, J. Vulevic, K. l'Anson, and G. R. Gibson. (2008). Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: a randomized,

double-blind, crossover, placebo-controlled intervention study. *Am J Clin Nutr*. 87: 785-791.

Dibb-Fuller, M. P., E. Allen-Vercoe, C. J. Thorns, and M. J. Woodward. (1999). Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. *Microbiology*. 145: 1023-1031.

Dobell, C. (1932). Antony van Leeuwenhoek and his "little animals". Harcourt Brace & Co, New York.

Donelli, G., A. Fabbri, and C. Fiorentini. (1996). *Bacteroides fragilis* enterotoxin induces cytoskeletal changes and surface blebbing in HT-29 cells. *Infect Immun*. 64: 113-119.

Droge, S., J. Frohlich, R. Radek, and H. Konig. (2006). *Spirochaeta coccoides* sp. nov., a novel coccoid spirochete from the hindgut of the termite *Neotermes castaneus*. *Appl Environ Microbiol*. 72: 392-397.

Duary, R. K., Y. S. Rajput, V. K. Batish, and S. Grover. (2011). Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J Med Res*. 134: 664-671.

Dugourd, D., C. Martin, C. R. Rioux, M. Jacques, and J. Harel. (1999). Characterization of a periplasmic ATP-binding cassette iron import system of *Brachyspira* (*Serpulina*) *hyodysenteriae*. *J Bacteriol*. 181: 6948-6957.

Duhamel, G. E. (1996). Porcine colonic spirochaetosis caused by *Serpulina pilosicoli*. *PIGS-Misset Int*. 12: 10-12.

Duhamel, G. E. (2001). Comparative pathology and pathogenesis of naturally acquired and experimentally induced colonic spirochetosis. *Anim Health Res Rev*. 2: 3-17.

Duhamel, G. E., J. M. Kinyon, M. R. Mathiesen, D. P. Murphy, and D. Walter. (1998a). *In vitro* activity of four antimicrobial agents against North American isolates of porcine *Serpulina pilosicoli*. *J Vet Diagn Invest*. 10: 350-356.

Duhamel, G. E., R. O. Elder, N. Muniappa, M. R. Mathiesen, V. J. Wong, and R. P. Tarara. (1997). Colonic spirochetal infections in nonhuman primates that were associated with *Brachyspira aalborgi*, *Serpulina pilosicoli*, and unclassified flagellated bacteria. *Clin Infect Dis*. 25: S186-188.

Duhamel, G. E., D. J. Trott, N. Muniappa, M. R. Mathiesen, K. Tarasiuk, J. I. Lee, and D. J. Hampson. (1998b). Canine intestinal spirochetes consist of *Serpulina pilosicoli* and a newly identified group provisionally designated "*Serpulina canis*" sp. nov. *J Clin Microbiol*. 36: 2264-2270.

Duke, G. E. (1977). Avian digestion. *In*: G. E. Duke (ed.), Duke's Physiology of Domestic Animals, 9th edn. Cornell University Press, New York.

Dunham, H. J., C. Williams, F. W. Edens, I. A. Casas, and W. J. Dobrogosz. (1993). *Lactobacillus reuteri* immune modulation of stress-associated diseases in newly hatched chickens and turkeys. *Poultry Sci*. 72 103.

Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F.

Shanahan, and J. K. Collins. (2001). *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am J Clin Nutr*. 73: 386S-392S.

Dwars, R. M., H. F. Smit, and F. G. Davelaar. (1990). Observations on avian intestinal spirochaetosis. *Vet Q*. 12: 51-55.

Dwars, R. M., F. G. Davelaar, and H. F. Smit. (1992a). Infection of broiler chicks (*Gallus domesticus*) with human intestinal spirochaetes. *Avian Pathol*. 21: 559-568.

Dwars, R. M., H. F. Smit, and F. G. Davelaar. (1992b). Influence of infection with avian intestinal spirochaetes on the faeces of laying hens. *Avian Pathol*. 21: 513-515.

Dwars, R. M., F. G. Davelaar, and H. F. Smit. (1993). Infection of broiler parent hens with avian intestinal spirochaetes: effects on egg production and chick quality. *Avian Pathol*. 22: 693-701.

Dwars, R. M., H. F. Smit, F. G. Davelaar, and W. V. Veer. (1989). Incidence of spirochaetal infections in cases of intestinal disorder in chickens. *Avian Pathol*. 18: 591-595.

Edens, F. W., C. R. Parkhurst, I. A. Casas, and W. J. Dobrogosz. (1997). Principles of *ex ovo* competitive exclusion and *in ovo* administration of *Lactobacillus reuteri*. *Poult Sci*. 76: 179-196.

EFSA. (2005). Scientific Colloquium Summary Report. Qualified Presumption of Safety of Microorganisms (QPS) in Food and Feed, efsa.europa.eu/en/supporting/doc/colloquiaqps.pdf 01/05/12.

EFSA. (2008). Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance, efsa.europa.eu/en/efsajournal/doc/732.pdf 01/05/12.

Egervarn, M., H. Lindmark, J. Olsson, and S. Roos. (2010). Transferability of a tetracycline resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal tract of humans. *Antonie van Leeuwenhoek*. 97: 189-200.

Ehrenberg, C. G. (1835). Dritter Beitrag zür erkenntniss grosser organisation in der richtung des kleinsten raumes, pp. 143-336, Abhandlungen der preussischen akademie der wissenschaftern (Berlin) aus dem Jahre 1833-1835. Abh Königl Akad Wiss Berlin, Berlin.

Ehrmann, M. A., P. Kurzak, J. Bauer, and R. F. Vogel. (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *J Appl Microbiol*. 92: 966-975.

Eschenbach, D. A., P. R. Davick, B. L. Williams, S. J. Klebanoff, K. Young-Smith, C. M. Critchlow, and K. K. Holmes. (1989). Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. *J Clin Microbiol*. 27: 251-256.

Euzeby, Y. J. P. (2008). List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet, www.bacterio.net 23/01/12.

Eveillard, M., V. Fourel, M. C. Barc, S. Kerneis, M. H. Coconnier, T. Karjalainen, P. Bourlioux, and A. L. Servin. (1993). Identification and characterization of adhesive

factors of *Clostridium difficile* involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT29 cells in culture. *Mol Microbiol*. 7: 371-381.

Fackler, O. T., and R. Grosse. (2008). Cell motility through plasma membrane blebbing. *J Cell Biol*. 181: 879-884.

Fantham, H. B. (1910). Observations on the parasitic protozoa of the red grouse (*Lagopus scoticus*), with a note on the grouse fly. *Proc Zool Soc Lond*. 692-708.

FAO/WHO. (2001). Health and nutritional properties of probiotics of food including powder milk with live lactic acid bacteria. Report of a joint FAO/WHO expert consultation, www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf 01/05/12.

Fayol-Messaoudi, D., C. N. Berger, M. H. Coconnier-Polter, V. Lievin-Le Moal, and A. L. Servin. (2005). pH-, lactic acid-, and non-lactic acid-dependent activities of probiotic lactobacilli against *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol*. 71: 6008-6013.

Feberwee, A., D. J. Hampson, N. D. Phillips, T. La, H. M. van der Heijden, G. J. Wellenberg, R. M. Dwars, and W. J. Landman. (2008). Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. *J Clin Microbiol*. 46: 593-600.

Feist, A. M., C. S. Henry, J. L. Reed, M. Krummenacker, A. R. Joyce, P. D. Karp, L. J. Broadbelt, V. Hatzimanikatis, and B. O. Palsson. (2007). A genome-scale metabolic reconstruction for *Escherichia coli* K-12 MG1655 that accounts for 1260 ORFs and thermodynamic information. *Mol Syst Biol*. 3: 121.

Felis, G. E., F. Dellaglio, and S. Torriani. (2009). The taxonomy of probiotic microorganism, pp. 591-636. *In*: D. Charalampopoulos and R. A. Rastall (eds.), Prebiotics and Probiotics Science and Technology. Springer, New York.

Fellstrom, C., and A. Gunnarsson. (1995). Phenotypical characterisation of intestinal spirochaetes isolated from pigs. *Res Vet Sci*. 59: 1-4.

Fellstrom, C., U. Zimmerman, A. Aspan, and A. Gunnarsson. (2001). The use of culture, pooled samples and PCR for identification of herds infected with *Brachyspira hyodysenteriae*. *Anim Health Res Rev*. 2: 37-43.

Fellstrom, C., B. Pettersson, M. Uhlen, A. Gunnarsson, and K. E. Johansson. (1995). Phylogeny of *Serpulina* based on sequence analyses of the 16S rRNA gene and comparison with a scheme involving biochemical classification. *Res Vet Sci*. 59: 5-9.

Fellstrom, C., T. Rasback, K. E. Johansson, T. Olofsson, and A. Aspan. (2008). Identification and genetic fingerprinting of *Brachyspira* species. *J Microbiol Methods*. 72: 133-140.

Fellstrom, C., B. Pettersson, J. Thomson, A. Gunnarsson, M. Persson, and K. E. Johansson. (1997). Identification of *Serpulina* species associated with porcine colitis by biochemical analysis and PCR. *J Clin Microbiol*. 35: 462-467.

Fellstrom, C., M. Karlsson, B. Pettersson, U. Zimmerman, A. Gunnarsson, and A. Aspan. (1999). Emended descriptions of indole negative and indole positive isolates of *Brachyspira* (*Serpulina*) *hyodysenteriae*. *Vet Microbiol*. 70: 225-238.

Fenno, J. C., M. Tamura, P. M. Hannam, G. W. Wong, R. A. Chan, and B. C. McBride. (2000). Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun*. 68: 1884-1892.

Ferhat, M., D. Atlan, A. Vianney, J. C. Lazzaroni, P. Doublet, and C. Gilbert. (2009). The TolC protein of *Legionella pneumophila* plays a major role in multi-drug resistance and the early steps of host invasion. *PLoS ONE*. 4: e7732.

Fineran, P. C., T. R. Blower, I. J. Foulds, D. P. Humphreys, K. S. Lilley, and G. P. Salmond. (2009). The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proc Natl Acad Sci U S A*. 106: 894-899.

Fisher, L. N., G. E. Duhamel, R. B. Westerman, and M. R. Mathiesen. (1997). Immunoblot reactivity of polyclonal and monoclonal antibodies with periplasmic flagellar proteins FlaA1 and FlaB of porcine *Serpulina* species. *Clin Diagn Lab Immunol*. 4: 400-404.

Floch, M. H., and D. C. Montrose. (2005). Use of probiotics in humans: an analysis of the literature. *Gastroenterol Clin North Am*. 34: 547-570.

Fossi, M., T. Saranpaa, and E. Rautiainen. (1999). *In vitro* sensitivity of the swine *Brachyspira* species to tiamulin in Finland 1995-1997. *Acta Vet Scand*. 40: 355-358.

Fossi, M., T. Pohjanvirta, A. Sukura, S. Heinikainen, R. Lindecrona, and S. Pelkonen. (2004). Molecular and ultrastructural characterization of porcine hippurate-negative *Brachyspira pilosicoli*. *J Clin Microbiol*. 42: 3153-3158.

Francis, M. S., and C. J. Thomas. (1997). The *Listeria monocytogenes* gene *ctpA* encodes a putative P-type ATPase involved in copper transport. *Mol Gen Genet*. 253: 484-491.

Franklin, A., M. Pringle, and D. J. Hampson. (2006). Antimicrobial resistance in *Clostridium* and *Brachyspira* spp. and other anaerobes, pp. 127-144. *In*: F. M. Aarestrup (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington, DC.

Fukunaga, M., Y. Yanagihara, and M. Sohnaka. (1992a). The 23S/5S ribosomal RNA genes (*rrl*/*rrf*) are separate from the 16S ribosomal RNA gene (*rrs*) in *Borrelia burgdorferi*, the aetiological agent of Lyme disease. *J Gen Microbiol*. 138: 871-877.

Fukunaga, M., I. Mifuchi, Y. Yanagihara, and N. Okuzako. (1992b). Comparison of flanking regions of the 5S ribosomal ribonucleic acid genes in *Leptospira biflexa* and *Leptospira interrogans*. *Chem Pharm Bull (Tokyo)*. 40: 544-546.

Fukunaga, M., N. Okuzako, I. Mifuchi, Y. Arimitsu, and M. Seki. (1992c). Organization of the ribosomal RNA genes in *Treponema phagedenis* and *Treponema pallidum*. *Microbiol Immunol*. 36: 161-167.

Fuller, R. (1977). The importance of lactobacilli in maintaining normal microbial balance in the crop. *Br Poult Sci*. 18: 85-94.

Fuller, R. (1989). Probiotics in man and animals. *J Appl Bacteriol*. 66: 365-378.

Fuller, R. (1992). Probiotics. The Scientific Basis. Chapman & Hall, London.

Fuller, R. (2004). What is a Probiotic? *Biologist*. 51: 232.

Gabastou, J. M., S. Kerneis, M. F. Bernet-Camard, A. Barbat, M. H. Coconnier, J. B. Kaper, and A. L. Servin. (1995). Two stages of enteropathogenic *Escherichia coli* intestinal pathogenicity are up and down-regulated by the epithelial cell differentiation. *Differentiation*. 59: 127-134.

Gad, A., R. Willen, K. Furugard, B. Fors, and M. Hradsky. (1977). Intestinal spirochaetosis as a cause of longstanding diarrhoea. *Ups J Med Sci*. 82: 49-54.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. C. Hinton, and F. van Immerseel. (2006). Butyrate specifically down-regulates *Salmonella* pathogenicity island 1 gene expression. *Appl Environ Microbiol*. 72: 946-949.

Ganzle, M. G. (2004). Reutericyclin: biological activity, mode of action, and potential applications. *Appl Microbiol Biotechnol*. 64: 326-332.

Gao, F., and C. T. Zhang. (2008). Ori-Finder: a web-based system for finding *oriC*s in unannotated bacterial genomes. *BMC Bioinformatics*. 9: 79.

Gerritsen, J., H. Smidt, G. T. Rijkers, and W. M. de Vos. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. *Genes Nutr*. 6: 209-240.

Gevers, D., G. Huys, and J. Swings. (2003). *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. *FEMS Microbiol Lett*. 225: 125-130.

Ghareeb, K., W. A. Awad, M. Mohnl, R. Porta, M. Biarnes, J. Bohm, and G. Schatzmayr. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of *Campylobacter jejuni* in broiler chickens. *Poult Sci*. 91: 1825-1832.

Gibson, G. R., and M. B. Roberfroid. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*. 125: 1401-1412.

Girard, F., I. Batisson, G. M. Frankel, J. Harel, and J. M. Fairbrother. (2005). Interaction of enteropathogenic and Shiga toxin-producing *Escherichia coli* and porcine intestinal mucosa: role of intimin and Tir in adherence. *Infect Immun*. 73: 6005-6016.

Glavits, R., E. Ivanics, A. Thuma, E. Kaszanyitzky, P. Samu, K. Ursu, L. Dencso, and A. Dan. (2011). Typhlocolitis associated with spirochaetes in duck flocks. *Avian Pathol*. 40: 23-31.

Goldin, B. R., and S. L. Gorbach. (1984). The effect of milk and *Lactobacillus* feeding on human intestinal bacterial enzyme activity. *Am J Clin Nutr*. 39: 756-761.

Goren, E., W. A. de Jong, P. Doornenbal, J. P. Koopman, and H. M. Kennis. (1984). Protection of chicks against *Salmonella* infection induced by spray application of intestinal microflora in the hatchery. *Vet Q*. 6: 73-79.

Grashorn, M. A., and S. Simonovic. (2009). Wholesome drinking water to prevent watery eggs. *World Poult*. 25: 18-19.

Greer, J. M., and M. J. Wannemuehler. (1989). Comparison of the biological responses induced by lipopolysaccharide and endotoxin of *Treponema hyodysenteriae* and *Treponema innocens*. *Infect Immun*. 57: 717-723.

Griffiths, I. B., B. W. Hunt, S. A. Lister, and M. H. Lamont. (1987). Retarded growth rate and delayed onset of egg production associated with spirochaete infection in pullets. *Vet Rec*. 121: 35-37.

Haeri, A., Z. Khodaii, S. M. H. Ghaderian, A. S. T. Panah, and R. A. Najar. (2012). Comparison of adherence patterns of a selection of probiotic bacteria to Caco-2, HEp-2, and T84 cell lines. *Ann Microbiol*. 62: 339-344.

Hafstrom, T., D. S. Jansson, and B. Segerman. (2011). Complete genome sequence of *Brachyspira intermedia* reveals unique genomic features in *Brachyspira* species and phage-mediated horizontal gene transfer. *BMC Genomics*. 12: 395.

Haghighi, H. R., J. Gong, C. L. Gyles, M. A. Hayes, B. Sanei, P. Parvizi, H. Gisavi, J. R. Chambers, and S. Sharif. (2005). Modulation of antibody-mediated immune response by probiotics in chickens. *Clin Diagn Lab Immunol*. 12: 1387-1392.

Halbedel, S., C. Hames, and J. Stulke. (2004). *In vivo* activity of enzymatic and regulatory components of the phosphoenolpyruvate:sugar phosphotransferase system in *Mycoplasma pneumoniae*. *J Bacteriol*. 186: 7936-7943.

Halliwell, B. (1978). Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: is it a mechanism for hydroxyl radical production in biochemical systems? *FEBS letters*. 92: 321-326.

Halter, M. R., and L. A. Joens. (1988). Lipooligosaccharides from *Treponema hyodysenteriae* and *Treponema innocens*. *Infect Immun*. 56: 3152-3156.

Hammes, W. P., and C. Hertel. (2009). *Lactobacillus*, pp. 465-511. *In*: P. de Vos, G. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. H. Schleifer and W. B. Whitman (eds.), Bergey's Manual of Systematic Bacteriology, vol. 3. Springer, New York.

Hampson, D. J. (1991). Slide-agglutination for rapid serological typing of *Treponema hyodysenteriae*. *Epidemiol Infect*. 106: 541-547.

Hampson, D. J., and A. J. McLaren. (1999). Experimental infection of laying hens with *Serpulina intermedia* causes reduced egg production and increased faecal water content. *Avian Pathol*. 28: 113-117.

Hampson, D. J., and G. E. Duhamel. (2006). Porcine colonic spirochetosis/intestinal spirochetosis. *In*: B. E. Straw, J. J. Zimmerman, S. d'Allaire and D. J. Taylor (eds.), Diseases of Swine, 9th edn. Blackwell Publishing, Oxford.

Hampson, D. J., and T. La. (2006). Reclassification of *Serpulina intermedia* and *Serpulina murdochii* in the genus *Brachyspira* as *Brachyspira intermedia* comb. nov. and *Brachyspira murdochii* comb. nov. *Int J Syst Evol Microbiol*. 56: 1009-1012.

Hampson, D. J., and D. E. Swayne. (2008). Avian intestinal spirochaetosis. *In*: Y. M. Saif (ed.), Diseases of Poultry, 12th edn. Blackwell Publishing, Oxford.

Hampson, D. J., I. D. Robertson, and J. R. Mhoma. (1993). Experiences with a vaccine being developed for the control of swine dysentery. *Aust Vet J*. 70: 18-20.

Hampson, D. J., S. L. Oxberry, and C. P. Stephens. (2002a). Influence of in-feed zinc bacitracin and tiamulin treatment on experimental avian intestinal spirochaetosis caused by *Brachyspira intermedia*. *Avian Pathol*. 31: 285-291.

Hampson, D. J., N. D. Phillips, and J. R. Pluske. (2002b). Dietary enzyme and zinc bacitracin reduce colonisation of layer hens by the intestinal spirochaete *Brachyspira intermedia*. *Vet Microbiol*. 86: 351-360.

Hampson, D. J., C. Fellstrom, and J. R. Thomson. (2006a). Swine dysentery, pp. 785-805. *In*: B. E. Straw, J. J. Zimmerman, S. d'Allaire and D. J. Taylor (eds.), Diseases of Swine, 9th edn. Blackwell Publishing, Oxford.

Hampson, D. J., S. L. Oxberry, and T. La. (2006b). Potential for zoonotic transmission of *Brachyspira pilosicoli*. *Emerg Infect Dis*. 12: 869-870.

Hampson, D. J., C. P. Stephens, and S. L. Oxberry. (2006c). Antimicrobial susceptibility testing of *Brachyspira intermedia* and *Brachyspira pilosicoli* isolates from Australian chickens. *Avian Pathol*. 35: 12-16.

Hampson, D. J., G. D. Lester, N. D. Phillips, and T. La. (2006d). Isolation of *Brachyspira pilosicoli* from weanling horses with chronic diarrhoea. *Vet Rec*. 158: 661-662.

Hanley, R. S., L. W. Woods, D. J. Stillian, and G. A. Dumonceaux. (1994). *Serpulina*-like spirochetes and flagellated protozoa associated with necrotizing typhlitis in the rheas (*Rhea amerciana*). *Proc Assoc Avian Vet*. 157-162.

Hansen, C. F., A. Hernandez, J. Mansfield, A. Hidalgo, T. La, N. D. Phillips, D. J. Hampson, and J. R. Pluske. (2011). A high dietary concentration of inulin is necessary to reduce the incidence of swine dysentery in pigs experimentally challenged with *Brachyspira hyodysenteriae*. *Br J Nutr*. 106: 1506-1513.

Hansen, C. F., N. D. Phillips, T. La, A. Hernandez, J. Mansfield, J. C. Kim, B. P. Mullan, D. J. Hampson, and J. R. Pluske. (2010). Diets containing inulin but not lupins help to prevent swine dysentery in experimentally challenged pigs. *J Anim Sci*. 88: 3327-3336.

Harish, K., and T. Varghese. (2006). Probiotics in humans – evidence based review. *Calicut Med J*. 4: e3.

Harland, W. A., and F. D. Lee. (1967). Intestinal spirochaetosis. *Br Med J*. 3: 718-719.

Harris, M. B. K. (1930). A study of spirochaetes in chickens with special reference to those of the intestinal tract. *Am J Hyg*. 12: 537-569.

Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. (1972a). Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. *Vet Med Small Anim Clin*. 67: 61-64.

Harris, D. L., J. M. Kinyon, M. T. Mullin, and R. D. Glock. (1972b). Isolation and propagation of spirochetes from the colon of swine dysentery affected pigs. *Can J Comp Med*. 36: 74-76.

Harris, D. L., T. J. Alexander, S. C. Whipp, I. M. Robinson, R. D. Glock, and P. J. Matthews. (1978). Swine dysentery: studies of gnotobiotic pigs inoculated with *Treponema hyodysenteriae*, *Bacteroides vulgatus* and *Fusobacterium necrophorum*. *J Am Vet Med Assoc*. 172: 468-471.

Hartland, E. L., A. S. Mikosza, R. M. Robins-Browne, and D. J. Hampson. (1998). Examination of *Serpulina pilosicoli* for attachment and invasion determinants of Enterobacteria. *FEMS Microbiol Lett*. 165: 59-63.

Herzog, A. M., A. Maderner, K. Fragner, and H. Weissenbock. (2005). The use of *in situ* hybridization for the detection of *Brachyspira* spp. in pigs. *Dtsch Tierarztl Wochenschr*. 112: 123-129.

Hidalgo, A., A. Carvajal, M. Pringle, P. Rubio, and C. Fellstrom. (2009). Characterization and epidemiological relationships of Spanish *Brachyspira hyodysenteriae* field isolates. *Epidemiol Infect*. 1-10.

Hidalgo, A., A. Carvajal, B. Vester, M. Pringle, G. Naharro, and P. Rubio. (2011). Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in Spain. *Antimicrob Agents Chemother*. 55: 3330-3337.

Higgins, S. E., G. F. Erf, J. P. Higgins, S. N. Henderson, A. D. Wolfenden, G. Gaona-Ramirez, and B. M. Hargis. (2007). Effect of probiotic treatment in broiler chicks on intestinal macrophage numbers and phagocytosis of *Salmonella enteritidis* by abdominal exudate cells. *Poult Sci*. 86: 2315-2321.

Higgins, S. E., J. P. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez, G. Tellez, and B. Hargis. (2008). Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella enteritidis* in neonatal broiler chicks. *Poult Sci*. 87: 27-31.

Holt, S. C. (1978). Anatomy and chemistry of spirochetes. *Microbiological reviews*. 42: 114-160.

Hommez, J., F. Castryck, F. Haesebrouck, and L. A. Devriese. (1998). Identification of porcine *Serpulina* strains in routine diagnostic bacteriology. *Vet Microbiol*. 62: 163-169.

Honer zu Bentrup, K., R. Ramamurthy, C. M. Ott, K. Emami, M. Nelman-Gonzalez, J. W. Wilson, E. G. Richter, T. J. Goodwin, J. S. Alexander, D. L. Pierson, N. Pellis, K. L. Buchanan, and C. A. Nickerson. (2006). Three-dimensional organotypic models of human colonic epithelium to study the early stages of enteric salmonellosis. *Microbes Infect*. 8: 1813-1825.

Hong, Y. S., Y. T. Ahn, J. C. Park, J. H. Lee, H. Lee, C. S. Huh, D. H. Kim, H. Ryu do, and G. S. Hwang. (2010). 1H NMR-based metabonomic assessment of probiotic effects in a colitis mouse model. *Arch Pharm Res*. 33: 1091-1101.

Hong, Y. S., K. S. Hong, M. H. Park, Y. T. Ahn, J. H. Lee, C. S. Huh, J. Lee, I. K. Kim, G. S. Hwang, and J. S. Kim. (2011). Metabonomic understanding of probiotic effects in humans with irritable bowel syndrome. *J Clin Gastroenterol*. 45: 415-425.

Hooper, L. V., T. Midtvedt, and J. I. Gordon. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr*. 22: 283-307.

Hovind-Hougen, K., A. Birch-Andersen, R. Henrik-Nielsen, M. Orholm, J. O. Pedersen, P. S. Teglbjaerg, and E. H. Thaysen. (1982). Intestinal spirochetosis: morphological characterization and cultivation of the spirochete *Brachyspira aalborgi* gen. nov., sp. nov. *J Clin Microbiol*. 16: 1127-1136.

Hsu, T., D. L. Hutto, F. C. Minion, R. L. Zuerner, and M. J. Wannemuehler. (2001). Cloning of a beta-hemolysin gene of *Brachyspira* (*Serpulina*) *hyodysenteriae* and its expression in *Escherichia coli*. *Infect Immun*. 69: 706-711.

Huang, M. K., Y. J. Choi, R. Houde, J. W. Lee, B. Lee, and X. Zhao. (2004). Effects of lactobacilli and an acidophilic fungus on the production performance and immune responses in broiler chickens. *Poult Sci*. 83: 788-795.

Humphrey, S. B., T. B. Stanton, and N. S. Jensen. (1995). Mitomycin C induction of bacteriophages from *Serpulina hyodysenteriae* and *Serpulina innocens*. *FEMS Microbiol Lett*. 134: 97-101.

Humphrey, S. B., T. B. Stanton, N. S. Jensen, and R. L. Zuerner. (1997). Purification and characterization of VSH-1, a generalized transducing bacteriophage of *Serpulina hyodysenteriae*. *J Bacteriol*. 179: 323-329.

Hunter, D., and T. Wood. (1979). An evaluation of the API ZYM system as a means of classifying spirochaetes associated with swine dysentery. *Vet Rec*. 104: 383-384.

Hutto, D. L., and M. J. Wannemuehler. (1999). A comparison of the morphologic effects of *Serpulina hyodysenteriae* or its beta-hemolysin on the murine cecal mucosa. *Vet Pathol*. 36: 412-422.

Huys, G., K. d'Haene, and J. Swings. (2006). Genetic basis of tetracycline and minocycline resistance in potentially probiotic *Lactobacillus plantarum* strain CCUG 43738. *Antimicrob Agents Chemother*. 50: 1550-1551.

Hyatt, D. R., A. A. ter Huurne, B. A. van der Zeijst, and L. A. Joens. (1994). Reduced virulence of *Serpulina hyodysenteriae* hemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain. *Infect Immun*. 62: 2244-2248.

Hyatt, D., G. L. Chen, P. F. Locascio, M. L. Land, F. W. Larimer, and L. J. Hauser. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics*. 11: 119.

Hyronimus, B., C. Le Marrec, A. H. Sassi, and A. Deschamps. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. *Int J Food Microbiol*. 61: 193-197.

IACUC. (2007). Poultry Module: Body systems of the chicken, www.iacuc.msu.edu/training/poultry_module/ 27/06/12.

Isenberg, H. D., E. R. Giugliano, K. France, and P. Alperstein. (1988). Evaluation of three disinfectants after in-use stress. *J Hosp Infect*. 11: 278-285.

Isolauri, E. (2001). Probiotics in human disease. *Am J Clin Nutr*. 73: 1142S-1146S.

Ito, Y., H. Moriwaki, Y. Muto, N. Kato, K. Watanabe, and K. Ueno. (1997). Effect of lactulose on short-chain fatty acids and lactate production and on the growth of faecal flora, with special reference to *Clostridium difficile*. *J Med Microbiol*. 46: 80-84.

Ivanics, E., M. Dobos-Kovacs, R. Glavits, E. Kaszanyitzky, C. Nemes, L. Szeredi, A. Beregszaszi, and L. Dencso. (2007). Experimental study on the role of *Brachyspira alvinipulli* in intestinal spirochaetosis of geese. *Acta Vet Hung*. 55: 315-326.

Jacobson, M., C. Fellstrom, R. Lindberg, P. Wallgren, and M. Jensen-Waern. (2004). Experimental swine dysentery: comparison between infection models. *J Med Microbiol*. 53: 273-280.

Jamshidi, A., and D. J. Hampson. (2002). Zinc bacitracin enhances colonization by the intestinal spirochaete *Brachyspira pilosicoli* in experimentally infected layer hens. *Avian Pathol*. 31: 293-298.

Jamshidi, A., and D. J. Hampson. (2003). Experimental infection of layer hens with a human isolate of *Brachyspira pilosicoli*. *J Med Microbiol*. 52: 361-364.

Jamshidian, M., T. La, N. D. Phillips, and D. J. Hampson. (2004). *Brachyspira pilosicoli* colonization in experimentally infected mice can be facilitated by dietary manipulation. *J Med Microbiol*. 53: 313-318.

Janardhana, V., M. M. Broadway, M. P. Bruce, J. W. Lowenthal, M. S. Geier, R. J. Hughes, and A. G. Bean. (2009). Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. *J Nutr*. 139: 1404-1409.

Jansson, D. S. (2009). Genus *Brachyspira* in Birds: Phenotypes, Phylogeny and Pathogenicity. Thesis. Doctor of Philosophy, Swedish University of Agricultural Sciences.

Jansson, D. S., and M. Pringle. (2011). Antimicrobial susceptibility of *Brachyspira* spp. isolated from commercial laying hens and free-living wild mallards (*Anas platyrhynchos*). *Avian Pathol*. 40: 387-393.

Jansson, D. S., C. Fellstrom, and K. E. Johansson. (2008). Intestinal spirochetes isolated from wild-living jackdaws, hooded crows and rooks (genus *Corvus*): provisionally designated "*Brachyspira corvi*" sp. nov. *Anaerobe*. 14: 287-295.

Jansson, D. S., T. Rasback, C. Fellstrom, and R. Feinstein. (2009a). Experimental challenge of mallards (*Anas platyrhynchos*) with *Brachyspira hyodysenteriae* and "*Brachyspira suanatina*" isolated from pigs and mallards. *J Comp Pathol*. 141: 211-222.

Jansson, D. S., M. Persson, U. Zimmerman, and K. E. Johansson. (2011). Phenotypic and genetic diversity among intestinal spirochaetes (genus *Brachyspira*) in free-living wild mallards (*Anas platyrhynchos*) sampled in southern Sweden. *Syst Appl Microbiol*. 34: 566-575.

Jansson, D. S., C. Brojer, D. Gavier-Widen, A. Gunnarsson, and C. Fellstrom. (2001). *Brachyspira* spp. (*Serpulina* spp.) in birds: a review and results from a study of Swedish game birds. *Anim Health Res Rev*. 2: 93-100.

Jansson, D. S., T. Broman, J. Waldenstrom, J. Bobnnedal, B. Olsen, and K. E. Johansson. (2009b). Intestinal spirochaete isolated from a snowy sheathbill (*Chionis alba*) in Antarctica. *In*: Proceedings of the 5th International Conference on Colonic Spirochaetal Infections in Animals and Humans, Leon, Spain.

Jansson, D. S., K. E. Johansson, T. Olofsson, T. Rasback, I. Vagsholm, B. Pettersson, A. Gunnarsson, and C. Fellstrom. (2004). *Brachyspira hyodysenteriae* and other strongly beta-haemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). *J Med Microbiol*. 53: 293-300.

JCICSB. (2008). The type strain of *Lactobacillus casei* is ATCC 393, ATCC 334 cannot serve as the type because it represents a different taxon, the name *Lactobacillus paracasei* and its subspecies names are not rejected and the revival of the name "*Lactobacillus zeae*" contravenes rules 51b (1) and (2) of the international code of nomenclature of bacteria. Opinion 82. *Int J Syst Evol Microbiol*. 58: 1764-1765.

Jenkinson, S. R., and C. R. Wingar. (1981). Selective medium for the isolation of *Treponema hyodysenteriae*. *Vet Rec*. 109: 384-385.

Jensen, N. S., T. B. Stanton, and D. E. Swayne. (1996). Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*). *Vet Microbiol*. 52: 259-269.

Jensen, T. K., K. Moller, M. Boye, T. D. Leser, and S. E. Jorsal. (2000). Scanning electron microscopy and fluorescent *in situ* hybridization of experimental *Brachyspira* (*Serpulina*) *pilosicoli* infection in growing pigs. *Vet Pathol*. 37: 22-32.

Jensen, T. K., M. Boye, P. Ahrens, B. Korsager, P. S. Teglbjaerg, C. F. Lindboe, and K. Moller. (2001). Diagnostic examination of human intestinal spirochetosis by fluorescent *in situ* hybridization for *Brachyspira aalborgi*, *Brachyspira pilosicoli*, and other species of the genus *Brachyspira* (*Serpulina*). *J Clin Microbiol*. 39: 4111-4118.

Jessup, J. M., M. Frantz, E. Sonmez-Alpan, J. Locker, K. Skena, H. Waller, P. Battle, A. Nachman, Bhatti, M. E. Weber, D. A. Thomas, R. L. Curbeam, Jr., T. L. Baker, and T. J. Goodwin. (2000). Microgravity culture reduces apoptosis and increases the differentiation of a human colorectal carcinoma cell line. *In Vitro Cell Dev Biol Anim*. 36: 367-373.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. (1996a). Effect of *Lactobacillus* culture on the digestive enzymes in chicken intestine. *In*: Proceedings of the 8th Animal Science Congress, Tokyo, Japan.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Lett Appl Microbiol*. 27: 183-185.

Jin, L. Z., Y. W. Ho, N. Abdullah, and S. Jalaludin. (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with *Lactobacillus* cultures. *Poult Sci*. 79: 886-891.

Jin, L. Z., Y. W. Ho, M. A. Ali, N. Abdullah, K. B. Ong, and S. Jalaludin. (1996b). Adhesion of *Lactobacillus* isolates to intestinal epithelial cells of chicken. *Lett Appl Microbiol*. 22: 229-232.

Joens, L. A. (1997). Virulence factors associated with *Serpulina hyodysenteriae*, pp. 151-172. *In*: D. J. Hampson and T. B. Stanton (eds.), Intestinal Spirochaetes in Domestic Animals and Humans. CAB International, Oxon.

Joens, L. A., and J. M. Kinyon. (1982). Isolation of *Treponema hyodysenteriae* from wild rodents. *J Clin Microbiol*. 15: 994-997.

Joens, L. A., I. M. Robinson, R. D. Glock, and P. J. Matthews. (1981). Production of lesions in gnotobiotic mice by inoculation with *Treponema hyodysenteriae*. *Infect Immun*. 31: 504-506.

Joerger, M. C., and T. R. Klaenhammer. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *J Bacteriol*. 167: 439-446.

Johansson, K. E., G. E. Duhamel, B. Bergsjo, E. O. Engvall, M. Persson, B. Pettersson, and C. Fellstrom. (2004). Identification of three clusters of canine intestinal spirochaetes by biochemical and 16S rDNA sequence analysis. *J Med Microbiol*. 53: 345-350.

Jordan, F. T. W., and D. J. Hampson. (2007). Some other bacterial diseases, pp. 243-249. *In*: M. Pattinson, P. McMullin, J. M. Bradbury and D. Alexander (eds.), Poultry Disease, 6th edn. Saunders Ltd., London.

Joseph, B., K. Przybilla, C. Stuhler, K. Schauer, J. Slaghuis, T. M. Fuchs, and W. Goebel. (2006). Identification of *Listeria monocytogenes* genes contributing to intracellular replication by expression profiling and mutant screening. *J Bacteriol*. 188: 556-568.

Kabir, S. M. L., M. M. Rahman, M. B. Rahman, M. M. Rahman, and S. U. Ahmed. (2004). The dynamics of probiotics on growth performance and immune response in broilers. *Int J Poult Sci*. 3: 361-364.

Kalavathy, R., N. Abdullah, S. Jalaludin, and Y. W. Ho. (2003). Effects of *Lactobacillus* cultures on growth performance, abdominal fat deposition, serum lipids and weight of organs of broiler chickens. *Br Poult Sci*. 44: 139-144.

Kandler, O., and N. Weiss. (1986). Genus *Lactobacillus beijerinck* 1901, 212AL, pp. 1209-1234, Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore.

Kanehisa, M., and S. Goto. (2000). KEGG: kyoto encyclopaedia of genes and genomes. *Nucleic Acids Res*. 28: 27-30.

Karlsson, M., A. Gunnarsson, and A. Franklin. (2001). Susceptibility to pleuromutilins in *Brachyspira* (*Serpulina*) *hyodysenteriae*. *Anim Health Res Rev*. 2: 59-65.

Karlsson, M., C. Fellstrom, K. E. Johansson, and A. Franklin. (2004). Antimicrobial resistance in *Brachyspira pilosicoli* with special reference to point mutations in the 23S rRNA gene associated with macrolide and lincosamide resistance. *Microb Drug Resist*. 10: 204-208.

Karlsson, M., C. Fellstrom, M. U. Heldtander, K. E. Johansson, and A. Franklin. (1999). Genetic basis of macrolide and lincosamide resistance in *Brachyspira* (*Serpulina*) *hyodysenteriae*. *FEMS Microbiol Lett*. 172: 255-260.

Karlsson, M., C. Fellstrom, A. Gunnarsson, A. Landen, and A. Franklin. (2003). Antimicrobial susceptibility testing of porcine *Brachyspira* (*Serpulina*) species isolates. *J Clin Microbiol*. 41: 2596-2604.

Kennedy, G. A., and A. C. Strafuss. (1976). Scanning electron microscopy of the lesions of swine dysentery. *Am J Vet Res*. 37: 395-401.

Kennedy, M. J., and R. J. Yancey, Jr. (1996). Motility and chemotaxis in *Serpulina hyodysenteriae*. *Vet Microbiol*. 49: 21-30.

Kennedy, M. J., E. L. Rosey, and R. J. Yancey, Jr. (1997). Characterization of *flaA*- and *flaB*- mutants of *Serpulina hyodysenteriae*: both flagellin subunits, FlaA and FlaB, are necessary for full motility and intestinal colonization. *FEMS Microbiol Lett*. 153: 119-128.

Kennedy, M. J., D. K. Rosnick, R. G. Ulrich, and R. J. Yancey, Jr. (1988). Association of *Treponema hyodysenteriae* with porcine intestinal mucosa. *J Gen Microbiol*. 134: 1565-1576.

Kerneis, S., M. F. Bernet, M. H. Coconnier, and A. L. Servin. (1994). Adhesion of human enterotoxigenic *Escherichia coli* to human mucus secreting HT-29 cell subpopulations in culture. *Gut*. 35: 1449-1454.

Kessler, A. C., A. Haase, and P. R. Reeves. (1993). Molecular analysis of the 3,6-dideoxyhexose pathway genes of *Yersinia pseudotuberculosis* serogroup IIA. *J Bacteriol*. 175: 1412-1422.

Kilic, A. O., L. Tao, Y. Zhang, Y. Lei, A. Khammanivong, and M. C. Herzberg. (2004). Involvement of *Streptococcus gordonii* beta-glucoside metabolism systems in adhesion, biofilm formation, and *in vivo* gene expression. *J Bacteriol*. 186: 4246-4253.

Kim, G. B., Y. M. Seo, C. H. Kim, and I. K. Paik. (2011). Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. *Poult Sci*. 90: 75-82.

Kim, Y., J. Y. Whang, K. Y. Whang, S. Oh, and S. H. Kim. (2008). Characterization of the cholesterol-reducing activity in a cell-free supernatant of *Lactobacillus acidophilus* ATCC 43121. *Biosci Biotechnol Biochem*. 72: 1483-1490.

Kinyon, J. M., and D. L. Harris. (1979). *Treponema innocens*, a new species of intestinal bacteria, and emended description of the type strain of *Treponema hyodysenteriae*. *Int J Syst Bacteriol*. 29: 102-109.

Kitai, K., M. Kashiwazaki, Y. Adachi, K. Kunugita, and A. Arakawa. (1987). *In vitro* antimicrobial activity against reference strains and field isolates of *Treponema hyodysenteriae*. *Antimicrob Agents Chemother*. 31: 1935-1938.

Kizerwetter-Swida, M., and M. Binek. (2009). Protective effect of potentially probiotic *Lactobacillus* strain on infection with pathogenic bacteria in chickens. *Pol J Vet Sci*. 12: 15-20.

Klaenhammer, T. R., and M. J. Kullen. (1999). Selection and design of probiotics. *Int J Food Microbiol*. 50: 45-57.

Klare, I., C. Konstabel, S. Muller-Bertling, R. Reissbrodt, G. Huys, M. Vancanneyt, J. Swings, H. Goossens, and W. Witte. (2005). Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Appl Environ Microbiol*. 71: 8982-8986.

Klitgaard, K., L. Molbak, T. K. Jensen, C. F. Lindboe, and M. Boye. (2005). Laser capture microdissection of bacterial cells targeted by fluorescence *in situ* hybridization. *Biotechniques*. 39: 864-868.

Klose, V., R. Bruckbeck, S. Henikl, G. Schatzmayr, and A. P. Loibner. (2009). Identification and antimicrobial susceptibility of porcine bacteria that inhibit the growth of *Brachyspira hyodysenteriae in vitro*. *J Appl Microbiol*. 108: 1271-1280.

Klose, V., K. Bayer, R. Bruckbeck, G. Schatzmayr, and A. P. Loibner. (2010). *In vitro* antagonistic activities of animal intestinal strains against swine-associated pathogens. *Vet Microbiol*. 144: 515-521.

Kobryn, K., and G. Chaconas. (2002). ResT, a telomere resolvase encoded by the Lyme disease spirochete. *Mol Cell*. 9: 195-201.

Koenen, M. E., J. Kramer, R. van der Hulst, L. Heres, S. H. Jeurissen, and W. J. Boersma. (2004). Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. *Br Poult Sci*. 45: 355-366.

Koski, L. B., M. W. Gray, B. F. Lang, and G. Burger. (2005). AutoFACT: an automatic functional annotation and classification tool. *BMC Bioinformatics*. 6: 151.

Kostman, J. R., M. Patel, E. Catalano, J. Camacho, J. Hoffpauir, and M. J. DiNubile. (1995). Invasive colitis and hepatitis due to previously uncharacterized spirochetes in patients with advanced human immunodeficiency virus infection. *Clin Infect Dis*. 21: 1159-1165.

Kraaz, W., B. Pettersson, U. Thunberg, L. Engstrand, and C. Fellstrom. (2000). *Brachyspira aalborgi* infection diagnosed by culture and 16S ribosomal DNA sequencing using human colonic biopsy specimens. *J Clin Microbiol*. 38: 3555-3560.

Krehbiel, C. R., S. R. Rust, G. Zhang, and S. E. Gilliland. (2003). Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. *J Anim Sci*. 81: E120–132.

Krzywinski, M., J. Schein, I. Birol, J. Connors, R. Gascoyne, D. Horsman, S. J. Jones, and M. A. Marra. (2009). Circos: an information aesthetic for comparative genomics. *Genome Res*. 19: 1639-1645.

Kunkle, R. A., and J. M. Kinyon. (1988). Improved selective medium for the isolation of *Treponema hyodysenteriae*. *J Clin Microbiol*. 26: 2357-2360.

Kunkle, R. A., D. L. Harris, and J. M. Kinyon. (1986). Autoclaved liquid medium for propagation of *Treponema hyodysenteriae*. *J Clin Microbiol*. 24: 669-671.

Kurtoglu, V., F. Kurtoglu, E. Seker, B. Coskun, T. Balevi, and E. S. Polat. (2004). Effect of probiotic supplementation on laying hen diets on yield performance and serum and egg yolk cholesterol. *Food Addit Contam*. 21: 817-823.

Kwon, H. S., E. H. Yang, S. W. Yeon, B. H. Kang, and T. Y. Kim. (2004). Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol Lett*. 239: 267-275.

La, T., and D. J. Hampson. (2001). Serologic detection of *Brachyspira* (*Serpulina*) *hyodysenteriae* infections. *Anim Health Res Rev*. 2: 45-52.

La, T., N. D. Phillips, and D. J. Hampson. (2003). Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. *J Clin Microbiol*. 41: 3372-3375.

La, T., N. D. Phillips, and D. J. Hampson. (2009). Evaluation of recombinant Bhlp29.7 as an ELISA antigen for detecting pig herds with swine dysentery. *Vet Microbiol*. 133: 98-104.

La, T., N. D. Phillips, M. P. Reichel, and D. J. Hampson. (2004). Protection of pigs from swine dysentery by vaccination with recombinant BmpB, a 29.7 kDa outer-membrane lipoprotein of *Brachyspira hyodysenteriae*. *Vet Microbiol*. 102: 97-109.

La Ragione, R. M., and M. J. Woodward. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Vet Microbiol*. 94: 245-256.

La Ragione, R. M., G. Casula, S. M. Cutting, and M. J. Woodward. (2001). *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. *Vet Microbiol*. 79: 133-142.

La Ragione, R. M., A. Narbad, M. J. Gasson, and M. J. Woodward. (2004). *In vivo* characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. *Lett Appl Microbiol*. 38: 197-205.

La Ragione, R. M., I. M. McLaren, G. Foster, W. A. Cooley, and M. J. Woodward. (2002). Phenotypic and genotypic characterization of avian *Escherichia coli* O86:K61 isolates possessing a gamma-like intimin. *Appl Environ Microbiol*. 68: 4932-4942.

Lamarche, M. G., C. M. Dozois, F. Daigle, M. Caza, R. Curtiss, 3rd, J. D. Dubreuil, and J. Harel. (2005). Inactivation of the Pst system reduces the virulence of an avian pathogenic *Escherichia coli* O78 strain. *Infect Immun*. 73: 4138-4145.

Lammers, K. M., P. Brigidi, B. Vitali, P. Gionchetti, F. Rizzello, E. Caramelli, D. Matteuzzi, and M. Campieri. (2003). Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS Immunol Med Microbiol*. 38: 165-172.

Lan, P. T., T. Binh le, and Y. Benno. (2003). Impact of two probiotic *Lactobacillus* strains feeding on fecal lactobacilli and weight gains in chicken. *J Gen Appl Microbiol*. 49: 29-36.

Lan, P. T., M. Sakamoto, and Y. Benno. (2004). Effects of two probiotic *Lactobacillus* strains on jejunal and cecal microbiota of broiler chicken under acute heat stress condition as revealed by molecular analysis of 16S rRNA genes. *Microbiol Immunol*. 48: 917-929.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*. 23: 2947-2948.

Laughton, J. M., E. Devillard, D. E. Heinrichs, G. Reid, and J. K. McCormick. (2006). Inhibition of expression of a staphylococcal superantigen-like protein by a soluble factor from *Lactobacillus reuteri*. *Microbiology*. 152: 1155-1167.

Lee, J. I., and D. J. Hampson. (1992). Intestinal spirochaetes colonizing aborigines from communities in the remote north of western Australia. *Epidemiol Infect*. 109: 133-141.

Lee, B. J., and D. J. Hampson. (1995). A monoclonal antibody reacting with the cell envelope of spirochaetes isolated from cases of intestinal spirochaetosis in pigs and humans. *FEMS Microbiol Lett*. 131: 179-184.

Lee, B. J., and D. J. Hampson. (1996). Production and characterisation of a monoclonal antibody to *Serpulina hyodysenteriae*. *FEMS Microbiol Lett*. 136: 193-197.

Lee, J. I., D. J. Hampson, A. J. Lymbery, and S. J. Harders. (1993). The porcine intestinal spirochaetes: identification of new genetic groups. *Vet Microbiol*. 34: 273-285.

Lelkes, P. I., E. Ramos, V. V. Nikolaychik, D. M. Wankowski, B. R. Unsworth, and T. J. Goodwin. (1997). GTSF-2: a new, versatile cell culture medium for diverse normal and transformed mammalian cells. *In Vitro Cell Dev Biol Anim*. 33: 344-351.

Lemcke, R. M., and M. R. Burrows. (1980). Sterol requirement for the growth of *Treponema hyodysenteriae*. *J Gen Microbiol*. 116: 539-543.

Lemcke, R. M., and M. R. Burrows. (1981). A comparative study of spirochaetes from the porcine alimentary tract. *J Hyg (Lond)*. 86: 173-182.

Leser, T. D., K. Moller, T. K. Jensen, and S. E. Jorsal. (1997). Specific detection of *Serpulina hyodysenteriae* and potentially pathogenic weakly beta-haemolytic porcine intestinal spirochetes by polymerase chain reaction targeting 23S rDNA. *Mol Cell Probes*. 11: 363-372.

Lester, C. H., N. Frimodt-Moller, T. L. Sorensen, D. L. Monnet, and A. M. Hammerum. (2006). *In vivo* transfer of the *vanA* resistance gene from an *Enterococcus faecium* isolate of animal origin to an *E. faecium* isolate of human origin in the intestines of human volunteers. *Antimicrob Agents Chemother*. 50: 596-599.

Li, R., Y. Li, K. Kristiansen, and J. Wang. (2008). SOAP: short oligonucleotide alignment program. *Bioinformatics*. 24: 713-714.

Lilly, D. M., and R. H. Stillwell. (1965). Probiotics: growth-promoting factors produced by microorganisms. *Science*. 147: 747-748.

Lin, W. H., B. Yu, S. H. Jang, and H. Y. Tsen. (2007). Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe*. 13: 107-113.

Liolios, K., K. Mavromatis, N. Tavernarakis, and N. C. Kyrpides. (2008). The genomes on line database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res*. 36: D475-479.

Lobova, D., J. Smola, and A. Cizek. (2004). Decreased susceptibility to tiamulin and valnemulin among Czech isolates of *Brachyspira hyodysenteriae*. *J Med Microbiol*. 53: 287-291.

Ludwig, W., J. Euzeby, and W. B. Whitman. (2008). Draft taxonomic outline of the Bacteroidetes, Planctomycetes, Chlamydiae, Spirochaetes, Fibrobacteres, Fusobacteria, Acidobacteria, Verrucomicrobia, Dictyoglomi and Gemmatimonadetes, www.bergeys.org/outlines/bergeys_vol_4_outline.pdf 01/05/12.

Lussier, G. (1962). Vibrionic dysentery in Swine in Ontario-part II: morphological, biochemical and serological characteristics of *Vibrio coli*. *Can Veterinary J*. 3: 267-278.

Lux, R., A. Moter, and W. Shi. (2000). Chemotaxis in pathogenic spirochetes: directed movement toward targeting tissues? *J Mol Microbiol Biotechnol*. 2: 355-364.

Lysons, R. J., and R. M. Lemcke. (1983). Swine dysentery: to isolate or to fluoresce? *Vet Rec*. 112: 203.

Lysons, R. J., K. A. Kent, A. P. Bland, R. Sellwood, W. F. Robinson, and A. J. Frost. (1991). A cytotoxic haemolysin from *Treponema hyodysenteriae*--a probable virulence determinant in swine dysentery. *J Med Microbiol*. 34: 97-102.

Macfarlane, G. T., H. Steed, and S. Macfarlane. (2008). Bacterial metabolism and health-related effects of galactooligosaccharides and other prebiotics. *J Appl Microbiol*. 104: 305-344.

Mack, D. R., S. Ahrne, L. Hyde, S. Wei, and M. A. Hollingsworth. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut*. 52: 827-833.

Mackiewicz, P., D. Mackiewicz, M. Kowalczuk, and S. Cebrat. (2001). Flip-flop around the origin and terminus of replication in prokaryotic genomes. *Genome Biol*. 2: 1004.

Makras, L., V. Triantafyllou, D. Fayol-Messaoudi, T. Adriany, G. Zoumpopoulou, E. Tsakalidou, A. Servin, and L. de Vuyst. (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Res Microbiol*. 157: 241-247.

Mantle, M., L. Basaraba, S. C. Peacock, and D. G. Gall. (1989a). Binding of *Yersinia enterocolitica* to rabbit intestinal brush border membranes, mucus, and mucin. *Infect Immun*. 57: 3292-3299.

Mantle, M., E. Thakore, J. Hardin, and D. G. Gall. (1989b). Effect of *Yersinia enterocolitica* on intestinal mucin secretion. *Am J Physiol*. 256: G319-327.

Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol*. 64: 795-799.

Marchoux, E., and A. Salimbeni. (1903). La spirillose des poules. *Ann Inst Pasteur*. 17: 569-580.

Margawani, K. R., I. D. Robertson, C. J. Brooke, and D. J. Hampson. (2004). Prevalence, risk factors and molecular epidemiology of *Brachyspira pilosicoli* in humans on the island of Bali, Indonesia. *J Med Microbiol*. 53: 325-332.

Margulis, L., J. B. Ashen, M. Sole, and R. Guerrero. (1993). Composite, large spirochetes from microbial mats: spirochete structure review. *Proc Natl Acad Sci U S A*. 90: 6966-6970.

Marteau, P. (2001). Safety aspects of probiotic products. *Scand J Nutr*. 45: 22-24.

Martin, R., N. Soberon, M. Vaneechoutte, A. B. Florez, F. Vazquez, and J. E. Suarez. (2008a). Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. *Int Microbiol*. 11: 261-266.

Martin, F. P., Y. Wang, N. Sprenger, I. K. Yap, T. Lundstedt, P. Lek, S. Rezzi, Z. Ramadan, P. van Bladeren, L. B. Fay, S. Kochhar, J. C. Lindon, E. Holmes, and J.

K. Nicholson. (2008b). Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol Syst Biol*. 4: 157.

Mathey, W. J., Jr., and D. V. Zander. (1955). Spirochetes and cecal nodules in poultry. *J Am Vet Med Assoc*. 126: 475-477.

McCracken, V. J., and H. R. Gaskins. (1999). Probiotics and the immune system, pp. 85-112. *In*: G. W. Tannock (ed.), Probiotics, A Critical Review. Horizon Scientific Press, Norfolk.

McLaren, A. J., D. J. Hampson, and S. L. Wylie. (1996). The prevalence of intestinal spirochaetes in poultry flocks in western Australia. *Aust Vet J*. 74: 319-321.

McLaren, A. J., D. J. Trott, D. E. Swayne, S. L. Oxberry, and D. J. Hampson. (1997). Genetic and phenotypic characterization of intestinal spirochetes colonizing chickens and allocation of known pathogenic isolates to three distinct genetic groups. *J Clin Microbiol*. 35: 412-417.

Mead, G. C. (1989). Microbes of the avian caecum: types present and substrates utilized. *J Exp Zool Suppl*. 3: 48-54.

Mead, G. C., P. A. Barrow, M. H. Hinton, F. Humbert, C. S. Impey, C. Lahellec, R. W. A. W. Mulder, S. Stavric, and N. J. Stern. (1989). Recommended assay for treatment of chicks to prevent *Salmonella* colonization by "competitive exclusion". *J Food Prot*. 52: 500-502.

Mellor, G. E., R. M. Goulter, T. W. Chia, and G. A. Dykes. (2009). Comparative analysis of attachment of Shiga-toxigenic *Escherichia coli* and *Salmonella* strains to cultured HT-29 and Caco-2 cell lines. *Appl Environ Microbiol*. 75: 1796-1799.

Merrick, M. J., and R. A. Edwards. (1995). Nitrogen control in bacteria. *Microbiol Rev*. 59: 604-622.

Metchnikoff, E. (1907). The Prolongation of Life. Butterworth-Heinemann, London.

Miao, R. M., A. H. Fieldsteel, and D. L. Harris. (1978). Genetics of *Treponema*: characterization of *Treponema hyodysenteriae* and its relationship to *Treponema pallidum*. *Infect Immun*. 22: 736-739.

Mikkelsen, L. L., M. Jakobsen, and B. B. Jensen. (2003). Effect of dietary oligosaccharides on microbial diversity and fructooligosaccharides degrading bacteria in faeces of piglets post-weaning. *Anim Feed Sci Technol*. 109: 133-150.

Mikosza, A. S., and D. J. Hampson. (2001). Human intestinal spirochetosis: *Brachyspira aalborgi* and/or *Brachyspira pilosicoli*? *Anim Health Res Rev*. 2: 101-110.

Mikosza, A. S., T. La, W. B. de Boer, and D. J. Hampson. (2001a). Comparative prevalences of *Brachyspira aalborgi* and *Brachyspira* (*Serpulina*) *pilosicoli* as etiologic agents of histologically identified intestinal spirochetosis in Australia. *J Clin Microbiol*. 39: 347-350.

Mikosza, A. S., T. La, K. R. Margawani, C. J. Brooke, and D. J. Hampson. (2001b). PCR detection of *Brachyspira aalborgi* and *Brachyspira pilosicoli* in human faeces. *FEMS Microbiol Lett*. 197: 167-170.

Milner, K. C., and M. F. Shaffer. (1952). Bacteriologic studies of experimental *Salmonella* infections in chicks. *J Infect Dis*. 90: 81-96.

Milner, J. A., and R. Sellwood. (1994). Chemotactic response to mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: potential role in intestinal colonization. *Infect Immun*. 62: 4095-4099.

Minato, Y., R. L. Siefken, and C. C. Hase. (2011). TolC affects virulence gene expression in *Vibrio cholerae*. *J Bacteriol*. 193: 5850-5852.

Molnar, L. (1996). Sensitivity of strains of *Serpulina hyodysenteriae* isolated in Hungary to chemotherapeutic drugs. *Vet Rec*. 138: 158-160.

Montrose, D. C., and M. H. Floch. (2005). Probiotics used in human studies. *J Clin Gastroenterol*. 39: 469-484.

Moran, N. A., and G. R. Plague. (2004). Genomic changes following host restriction in bacteria. *Curr Opin Genet Dev*. 14: 627-633.

Mortimer-Jones, S. M., N. D. Phillips, T. La, R. Naresh, and D. J. Hampson. (2008). Penicillin resistance in the intestinal spirochaete *Brachyspira pilosicoli* associated with OXA-136 and OXA-137, two new variants of the class D beta-lactamase OXA-63. *J Med Microbiol*. 57: 1122-1128.

Motro, Y., D. S. Dunn, T. La, N. D. Phillips, D. J. Hampson, and M. I. Bellgard. (2008). Intestinal spirochaetes of the genus *Brachyspira* share a partially conserved 26 kilobase genomic region with *Enterococcus faecalis* and *Escherichia coli*. *Microbiol Insights*. 1: 3-11.

Motro, Y., T. La, M. I. Bellgard, D. S. Dunn, N. D. Phillips, and D. J. Hampson. (2009). Identification of genes associated with prophage-like gene transfer agents in the pathogenic intestinal spirochaetes *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli* and *Brachyspira intermedia*. *Vet Microbiol*. 134: 340-345.

Mountzouris, K. C., P. Tsirttsikos, E. Kalamara, S. Nitsch, G. Schatzmayr, and K. Fegeros. (2007). Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating caecal microflora composition and metabolic activities. *Poult Sci*. 86: 309-317.

Movahedi, A., and D. J. Hampson. (2009). A reverse vaccinology approach to *Brachyspira pilosicoli* vaccine development. *In*: Proceedings of the 5th International Conference on Colonic Spirochaetal Infections in Animals and Humans, Leon, Spain.

Muir, S., M. B. Koopman, S. J. Libby, L. A. Joens, F. Heffron, and J. G. Kusters. (1992). Cloning and expression of a *Serpula* (*Treponema*) *hyodysenteriae* hemolysin gene. *Infect Immun*. 60: 529-535.

Muniappa, N., M. R. Mathiesen, and G. E. Duhamel. (1997). Laboratory identification and enteropathogenicity testing of *Serpulina pilosicoli* associated with porcine colonic spirochetosis. *J Vet Diagn Invest*. 9: 165-171.

Muniappa, N., G. E. Duhamel, M. R. Mathiesen, and T. W. Bargar. (1996). Light microscopic and ultrastructural changes in the ceca of chicks inoculated with human and canine *Serpulina pilosicoli*. *Vet Pathol*. 33: 542-550.

Muniappa, N., M. R. Ramanathan, R. P. Tarara, R. B. Westerman, M. R. Mathiesen, and G. E. Duhamel. (1998). Attachment of human and rhesus *Serpulina pilosicoli* to cultured cells and comparison with a chick infection model. *J Spiroch Tick-borne Dis*. 5: 44-53.

Munshi, M. A., N. M. Taylor, A. S. Mikosza, P. B. Spencer, and D. J. Hampson. (2003). Detection by PCR and isolation assays of the anaerobic intestinal spirochete *Brachyspira aalborgi* from the feces of captive nonhuman primates. *J Clin Microbiol*. 41: 1187-1191.

Munshi, M. A., R. J. Traub, I. D. Robertson, A. S. Mikosza, and D. J. Hampson. (2004). Colonization and risk factors for *Brachyspira aalborgi* and *Brachyspira pilosicoli* in humans and dogs on tea estates in Assam, India. *Epidemiol Infect*. 132: 137-144.

Myers, S. E., P. A. Dunn, N. D. Phillips, T. La, and D. J. Hampson. (2009). *Brachyspira intermedia* and *Brachyspira pilosicoli* are commonly found in older laying flocks in Pennsylvania. *Avian Dis*. 53: 533-537.

Nahanshon, S. N., H. S. Nakaue, and L. W. Mirosh. (1992). Effect of direct-fed microbials on nutrient retention and parameters of laying pullets. *Poultry Sci*. 71: 111.

Nahanshon, S. N., H. S. Nakaue, and L. W. Mirosh. (1994). Performance of single comb white leghorn laying pullets fed diets supplemented with direct – fed microbials. *Poultry Sci*. 73: 1699-1711.

Nahanshon, S. N., H. S. Nakaue, and L. W. Mirosh. (1996). Performance of single comb white leghorn layers fed with a live microbial during the growth and egg laying phases. *Anim Sci Technol*. 57: 25-38.

Naikare, H., K. Palyada, R. Panciera, D. Marlow, and A. Stintzi. (2006). Major role for FeoB in *Campylobacter jejuni* ferrous iron acquisition, gut colonization and intracellular survival. *Infect Immun*. 74: 5433-5444.

Nakamura, S., Y. Adachi, T. Goto, and Y. Magariyama. (2006). Improvement in motion efficiency of the spirochete *Brachyspira pilosicoli* in viscous environments. *Biophys J*. 90: 3019-3026.

Nakamura, A., Y. Ota, A. Mizukami, T. Ito, Y. B. Ngwai, and Y. Adachi. (2002). Evaluation of aviguard, a commercial competitive exclusion product for efficacy and after-effect on the antibody response of chicks to *Salmonella*. *Poult Sci*. 81: 1653-1660.

Nakphaichit, M., S. Thanomwongwattana, C. Phraephaisarn, N. Sakamoto, S. Keawsompong, J. Nakayama, and S. Nitisinprasert. (2011). The effect of including *Lactobacillus reuteri* KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. *Poult Sci*. 90: 2753-2765.

Naresh, R., and D. J. Hampson. (2010). Attraction of *Brachyspira pilosicoli* to mucin. *Microbiology*. 156: 191-197.

Naresh, R., Y. Song, and D. J. Hampson. (2009). The intestinal spirochete *Brachyspira pilosicoli* attaches to cultured Caco-2 cells and induces pathological changes. *PLoS ONE*. 4: e8352.

Nathues, H., C. J. Oliveira, M. Wurm, E. Grosse Beilage, and P. E. Givisiez. (2007). Simultaneous detection of *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*

and *Lawsonia intracellularis* in porcine faeces and tissue samples by multiplex-PCR. *J Vet Med*. 54: 532-538.

Neef, N. A., R. J. Lysons, D. J. Trott, D. J. Hampson, P. W. Jones, and J. H. Morgan. (1994). Pathogenicity of porcine intestinal spirochetes in gnotobiotic pigs. *Infect Immun*. 62: 2395-2403.

Nelson, E. J., A. Tanudra, A. Chowdhury, A. V. Kane, F. Qadri, S. B. Calderwood, J. Coburn, and A. Camilli. (2009). High prevalence of spirochetosis in cholera patients, Bangladesh. *Emerg Infect Dis*. 15: 571-573.

Nemes, C. S., R. Glavits, M. Dobos-Kovacs, E. Ivanics, E. Kaszanyitzky, A. Beregszaszi, L. Szeredi, and L. Dencso. (2006). Typhlocolitis associated with spirochaetes in goose flocks. *Avian Pathol*. 35: 4-11.

Nissle, A. (1916). Uber die grundlagen einer neuen ursachlichen bekampfung der pathologischen darmflora. *Dtsch Med Wschr*. 42: 1181-1184.

Nomoto, K. (2005). Prevention of infections by probiotics. *J Biosci Bioeng*. 100: 583-592.

Nuessen, M. E., L. A. Joens, and R. D. Glock. (1983). Involvement of lipopolysaccharide in the pathogenicity of *Treponema hyodysenteriae*. *J Immunol*. 131: 997-999.

Nuotio, L., C. Schneitz, U. Halonen, and E. Nurmi. (1992). Use of competitive exclusion to protect newly-hatched chicks against intestinal colonisation and invasion by *Salmonella enteritidis* PT4. *Br Poult Sci*. 33: 775-779.

Nurmi, E., and M. Rantala. (1973). New aspects of *Salmonella* infection in broiler production. *Nature*. 241: 210-211.

Ochiai, S., Y. Adachi, and K. Mori. (1997). Unification of the genera *Serpulina* and *Brachyspira*, and proposals of *Brachyspira hyodysenteriae* comb. nov., *Brachyspira innocens* comb. nov. and *Brachyspira pilosicoli* comb. nov. *Microbiol Immunol*. 41: 445-452.

Olson, L. D., K. I. Dayalu, and G. T. Schlink. (1994). Exacerbated onset of dysentery in swine vaccinated with inactivated adjuvanted *Serpulina hyodysenteriae*. *Am J Vet Res*. 55: 67-71.

Osorio, J., A. Carvajal, G. Naharro, T. La, N. D. Phillips, P. Rubio, and D. J. Hampson. (2012). Dissemination of clonal groups of *Brachyspira hyodysenteriae* amongst pig farms in Spain, and their relationships to isolates from other countries. *PLoS One*. 7: e39082.

Otto, T. D., M. Sanders, M. Berriman, and C. Newbold. (2010). Iterative correction of reference nucleotides (iCORN) using second generation sequencing technology. *Bioinformatics*. 26: 1704-1707.

Oxberry, S. L., D. J. Trott, and D. J. Hampson. (1998). *Serpulina pilosicoli*, waterbirds and water: potential sources of infection for humans and other animals. *Epidemiol Infect*. 121: 219-225.

Parker, R. B. (1974). Probiotics: the other half of the antibiotics story. *Anim Nutr Health*. 29: 4-8.

Parkhill, J., B. W. Wren, N. R. Thomson, R. W. Titball, M. T. Holden, M. B. Prentice, M. Sebaihia, K. D. James, C. Churcher, K. L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeno-Tarraga, T. Chillingworth, A. Cronin, R. M. Davies, P. Davis, G. Dougan, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. Oyston, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell. (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature*. 413: 523-527.

Parkhill, J., M. Sebaihia, A. Preston, L. D. Murphy, N. Thomson, D. E. Harris, M. T. Holden, C. M. Churcher, S. D. Bentley, K. L. Mungall, A. M. Cerdeno-Tarraga, L. Temple, K. James, B. Harris, M. A. Quail, M. Achtman, R. Atkin, S. Baker, D. Basham, N. Bason, I. Cherevach, T. Chillingworth, M. Collins, A. Cronin, P. Davis, J. Doggett, T. Feltwell, A. Goble, N. Hamlin, H. Hauser, S. Holroyd, K. Jagels, S. Leather, S. Moule, H. Norberczak, S. O'Neil, D. Ormond, C. Price, E. Rabbinowitsch, S. Rutter, M. Sanders, D. Saunders, K. Seeger, S. Sharp, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, L. Unwin, S. Whitehead, B. G. Barrell, and D. J. Maskell. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet*. 35: 32-40.

Parr, L. W. (1923). Intestinal spirochaetes. *J Infect Dis*. 33: 369-383.

Parracho, H., A. L. McCartney, and G. R. Gibson. (2007). Probiotics and prebiotics in infant nutrition. *Proc Nutr Soc*. 66: 405-411.

Pascual, M., M. Hugas, J. I. Badiola, J. M. Monfort, and M. Garriga. (1999). *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* colonization in chickens. *Appl Environ Microbiol*. 65: 4981-4986.

Paster, B. J., and F. E. Dewhirst. (2000). Phylogenetic foundation of spirochetes. *J Mol Microbiol Biotechnol*. 2: 341-344.

Paster, B. J., F. E. Dewhirst, W. G. Weisburg, L. A. Tordoff, G. J. Fraser, R. B. Hespell, T. B. Stanton, L. Zablen, L. Mandelco, and C. R. Woese. (1991). Phylogenetic analysis of the spirochetes. *J Bacteriol*. 173: 6101-6109.

Pati, A., J. Sikorski, S. Gronow, C. Munk, A. Lapidus, A. Copeland, T. Glavina Del Tio, M. Nolan, S. Lucas, F. Chen, H. Tice, J.-F. Cheng, C. Han, J. C. Detter, D. Bruce, R. Tapia, L. Goodwin, S. Pitluck, K. Liolios, N. Ivanova, K. Mavromatis, N. Mikhailova, A. Chen, K. Palaniappan, M. Land, L. Hauser, Y.-J. Chang, C. D. Jeffries, S. Spring, M. Rohde, M. Goker, J. Bristow, J. A. Eisen, V. Markowitz, P. Hugenholtz, N. C. Kyrpides, and H.-P. Klenk. (2010). Complete genome sequence of *Brachyspira murdochii* type strain (56-150^{T}). *Stand Genomic Sci*. 2: 260-269.

Patterson, J. A., and K. M. Burkholder. (2003). Application of prebiotics and probiotics in poultry production. *Poult Sci*. 82: 627-631.

Peirs, P., P. Lefevre, S. Boarbi, X. M. Wang, O. Denis, M. Braibant, K. Pethe, C. Locht, K. Huygen, and J. Content. (2005). *Mycobacterium tuberculosis* with disruption in genes encoding the phosphate binding proteins PstS1 and PstS2 is deficient in phosphate uptake and demonstrates reduced *in vivo* virulence. *Infect Immun*. 73: 1898-1902.

Perdigon, G., S. Alavares, M. Rachid, G. Aguero, and N. Gobbato. (1995). Immune system stimulation by probiotics. *J Dairy Sci*. 78: 1597-1606.

Perez, A., M. Poza, A. Fernandez, M. Del Carmen Fernandez, S. Mallo, M. Merino, S. Rumbo-Feal, M. P. Cabral, and G. Bou. (2012). Involvement of the AcrAB-TolC efflux pump in the resistance, fitness, and virulence of *Enterobacter cloacae*. *Antimicrob Agents Chemother*. 56: 2084-2090.

Pernice, M., S. Wetzel, O. Gros, R. Boucher-Rodoni, and N. Dubilier. (2007). Enigmatic dual symbiosis in the excretory organ of *Nautilus macromphalus* (Cephalopoda: Nautiloidea). *Proc Royal Soc Lond Ser Biol Sci*. 274: 1143-1152.

Perreten, V., L. Vorlet-Fawer, P. Slickers, R. Ehricht, P. Kuhnert, and J. Frey. (2005). Microarray-based detection of 90 antibiotic resistance genes of Gram-positive bacteria. *J Clin Microbiol*. 43: 2291-2302.

Pettersson, B., M. Wang, C. Fellstrom, M. Uhlen, G. Molin, B. Jeppsson, and S. Ahrne. (2000). Phylogenetic evidence for novel and genetically different intestinal spirochetes resembling *Brachyspira aalborgi* in the mucosa of the human colon as revealed by 16S rDNA analysis. *Syst Appl Microbiol*. 23: 355-363.

Phillips, N. D., T. La, and D. J. Hampson. (2003). Survival of intestinal spirochaete strains from chickens in the presence of disinfectants and in faeces held at different temperatures. *Avian Pathol*. 32: 639-643.

Phillips, N. D., T. La, and D. J. Hampson. (2005). A cross-sectional study to investigate the occurrence and distribution of intestinal spirochaetes (*Brachyspira* spp.) in three flocks of laying hens. *Vet Microbiol*. 105: 189-198.

Phillips, N. D., T. La, and D. J. Hampson. (2006). Development of a two-step nested duplex PCR assay for the rapid detection of *Brachyspira pilosicoli* and *Brachyspira intermedia* in chicken faeces. *Vet Microbiol*. 116: 239-245.

Phillips, N. D., T. La, J. R. Pluske, and D. J. Hampson. (2004a). The wheat variety used in the diet of laying hens influences colonization with the intestinal spirochaete *Brachyspira intermedia*. *Avian Pathol*. 33: 586-590.

Phillips, N. D., T. La, J. R. Pluske, and D. J. Hampson. (2004b). A wheat-based diet enhances colonization with the intestinal spirochaete *Brachyspira intermedia* in experimentally infected laying hens. *Avian Pathol*. 33: 451-457.

Phillips, N. D., T. La, M. M. Amin, and D. J. Hampson. (2010). *Brachyspira intermedia* strain diversity and relationships to the other indole-positive *Brachyspira* species. *Vet Microbiol*. 143: 246-254.

Piddock, L. J. (2006a). Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev Microbiol*. 4: 629-636.

Piddock, L. J. (2006b). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev*. 19: 382-402.

Piotrowski, J., A. Slomiany, V. L. Murty, Z. Fekete, and B. L. Slomiany. (1991). Inhibition of *Helicobacter pylori* colonization by sulfated gastric mucin. *Biochem Int*. 24: 749-756.

Pivnick, H., and E. Nurmi. (1982). The Nurmi concept and its role in the control of *Salmonella* in poultry, pp. 41-70. *In*: R. Davies (ed.), Developments in Food Microbiology. Applied Science Publishers, London.

Plague, G. R., H. E. Dunbar, P. L. Tran, and N. A. Moran. (2008). Extensive proliferation of transposable elements in heritable bacterial symbionts. *J Bacteriol*. 190: 777-779.

Poole, K. (2001). Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol*. 3: 255-264.

Porcella, S. F., T. G. Popova, K. E. Hagman, C. W. Penn, J. D. Radolf, and M. V. Norgard. (1996). A *mgl*-like operon in *Treponema pallidum*, the syphilis spirochete. *Gene*. 177: 115-121.

Prapasarakul, N., K. Lugsomya, S. Disatian, T. Lekdumrongsak, W. Banlunara, P. Chetanachan, and D. J. Hampson. (2011). Faecal excretion of intestinal spirochaetes by urban dogs, and their pathogenicity in a chick model of intestinal spirochaetosis. *Res Vet Sci*. 91: e38-43.

Prim, N., R. Pericas, M. Espanol, A. Rivera, B. Mirelis, and P. Coll. (2011). Bloodstream infection due to *Brachyspira pilosicoli* in a patient with multiorgan failure. *J Clin Microbiol*. 49: 3697-3699.

Pringle, M., A. Landen, and A. Franklin. (2006). Tiamulin resistance in porcine *Brachyspira pilosicoli* isolates. *Res Vet Sci*. 80: 1-4.

Pringle, M., J. Poehlsgaard, B. Vester, and K. S. Long. (2004). Mutations in ribosomal protein L3 and 23S ribosomal RNA at the peptidyl transferase centre are associated with reduced susceptibility to tiamulin in *Brachyspira* spp. isolates. *Mol Microbiol*. 54: 1295-1306.

Rabe, L. K., and S. L. Hillier. (2003). Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. *J Clin Microbiol*. 41: 3260-3264.

Rabiu, B. A., A. J. Jay, G. R. Gibson, and R. A. Rastall. (2001). Synthesis and fermentation properties of novel galactooligosaccharides by beta-galactosidases from *Bifidobacterium* species. *Appl Environ Microbiol*. 67: 2526-2530.

Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. 118: 229-241.

Randall, L. P., S. W. Cooles, N. C. Coldham, K. S. Stapleton, L. J. Piddock, and M. J. Woodward. (2006). Modification of enrofloxacin treatment regimens for poultry experimentally infected with *Salmonella enterica* serovar Typhimurium DT104 to minimize selection of resistance. *Antimicrob Agents Chemother*. 50: 4030-4037.

Rantala, M. (1974). Cultivation of a bacterial flora able to prevent the colonization of *Salmonella infantis* in the intestines of broiler chickens, and its use. *Acta Pathol Microbiol Scand B Microbiol Immunol*. 82: 75-80.

Rantala, M., and E. Nurmi. (1973). Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *Br Poult Sci*. 14: 627-630.

Rasback, T., C. Fellstrom, A. Gunnarsson, and A. Aspan. (2006). Comparison of culture and biochemical tests with PCR for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli*. *J Microbiol Methods*. 66: 347-353.

Rasback, T., D. S. Jansson, K. E. Johansson, and C. Fellstrom. (2007a). A novel enteropathogenic, strongly haemolytic spirochaete isolated from pig and mallard, provisionally designated "*Brachyspira suanatina*" sp. nov. *Environ Microbiol*. 9: 983-991.

Rasback, T., K. E. Johansson, D. S. Jansson, C. Fellstrom, M. Y. Alikhani, T. La, D. S. Dunn, and D. J. Hampson. (2007b). Development of a multilocus sequence typing scheme for intestinal spirochaetes within the genus *Brachyspira*. *Microbiology*. 153: 4074-4087.

Rasback, T., C. Fellstrom, B. Bergsjo, A. Cizek, K. Collin, A. Gunnarsson, S. M. Jensen, A. Mars, J. Thomson, P. Vyt, and M. Pringle. (2005). Assessment of diagnostics and antimicrobial susceptibility testing of *Brachyspira* species using a ring test. *Vet Microbiol*. 109: 229-243.

Reeves, S. A., A. G. Torres, and S. M. Payne. (2000). TonB is required for intracellular growth and virulence of *Shigella dysenteriae*. *Infect Immun*. 68: 6329-6336.

Reiner, G., S. Hillen, S. von Berg, M. Kixmoller, and H. Willems. (2011). Analysis of bacterial load and prevalence of mixed infections with *Lawsonia intracellularis*, *Brachyspira hyodysenteriae* and/or *Brachyspira pilosicoli* in German pigs with diarrhoea. *Berl Munch Tierarztl Wochenschr*. 124: 236-241.

Rettger, L. R., and H. A. Chaplin. (1921). Treatise on the transformation of the intestinal flora with special reference to the implantation of *Bacillus acidophilus*. Yale University Press, Connecticut.

Rettger, L. F., M. N. Levy, L. Weinstein, and J. E. Weiss. (1936). *Lactobacillus acidophilus* and its therapeutic application. *Am J Med Sci*. 191: 123.

Ristow, P., P. Bourhy, F. W. da Cruz McBride, C. P. Figueira, M. Huerre, P. Ave, I. S. Girons, A. I. Ko, and M. Picardeau. (2007). The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS pathogens*. 3: e97.

Rodriguez, E., J. L. Arques, R. Rodriguez, M. Nunez, and M. Medina. (2003). Reuterin production by lactobacilli isolated from pig faeces and evaluation of probiotic traits. *Lett Appl Microbiol*. 37: 259-263.

Rohde, J., and K. Habighorst-Blome. (2012). An up-date on the differentiation of *Brachyspira* species from pigs with nox-PCR-based restriction fragment length polymorphism. *Vet Microbiol*. 158: 211-215.

Romano, A. H., and T. Conway. (1996). Evolution of carbohydrate metabolic pathways. *Res Microbiol*. 147: 448-455.

Ronne, H., and J. Szancer. (1990). *In vitro* susceptibility of Danish field isolates of *Treponema hyodysenteriae* to chemotherapeutics in swine dysentery (SD) therapy. Interpretation of MIC results based on the pharmacokinetic properties of the antibacterial agents. *In*: International Pig Veterinary Society, 11th Congress, Lausanne, Switzerland.

Roper, D. I., T. Huyton, A. Vagin, and G. Dodson. (2000). The molecular basis of vancomycin resistance in clinically relevant enterococci: crystal structure of D-alanyl-D-lactate ligase (VanA). *Proc Natl Acad Sci U S A*. 97: 8921-8925.

Rosander, A., E. Connolly, and S. Roos. (2008). Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Appl Environ Microbiol*. 74: 6032-6040.

Rosey, E. L., M. J. Kennedy, and R. J. Yancey, Jr. (1996). Dual *flaA1 flaB1* mutant of *Serpulina hyodysenteriae* expressing periplasmic flagella is severely attenuated in a murine model of swine dysentery. *Infect Immun*. 64: 4154-4162.

Rosey, E. L., M. J. Kennedy, D. K. Petrella, R. G. Ulrich, and R. J. Yancey, Jr. (1995). Inactivation of *Serpulina hyodysenteriae flaA1* and *flaB1* periplasmic flagellar genes by electroporation-mediated allelic exchange. *J Bacteriol*. 177: 5959-5970.

Rotterdam, H. (1997). Intestinal spirochetosis, pp. 583-588. *In*: D. H. Conner, F. W. Chandler, D. A. Schwartz, H. J. Manz and E. E. Lack (eds.), Pathology of Infectious Diseases. McGraw-Hill Professional, New York.

Royal, W. A., and M. D. Mutimer. (1972). Inhibition of *Salmonella* Typhimurium by fowl caecal cultures. *Res Vet Sci*. 13: 184-185.

Ruas-Madiedo, P., M. Gueimonde, A. Margolles, C. G. de los Reyes-Gavilan, and S. Salminen. (2006). Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. *J Food Prot*. 69: 2011-2015.

Ryter, A., and J. Pillot. (1965). Structure of spirochetes, II: study of the genus *Cristispira* by optic microscope and electron microscope. *Ann Inst Pasteur*. 109: 552-562.

Sacco, R. E., D. W. Trampel, and M. J. Wannemuehler. (1997). Experimental infection of C3H mice with avian, porcine, or human isolates of *Serpulina pilosicoli*. *Infect Immun*. 65: 5349-5353.

Sagartz, J. E., D. E. Swayne, K. A. Eaton, J. R. Hayes, K. D. Amass, R. Wack, and L. Kramer. (1992). Necrotizing typhlocolitis associated with a spirochete in rheas (*Rhea americana*). *Avian Dis*. 36: 282-289.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 239: 487-491.

Sakharoff, M. N. (1891). *Spirochaeta anserina* et la septicemie des oies. *Ann Inst Pasteur*. 5: 564-566.

Sako, T., K. Matsumoto, and R. Tanaka. (1999). Recent progress on research and applications of non-digestible galactooligosaccharides. *Int Dairy J*. 9: 69-80.

Salminen, S., A. von Wright, L. Morelli, P. Marteau, D. Brassart, W. M. de Vos, R. Fonden, M. Saxelin, K. Collins, G. Mogensen, S. E. Birkeland, and T. Mattila-Sandholm. (1998). Demonstration of safety of probiotics -- a review. *Int J Food Microbiol*. 44: 93-106.

SCAN. (2001). Guidelines for the assessment of additives in feedingstuffs, part II: enzymes and microorganisms. European Commission, Health and Consumer Protection Directorate-General, ec.europa.eu/food/fs/sc/scan/out68_en.pdf 01/05/12.

SCAN. (2003a). Opinion of the scientific committee on animal nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human and veterinary importance. European Commission, Health and Consumer protection Directorate-General, ec.europa.eu/food/fs/sc/scan/out108_en.pdf 01/05/12.

SCAN. (2003b). Opinion on the use of certain micro-organisms as additives in feedingstuffs, ec.europa.eu/food/fs/sc/scan/out93_en.pdf 14/07/12.

Schachtsiek, M., W. P. Hammes, and C. Hertel. (2004). Characterization of *Lactobacillus coryniformis* DSM 20001^T surface protein Cpf mediating coaggregation with and aggregation among pathogens. *Appl Environ Microbiol*. 70: 7078-7085.

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. *Gut*. 35: S35-38.

Schmitz-Esser, S., T. Penz, A. Spang, and M. Horn. (2011). A bacterial genome in transition--an exceptional enrichment of IS elements but lack of evidence for recent transposition in the symbiont *Amoebophilus asiaticus*. *BMC Evol Biol*. 11: 270.

Schneitz, C. (2005). Competitive exclusion in poultry - 30 years of research. *Food Control*. 16: 657-667.

Schneitz, C., L. Nuotio, G. Mead, and E. Nurmi. (1992). Competitive exclusion in the young bird: challenge models, administration and reciprocal protection. *Int J Food Microbiol*. 15: 241-244.

Schneitz, C., M. Hakkinen, L. Nuotio, E. Nurmi, and G. Mead. (1990). Droplet application for protecting chicks against *Salmonella* colonisation by competitive exclusion. *Vet Rec*. 126: 510.

Schoeni, J. L., and A. C. Wong. (1994). Inhibition of *Campylobacter jejuni* colonization in chicks by defined competitive exclusion bacteria. *Appl Environ Microbiol*. 60: 1191-1197.

Scholtens, P. A. M. J., P. Alliet, M. Raes, M. S. Alles, H. Kroes, G. Boehm, L. M. J. Knippels, J. Knol, and Y. Vandenplas. (2008). Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galactooligosaccharides and long-chain fructooligosaccharides. *J Nutr*. 138: 1141-1147.

Schrezenmeir, J., and M. de Vrese. (2001). Probiotics, prebiotics, and synbiotics--approaching a definition. *Am J Clin Nutr*. 73: 361S-364S.

Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A*. 95: 5857-5864.

Schultz, M., H. J. Linde, N. Lehn, K. Zimmermann, J. Grossmann, W. Falk, and J. Scholmerich. (2003). Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J Dairy Res*. 70: 165-173.

Schwab, C. G., J. J. Moore, P. M. Hoyt, and J. L. Prentice. (1980). Performance and caecal flora of calves fed nonviable *Lactobacillus bulgaricus* fermentation product. *J Dairy Sci*. 63: 1412-1423.

Se, C. Y., F. F. Yong, H. M. Tan, W. M. Yeo, and A. Y. L. Teo inventors. (2008). Broad-spectrum antibacterial and antifungal activity of *Lactobacillus johnsonii* D115. Patent. United States of America.

Searle, L. E., W. A. Cooley, G. Jones, A. Nunez, B. Crudgington, U. Weyer, A. H. Dugdale, G. Tzortzis, J. W. Collins, M. J. Woodward, and R. M. La Ragione. (2010). Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*. *J Med Microbiol*. 59: 1428-1439.

Searle, L. E., A. Best, A. Nunez, F. J. Salguero, L. Johnson, U. Weyer, A. H. Dugdale, W. A. Cooley, B. Carter, G. Jones, G. Tzortzis, M. J. Woodward, and R. M. La Ragione. (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium infection in mice. *J Med Microbiol*. 58: 37-48.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol*. 51: 873-884.

Sellwood, R., and A. P. Bland. (1997). Ultrastructure of intestinal spirochaetes, pp. 109-149. *In*: D. J. Hampson and T. B. Stanton (eds.), Intestinal Spirochaetes in Domestic Animals and Humans. CAB International, Oxon.

Seuna, E., M. Raevuori, and E. Nurmi. (1978). An epizootic of *Salmonella* Typhimurium var. Copenhagen in broilers and the use of cultured chicken intestinal flora for its control. *Br Poult Sci*. 19: 309-314.

Shah, P., D. G. Romero, and E. Swiatlo. (2008). Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia. *Microb Pathog*. 45: 167-172.

Sharma, A., H. T. Sojar, I. Glurich, K. Honma, H. K. Kuramitsu, and R. J. Genco. (1998). Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *Bacteroides forsythus* ATCC 43037. *Infect Immun*. 66: 5703-5710.

Shivaprasad, H. L., and G. E. Duhamel. (2005). Cecal spirochetosis caused by *Brachyspira pilosicoli* in commercial turkeys. *Avian Dis*. 49: 609-613.

Shortt, C. (1999). The probiotic century: historical and current perspectives. *Trends Food Sci Tech*. 10: 411-417.

Sierig, G., C. Cywes, M. R. Wessels, and C. D. Ashbaugh. (2003). Cytotoxic effects of streptolysin O and streptolysin S enhance the virulence of poorly encapsulated group a streptococci. *Infect Immun*. 71: 446-455.

Silver, S., and M. Walderhaug. (1992). Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microb Mol Biol Rev*. 56: 195-228.

Simon, O., A. Jadamus, and W. Vahjen. (2001). Probiotic feed additives - effectiveness and expected modes of action. *J Anim Feed Sci*. 10: 51-67.

Sissons, J. W. (1989). Potential of probiotic organisms to prevent diarrhea and promote digestion in farm animals; a review. *J Food Agric Sci*. 49: 1-13.

Skrivanova, E., and M. Marounek. (2007). Influence of pH on antimicrobial activity of organic acids against rabbit enteropathogenic strain of *Escherichia coli*. *Folia Microbiol (Praha)*. 52: 70-72.

Smit, H. F., R. M. Dwars, F. G. Davelaar, and G. A. Wijtten. (1998). Observations on the influence of intestinal spirochaetosis in broiler breeders on the performance of their progeny and on egg production. *Avian Pathol*. 27: 133-141.

Smith, J. L. (2005). Colonic spirochetosis in animals and humans. *J Food Prot*. 68: 1525-1534.

Soerjadi, A. S., G. H. Snoeyenbos, and O. M. Weinack. (1982a). Intestinal colonization and competitive exclusion of *Campylobacter fetus* subsp. *jejuni* in young chicks. *Avian Dis*. 26: 520-524.

Soerjadi, A. S., R. Rufner, G. H. Snoeyenbos, and O. M. Weinack. (1982b). Adherence of salmonellae and native gut microflora to the gastrointestinal mucosa of chicks. *Avian Dis*. 26: 576-584.

Soerjadi-Liem, A. S., G. H. Snoeyenbos, and O. M. Weinack. (1984). Comparative studies on competitive exclusion of three isolates of *Campylobacter fetus* subsp. *jejuni* in chickens by native gut microflora. *Avian Dis*. 28: 139-146.

Song, Y., B. Frey, and D. J. Hampson. (2012). The use of ELISAs for monitoring exposure of pig herds to *Brachyspira hyodysenteriae*. *BMC Vet Res*. 6.

Song, H., J. Hwang, H. Yi, R. L. Ulrich, Y. Yu, W. C. Nierman, and H. S. Kim. (2010). The early stage of bacterial genome-reductive evolution in the host. *PLoS pathogens*. 6: e1000922.

Songer, J. G., J. M. Kinyon, and D. L. Harris. (1976). Selective medium for isolation of *Treponema hyodysenteriae*. *J Clin Microbiol*. 4: 57-60.

Sonnhammer, E. L., and R. Durbin. (1995). A dot-matrix program with dynamic threshold control suited for genomic DNA and protein sequence analysis. *Gene*. 167: GC1-10.

Sonnhammer, E. L., S. R. Eddy, and R. Durbin. (1997). Pfam: a comprehensive database of protein domain families based on seed alignments. *Proteins*. 28: 405-420.

Sorek, R., V. Kunin, and P. Hugenholtz. (2008). CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat Rev Microbiol*. 6: 181-186.

Sperti, G. S. (1971). Probiotics. Avi Publishing Co, West Point.

Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. (2000). The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. *Poult Sci*. 79: 205-211.

Stanton, T. B. (1989). Glucose metabolism and NADH recycling by *Treponema hyodysenteriae*, the agent of swine dysentery. *Appl Environ Microbiol*. 55: 2365-2371.

Stanton, T. B. (1992). Proposal to change the genus designation *Serpula* to *Serpulina* gen. nov. containing the species *Serpulina hyodysenteriae* comb. nov. and *Serpulina innocens* comb. nov. *Int J Syst Bacteriol*. 42: 189-190.

Stanton, T. B. (2006). The Genus *Brachyspira*, pp. 330-356. *In*: M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer and E. Stackebrandt (eds.), The Prokaryotes, 3rd edn. Springer, New York.

Stanton, T. B., and C. P. Cornell. (1987). Erythrocytes as a source of essential lipids for *Treponema hyodysenteriae*. *Infect Immun*. 55: 304-308.

Stanton, T. B., and D. F. Lebo. (1988). *Treponema hyodysenteriae* growth under various culture conditions. *Vet Microbiol*. 18: 177-190.

Stanton, T. B., and N. S. Jensen. (1993). Purification and characterization of NADH oxidase from *Serpulina* (*Treponema*) *hyodysenteriae*. *J Bacteriol*. 175: 2980-2987.

Stanton, T. B., B. L. Hanzelka, and N. S. Jensen. (1995). Survey of intestinal spirochaetes for NADH oxidase by gene probe and enzyme assay. *Microb Ecol Health Dis*. 8: 93-100.

Stanton, T. B., D. Postic, and N. S. Jensen. (1998). *Serpulina alvinipulli* sp. nov., a new *Serpulina* species that is enteropathogenic for chickens. *Int J Syst Bacteriol*. 48: 669-676.

Stanton, T. B., M. G. Thompson, S. B. Humphrey, and R. L. Zuerner. (2003). Detection of bacteriophage VSH-1 svp38 gene in *Brachyspira* spirochetes. *FEMS Microbiol Lett*. 224: 225-229.

Stanton, T. B., S. B. Humphrey, D. O. Bayles, and R. L. Zuerner. (2009). Identification of a divided genome for VSH-1, the prophage-like gene transfer agent of *Brachyspira hyodysenteriae*. *J Bacteriol*. 191: 1719-1721.

Stanton, T. B., E. L. Rosey, M. J. Kennedy, N. S. Jensen, and B. T. Bosworth. (1999). Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete *Brachyspira* (*Serpulina*) *hyodysenteriae*, etiologic agent of swine dysentery. *Appl Environ Microbiol*. 65: 5028-5034.

Stanton, T. B., N. S. Jensen, T. A. Casey, L. A. Tordoff, F. E. Dewhirst, and B. J. Paster. (1991). Reclassification of *Treponema hyodysenteriae* and *Treponema innocens* in a new genus, *Serpula* gen. nov., as *Serpula hyodysenteriae* comb. nov. and *Serpula innocens* comb. nov. *Int J Syst Bacteriol*. 41: 50-58.

Stanton, T. B., D. J. Trott, J. I. Lee, A. J. McLaren, D. J. Hampson, B. J. Paster, and N. S. Jensen. (1996). Differentiation of intestinal spirochaetes by multilocus enzyme electrophoresis analysis and 16S rRNA sequence comparisons. *FEMS Microbiol Lett*. 136: 181-186.

Stanton, T. B., E. Fournie-Amazouz, D. Postic, D. J. Trott, P. A. Grimont, G. Baranton, D. J. Hampson, and I. Saint Girons. (1997). Recognition of two new species of intestinal spirochetes: *Serpulina intermedia* sp. nov. and *Serpulina murdochii* sp. nov. *Int J Syst Bacteriol*. 47: 1007-1012.

Stavric, S. (1987). Microbial colonization control of chicken intestine using defined cultures. *Food Technol*. 43: 93-98.

Stavric, S., and J. Y. d'Aoust. (1993). Undefined and defined bacterial preparations for the competitive exclusion of *Salmonella* in poultry – a review. *J Food Prot*. 56: 173-180.

Stavric, S., B. Buchnan, and T. M. Gleeson. (1991). Competitive exclusion of *Escherichia coli* O157:H7 from chicks with anaerobic cultures of faecal microflora. *Lett Appl Microbiol*. 14: 191-193.

Stavric, S., B. Buchanan, and T. M. Gleeson. (1993). Intestinal colonization of young chicks with *Escherichia coli* O157:H7 and other verotoxin-producing serotypes. *J Appl Bacteriol*. 74: 557-563.

Stephens, C. P., and D. J. Hampson. (1999). Prevalence and disease association of intestinal spirochaetes in chickens in eastern Australia. *Avian Pathol*. 28: 447-454.

Stephens, C. P., and D. J. Hampson. (2001). Intestinal spirochete infections of chickens: a review of disease associations, epidemiology and control. *Anim Health Res Rev*. 2: 83-91.

Stephens, C. P., and D. J. Hampson. (2002a). Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli* causes reduced egg production. *Avian Pathol*. 31: 169-175.

Stephens, C. P., and D. J. Hampson. (2002b). Evaluation of tiamulin and lincomycin for the treatment of broiler breeders experimentally infected with the intestinal spirochaete *Brachyspira pilosicoli*. *Avian Pathol*. 31: 299-304.

Stephens, C. P., S. L. Oxberry, N. D. Phillips, T. La, and D. J. Hampson. (2005). The use of multilocus enzyme electrophoresis to characterise intestinal spirochaetes (*Brachyspira* spp.) colonising hens in commercial flocks. *Vet Microbiol*. 107: 149-157.

Stern, N. J., N. A. Cox, J. S. Bailey, M. E. Berrang, and M. T. Musgrove. (2001). Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. *Poult Sci*. 80: 156-160.

Stern, N. J., E. A. Svetoch, B. V. Eruslanov, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. D. Pokhilenko, V. P. Levchuk, O. E. Svetoch, and B. S. Seal. (2006). Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. *Antimicrob Agents Chemother*. 50: 3111-3116.

Stoutenburg, J. W. (1993). Studies of intestinal spirochetes in avian species. Thesis. Master of Science, Ohio State University.

Stoutenburg, J. W., and D. E. Swayne. (1992). Comparison of microbiologic characteristics of spirochaetes isolated from pigs, rats, chickens and ratites. *Poult Sci*. 71: 69.

Stoutenburg, J. W., D. E. Swayne, T. M. Hoepf, R. Wack, and L. Kramer. (1995). Frequency of intestinal spirochaetes in avian species from a zoological collection and private rhea farms in Ohio. *J Zoo Wildl Med*. 26: 272-278.

Strus, M., M. Brzychczy-Wloch, P. Kochan, and P. Heczko. (2004). Hydrogen peroxide produced by *Lactobacillus* species as a regulatory molecule for vaginal microflora. *Med Dosw Mikrobiol*. 56: 67-77.

Stuart, R. L., H. C. Surprise, and L. W. Davis. (1978). Response of growing rats to diets supplemented with a liquid nonviable *Lactobacillus* fermentation product. *J Animal Sci*. 47: 322.

Su, J., H. Gong, J. Lai, A. Main, and S. Lu. (2009). The potassium transporter Trk and external potassium modulate *Salmonella enterica* protein secretion and virulence. *Infect Immun*. 77: 667-675.

Sueyoshi, M., and Y. Adachi. (1990). Diarrhea induced by *Treponema hyodysenteriae*: a young chick cecal model for swine dysentery. *Infect Immun*. 58: 3348-3362.

Sueyoshi, M., Y. Adachi, and S. Shoya. (1987). Enteropathogenicity of *Treponema hyodysenteriae* in young chicks. *Zentralbl Bakteriol Mikrobiol Hyg A*. 266: 469-477.

Sueyoshi, M., Y. Adachi, S. Shoya, E. Miyagawa, and H. Minato. (1986). Investigations into the location of *Treponema hyodysenteriae* in the cecum of experimentally infected young broiler chicks by light- and electronmicroscopy. *Zentralbl Bakteriol Mikrobiol Hyg A*. 261: 447-453.

Surawicz, C. M. (2008). Role of probiotics in antibiotic-associated diarrhea, *Clostridium difficile*-associated diarrhea, and recurrent *Clostridium difficile*-associated diarrhea. *J Clin Gastroenterol*. 42: S64-70.

Suzek, B. E., H. Huang, P. McGarvey, R. Mazumder, and C. H. Wu. (2007). UniRef: comprehensive and non-redundant UniProt reference clusters. *Bioinformatics*. 23: 1282-1288.

Svetoch, E. A., B. V. Eruslanov, V. P. Levchuk, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, J. Stepanshin, I. Dyatlov, B. S. Seal, and N. J. Stern. (2011). Isolation of *Lactobacillus salivarius* 1077 (NRRL B-50053) and characterization of its bacteriocin, including the antimicrobial activity spectrum. *Appl Environ Microbiol*. 77: 2749-2754.

Swayne, D. E. (1994). Pathobiology of intestinal spirochetosis in mammals and birds. *Proc Ann Meet Am Coll Vet Pathol*. 45: 224-238.

Swayne, D. E. (1997). Avian intestinal spirochaetosis, pp. 325-332. *In*: B. W. Calnek (ed.), Disease of Poultry. Mosby-Wolfe, Iowa.

Swayne, D. E., and A. J. McLaren. (1997). Avian intestinal spirochaetes and avian intestinal spirochaetosis, pp. 267-300. *In*: D. J. Hampson and T. B. Stanton (eds.), Intestinal Spirochaetes in Domestic Animals and Humans. CAB International, Oxon.

Swayne, D. E., K. A. Eaton, J. W. Stoutenburg, and E. L. Buckles. (1993). Comparison of the ability of orally inoculated avian-, pig-, and rat-origin spirochetes to produce enteric disease in 1-day-old chickens. *Proc Amer Vet Med Assoc*. 130: 155.

Swayne, D. E., K. A. Eaton, J. Stoutenburg, D. J. Trott, D. J. Hampson, and N. S. Jensen. (1995). Identification of a new intestinal spirochete with pathogenicity for chickens. *Infect Immun*. 63: 430-436.

Swayne, D. E., A. J. Bermudez, J. E. Sagartz, K. A. Eaton, J. D. Monfort, J. W. Stoutenburg, and J. R. Hayes. (1992). Association of cecal spirochetes with pasty vents and dirty eggshells in layers. *Avian Dis*. 36: 776-781.

Szylit, O., M. Champ, N. Ait-Abdelkader, and P. Raibaud. (1980). Role of five *Lactobacillus* strains on carbohydrate degradation in monoxenic chickens. *Reprod Nutr Dev*. 20: 1701-1706.

Tachibana, H., S. Nakamura, and Y. Adachi. (2003). Proposal for *Brachyspira ibaraki* sp. nov. for Japanese human intestinal spirochaetes closely related to *Brachyspira aalborgi*. *In*: Proceedings of the 2nd International Conference on Colonic Spirochaetal Infections in Animals and Humans, Scotland, UK.

Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. (1976). Bacteriocins of Gram-positive bacteria. *Bacteriol Rev*. 40: 722-756.

Takeda, K., H. Miyatake, N. Yokota, S. Matsuyama, H. Tokuda, and K. Miki. (2003). Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. *EMBO J*. 22: 3199-3209.

Tamura, G. S., A. Nittayajarn, and D. L. Schoentag. (2002). A glutamine transport gene, *glnQ*, is required for fibronectin adherence and virulence of group B streptococci. *Infect Immun*. 70: 2877-2885.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 28: 2731-2739.

Tang, Y. P., M. M. Dallas, and M. H. Malamy. (1999). Characterization of the Batl (*Bacteroides* aerotolerance) operon in *Bacteroides fragilis*: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in *in vivo* model systems. *Mol Microbiol*. 32: 139-149.

Tannock, G. W., and D. C. Savage. (1974). Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. *Infect Immun*. 9: 591-598.

Tatusov, R. L., E. V. Koonin, and D. J. Lipman. (1997). A genomic perspective on protein families. *Science*. 278: 631-637.

Taylor, D. J., and T. J. Alexander. (1971). The production of dysentery in swine by feeding cultures containing a spirochaete. *Br Vet J*. 127: 58-61.

Taylor, D. J., J. R. Simmons, and H. M. Laird. (1980). Production of diarrhoea and dysentery in pigs by feeding pure cultures of a spirochaete differing from *Treponema hyodysenteriae*. *Vet Rec*. 106: 326-332.

Teige, J., Jr., T. Landsverk, A. Lund, and H. J. Larsen. (1981). Swine dysentery: a scanning electron microscopic investigation. *Acta Vet Scand*. 22: 218-225.

Tenaya, I. W., W. J. Penhale, and D. J. Hampson. (1998). Preparation of diagnostic polyclonal and monoclonal antibodies against outer envelope proteins of *Serpulina pilosicoli*. *J Med Microbiol*. 47: 317-324.

ter Huurne, A. A., M. van Houten, S. Muir, J. G. Kusters, B. A. van der Zeijst, and W. Gaastra. (1992). Inactivation of a *Serpula* (*Treponema*) *hyodysenteriae* hemolysin gene by homologous recombination: importance of this hemolysin in pathogenesis in mice. *FEMS Microbiol Lett*. 71: 109-113.

Teuber, M., L. Meile, and F. Schwarz. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek*. 76: 115-137.

Thomson, J. R., B. P. Murray, L. E. Henderson, J. Thacker, and D. G. S. Burch. (2007). *Brachyspira* species isolated from UK poultry samples. *In*: Proceedings of the 4th International Conference on Colonic Spirochaetal Infections in Animals and Humans, Prague, Czech Republic.

Thuma, A., A. Dan, E. Kaszanyitzky, B. Fazekas, A. Toth, and R. Glavits. (2011). Experimental inoculation of day-old ducks with *Brachyspira pilosicoli* and *B. alvinipulli*. *Acta Vet Hung*. 59: 165-174.

Tillier, E. R., and R. A. Collins. (2000). Genome rearrangement by replication-directed translocation. *Nat Genet*. 26: 195-197.

Timmerman, H. M., C. J. Koning, L. Mulder, F. M. Rombouts, and A. C. Beynen. (2004). Monostrain, multistrain and multispecies probiotics--A comparison of functionality and efficacy. *Int J Food Microbiol*. 96: 219-233.

Timmerman, H. M., A. Veldman, E. van den Elsen, F. M. Rombouts, and A. C. Beynen. (2006). Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. *Poult Sci*. 85: 1383-1388.

Tissier, H. (1900). Recherches sur la flore intestinale normale et pathologique du nourisson. Thesis. Doctor of Philosophy, University of Paris.

Tissier, H. (1906). Tritement des infections intestinales par la methode de translormation de la flore bacterienne de l'intestin. *C R Soc Biol*. 60: 359-361.

Tortuero, F. (1973). Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. *Poult Sci*. 52: 197-203.

Tortuero, F., and E. Fernandez. (1995). Effects of inclusion of microbial cultures in barley-based diets fed to laying hens. *Anim Feed Sci Technol*. 53: 255-265.

Trampel, D. W., N. S. Jensen, and L. J. Hoffman. (1994). Cecal spirochetosis in commercial laying hens. *Avian Dis*. 38: 895-898.

Trampel, D. W., J. M. Kinyon, and N. S. Jensen. (1999). Minimum inhibitory concentration of selected antimicrobial agents for *Serpulina* isolated from chickens and rheas. *J Vet Diagn Invest*. 11: 379-382.

Trivett-Moore, N. L., G. L. Gilbert, C. L. Law, D. J. Trott, and D. J. Hampson. (1998). Isolation of *Serpulina pilosicoli* from rectal biopsy specimens showing evidence of intestinal spirochetosis. *J Clin Microbiol*. 36: 261-265.

Trott, D. J., and D. J. Hampson. (1998). Evaluation of day-old specific pathogen-free chicks as an experimental model for pathogenicity testing of intestinal spirochaete species. *J Comp Pathol*. 118: 365-381.

Trott, D. J., A. J. McLaren, and D. J. Hampson. (1995). Pathogenicity of human and porcine intestinal spirochetes in one-day-old specific-pathogen-free chicks: an animal model of intestinal spirochetosis. *Infect Immun*. 63: 3705-3710.

Trott, D. J., C. R. Huxtable, and D. J. Hampson. (1996a). Experimental infection of newly weaned pigs with human and porcine strains of *Serpulina pilosicoli*. *Infect Immun*. 64: 4648-4654.

Trott, D. J., T. B. Stanton, N. S. Jensen, and D. J. Hampson. (1996b). Phenotypic characteristics of *Serpulina pilosicoli* the agent of intestinal spirochaetosis. *FEMS Microbiol Lett*. 142: 209-214.

Trott, D. J., A. S. Mikosza, B. G. Combs, S. L. Oxberry, and D. J. Hampson. (1998). Population genetic analysis of *Serpulina pilosicoli* and its molecular epidemiology in villages in the eastern Highlands of Papua New Guinea. *Int J Syst Bacteriol*. 48: 659-668.

Trott, D. J., R. F. Atyeo, J. I. Lee, D. A. Swayne, J. W. Stoutenburg, and D. J. Hampson. (1996d). Genetic relatedness amongst intestinal spirochaetes isolated from rats and birds. *Lett Appl Microbiol*. 23: 431-436.

Trott, D. J., T. B. Stanton, N. S. Jensen, G. E. Duhamel, J. L. Johnson, and D. J. Hampson. (1996d). *Serpulina pilosicoli* sp. nov., the agent of porcine intestinal spirochetosis. *Int J Syst Bacteriol*. 46: 206-215.

Trott, D. J., N. S. Jensen, I. Saint Girons, S. L. Oxberry, T. B. Stanton, D. Lindquist, and D. J. Hampson. (1997a). Identification and characterization of *Serpulina pilosicoli* isolates recovered from the blood of critically ill patients. *J Clin Microbiol*. 35: 482-485.

Trott, D. J., B. G. Combs, A. S. Mikosza, S. L. Oxberry, I. D. Robertson, M. Passey, J. Taime, R. Sehuko, M. P. Alpers, and D. J. Hampson. (1997b). The prevalence of *Serpulina pilosicoli* in humans and domestic animals in the eastern highlands of Papua New Guinea. *Epidemiol Infect*. 119: 369-379.

Tsai, I. J., T. D. Otto, and M. Berriman. (2010). Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps. *Genome Biol*. 11: R41.

Tsai, C. C., H. Y. Hsih, H. H. Chiu, Y. Y. Lai, J. H. Liu, B. Yu, and H. Y. Tsen. (2005). Antagonistic activity against *Salmonella* infection *in vitro* and *in vivo* for two *Lactobacillus* strains from swine and poultry. *Int J Food Microbiol*. 102: 185-194.

Tsinganou, E., and J. O. Gebbers. (2010). Human intestinal spirochetosis--a review. *Ger Med Sci*. 8: Doc01.

Turpin, W., C. Humblot, M. L. Noordine, M. Thomas, and J. P. Guyot. (2012). *Lactobacillaceae* and cell adhesion: genomic and functional screening. *PLoS One*. 7: e38034.

Tzortzis, G., A. K. Goulas, J. M. Gee, and G. R. Gibson. (2005). A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs *in vivo*. *J Nutr*. 135: 1726-1731.

Upreti, G. C., and R. D. Hinsdill. (1975). Production and mode of action of lactocin 27: bacteriocin from a homofermentative *Lactobacillus*. *Antimicrob Agents Chemother*. 7: 139-145.

van de Peer, Y., P. de Rijk, J. Wuyts, T. Winkelmans, and R. de Wachter. (2000). The European small subunit ribosomal RNA database. *Nucleic Acids Res*. 28: 175-176.

van Immerseel, F., J. B. Russell, M. D. Flythe, I. Gantois, L. Timbermont, F. Pasmans, F. Haesebrouck, and R. Ducatelle. (2006). The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathol*. 35: 182-188.

Vanderpool, C., F. Yan, and D. B. Polk. (2008). Mechanisms of probiotic action: implications for therapeutic applications in inflammatory bowel diseases. *Inflamm Bowel Dis*. 14: 1585-1596.

Velayudhan, J., N. J. Hughes, A. A. McColm, J. Bagshaw, C. L. Clayton, S. C. Andrews, and D. J. Kelly. (2000). Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol*. 37: 274-286.

Verlinden, M., F. Boyen, F. Pasmans, A. Garmyn, F. Haesebrouck, and A. Martel. (2011). Antimicrobial susceptibility pattern of *Brachyspira intermedia* isolates from European layers. *Microb Drug Resist*. 17: 485-488.

Vicente, J. L., A. Torres-Rodriguez, S. E. Higgins, C. Pixley, G. Tellez, A. M. Donoghue, and B. M. Hargis. (2008). Effect of a selected *Lactobacillus* spp.-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Dis*. 52: 143-146.

Vimal, D. B., M. Khullar, S. Gupta, and N. K. Ganguly. (2000). Intestinal mucins: the binding sites for *Salmonella* Typhimurium. *Mol Cell Biochem*. 204: 107-117.

von Wright, A. (2005). Regulating the safety of probiotics - the European approach. *Curr Pharm Des*. 11: 17-23.

Vos, A. P., L. M'Rabet, B. Stahl, G. Boehm, and J. Garssen. (2007). Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. *Crit Rev Immunol*. 27: 97-140.

Wagenaar, J. A., M. A. P. van Bergen, I. van der Graaf, and W. J. M. Landman. (2003). Free-range chickens show a higher incidence of *Brachyspira* infections in the Netherlands. *In*: Proceedings of the 2nd International Conference on Colonic Spirochaetal Infections in Animals and Humans, Scotland, UK.

Wallace, R. J. (1994). Ruminal microbiology, biotechnology, and ruminant nutrition: progress and problems. *J Anim Sci*. 72: 2992-3003.

Wanchanthuek, P., M. I. Bellgard, T. La, K. Ryan, P. Moolhuijzen, B. Chapman, M. Black, D. Schibeci, A. Hunter, R. Barrero, N. D. Phillips, and D. J. Hampson. (2010). The complete genome sequence of the pathogenic intestinal spirochete *Brachyspira pilosicoli* and comparison with other *Brachyspira* genomes. *PLoS ONE*. 5: e11455.

Wang, J., H. Zhang, X. Chen, Y. Chen, Menghebilige, and Q. Bao. (2012). Selection of potential probiotic lactobacilli for cholesterol-lowering properties and their effect on cholesterol metabolism in rats fed a high-lipid diet. *J Dairy Sci*. 95: 1645-1654.

Warnecke, F., and P. Hugenholtz. (2007). Building on basic metagenomics with complementary technologies. *Genome Biol*. 8: 231.

Webb, D. M., G. E. Duhamel, M. R. Mathiesen, N. Muniappa, and A. K. White. (1997). Cecal spirochetosis associated with *Serpulina pilosicoli* in captive juvenile ring-necked pheasants. *Avian Dis*. 41: 997-1002.

Webber, M. A., and L. J. Piddock. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother*. 51: 9-11.

Weinack, O. M., G. H. Snoeyenbos, C. F. Smyser, and A. S. Soerjadi. (1981). Competitive exclusion of intestinal colonization of *Escherichia coli* in chicks. *Avian Dis*. 25: 696-705.

Weinack, O. M., G. H. Snoeyenbos, C. F. Smyser, and A. S. Soerjadi. (1982). Reciprocal competitive exclusion of *Salmonella* and *Escherichia coli* by native intestinal microflora of the chicken and turkey. *Avian Dis*. 26: 585-595.

Weisman, Y., A. Herz, Y. Yegana, M. N. Egyed, and A. Shlosberg. (1983). The effect of tiamulin administered by different routes and at different ages to turkeys receiving monensin in their feed. *Vet Res Commun*. 6: 189-198.

Whipp, S. C., I. M. Robinson, D. L. Harris, R. D. Glock, P. J. Matthews, and T. J. Alexander. (1979). Pathogenic synergism between *Treponema hyodysenteriae* and other selected anaerobes in gnotobiotic pigs. *Infect Immun*. 26: 1042-1047.

Whitfield, C. (1995). Biosynthesis of lipopolysaccharide O antigens. *Trends Microbiol*. 3: 178-185.

Wier, A., M. Dolan, D. Grimaldi, R. Guerrero, J. Wagensberg, and L. Margulis. (2002). Spirochete and protist symbionts of a termite (*Mastotermes electrodominicus*) in miocene amber. *Proc Natl Acad Sci U S A*. 99: 1410-1413.

Wierup, M., M. Wold-Troell, E. Nurmi, and M. Hakkinen. (1988). Epidemiological evaluation of the *Salmonella*-controlling effect of a nationwide use of a competitive exclusion culture in poultry. *Poult Sci*. 67: 1026-1033.

Wildschutte, H., D. M. Wolfe, A. Tamewitz, and J. G. Lawrence. (2004). Protozoan predation, diversifying selection, and the evolution of antigenic diversity in *Salmonella*. *Proc Natl Acad Sci U S A*. 101: 10644-10649.

Wilson, K. (1990). Preparation of genomic DNA from bacteria, pp. 241- 242. *In*: F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith and K. Struhl (eds.), Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Wilson, K. H., and F. Perini. (1988). Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. *Infect Immun*. 56: 2610-2614.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews*. 51: 221-271.

Wolfenden, A. D., C. M. Pixley, J. P. Higgins, S. E. Higgins, B. M. Hargis, G. Tellez, J. L. Vicente, and A. Torres-Rodriguez. (2007). Evaluation of spray

application of a *Lactobacillus*-based probiotic on *Salmonella enteritidis* colonization in broiler chickens. *Int J Poult Sci*. 6: 493-496.

Wood, E. J., R. J. Seviour, A. B. Siddique, R. W. Glaisher, R. I. Webb, and D. J. Trott. (2006). Spherical body formation in the spirochaete *Brachyspira hyodysenteriae*. *FEMS Microbiol Lett*. 259: 14-19.

Wu, J. A., C. Kusuma, J. J. Mond, and J. F. Kokai-Kun. (2003). Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. *Antimicrob Agents Chemother*. 47: 3407-3414.

Xu, J., and J. I. Gordon. (2003). Inaugural Article: Honor thy symbionts. *Proc Natl Acad Sci U S A*. 100: 10452-10459.

Yeh, Y. H., and D. F. Hwang. (2001). High-performance liquid chromatographic determination for bile components in fish, chicken and duck. *J Chromatogr B Biomed Sci Appl*. 751: 1-8.

Yeung, P. S., M. E. Sanders, C. L. Kitts, R. Cano, and P. S. Tong. (2002). Species-specific identification of commercial probiotic strains. *J Dairy Sci*. 85: 1039-1051.

Yu, B., and H. Y. Tsen. (1993). *Lactobacillus* cells in the rabbit digestive tract and the factors affecting their distribution. *J Appl Bacteriol*. 75: 269-275.

Zgurskaya, H. I., G. Krishnamoorthy, A. Ntreh, and S. Lu. (2011). Mechanism and function of the outer membrane channel TolC in multidrug resistance and physiology of Enterobacteria. *Front Microbiol*. 2: 189.

Zhang, P., X. Cheng, and G. E. Duhamel. (2000). Cloning and DNA sequence analysis of an immunogenic glucose-galactose MglB lipoprotein homologue from *Brachyspira pilosicoli*, the agent of colonic spirochetosis. *Infect Immun*. 68: 4559-4565.

Zhang, G., L. Ma, and M. P. Doyle. (2007). Salmonellae reduction in poultry by competitive exclusion bacteria *Lactobacillus salivarius* and *Streptococcus cristatus*. *J Food Prot*. 70: 874-878.

Zhang, G., L. Ma, and M. P. Doyle. (2007). Potential competitive exclusion bacteria from poultry inhibitory to *Campylobacter jejuni* and *Salmonella*. *J Food Prot*. 70: 867-873.

Zuerner, R. L., and T. B. Stanton. (1994). Physical and genetic map of the *Serpulina hyodysenteriae* B78^T chromosome. *J Bacteriol*. 176: 1087-1092.

Zulkifli, I., N. Abdulllah, N. M. Azrin, and Y. W. Ho. (2000). Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. *Br Poult Sci*. 41: 593-597.

Appendices

Appendix I. Type and copy number of mobile genetic elements (MGE) in the genomes of *B. pilosicoli* 95/1000, B2904 and WesB. A combination of protein markov cluster analysis and reciprocal blast searches against the conserved domain database (CDD) was used to determine the copy number of each type of MGE across the three *B. pilosicoli* genomes, using a cut-off e-value of 1e-20. The open reading frame (ORF) number, position, and size of all MGEs identified in each of the *B. pilosicoli* genomes is displayed.

Appendix II. Comparison of the utilisation of unique carbon sources by four strains each of *B. alvinipulli*, *B. hyodysenteriae*, *B. innocens*, *B. intermedia*, *B. murdochii* and *B. pilosicoli* and two strains each of "*B. canis*" and "*B. pulli*". Biolog Phenotype MicroArray^{TM} (PM) technology was employed for these studies and OmniLog apparatus was used to detect formazan formation and hence, respiration due to utilisation of the carbon source; +, able to utilise the compound; −, unable to utilise the compound.

Appendix I. (cont'd p. 270).

Type of MGE in cluster	95/1000					B2904						WesB				
	No. of copies	ORF number (BP951000)	Position Start	End	Size (bp)	No. of copies	ORF number (B2904_orf)	Position Start	End	Size (bp)		No. of copies	ORF number (wesB_)	Position Start	End	Size (bp)
Insertion element IS1016 transposase	0	-	-	-	-	15	131	139589	139897	308		17	164	177409	178065	656
							132	139946	140251	305			285	302068	302319	251
							862	874327	874644	317			286	302375	302722	347
							1758	1817867	1818313	446			344	349841	350158	317
							1759	1818255	1818515	260			508	541858	542514	656
							1865	1931503	1931949	446			895	964112	964429	317
							1866	1931891	1932151	260			991	1074208	1074864	656
							2118	2164601	2165029	428			1190	1298341	1298997	656
							2119	2164971	2165231	260			1481	1638395	1639051	656
							2175	2220218	2220673	455			1555	1715503	1715763	260
							2176	2220615	2220875	260			1661	1843494	1843775	281
							2574	2645754	2646200	446			2013	2233848	2234165	317
							2575	2646142	2646402	260			2035	2255602	2255919	317
							2609	2677670	2678116	446			2036	2255986	2256267	281
							2610	2678058	2678318	260			2135	2374103	2374423	320
													2136	2374413	2374760	347
													2193	2433494	2434150	656
Integrase	0	-	-	-	-	43	62	67238	68143	905		7	293	312156	312326	170

Appendix I. (cont'd p. 271).

266	257036	257383	347	371	378714	379349	635
267	257427	257873	446	1309	1433802	1434218	416
430	412755	413591	836	1331	1460303	1460905	602
509	496965	497801	836	1373	1506314	1506607	293
559	548359	549195	836	2030	2249777	2250970	1193
578	566443	567336	893	2595	2834743	2835240	497
646	643066	643971	905				
681	684734	685570	836				
703	707119	707955	836				
864	875534	876274	740				
1081	1112697	1113755	1058				
1082	1113777	1114241	464				
1083	1114189	1114578	389				
1167	1211169	1211951	782				
1187	1228253	1229089	836				
1227	1267962	1268867	905				
1261	1302499	1303404	905				
1290	1338640	1339482	842				
1302	1348409	1349245	836				
1357	1403944	1404849	905				
1358	1404985	1405890	905				
1379	1426063	1426899	836				

Appendix I. (cont'd p. 272).

							1538	1594748	1595164	416					
							1539	1595233	1595652	419					
							1568	1627747	1628583	836					
							1686	1751629	1752534	905					
							1881	1946934	1947839	905					
							1914	1979779	1980600	821					
							2019	2067987	2068892	905					
							2068	2113372	2114277	905					
							2165	2207793	2208629	836					
							2206	2255145	2256050	905					
							2216	2268934	2269755	821					
							2262	2320210	2321046	836					
							2304	2361461	2362297	836					
							2382	2446227	2447132	905					
							2436	2504446	2505282	836					
							2559	2630021	2630539	518					
							2622	2687361	2688197	836					
							2637	2709835	2710740	905					
							2639	2711887	2712792	905					
							2675	2746351	2747187	836					
Integrase	0	-	-	-	-	0	-	-	-	-	1	319	327900	329609	1709
Integrase	0	-	-	-	-	0	-	-	-	-	1	739	785668	786816	1148

Appendix I. (cont'd).

Integrase	0	-	-	-	-	0	-	-	-	-	1	2567	2797020	2798153	1133
Transposase	1	627	697109	697405	296	1	739	748759	748941	182	1	1932	2147424	2147720	296
Transposase	0	-	-	-	-	0	-	-	-	-	1	1495	1656211	1656804	593
Transposase	1	1186	1309277	1310314	1037	0	-	-	-	-	0	-	-	-	-
XerD site-specific tyrosine recombinase	1	451	505412	506278	866	1	941	958688	959614	926	1	1745	1939246	1940172	926
XerD site-specific tyrosine recombinase	1	2141	2347639	2348562	923	1	1037	1063318	1064241	923	1	1655	1834931	1835854	923

Appendix II. (cont'd p. 274).

	B. alvinipulli				*B. canis*		*B. hyodysenteriae*				*B. innocens*				*B. intermedia*				*B. murdochii*				*B. pilosicoli*				*B. pulli*	
	A N1 26 3/2 /04	**A N3 38 2/2 /03**	**C 1 T**	**C 5**	**D o g B**	**D 2 4**	**B 7 8 T**	**P 1 8 A**	**Q 93 48 .6**	**W A 1**	**A N3 16 5/2 /03**	**A N4 11 3/ 03**	**A N4 34 1/ 03**	**Q9 7.3 28 9.5 .5**	**A N3 37 0/0 3**	**P 2 8 0- 1**	**Q9 8.0 44 6.2**	**U N L - 2**	**1 5 5/ 2 0**	**5 6/ 1 5 0 T**	**A N1 81/ 1/0 4**	**A N3 54 9/1 /03**	**95 /1 00 0**	**B 2 9 0 4**	**W e s B**	**C P S p 1**	**B 3 7 ii**	**Bp 60 5**
N-Acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Saccharic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−
Succinic acid	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Aspartic acid	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	+	−	+	+	−	−	−	−	+	+
L-Proline	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	+	+
D-Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+	−	+	+	+	−	+	−	−	−	−	−	+	−
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	−
Dulcitol	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−
D-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
D-Sorbitol	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−
Glycerol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−
L-Fucose	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
D-Glucuronic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
D-Gluconic acid	+	+	+	−	+	+	+	+	+	+	+	−	+	−	+	−	+	+	+	+	+	+	−	−	−	−	−	−

Appendix II. (cont'd p. 275).

D,L-α-Glycerol-phosphate	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Lactic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Formic acid	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
D-Mannitol	−	−	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
L-Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+
D-Glucose-6-phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactonic acid-γ-lactone	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D,L-Malic acid	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	+	+	+	+	−	+
Tween 20	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	−	−	+
L-Rhamnose	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	+	+	−	−	+	−	+	+	+	+	+	+	−	+	−	+	+	+	+	−	−	+	+	+	+	+	−	+
α-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thymidine	−	−	−	−	−	−	+	−	+	+	+	−	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+	−	−
L-Asparagine	−	−	−	−	−	−	+	+	+	+	+	+	+	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−
D-Aspartic acid	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−	−	+	+
D-Glucosaminic acid	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1,2-Propanediol	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−

Appendix II. (cont'd p. 276).

Tween 40	+	+	+	−	−	−	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	−	−	+
α-Ketoglutaric acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Ketobutyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Methyl-D-galactoside	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	+	+
α-D-Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactulose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
Uridine	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
L-Glutamine	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	−	−
m-Tartaric acid	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+	−	−	−	−	−	+
D-Glucose-1-phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose-6-phosphate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	+
α-Hydroxyglutaric acid-γ-lactone	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Hydroxybutyric acid	+	+	+	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+
β-Methyl-D-glucoside	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	+
Adonitol	+	−	−	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Maltotriose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2`-Deoxyadenosine	+	+	+	+	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Adenosine	+	+	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−
Glycyl-L-aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+

Appendix II. (cont'd p. 277).

Citric acid	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
m-Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	−	−	−	−	+	+
D-Threonine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fumaric acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Bromosuccinic acid	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Propionic acid	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mucic acid	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−
Glycolic acid	+	+	+	+	−	−	−	+	+	−	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−	−	−	−
Glyoxylic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inosine	+	+	+	+	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	+	−	+	+	+	+	−	+
Glycyl-L-glutamic acid	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	+
Tricarballylic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Threonine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Alanyl-glycine	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	+	−	−	−	−	+	+
Acetoacetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−
N-Acetyl-β-D-mannosamine	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
Monomethyl succinate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Methyl pyruvate	+	+	+	+	−	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	−	−	+	−

Appendix II. (cont'd p. 278).

D-Malic acid	+	+	−	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	+
L-Malic acid	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+
Glycyl-L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	+	+
p-Hydroxyphenylacetic acid	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	−	−	−	−	−	−	−
m-Hydroxyphenylacetic acid	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+	−	−	−	−	−	−	−
Tyramine	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+
D-Psicose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Glucuronamide	−	−	−	−	+	+	−	+	+	+	−	−	−	−	+	−	+	+	+	+	+	−	+	−	+	+	+	−	−
Pyruvic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Galactonic acid-γ-lactone	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Galacturonic acid	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-Phenylethylamine	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	−	−	+	+
Ethanolamine	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	+	+
Chondroitin sulfate C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Cyclodextrin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Cyclodextrin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
γ-Cyclodextrin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Dextrin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	
Gelatin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Appendix II. (cont'd p. 279).

Glycogen	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inulin	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Laminarin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannan	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pectin	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	−	+	+
N-Acetyl-D-galactosamine	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	−	−
N-Acetylneuraminic acid	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−
β-D-Allose	−	+	+	−	+	−	+	+	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	+	−	+	−
Amygdalin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Arabitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
i-Erythritol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Fucose	−	+	−	−	+	−	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−	−	+	+	+	+	−
3-*O*-β-D-Galacto pyranosyl-D-arabinose	+	−	−	−	−	−	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	+
Gentiobiose	−	−	−	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	+	+	+	+	+	−
L-Glucose	−	−	−	−	+	−	+	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	+	+	+	+	+	−
D-Lactitol	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	−	+	−
D-Melezitose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Maltitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Appendix II. (cont'd p. 280).

α-Methyl-D-glucoside	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Methyl-D-galactoside	+	−	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−
3-*O*-Methyl-glucose	+	+	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−
β-Methyl-D-glucuronic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
α-Methyl-D-mannoside	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Methyl-D-xyloside	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
D-Raffinose	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−
Salicin	+	+	+	+	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−
Sedoheptulosan	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Sorbose	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Stachyose	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−
Turanose	+	−	−	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−	+	+	+	−	+	+	+	+	+	+
Xylitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
N-Acetyl-D-glucosaminitol	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−
γ-Aminobutyric acid	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
δ-Aminovaleric acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Butyric Acid	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
Capric Acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Caproic Acid	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Citraconic acid	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Appendix II. (cont'd p. 281).

D,L-Citramalic acid	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
2-Hydroxybenzoic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4-Hydroxybenzoic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Hydroxybutyric acid	−	−	+	−	−	−	+	+	+	+	−	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−
γ-Hydroxybutyric acid	+	+	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-Ketovaleric acid	+	+	−	−	+	−	+	+	+	+	−	−	+	+	−	−	−	−	+	+	−	+	−	−	−	−	+	−
Itaconic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Lactic acid methyl ester	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Malonic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Melibionic acid	−	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−
Oxalic acid	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Oxalomalic acid	−	−	−	−	−	−	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−
Quinic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Ribono-1,4-lactone	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sebacic acid	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Succinamic acid	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Tartaric acid	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−
L-Tartaric acid	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−
Acetamide	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Alaninamide	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N-Acetyl-L-glutamic acid	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Appendix II. (cont'd).

L-Arginine	−	−	−	−	+	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
Glycine	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−
L-Histidine	−	−	+	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
L-Homoserine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
trans-4-Hydroxy-L-proline	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Isoleucine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Leucine	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
L-Lysine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Methionine	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Ornithine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Phenylalanine	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
L-Pyroglutamic acid	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Valine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
D,L-Carnitine	−	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
sec-Butylamine	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	−	+
D,L-Octopamine	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
Putrescine	−	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
2,3-Butanediol	−	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−
2,3-Butanone	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3-Hydroxy-2-butanone	−	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−