Investigating the role of intestinal microflora in the aetiology, symptom scores and life quality of people with coeliac disease

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Investigating the Role of Intestinal Microflora in the Aetiology, Symptom Scores and Life Quality of People with Coeliac Disease

Joanna Harnett
Statement

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. **I certify that I have complied** with the rules, requirements, procedures and policy of the University (as they may be from time to time).

Print Name: Joanna Elizabeth Harnett

Signature:

Date: 24\textsuperscript{th} of May, 2013
Dedication

To my parents, the late Margaret and Brian Harnett
Acknowledgements

“It is the glory of God to conceal a matter; to search out a matter is the glory of kings”
(Proverbs 25.2)

There have been many ‘kings’ involved in this gloriously painful journey. Acknowledging the wonderful people that have played a role in this thesis is the easiest part of all.

To the gracious and willing people with coeliac disease who participated in the research projects in this study, thank you, without you, none of this would have been possible.

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Abstract

Coeliac disease (CD) is a disorder resulting from the interaction between diet, genome and immunity. CD can present at any age, affecting intestinal and extra-intestinal organ systems. Treatment of CD is a life-long adherence to a gluten-free diet (GFD), resulting in both clinical and histological improvement. The aim of this thesis was to investigate the role of intestinal microflora in the aetiology and ongoing clinical management of patients with CD.

The indigenous intestinal microflora plays a critical role in host antigen responses, thus influencing immune homoeostasis. Genetic predisposition cannot solely be responsible for CD aetiology. For instance, maternal and early childhood health factors that affect the development of the intestinal milieu have also been suggested to be environmental risk factors for CD.

An online survey was administered to gather data regarding early childhood health and clinical management. The survey results suggested CD individuals were breast-fed less compared to the general population (p=0.05). Being born by caesarean section was more frequent in those < 30 years of age which coincides with a general increase in CD diagnosis. Broad clinical presentations, considerable diagnostic delay (mean 8.4 years), only partial symptom resolution (44.7%) despite adherence to a GFD were reported. Family history of CD was reported by 38.1%. The incidence of food allergies (p=0.000), complementary medicine use (p=0.05), and early childhood infection was higher in those reporting persistent symptoms.

To investigate if intestinal dysbiosis was associated with persistent symptoms, and if bacteriotherapy would be an effective intervention, a randomised controlled trial was conducted. The intestinal microbial measures of CD participants were compared to non-coeliac controls. CD participants were then allocated to receive either the probiotic VSL#3™ or placebo twice daily for twelve weeks. A CD symptom and quality of life questionnaire was administered at baseline and weeks 4, 8 and 12. The CD group had higher total bacterial counts compared to the non-coeliac controls (p=0.003). Higher counts of Clostridia sp. (p=0.002), Prevotella sp. (p=0.009) and Bifidobacteria sp. (p=0.02) were detected in the CD group. Detection of Candida sp (p=0.001) and Saccharomyces sp. (p=0.030) were also specific to the CD group. There were no differences in the measures of microbial species or symptom scores and quality of life measures in the VSL#3™ group compared to the placebo group at week 12. Were this hypothesis proven, it would contribute to potential environmental preventative directives, and effective bacteriotherapy would contribute to interventions that would enhance the efficacy of dietary treatment as well as quality of life.
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## List of Abbreviations

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<th>Description</th>
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</thead>
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<tr>
<td>AID</td>
<td>Autoimmune Disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-Presenting Cells</td>
</tr>
<tr>
<td>ASA</td>
<td>Anti-Saccharomyces Antibodies</td>
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<td>ASCA</td>
<td>Anti-Saccharomyces Cerevisiae Antibodies</td>
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<td>AST</td>
<td>Aspartate Transaminase</td>
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<tr>
<td>BAPEN</td>
<td>British Association of Parenteral and Enteral Nutrition</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and Alternative Medicine</td>
</tr>
<tr>
<td>CD</td>
<td>Coeliac Disease</td>
</tr>
<tr>
<td>CDN</td>
<td>Content Delivery Network</td>
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<tr>
<td>CDQ</td>
<td>Celiac Disease Questionnaire</td>
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<tr>
<td>CDSQOL</td>
<td>Coeliac Disease Symptom Score and Quality of Life</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-Forming Unit</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type Lectin Receptors</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>C-section</td>
<td>Caesarean section</td>
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<tr>
<td>DB</td>
<td>Double Blind</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>DRG</td>
<td>Drug-resistant Genes</td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive Escherichia coli</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EMA</td>
<td>Ethidium Monoxide</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>FCM</td>
<td>Flow Cytometry</td>
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<tr>
<td>FGD</td>
<td>Functional Gastrointestinal Disorders</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in Situ Hybridisation</td>
</tr>
<tr>
<td>FODMAP</td>
<td>Fermentable Oligo-, Di- and Mono-Saccharides and Polyols</td>
</tr>
<tr>
<td>GA</td>
<td>Georgia</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-Associated Lymphoid Tissue</td>
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<td>GB</td>
<td>Gigabases</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practices</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GEE</td>
<td>Generalised Estimated Equations</td>
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<tr>
<td>GERD</td>
<td>Gastro-Oesophageal Reflux Disease</td>
</tr>
<tr>
<td>GFD</td>
<td>Gluten-Free Diet</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-Like Peptide-1</td>
</tr>
<tr>
<td>HGP</td>
<td>Human Genome Project</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Lymphocyte Antigen</td>
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<tr>
<td>HMP</td>
<td>Human Microbiome Project</td>
</tr>
<tr>
<td>HPAA</td>
<td>Hydroxyphenylacetic Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPPA</td>
<td>Hydroxyphenylpropionate</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research Ethics Committee</td>
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<tr>
<td>HRQOL</td>
<td>Health-Related Quality of Life</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IDO</td>
<td>Indolamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IE</td>
<td>Intestinal epithelium</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFR</td>
<td>Inter-follicular T-cell regions</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin-A</td>
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<tr>
<td>IgD</td>
<td>Immunoglobulin-D</td>
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<td>Immunoglobulin-E</td>
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<td>Immunoglobulin-G</td>
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<td>IgM</td>
<td>Immunoglobulin-M</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LPS</td>
<td>Lipo-polysaccharide</td>
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<td>MCAD</td>
<td>Medium chain acyl-coenzyme A dehydrogenase deficiency</td>
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<td>MDREC</td>
<td>Multi-drug resistant E-coli</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MUC1</td>
<td>MUCIN-1</td>
</tr>
<tr>
<td>NA</td>
<td>Not available</td>
</tr>
<tr>
<td>NASH</td>
<td>Nonalcoholic Steatohepatitis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NATA</td>
<td>National Association of Testing Authorities</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health Medical Research Council</td>
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<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerisation domain protein-like receptor</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>OCP</td>
<td>Oral contraceptive pill</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomical units</td>
</tr>
<tr>
<td>PAA</td>
<td>Phenylacetic acid</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEP</td>
<td>Prolyl endopeptidases</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's Patches</td>
</tr>
<tr>
<td>PPA</td>
<td>Phenylpropionate</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton Pump Inhibitors</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition</td>
</tr>
<tr>
<td>QIME</td>
<td>Quantities Insights into Microbial Ecology</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>RCD</td>
<td>Refractory Coeliac Disease</td>
</tr>
<tr>
<td>RCPA</td>
<td>Royal College of Pathologists of Australasia</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Controlled Trial</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosomal Database Projects pyrosequencing</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribosomal Nucleic Acid</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short Chain Fatty Acids</td>
</tr>
<tr>
<td>SCU</td>
<td>Southern Cross University</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SIBO</td>
<td>Small Intestinal Bacterial Overgrowth</td>
</tr>
<tr>
<td>TCR</td>
<td>T-Cell Receptor</td>
</tr>
<tr>
<td>TGA</td>
<td>Therapeutic Goods Administration</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumour Growth Factor Beta</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>Th3</td>
<td>T-helper 3</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor Metallopeptidase</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TMAC</td>
<td>Tetramethyl Ammonium Chloride</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzenesulfonic Acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>T-reg</td>
<td>T-regulatory</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoiëtin</td>
</tr>
<tr>
<td>TTTGE</td>
<td>Temperol Temperature Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>UAC</td>
<td>Unable to Calculate</td>
</tr>
<tr>
<td>UEA</td>
<td>Ulex Europaeus Agglutinin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonulins</td>
</tr>
<tr>
<td>ZOT</td>
<td>Zonula Occludens Toxin</td>
</tr>
</tbody>
</table>
1 Introduction

The aim of this thesis was to investigate the role of intestinal microflora in the aetiology and ongoing clinical management of patients with Coeliac Disease (CD). CD is a chronic inflammatory autoimmune T-cell mediated disorder of the small intestinal mucosa, triggered by the ingestion of the dietary protein gluten, in a genetically predisposed population. However, genetic predisposition cannot be solely responsible for disease development as an estimated 30–40% of the general population carry the gene, yet never develop CD. This has prompted scientists to investigate other environmental risk factors, including caesarean section births, feeding practices and frequent infections as potential triggers or risk factors.

CD affects approximately one in a hundred Australians. A low diagnostic rate exists and has been attributed to CD having a broad spectrum of clinical presentations, and CD onset can be at any age. The consequences of a delay in diagnosis and delayed treatment are associated with poor histological and clinical recovery, and a greater risk of developing conditions associated with CD. Approximately 30% of patients on a gluten-free diet (GFD) still report a reduced quality of life.

The focus of this thesis was to investigate the role of intestinal microflora in the aetiology, symptom scores and quality of life of patients with CD. A literature review was conducted in the following areas:

- The gastrointestinal immune system
- The composition and assessment of the intestinal microbiome
- The mechanisms and application of probiotics
- The pathogenesis of CD
- Environmental risk factors
- Potential non-dietary therapies

This review led to the construction of a research plan that involved the administration of a survey to people with CD residing in New South Wales, Australia. The questions pertained to the possible environmental risk factors, clinical management and the social issues experienced by people with CD. A sub-group of survey respondents with persistent symptoms were then enrolled in a double-blind randomised controlled trial (RCT). The study compared the composition of the intestinal microbiota of patients with CD to non-coeliac individuals, followed by a clinical trial. The trial involved two parallel arms, and tested the effects of the probiotic VSL#3™ versus a placebo on gastrointestinal microbial measures, symptom scores and the quality of life (QOL) of CD participants.
The thesis concludes with a general discussion of this research and suggestions for direction of further research.
2 Literature Review

2.1 Coeliac Disease

CD is a chronic inflammatory autoimmune T-cell mediated disorder of the small intestinal mucosa, triggered by the ingestion of the dietary protein gluten, in a genetically predisposed population. CD is estimated to affect up to 1% of the Australian population. Approximately 75% of the CD population remain undiagnosed. The incidence of CD in females is twice that in males (Tye-Din and Anderson 2008; Chin, Mallon et al. 2009; Gibson, Shepherd et al. 2012). Results of a prevalence study led by Tye-Din reported 1 in 60 Australians residing in Geelong, Australia, have CD (Tye-Din 2012).

2.1.1 History of Coeliac disease

The first description of CD comes from the writings of the Greek physician Aretaeus of Cappadocia (131-201 AD) and colleagues who wrote, “If the stomach be irretentive of food and pass through undigested and crude, and nothing ascends into the body, we call such persons koiliakos.” Aretaeus named it koiliakos after the Greek word koelia (abdomen). The word koiliakos translates as ‘suffering in the bowels’ (Guandalini 2008) and has given rise to the currently used word ‘coeliac’.

Seventeen centuries later Dr Matthew Baillie, who is rarely mentioned in the history of CD, published his observations on a chronic diarrhoeal disorder of adults causing malnutrition and characterised by a gas-distended abdomen stating: “....some patients have appeared to derive considerable advantage from living almost entirely upon rice” (Guandalini 2008).

The first clear description of CD and its association with diet was given in 1888 by the British physician Samuel Gee. Gee was noted for his ability to research the literature and for this reason, and due to the similarities in choice of phrases in his writings, it is thought that Gee had referred to the writings of his Greek predecessors (Guandalini 2008; Losowsky 2008). Gee’s opening paragraph in his book The Coeliac Affection (1888) states: “There is a kind of chronic indigestion which is met with in persons of all ages, yet is especially apt to affect children between one and five years old. Signs of the disease are yielded by the faeces; being loose, not formed, but not watery; more bulky than the food taken would seem to account for; pale in colour, as if devoid of bile; yeasty, frothy, an appearance probably due to fermentation; stinking, stench often very great, the food having undergone putrefaction rather than concoction......... But if the patient can be cured at all, it must be by means of diet.” (Guandalini 2008)
The contribution of Gee, who identified diet as the causative factor in his coeliac patients, was noted (Guandalini 2008), but the toxic compounds in the diet had still not been identified. During the period 1908–1918 paediatricians in the United Kingdom observed that fats were better tolerated than carbohydrates in the diet of patients meeting Gee’s description. At this time introducing foods by stages was the only available treatment. Gee and his associates took several months or even years to complete these elimination diets with their patients (Guandalini 2008). Breads, cereals and potatoes were the final foods to be reintroduced to the diet. In 1920 a new dietetic treatment was proposed by Sidney Haas (Guandalini 2008). Haas described a successful treatment of eight children previously diagnosed with CD. His treatment, popularised as ‘the banana diet’, enjoyed wide popularity and was thought to have prevented many deaths. The diet specifically excluded bread, crackers, potatoes and all cereals. However, there was still no clear identification of the toxic components in a diet that caused the symptoms of CD. It was not until the 1950s when a doctoral thesis by Dutch paediatrician Wim Dicke firmly established the association between dietary intake of wheat, rye, barley and oats and the symptoms and signs attributed to CD (Losowsky 2008). Dicke showed that the toxicity was caused by the protein component now known as gluten (Losowsky 2008).

The identification of gluten was followed by another breakthrough in 1954, when British physician John W Paulley recognised the characteristic abnormality of the lining of the upper part of the bowel in coeliac patients known as villous atrophy (Losowsky 2008). Paulley’s observation of villous atrophy has become the most essential single feature on which the diagnosis of CD is made (Guandalini 2008). In 1956 Margot Shiner described a new jejunal biopsy apparatus with which she successfully reached and biopsied the distal duodenum (Guandalini 2008). This, and the less cumbersome capsule developed shortly after by Crosby, finally allowed doctors to link the disease with a specific, recognisable pattern of damage to the proximal small intestinal mucosa (Guandalini 2008). They concluded that after the prescription of a strict GFD the patients’ small intestinal villi that were previously flat were returning to normal.

The journey and discovery of physicians and clinicians from 2nd century AD until the 1960s has contributed three important elements to the knowledge of CD:

1. The understanding that gluten acts as a dietary toxin in susceptible individuals, thereby causing CD, and that its removal results in clinical improvement.
2. The identification of a consistent lesion in the small intestine of patients with CD.
3. A technological breakthrough in the availability of an instrument to obtain biopsies and monitor histological progress.
The discovery in the late 1960s that CD could be diagnosed by jejunal biopsy showing atrophy of the villi was ground-breaking. However, it was acknowledged that villous atrophy can occur in non-coeliac patients. Therefore, doctors were hesitant to diagnose a patient with CD until it could be proved that gluten was indeed the cause. It was clear that a diagnostic criterion for CD was required and in 1969 the development of the “Interlaken criteria” was formalised by the European Society for Paediatric Gastroenterology. The diagnostic criteria demanded the following steps be taken to diagnose CD.

1. Jejunal biopsy showing villous atrophy.
2. A strict GFD resulting in clinical remission of symptoms.
3. Repeat jejunal biopsy proving normalization of the lesion.
4. Finally, patients were challenged by re-introducing a diet containing gluten to see if this caused a lesion to recur.

However, the Interlaken criteria had overlooked an earlier discovery that children with CD had antibodies caused by the ingestion of gluten that were detected in their blood samples (Guandalini 2008). An appreciation of the utility of these tests took some time to achieve general consensus. Antibody testing evolved in two stages: first, Berger discovered in 1964 that there were anti-food proteins known as the anti-gliadin antibodies (Bürgin-Wolff, Bertele et al. 1983), then in 1971 Seah et al. (1971) identified an actual intestinal auto-antibody, the anti-reticulins, in the serum of children with CD.

In the late 1980s Dr Stefano Guandialini conducted a large multi-centre Italian study that resulted in a new diagnostic guideline that was published by the European Society for Paediatric Gastroenterology. The new diagnostic criteria was limited to an initial jejunal biopsy in conjunction with serum antibodies at diagnosis and both the serum antibodies and jejunal biopsy to be repeated as a follow-up measure of improvement. These diagnostic guidelines were reconsidered again in 2009. Throughout the 1980s it became apparent that CD was frequently accompanied by other conditions, mostly autoimmune disorders such as type 1 diabetes, but also with some syndromes such as trisomy 21, Turners Syndrome and irritable bowel syndrome (IBS). It was also apparent that CD was changing patterns of presentation, becoming less a pronounced intestinal disorder and more a variety of vague intestinal and extra-intestinal signs and symptoms (Preussner 1998).

In the 1990s it was discovered that CD was an autoimmune disease with a specific associated gene known as HLA DQ2 or HLA DQ8 (Barbeau, Novascone et al. 1997; Tye-Din and Anderson 2008). Also, in that decade the missing auto-antigen, the enzyme tissue transglutaminase, was discovered (Dieterich, Ehnis et al. 1997). By 2010, Tye-Din et al. (2010) had demonstrated that pathogenic T-cells in CD show limited diversity with only three
peptides accounting for the majority of gluten-specific T-cells, prompting the investigation of peptide-based therapeutics.

In summary, from the middle of last century physicians and scientists have made vast and significant progress in understanding the aetiology, pathogenesis and management of CD. They have demonstrated that the dietary ingestion of the protein gluten in some genetically predisposed individuals elicits an autoimmune response that can be detected serologically and represented histologically as inflammation and villous atrophy of the small intestine. We know that patients with CD present clinically with both asymptomatic and symptomatic intestinal and extra-intestinal symptoms that respond serologically and histologically to the removal of gluten proteins from the diet.

Not all individuals carrying the HLA DQ2 or DQ8 halotype will go on to develop CD. It is estimated that up to 40% of the Australian population carry these halotypes (Bourgey, Calcagno et al. 2007). Discovery of what triggers the genetically predisposed population to express the gene and develop is yet to be fully elucidated. Investigators in the 21st century still have their role to play in contributing to the body of knowledge regarding the risk factors involved in the genetic expression of CD.

2.1.2 Epidemiology
Our understanding of the epidemiology of CD has changed substantially in the last three decades (Lohi, Mustalahti et al. 2007). During the 1970s CD was considered a rare disorder affecting 1:1000 individuals, predominantly of European origin (Lohi, Mustalahti et al. 2007). We now know that CD occurs in both adults and children at rates approaching 1% of the population, and is recognised not only throughout Europe and in countries populated by persons of European ancestry, but also in North and South America, Australia, New Zealand, South-West Asia and North Africa (Accomando and Cataldo 2004; Lohi, Mustalahti et al. 2007; Lohi 2010). Most individuals with CD remain undiagnosed (West, Logan et al. 2003) although the rate of diagnosis is increasing (Melton 2003).

2.1.3 Clinical picture
CD onset is not exclusive to childhood and may manifest at any stage of life (Ciclitira and Moodie 2003). Biochemical markers suggestive of CD may also occur at any age, including anaemia, iron deficiency, folic acid, B12, zinc, vitamin A and D deficiencies, hypocalcaemia, hypoproteinaemia and transaminasaemia (Giorgi, Catassi et al. 1984; Alwitry 2000; Lim, Tzemos et al. 2002; Catassi and Cobellis 2007). It has been demonstrated that the biochemical aberrations of CD are ameliorated with implementation of a GFD (Hallert, Grant et al. 2002; Lim, Tzemos et al. 2002). CD has been referred to as a disease of ‘protean’ presentations, or a
disease of many faces, and this may be attributed to age and gender at the time of diagnosis (Preussner 1998).

2.1.3.1 Paediatric clinical presentation
In infancy, the onset of CD is more likely to present with a classic cluster of malabsorption signs and symptoms including steatorrhoea, anorexia, vomiting, rickets, irritability and extreme lethargy with visible abdominal distension and failure to thrive (Fasano 2005). Indeed, this classic presentation may occur at any age but in older children the presentation may be less acute, with milder signs of malnutrition such as anaemia and growth failure. Several groups have reported an increasing prevalence of non-classical or atypical presentations during childhood, suggesting that malabsorption may often be mild, non-specific or absent, with less than one-third of patients presenting with diarrhoea and up to half being overweight (Ciclitira and Moodie 2003; Ravikumara, Tuthill et al. 2006; Valletta, Fornaro et al. 2010). Recurrent apthous ulcers and dental enamel defects are common in untreated CD children (Bucci, Carile et al. 2006). The neurological presentations of epilepsy may also be associated with untreated CD (Grossman 2008).

2.1.3.2 Adolescent clinical presentation
Young adults may also experience any of the common infant childhood affections. However, during adolescence an increased prevalence of mental health issues, including depressive and disruptive behavioural disorders, particularly in the phase before treatment, has been demonstrated (Pynnonen, Isometsa et al. 2004; Pynnonen, Isometsa et al. 2005). Primary and secondary amenorrhoea may present in adolescent females, and a delayed pubescent growth spurt in males (Preussner 1998).

2.1.3.3 Adult clinical presentation
The clinical picture of adult onset CD is broad. Adult CD may present with any of the clinical features observed in childhood or adolescence, in addition to a broad array of atypical extra-intestinal features. Anaemia, fatigue and diarrhoea continue to be the most common symptoms (Fasano 2005), with the gastrointestinal symptoms of CD often being less acute, presenting as mild or atypical complaints such as abdominal discomfort, poor appetite, malaise, stomach growling and flatulence, all now recognised as being associated with CD.

2.1.4 The extra-intestinal presentations and complications of Coeliac disease
While the strength of association between extra-intestinal presentations and CD vary, approximately 25% of CD patients are diagnosed by extra-intestinal manifestations of the disease (Collin 1994). Many of these extra-intestinal symptoms are technically classified as
complications of the disease. Table 2.1 on page 32 highlights the multi-system effects of CD that were first summarised by Lohi et al. (2007).

2.1.5 Associated conditions
CD has been associated with a number of conditions with varying strengths of association, as outlined in Table 2.2. Co-morbid autoimmune disorders arise ten times more often in patients with CD than they do in the general population (Green and Jabri 2003). The association with autoimmune disorders appears to be greater than other co-morbid conditions, disorders, with an estimated range of 15–30% of CD patients having at least one associated autoimmune disorder, compared to 1–11% in controls (Ventura, Magazzù et al. 1999; Bonamico, Mariani et al. 2001; Viljamaa, Kaukinen et al. 2005; Guariso, Conte et al. 2007). CD patients most at risk for autoimmune disease are those diagnosed early in life and having a family history of autoimmunity. The GFD has been demonstrated to confer a protective effect (Cosnes, Cellier et al. 2008). The most widely published autoimmune disorders associated with CD are autoimmune thyroid disease (Collin, Kaukinen et al. 2002; Li Voon Chong, Leong et al. 2002; Kohler and Wass 2009) and IgA deficiency (Cataldo, Marino et al. 1998a). Genetic diseases such as Down syndrome and Turner’s syndrome are also associated with CD (Bonamico, Mariani et al. 2001; Mortensen, Cleemann et al. 2009).
Table 2.1 Extra-intestinal symptoms and complications of Coeliac Disease

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychiatric conditions</td>
<td>33% of patients with CD have psychiatric symptoms, an association with depression, anxiety and even psychosis; the efficacy of the (GFD) for these conditions is yet to be elucidated (Pynnonen, Isometsa et al. 2004; Pynnonen, Isometsa et al. 2005; Cascella, Kryszak et al. 2011).</td>
</tr>
<tr>
<td>Dental enamel defects and aphthous stomatitis</td>
<td>10–96% of patients with CD have dental enamel defects in permanent dentition (Farmakis, Puntis et al. 2005; Bucci, Carile et al. 2006).</td>
</tr>
<tr>
<td>Neurological disorders</td>
<td>17% of patients with neurological symptoms of unknown origin have been found to have biopsy proven CD (Hadjivassiliou, Gibson et al. 1996; Hadjivassiliou, Grunewald et al. 2003). Patients with CD have a higher prevalence of epilepsy (Lionetti, Francavilla et al. 2010; Licchetta, Bisulli et al. 2011) Up to 49% of patients with CD have symptoms of peripheral neuropathy, but the effect of the GFD is controversial (Cicarelli, Della Rocca et al. 2003; Kron 2008), likely due to the need for correcting an associated vitamin B12 deficiency. An association between CD and a higher incidence of headaches (Lionetti, Francavilla et al. 2009).</td>
</tr>
<tr>
<td>Osteopaenia and Osteoporosis</td>
<td>The risk of fractures in CD patients is increased by an average of 40% according to a meta-analysis (Mustalahti, Collin et al. 1999; Kurppa, Collin et al. 2010). This likely due to malabsorption of vitamins A and D and the minerals calcium and magnesium.</td>
</tr>
<tr>
<td>Infertility and recurrent miscarriage</td>
<td>CD may be associated with infertility in men and women. CD females have been shown to have a shorter reproductive period and 30% more miscarriages, with the effect of the GFD unclear (Farthing, Edwards et al. 1982; Farthing, Rees et al. 1983; Dondorp and de Groot 1998; Martinelli, Troncone et al. 2000).</td>
</tr>
<tr>
<td>Musculoskeletal problems</td>
<td>Non-specific arthralgia and arthritis can also be manifestations of CD; the effect of the GFD is unclear (Molberg and Sollid 2006).</td>
</tr>
<tr>
<td>Functional liver problems</td>
<td>Reversibility via a GFD of abnormal liver biochemistry with elevated transaminases in approximately half of CD patients; CD is over-represented in patients with chronic liver disease, often autoimmune in origin; a GFD may be beneficial (Kaukinen, Collin et al. 2007; Rubio-Tapia, Rahim et al. 2010).</td>
</tr>
<tr>
<td>Splenic hypo-function</td>
<td>Frequently found in CD patients; a GFD seems to be protective (Di Sabatino, Carsetti et al. ; O’Grady, Harding et al. 1985; Corazza, Sarchielli et al. 1986).</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>The risk of pancreatitis of any type was threefold among CD patients hospitalised for any reason; the risk of chronic pancreatitis was especially high, being nineteen times greater than in non-CD patients (Ludvigsson, de Faire et al. 2007).</td>
</tr>
<tr>
<td>Cardiac diseases</td>
<td>There is conflicting evidence for association with ischaemic disease of the circulatory system as well as with cardiomyopathy, pericarditis and myocarditis (Ludvigsson, de Faire et al. 2007).</td>
</tr>
</tbody>
</table>
2.1.6 The efficacy of the GFD on associated conditions

Evaluating the effect of the GFD on the development and course of autoimmune disorders that are related to CD remains inconclusive (Cosnes, Cellier et al. 2008; Volta, Vincentini et al. 2011; Metso, Hyytia-Illonen et al. 2012). A GFD has been demonstrated to have a favourable effect on some reversible clinical symptoms of autoimmune disease. However, the GFD is unable to influence the organ and non-organ specific auto-antibody production (Guariso, Conte et al. 2007). This clinical response suggests a GFD may confer protection over the development of new clinical or subclinical features of autoimmune disease, but has no effect on autoimmune antibodies (Guariso, Conte et al. 2007). Cosnes et al. (2008) demonstrated that the cumulative risk of subsequent autoimmune disease was lowered by half in patients being compliant versus being non-compliant with a GFD and this risk reduction was independent of age, concluding that a GFD protects against the development of Autoimmune Disease (AID). These findings would infer that gluten proteins can trigger the development of other autoimmune conditions in patients with CD.

Table 2.2 Autoimmune conditions associated with Coeliac Disease (Cosnes, Cellier et al. 2008)

<table>
<thead>
<tr>
<th>Associated Condition</th>
<th>Strength of Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune thyroid disease</td>
<td>15% of patients with CD have autoimmune thyroid disease (Collin, Kaukinen et al. 2002; Buysschaert 2003; Metso, Hyytia-Illonen et al. 2012).</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>Type 1 diabetes occurs in 1–7% CD cases. Approximately 4% of patients with Type 1 diabetes have CD (Buysschaert 2003).</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
<td>5–15% of Sjogren’s syndrome patients have CD (Szodoray, Alex et al. 2004).</td>
</tr>
<tr>
<td>Autoimmune liver disorders</td>
<td>3–7% of patients with either primary biliary cirrhosis or autoimmune hepatitis have CD (Di Biase, Colecchia et al.; Villalta, Girolami et al. 2005).</td>
</tr>
<tr>
<td>Down Syndrome</td>
<td>An estimated 5% of individuals with Down syndrome have CD (Bonamico, Mariani et al. 2001; Bhat, Chaturvedi et al. 2012).</td>
</tr>
<tr>
<td>Selective IgA deficiency</td>
<td>Selective immuno-globulin deficiency occurs in approximately 2% of CD cases, with 10% of IgA deficient patients having CD (Cataldo, Marino et al. 1998b; Korponay-Szabo, Dahlbom et al. 2003).</td>
</tr>
<tr>
<td>Alopecia aerata</td>
<td>Approximately 2% of patients with alopecia areata have CD (Corazza, Di Sario et al. 1996; Collin, Kaukinen et al. 2002)</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>The risk of developing Addison’s disease in individuals with CD is fivefold (Elfrstrom, Montgomery et al. 2007).</td>
</tr>
<tr>
<td>End-stage renal disease</td>
<td>Approximately 3% of individuals with end-stage renal failure have CD (Welander, Pritz et al. 2012).</td>
</tr>
<tr>
<td>Turner’s syndrome</td>
<td>An estimated 4.7% to 5.1% of patients with Turner’s syndrome have CD (Frost, Band et al. 2009).</td>
</tr>
</tbody>
</table>
2.1.7 Diagnosis
The diagnostic process from clinical presentation to a final diagnosis requires careful consideration of the clinical and laboratory findings at each step in the diagnostic process. Figure 2.1 presents the current standard practice for exclusion and diagnosis of CD. The final diagnosis of CD is made on the basis of an abnormal small intestinal biopsy that normalises after the removal of gluten from the diet. Confirmation of the diagnosis of CD is by clinical and histological regression on dietary challenge with gluten (Anderson 2008).

![Diagnostic flowchart for Coeliac Disease](http://www.coeliac.org.au/content/downloads/Diagnosis%20Card.pdf, accessed 12/12/12)
2.1.8 Serology
Identification of anti-endomysial and anti-tissue transglutaminase auto-antibodies in patients with CD has improved the reliability of serological markers and shifted the first stage of the diagnostic process towards primary care. However, neither the presence nor the absence of anti-endomysial and/or anti-tissue transglutaminase auto-antibodies in serum samples provides a definitive diagnosis of the disease (Sweis, Pee et al. 2009). Approximately 10% of CD patients are sero-negative, particularly those without overt villous atrophy. In addition, testing for sera anti-gliadin antibodies is not suitable for screening of children less than two years old due to lack of sensitivity (Anderson 2009); in addition, children’s immunological response may vary between tolerance and non-tolerance before settling into an autoimmune disease (Bizzaro 2007; Anderson 2008; Bizzaro 2008). The auto-antibody to endomysium may be produced before histological change and the immune response to transglutaminase is crucial to the disease process. Nevertheless, first stage clinical suspicion of CD is routinely followed by serological screening.

2.1.9 Histology of small bowel biopsy
If there is a strong clinical suspicion of CD, regardless of positive or negative serological findings, it should be followed by small bowel biopsy and histological assessment. The histological diagnosis of CD no longer requires small intestine villous atrophy (Walker and Murray 2011). The Marsh Classification recognises increased intraepithelial lymphocytes and crypt hyperplasia with intact villi as part of the gluten enteropathy spectrum, while some individuals have more subtle abnormalities identified only on electron microscopy (Dickey 2009; Walker and Murray 2011). Histological findings of a small bowel biopsy remain the gold standard of diagnosis for CD (Ludvigsson, Brandt et al. 2009; Walker and Murray 2011). Although no studies have examined the number of biopsies required for diagnosis, discussion has suggested that a minimum of four to six endoscopic-biopsy specimens should be obtained from the duodenum, due to the patchy nature of the disease. In addition, due to the difficulty in obtaining suitable specimens for assessment of villous morphology, obtaining several specimens ensures a suitable specimen is available (Green, Rostami et al. 2005; Green and Cellier 2007).

2.1.10 Coeliac genotyping
A negative test result for serology or small bowel histopathology does not preclude CD development at a later stage (Anderson 2008). HLA testing may allow ‘once and for all’ exclusion as 99% of patients with CD carry one or both of the genes. Genotyping for HLA DQ2 or DQ8 haplotypes has an excellent negative predictive value but a relatively poor
predictive value (Tye-Din and Anderson 2008; Kneepkens and von Blomberg 2012). Genotyping can be performed from a buccal swab or serum sample of a patient considered at risk of developing CD. Genotyping may also be undertaken by patients who have already commenced a GFD and attained clinical benefits and who are unwilling about undertaking a gluten challenge for serology or histopathology testing. For patients who are positive for HLA DQ2 or DQ8 halotypes and who wish to proceed with a formal diagnostic process, a gluten challenge is required. Four slices of bread for an adult and two or more slices of bread per day, consumed for six weeks, is considered enough to produce the serological and histological evidence required for a diagnosis of CD (Gibson, Shepherd et al. 2012).

2.1.11 Latent and potential CD
A recent review of definitions and related terms for CD by an expert review committee in Oslo highlighted that the term ‘potential CD’ is often used with different meanings (Ludvigsson, Leffler et al. 2012). The current recommendation is that this term be used for people with normal small intestinal mucosa who are at increased risk of developing CD as indicated by positive coeliac serology (Ludvigsson, Leffler et al. 2012).

2.1.12 Differential diagnosis
Inflammation and villous atrophy in the small bowel mucosa should prompt consideration of non-coeliac states, especially in individuals who are sero-negative (Green and Cellier 2007). Non-coeliac states to consider are presented in Table 2.3 and include intestinal illnesses, food allergy induced enteropathy, intestinal infections, immunodeficiency states and drug-induced enteropathy.

2.1.13 Unresponsive or refractory Coeliac disease
Refractory CD (RCD) is diagnosed after other small bowel diseases with villous atrophy have been excluded. RCD is sub-divided into two sub-groups: type I RCD and type II RCD. The latter condition is considered a low-grade intraepithelial lymphoma and has a poor prognosis (Malamut, Murray et al. 2012). RCD affects less than 5% of the CD population and is more frequent in adults than children (Vivas and Ruiz 2008). Detection of alterations in the intraepithelial lymphocyte population is essential for diagnosis. Some alterations in these lymphocytes, such as the absence of T-cell surface receptor expression (CD3 and CD4), indicate an aggressive form of the disease with the potential for malignant transformation (type II RCD). The main cause of RCD is usually inadvertent continued ingestion of gluten. No treatment for RCD has demonstrated long-term efficacy although prednisolone combined with azathioprine has been demonstrated to successfully treat type II RCD (Donnelly, Ho-Yen et al. 2011). Therefore current treatment is based on adequate nutritional support and on the
use of corticosteroids, either with or without immuno-suppressive agents. The combination of more aggressive therapeutic strategies is sometimes required for RCD II. Immunotherapy and bone marrow transplantation hold promise for the treatment of RCD (Vivas and Ruiz 2008).

Table 2.3 Differential diagnosis (Green and Cellier 2007)

<table>
<thead>
<tr>
<th>Intestinal Illness</th>
<th>Food Allergy Other Than Gluten</th>
<th>Intestinal Infections</th>
<th>Iatrogenic Aetiology</th>
<th>Immunodeficiency Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crohn’s disease</td>
<td>Milk</td>
<td>Rotavirus</td>
<td>Radiotherapy enteritis</td>
<td>Common-variable immunodeficiency</td>
</tr>
<tr>
<td>Autoimmune enteropathy</td>
<td>Soya</td>
<td>Giardiasis</td>
<td>Chemotherapy drugs</td>
<td>Autoimmune enteropathy</td>
</tr>
<tr>
<td>Bacterial overgrowth syndrome</td>
<td>Chicken</td>
<td>Cryptosporidium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenous sprue</td>
<td>Tuna</td>
<td>Tuberculosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophilic gastroenteritis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Zollinger – Ellison Syndrome</td>
<td>Intestinal Lymphoma</td>
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</tbody>
</table>

2.1.14 Health-related quality of life

There are several significant Health Related Quality of Life (HRQOL) issues for individuals with CD. Overall they have a low positive health perception. Validated diet and disease-specific questionnaires reveal a significant negative impact on quality of life in social settings. Specifically, the areas of travel, dining out and family life are most affected (Hauser, Gold et al. 2007; Black and Orfila 2011). The negative impact of GFD adherence significantly decreases over time, although it does not resolve for the domains of dining out of the home and travel. It has been suggested that women perceive a greater burden of CD, possibly due to women more frequently reporting a poor response to therapy (Hallert, Grant et al. 1998; Hallert, Sandlund et al. 2003). Those diagnosed in childhood and maintained on the diet experience smaller effects on their quality of life as adults (Lee, Ng et al. 2012a). This may be due to a life-time familiarity with following a GFD. In addition, early diagnosis at a young age is associated with improved clinical outcomes and QOL.

2.1.14.1 Impact of a GFD and health-related quality of life

In untreated CD the most important factors that influence patient perception of health are the presence of symptoms and a gluten-containing diet. HRQOL improves to levels similar to
those described in the general population in CD patients well controlled with a GFD (Casellas, Rodrigo et al. 2008). Improved HRQOL has been demonstrated after one year on a GFD regardless of the severity of signs and symptoms at baseline diagnosis (Lewis, Hubbard et al. 2011). These findings reinforce the important HRQOL factors related to appropriate diagnosis and treatment. Furthermore, Nachman et al. (2009) demonstrated that treatment with a GFD induces a rapid and significant improvement in life quality in patients who are symptomatic at diagnosis. However, in CD patients who were asymptomatic at diagnosis the GFD did not change their life quality. After one year of GFD adherence both these sub-groups had comparable scores to those of healthy controls, suggesting symptom improvement was significantly related to HRQOL. Further work by Nachman et al. (2010) reinforced these findings demonstrating a long-term deterioration of HRQOL outcomes in CD people who did not comply with a strict GFD (Nachman, del Campo et al. 2010). Strict adherence to a GFD is considered the most effective and improves symptom scores and HRQOL in patients with CD.

2.1.14.2 Belief systems related to CD and the HRQOL
The perceived benefits and costs of adherence to a GFD in the treatment of CD impacts individuals’ health-related quality of life. The theory of planned behaviour in predicting GFD adherence has been explored by Sainsbury et al. (2011). These findings suggest that a poorer intention to adhere to a GFD was associated with poorer attitudes and perceptions of dietary control. Furthermore, poor GFD adherence was associated with depression and poor HRQOL. Interventions to improve attitudes, food control and psychological conditions may improve HRQOL (Sainsbury and Mullan 2011). However, Barrat et al. (2011) demonstrated that while most people with CD adhere to a GFD the perceived degree of difficulty and psychological stress of doing so had a greater impact on HRQOL than the degree of GFD adherence. These findings reinforce the need for patients with CD to be equipped with practical, social and psychological skills to adhere to a GFD and so improve their HRQOL.

2.1.14.3 HRQOL: Functional gastrointestinal disorders and CD
The functional gastrointestinal disorders (FGD) reflux and IBS are both associated with a lower HRQOL and are more prevalent in the CD population than in age-matched and sex-matched controls (Barratt, Leeds et al. 2011). Furthermore, IBS and reflux symptoms are associated with reduced HRQOL and increase the likelihood of anxiety and depression (Barratt, Leeds et al. 2011). Therefore, detection and management of these symptoms in patients with CD is important in improving their HRQOL (Welander, Tjernberg et al. 2010).
2.2 The function of Gut-Associated Lymphoid Tissue

A review of the function of the gut-associated lymphoid tissue (GALT) is important when considering the role of the microflora in CD. Approximately 70% of the total immunologically active cells in the body are located in the GALT, which is the largest mass of mucosal lymphoid tissue in the human body (Vighi, Marcucci et al. 2008). Whilst, lymphoid tissue can be found in mucous membranes including the tonsils, adenoids and regions of the gastrointestinal tract, it is in highest numbers and most diffusely distributed in the lamina propria of the small intestine.

The GALT is made up of several types of lymphoid tissue that store T- and B-lymphocytes. Its specialised cells, tissues and signalling systems detect, protect and remember harmful substances and organisms while identifying and tolerating harmless ones and mediating inflammation (Tlaskalová-Hogenová, Stepánková et al. 2004). Tlaskalova et al. (2004) delineate the characteristic features of mucosal immunity that distinguish it from systemic immunity, which include mechanisms of innate defence, populations of unique lymphocytes, colonisation of mucosal glands by cells originating from mucosal associated tissues, and the preferential induction of inhibitory responses to non-pathogenic antigens. Mucosal immunity may be fundamentally biased towards immune tolerance.

The gastrointestinal tracts defence system is a dynamically complex and intricate orchestration of surveillance and responses. The intestinal epithelial cells (enterocytes) form a single layered wall that protects the entry of harmful substances from the luminal contents entering the sub-mucosa by secreting mucins and defensins (Söderholm and Perdue 2001). In addition, the intestinal T-cells are capable of generating an autogenous innate immune response that triggers the production of antibodies that bind, deactivate and eliminate antigen antibodies (Guarner and Malagelada 2003a).

One important physiological function of the intestinal epithelial cells is the capacity to communicate with commensal microflora to coordinate an immune response (Wells, Rossi et al. 2011). Therefore the composition and quantities of commensal organisms are thought to play a critical role in the interplay between the epithelial barrier's capacity to prevent pathogenic microorganisms from entering into systemic circulation (Conte, Schippa et al. 2006; Wells, Rossi et al. 2011).

Occasionally the GALT can orientate the immune system towards antigens in the luminal content, thereby provoking damage to the intestinal mucosa, as reflected in CD and allergic gastritis (Vighi, Marcucci et al. 2008). The immune mechanisms in this process are very complex and can be categorised relatively crudely into either (a) adaptive immunity,
characterised by specificity and memory or (b) innate immunity which is less specific, with no developmental memory (Vighi, Marcucci et al. 2008).

2.2.1 The intestinal microflora and GALT relationship

The gastrointestinal tract is normally colonised with trillions of commensal microorganisms that provide metabolomic and nutritional benefits to the host. While the role of pathogenic bacteria in inducing gastric inflammation is well understood, even commensal organisms under certain micro-ecological conditions have the capacity to be pathogenic, especially when factors allow them to overgrow usual population numbers (Quigley and Quera 2006). All micro-organisms have the ability to elicit an immune response. The intestinal mucus and its secretion of antimicrobial agents is designed to limit or prevent direct interaction of the luminal contents with the intestinal mucosa (Atkins and Furuta 2010). Given a certain microbial environment, potential pathogens may penetrate and interact with cellular elements of the mucus and induce an inflammatory response (Wilson 2008). A critical role of the commensal microflora is in forming a mucosal barrier effect that interacts with the mucosa via a lympho-epithelial and bacterial cross-talk, inducing immuno-tolerance and maintaining homeostasis (Wells, Rossi et al. 2011). Tolerance is the process by which the immune system does not attack an antigen. Not all the molecular mechanisms underlying bacterial signalling and tolerance have been fully elucidated. However, some of the mechanisms involved in the GALT's immune relationship with microorganisms are discussed further in Chapter 2.3. These mechanisms play a critical role in allowing colonisation of the gastrointestinal tract with commensal microflora, and so become the host's allies in warding off colonisation by transient pathogens (Lu and Walker 2001). The development of this relationship between commensal microorganisms and the host is orchestrated by toll-like receptors and nucleotide-binding oligomerisation domain proteins (NODs)(Wells, Rossi et al. 2011). The role of these specialised immune cells is presented in detail in Appendix A1.

2.2.2 Commensal bacteria and immune function

The intestinal microflora initiate and regulate intestinal inflammation and tolerance (Round and Mazmanian 2009). Commensal organisms induce immune tolerance by stimulating the production of lymphocytes from the Peyer's patches. This is followed by a key feature of intestinal adaptive immunity, which is the generation of massive amounts of non-inflammatory immunoglobulin A (IgA) antibodies through multiple follicular and extra-follicular pathways. These operate in the presence or absence of cognate T-B-cell interactions (Cerutti and Rescigno 2008). Between three to five grams of IgA is secreted into the intestinal
lumen each day. This accumulates to 75% of the total immunoglobulin produced in the human body. The role of B- and T-lymphocytes in normal and aberrant immune responses is discussed in detail in Appendix A1. Importantly, in the absence of normal predominant enteral microflora appropriate populations of B- and T-lymphocytes do not locate in the lamina propria and IgA is not secreted (Cerutti and Rescigno 2008). If tolerance of the normal commensal bacteria is not established in the GALT persistent inflammation may ensue (Wiedermann 2003).

Reduced numbers and diversity of commensal microorganisms are associated with a number of other processes that assist in the homeostasis of the GALT (Mulder, Schmidt et al. 2009). Mulder et al (2009) point out that decreases in gastrointestinal epithelial cell turnover, a reduction in smooth muscle function, a decreased vascularisation and a diminished development of GALT are all thought to be related to a lack of numbers and microbial diversity. The indigenous commensal microflora may also stimulate adaptive immunity via their ability to stimulate the production of antigen-presenting and intestinal epithelial cells, and these specialised cells are discussed in detail in Appendix A1.

2.2.3 Response of the immune system to intestinal microbiota

It is still not fully understood why commensal organisms do not trigger an inflammatory cascade similar to that of notorious pathogens. However, over the last decade our understanding of how the intestinal epithelial cells recognise commensal organisms and how they maintain host-bacterial symbiosis has advanced considerably. Endocrine, goblet cells and enterocytes of the intestinal epithelium express a range of pattern-recognition receptors to sense the presence of microbes. The toll-like receptors and nucleotide oligomirisation domain receptors are well known for their roles in pathogen recognition and the induction of innate effectors and inflammation (Wells, Rossi et al. 2011).

The innate barrier functions of the epithelium play an important role in maintaining a harmonious relationship with the commensal community (Maciej and Laura 2008). These innate effectors are regulated by the signalling of pattern-recognition receptors, which explains why defects in NF-kappa B (NF-κ B) pathways of toll-like-receptor signalling are more likely to result in the development of inflammatory bowel disease (Rakoff-Nahoum and Medzhitov 2008). In addition, secretory immunoglobulin-A (sIgA) antibodies to the microbiota limit epithelial contact and invasion of host cells (Cerutti and Rescigno 2008). Epithelial cells produce a B-cell-activating factor of tumour necrosis factor (TNF) family (BAFF) and a proliferation-inducing ligand, which promote B-cell recruitment in the lamina propria and class switching in response to toll-like receptors. Therefore the host’s recognition
of intestinal microbes is inextricably linked to the production of sIgA and immune exclusion of microbes (Cerutti and Rescigno 2008). Despite the existence of several mechanisms to avoid intimate contact of the epithelium with intestinal bacteria, the lamina propria has a distinctly immunosuppressive tone so as to inhibit over-reaction to innocuous luminal antigens, including the commensal microbiota. This mechanism of ‘oral tolerance’ depends largely on the development of T-regulatory cells in draining lymph nodes.

The epithelial cells produce thymic stromal lymphopoietin (TSLP) (Zhang, Zhou et al. 2012). TSLP is involved in immune homeostasis or inflammation. TSLP and tumour growth factor- beta (TGF-β), and possibly other factors that abolish the ability of dendritic cells to produce inflammatory cytokine responses, promote the induction of T-regulatory (T-reg) cells in the mesenteric lymph nodes (Taylor, Zaph et al. 2009). TSLP is up-regulated by NF-κ B-dependent pathways, suggesting that pattern-recognition receptors signalling from the luminal side of the epithelium would enhance the suppressive tone in the gastrointestinal tract, normally keeping inflammation under control. However, in the case of infection, chemokine secreted by epithelial cells would recruit unconditioned dendritic cells to mucosal sites, which changes the response to a more pro-inflammatory character (Tlaskalová-Hogenová, Stepánková et al. 2004). Understanding the interactions between the microbial-intestinal epithelial cells' interactions in immuno-regulation and inflammation is important for directing investigations concerning the gastrointestinal microbiota and CD. The equally important role of the mucous layer that covers the epithelium is discussed in detail in Appendix A.1.

2.2.4 Tight Junctions

The epithelial tight junctions constitute the major component of the gastrointestinal tract barrier. They act as a physical functional barrier against para-cellular penetration of macromolecules from luminal contents, including microorganisms and dietary antigens (Mitic and Anderson 1998). The tight junction is organised by four types of trans-membrane proteins, occludins, claudins, tricellulin and junctional adhesion molecules, which interact with scaffold proteins such as Zonulins (ZO) - ZO-1, ZO-2 and ZO-3 (Anderson and Van Itallie 1995; van Itallie and Anderson 2006). The role of ZO-1 is to anchor the trans-membrane protein of tight junctions to the actin cytoskeleton and it interacts with other tight junction proteins. Tight junction proteins interact with numerous signalling proteins that regulate tight junction assembly and maintenance, indicating the potential role of intra-cellular signalling pathways in the regulation of epithelial tight junction and barrier function (Capaldo and Nusrat 2009).
The interplay of tight junctions is influenced by events in the intestinal epithelium, lamina propria and lumen and has potential to open and transfer antigenic material. Paracellular transport of larger molecules is restricted by which therefore in a normal regulatory state protect the underlying mucosa from antigen exposure (Snoeck, Goddeeris et al. 2005). The structural organisation of all the intestinal epithelial cell junction complexes contains includes both tight junctions, as well as junction types found in other epithelial tissue (adherens junctions and desmosomes).

The tight junctions between M-cells can be differentiated from enterocytes by the greater depth and enhanced expression of catenin actinin, polymerised actin and, in some cases, cadherin. M-cells may require a more rigid cytoskeleton and a more stable intercellular adhesion to cope with increased interactions with and transcytosis of enteral bacteria.

Cytokines are small cell signalling protein molecules that are secreted by numerous cells and are used extensively in intercellular communication. Although a cellular response to cytokines show cell type specific, pleitropic and time dependent dose-effects, common mechanisms for tight junction modulation emerge. These include cytokine induced actin remodelling and changes in tight junction structure. The effects of cytokines on tight cell junctions appear consistently to be independent of apoptosis. Epithelial and endothelial barrier continuity, when disrupted by cell apoptosis, leads to increases in tissue permeability (Bruewer, Luegering et al. 2003). Cytotoxic effects due to cytokines have been described in a variety of cells lines and are dependent on dose and duration of tumour necrosis factor-α (TNF-α) and IFN-γ exposure (Madara and Stafford 1989; Adams, Planchon et al. 1993; Bruewer, Luegering et al. 2003; Ma, Iwamoto et al. 2004). An example of this is when pharmacological inhibition of apoptosis fails to attenuate increases in monolayer permeability (Li, Zhang et al. 2008). Therefore cytokines are capable of directly modifying tight junction composition and structure through signalling pathways independent of cell death (Capaldo and Nusrat 2009).

Cytokines can be classified as proteins, peptides or glycoproteins. The term cytokine encompasses a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin (Ausiello and Mastrantonio 2000). The term cytokine refers to the immuno-modulating agents, such as interleukins and interferons (Anne and Arai 2000). All nucleated cells have the capacity to produce cytokines but the endothelial/epithelial cells and resident macrophages near the interface of the external environment are the most potent producers of interleukin-1, interleukin-6 and TNF-α (Kayama and Takeda 2012).
2.2.4.1 Cytokine profiles in coeliac disease

A number of groups have demonstrated cytokine profiles for patients with CD. Lahat et al. (1999) measured specific cytokine levels in the intestinal and peripheral samples of CD patients and those of healthy controls. Both compartments showed significant differences in the cytokine levels of the two groups. The percentage of samples expressing IL-2, IFN-γ, TNF-β, IL-10, IL1-β, TNF-α and TGF-β transforming growth factor-β from CD was higher than in controls. A prominent finding was the expression of both Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) associated cytokine transcripts in the same biopsies and peripheral blood cells from patients with active CD, implying the activation of Th0 cells (Lahat, Shapiro et al. 1999). More recently, Lahdenpera (2011) assessed the Th1-Th2-Th3 gene activation profile in the small intestine of seven children, initially at CD diagnosis and again one year after adherence to a GFD. They demonstrated that Th1 gene expression profiles, including IFN-γ signal inducer, activator transcription (STAT) 1, and interferon regulatory factor (IRF) 1, together with reduced interleukin (IL)-2 expression, were pronounced in small intestinal biopsies from children with untreated CD. A down-regulation of IFN-γ transcripts was observed one year after a GFD, but there was still increased expression of STAT1 and IRF1 in association with low IL-2 expression despite strict adherence to the GFD treatment. The authors concluded that the GFD did not correct the increased activation of the Interferon-γ signalling pathway related markers or did it reduce IL-2 expression, suggesting that they represent an immune dysregulation not dependent on gluten exposure (Lahdenperä, Ludvigsson et al. 2011). Furthermore, Kapoor et al. (2012) suggested that serum-soluble IL-2 receptor and IL-6 have a good correlation with CD activity and can be used as reliable markers for detecting minimal transgression from a GFD.

IL-1 is a type 1 pro-inflammatory cytokine that is elevated in the intestinal mucosa of patients with CD (Cherñavsky, Páez et al. 2008). In both epithelial and endothelial in vitro cell culture systems, IL-1 addition to growth media directly increases para-cellular permeability to ions and small molecules (Marcus, Wyble et al. 1996; Al-Sadi and Ma 2007). Chernavsky et al. (2008) suggested that the co-existence of both TNF-α and IL-1-β may enhance the risk of CD when they compared the cytokines individually and synergistically in 288 CD patients and 224 healthy non-HLA positive individuals.

Cytokine restructuring of tight junctions proceeds through a variety of different signalling pathways to affect para-cellular permeability. A number of groups have sought to distil common mechanisms by which cytokines affect tight junctions and the association with disease, related to increased intestinal permeability. However, pleiotropic cytokine actions as well as temporal and dose-dependent variations in cell culture systems make it complicated.
Modulation of tight junction properties by cytokines appears to proceed through two distinct processes (Capaldo and Nusrat 2009). First, by remodelling of tight junctions by selectively removing or introducing tight junction components, or secondly, by the wholesale restructuring of tight junction and actin networks. Several lines of evidence indicate that these processes may function simultaneously or sequentially (Capaldo and Nusrat 2009).

2.2.5 The mechanisms of immune tolerance

The mammalian immune system protects the host from a broad range of pathogenic microorganisms while avoiding misguided or excessive immune reactions that would be deleterious to it. Both protective and harmful immune responses are principally mediated by T- and B-cells, with each having diverse skills in antigen recognition, high antigen-specificity, potent effector activity and long lasting immunologic memory. These efficacious mechanisms of immune cells also have the potential to cause serious damage in the event of an aberrant immune response, such as autoimmunity or allergy, being triggered. How the adaptive immune system discriminates between self and foreign antigens is critical to our understanding of autoimmune conditions including CD. There are two major groups of mechanisms for achieving self-tolerance: recessive and dominant.

Recessive tolerance, also known as cell-intrinsic mechanisms, leads to the physical elimination or functional inactivation of a given self-reactive T-cell clone. The recessive mechanism of tolerance operates in the thymus and in the periphery and is likely to be responsible for neutralisation of the majority of high-affinity T-cells recognising self (Lu and Rudensky 2009). Immature CD4 and CD8 double-positive (DP), and single-positive (SP) thymocytes undergo apoptosis or ‘negative selection’ upon high affinity T-cell receptor (TCR) engagement by self-antigen displayed by thymic dendritic cells and thymic medullary epithelial cells (Lu and Rudensky 2009). The self-reactive thymocytes that escape clonal deletion become “anergic”, or incapable of efficient proliferation in response to ‘self’ antigen, and differentiate into functional effector cytokine-producing or cytotoxic T-cells (Starr, Jameson et al. 2003; Lu and Rudensky 2009). Beyond the thymus, chronic engagement of TCR displayed by peripheral T-cells by self antigens can also lead to activation-induced cell death or anergy induction. Peripheral anergy induction is reinforced by two-signal requirements for initiation of the naïve T-cell responses. This requirement is fulfilled upon simultaneous engagement of TCR and T-cell co-stimulatory receptor CD28 by cognate peptide-MHC complex and inducible CD28 ligands CD80 and CD86 displayed on the surface of antigen presenting cells (APC) (Starr, Jameson et al. 2003). High levels of CD80 and CD86 expression are induced upon activation of a diverse set of dedicated sensors...
directly in response to microbial or viral products, or indirectly by metabolic or biochemical changes that they induce (Lu and Rudensky 2009).

Recessive tolerance is insufficient to counter the threat of immune-mediated pathology without dominant tolerance. In the dominant or cell-extrinsic mechanism certain T-cells actively keep in check the activation and expansion of aberrant or over active lymphocytes, in particular other types of T-cells (Sakaguchi, Yamaguchi et al. 2008). It is thought that disruption in the development or function of T-regulatory cells is a primary cause of autoimmune and inflammatory diseases (Sakaguchi, Yamaguchi et al. 2008; Saurer and Mueller 2009). Every adaptive immune response involves recruitment and activation of not only effector T- and B-cells but also T-regulatory cells, and the balance between the two populations is critical for the proper control of the quality and magnitude of adaptive immune responses and for establishing or breaching tolerance to self and non-self antigens (Sakaguchi, Yamaguchi et al. 2008; Saurer and Mueller 2009).

2.2.6 The mechanism of intestinal inflammation

Inflammation is essentially a protective tissue response to injury or destruction of tissues, which serves to destroy, dilute, or wall off both the injurious agent/pathogen and the injured tissues. Intestinal inflammation is a localised or general defensive response to a pathogenic organism or antigen which in turn leads to its elimination. As discussed elsewhere, an inflammatory response triggered by the release of mediators is essential for host defence. However, unregulated or excessive inflammation is harmful as is demonstrated in CD (Schulzke, Ploeger et al. 2009). Control mechanisms directed from cells of the intestinal epithelium in response to luminal contents have the ability to maintain homeostasis, maintain inflammatory response or terminate immune responses (Schulzke, Ploeger et al. 2009). It is well understood that the inflammatory response orchestrates the activation of both innate and adaptive immunity through the release of cytokines (Iwasaki and Medzhitov 2010). In normal health there is usually active down-regulation of inflammatory signals to maintain a state of controlled tolerance in order to co-exist with commensal bacteria (Ashida, Ogawa et al. 2012). Even small changes in the heavily regulated system may lead to chronic inflammation and functional disturbances of the intestinal tract. Inflammation induces blood vessel dilation, increased expression of adhesion molecules, exudation of fluids and extravasations of the neutrophils, macrophages and mast cells, activated Th-cells, cytotoxic T-cells, and memory T- and B-cells to tissues (Duchmann, Kaiser et al. 1995). Activation of inflammation at a molecular level is triggered by TNF- alpha, IL-6 and IL-1 β, which stimulate endothelial cells to up-regulate receptors for immune cells (Westerholm-Ormio, Garioch et al. 2002).
The innate immune response is inextricably intertwined with the mechanisms of inflammation. Inflammatory responses mediated by the innate immune system are immediate. Innate immune mechanisms that elicit an inflammatory response employ a number of transiently synthesised preformed non-specific a-effector molecules and phagocytes (Vesely, Kershaw et al. 2011). In the event of innate immune overload an adaptive response is triggered (McCormick and Weinberg 2010).

An adaptive response is based on the recognition of the antigen presented by antigen presenting cells. Pattern recognition receptor signalling in response to the pathogen associated molecular pattern molecules (PAMPs) of microorganisms activates immune mechanisms, causing a release of TLRs and nucleotide-binding oligomerisation domains (NODs). This triggers a signalling cascade which alerts and protects the host. However, an aberrant response can turn normal physiological function into a pathological situation, leading to inflammation (Ospelt and Gay 2010). A number of groups have proposed that host-derived negative regulators cross-talk to TLR and NOD signalling cascades and shape the magnitude and duration of inflammatory processes (D 2006; Ospelt and Gay 2010; Wells, Rossi et al. 2011).

The role of cytokines in inflammatory bowel disease has been reviewed and is presented in Table 2.4. Up-regulation of TNF-α, IL-1 β, IL-12 and IL-23 by macrophages and dendritic cells is present in inflammatory bowel disease (IBD) patients (Sanchez, Munoz et al. 2008). In addition to Th1, Th2 and NK cells up-regulated IL-5 and IL-13 and Th17 up-regulating IL-17 (Sanchez, Munoz et al. 2008). The abrogation of TGF-β signalling in T-cells is instrumental in inflammatory response and has a dual role as a potent suppressor of T- and B-cell responses while promoting the production of Immunoglobulin A (Cerutti and Rescigno 2008). Strobel and Mowat et al (2006) suggests that unresponsiveness of T-cells to commensal bacteria can actually be reversed by depletion of IL-10 and TGF-β resulting in inflammation, while Lee et al (2003) describe the action of effector cytokines TNF-α and IFN-γ and IL-23 in inflammatory processes. T-regulatory cells are crucial to the preservation of immune homeostasis because they prevent uncontrolled expansion of effector T-cells after exposure to bacterial antigen (Sakaguchi, Yamaguchi et al. 2008). A reduced number of T-regulatory cells is associated with increased intestinal inflammation that is associated with activated TLR-4 and subsequent NF-κB activation (Himmel, Hardenberg et al. 2008).

In summary, the small intestine in humans plays a critical role in the digestion and assimilation of nutrients and in facilitating normal digestive function. In addition the small intestine is home to a complex and intricate immune system that regulates both adaptive and innate responses. Normal function of the GALT is critical in differentiating between...
beneficial and pathogenic microorganisms. Appropriate responses by the GALT are known to play an important role in the balance between pro- and anti-inflammatory responses. Polarisation in these responses can lead to a number of pathologies, including autoimmunity.

Figure 2.2 serves as a graphical summary of the content in this chapter and Appendix A1, in addition to introducing Chapter 2.3, and is taken from Janoff and Gustafson (2012).

Table 2.4 The role of cytokines and the cell lines involved in their production in patients with Inflammatory Bowel Disease (adapted from (Sanchez, Munoz et al. 2008))

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Ulcerative Colitis</th>
<th>Crohn’s Disease</th>
<th>Cells Involved in Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Macrophages</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Unknown</td>
<td>Upregulated</td>
<td>Th1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1ra/IL-1 ratio</td>
<td>IL-1ra/IL-1 ratio</td>
<td>Macrophages</td>
</tr>
<tr>
<td>IL-6</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>others</td>
</tr>
<tr>
<td>IL-18</td>
<td>Not clear; possibly defective</td>
<td>Not clear; possibly defective</td>
<td>Macrophages</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Not clear; possibly defective signalling</td>
<td>Not clear; possibly defective signalling</td>
<td>Th0, Th3, T-reg</td>
</tr>
<tr>
<td>IL-10</td>
<td>Not clear</td>
<td>Yes, upregulated</td>
<td>Tr1 T-reg</td>
</tr>
<tr>
<td>IL-4</td>
<td>Not clear</td>
<td>Not clear</td>
<td>Th2 NK</td>
</tr>
<tr>
<td>IL-12</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Macrophages and dendritic cells</td>
</tr>
<tr>
<td>IL-23</td>
<td>Yes</td>
<td>Yes</td>
<td>Macrophages and APCs</td>
</tr>
<tr>
<td>IL-27</td>
<td>Not clear</td>
<td>Upregulated</td>
<td>APCs</td>
</tr>
<tr>
<td>IL-17</td>
<td>Upregulated</td>
<td>Upregulated</td>
<td>Th17</td>
</tr>
<tr>
<td>IL-13</td>
<td>Upregulated</td>
<td>Not clear</td>
<td>Th1, NK</td>
</tr>
<tr>
<td>IL-5</td>
<td>Upregulated</td>
<td>Not clear</td>
<td>Th2, NK</td>
</tr>
</tbody>
</table>
Figure 2.2 The complex interplay between luminal microorganisms and innate and adaptive immune molecules. **Specific colonizing bacteria elicit innate immune responses and development of T-cells and IgA-producing cells in the intestine** (Janoff, Gustafson et al. 2012).
2.3 The intestinal microbiome

2.3.1 Introduction
In 2001 Joshua Lederberg coined the term ‘human microbiome’ (Lichtman and Sanes 2008). The human microbiome is defined as the collection of microbes (bacteria, viruses and single cell eukaryotes) that inhabit the human body. The terms microbiome, microbiota and microflora are often used interchangeably. For the purpose of this thesis these terms will be referred to with equivalent meaning (Ursell, Metcalf et al. 2012). The human microbiome consists of the 10–100 trillion symbiotic microbial cells, mainly bacterial cells, harboured by every individual and predominantly found in the gut (Turnbaugh, Ley et al. 2007; Ursell, Metcalf et al. 2012). Interest in the diversity of the human microbiota dates back to the discoveries first made by Van Leewenhoek in 1680 (Dobell 1920). Van Leewenhoek designed the first lens for the microscope that was able to identify distinct microbial differences between his own oral and faecal microbiota (Dobell 1920; Porter 1976). During the last decade publications dedicated to the identification and the inter-individual and intra-individual diversity of the human microbiota have gained momentum. This increase in publications is mainly attributed to the development of molecular technologies since the late 1970s (Ouwehand 2010). Much of the growth of our knowledge of the microbiota over the last four years has been contributed to by publications originating from the Human Microbiome Project (HMP).

The HMP was a collaborative effort between 80 universities, spanning the years 2007 to 2011. It was a 173 million dollar project funded by the United States National Institute of Health (http://hmpdacc.org/, accessed on 20/3/2013). The project involved recruiting over 300 healthy individuals and collecting over 11,000 specimens from 18 different sites of the human body. Its primary objective was to categorise the microbial species that inhabit different sites of the human body with a view to establishing what constitutes the parameters of a normal microbiome in healthy individuals. The results of these findings were to provide the basis for future research that assesses the microbiome in various disease states, thereby forming a basis for interventions that target disease associated aberrations in the microbiome.

Seven years before the HMP Hooper et al. (2001) estimated that the hundreds of microbial species native to the colon, where microbial richness and abundance are maximal, would contain ten times more genes than does the human genome. Before the Human Genome Project (HGP) was completed some researchers predicted that 100,000 human genes would be found. It was both a surprise and humbling that the human genome contained a comparative number of genes to that of the fruit-fly genome (i.e. 20,000). It soon became apparent that the
definition of what forms a human being should be revised to include microbial genes and this was confirmed by the HMP, estimating 100,000 microbial genes inhabit the human body (Cani and Delzenne 2009).

The discovery that the inclusion of genes co-inhabiting the gastrointestinal tract would far exceed original estimates of 100,000 has profound implications for the future direction of research in human health and disease states. The conclusion of the HMP was that humans are a ‘super-organism’, with microbes inhabiting their blood stream, tissues and cells. Furthermore, millions of genes belonging to thousands of species interact with a mere 20,000 genes of the human body and with this interaction there is potential for dysfunction (Cani and Delzenne 2009; Vardakas, Konstantelias et al. 2012). Another conclusion of the HMP, one that in fact confirms earlier suggestions (Hooper, Midveldt et al. 2002; Dethlefsen, Eckburg et al. 2006), was that everyone has a unique microbiome and that variations in this microbiome occur over both long and short periods of time (Dethlefsen, Huse et al. 2008; Cani and Delzenne 2009). This chapter will discuss the role, the development, the composition, the assessment and the impact of the microbiome in health and disease.

### 2.3.2 The role of the intestinal microbiome

The intestinal microflora plays several key roles in human health. These roles include gut maturation, host nutrition and resistance to pathogens (Versalovic, Wilson et al. 2009). In addition we now understand that the intestinal microbes regulate intestinal epithelial proliferation (Rakoff-Nahoum and Medzhitov 2008), host energy metabolism (Noverr and Huffnagle 2004; Cani and Delzenne 2007; Cani and Delzenne 2009) and inflammatory immune responses (Noverr and Huffnagle 2004). The associations between aberrations in the intestinal microbiota have become well recognised but are still relatively poorly understood. The last seven years have seen associations made between microbial communities and diseases, including inflammatory bowel disease (IBD) (Achkar and Duerr 2008; Alverdy and Chang 2008), cancer (McGarr, Ridlon et al. 2005; Davis and Milner 2009), allergies (Abrahamsson, Jakobsson et al. 2007), mental health (Addolorato, Mirijello et al. 2008), obesity (Cani and Delzenne 2009), and autoimmune disorders including rheumatoid arthritis, multiple sclerosis and type 1 diabetes (Blander, Torchinsky et al. 2012).

The capacity of the intestinal microbiome to fulfil important roles depends largely on the composition of the microbiota. This composition co-develops with the host from birth and is subject to a complex interplay that depends on the host genome, nutrition and lifestyle. The gut microbiota are involved in regulating multiple-host metabolic pathways, giving rise to interactive host microbiota metabolic signalling and immune-inflammatory axes that
physiologically connect the gut, liver, muscle and brain (Cani and Delzenne 2007; Cani and Delzenne 2009). It has been suggested that a deeper understanding of these axes is a prerequisite for optimizing therapeutic strategies to manipulate the gut microbiota (Nicholson 2012).

2.3.3 The development of the intestinal microbiome

It is thought that the development and interactions between the gut microbiota and the host immune system begin at birth, but more recently it has been suggested the maternal uterine environment is not as sterile as was previously thought and plays an important role in early microbial programming (Gregory 2011; Nicholson 2012; Rautava, Luoto et al. 2012). The development of microbial programming in utero and during birth shapes the development of the immune system, and the immune system in turn shapes the composition of the microbiota (Li, Wang et al. 2008). The immune-mediated signalling processes, together with direct chemical reactions between the microbe and host, act upon multiple organs such as the gut, liver, muscle and brain (Cani and Delzenne 2009). Together these complex interactions comprise a series of host metabolic axes. Figure 2.3, taken from Nicholson et al. (2012), illustrates contributions to the development of the intestinal microbiome and can be described as follows: “The influence of the gut microbiota on human health is continuous from birth to old age. The maternal microbiota may influence both the intrauterine environment and the post-natal health of the foetus. At birth about 100 microbial species populate the colon. Early environmental factors (e.g. method of delivery), nutritional factors (e.g. breast- or bottle-feeding), and epigenetic factors have been implicated in the development of a healthy gut and its microbial symbionts. Changes in gut microbial composition in early life can influence risk for developing disease later in life. During suckling the microbial community develops rapidly; shifts in microbial diversity occur throughout childhood and adult life, and in old age there is a decrease in Bacteroidetes and an increase in Firmicutes species. The gut microbiota are important for maintaining normal physiology and energy production throughout life. Body temperature regulation, reproduction and tissue growth are energy-dependent processes that may rely in part on gut microbial energy production. Extrinsic environmental factors (such as antibiotic use, diet, stress, disease and injury) and the mammalian host genome continually influence the diversity and function of the gut microbiota with implications for human health. Disruption of the gut microbiota (dysbiosis) can lead to a variety of different diseases, including (a) IBD, colon cancer, and IBS; (b) gastric ulcers, non-alcoholic fatty liver disease, and obesity and metabolic syndrome; (c) asthma, atopy, and hypertension; and (d) mood and behaviour through hormone signalling e.g. glucagon-like peptide-1 (GLP-1). The gut
microbiota are also important for drug metabolism and preventing the establishment of pathogenic microbes.”

Figure 2.3 The gut microbiota in development and disease (Nicholson 2012)

2.3.3.1 The development of the bacterial consortia in the human gut during childhood
An essential role of the HMP was to understand the assembly and community composition of the microbiota in various body sites including the gastrointestinal tract (Turnbaugh, Ley et al. 2007; Koenig, Spor et al. 2011). Adult microbiota are thought to be relatively stable over time (Ley, Peterson et al. 2006; Turnbaugh, Ley et al. 2007; Dethlefsen, Huse et al. 2008); this stability imparts resilience to disturbance, ensuring continued gut function. However, in a disease context such stability and resilience could be detrimental if the gut community is pathogenic (Koenig, Spor et al. 2011). Koenig et al. (2011) proposed that by understanding the succession of the bacterial consortia in the human gut during childhood the development
of strategies that establish health-promoting microbiomes could theoretically prevent microbiome-associated disease in later life. To begin developing this hypothesis Koenig’s group employed molecular techniques to conduct a time series study of the microbiota of an infant from birth to 2.5 years. The authors acknowledged that the results of this ‘case study’ require a follow-up study assessing the intestinal microbiota of multiple infant participants. Nevertheless, Koenig’s group did find a gradual increase in diversity over time that was related to a change in community diversity. Superimposed on these patterns of gradual change were the effects of life events, such as drastic diet changes or antibiotic treatments, which resulted in large shifts in the relative abundance of taxonomic groups. The temporal impact of antibiotics and diet on the composition of the microbiome have been observed by other groups (Beaugerie and Petit 2004; Dethlefsen, Huse et al. 2008). Other significant impacts on the infant’s microbiota included fever during the exclusive breast-feeding stage, which was followed by a shift in community composition with fungal and viral genes increasing (Koenig, Spor et al. 2011). Interestingly, the genes that facilitate the breakdown of plant-derived polysaccharides were present during the exclusive breast-feeding period. The presences of microbial enzymes that degrade non-digestible polysaccharides of plant origin have also been reported by other groups, suggesting a microbial priming in preparation for the introduction of solid foods (Kurokawa, Itoh et al. 2007; Vaishampayan, Kuehl et al. 2010). After the introduction of solid foods there appears to be another shift, with an increase in the dominance of the phyla classes Bacteroidetes and Firmicutes. This increase was correlated with enrichment in functional genes necessary for the breakdown of xenobiotic compounds and for vitamin biosynthesis. Together, the results of Koenig et al.’s (2011) study and the observations of other inter-related studies (Preidis and Versalovic 2009; Hunt, Foster et al. 2011; Sellitto, Bai et al. 2012) suggest that the human microbiome at 2.5 years of age has many of the functional attributes of the adult microbiome.

2.3.3.2 Mode of delivery and microbiome
The delivery mode of an individual is thought to shape their unique microbiome. During pregnancy infants reside in a semi-sterile uterine environment, and during a vaginal birth they are inoculated with a wide variety of microbes from the mother’s vaginal microbiome, or with the microbiome of the mother’s skin if they are born via caesarean section (C-section) (Domínguez-Bello, Costello et al. 2010). The hypothesis that the two delivery modes may result in different microbial compositions in infants has been supported by a number of groups (Grölund, Lehtonen et al. 1999; Fanaro, Chierici et al. 2003; Guarner and Malagelada 2003b; Penders, Thijs et al. 2006). Furthermore, it is thought that the initial inoculation of
microbiota from the birth canal is important in defining the successional trajectories leading to more complex and stable ecosystems (Connell and Slatyer 1977; Biasucci, Benenati et al. 2008). Importantly, it is thought that only a subset of the microbes to which the newborn is initially exposed will permanently colonise available niches and contribute to the distinctive microbiota harboured by body habitats of adults (Dominguez-Bello, Costello et al. 2010). Dethlefsen et al. (2006) propose that the diverse and personalised gut communities of humans are shaped by historical contingencies during community assembly and temporal dynamics arising from interactions within the microbiota, including during birth.

In Australia in 2009 31.5% of infants were delivered by C-section (http://www.aihw.gov.au/access/201204/feature/mums-and-bubs.cfm, accessed on 20/03/2013). The long-term sequel to the increase in C-section deliveries has not yet been fully elucidated. However, it has been suggested that the delivery mode may lead to fundamental differences in microbiome development, which may in turn contribute to variations in normal physiology and predisposition to disease (Pflughoeft and Versalovic 2012). Decker et al. (2010) explored the prevalence of C-section delivery in children with IBD. They gathered data on the mode of delivery in children with Crohn’s disease, ulcerative colitis and CD and on healthy children. The results demonstrated a significantly enhanced likelihood of developing CD in children born by C-section compared with control subjects, suggesting a potential causal risk factor in the development of CD. It is noteworthy that the significant increase in C-section births in Australia and the USA over the last 30 years (Robson, Tan et al. 2009; Stavrou, Ford et al. 2011) has coincided with an exponential increase in the number of children diagnosed with CD (Lionetti and Catassi 2011).

While the mode of delivery appears to be an important foundational step in establishing an individual’s microbiome, other influential factors in infancy are known to play an integral role in what appears to be a cohesive progression in establishing a unique microbiome in the first years of life (Penders, Thijs et al. 2006). The birth environment, infant feeding practices, number of siblings, exposure to animals, infection at an early age and psychological and physical stressors are proposed as a set of unified environmental influences in the development of an individual’s intestinal microbiota. This unified environmental influence theory has been suggested by a number of groups (Penders, Thijs et al. 2006; Cabrera-Rubio, Collado et al. 2012; Costello, Stagaman et al. 2012).

2.3.3.3 Microbiome of breast milk
Breast-feeding has regained recognition as the most important postpartum element in the metabolic and immunological programming of the infant. The constituents of breast-feeding
are considered the gold standard for conferring protection from gastrointestinal and respiratory infections (Duijts, Jaddoe et al. 2010; Natchu, Liu et al. 2012), obesity (Gillman, Rfas-Shiman et al. 2001; Miralles, Sánchez et al. 2012) and for the maturation of the gut immune system in newborn infants (Walker 2010). Breast-feeding is a significant factor in the determination of neonatal gut microbiota. During lactation cells from gut-associated lymphoid tissue of the mother travel to the breast via the lymphatics and peripheral blood (Rigon, Vallone et al. 2012). Sustained long-term mucosal protection after breast-feeding has ceased has also been demonstrated (Eidelman, Schanler et al. 2012).

Once an infant commences breast-feeding the previously immature pre-natal small intestinal epithelium with characteristic delayed enterocyte proliferation and sparse lymphoid cells begins to actively proliferate (Walker 2010). In the small intestine of breast-fed infants mature epithelium and all subclasses of enterocytes are represented, with an abundance of lymphoid tissues (Walker 1999). In addition, breast milk confers an anti-inflammatory effect on the immature and excessive inflammatory response in newborns (Hanson, Silfverdal et al. 2009). TGF-β, IL-10, erythropoiten, and human and bovine lactoferrin act independently or synergistically to reduce the cascade of inflammatory cytokines, and so reduce the immature neonatal inflammatory response.

The factors influencing the milk microbiome and the potential impact of microbes on infant health have not yet been fully elucidated. Hunt et al. (2011) have reported pre- and post-natal factors that are thought to influence the bacterial communities inhabiting human milk by characterising the milk microbial community in 18 women over three different time points. They found that human milk microbiome changed over the period of lactation, with *Weisella, Leuconostoc, Staphylococcus, Streptococcus* and *Lactococcus* predominating in colostrum samples. At one month and again at six months the microbiome was more characteristic of the oral microbiome, with *Veillonella, Leptotrichia, and Prevotella* increasing significantly over time. The microbiota of obese women’s milk was less diverse than the milk of women of normal weight. The authors also noted that women who undertook elective, as opposed to emergency, C-section had different microbes in their milk, suggesting that the physiological stressors of labour experienced prior to the emergency C-section could influence the microbial transmission process to milk (Hunt, Foster et al. 2011). They concluded that milk bacteria are not contaminants and are likely to be influenced by a number of factors, including the mode of delivery and the mother’s weight (Hunt, Foster et al. 2011).

The demonstrated health benefits of breast-feeding, i.e. prevention of allergies and maintenance of normal weight, are thought to be related to the microbiome of breast milk. In a sequence of microbial inoculations in early infancy breast milk plays a critical role in
priming the infant gut (Tringe and Rubin 2005; Hunt, Foster et al. 2011). Furthermore, the environmental factors that influence the milk microbiome, including the mother’s weight and mode of delivery, and their potential effect on the biological capacity of the milk microbiome need to be elucidated.

2.3.3.4 Collective influences on the microbiome in early infancy
As discussed, pregnancy, delivery method and breast-feeding are important factors influencing the development of the intestinal microbiome. Less is known about the role of stress, medications, size of family, hygiene practices and domestic animals in the role of developing the intestinal microbiome during infancy. Adlerberth et al. (2008) investigated a number of these influences and observed that differences in the colonisation pattern can be observed between vaginally and C-section delivered infants, and between infants in industrialised and developing countries. Children who were born vaginally, at home, who had a greater number of siblings and who were breast-fed had greater microbial diversity and fewer infections than those born in hospital and who were bottle-fed and had fewer siblings.

2.3.3.5 The ‘old friends hypothesis’
The ‘hygiene hypothesis’/ ‘old friends hypothesis’ can be traced back to the 1870s when Charles Harrison Blackley noticed that aristocrats and city dwellers were more likely to get hayfever than were farmers (Rook 2010). Similarly, Leibowitz et al. (1966) noted that the incidence of multiple sclerosis in Israel was positively related to sanitation. The term ‘hygiene hypothesis’ was coined and popularised by the media when Strachan et al. (1989) noted that hayfever was less frequent in larger families containing many siblings. This directed focus towards allergic disorders despite the clear evidence that other inflammatory conditions were increasing in parallel in western societies. Popular media provided a polarised interpretation of these findings, suggesting that all early childhood illness and poor hygiene practices were a prerequisite for the normal development of the immune system.

The ‘old friends hypothesis’ was presented to provide a Darwinian synthesis and to focus attention on the fact that modern domestic hygiene practices are not the primary issue (Rook 2010). The twentieth century witnessed an unprecedented rise in the incidence of many chronic inflammatory disorders in wealthy developed countries. These included autoimmune disorders such as CD, diabetes mellitus and multiple sclerosis (Ponsonby, Hughes et al. 2011). Although genetics and specific triggering mechanisms such as molecular mimicry and viruses are involved, the increase in these diseases has been so rapid that an explanation that omits environmental change is incomplete. These environmental factors, most of them microbial, have led to a decrease in the efficacy of our immuno-regulatory mechanisms. This
is probably because we are in a state of evolved dependence on organisms with which we have co-evolved and which have been tolerated as inducers of immuno-regulatory circuits. These organisms have been referred to as ‘old friends’ whose numbers are depleted in the modern urban environment (Janoff, Gustafson et al. 2012). Rook (2010) cautioned against a reductionist approach when investigating depletion of our ‘old friends’. He suggested an integrative study model that considers a range of factors that influence microbial foetal and neonatal programming, including the family and social environment, hygiene practices, psychological and physical stressors, medication use and dietary practices.

2.3.4 Composition of the intestinal microbiome
Although the exact composition of the gut microbiota is not known, advances in metagenomic technologies have recently begun to unravel the human microbiome. As the name implies, metagenomics is the study of a collection of genetic material (genomes) from a mixed community of organisms. Until the 1970s assessment of the composition of the microbiota relied largely on culture-based techniques that characterise only a small fraction of the microbial diversity present in the gut (Holdeman, Good et al. 1976; Qin, Li et al. 2010). The last 40 years have witnessed advances in techniques applied to assess the composition of the intestinal microbiome. The introduction of molecular techniques, e.g. DNA sequencing of polymerase chain reaction (PCR) amplicons from mRNA genes, allowed the detection and enumeration of microorganisms that are refractory to cultivation. Each sequence serves as a proxy for the occurrence of a microbial genome in a microbial community (Eckburg, Bik et al. 2005). Recent findings by Qin et al. (2010) estimate that each individual gives a home to at least 160 species of bacteria from a consortium of 1000 to 1150 prevalent species. Surprisingly, given the complex microbial community of the gut, only a small number of phylotypes (the phylotypic stage is where all members in the taxon look essentially the same) dominate population structures and mask the appearance of many distinct but low abundance taxa in most molecular surveys (Antonopoulos, Huse et al. 2009). Among these bacteria, 90% of the bacterial phylotypes are members of two phyla, the Bacteroidetes and Firmicutes (Qin, Li et al. 2010), with the remaining bacteria belonging to the Actinobacteria or Proteobacteria (Eckburg, Bik et al. 2005; Ley, Peterson et al. 2006; Qin, Li et al. 2010). The complexity of Firmicutes and Bacteroidetes exceeds 15,000 different operational taxonomical units (OTUs) (Antonopoulos, Huse et al. 2009). Within these two phyla the most prominent clusters of intestinal microbial species identified by Qin’s group were Dorea/Eubacteria/Ruminococcus groups and also Bifidobacteria, Proteobacteria and Streptococci/Lactobacilli groups, indicating that similar constellations of bacteria are present in different individuals for reasons
yet to be established (Qin, Li et al. 2010). They conducted a deep sequencing of total DNA extracted from 124 faecal samples employing Genome Analyser technology. Deep sequencing refers to the number of times a nucleotide is read during the sequencing process. Deep sequencing indicates that the coverage, or depth, of the process is many times larger than the length of the sequence under study. Qin et al. (2010) generated 576.7 gigabases (Gb) containing virtually all of the prevalent gut microbial genes. The magnitude of their work is highlighted by the fact that previous groups had managed to generate only 3 Gb of microbial sequence from faecal samples of 33 individuals from the United States and Japan (Tringe and Rubin 2005; Qin, Li et al. 2010). Qin compared their contigs (a set of overlapping DNA segments that together represent a consensus region of DNA) with that of these two largest known intestinal microbiome studies originating from the American and Japanese cohorts. Comparatively, a total of 70% and 85.9% of the reads from the Japanese and US samples, respectively, aligned. An additional 85% and 69% of the contigs were novel to Qin et al (Qin, Li et al. 2010). Importantly, only 31–48% of the reads in Qin’s and the two previous studies could be aligned to 194 public human gut bacterial genomes, and 7.6–21% to the bacterial genomes deposited in GenBank, indicating that the reference gene set obtained by sequencing genomes of isolated bacterial strains is still of a limited scale.

With a view to identifying a common core of microbial species Qin et al. (2010) group conducted deep sequencing of their own cohort of 124 individuals, which revealed 18 species common to all individuals, 57 species in > 90% of individuals, 75 in > 50% of individuals and 35 in > 50% of individuals. They reported an inter-individual variability of abundance between 12- and 2,187-fold for the 57 most common species common to >90% of their cohort. These findings highlight the variations in the numbers of microbial species in individuals, which greatly influences our perception of the common core microbiome.

Before Qin et al.’s (2010) deep sequencing study, Tap et al. (2009) had also explored the existence of a phylogenetic core by conducting an assessment of 17 human faecal samples, employing 16S rRNA molecular technology. The 16S rRNA sequences mainly belonged to the phyla Firmicutes (79.4%), Bacteroidetes 16.9%, Actinobacteria (2.5%), Proteobacteria (1%) and Verrumicrobia (0.1%). While most of the operational taxonomical units appeared individual-specific, 2.1% were present in more than 50% of the samples and accounted for 35.8% of the total sequences. Despite the species’ richness and a high individual specificity a limited number of operational taxonomical units is shared among individuals and might represent the phylogenetic core of the human intestinal microbiota. The authors of the study concluded that a small but common bacterial species or ‘core’ was shared among 50% of their cohort.
Clearly, valuable steps have been made in elucidating the composition of the intestinal microbiome and the possibility of a ‘core’ intestinal microbiome. Collectively from the HMP studies, 99.1% of the genes catalogued were of bacterial origin, with only 0.1% of eukaryotic and viral origins. The bacterial genes were classified into predominantly two major phyla classes, the Bacteroidetes and the Firmicutes, which confirmed studies conducted before the HMP (Wang, Heazlewood et al. 2003; Eckburg, Bik et al. 2005). This information will contribute to comparative studies assessing the composition in disease states. In a comparative study of healthy individuals and individuals with IBD, the results of the routine sampling indicated that approximately 38% of an individual’s total gene pool is shared and that patients with IBD had on average 25% fewer genes than non-IBD individuals (Qin, Li et al. 2010). These results suggested lower microbial diversity among patients with IBD.

In summary, the composition of the gastrointestinal microbiota, as identified primarily by faecal samples, is a highly diverse, hierarchical complex community of microorganisms. It appears that a small core-dominant cluster of microbiota is shared by most individuals. However, the significance and role of inter-individual variations of many less dominant microbiota are still being explored. These intestinal microbial communities are referred to as the indigenous microbiota and exist in a delicate symbiosis with the host (Bäckhed, Ley et al. 2005; Frank and Pace 2008; Koren, Goodrich et al. 2012). The relationship between the indigenous microbiota and a host involves multiple interactions that are thought to take place in various regions of the GI tract (Ley, Peterson et al. 2006).

2.3.5 Regional composition and sampling of the small intestine
The different methods of investigating the composition of the intestinal microbiota in humans all have their advantages and limitations. Sampling of the gastrointestinal tract in humans is far more difficult than in animal models. The sampled area in both animals and humans is often relatively small in comparison with the total area (Ouwehand 2010). In general, patients prefer non-invasive methods. Non-invasive methods are of particular importance for very young paediatric patients, pregnant women and the elderly, as well as for research purposes. While the difficulties of sampling the entire gastrointestinal tract are reduced by non-invasive tests they tend to be less sensitive and specific. Invasive methods such as endoscopy may be unpleasant, but yield highly sensitive and site-specific information. The limitations of diagnostic tests are outlined in Table 2.5. For the focus of this review, i.e. CD, sampling techniques employed for microbial compositional studies of the small intestine in disordered and normal states will be discussed.
2.3.6 Normal microbiota of the small intestine

Both the lumen and mucosa of the upper regions of the small intestine (duodenum and jejunum) are generally sparsely populated. This is because the pH is low and rapid peristalsis in the duodenum, jejunum and proximal region of the lumen results in a short transit time (three to five hours) for the luminal contents of these regions. However, peristalsis in the distal section of the ileum is much slower. In an adult a total of approximately 9.0 litres of fluid per day enters the small intestine, and this exerts a flushing action that hinders microbial colonisation, particularly in the upper regions. This fluid consists of 2 litres of drinks, 1.5 litres of saliva, 2.5 litres of gastric juice, and 1 litre of intestinal fluid. More than 80% of this is absorbed by the small intestine. Other factors limiting microbial colonisation are the high concentrations of bile salts and proteolytic enzymes, other host antimicrobial defences and low pH of the upper regions (Wilson 2008). Although the short residence time limits microbial growth in the lumen, mucosal populations might be important for colonisation of luminal material and for pathogen resistance. Bacterial populations increase from $10^4$ colony-forming units (CFU) per mL near the stomach to $10^7$ CFU per mL near the colon, where transit slows; their composition also changes, with an increasing proportion of anaerobic species. Both culture-independent and molecular studies have shown that the number of organisms in the lumen and on the mucosa, as well as the complexity of the resident communities, gradually increase along the small intestine (Wilson 2008). This is why the microbial composition of faecal samples is thought to be in part a reflection of the small intestinal microbial ecology.

The most commonly detected organisms cultured from samples taken from the duodenum are mainly acid-tolerant, facultative anaerobes. These include Streptococcus spp., Lactobacillus spp., and Enterococcus spp. However, Bacteroides spp., Veillonella spp., Bifidobacterium spp., Enterobacteria, Staphylococcus spp, and some yeasts may also be isolated (Wilson 2008). Skar et al. (1989) analysed the microbiota of samples obtained from duodenal mucosa in 26 healthy individuals during endoscopy by culture techniques. The number of strains isolated from each of 26 healthy individuals ranged from zero to seven, with a median value of three. A total of 65 isolates were obtained, with Viridians streptococci (41%) the most common culture organism, followed by Enterococcus spp (8%), Lactobacillus spp (8%), Escherichia coli (7%) and Staphylococcus sp (6%). Other organisms identified less frequently were Bacteroides spp., Veillonella spp., and Bifidobacterium spp and Enterobacteria and Candida spp.
Table 2.5 Limitations of samples used for diagnosis of bacterial abnormalities in the small intestine (Malik, Xie et al. 2011)

<table>
<thead>
<tr>
<th>Test</th>
<th>Limitations</th>
</tr>
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<tbody>
<tr>
<td>Major limitations of direct aspirate culture (taken from biopsy during endoscopy)</td>
<td>Reflects only a single site of the gut. However, bacterial overgrowth may be patchy (i.e., samples may not be representative of the entire intestine, thereby compromising reproducibility) (Malik, Xie et al. 2011). Increased potential of contamination by oropharyngeal bacteria during intubation (Malik, Xie et al. 2011). Not all bacteria are easy to culture; longer incubation periods and specialised chamber conditions may be required. Of the 500 to 1000 identified species, less than 20% can be grown in laboratory (Citters and Lin 2005). Reproducibility of small bowel aspiration and culture is low compared with hydrogen breath testing (Tillman, King et al. 1981; Citters and Lin 2005). Invasive, especially for patients who require repeated testing. Lack of standardisation of diagnostic test protocol (intra- and inter-individual variations impose significant limitations on developing protocols to increase sensitivity and specificity for detecting bacterial overgrowth) (Khoshini, Dai et al. 2008). Labour-intensive and expensive. Centres may lack the appropriate equipment and laboratory support for proper collection and analysis.</td>
</tr>
<tr>
<td>Major limitations of hydrogen breath testing</td>
<td>Requires the presence of an active bacterial flora capable of metabolizing carbohydrate to hydrogen (Shah, Basseri et al. 2010). Breath excretion of intestinally derived gases reflects only net excretion and does not account for metabolic consumption of hydrogen by the lumen (Malik, Xie et al. 2011). Test is inaccurate when paediatric patients cannot breathe into the collecting devices. Ineffective for patients with respiratory distress (Malik, Xie et al. 2011). Prolonged testing required for patients with slow small intestinal transit time. Need for cut off values for age-, sex- and disease-matched controls (most paediatric procedures are an extension of adult studies; the diagnostic values may be misrepresented). Specificity can be compromised by excessive substrate dosage, leading to osmotic fluid shifts, shortened transit time and, subsequently, a faster delivery of substrate to the colon (Bishop 1997). Specialised equipment and trained technicians required.</td>
</tr>
<tr>
<td>Major limitations of polymerase chain reaction-denaturing gradient gel electrophoresis from faecal samples</td>
<td>Single population may have multiple ribosomal RNA operons with different 16S ribosomal RNA gene sequence. DNA purification required for polymerase chain reaction detection of bacteria in faeces (i.e., faeces contain bilirubin and bile salts, which can interfere with polymerase chain reaction analysis) (Wilde, Eiden et al. 1990).</td>
</tr>
</tbody>
</table>
The jejunum also appears to have relatively sparse microbiota, although viable organisms tend to be recovered more frequently, and in greater numbers, from this region than from the duodenum. Importantly for the focus of this review, organisms present in the contents of the lumen are similar to those found in the duodenum and include members of the oral and respiratory microbiota, e.g. *Streptococci, Lactobacilli, Fusobacterium nucleatum, Neisseria* spp., *Actinomyces* spp., and *Veillonella* spp (Sullivan, Törnblom et al. 2003). As in the duodenum, *Viridians streptococci* are among the dominant organisms, but *Neisseria* spp. and *F. nucleatum* are also present in high proportions (Wilson 2008). It is also important to note that the range of microbes cultured from the jejuna mucosa is similar to that found in the lumen, but *Viridians streptococci* dominate the microbiota (Sullivan, Törnblom et al. 2003). In analysis of mucosal microbiota of the jejunum in one healthy adult, employing 16S rRNA gene libraries of the duodenal microbiota, 88 clones were analysed, and these corresponded to 22 different phylotypes, most of which are members of the oral or respiratory microbiota (Wang, Ahrné et al. 2005). These are similar to those obtained from culture-based studies. Of the sequences, 68% correspond to *Streptococci*, among which *Streptococcus mitis* predominated. In contrast, another study employing 16S rRNA gene libraries of the jejunal contents of three healthy adults produced results that were very different from those generated in culture-based studies (Hayashi, Takahashi et al. 2005). *Streptococci* were not found to be the dominant organism in any of the individuals: one was dominated by *Lactobacillus*, another by *Enterobacter asburiae* sub-group, and the third by *Klebsiella planticola* sub-group. The composition of the microbial community in each individual was relatively simple. Significant differences in composition and diversity have also been demonstrated between underdeveloped and developed countries (Bhat, Albert et al. 1980), suggesting likely dietary and environmental influences in composition.

In the region of the ileum conditions are more conducive to the establishment of resident microbiota due to:

- slower peristaltic activity;
- intestinal juice diluting the microbial effects of pancreatic enzymes and bile;
- the content of bile acids being severely reduced because most are absorbed in the jejunum; and
- the pH being neutral or slightly alkaline.

The ileal microbiota are derived from organisms arriving in material from the jejunum and the reflux of caecal contents through the ileocaecal sphincter, which separates the ileum from the large intestine. In terms of its cultivable microbiota, its composition can be regarded as being intermediate between that of the upper intestine (sparse, mainly gram-positive species, with
many facultative anaerobes) and the large intestine (large numbers of microbes, mainly Gram-negative species dominated by obligate anaerobes) (Wilson 2008). The majority of data available regarding the microbial composition of the ileum has been derived from studies of patients post-ileostomy. The luminal contents of the ileum contain between $10^6$ and $10^8$ CFU/mL and cultivable microbiota are dominated by facultative bacteria (Berghouse, Hori et al. 1984), which are 20 and 50 times more numerous than obligate anaerobes (mainly Veillonella spp., Clostridium spp., and Bacteroides spp.). Facultative organisms (mainly Streptococci, Enterococci, and coliforms) dominate the luminal microbiota for up to six hours following a meal. The findings of Berghouse et al. (1984) may be argued to be limited by the culture-dependent method employed. However, Hayashi et al. (2005) obtained similar results from culture-independent studies. They found the ileal contents were dominated by facultative anaerobes, mainly Enterobacteriaceae, Streptococci and Enterococci species.

In contrast to findings of studies of luminal contents, obligately anaerobic species comprise an appreciable proportion (approximately 50%) of the cultivable microbiota associated with the mucosal surface of the ileum – findings that are supported by the results of culture-independent studies. Organisms isolated from the ileal mucosa include Bacteroides spp., col. aerofaciens, Clostridia, Bifidobacteria, Peptostreptococci, Eubacteria and Propionibacteria (Wilson 2008). In keeping with results of culture-dependent studies, Wang et al. (2003) found that obligate anaerobes were the dominant organisms present in the ileal mucosa, using 16S rRNA gene clone libraries. Bacteroides vulgatus constituted 88% of the Bacteroidetes, while all the sequences in clostridium clusters XIVa and XI correspond to those of uncultured bacteria published in GenBank. The remaining clones had sequences corresponding to Clostridium ramsorum, Haemophilus influenza and Streptococcus salvarius (Bacillus- Lactobacillus-Streptococcus). A study by Kleeson et al. (2002) employed fluorescent in situ hybridisation (FISH) of healthy individuals and found obligate anaerobes to comprise 50% of ileal mucosal specimens studied. Interestingly, in 50% of the subjects no bacteria were detected at all.

Fernandez et al. (1985) demonstrated that the host’s diet has a dramatic effect on the relative proportions of microbes in the ileal contents. In patients post-ileostomy an increase in dietary protein resulted in increased proportions of Streptococci, Enterococci and coliforms, whereas a high fat diet favoured increased proportions of Bacteroides spp. and Clostridium spp. Significant temporal changes can be seen in response to diet and for this reason recent compositional studies have assessed only pre-prandial faecal samples or overnight fasting duodenal aspirates (Di Cagno, Rizzello et al. 2009).
In summary the composition and luminal concentrations of dominant microbial species vary throughout the intestinal tract and develop from early in life. The dominant microbial species by region of the GIT and time period are represented graphically in Figure 2.4 (Sekirov and Finlay 2009).

Figure 2.4 Spatial and temporal aspects of intestinal microbiota composition
A: Variations in microbial numbers and composition across the length of the gastrointestinal tract
B: Longitudinal variations in microbial composition in the intestine
C: Temporal aspects of microbiota establishment and maintenance and factors influencing microbial composition (Sekirov and Finlay 2009)

2.3.7 The composition of the microbiota in coeliac disease

Studies investigating the composition of the intestinal microbiota in CD patients and control subjects have been conducted primarily to identify associations between specific microbial groups and this disorder. These studies have been extensively reviewed by others (Sanz, Palma et al. 2011).

Nadal et al. (2007) conducted a comparative analysis of the duodenal biopsies from children with CD and healthy controls by FISH coupled with flow cytometry (FCM). They showed that the ratio of ‘harmless’ Lactobacillus-Bifidobacteria to the potentially harmful Bacteroides-Prevotella- E.coli was significantly lower in CD patients than in controls, regardless of whether CD was active or inactive. They also detected a greater abundance of Bacteroides-Prevotella and E.coli groups in untreated CD patients than in controls. These bacterial deviations were normalised in treated CD patients following a long-term GFD (Nadal, Donant et al. 2007b).
These findings have been supported by De Palma et al. (2010) who analysed the faecal microbiota composition of children with CD by FISH-FCM. They showed the Gram-positive to Gram-negative bacteria ratio was significantly reduced in both untreated and treated CD patients compared with controls. In addition the *Bifidobacterium* genus was less abundant in untreated CD patients than in healthy controls, while the *Bacteroides-Prevotella* group was more abundant in untreated CD patients than in controls (De Palma, Nadal et al. 2010).

Collado et al. (2009a) analysed the faeces and duodenal biopsies of paediatric patients with CD by quantitative PCR and showed that reductions in *Bifidobacterium* and *B.longum* numbers were associated with both active and inactive CD when compared with controls. They also showed that *Bacteroides fragilis* group and *Clostridium leptum* group numbers were higher in both the faeces and biopsies of CD patients than in controls, regardless of the stage of the disease. As shown by De Palma et al. (2010), abnormalities in other bacterial groups, i.e. *Staphylococcus* and *E.coli*, normalised after a GFD had been implemented (Collado, Donat et al. 2009a).

In another study, Sánchez et al. (2010) characterised the predominant bacterial species of duodenal biopsies taken from children with active and treated CD. They analysed the samples by PCR-denaturing gradient gel electrophoresis. They showed reduced diversity of *Bacteroides* spp. in the biopsy specimens of patients with both active and treated CD compared with controls, whereas *Bifidobacterium* species diversity was significantly higher in CD patients than in controls. A higher diversity of lactic acid bacteria species was also found in the treated CD and control group; however, the species identified were food-related lactic acid bacterial species and were thought to be related to transient diet-induced microbial changes (Sánchez, Donat et al. 2009a).

A separate study conducted by Sánchez et al. (2008) demonstrated a reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of children with both treated and untreated CD. The authors suggested an association between the pathogenic potential of enterobacteria and CD. In addition, the diversity of the phylogenetic groups (A, B1, B2 and D) of *E.coli* clones was higher in healthy children than in the CD patients. The four phylogenetic *E.coli* groups were equally distributed in healthy children. h, in both CD patient groups most commensal isolates belonged to group A and the *E.coli* clones of the virulent phylogenetic groups (B2 and D), and carried a higher number of virulence genes than those from healthy children (Sanchez, Nadal et al. 2008).

Schippa et al. (2010) analysed the mucosa-associated microbiota of CD children before and after GFDs by temporal temperature gradient gel electrophoresis (TTTGE) and compared it to healthy controls. Total bacterial diversity was higher in active CD patients than in those
in remission and in the controls. The prevalence of *Bacteroides vulgatus* and *E.coli* in CD patients was also significantly higher than in controls, but not that of *Bifidobacterium spp.* As noted in a review by Sanz et al. (2011), the data on *E.coli* prevalence in this study followed the same trend as quantitative data of *E.coli* reported by both Nadal et al. (2007) and Collado et al. (2008), but was contrary in regard to the data on *Bacteroides vulgatus* prevalence.

Ou et al. (2009) conducted a small retrospective analysis of nine frozen mucosal samples by 16S rDNA sequencing and identified *Clostridium* spp., *Prevotella* spp. and *Actinomyces* spp. as the main components of the small intestinal microbiota of children born during the Swedish CD epidemic. While the authors concluded that these bacteria may have played a role in the four-fold increase in paediatric CD diagnosis at that time, others employing a larger and more appropriate sample size have suggested that further support of this hypothesis is indicated (Sanz, Palma et al. 2011).

A number of groups have assessed the gut ecosystem of patients with CD by measuring faecal levels of short chain fatty acids (Tjellstrom, Stenhammar et al. 2005a); (Bertini, Calabrò et al. 2009). Tjellstrom et al. (2005b) and Tjellstrom (2007) reported alterations in the composition of short chain fatty acids (SCFA) generated from microbial fermentation in CD patients and first-degree relatives. They showed increased levels of total SCFA and acetic, valeric and butyric acids compared with healthy controls. Compared with healthy controls first-degree relatives of CD patients had lower levels of acetic acid and total SCFAs but higher levels of butyric acid, suggesting that increased butyric acid production could be a feature common to patients and their relatives. In another study by Tjellstrom et al. (2010) SCFAs were analysed in the faeces of asymptomatic screen-detected CD children and compared with symptomatic CD children and healthy controls. They found that asymptomatic screen-detected CD children and symptomatic CD children had significantly different SCFAs profiles compared with healthy children suggesting that distinct CD associated alterations in gastrointestinal microbial metabolism exists (Tjellström, Stenhammar et al. 2010). Conversely, Bertini et al. (2009) identified a characteristic metabolic signature only in untreated CD patients, with a further observation that implementation of a GFD for 12 months resulted in a similar metabolic signature to healthy controls.

Small intestinal bacterial overgrowth has been identified as a cause of persistent symptoms despite adherence to a GFD. One group conducting a culture study of the intestinal aspirates of both symptomatic treated and untreated CD patients found that both groups had small intestinal bacterial overgrowth (Rubio-Tapia, Barton et al. 2009; Rubio-Tapia, Rahim et al. 2010).
In summary, the studies above provide evidence that aberrations in the composition and behaviour of the microbiota in patients with CD exist. Some of these aberrations, such as increased levels of *Staphylococci* and enterobacteria, appear to be secondary consequences of CD in response to untreated CD, as evidenced by normalisation of levels after dietary treatment. However, other microbiota, such as increased levels of *Bacteroides* species numbers, virulent *E coli* clones and decreased *Bifidobacterium* species numbers, appear to be associated with CD regardless of clinical presentation and degree of inflammation.

### 2.3.8 Temporal variations in the adult intestinal microbiome

In response to microbiome and microbiota compositional studies, the question of variations from temporary influences has been assessed (Caporaso, Lauber et al. 2011). Several groups have attributed the qualities of stability and resilience to the adult intestinal microbiome (Turnbaugh, Ley et al. 2007; Dethlefsen, Huse et al. 2008). While these studies were sufficiently powered, the results were not based on extensive time series analysis to elucidate whether a core temporal adult microbiome exists. Caporaso et al. (2011) conducted a large human microbiota time series analysis, covering two individuals at four body sites over 396 time points (Caporaso, Lauber et al. 2011). The findings supported previous reports by Qin et al. (2010) and Dethlefsen et al. (2006) of body site microbiome distinction, inter-individual variation and body site microbiome stability over times greater than one year. However, there was intra-individual variation across months, weeks and even days, with only a small number of the total taxa identified in the intestinal microbiome being present at all-time points. The authors suggested that no core temporal microbiome exists in high abundance, with many more taxa being persistent but non-permanent community members (Qin, Li et al. 2010). Another way to consider these findings could be that if a core abundant temporal microbiome exists it is indeed small in relation to the dynamic interplay of visiting species.

### 2.3.9 Tools utilised for microbial analysis

The tools utilised for microbial analysis have evolved and developed from the time Luenhooks first produced the lens now known as a microscope, and from the cultivation techniques of Pasteur through to modern discoveries of molecular technology (Wilson 2008). Culture, microscopy and molecular techniques all share varying degrees of strengths and limitations. The following section aims to present the strengths and limitations of culture-dependent and molecular approaches that are currently employed to identify microbiota obtained from samples from the gastrointestinal tract.
2.3.9.1 Culture-dependent approaches

Many of the problems inherent in culture-dependent approaches to analysing microbial communities can be circumvented by the use of molecular techniques, although these are not immune from problems. Even the best cultivation methods seriously underestimate the number of organisms present in the microbiota of certain body sites, particularly those from the GIT and oral cavity (Wilson 2008). The explanations for this are many but the most important causes of problems associated with culture-dependent analysis of microbial communities are, as listed by Wilson (2008):

- Failure to satisfy the nutritional requirements of some of the organisms present;
- Failure to satisfy the environmental requirements of some of the organisms present;
- Failure to detect organisms in a “viable but not cultivable state”;
- Failure to disrupt chains or clusters of organisms before plating out (this results in the production of only one colony-forming a unit from a cluster or chain consisting of many viable bacteria, thereby underestimating their proportions);
- Death of viable cells during transportation and processing of the sample;
- Overgrowth of culture plates by fast-growing organisms;
- “Diluting-out” of organisms present in small proportions;
- Possible inhibitory effect of selective media;
- The labour-intensive nature of the whole process.

Collectively these difficulties have resulted in a serious underestimation of the number and variety of organisms in a sample taken from any environment, and it has been estimated that only 1–2% of microbial species in existence on earth have been cultured (Wilson 2008).

Other phenotypic tests that have been used for microbial identification purposes include cell wall protein analysis, serology, and fatty acid methyl ester analysis.

2.3.9.2 Molecular approaches

In the last decade the advent of molecular technologies such as 16S rRNA gene analysis has allowed a more complete assessment of the complex gastrointestinal microbial ecosystem. It has made it possible to identify specific microbial taxa found in the human gut that were previously challenging or simply impossible to cultivate (Wilson 2008). The first stage in the analysis of a microbial community by a molecular technique is to isolate either DNA or RNA from the sample, and herein lies the first challenge. Extraction of nucleic acids from microbes requires that cells are lysed, and the ease of lysis varies significantly among different organisms. Numerous protocols for the lysis of microbes present in samples have been devised and include the use of enzymes, chemicals and mechanical methods (Wilson 2008).
The extracted nucleic acids can be used in a variety of ways to reveal the identity of the microbes originally present in the sample and/or to produce a “profile” or “fingerprint” of the microbial community.

Universal primers can be used to amplify all the 16S rRNA genes present in the DNA extracted from the sample, and the amplified sequences are then cloned. The sequences of the cloned amplicons are then determined and compared with sequences deposited in databases such as the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) or in BLAST (basic alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST/), accessed on 20/11/12. In this way the sequences of the 16S rRNA genes of all the organisms present in the community (including those that cannot be grown in the laboratory) can be determined and, if these sequences match those of known organisms in databases, the identities of all the organisms present will be revealed. However, up until 2008 studies of the faecal microbiota, for example, have revealed that not all the sequences of the 16S rRNA genes obtained correspond to sequences in databases. According to Wilson (2008) as many as 75% of the sequences did not match those of known organisms. Some of the problems with this approach are that both the PCR and cloning steps in the procedure have their biases, and the technique is expensive and labour-intensive, so limiting output. To overcome some of these limitations, researchers assessing indigenous microbial communities of humans are employing real-time PCR (Malinen, Rinttila et al. 2005; Chang, Nam et al. 2007; Ahlroos and Tynkkynen 2009).

Another useful approach is to separate the amplicons on a denaturing gradient gel. Although all the amplicons have the same length their different base compositions results in their having different melting points, so each will melt at a different point when run on gel along which there is either a temperature gradient (temperature gradient gel electrophoresis – TGGE) or a gradient in the concentration of a denaturing agent such as urea or formamide (DGGE). The altered conformation of the DNA due to denaturing slows the migration, and this results in separation of the various amplicons. Staining of the DNA in the resulting gel reveals a banding pattern, or ‘fingerprint’, that is characteristic of that particular community. The individual bands can be cut out and each amplicon eluted, re-amplified, sequenced and identified using the aforementioned databases (Wilson 2008). Alternatively, the fingerprints produced from samples from the same individual obtained on different occasions can be compared and analysed for differences. Hence, bands appearing or disappearing with time can be sequenced to determine the gain or loss of an organism from the community. The method is also useful for comparing microbiota present at the same body site in different individuals, and has been used extensively for comparing microbiota in different regions of the GIT
The DNA extracted from a microbial community can also be used in dot-blot hybridisation assays. In such assays, aliquots of the extracted DNA are spotted onto a nitrocellulose membrane to form a gridded array. This array can then be probed with labelled oligonucleotide probes designed to recognise a single species, a genus or a group of related organisms. Alternatively, gridded arrays of DNA from a range of organisms can be prepared, and these can be probed with the DNA extracted from the sample once it has been labelled in some way (Pawel, Ralph et al. 2004).

A significant criticism of DNA-based methods of analysing the composition of microbial communities is that the DNA extracted from a body site is likely to consist not only of DNA from the resident community, but also that derived from allochthonous species from dead organisms (Wilson 2008). Adams et al. (2010) demonstrated that both dead and live bacterial cells have the capacity to exert a biological effect, which raises the question of whether this differentiation is required for understanding microbial compositions, particularly in relation to disease states. Nevertheless, in response to this concern Rudi et al. (2005) developed a technique that is employed by some laboratories to differentiate between live and dead cells, thus preventing the reporting of false live positives. A staining chemical by the name of ethidium monoxide (EMA) is used in combination with real-time PCR to inhibit amplification of DNA from dead cells that have taken up EMA. Figure 2.5 graphically describes the method.

![Figure 2.5 Differentiation between live and dead cells](Rudi, Moen et al. 2005)
Since 2005 a number of additional methods have been developed which build on the EMA model using additional cross-linking agents such as psoralen (Soejima, Iida et al. 2008). These methods are employed by some laboratories to overcome the limitation of distinguishing live from dead cells in faecal specimens.

From the 1970s to the early part of this century the capacity of molecular technology to identify microbial taxa with greater accuracy than traditional techniques has been established. However, identification is not the only benefit of molecular technology, with millions of sequences per sample now able to be generated (Kurokawa, Itoh et al. 2007). To accompany the vast amount of sequencing data equally powerful computational tools have also been developed, including mothur (Kurokawa, Itoh et al. 2007), Workflow for Alignment, Taxonomy, and Ecology of the Microbial Environment (WATERS) (Suau, Bonnet et al. 1999), the Ribosomal Database Projects pyrosequencing tools (RDP), and Quantities Insights into Microbial Ecology (QIME) (Noguchi, Park et al. 2006). QIME is a platform for the analysis of high throughput sequencing data that enables users to import raw sequence data and readily produce measures of inter- and intra-sample diversity. Consistency in the identification of operational taxonomical units and the establishment of agreed-upon measures of diversity within and between samples are crucial for the comparison of results across studies (Ursell, Metcalf et al. 2012).

The broad overview above has described a number of microbial sampling methods and techniques that are currently in use and these are summarised in Table 2.6. For the purposes of this review further discussion will focus on gastrointestinal 16S rRNA gene analysis and new molecular diversity approaches. Ouwehand et al. (2010) provide a comprehensive review of methods employed in evaluating intestinal microbiota.

2.3.9.3 Gastrointestinal microbiota as identified by 16S rRNA gene analysis
The human GI tract comprises bacteria, archaea and eukarya, with bacteria being by far the most dominant. The comparative analysis of environmentally retrieved nucleic acid sequences, most notably rRNA molecules and genes encoding them, has become the standard over the last decade for cultivation-independent assessment of bacterial diversity in environmental samples (Pawel, Ralph et al. 2004). The 16S rRNA gene comprises highly variable to highly conserved regions, and the differences in sequence are used to distinguish bacteria at different levels from species to domain and to determine phylogenetic relationships. rRNA are today routinely retrieved without prior cultivation of the microbes by constructing 16S ribosomal DNA (rDNA) libraries. The procedure is based upon PCR-mediated amplification of 16S rRNA genes or gene fragments, isolated from the
environmental sample, followed by segregation of individual gene copies by cloning into *Escherichia coli*. In this way a library of community 16S RNA genes is generated, the composition of which can be estimated by screening clones, in full or partial sequence analysis, and comparing them with adequate appropriate reference sequences in data bases to infer their phylogenetic affiliation. Large databases of 16S rRNA gene sequence information (>200,000 sequences) for described as well as uncultured microorganisms are available, and provide a high resolution platform for the assignment of those new sequences obtained in 16S rDNA libraries. Databases harbouring 16S rRNA sequences include the Arbor (ARB) software package, the Ribosomal Database Project and European Molecular Biology Laboratory (EMBL) (Ouwehand 2010). Even though sequencing of cloned 16S rDNA amplicons provides relevant information about the identity of uncultured bacteria, the data are not quantitative. Moreover, PCR and cloning steps are not without bias (Ouwehand 2010).

Table 2.6 Potential and limitations of various methods of investigating the diversity of the human intestinal microbiota (Ouwehand 2010)

<table>
<thead>
<tr>
<th>Method</th>
<th>Application</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culturing</td>
<td>Isolation of pure cultures, enumeration</td>
<td>Not representative for microbiota: insufficient selective media; time-consuming</td>
</tr>
<tr>
<td>16S rRNA gene libraries and sequencing</td>
<td>Identification and phylogeny</td>
<td>Large scaling cloning is laborious; primer bias can be an issue</td>
</tr>
<tr>
<td>Dot-Blot hybridisation</td>
<td>Detection, quantification and activity</td>
<td>Gives information about activity of microbiota; of rRNA; comprehensive set of probes published</td>
</tr>
<tr>
<td>FISH</td>
<td>Single cell detection and enumeration</td>
<td>High throughput with image analysis software and flow cytometry; requires probe design; comprehensive set of probes published</td>
</tr>
<tr>
<td>PCR-DGGE/TGGE</td>
<td>Rapid profiling of total microbiota</td>
<td>Detection of specific groups possible; semi-quantitative identification by band extraction and sequencing</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Rapid profiling of total microbiota</td>
<td>Identification by cloning and sequencing; bank of T-RF under construction</td>
</tr>
<tr>
<td>Quantitative real-time PCR</td>
<td>Detection and quantification</td>
<td>Requires probe/primers design; very high throughput</td>
</tr>
</tbody>
</table>

2.3.9.4 New molecular diversity approaches: real-time PCR

Real-time quantitative PCR (qPCR), which has the advantages of being high throughput and measuring from 1 to up to $10^8$ CFU, has been developed over the last decade for the detection and quantification of human intestinal microbiota (Ouwehand 2010). As cited by Ouwehand
(2010) both SYBR Green1 and TaqMan chemistries have been used to target *Bacteroides fragilis*, *Bifidobacterium* species, *E.coli*, *L.acidophilus* and *Ruminococcus productus*, and have been demonstrated to be easier and faster than dot-blot hybridisation methodology (Malinen, Kassinen et al. 2003). Real-time quantitative PCR has been reported to be very sensitive, with an ability to detect as little as 1 and 9 CFU of *E.coli* and *B.vulgatus* respectively (Huijsdens, Linskens et al. 2002). Employment of qPCR is used in the functional food industry for the identification of *Lactobaccilli* and *Bifidobacterium* species (Bartosch, Fite et al. 2004; Matsuki, Watanabe et al. 2004). The qRT-PCR has been used for various applications, such as comparison of healthy persons with patients suffering from IBS (Malinen, Rinttila et al. 2005) and in patients with active IBD (Ott, Musfeldt et al. 2004). Ott et al. (2004) developed a TAQ Man real-time PCR-based method for the quantification of two dominant bacterial species and groups of microbiota. This method involved a pair of conserved primers, as well as universal and specific quantification probes for species, group or genus in question, in a single reaction, and allowed relative and absolute quantification of bacteria in human biopsy and faecal samples.

**2.3.9.5 Metabolomics and the gastrointestinal tract: urinary markers**

Despite the limitations, culture analysis of duodenal aspirates are still the most commonly employed method for diagnosing small intestinal bowel bacterial overgrowth and identifying the related microbes (Guarner and Malagelada 2003a; Choung, Ruff et al. 2011). Duodenal aspirates are limited by their invasive nature and the cost of the procedure. Hydrogen and methane breath testing are employed to assess the potential source of chronic unexplained gastrointestinal symptoms. However, conclusions drawn from the studies are highly controversial and divergent results exist (Simrén and Stotzer 2006; Khoshini, Dai et al. 2008; Lord and Bralley 2008). The transitional or mid-gut is more difficult to assess by direct examination and is the primary origin of urinary microbial products because the passing of chyme to the lower ileum corresponds to the lag phase for the onset of logarithmic growth rates characteristic of most bacteria (Child, Kennedy et al. 2006). Metabolic products are actively produced when microbial counts rise from $10^5$ to $10^{11}$/g. It is thought that information about the metabolism of the microbial mass in regions of the gut prior to the descending colon can be obtained by a fasting overnight urine sample and measuring organic acids (Ewaschuk, Zello et al. 2002). Urinary organic acid markers provide indirect evidence to support transitional gut bacterial overgrowth (Lord and Bralley 2008). Absorbed microbial metabolic products are distributed by the circulatory and lymphatic systems to the hepatic and renal detoxification systems, followed by excretion as unaltered or modified metabolites of
bacterial metabolism in the urine. Through the indirect procedure of examining microbial products in urine, the in vitro growth conditions are completely unaltered by the assay (Lord and Bralley 2008).

Early in vitro work demonstrated various metabolic potentials for specific bacteria (Gale 1940a; Gale 1940b; Gale 1941; Gale 1942). Measuring products formed by ruminants when dietary intake of specific compounds was varied yielded information about rumen bacteria (Lord 2008). The proximity of the rumen to the oxygen content of swallowed air means that organisms thriving there are generally aerobic and microaerophilic, in contrast to the highly anaerobic environment below the stomach (Martin 1982; Martin, Milne et al. 1983). This ruminal stage of microbial activity is most closely approximated in the human jejunum. Absorbed bacterial metabolic products may undergo further metabolism in host tissues, mainly liver and kidney tissue. For example, urinary benzoic acid was found in ruminant urine as the result of hepatic action on the phenylpropionate, a principal product of microbial metabolism of dietary cinnamic acid (Martin 1982). As described by Lord and Bralley 2008, normal hepatic oxidase activity converts any absorbed phenylpropionate into benzoate and hippurate (Lord and Bralley 2008). The specific compounds excreted by a given individual depend on the available substrates and the species of organism present (Rios, Gonthier et al. 2003; Rechner, Smith et al. 2004). Recent combinations of HPLC-MC and nuclear magnetic resonance-based metabolomic studies have revealed great differences between individuals, whereas single patients show quite stable patterns across all types of molecules, including microbial products (Lenz, Bright et al. 2003; Williams, Lenz et al. 2005). In addition, high predictive values of urinary markers have been established for some conditions by employing a validated analysis of urinary aromatic acids by liquid chromatography tandem mass spectrometry (Crow, Bishop et al. 2008). Urinary metabolites of gastrointestinal organisms are presented in Figure 2.6.

While the methodology for the analysis of urinary organic acid analytes has been published (Crow, Bishop et al. 2008), the literature regarding the relationship between individual analytes and specific microorganisms is sparse. Nevertheless, the literature that is available is of interest to investigations of disorders of the GIT and a detailed review is provided in Appendix A2.
Figure 2.6 Origins of urinary dysbiosis markers: bacteria and yeasts in the intestine produce by-products that are excreted in the urine. An elevated level of these products may identify an overgrowth of one or more bacteria or of yeast in the intestine (Lord and Bralley 2008)

2.3.10 Niches of predominant microbial taxa in the colon

There have been undeniable successes in demonstrating the physiochemical factors involved in determining microbial communities (Mead 2000; Ohland and MacNaughton 2010; van Meurs, Shapiro et al. 2010). These more deterministic studies form a ‘competition-centred’ view: where there is interspecies competition involving resources, attachment sites and interference mechanisms that account for colonisation resistance of intact gut communities, and variation between intestinal compartments in physiochemical factors such as retention time, oxygen availability and pH accounts for characteristic microbial communities (Versalovic, Wilson et al. 2009). However, microbial interactions are not limited to competitive exclusion in pre-existing niches; they are also crucial factors in generating the diversity of niches within an intestinal compartment. The assembly of the microbiota is partially recursive, as it creates and responds to many of its own selective pressures (Dethlefsen, Eckburg et al. 2006; Costello, Stagaman et al. 2012). These authors argued that the traditional explanations of community structure need to be evaluated along with newer ecological concepts, such as the importance of historical contingency and the potential for intrinsic population dynamics. One such concept, suggested that organisms alter niches for both themselves and other organisms by changing the local environment, which can extend
the effects of historical contingencies. The development of a stable climax is no longer assumed with even long standing communities potentially remaining out of equilibrium owing to their internal dynamics or changing external conditions. In addition to competitive interactions, colonisation history is now recognised as a potential influence on community composition. The ‘niche construction perspective’ emphasises that organisms alter niches for themselves and other organisms by changing the local environment, which can extend the effects of historical contingencies. Therefore it is important to understand historical contingencies such as environmental and nutritional influences when exploring Dethlefsen et al. (2006) and Costello et al. (2012) theories.

2.3.11 Environmental and nutritional influences on the intestinal microbiota

The influence of the gut microbiota on human health is continuous from birth to old age. The maternal microbiota may influence both the intrauterine environment and the post-natal health of the foetus. At birth approximately 100 microbial species populate the colon. Early environmental factors including method of delivery and nutritional factors such as breast- or bottle-feeding in addition to epigenetic factors have all been suggested to play a role in the development of a healthy gut and its microbial symbionts. Changes in gut microbial composition in early life are now being considered as a risk factor for developing disease later in life. During suckling the microbial community develops rapidly; shifts in microbial diversity occur throughout childhood and adult life, and in old age there is a decrease in the Bacteroidetes and an increase in the Firmicutes species. The gut microbiota are important for maintaining normal physiology and energy production throughout life. Body temperature regulation, reproduction, and tissue growth are energy-dependent processes that may rely in part on gut microbial energy production. Extrinsic environmental factors, including antibiotic use, diet, stress, disease, injury and the human host genome continually influence the diversity and function of the gut microbiota with implications for human health. Disruption of the gut microbiota (dysbiosis) can lead to a variety of different diseases including IBD, colon cancer and IBS, gastric ulcers, non-alcoholic fatty liver disease, obesity and metabolic syndromes, asthma, atopy, hypertension, and mood and behaviour disorders through hormone signalling. The gut microbiota are also important for drug metabolism and preventing establishment of pathogenic microbes.

2.3.11.1 Antimicrobial agents and the gastrointestinal microbiota

Undoubtedly, antibiotics have made a historical and significant contribution to the medical management of infectious disease. An understanding of the impact on the ecological environment in which antibiotics exert their pharmacological effects is arguably of equal
significance in the long-term medical management of human health. A number of the species of the normal human gastrointestinal microbiota are potential pathogens that may cause disease under certain circumstances (Guarner and Malagelada 2003a). One of the functions of the gastrointestinal microbiota is to act as a barrier against overgrowth of such organisms and to prevent colonisation of pathogenic bacteria from the environment. This phenomenon is termed ‘colonisation resistance’ (Høiby 2000). Treatment with antimicrobial agents disturbs the ecological balance between the host and the normal microbiota, and overgrowth of yeasts and Clostridium difficile, or of intrinsically or acquired resistant microorganisms, may occur. Horizontal spread of resistance genes by conjugation or transformation to other microbial species can take place. The gastrointestinal microbiota play an important role in this development (Ouwehand, Salminen et al. 2003).

Orally administered antimicrobial drugs that are incompletely absorbed or excreted via bile or transluminally frequently give rise to a reduced colonisation resistance. Other factors of importance include the antimicrobial spectrum of the agents, the dose and the pharmacokinetic properties of the agents (Ouwehand, Salminen et al. 2003).

Recent studies have highlighted the profound changes in microbial populations that result from antimicrobial medications (Neu 2007; Jakobsson, Jernberg et al. 2010). The assessment of infants’ faecal microbiota after antibiotic treatment has shown significant reductions in both the total bacterial densities and the composition of species (Palmer, Bik et al. 2007). While the changes to the microbiota appear to be mostly temporary, rarely lasting > 2 months, some long-term microbial perturbations have been reported in otherwise healthy adults. These variations in response by the microbial community to antibiotics has been supported by Huse et al. (2008) and Dethlefsen et al. (2008) who demonstrated that, while intestinal microbial populations show resilience as whole communities, several microbial taxa can remain depleted for six months after completion of ciprofloxacin therapy.

The aforementioned significant changes in the composition of gastrointestinal microbial communities induced by antibiotic therapy can result in important functional differences in the host metabonome (Preidis and Versalovic 2009). Some of the potential functional consequences of these alterations in microbial populations are being revealed by animal studies. Depletion of Lactobacillus genus by amoxicillin was associated with significant changes in the expression of nearly one-third of developmentally regulated genes (Schumann, Nutten et al. 2005). Furthermore, Yap et al. (2008) demonstrated that changes to the microbial community caused disruption in carbohydrate metabolism. Disruption of carbohydrate metabolism was defined as increased quantities of unfermented oligosaccharides in the faeces.
and reduced concentrations of short chain fatty acids, including acetate, n-butyrate, propionate and lactate.

Antibiotic-induced compositional changes and functional disruptions to the microbial community increase the risk of gastrointestinal infections (Vardakas, Konstantelias et al. 2012). Antibiotic-associated diarrhoea and colitis can be caused by Clostridia pathogens such as Clostridia difficile or Clostridium perfringens (Bartlett 2002). Predictive patterns within the microbial community of the gastrointestinal tract have been demonstrated (de la Cochetiare, Durand et al. 2005). Similarly, increased susceptibility of animals to invasive salmonellosis after disruption to the microbial community have been reported after administration of streptomycin and vancomycin (Sekirov and Finlay 2009).

The acquisition of a fungal infection is another problem reported as a side effect of antibiotic treatment (Pultz, Stiefel et al. 2005). Fungal overgrowth has been observed, specifically after treatment with antibiotics that reduce the population of anaerobic bacteria, in experimental animals, healthy individuals and neutropenic patients (Kennedy and Volz 1985; Pletz, Rau et al. 2004). These authors concluded that the preservation of anaerobic bacteria appears critical in the prevention of fungal acquisition in neutropenic patients. The immuno-compromised patient is at highest risk of a disseminated fungal infection in various sites, which can result in serious complications and death (Hube 2004). It has been observed that the fungal cells in antibiotic-treated mice are able to penetrate deep into the mucosa of the intestinal tract, with many remaining in the caecal mucosa. However, this was not observed in the untreated mice, which had more indigenous bacterial flora (Kennedy and Volz 1985).

Another undeniable medical breakthrough has been the identification and association between Helicobacter pylori and gastric ulcers and carcinoma. The impact on the intestinal microbiome by treatment for Helicobacter pylori has been investigated by assessment of bacterial populations in faeces before and after the administration of the standard triple therapy plus metronizadole (Bühling, Radun et al. 2001). Significant reductions in the levels of Lactobacillus and Bifidobacteria were reported between pre-treatment and Helicobacter pylori treatment (Bühling, Radun et al. 2001). Conversely, overgrowth of drug resistant Enterobacteriaceae and yeasts (Adamsson, Nord et al. 1999), and antibiotic induced colitis caused by C.difficile have also been reported in response to H.pylori treatment (Nawaz, Mohammed et al. 1998).

Tables 2.7 to 2.11 present a summary of the classes of antibiotics and their effect on the gastrointestinal microflora. This data is reprinted with the permission of Rafi et al. (2008).
Table 2.7 The effects of penicillin alone or in combination with other drugs on intestinal microflora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bacteria Suppressed</th>
<th>Bacteria Proliferated</th>
<th>Overgrowth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Enterobacteria, enterococci, and anaerobic bacteria</td>
<td></td>
<td>Candida in some</td>
<td></td>
</tr>
<tr>
<td>Ampicillin and sulbactam</td>
<td>Enterobacteria, enterococci, and anaerobic bacteria</td>
<td></td>
<td>Candida or C. Difficile in some</td>
<td>(Kager, Liljeqvist et al. 1982)</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>Enterobacteria, enterococci, and anaerobic bacteria</td>
<td></td>
<td>Candida or C. Difficile in some</td>
<td>(Kager, Liljeqvist et al. 1982)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Enterobacteria</td>
<td>Resistant enterobacteria</td>
<td>Candida or C. Difficile in some</td>
<td>(Gipponi M. 1985)</td>
</tr>
<tr>
<td>Ticarcillin and clavulanic acid</td>
<td>Enterococci</td>
<td></td>
<td>Candida or C. Difficile in some</td>
<td>(Nord, Brismar et al. 1993)</td>
</tr>
<tr>
<td>Phenoxyymethyl-penicillin</td>
<td>Little effect</td>
<td></td>
<td></td>
<td>(Adamsson, Edlund et al. 1997)</td>
</tr>
</tbody>
</table>
Table 2.8 Effects of selected beta-lactams other than Cephalosporins, including those in combination with other drugs, on intestinal microflora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment (mg per day)</th>
<th>Number of Days and Number of Treatments Per Day</th>
<th>Number of subjects</th>
<th>Bacteria Suppressed</th>
<th>Bacteria Proliferated</th>
<th>Resistant Strains Developed or Other Results</th>
<th>Days to Normal after Abs Treatment Discontinued</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin/ tazobactam</td>
<td>4000/500</td>
<td>4–8</td>
<td>20</td>
<td>Enterobacteria, Enterococci</td>
<td></td>
<td>Enterobacterial β-lactamase production</td>
<td></td>
<td>(Stark, Adamsson et al.)</td>
</tr>
<tr>
<td>Omeprazole/amoxicillin</td>
<td>20/100</td>
<td>14/2</td>
<td>14</td>
<td>Alteration to microflora</td>
<td></td>
<td></td>
<td></td>
<td>(Hartmut 2001)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>1000</td>
<td>7</td>
<td>12</td>
<td>Bifidobacteria, Lactobacillus, Clostridia</td>
<td>Enterococci, Escherichia coli</td>
<td>Clostridium difficile isolated from three volunteers</td>
<td></td>
<td>(Hartmut 2001)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>500</td>
<td>7/3</td>
<td>10</td>
<td></td>
<td></td>
<td>Amoxicillin-resistant bacteria including E.coli, Klebsiella and Enterobacter</td>
<td></td>
<td>(Brismar, Edlund et al. 1993)</td>
</tr>
<tr>
<td>Omeprazole/amoxicillin/metronizadol</td>
<td>500/1000</td>
<td>2/4</td>
<td>20</td>
<td>Marked changes</td>
<td>Total anaerobic bacteria</td>
<td>Streptococcus spp, Enterococcus spp, Enterococcus spp, Enterobacteria Yeast colonisation in nine subjects</td>
<td>35 days</td>
<td>(Adamsson, Nord et al. 1999)</td>
</tr>
<tr>
<td>Imipenem (surgical prophylaxis)</td>
<td>500/100</td>
<td>2/4</td>
<td>20</td>
<td>Staphylococci, Streptococci Enterococci, Enterobacteria, Anaerobic cocci, Bifidobacteria, Eubacteria, Lactobacilli Clostridia, Fusobacteria Bacteroides</td>
<td></td>
<td>No normal colonisation after 14 days</td>
<td></td>
<td>(Dunne, Murphy et al. 1999)</td>
</tr>
<tr>
<td>Pivmecillinam</td>
<td>400</td>
<td>14–41</td>
<td>15</td>
<td></td>
<td>E.coli</td>
<td></td>
<td></td>
<td>(Sullivan, Edlund et al. 2001)</td>
</tr>
<tr>
<td>Compound</td>
<td>Treatment (mg per day)</td>
<td>Number of Days and Number of Treatments Per Day</td>
<td>Number of Subjects</td>
<td>Bacteria Suppressed</td>
<td>Bacteria Proliferated</td>
<td>Resistant Strains Developed or Other Results</td>
<td>Days to Normal after Abs Treatment Discontinued</td>
<td>References</td>
</tr>
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<td>-------------------</td>
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<td>------------------------------------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------------------</td>
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<td>-----------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>1000</td>
<td>7</td>
<td>10</td>
<td><em>Lactobacilli, Bifidobacteria, Clostridia, Bacteroides, E.coli</em></td>
<td>Enterococci</td>
<td>Overgrowth of yeasts</td>
<td>21–35 days</td>
<td>(Pletz, Rau et al. 2004)</td>
</tr>
<tr>
<td>Aztreonam plus tobramycin</td>
<td>15 febrile neutropenic patients</td>
<td>Enteric Gram-negative bacilli, Faecal anaerobes</td>
<td>Fungal contamination</td>
<td>(Louie, Chubb et al. 1985)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aztreonam plus cloxacillin</td>
<td>14 febrile neutropenic patients</td>
<td>Enteric Gram-negative bacilli, Faecal anaerobes</td>
<td>Fungal contamination</td>
<td>(Louie, Chubb et al. 1985)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moxalactam plus tobramycin</td>
<td>500–1000</td>
<td>4–11/4</td>
<td>10</td>
<td>Enterobacteria Anaerobic cocci, Bacteroides</td>
<td>Fungal contamination</td>
<td>(Louie, Chubb et al. 1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>500–1000</td>
<td>4–11/4</td>
<td>10</td>
<td>Enterobacteria, Streptococci, Clostridia, Bacteroides, Gram-negative cocci</td>
<td>Enterococci</td>
<td>14 days</td>
<td>(Kager, Brismar et al. 1988)</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>500</td>
<td>7/3</td>
<td>10</td>
<td>Enterobacteria, Streptococci, Clostridia, Bacteroides, Gram-negative cocci</td>
<td>Enterococci</td>
<td></td>
<td>(Bergan, Nord et al. 1991)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.9 Effects of Cephalosporins on intestinal microflora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment (mg per day)</th>
<th>Number of Days and Number of Treatments Per Day</th>
<th>Number of Subjects</th>
<th>Bacteria Suppressed</th>
<th>Bacteria Proliferated</th>
<th>Resistant Strains Developed or Other Side-Effects</th>
<th>Days to Normal after Treatment Discontinued</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefprozil</td>
<td>500</td>
<td>8/2</td>
<td>8</td>
<td>Enterobacteria</td>
<td></td>
<td></td>
<td></td>
<td>(Hartmut 2001)</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>500</td>
<td>10</td>
<td>20</td>
<td>Not much changed</td>
<td></td>
<td></td>
<td></td>
<td>(Adamsson, Edlund et al. 1997)</td>
</tr>
<tr>
<td>Cefpodoxime proxitel</td>
<td>200</td>
<td>7/2</td>
<td>10</td>
<td>Streptococci, Enterobacteria, Clostridia</td>
<td>Enterococci Clostridium difficile</td>
<td>B-lactamase activity in the Flora of some subjects</td>
<td></td>
<td>(Brismar, Edlund et al. 1993)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2000</td>
<td>7</td>
<td>10</td>
<td>Lactobacilli Bifidobacteria Clostridia Bacteroides E Coli</td>
<td>Enterococci</td>
<td>Overgrowth of Yeast</td>
<td>1 to 35 days</td>
<td>(Pletz, Rau et al. 2004)</td>
</tr>
<tr>
<td>Ceftibutan</td>
<td>400</td>
<td>10</td>
<td>14</td>
<td>Enterococci</td>
<td>E.coli and anaerobic cocci</td>
<td>Increased β-lactamase C.Difficile colonisation in six individuals</td>
<td></td>
<td>(Brismar, Edlund et al. 1993)</td>
</tr>
</tbody>
</table>
Table 2.10 Effects of Fluroquinolones on intestinal microflora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment mg per day</th>
<th>Number of Days</th>
<th>Number of Subjects</th>
<th>Bacteria Suppressed</th>
<th>Bacteria Proliferated</th>
<th>Resistant Strains Developed</th>
<th>Days to Normal after Treatment Discontinued</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garenoxacin</td>
<td>600</td>
<td>6</td>
<td>16</td>
<td><em>Enterobacteria</em>, <em>Bacilli</em>, <em>Corynebacteria</em>, <em>Enterobacteria</em>, <em>Bifidobacteria</em>, <em>Bifidobacteria</em>, <em>Lactobacilli</em>, <em>Clostridia</em>, <em>Bacteroides</em></td>
<td>Eubacteria</td>
<td>Garenoxacin-resistant Eubacterium lentum</td>
<td>14 days</td>
<td>(Nord, Brismar et al. 1993)</td>
</tr>
<tr>
<td>Gemi-floxacin</td>
<td>320</td>
<td>7</td>
<td>10</td>
<td><em>Enterobacteria</em>, <em>Enterococci</em>, <em>Streptococci</em>, <em>Anaerobic cocci</em>, <em>Lactobacilli</em></td>
<td></td>
<td></td>
<td>49 days</td>
<td>(Barker, Sheehan et al. 2001)</td>
</tr>
<tr>
<td>Clinafloxac-acin 200</td>
<td>200</td>
<td>7</td>
<td>12</td>
<td><em>Aerobic bacteria</em></td>
<td><em>Anaerobic bacteria</em></td>
<td><em>Bacteroides spp.</em></td>
<td></td>
<td>(Oh, Nord et al. 2000)</td>
</tr>
</tbody>
</table>
Table 2.11 Effects of Dirithromycin, Clarithromycin, Tigecycline and Erythromycin on intestinal microflora

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment (mg per day)</th>
<th>Number of Days and Number of Treatments</th>
<th>Number of Subjects</th>
<th>Bacteria Suppressed</th>
<th>Bacteria and Yeasts Proliferated</th>
<th>Resistant Strains Developed</th>
<th>Days to Normal after Treatment Discontinued</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dirithromycin</td>
<td>500</td>
<td>7</td>
<td>20</td>
<td>Enterobacteria, Anaerobes, gram-positive cocci, Bifidobacteria, Eubacteria, Bacteroides spp., Clostridia, Lactobacilli</td>
<td>S. pyogenes, enterococci, S. aureus, and other streptococci</td>
<td>None</td>
<td>35 days</td>
<td>(Eckernäs, Grahnén et al. 1991)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>500</td>
<td>7/2</td>
<td>12</td>
<td>Escherichia coli, Bifidobacteria, Lactobacilli, Clostridia</td>
<td>Enterococci, Enterobacter, Citrobacter, Klebsiella, Psedomonas, S. pyogenes</td>
<td>None</td>
<td>35 days</td>
<td>(Edlund, Álván et al. 2000)</td>
</tr>
<tr>
<td>Omeprazole/clarithromycin/metronidazole</td>
<td>20/250/400</td>
<td>7</td>
<td>16</td>
<td>Anaerobic bacteria, Total anaerobic microflora</td>
<td>S. pyogenes, enterococci, S. aureus, and other streptococci</td>
<td>None</td>
<td>35 days</td>
<td>(Adamsson, Nord et al. 1999)</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>100/ then 50/2</td>
<td>10</td>
<td>13</td>
<td>Enterococci Escherichia, Lactobacilli, Bifidobacteria,</td>
<td>Enterobacteria and yeasts, Klebsiella pneumonia, Five resistant Enterobacter cloacae strains</td>
<td>None</td>
<td>35 days</td>
<td>(Nord, Sillerström et al. 2006)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1000/500</td>
<td>7/2</td>
<td>10</td>
<td>Streptococci, Enterococci, Enterobacteria, Anaerobic bacteria were also affected</td>
<td>S. pyogenes, enterococci, S. aureus, and other streptococci</td>
<td>None</td>
<td>35 days</td>
<td>(Brismar, Edlund et al. 1991)</td>
</tr>
</tbody>
</table>
2.3.11.2 Proton pump inhibitors

Undoubtedly, another important piece of knowledge is that Helicobacter pylorus is an acid-loving microorganism and its survival relies on a low gastric pH environment. Therefore the prescribing of proton pump inhibitors (PPIs) has become an important part of the medical management of H. pylori infection. However, PPIs’ use is not limited to H. pylori management; they are commonly prescribed for gastro-oesophageal reflux disease (GERD), heartburn and dyspepsia (Naunton, Peterson et al. 2000), resulting in PPIs being the most prescribed class of drug globally (Vine, Philpott et al. 2012). Australians’ use of PPIs increased a staggering 1300% between 1995 and 2006 (Cundy and Mackay 2011).

Despite the proven efficacy of PPIs as a part of H pylori management there are concerns about the association between long-term and often inappropriate use of PPIs and their potential side-effects. These concerns include an increased risk of fractures (Vine, Philpott et al. 2012), vitamin B12 deficiency (O’Leary, Wai et al. 2011), severe magnesium deficiency (Cundy and Mackay 2011; Swaminathan and Wilson 2011), an increased risk of gastric carcinoma (Naunton, Peterson et al. 2000), increased susceptibility to respiratory infections (Leonard, Ho et al. 2012) and alterations in the normal composition of the intestinal microbiota (Aseeri, Schroeder et al. 2008; Choudhry, Soran et al. 2008; Khoruts, Dicksved et al. 2010).

In the context of the gastrointestinal microbiome, the exponential increase of PPIs’ use coincides with a significant increase in the prevalence of Clostridium difficile colitis in hospitalised patients (Choudhry, Soran et al. 2008). Choudhry et al. (2008) found that 64% (88 of 138) of patients who developed C. difficile infections were on PPIs. Perhaps more concerning was the fact that 63% of those patients prescribed PPIs had no valid indication for their use (Choudhry, Soran et al. 2008). The authors suggested that reduction of unnecessary PPIs use may be an additional strategy to reduce the incidence of infection in hospitalised patients.

2.3.11.3 The impact of stress on the intestinal microbiome

Stress, which is defined as an acute threat to homeostasis, has both short and long-term effects on the functions of the gastrointestinal tract. Recognition of the significant impact of psychological stress on human health is well accepted. The major effects of stress on gut function are a) increase in visceral perception; b) changes in gastrointestinal secretion; c) an increase in intestinal permeability; d) altered colonic motility; e) suppression of mucosal defences; and f) significant alterations to the indigenous intestinal microbiota.
Furthermore, specific associations between the role of the stress response in the aetiology and severity of intestinal disorders such as IBS (Clark and DeLegge 2008), GERD and IBD (Konturek, Brzozowski et al. 2011) have been investigated. Associations between early life stressors and sustained stressful events in the development of these disorders have also been made (Hewson-Bower and Drummond 2001). It is thought that corticotrophin-releasing factor signalising pathways are the main coordinators of the endocrine, behavioural and immune responses to stress (Taché, Mönnikes et al. 1993). Chronic activation of these pathways has been linked to alterations of gut motor function and regulation of barrier functions.

While clear associations between the stress response and altered gastrointestinal function have been demonstrated, how these alterations in motility and barrier function actually disturb the gastrointestinal microbiota is not fully understood. A number of groups have demonstrated that stress can increase the permeability of the intestine, allowing luminal antigens to cross the epithelium and activate the immune system (Saudi, Islam et al. 2009; Mayer and Tillisch 2011; Alonso, Guilarte et al. 2012). In prolonged or excessive stress the confrontation between the luminal contents to the immune system may lead to chronic inflammation and/or possible systemic bacterial or yeast infections (Georgopoulos, Feistauer et al. 1996; Konturek, Brzozowski et al. 2011). Alterations in the GI microbiota induced by the stress response have been demonstrated by a number of groups, showing relative increases in the number of Gram-negative bacteria being found in dense association with the mucosal epithelium (Spitz, Ghandi et al. 1996; Martin, Campbell et al. 2004). The stress of maternal separation has been linked to significantly reduced Lactobacilli for several days in babies/children following removal from the mother (Bailey and Coe 1999). Explanatory models for understanding the mechanisms by which Lactobacilli respond to stress include (a) Lactobacilli sp. are acid-loving and the stress response results in the inhibition of gastric acid release, thereby lowering proliferation of Lactobacilli sp; (b) stress-induced alterations in gastric motility prevent adherence and proliferation of Lactobacilli; and (c) the stress response may increase duodenal bicarbonate alkali, so further alkalising the pH of the intestine and making a less conducive environment for Lactobacilli sp. to survive in.

Liz’ko et al. (1979) assessed the microbiota of twelve individuals preparing for flight into space. This emotionally intense event was associated with significant reductions in faecal counts of Lactobacilli and Bifidobacteria and significant increases in Escherichia coli, with resultant decreases in colonisation resistance and increased numbers of pathogenic organisms. High levels of norepeniphrine have been found in the luminal contents of the gastrointestinal tract, effecting significant alterations to a number of indigenous microbiota (Lyte and Bailey 1997). Norepeniphrine increases bile secretion and flow, thereby providing an opportunistic
environment for the bile-loving *Bacteroides fragilis* subsp. *thetaiotaomicron* to proliferate in (Varel and Bryant 1974). In addition norepinephrine provides a growth-enhancing substrate for *E.coli, Yersinia enterocolitica* and *Pseudomonas aeruginosa*, which have all been associated with autoimmune and inflammatory disease of both the gastrointestinal tract and extra-intestinal organs (Karimi and Peña 2008; Pineton de Chambrun, Colombel et al. 2008).

Stress has been associated with a decreased presence of the GI defence molecules mucin, mucopolysaccharide and secretory immunoglobulin A (sIgA) in the presence of epinephrine and norepinephrine (Söderholm and Perdue 2001; Collins 2012). These molecules play a critical role in the inhibition of pathogenic organisms adhering to and subsequently colonising on the intestinal epithelium. A decrease in colonisation resistance has been demonstrated in a mouse study, where dexamethsone stress-induced mice had marked reductions in sIgA production and an increase in pathogenic bacterial adhesion (Spitz, Ghandi et al. 1996). This result has been supported in a paediatric study in which it was suggested that sympathetic nervous system activation suppresses the production of sIgA in children during times of stress, thereby increasing their predisposition to infection (Hewson-Bower and Drummond 2001). Following stress reduction techniques these children showed a marked increase in their sIgA levels and a significant reduction in the number of infections (Hewson-Bower and Drummond 2001).

A number of groups have reported intestinal pathophysiology in critically ill patients, e.g. following trauma and sepsis or after major surgery (Boudry, Jury et al. 2007; Clark, Clark et al. 2008). The intestinal pathology is thought to be stress-induced by the following mechanisms: (a) suppression of mucin, IgA and mucopolysaccharides, and (b) changes in prostaglandin synthesis, resulting in increased intestinal permeability. It is thought that these two stress-induced mechanisms are principally responsible for placing the critically ill at greater risk of bacterial translocation resulting in systemic infection.

In relation to CD, another possible mechanism by which stress exerts its damage is the extensive focal apoptosis observed in the small intestine of rats subjected to chronic stress as well as in sepsis models (Hotchkiss, Schmieg et al. 2000; Boudry, Jury et al. 2007). Physical and psychological stresses clearly play a significant role in altering the human gastrointestinal microbial environment, resulting in a patho-physiological basis for a number of gastrointestinal and immune-mediated diseases.

### 2.3.11.4 Dietary influences on the microbiome

The contribution of diet to establishing and maintaining the homoeostasis of the intestinal microbiota has been explored by a number of groups. The transition from breast milk to
formula feeding and solid foods has repeatedly been demonstrated to cause changes in the microbial communities. Infants who are breast-fed have higher faecal counts of *Lactobacilli, Bifidobacteria and Enterococcus* at six months than formula-fed infants (Rinne, Kalliomaki et al. 2005). Penders et al. (2006) found that infants who were exclusively formula-fed were more often colonised with *E.coli, C.difficle, Bacteroides* and *Lactobacilli* than breast-fed infants, who had a dominance of *Bifidobacteria*. Harmsen et al. (2000) produced similar findings when assessing the faecal microbiota of breast-fed and formula-fed infants with all breast-fed infants having a dominance of *Bifidobacteria* and formula-fed infants having less *Bifidobacteria*, more *Bacteroides, Streptococci, Escherichia coli, Clostridia* and *Staphylococci*.

Until recently, the distinction between specific dietary practices and microbial patterns after infancy have been unclear. A number of groups have compared broadly defined diets such as western and Asian, or investigated the impact of changing the intake of food categories, and have found only small effects involving a few genera. However, comparative studies with chemically well-defined diets have shown more pronounced effects. For example, dietary sulphate favours sulphate-reducing bacteria over methanogenic bacteria (Gibson, Cummings et al. 1991), and inulin and related fibres stimulate the proliferation of *Bifidobacteria* when the genus is rare (Gibson and Roberfroid 1995). The influence of periodic diet changes on the composition of intestinal bacteria are thought to be minimal and transient at a phyla level.

More recent work by de Filippo et al. (2010) argued against the notion of a minimal impact of dietary practice, proposing that diet has a dominant role over other possible variables such as ethnicity, sanitation, hygiene, geography and climate in shaping the gut microbiota. This statement is based on the comparison of the faecal microbiota of European children and children from a rural African village at both phyla and genus levels. The diet of the African children was high in fibre content, similar to the diet of early human settlements at the time of the birth of agriculture. Using 16S rDNA sequencing and biochemical analysis they found a significant enrichment in *Bacteroides* and depletion in *Firmicutes* (p<0.01), with a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, in addition to significantly more short chain fatty acids. Conversely, the faeces of children living in industrialised European societies and consuming a diet high in sugars, animal fats and calorie-dense foods were completely lacking in *Prevotella* and *Xylanibacter*. The authors hypothesised that gut microbiota co-evolved with the polysaccharide-rich diet of rural African village children, allowing them to maximise energy intake from fibres while also protecting them from
inflammations and non-infectious colonic disease (de Filippo, Cavalieri et al. 2010). Furthermore, microbial simplification as a result of industrialised dietary practices could deprive the microbial gene pool of potentially important gene reservoirs that allow adaptation to food sources in geographic locations, as demonstrated by algae-consuming Japanese (Sugita and Ito 2006). These two projects led by de Filippo et al. (2010) and Sugita et al. (2006) highlight the importance of preserving the treasure of microbial diversity from ancient rural communities worldwide. Increased gastrointestinal microbial diversity and reduced quantities of potentially pathogenic strains *Shigella* and *Escherichia coli* observed in the children from the African rural village gives further support to the ‘old friend’ hypothesis.

**2.3.12 Microbial diversity and disease predisposition**

Our understanding that rich intestinal microbial diversity encodes bacterial genes required for the hydrolysis of dietary compounds, in addition to the production of short chain fatty acids required for the integrity and normal cell turnover of the intestinal epithelium, has led to specific inquiries regarding the gastrointestinal microbiome in specific disease states. Sellitto et al. (2012) hypothesised that the intestinal microbiota as a whole, rather than specific infections, dictates the switch from tolerance to immune response in genetically predisposed individuals. To test this hypothesis, infants who were HLA DQ2 and/or DQ8 positive were assessed for longitudinal changes in their faecal microbial communities from birth to 24 months and compared with non-genetically predisposed children. In addition, the impact on the gastrointestinal microbiome of introducing gluten at six months or at >12 months of age was assessed. The results indicated that a lack of bacterial diversity in the phylum *Bacteroidetes* relative to *Firmicutes* in the genetically predisposed children, and an overall microbial profile that did not resemble that of an adult even by two years of age, correlated with a greater risk of developing CD in children exposed to gluten before twelve months of age. The molecular mechanisms underlying the benefit of a mature and diverse intestinal microbiome in preventing autoimmunity in at risk children is not fully understood. In addition, the early childhood influences on gastrointestinal microbiome patterns of adult patients with established CD has not been assessed.

The literature discussed in 2.3.11.4 pertaining to dietary influences suggests that the interaction of the human genome with the human gastrointestinal microbiome is established early in life through delivery methods, feeding practice and exposure to a broad range of microbes, thereby providing a blueprint for the capacity of the host to respond to a broad number of dietary influences that interact with the gastrointestinal and immune system (de Filippo, Cavalieri et al. 2010). The significance of temporal gastrointestinal microbiome
changes in response to dietary practice remains less clear. However, the temporal change induced by medications has attracted considerable attention and is presented in 2.3.11 and 2.3.12.

To what degree the collective influences of factors that are known to impact the diversity and development of the microbiome play in CD predisposition has not been fully elucidated.

2.4 Probiotics

The term ‘probiotic’ means ‘for life’. The use of probiotics in human medicine is a relatively recent development. However, many of the current concepts and beliefs regarding probiotic use are a culmination of research dating back to the 1800s. With a view to understand whether there is a potential role for probiotic use in patients with CD, a review of the history, proposed mechanisms of action and their current use in other conditions was undertaken.

2.4.1 History of probiotics

Pasteur was the first to introduce the notion of bacteriotherapy: the utilisation of ‘harmless’ bacteria to displace pathogenic organisms as a means of treating infection (Pasteur 1877). Throughout the 1900s physicians placed bacteria against bacteria in both test tubes and infected patients in an attempt to find an effective therapy (Florey 1946). However, the 1940s saw the discovery of antibiotics that arguably changed the way we view the microbial kingdom. The practices of bacteriotherapy and bacterio-prophylaxis were largely discontinued and a sense of triumph and complacency towards the risk of microbial adversaries became commonplace. It is clear that antibiotics have played a valuable role in human health and medicine. However, in the face of persistent exposure to antibiotic laced ecosystems, microbes have waged a war, and variant forms now flourish with apparent impunity despite technological advances in antimicrobial medicine development (Deitch 1988). This new challenge to modern medicine has encouraged many researchers to reconsider the Pasteurian creed that bacteria themselves could prove to be our most effective allies in microbial medicine (Versalovic, Wilson et al. 2009).

A review of probiotics is not complete without a brief description of the work of Elie Metchnikoff (1845–1916). Metchnikoff was a Nobel Prize winner for his pioneering observations and descriptions of phagocytosis at the turn of the 20th century. He is frequently referred to in regard to the origin and concept of probiotic food sources and their proposed benefits in conferring gastrointestinal microbial ‘balance’. Metchnikoff proposed that the cause of autointoxication were the gastrointestinal microflora. He noted that bacteria were
capable of protein degradation (putrefaction), releasing ammonia, amines and indole and that these were toxic metabolites to human tissue in varying concentrations. Furthermore, he proposed that these by-products would escape detoxification by the liver and enter the systemic circulation, thus causing autointoxication (Metchnikoff 1908). His treatment for the prevention of autointoxication was radical, to say the least, and involved surgical removal of the large intestine. The less radical approach, and the one that gained greater acceptance, was to change the microbial environment of the intestine through the introduction of fermented milks. Metchnikoff had observed that the lactic acid-producing bacteria present in fermented milk products prevents the growth of non-acid-tolerant bacteria, including proteolytic species in milk (Metchnikoff 1908). He extrapolated from this observation that lactic acid fermentation would confer the same protection in the digestive tract. This concept was supported by Metchnikoff’s epidemiological observations of many western Europeans who drank fermented milk and had unusual longevity. Thus the concept of ‘microbial food therapy’ was conceived.

In the last 20 years a significant body of research has been directed towards the concepts introduced by Metchnikoff and to the later work of British microbiologist Roy Fuller whose work is discussed by Florey (1946). By 2001, a ‘modern’ definition had been drafted by joint expert consultation of the Food and Agricultural Organisation in Argentina, which defined probiotics as ‘Live organisms which, when consumed in adequate amounts, confer a health benefit on the host’ (Pineiro and Stanton 2007).

2.4.2 Microbes used as probiotics in clinical trials

The discoveries of Pasteur and Metchnikoff in the 1800s and Florey in the 1940s pioneered the concepts of bacteriotherapy. From the 1990s to the present, these concepts regained popularity among both the public and many health care professionals. The evidence for probiotic indications, safety and efficacy was initially based on historical use and the findings of exploratory studies that have employed culture and microscopy to identify commensal microbiota of the gastrointestinal tract (Florey 1946; Versalovic 2006). A criticism of these earlier composition studies is that they have over-simplified a complex ecosystem into a crude division between ‘good’ and ‘bad’ (Tannock 2000).

However, over the last decade, with the advent of molecular technology, exploratory studies have revealed a dynamic and metabolically complex gastrointestinal microbial composition. This is discussed in detail by Ouwehand et al. (2010). From 2008, in particular there has been a copious growth in knowledge arising from the completion of the four-year HMP, which has confirmed the complexity of the composition and metabolism of the
intestinal milieu. In addition to microbiome compositional studies in specific disease states, intervention studies have assessed the influences of probiotic effector organisms (Proctor 2011). Table 2.12 presents the microorganisms most commonly used as probiotics in human clinical trials for both intestinal and extra-intestinal conditions from 2006 to 2011. No clinical studies specific to CD were identified in this review. However, conditions that share either some of the immunological or clinical features of CD are presented in Table 2.12 with a view to extrapolate the findings and apply them to future trials investigating CD.

2.4.3 Considerations for probiotic use in gastroenterology and nutrition

Probiotics have been defined as a preparation of viable microorganisms of a specific genus and species that in sufficient numbers has the ability to alter the microbiota either transiently or by colonisation in a specific niche of the human body and that confers benefit to the host (Barbara, Stanghellini et al. 2008). There are numerous considerations when choosing probiotics for gastroenterology and nutrition including functional, technological and safety aspects.

2.4.3.1 Functional considerations

From a functional aspect, the strains reported to be most efficacious are of human origin, probably due to the increased likelihood of being able to grow under the physiological conditions of the human gastrointestinal tract (Versalovic, Wilson et al. 2009). The ability of probiotics to exert an immunological effect is closely related to the ability of the microorganism to adhere and colonise, and so have a prolonged contact with gut-associated lymphoid tissue (GALT) (Dunne, Murphy et al. 1999; Szymanski, Chmielarczyk et al. 2006). The ability of a microorganism to prevent pathogen growth is largely thought to be related to its ability to produce antimicrobial substances, including bacteriocins, and bacteriocin-like substances, as well as lactic acid and hydrogen peroxide (Gotteland, Brunser et al. 2006).
Table 2.12 Microorganisms commonly used as probiotics in human trials between 2006 and 2011 (adapted from (Rauch and Lynch 2012))

<table>
<thead>
<tr>
<th>Species</th>
<th>Disease Targeted</th>
<th>Study</th>
<th>Probiotic Formulation and Dosage</th>
<th>Study Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus</td>
<td>Type 2 diabetes</td>
<td>R, DB, PC</td>
<td>Capsules with freeze dried Lactobacillus NCFM; approx 10^10 CFU per capsule</td>
<td>Ingestion of Lactobacillus preserved insulin sensitivity versus placebo, but did not affect systemic inflammatory response.</td>
<td>Andreasen et al. (2010)</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>Incidence of cold and influenza-like symptoms in healthy children</td>
<td>R, DB, PC</td>
<td>Dried Lactobacillus alone (10^10 CFU/day) or in combination with Bifidobacterium animalis subsp. Lactis (10^10 CFU/day combined) mixed into milk</td>
<td>Lactobacillus alone or in combination with B. animalis subsp. Lactis reduced incidence and duration of fever, coughing, rhinorrhoea in healthy children.</td>
<td>Leyer et al. (2009)</td>
</tr>
<tr>
<td>Lactobacillus fermentum</td>
<td>Mucosal immunity in endurance athletes</td>
<td>DB, PC, CO</td>
<td>Daily dose of 1.26 x 10^10 CFU of L. fermentum VR1-003 as freeze dried powder in gelatine capsule</td>
<td>Substantial reduction in the number of days and severity of respiratory illness as reported by participants.</td>
<td>Cox et al. (2010)</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii</td>
<td>Immune senescence and resistant to the common cold</td>
<td>NA</td>
<td>Yoghurt fermented with L.delbrueckii subsp. bulgaricus</td>
<td>Augmented natural killer cell activity and reduced the risk of catching the common cold in elderly individuals.</td>
<td>Makino et al. (2010)</td>
</tr>
<tr>
<td>Lactobacillus johnsonii</td>
<td>Crohns disease</td>
<td>R, DB, PC</td>
<td>L.johnsonii LA1 (Nestec) in freeze dried form and blended with maltodextrin at 10^10 CFU/day</td>
<td>No effect on endoscopic recurrence after ileocaecal resection.</td>
<td>Rahimi et al. (2008)</td>
</tr>
<tr>
<td>Lactobacillus johnsonii</td>
<td>Skin immune status following immune status</td>
<td>R, DB, PC</td>
<td>1 x 10^10 CFU of L.johnson LA1 (NCC533) per day</td>
<td>Accelerated recovery of skin immune homeostasis after UV induced immuno-suppression.</td>
<td>Peguet-Navarro et al. (2008)</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td>Allergic sensitisation and asthma in infants at risk</td>
<td>R, DB, PC</td>
<td>6–24 months old infants at risk received 1010 CFU L.rhamnosus strain GG (LGG; ATCC 53103) twice daily for 6 months</td>
<td>No clinical effect on atopic dermatitis or asthma related events, and only mild effects on allergic sensitisation.</td>
<td>Rose et al. (2010)</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td>Prevention of atopic eczema in children</td>
<td>R, BD, PC</td>
<td>Daily dose of 1 x 1010 CFU of L.rhamnosus strain GG (LGG) post-natally for 6 months</td>
<td>Frequency of eczema reduced during the first 7 years of life.</td>
<td>Kalliomaki et al. (2007)</td>
</tr>
<tr>
<td>Species</td>
<td>Disease Targeted</td>
<td>Study</td>
<td>Probiotic Formulation and Dosage</td>
<td>Study Outcome</td>
<td>Reference</td>
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</tr>
<tr>
<td>Lactobacillus reuteri</td>
<td>Prevention of antibiotic-associated diarrhoea</td>
<td>R, DB, PC</td>
<td>Patients receiving antibiotics were given $1 \times 10^{10}$ CFU of <em>L.reuteri</em> twice daily</td>
<td>Significant decrease of antibiotic induced diarrhoea.</td>
<td>Cimperman et al. (2011)</td>
</tr>
<tr>
<td>Lactobacillus reuteri</td>
<td>Infantile colic</td>
<td>R, DB, PC</td>
<td>Suspension of freeze dried <em>L.reuteri</em> DSM 17938 in a mixture of sunflower oil and medium chain triglyceride oil; $10^8$ CFU/day</td>
<td>Improved symptoms of infantile colic.</td>
<td>Savino et al. (2010)</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>Allergic asthma and/or rhinitis in children</td>
<td>R, DB, PC</td>
<td>Long-term consumption of fermented milk containing $10^8$ CFU/ml of <em>L.casei</em></td>
<td>Improved allergic rhinitis but no effect in asthmatic children.</td>
<td>Giovannini et al. (2007)</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>Small bowel injury in chronic low dose aspirin users</td>
<td>R, PC</td>
<td><em>L.casei</em> for 3 months</td>
<td>Effective for the treatment of aspirin associated small bowel injury.</td>
<td>West et al. (2009)</td>
</tr>
<tr>
<td>Lactobacillus paracasei</td>
<td>Incidence of eczema in infants</td>
<td>R, DB, PC</td>
<td>Infants were fed cereals with <em>L.paracasei</em> strain F19 from 4 to 13 months of age</td>
<td>Decreased incidence of eczema and increased Th1/Th2 ratio.</td>
<td>West et al. (2009)</td>
</tr>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>Allergic rhinitis</td>
<td>R, DB, PC</td>
<td>Combination of <em>B.adolescentis</em> and <em>L.rhamnosus</em> mixed into yoghurt</td>
<td>Potentially effects on cytokine profile but few clinical benefits and no significant effect on quality of life score.</td>
<td>Koyama et al. (2010)</td>
</tr>
<tr>
<td>Bifidobacterium breve</td>
<td>Necrotizing enterocolitis (NEC) in preterm infants</td>
<td>R, DB, PC</td>
<td>Hydrolysed formula with <em>B.breve</em> alone or in combination with galacto/fructo-oligo-saccharide mixture</td>
<td>Reduced occurrence of NEC.</td>
<td>Braga et al. (2011)</td>
</tr>
<tr>
<td>Bifidobacterium breve</td>
<td>Prevalence of asthma like symptoms in infants with atopic dermatitis</td>
<td>R, DB, PC</td>
<td>Hydrolysed formula with <em>B.breve</em> alone or in combination with galacto/fructo-oligo-saccharide mixture</td>
<td>Combination prevented asthma like symptoms.</td>
<td>Van der Aalderen et al. (2011)</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>IBS</td>
<td>R, DB, PC</td>
<td>One capsule containing $1 \times 10^9$ CFU of freeze dried <em>B.bifidum</em> MIMBb75 daily for 4 weeks</td>
<td>Significantly alleviated IBS symptoms and improved quality of life.</td>
<td>Gugliemetti et al. (2011)</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>Incidence of eczema in high risk children</td>
<td>R, DB, PC</td>
<td>Mixture of <em>B.bifidum</em>, <em>B.animalis</em> subsp. lactis, and <em>Lactococcus lactis</em></td>
<td>Reduction in parental reported eczema during the first 3 months.</td>
<td>Niers et al. (2009)</td>
</tr>
<tr>
<td>Species</td>
<td>Disease Targeted</td>
<td>Study</td>
<td>Probiotic Formulation and Dosage</td>
<td>Study Outcome</td>
<td>Reference</td>
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<td>Niers et al. (2009)</td>
</tr>
<tr>
<td><em>Bifidobacterium animalis</em> subsp. <em>lactis</em></td>
<td>Risk of infections in infancy</td>
<td>R, DB, PC</td>
<td>Tablets containing <em>B. animalis</em> subsp. <em>lactis</em> BB-12 (10^10 CFU/d)</td>
<td>Decreased incidence of respiratory infections but no significant differences between the groups in reported gastrointestinal symptoms, otitis media or use of antibiotics.</td>
<td>Taipale et al. (2011)</td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td>IBS</td>
<td>R, DB, PC</td>
<td>Freeze dried, encapsulated <em>B. Infantis</em> at daily doses of 10^6, 10^8, or 10^9 CFU for 4 weeks</td>
<td>Significant improvement of IBS symptoms with 10^8 CFU but not with other doses.</td>
<td>Moayyedi et al. (2010a)</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td>Allergic rhinitis</td>
<td>R, DB, PC</td>
<td><em>B. longum</em> BB536</td>
<td>Significant decrease in subjective symptom scores; marked improvements in medical scores.</td>
<td>Xiao et al. (2006)</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em></td>
<td>Bowel movements in the elderly</td>
<td>R, DB, C</td>
<td>Fermented oat drink with 10^9 CFU/day of <em>B. longum</em> strains BL46 and BL2C or 10^9 CFU/day <em>B. animalis</em> subsp. <em>lactis</em> strain Bb 12 or without viable bacteria</td>
<td>Probiotic supplements normalised bowel movements.</td>
<td>Pitkala et al. (2007)</td>
</tr>
</tbody>
</table>
Table 2.12 (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Disease Targeted</th>
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<th>Probiotic Formulation and Dosage</th>
<th>Study Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Impact on carriage of multi-drug-resistant <em>E. coli</em> (MDREC)</td>
<td>R, DB, PC</td>
<td>Encapsulated <em>E. coli</em> strain Nissle 1917 (5x 10^7 to 5x 10^10 CFU/day)</td>
<td>No effect on MDREC carriage.</td>
<td>Pitkala et al. (2007)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Persisting diarrhoea in infants and toddlers</td>
<td>R, DB, PC</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Significant reduction of daily stool frequency after 14 days.</td>
<td>Henker et al. (2008)</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>IBS in children</td>
<td>R, DB, PC, CO</td>
<td>Mix of eight lyophilised strains (<em>S. thermo-philus, B. breve, B. longum, B. infantis, Lacidophilus, L.plantarum, Lparacasei, L.bulgaricus</em>; commercial product VSL#3™ daily dose of 4.5 x 10^11 total CFU)</td>
<td>Significantly ameliorated IBS symptoms and improved quality of life.</td>
<td>Guandalini et al. (2010)</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>Intestinal gas symptoms in adults with no gastrointestinal diagnosis</td>
<td>R, DB, PC</td>
<td><em>B. coagulans</em> GBI-30, 6086 (commercial product GanedenBC 30™)</td>
<td>Reduced GI symptoms; strong placebo effect was evident.</td>
<td>Kalman et al. (2009)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Intestinal permeability in patients with Crohn’s disease in remission</td>
<td>R, PC</td>
<td>Encapsulated, lyophilised <em>S. cerevisiae</em> subsp. <em>boulardii</em> (S. Boulardii-17; commercial product Floratil™); one capsule (4 x 10^7 CFU) every 8 hours for 3 months</td>
<td>Improved intestinal permeability; however, complete normalisation was not achieved.</td>
<td>Garcia et al. (2008)</td>
</tr>
</tbody>
</table>

a. R, randomised; DB, double-blind; PC, placebo-controlled; CO, crossover; NA, data not available.
b. CFU, colony-forming unit.
c. As reported in the referenced studies.
d. Commonly referred to as *S. boulardii* in probiotic products. Currently classified as a sub-species of *S. cerevisiae* due to its highly similar genotype, *S. cerevisiae* subsp. boulardii differs from other strains of *S. cerevisiae* by several key metabolic and genetic characteristics and may represent a unique species.
2.4.3.2 Technological considerations

Technological considerations are largely related to the ability of a chosen strain to remain viable and retain its desirable characteristics during manufacture and storage (Vinderola, Prosello et al. 2000; Klayraung, Viernstein et al. 2009). Factors such as pH (Ding and Shah 2009), the presence of other microorganisms, storage temperature and the presence or absence of microbial inhibitors in the substrate will all have a significant influence on whether a strain survives or not (Tomas, Bru et al. 2009). The taste, smell and palatability of a probiotic needs to be considered in the development of formulations (Molin 2001). Figure 2.7 illustrates the technological considerations of probiotic formulations.

![Technological considerations in probiotic manufacturing](Current_Opinion_in_Biotechnology)

**Figure 2.7 Technological considerations in probiotic manufacturing (Rauch and Lynch 2012)**

2.4.3.3 Rethinking viability: live or dead bacteria, or bacterial DNA

The manufacturers of probiotics make a considerable effort to ensure the bacteria remain viable to achieve the desired clinical outcome. However, recent studies suggest that bacterial DNA sequences may provide the same effects as live bacteria (Jijon, Backer et al. 2004;
Rachmilewitz, Katakura et al. 2004). Bacterial DNA motifs can bind to toll like receptor-9 (TLR-9). TLR-9 signalling depends on the adaptor protein myeloid differentiation primary response 88 gene (MyD88). MyD88 is the gene that encodes a cytosolic adapter protein and plays a central role in the innate and adaptive immune response. This protein functions as an essential signal transducer in the IL-1 and TLR signalling pathways. These pathways regulate that activation of numerous pro-inflammatory genes and non-viable bacteria may be able to signal and elicit beneficial effects. In an experiment that used methylated and non-methylated genomic DNA isolated from the probiotic preparation VSL#3™ (a proprietary blend of eight strains of live freeze dried bacteria including Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus and Streptococcus thermophilus). Rachmilewitz et al. (2004) demonstrated that genomic DNA but not methylated DNA, calf thymus DNA or DNAse treated probiotics ameliorated the severity of colitis in dextran sulphate and 2, 4, 6-trinitrobenzene sulfonic acid induced colitis and spontaneous colitis in IL-10 knock-out mice. Furthermore, they showed that intra-gastric and subcutaneous administration of gamma-irradiated non-viable bacteria and live bacteria had similar beneficial effects. Lammers et al. (2002) have also demonstrated that bifidobacterium-genomic DNA induced the secretion of IL-10 by peripheral blood mononuclear cells from healthy donors, suggesting that bacterial DNA has an immuno-modulatory effect. In another study, when a VSL#3™ conditioned medium was used, NF-κB was inhibited in a cell line from the intestinal epithelium of mice (Petrof, Kojima et al. 2004). The use of VSL#3™-conditioned medium (non-viable bacterial cells) further challenges the concept that to exert beneficial effects probiotics must be live bacteria (Ng, Hart et al. 2009).

2.4.3.4 Safety considerations
Probiotics have a long history of safe use, and most microorganisms are considered commensals with little to no pathogenic potential (Versalovic, Wilson et al. 2009). However, this generalised statement is based on relatively few studies and does not include many of the new strains being isolated for potential use in gastrointestinal medicine. The safety of probiotic use in gastroenterology and nutritional medicine needs to be clearly established, especially as single and new strains are developed for specific conditions. Versalovic et al. (2009) proposes three approaches that can be used to assess the safety of potential probiotic strains:

5. Studies on the intrinsic properties of the strain such as antibiotic resistance, excessive deconjugation of bile acids, or degradation of mucus;
6. Studies on the pharmacokinetic properties of the strain, determining the efficacy of ingested probiotic bacteria and assessing the effect of massive probiotic dose on the composition of the human microbiota; and

7. Studies investigating the interaction between the strain and the hosts. The last approach consists in verifying that the proposed probiotic does not possess any invasion potential and does not harm the host in any way (Versalovic, Iyer et al. 2008; Versalovic, Wilson et al. 2009).

2.4.3.5 *Proposed mechanisms of probiotic action*

Probiotic bacteria have multiple and diverse influences on the host. Different organisms can influence the intestinal luminal environment, epithelial and mucosal barrier function, and the mucosal immune system. They exert their effects on numerous cell types involved in the innate and adaptive immune responses, such as epithelial, dendritic cells, monocytes/macrophages, B-cells, T-cells, including T-cells with regulatory properties, and NK-cells (Ng, Hart et al. 2009). A detailed review of the cell types in relation to the GALT is provided in Appendix A1. The evidence for these mechanisms will be reviewed in relation to inflammatory conditions of the gastrointestinal tract.

2.4.3.6 *Modification of the intestinal microbiota*

Modification of the intestinal microbiota through competitive exclusion of pathogens is achieved by the ability of the chosen probiotic to:

1. Adhere to mucus and co-aggregate, thereby forming a barrier which prevents colonisation by pathogens or competition for adhesion receptors of the epithelial cells;

2. Produce antimicrobial compounds such as hydrogen peroxide, lactic acid, bacteriocins or bacteriocin-like substances, bio-surfactants and a number of partially purified but unnamed compounds (Mead 2000; Sheil, Shanahan et al. 2007); and

3. Reduce luminal pH (Venturi, Gionchetti et al. 1999; Asahara, Shimizu et al. 2004). The ability of probiotic microorganisms to adhere to the epithelial cells influences its colonisation potential. The process of adherence depends on components of the bacterial cell surface such as glycoproteins, carbohydrates and lipoteichoic acid (Sanz, Nadal et al. 2007). It has been suggested that bio-surfactants may also play an important role in microbial adhesion (Frey, Giannasca et al. 1996; Marcus, Wyble et al. 1996; Banat, Franzetti et al. 2010). Bio-surfactants are surface-active compounds produced by microorganisms that are able to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. The main physiological role of bio-surfactants is to facilitate the uptake of water-immiscible substrates, although they can also exert antimicrobial activity against a variety of microbes.
Bio-surfactants include glycolipids, lipopeptides, phospholipids, substituted fatty acids and lipo-polysaccharide (LPS). LPS is the most extensively researched bio-surfactants (Banat, Franzetti et al. 2010).

Antimicrobial bacteriocins are also likely to contribute to the beneficial activity of probiotics. Several bacteriocins produced by different species from the genus *Lactobacillus* have been described (Klaenhammer 1988). The inhibitory activity of these bacteriocins varies: some inhibit other lactobacilli or taxonomically related Gram-positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria as well as yeasts and moulds (Nemcova 1997). *L. salivarius* subsp. *salivarius* UCC118 produces a peptide that inhibits a broad range of pathogens such as *Bacillus, Staphylococcus, Enterococcus, Listeria* and *Salmonella* species (Flynn, van Sinderen et al. 2002). *Lactococcus lactis* subsp. produces a broad spectrum bacteriocin called Lacticin 3147 that has been shown to inhibit a range of genetically distinct *C. difficile* isolates from healthy subjects and patients with IBD (Rea, Clayton et al. 2007). *Lactobacillus* species have been shown to decrease the adherence of *H. pylori* to the epithelial cells of the gastric mucosa through the release of bacteriocins (Gotteland, Brunser et al. 2006). Pre-treatment of intestinal (T84) cells with lactic acid-producing bacteria reduces the ability of pathogenic *E. coli* to inject virulence factors into the cells or to breach the intra-cellular tight junctions (Sherman, Johnson-Henry et al. 2005). Virulence factors are molecules expressed and secreted by pathogens that enable them to achieve invasion of the host by colonising a niche in the host, evading or inhibiting the host’s immune response, and allowing entry of the pathogen into and out of the cell.

In another study, the adherence potential to intestinal epithelial cells isolated from Crohn’s disease patients was examined in the presence of probiotic strain *E. coli* Nissle 1917. The outcome of this study was promising, with the authors concluding that *E. coli* Nissle strain prevented epithelial injury by competitive exclusion of invasive *E. coli* (Malchow 1997; Rembacken, Snelling et al. 1999b).

Defensins are antimicrobial peptides involved in innate defence mechanisms (Ng, Hart et al. 2009). Human beta-defensins 2 expression was increased in caco-2 intestinal epithelial cells by *E. coli* Nissle 1917 (Wehkamp, Harder et al. 2004). It is thought that the increase in human beta-defensins 2 expression could improve mucosal barrier and thereby reduce intestinal permeability and trans-cellular transport of pathogens (Ng, Hart et al. 2009).

2.4.3.7 Enhancement of barrier function

Disruption of barrier function is seen in several conditions, including active and inactive IBD (Hollander, Vadheim et al. 1986; Wyatt, Vogelsang et al. 1993; Hilsden, Meddings et al.
Interestingly, barrier function can also be disrupted in the healthy relatives of patients with IBD (Sakaguchi, Köhler et al. 2002), in patients with CD (van Elburg, Uil et al. 1993; Perry, Iqbal et al. 2001; Watts, Berti et al. 2005) and patients with other autoimmune diseases such as Type 1 diabetes (Watts, Berti et al. 2005).

Enhancing mucosal membrane integrity and function in these conditions is important. Probiotic therapies have been demonstrated to improve intestinal bacteria function both in vitro and in vivo. As described by Ng et al (2009), the probiotic mixture VSL#3™ normalised barrier integrity as assessed by short circuit currents, trans-epithelial potential differences and mannitol fluxes in excised tissue from mice (Madsen, Cornish et al. 2001). In another study, in an in vitro culture using T84 epithelial cells, VSL#3™, but not L.reuteri, S.bovis or non-pathogenic E.coli, decreased monolayer permeability and conductance, indicating that the increase in resistance was specific to one or more of the bacteria in VSL#3™. In a further study VSL#3™ probiotic therapy decreased tight junction protein expression and increased the apoptotic ratio in colitis-induced mice (Mennigen, Nolte et al. 2009). Garcia-Lafuente et al. (2001) demonstrated that L. brevis reduced intestinal permeability, as evidenced by permeability to mannitol in rats. In a separate study, IL-10 deficient mice with chronic colitis were used to prove the benefits to barrier function by the administration of VSL#3™ (Madsen, Cornish et al. 2001). Methotrexate can induce colitis and was used in a rat model to test the potential of L.plantarum and L.reuteri in enhancing barrier function (Mao, Nobaek et al. 1996). However, Kennedy et al. (2000) did not see the same results with L.plantarum and was not successful in enhancing barrier function in the context of 2, 4, 6-trinitrobenzene sulfonic acid colitis (drug-induced colitis). The exact mechanisms by which probiotic bacteria enhance barrier function are unclear but may relate to alterations in mucus or chloride secretion or changes in tight junction protein expression by epithelial cells (Ng, Hart et al. 2009).

Several groups have assessed the potential of probiotic bacteria to modify Mucin-1 (MUC1) gene expression and mucus secretion. Over-expression of MUC1 gene is often associated with ovarian, breast, prostate, lung and colon cancer. L.plantarum 299v increased Mucin2 (MUC2) and Mucin3 (MUC3) mRNA expression when incubated with the epithelial cell line HT-29 (Mack, Michail et al. 1999). MUC2 is particularly prominent in the gut where it is secreted from goblet cells in the epithelial lining into the lumen of the large intestine. MUC3 is expressed in the large intestine, and also in the gallbladder. Otte et al.(2004) assessed the composite probiotic formula VSL#3™ and E.coli Nissle strain and were able to demonstrate an increase in MUC2, MUC3 and MUC5AC gene and protein expression. The components of VSL#3™ S. thermophilus and L.acidophilus have been shown to limit
chloride and water secretion and thereby to reverse the increase in entero-invasive \textit{E. coli}-induced chloride secretion by an epithelial cell line in mice (Resta-Lenert and Barrett 2003).

The effect of the probiotic formula VSL\textsuperscript{3} on the distribution of the protein zonula occludens-1 was assessed by Otte et al. (2004). Co-culture of epithelial cells with VSL\textsuperscript{3} probiotic bacteria and pathogenic \textit{S. dublin} prevented redistribution of zonula occludens-1 and stabilised the barrier function. \textit{L. acidophilus} has also been shown to protect cytoskeleton structure F-actin arrangement induced by pathogenic \textit{E. coli} (Otte and Podolsky 2004). Other probiotic organisms such as \textit{S. thermophilus} and \textit{L. acidophilus} have been shown to maintain or enhance the microfilament protein actinin, and the essential protein occludin, for cytoskeletal and tight junction protein structures in epithelial lines in the presence of pathogenic \textit{E. coli} (Liévin-Le Moal, Amsellem et al. 2002). \textit{E. coli} Nissle 1917 can counteract the disruptive effects of enteropathic \textit{E. coli} on T-84 epithelial cells monolayers. This effect is achieved by altering protein kinase-C signalling and increasing the redistribution and expression of zonula occludens-2, a crucial factor in maintaining epithelial tight junctions.

\textbf{2.4.3.8 Immune modulation by probiotics}

Another role of indigenous enteric microbes, including those commonly used as probiotic organisms, is vigilant priming of the innate immune system, thereby enabling a prompt response to pathogens (Clarke, Davis et al. 2010). Appropriate microbial colonisation of these microorganisms plays a key role in the development of GALT, a primary mechanism of defence against enteric pathogens. In the last seven years a number of groups have focussed on the relationship between early life events, the assembly of the GI microbiota and the maturation of the immune system (Cebra, Jiang et al. 2005; Rinne, Kalliomaki et al. 2005; Penders, Thijs et al. 2006; Amy and Philip 2009; Hill and Artis 2010). In addition, these studies have investigated whether manipulation of the microbiota during this key developmental stage can influence inflammatory disease outcome. These studies are included in Table 2.12. Intestinal colonisation with probiotic microorganisms, including \textit{Lactobacilli}, \textit{Bifidobacteria}, \textit{Streptococcus thermophilus} and the yeast \textit{Saccharomyces} (Bizzini and Fattal-German 1990), have been demonstrated to stimulate the production of effector molecules such as secretory IgA (Bakker-Zierikzee, Tol et al. 2006). It has also been reported that early stimulation of the immature immune system by a diversity of appropriate commensal microbes is fundamental to establishing and maintaining the essential balance between Th1, Th2 and Th17 cytokine T-cells (Dalmasso, Loubat et al. 2006; Dethlefsen, Eckburg et al. 2006; Strobel and Mowat 2006; Collado, Isolauri et al. 2009; Tomas 2009).
It is thought that there are likely to be intrinsic differences in how epithelial cells sense commensal or probiotic bacteria as opposed to pathogenic bacteria at the level of signal transduction pathways and cytokine production (Ng, Hart et al. 2009). The formula VSL#3™ was employed to test this hypothesis and the results showed that the bacteria in VSL#3™ did not induce IL-8 secretion by epithelial cells, whereas the enteropathogens *E. coli, Salmonella dublin, Shigella dysenteriae,* and *Listeria monocytogenes* all induce IL-8 secretion (Lammers, Helwig et al. 2002; Otte and Podolsky 2004).

Interestingly, the probiotic *E. coli* Nissle 1917 induced IL-8 secretion by intestinal epithelial cell lines in a dose dependent manner, suggesting that the ability to secrete IL-8 from epithelial cells is not a feature of all probiotic bacteria. One other effect of probiotic bacteria on epithelial cells is the ability of commensal organisms to act through pattern-recognition molecules (PRR) or Toll-like receptors (TLR), such as TLR-2 and TLR-4 possibly on epithelial cells. The interaction between probiotic bacteria, PRRs, TLRs and epithelial cells produces protective cytokines that enhance epithelial cell regeneration and inhibit epithelial cell apoptosis (Rakoff-Nahoum and Medzhitov 2008). Prevention of drug-induced colitis in TLR-4 mutant mice by *L. casei* has been demonstrated (Chung, Choi et al. 2008). Administering *L. casei* to mice inhibited myeloperoxidase activity and IL-12p40, and increased TGF-β and IL-10 mRNA. These effects suggest that the mechanism of action of *L. casei* depends largely on the TLR-4 status (Chung, Choi et al. 2008).

There appear to be two distinct pathways that allow epithelial cells to be able to distinguish commensal and probiotic organisms from pathogenic organisms. First, pathogenic bacteria induce a pro-inflammatory response in intestinal epithelial cells by activating the transcription factor NF-κB (Ng 2009). Conversely, probiotic and commensal bacteria attenuate pro-inflammatory responses, thereby blocking the degradation of the counter-regulatory factor 1κB. The method of blocking pro-inflammatory responses of pathogenic bacteria by non-pathogenic bacteria through attenuation of IL-8 secretion has been demonstrated by other groups who have found that co-administration of non-pathogenic *Salmonella pullorum* attenuated IL-8 secretion induced by *Salmonella typhimurium* (Neish, Gewirtz et al. 2000; Shibolet and Podolsky 2007). VSL#3™ probiotic bacteria produced soluble factors that inhibited chymotrypsin-like activity of the proteosome in intestinal epithelial cells, thereby inhibiting the NF-κB pathway and inducing expression of cytoprotective heat shock proteins (Petrof, Kojima et al. 2004). In addition, it has been reported that DNA derived from VSL#3™ delayed NF-κB activation, stabilised levels of 1 κB and inhibited proteasome function (Jijon, Backer et al. 2004). Cytokine-induced apoptosis was prevented in the presence of *Lactobacillus rhamnosus* GG. Culture of probiotic bacteria with
either mouse or human colon cells activated anti-apoptotic proteins and inhibited activation of the pro-apoptotic proteins by TNF-α, IL-1, or IFN-γ. Inhibition of apoptosis may enhance survival of intestinal cells and promote proliferation during recovery from epithelial injury (Neish, Gewirtz et al. 2000).

2.4.3.9 Effects of probiotic bacteria on dendritic cells

The role of dendritic cells is discussed in Appendix A1. To briefly recap, dendritic cells are antigen-presenting cells that are important in earliest bacterial recognition and in shaping the subsequent T-cell responses (Colonna, Pulendran et al. 2006). In the intestine, dendritic cells have specialised functions, contributing to oral tolerance induction by generating regulatory T-cells and IgA producing B-cells through production of cytokines such as IL-10 and TGF-β (Iwasaki and Kelsall 2000; Colonna, Pulendran et al. 2006; Iwasaki and Medzhitov 2010). Intestinal dendritic cells interact directly with luminal bacteria by passing their dendrites between epithelial tight junctions into the gut lumen (Rescigno 2010; Ng, Benjamin et al. 2011), and indirectly with bacteria that have gained access via M-cells (Stagg, Hart et al. 2003). The roles of the dendritic cells to recognise and respond to bacterial components, initiate primary immune responses and direct developing T- and B-cells place them at the interface between innate and adaptive immunity (Ng, Benjamin et al. 2011).

Given the pivotal role of dendritic cells, the effect of different probiotic therapies on their behaviour is clearly an important line of inquiry. Therefore, different experimental models have been employed to understand the potential effects of probiotics on dendritic cells, including both human and mouse models that assess whole blood dendritic cells, freshly isolated lamina propria dendritic cells, monocyte derived dendritic cells and bone marrow derived dendritic cells and their behaviour in response to probiotics (Ng, Hart et al. 2009).

VSL#3™ is a potent inducer of IL-10, as evidenced by both blood and lamina propria dendritic cells in vitro (Hart, Lammers et al. 2004). Ng and Plamondon et al. (2010) followed through with this line of inquiry by conducting a clinical study. They demonstrated that patients with ulcerative colitis had increased IL-10 and a reduced IL-12 p40 production by colonic dendritic cells after taking VSL#3™ (Ng, Plamondon et al. 2010). These effects were not seen in the placebo-treated control group. Dendritic cells were defined as a population of cells that were HLA-DR positive and negative for a set of lineage markers (CD3, CD14, CD16, CD19, CD34), thereby excluding T-cells, B-cells, macrophages, NK-cells, and myeloid progenitor cells.

Later studies have supported the findings that VSL#3™ can enhance levels of IL-10 and decrease TNF-α, IL-1, inducible nitric oxide synthase and matrix metalloproteinase in patients
with pouchitis (Ulisse, Gionchetti et al. 2001). Ng and Plamondon et al. (2010) concluded that the in vitro findings supported in part the findings of their clinical study, although the cellular source of the IL-10 was not known. In further support of VSL#3™ as a potent inducer of IL-10 by dendritic cells, it was demonstrated that bone marrow derived dendritic cells incubated with VSL#3™ increased IL-10 detected by enzyme linked immunosorbent assay (ELISA) (Drakes, Blanchard et al. 2004). Young and Simon et al. (2004) employed human cord blood monocytes, and the Bifidobacteria strains, B.longum, B.pseudocatenulatum, and B.breve. They found that the bifidobacteria strains had a distinct immuno-modulatory effect on dendritic cells by up-regulating IL-10 production. Two of these strains are constituents of VSL#3™. Incubated fresh lamina propria dendritic cells from mice with B.longum showed greater secretion of IL-10 than of IL-12. In another experiment, purified human monocytes and monocyte derived dendritic cells were stimulated with ultraviolet inactivated Gram-positive (L.plantarum and B.adolescentis) and Gram-negative (E.coli and Veillonella parvula) bacterial strains. B.adolescentis induced low amounts of IL-12, TNF-α, IL-6 and IL-8; L.reuteri and L.casei, but not L.plantarum, primed monocyte derived dendritic cells to drive the development of T-cells with regulatory properties. The regulatory T-cells produced increased levels of IL-10 and inhibited proliferation of bystander T-cells in an IL-10 dependent manner (Braat, van den Brande et al. 2004; Karlsson, Larsson et al. 2004).

It would appear that not all strains of bifidobacteria and lactobacilli induce anti-inflammatory effects. Mohamadzadeh and Olson et al. (2005) showed that when L.reuteri, L.gasseri and L.johnsonii were cultured with human monocyte derived dendritic cells; the production of IL12 was increased and induced T-cell priming. These varying effects of probiotic bacteria may be attributed to results that can occur when different experimental systems are applied in different animals. Another explanation that has been proposed is that different responses of different bacterial strains occur even within a genus. This later explanation was highlighted by Mohamadzadeh’s group who found L.plantarum and L.rhamnosus induced no or low levels of pro-inflammatory IL-12, whereas L.gasseri and L.johnsonii induced high levels (Mohamadzadeh, Olson et al. 2005). In another study, it was suggested that L.rhamnosus may ‘educate’ dendritic cells to stimulate proliferation of peripheral CD4+ T-cells and reduce CD3/CD28 stimulated cytokine production in vitro (Braat, van den Brande et al. 2004). L.rhamnosus resulted in a reduction of IL-4 from peripheral CD4+ T-cells of normal individuals and a decrease in IFN-γ and IL-2 production by CD4+ T-cells of normal cells from patients with Crohn’s disease. These studies support the interpretation that there is an anti-inflammatory action attributed to some probiotic strains
that, apart from influencing both Th1 and Th2 immune responses, also have an indirect effect via antigen presenting cells in the gut (Ng, Plamondon et al. 2010).

2.4.3.10 Effects of probiotic bacteria on monocytes and macrophages

Blood monocytes and tissue macrophages are secondary presenters of antigens to memory T-cells. The secretion and synthesis of IL-10 by macrophages taken from the inflamed colons of mice was increased by *L.plantarum* (Pathmakanthan, Li et al. 2004). In another mouse study, macrophage-like cell lines were used to assess the effect of the *bifidobacteria* strains *B.bifidum, B.breve, B.infantis and B.adolescentis* (He, Morita et al. 2002). *B.bifidum, B.breve* and *B.infantis* all stimulated more IL-10 and less IL-12 and TNF-α than *B.adolescentis*, once again highlighting the apparent strain specificity within a genus.

In addition to strain specificity is the question of species and strain viability. Rachmilewitz et al. (2004) found that the DNA derived from VSL#3™ could activate NF-κB and induce low levels of IL-6 and IL-12 in bone marrow derived macrophages compared to those induced by immuno-stimulatory oligonucleotides. The authors concluded that the protective effects of probiotics are mediated by their own DNA rather than by their metabolites or their ability to colonise the colon, and that Toll-like receptor 9 signalling is essential in mediating the anti-inflammatory effect of probiotics. Importantly, live microorganisms were not required to attenuate experimental colitis because non-viable probiotics are equally effective.

2.4.3.11 Effects of probiotic bacteria on B-lymphocytes

Probiotic bacteria may affect lymphocytes directly or secondarily via changes in stimulation induced by alterations in antigen presenting dendritic cells or macrophages. The effects of probiotics have been the subject of studies assessing different types of lymphocytes; these studies will be presented here. *L. Rhamnosus GG* administered to children with acute gastroenteritis enhanced a non-specific humoral response, as evidenced by an increase in IgG, IgA and IgM secretion from circulating lymphocytes (Kaila, Isolauri et al. 1992). Tejada-Simon et al. (1999) found by feeding mice yoghurts containing *L.acidophilus, L.bulgaricus, S.thermophilus, B.bifidum, and B.infantis* that secretory IgA (sIgA) production was enhanced in response to the cholera toxin, compared to the production of sIgA levels produced by cholera toxin itself. The effect of probiotics on B lymphocytes and antibody production have been assessed in vaccination trials (Isolauri, Joensuu et al. 1995). Isolauri et al. (1995) administered *L.casei* to children receiving a rotovirus vaccine and found the immunogenicity of the rotovirus vaccine was enhanced in the supplemented group compared to the placebo. In another vaccine trial *L.rhamnosus GG* enhanced *Salmonella* specific IgA antibodies in
subjects receiving a Salmonella vaccine compared to those who received the vaccine alone (Fang, Elina et al. 2000).

2.4.3.12 Effects of probiotic bacteria on natural killer cells
Synbiotic formulas are a combination of a prebiotic substance and probiotic bacteria. These combinations are thought to increase the efficacy of certain probiotic bacteria. The probiotic L. casei ssp. casei, when administered with the prebiotic substance dextran, significantly elevated natural killer cell (NK-cell) activity in spleen mononuclear cells from BALB/c mice. In addition, when the synbiotic was orally administered to healthy humans, an increase in NK-cell activities and increased production of IL-12 was found in peripheral blood mononuclear cells (Ogawa, Asai et al. 2006). Takeda et al. (2006) found that L. casei Shirota can also enhance NK-cell activity in humans.

2.4.3.13 Effects of probiotic bacteria on T-cells
The type of T-cell response, whether it be a Th1, Th2 or Th3/Tr1 response, is controlled predominantly by interactions between dendritic cells and T-cells (Ng, Hart et al. 2009). VSL#3™ potently induced IL-10 by DCs (Hart, Stagg et al. 2002). Furthermore, co-culture of naïve T-cells with the probiotic-treated DCs led to a decrease in Th1 polarised cells (Hart, Lammers et al. 2004). Braat et al. (2004) assessed the effect on T-cells by culturing monocyte derived DCs with L. rhamnosus. They found decreased T-cell proliferation and T-cell cytokine production, specifically of IL-2, IL-4, and IL-10. Similar results were found in an a clinical trial where healthy controls and Crohn’s disease patients were administered L. rhamnosus for two weeks. The results showed that ingestion of L. rhamnosus reduced interferon gamma and IL-2 production by peripheral T-cells in the Crohn’s disease patients and reduced IL-4 production in the control group (Braat, van den Brande et al. 2004). In an in vitro study by von der Weid et al.(2001), L. paracasei NCC2461 induced the development of a population of CD4+ T-cells with regulatory properties, i.e. they had a low proliferative capacity and produced TGF-α and IL-10. It has also been shown that probiotic bacteria also induce regulatory T-cells in the context of intestinal inflammation. In 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis (TNBS is the most common agent used to induce colitis in animal studies), VSL#3™ reduced intestinal inflammation. This reduction in inflammation was attributable to IL-10 dependent regulatory CD4+ T-cells bearing surface TGF-γ (Di Giacinto, Marinaro et al. 2005).

2.4.3.14 Effects of probiotic bacteria on T-cell redistribution
A more recent line of inquiry has explored the biological properties of the yeast S. boulardii in mice (Dalmasso, Cottrez et al. 2006a). Dalmasso (2006b) found that S. boulardii affected
immune cell redistribution by improving the competence of lymphatic endothelial cells to trap T-lymphocytes. In *S. boulardii*-fed mice, IFN-γ production by CD4+ T-cells was reduced in the colon but increased in the mesenteric lymph nodes. The authors concluded that *S. boulardii* has a unique action on inflammation by a specific alteration of the migratory behaviour of T-cells causing accumulation of these cells in mesenteric lymph nodes.

### 2.4.4 Mechanism of probiotic action in inflammatory bowel disease

Studies exploring the mechanism of the action of probiotics in a number of clinical diseases have steadily increased over the last decade. A number of studies in (IBD) have shown that probiotics can induce regulatory cytokines, including IL-10 and TGF-β and suppress pro-inflammatory cytokines, such as TNF, in the mucosa of patients with Crohn’s disease and pouchitis (Ulisse, Gionchetti et al. 2001; Borruel, Carol et al. 2002; Pathmakanthan, Li et al. 2004). In another study VSL#3™ and corticosteroids induced IL-10 and down-regulated IL-12p40 production by the lamina propria DCs, suggesting similar cytokine profiles can be produced by either corticosteroids or the probiotic VSL#3™ (Ng, Plamondon et al. 2010).

*L. reuteri RC-14* and *L. rhamnosus GR1* supplemented yoghurt was administered to IBD patients for 30 days (Lorea Baroja, Kirjavainen et al. 2007). Interestingly, the proportion of putative regulatory CD4+ CD25 T-cells in peripheral blood increased significantly in IBD patients after 30 days but not in controls. The baseline proportions of TNF-α (+)/interleukin (IL-12 (+), monocytes and myeloid DCs decreased in both subject groups, but those of stimulated cells only in IBD patients. In addition, serum IL-12 concentrations and the proportion of IL-2 (+) and CD69 (+) T-cells from stimulated cells decreased in IBD patients. The increase in CD4(+), CD25(high) T-cells correlated with a decrease in the percentage of TNF-α or IL-12 producing monocytes and DCs. This study demonstrated that probiotic yoghurt intake was associated with significant anti-inflammatory effects that paralleled the expansion pool of putative T-regulatory cells in IBD patients, but with few effects in controls (Lorea Baroja, Kirjavainen et al. 2007; Ng, Hart et al. 2009).

Pathogenic enterobacteria *E. coli* has been associated with ulcerative colitis. MacFarlane and Furrie et al(2005) employed 16S rRNA technique to assess the faecal composition of the microbiota in patients with ulcerative colitis where they found a reduction in *Bifidobacteria.* MacFarlane et al.(2005) followed up their findings by administering a synbiotic consisting of *Bifidobacterium longum* and oligofructose enriched inulin for one month to 18 ulcerative colitis patients. The results demonstrated that the short term synbiotic treatment resulted in a significant increase in *Bifidobacteria* colonisation of the rectal mucosa and induced significant reductions in the expression of molecules that control inflammation in active
ulcerative colitis. In a separate experiment colonic biopsies from patients with ulcerative colitis were co-cultured for 24 hours with *B. longum*. The concentrations of TNF and IL-8 in supernatants of inflamed ulcerative colitis tissue co-cultured with *B. longum* were lower than in those cultured alone. The number of lamina propria mononuclear cells with NK-κB p65+ in co-cultured tissues was also reduced (Bai, Ouyang et al. 2006). *Lactobacilli* species was also found to be lower in ulcerative colitis patients than in healthy controls. In a compositional study *Lactobacilli* were found to be lower in the faeces of patients with active ulcerative colitis (Bullock, Booth et al. 2004). Borruel et al. (2002) found that the release of TNF-α by inflamed mucosa was significantly reduced by co-culture with *L. casei* or *L. bulgaris*, but not with *L. crispatus* and *E. coli*. No change was observed in the production of TNF-α between non-inflamed Crohn’s patients’ mucosa and control mucosa. It has been suggested that this study this study highlights the possibility that certain probiotic bacteria interact with immunocompetent cells at the mucosal interface and thus modulate local production of pro-inflammatory cytokines by inflamed tissue (Ng, Hart et al. 2009).

### 2.4.5 Mechanism of probiotics for extra-intestinal inflammation

The concept that the anti-inflammatory effects of probiotic bacteria could be systemic has attracted the attention of a number of groups. Rachmilewitz et al. (2004) observed beneficial effects after parenteral administration of inactivated and fractionated bacteria. Borruel et al. (2002) co-cultured either *L. casei* or *L. bulgaris* with mucosal explants from affected intestinal mucosa of Crohn’s disease patients and found that the inflammatory response induced by bacteria was reduced by the probiotics. This reduction in inflammation was evidenced by a significant reduction in the number of CD4-cells as well as TNF-α expression among intraepithelial lymphocytes, suggesting that the anti-inflammatory response was systemic. In another study, IL-10 knock-out mice were given subcutaneous *L. salivarius* for which the authors reported a non-specific anti-inflammatory effect. Faecal microflora remained unchanged following the injection, but TNF and IL-12 levels from splenocytes stimulated by *S. typhimurium* decreased and TGF-α levels were maintained, suggesting a mechanism of action distinct from colonic flora modulation (Sheil, McCarthy et al. 2004). Probiotics may not need to encounter the mucosal immune system directly to exert an effect. Parenteral administration of *L. salivarius* in IL-10 knock-out mice ameliorated the severity of colitis (Sheil, Shanahan et al. 2007). This beneficial effect had been demonstrated by the same group previously with oral administration of *L. salivarius*. In addition, this group showed that the probiotic effect is not disease-specific, with a similar beneficial effect demonstrated in collagen-induced murine arthritis.
2.4.6 Clinical outcome studies: indications for probiotic use

As presented above, numerous groups have explored potential mechanisms by which probiotic organisms affect biological processes associated with disease states. The most frequently scrutinised organisms are from the genera *Bifidobacterium* and *Lactobacillus*. To a lesser degree *Streptococcus thermophilus*, *Enterococcus faecium*, *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces boulardii* as single strains or as part of a multi-species probiotic formulation have been evaluated (Preidis and Versalovic 2009). Probiotic organisms have been assessed in human medicine for disorders of the oral cavity (Caglar, Cildir et al. 2006; Caglar, Kuscu et al. 2008), intestinal tract (Bezkorovainy 2001; Bohm and Kruis 2006), immune system (Tlaskalová-Hogenová, Stepánková et al. 2004), genitourinary tract (Colodner, Edelstein et al. 2003; Hoesl and Altwein 2005) and in the fields of neurology and psychiatry (Desbonnet, Garrett et al. 2008; Dinan and Cryan 2012). An overview of clinical trials and systematic reviews for both extra-intestinal and intestinal conditions published between 2006 and 2012 (Rauch and Lynch 2012) was presented in Table 2.12. To our knowledge, no clinical trials have been conducted assessing the effects of probiotic microorganisms in patients with CD. Therefore, in the following section a general review of the outcomes of clinical trials in gastroenterology will be the central focus. This is due to a number of shared or similar aberrations of immune function being characteristic of both CD and the disorders of the gastrointestinal tract discussed below and therefore may also apply to CD as well.

2.4.6.1 Probiotics for acute gastroenteritis

The rationale for using probiotics in infectious diarrhoea is that they act against enteric pathogens by competing for available nutrients and binding sites, making the gastrointestinal contents acid, producing a variety of chemicals and increasing specific and non-specific immune responses (Gismondo, Drago et al. 1999; Vanderhoof 2000; Goldin and Gorbach 2008). No serious adverse effects of probiotics have been suggested in non-immune compromised individuals, with rare occurrences of infection in patients with indwelling catheters and impaired immune systems (Sussman, Baron et al. 1986; Hata, Yoshida et al. 1988; Salminen, von Wright et al. 1998; Piarroux, Millon et al. 1999; Saxelin, Lassig et al. 2010).

Allen et al. (2004) conducted a systematic review that reported the combined outcome of interventions with *Lactobacillus* spp. *Streptococcus* spp. *E. faecium* and *Saccharomyces boulardii* in 1917 adults and children who had been suffering from diarrhoea for less than 14 days. The combined outcomes of these studies suggested a reduced risk of diarrhoea by day
three with a significant reduction in duration of 30.5 hours. However, Huang and Bousvaros et al. (2002) reported less convincing benefits when they systematically reviewed the combined efficacy of *Lactobacillus* spp., *Bifidobacterium* spp. *S.thermophilus*, *B.subtilis*, *E. faecium* and *S.boulardii* in children one to 60 months of age who had been suffering from diarrhoea for less than one week. The results of this meta-analysis indicated that in this age group of children a reduced duration of diarrhoea by 0.8 days was achieved in children taking the probiotics.

### 2.4.6.2 Probiotics for *C. difficile* antibiotic-induced diarrhoea

The rationale for the use of probiotics in antibiotic-induced *C.difficile* diarrhoea and colitis is based on experiments in murine models and clinical observations in humans that suggest the luminal contents provide constant antigenic stimulus for intestinal inflammation (Sartor 2004; Weese, Debra et al. 2007). A dysbiosis theory suggests that a breakdown in the balance between putative species of protective versus harmful intestinal bacteria leads to chronic intestinal inflammation (Tamboli, Caucheteux et al. 2003). The diversity and numbers of commensal bacteria are altered in IBD with notably reduced levels of *bifidobacterium* and *lactobacillus* species (Swidsinski, Ladhoff et al. 2002). Probiotics assist in re-establishing the antibiotic-disrupted intestinal microflora, enhancing immune response and clearing pathogens and their toxins from the host (Elmer, McFarland et al. 1999; McFarland 2006). Johnston et al. (2007) assessed publications that included the results of 1986 children under the age of 18 years who were receiving antibiotic therapy for any reason. A significant reduction in antibiotic-associated diarrhoea was found in patients administered probiotics. Another meta-analysis pooled the results of publications assessing probiotic intervention in 1214 antibiotic-treated children and adults and found a reduced risk of antibiotic-associated diarrhoea, accompanied by a reduced risk of the bacteria *Clostridium difficile* and antibiotic-associated yeast infection (D'Souza, Rajkumar et al. 2002).

### 2.4.6.3 Probiotics for ulcerative colitis and pouchitis

Several probiotic strains have been tested in controlled clinical trials with patients with IBD, including ulcerative colitis. Kruis and Fric et al. (2004) compared the efficacy in maintaining remission of the probiotic preparation *Escherichia coli* Nissle 1917 verse placebo and established mesalazine therapy in 327 patients with ulcerative colitis. The authors concluded that the probiotic *E.coli* Nissle 1917 shows efficacy and safety in maintaining remission equivalent to the gold standard mesalazine in patients with ulcerative colitis. Rembacken and Snelling et al. (1999a) found similar results, concluding that non-pathogenic *E.coli* was as efficacious as mesalazine in maintaining remission of ulcerative colitis.
In a randomised controlled trial, *Lactobacillus* GG was administered either alone or in combination with mesalazine or mesalazine alone to 187 patients with ulcerative colitis. *Lactobacillus* GG was found to be superior to treatment with mesalazine in prolonging relapse-free time (Zocco, dal Verme et al. 2006).

Tursi et al. (2010) assessed the effects of supplementing 144 patients affected by relapsing ulcerative colitis, who were already under treatment with 5-aminosalicylic acid (ASA) and/or immuno-suppressants, with VSL#3™ or placebo. They reported that VSL#3™ supplementation was safe and able to reduce ulcerative colitis disease assessment index scores in patients affected by relapsing mild to moderate ulcerative colitis who were under treatment with 5-ASA and/or immuno-suppressants. Moreover VSL#3™ reduced rectal bleeding and appeared to induce remission in relapsing ulcerative colitis patients who were under treatment with ASA and/or immuno-suppressants. Another study also demonstrated a positive outcome employing VSL#3™ in ulcerative colitis patients (Turcotte and Huynh 2011). They found that treatment with the probiotic VSL#3™ as an adjunctive therapy in relapsing mild to moderate ulcerative colitis significantly reduced ulcerative colitis disease activity.

Sood and Midha et al. (2009) conducted a multi-centre randomised double-blind, placebo-controlled, trial of the probiotic VSL#3™ for the treatment of mildly to moderately active ulcerative colitis in 147 active patients for twelve weeks. The VSL#3™ groups had significantly greater decreases in ulcerative colitis disease activity index and individual symptoms at weeks six and twelve when compared with the placebo group.

A prospective, one year, placebo-controlled, double-blind, study assessed the safety and efficacy of VSL#3™ on the induction and maintenance of remission of ulcerative colitis in 29 children with the active disease. The authors found that VSL#3™, when administered to children with ulcerative colitis, was safe and efficacious for maintaining disease remission (Miele 2009).

Pouchitis is a major long-term complication after ileal pouch anal anastomosis for ulcerative colitis. Broad spectrum antibiotics are the mainstay of treatment in this condition. Gionchetti and Rizzello et al. (2003) demonstrated that VSL#3™ was efficacious in preventing relapse of chronic pouchitis and in preventing pouchitis onset. While the study was small (n=23) other groups have also reported positive outcomes in preventing recurrent pouchitis. Mimura and Rizzello et al. (2004) evaluated the effectiveness of VSL3# in maintaining antibiotic-induced remission and quality of life (QOL) for one year in patients with a history of recurring pouchitis. The IBD questionnaire score remained high in the VSL#3™ group but deteriorated in the placebo group. The authors concluded that VSL#3™
is effective in maintaining antibiotic-introduced remission for at least a year in patients with recurrent or refractory pouchitis. This was associated with improved quality of life.

A review of VSL#3™ use in IBD was conducted by Chapman and Plosker et al. (2006). While the studies above were acknowledged as positive outcomes the authors of the review suggested larger, well designed, controlled confirmatory clinical trials were necessary to further define the value of VSL#3™ in the treatment of ulcerative colitis. However, it should be noted that the treatment guidelines from the United States of America and the United Kingdom list VSL#3™ as a therapeutic option for the prevention of pouchitis relapse.

2.4.6.4 Probiotics for Crohn’s disease

The outcomes of randomised placebo-controlled clinical trials assessing the efficacy of probiotics in Crohn’s disease patients are less positive for Lactobacillus rhamnosus GG (Prantera and Scribano 2002; Schultz, Linde et al. 2003) and Lactobacillus johnsonii (van Gossum 2000). A Cochrane review also reported no significant benefits from Lactobacillus GG ingestion in reducing relapse after surgically induced remission compared to placebo (Rolfe, Fortun et al. 2006a). Furthermore, both poor efficacy and adverse events were associated with Lactobacillus GG in children with Crohn’s disease. Lactobacillus species are not the only microorganism to have shown negative outcomes in studies. The results of a Cochrane review conducted by Rolfe et al. (2006b) reported no significant benefit of E. coli Nissle for reducing the risk of relapse compared to placebo or control. Differences were noted between the yeast probiotic Saccharomyces boulardii and probiotics but these were not statistically significant. These findings have been confirmed by a more recent systematic review of probiotic use in IBD, including Crohn’s disease (Jonkers, Penders et al. 2012). They concluded that while there are well designed randomised controlled trials supporting the application of IBD, they are still limited. They suggested the role of a number of probiotics in reducing relapse in ulcerative colitis and pouchitis are favourable, however, in keeping with the findings of Rolfe et al. (2006b), there was no evidence to support the use of probiotics in Crohn’s disease.

2.4.6.5 Probiotics for irritable bowel syndrome

Microbial manipulation could provide tangible benefits for the intestinal fermentation and gas production, intestinal motility patterns and anti-nociceptive or inflammatory effects that result from changes in the host associated microbiome thought to be associated with IBS.

Colonic fermentation results in the generation of variable gas volumes in the intestine. Not all gastrointestinal bacteria are gas producers, with some microbial species actually consuming gas, particularly hydrogen (Versalovic, Wilson et al. 2009). Symptoms of
abdominal pain, bloating and flatulence are commonly seen in patients with IBS. Versalovic and Wilson (2009) propose that administration of appropriate bacterial strains could reduce gas accumulation within the bowel in these patients and induce symptomatic improvement. A number of double-blind clinical trials testing this hypothesis have resulted in varying outcomes. A systematic review examining 20 randomised controlled trials conducted by McFarland et al. (2008) found that probiotic consumption was associated with an improvement in global IBS symptoms, especially a reduction in abdominal pain. However, in most studies both probiotic and placebo treatments decreased abdominal pain to some extent (McFarland and Elmer 1997). Such equivocal findings are common observations in trials of patients with IBS, who respond to placebo formulations at variable rates (Versalovic, Wilson et al. 2009).

Not all clinical trials assessing efficacy of probiotics in IBS have been without positive outcome. In general, more positive outcomes are reported with administration of *Bifidobacterium* strains. Guyonnet et al. (2007) conducted a placebo-controlled randomised controlled trial, administering a fermented drink containing *B. animalis* or placebo twice daily to 274 IBS patients. They reported significant improvements in health-related quality of life, bloating and stool frequency for those with less than three stools per week in the *B. animalis* DN-173010 supplemented group.

Whorwell et al. (2006) conducted a randomised placebo-controlled trial to assess the efficacy of administering *B. infantis* 35624 to 362 women with IBS. They reported a significant improvement in abdominal pain scores and overall composite scores which included bloating, bowel dysfunction, incomplete defecation, straining and flatulence after four weeks of taking *B. infantis* 35264. The responses of symptoms and cytokine ratios in 77 IBS patients was compared between ingestion of either *Lactobacillus salivarius* UCC4331 or *Bifidobacterium infantis* 35624 versus placebo for eight weeks (O'Mahony, McCarthy et al. 2005). A significantly greater reduction in total composite score and individual scores for pain/discomfort/ bloating/distension and bowel movement difficulty was reported in the *B. infantis* 35624 group. In addition, the improvement in the *B. infantis* group was associated with a normalisation of the ratio of an anti-inflammatory to pro-inflammatory cytokine.

The efficacy of *Bifidobacterium bifidum* MIMBb75 was investigated in 122 patients with IBS in a randomised placebo-controlled trial (Guglielmetti, Mora et al. 2011). *Bifidobacterium* MIMBb75 significantly improved IBS symptoms of pain/discomfort, distension/bloating, urgency and digestive disorder compared to placebo.

The efficacies of probiotic formulations for IBS that contain a number of species including *Bifidobacterium* have also been assessed. Kim et al. (2005) conducted a double-
blind placebo-controlled trial to evaluate VSL#3™ efficacy in improving colonic transit in 48 patients with IBS and significant bloating over three time points, baseline, week four and week eight. They found VSL#3™ significantly reduced flatulence scores and retarded colonic transit without altering bowel function in patients with IBS and bloating. However, an earlier study conducted by the same group found the administration of VSL#3™ resulted in no significant differences in mean gastrointestinal transit measures, bowel function scores or satisfactory global symptom relief (Kim, Camilleri et al. 2003).

Kajander et al. (2005) conducted a double-blind placebo controlled trial to investigate whether the administering a probiotic mixture containing *Lactobacillus rhamnosus* GG, *L.rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. *Shemanii* JS for six months was effective in alleviating IBS symptoms in 103 patients. They concluded that the probiotic formula was effective in alleviating total IBS symptom scores.

The overall results for single strain *Lactobacillus* strains appear less promising; however, some studies have reported positive outcomes. Gawronska et al. (2007) investigated the efficacy of *Lactobacillus* GG in 104 children with IBS by conducting a double-blind randomised controlled trial. The results suggested *Lactobacillus* GG was more likely than placebo to reduce pain compared to placebo, with the authors concluding that the probiotic moderately improved treatment success among children with IBS. In another double-blind randomised controlled study assessing the efficacy of *Lactobacillus* GG in the treatment of paediatric IBS conducted by Bausserman et al. (2005), 50 children with IBS were assigned to *Lactobacillus* GG or placebo for six weeks. The probiotic was not superior to placebo in the treatment of abdominal pain.

Another double blind placebo controlled trial conducted over six months assessed the short and long-term effects on clinical symptoms and quality of life in 54 IBS patients of administering *Lactobacillus reuteri* ATCC 55730 (Niv, Naftali et al. 2005). Both groups improved significantly in all the studied parameters, with no significant differences between groups. The authors concluded that a strong placebo effect and a lack of uniformity in the IBS population may have hindered a clearer demonstration of the effect. *Lactobacillus plantarum* has also been investigated for potential clinical benefits in 60 IBS patients, with positive results for overall symptom scores and flatulence scores. However, there was no significant difference between the probiotic and the placebo for individual scores in bloating or pain (Nobaek, Johansson et al. 2000).
2.4.7 Summary

As discussed above, a number of groups have contributed to our understanding regarding the mechanisms and clinical outcomes of probiotic use in gastrointestinal disorders. As presented in chapter 2.3, the composition of the gastrointestinal microflora in children with CD have been investigated (Sanz, Palma et al. 2011). In addition, *in vitro* studies exploring the potential of *Bifidobacteria* species to ameliorate the inflammatory profile characteristic of CD have been promising (Medina, De Palma et al. 2008; Laparra and Sanz 2010; Laparra, Olivares et al. 2012). However, to our knowledge no clinical studies employing either *Bifidobacteria* species or other common probiotic microorganisms in CD have been conducted.

2.5 The pathogenesis of Coeliac Disease

Our knowledge of CD pathogenesis has made considerable progress over the last decade. CD is currently considered the result of a complex interplay of intrinsic (genetic) and variable extrinsic (environmental) factors. A graphical representation of the interplay between gluten, the immune system and genetic predisposition is presented in Figure 2.8 (Maresca and Fantini 2010).

![Figure 2.8 The pathogenesis of Coeliac Disease (Maresca and Fantini 2010)]
2.5.1 The role of gluten

Gluten is responsible for inducing CD in the genetically predisposed individual, after the ingestion of the glutenous grains: wheat, rye and barley. Gluten (from the latin *gluten*, ‘glue’) is a protein composite found in foods processed from wheat and related grain species, including barley and rye. Gluten provides elasticity and the ‘fluffy’ texture characteristic of gluten-containing baked goods. Gluten is the composite of gliadin and glutenin which is co-joined with starch in the endosperm of various grass-related grains. The prolamin and glutelin from wheat (gliadin, which is alcohol soluble, and glutenin, which is only soluble in dilute acids or alkalis) and constitutes approximately 80% of the protein contained in wheat fruit.

Figure 2.9 graphically presents the sub-units of gliadins and glutenins. It has been shown that α-gliadins harbour several major epitopes involved in CD pathogenesis (Tye-Din, Stewart et al. 2010; Anderson and Tye-Din 2012). Gluten proteins are enriched in proline and glutamine and are poorly digested in the upper gastrointestinal tract. Undigested molecules of gliadin, such as the peptide from an α-gliadin fraction which is made up of 33 amino acids, are resistant to degradation by gastric, pancreatic and intestinal brush border membrane proteases in the human intestine, therefore they remain in the intestinal lumen after gluten ingestion (Green and Cellier 2007). These peptides pass through the epithelial barrier of the intestine, possibly during intestinal infections or when there is an increase in intestinal permeability, and interact with antigen-presenting cells in the lamina propria.

2.5.2 Mucosal immune responses in coeliac disease

Individuals with CD have an aberrant immune response to gliadin fractions, thus promoting an inflammatory reaction in the mucosa of the small intestine. This inflammation is characterised by infiltration of the lamina propria and the epithelium by chronic inflammatory
cells and small intestinal villous atrophy (Green and Cellier 2007). The inflammatory response is thought to be mediated by the activity of both the innate and adaptive immune systems (Green 2009).

First, gliadin reactive CD4+ T-cells in the lamina propria recognise gliadin peptides that are bound to the HLA class II molecules DQ2 or DQ8 on antigen-presenting cells (Tye-Din and Anderson 2008); T-cells subsequently produce pro-inflammatory cytokines, particularly interferon-γ (Tye-Din and Anderson 2008). Gliadin peptides are deaminated by the intestinal enzyme tissue transglutaminase, which further increases the peptides’ immunogenicity. The subsequent inflammatory cascade releases metallo-proteinases and other tissue-damaging mediators that indicate crypt hyperplasia and villous injury (Green 2009).

Secondly, gliadin peptides also activate an innate immune response in the intestinal epithelium that is characterised by increased expression of interleukin 15 by enterocytes, resulting in activation of intraepithelial lymphocytes expressing the activating receptor NK-G2D, a natural killer cell marker (Green and Cellier 2007). These activated cells become cytotoxic and kill enterocytes with surface expression of major histocompatibility-complex class 1 chain-related A, a cell surface antigen induced by stress, such as an infection (Pott, Mahlakõiv et al. 2011).

2.5.3 Genetics

The genetic influence in the pathogenesis of CD is evidenced by its familial occurrence (Bevan, Popat et al. 1999). For an individual to develop CD they must have the alleles that encode for HLA-DQ2 or HLA-DQ8 proteins. However, not all individuals carrying these alleles will develop CD, indicating that genetic predisposition alone is not sufficient for CD development. Furthermore, twin studies indicate that the contribution of HLA genes to CD is less than 50% (Bourgey, Calcagno et al. 2007).

Genetic studies implicating HLA-DQ2 and HLA-DQ8 have been an integral platform for scientists to understand the link between gluten peptides and CD4+ T-cells. In vitro studies using cultured T-cells have provided the principles that explain the interaction between gluten and the immune system. Furthermore, the results of these studies have provided a proof of concept for the development of immune modulating intervention trials in humans (Tye-Din, Anderson et al. 2010).

2.5.4 Environmental risk factors

CD results from an interaction between ingested gluten and immune, genetic and environmental factors. While gluten ingestion is an essential environmental factor in the
pathogenesis of CD, the roles of other environmental triggers have also been explored in this complex interplay. An association between a person’s season of birth (Ivarsson, Hernell et al. 2003a; Lewy, Meirson et al. 2009), mode of delivery (Decker, Engelmann et al. 2010), birth weight (Mårild, Stephansson et al. 2011), feeding practices (Szajewska and Chmielewska 2012) infection history (Collado, Donat et al. 2009a) have all been implicated as risk factors in CD development.

2.5.5 Season of birth

Season of birth has been demonstrated as a risk factor for CD (Ivarsson, Hernell et al. 2003b; Lewy, Meirson et al. 2009), particularly in boys diagnosed before the age of 15 years (Tanpowpong, Obuch et al. 2012). This finding is part of a new theoretical model that integrates potential environmental factors such as timing of gluten introduction, ultraviolet-B exposure, vitamin D status and acute viral gastrointestinal infections in early childhood. The model suggests that people born in summer months are more likely to be weaned in the winter months when there is a greater risk of infection, are exposed to less sunlight and are at the recommended age for the introduction of solids, including gluten. The CD populations that have contributed to the data presented in these findings have all resided in the northern hemisphere where the characteristics of the winter months are different from those of New South Wales, Australia (as represented in the data in the experimental chapters of this thesis). Daylight hours in winter are significantly shorter in Northern Europe and the USA, and the ultraviolet rays are also considerably lower than in the countries of the southern hemisphere, including Australia. Australians enjoy long daylight hours all year round with plenty of vitamin D provision from the sun, thus challenging the season of birth and vitamin D hypothesis. However, due to the higher rates of ultraviolet rays and skin-related cancers, Australians adhere to a sunscreen policy that has inadvertently increased the rate of vitamin D deficiency in children comparative to that of children in the northern hemisphere (Holick, Binkley et al. 2011). In addition, the prevalence of infection and vitamin D deficiency in winter months has found to be the same between hemispheres (de Gruijl and Pavel 2012). The timing of introduction of solids is similar in both hemispheres, further supporting the season of birth, vitamin D deficiency and month of birth hypothesis.

2.5.6 Breast-feeding and introduction of gluten

The effect of breast-feeding in protecting against the development of CD has been explored by a number of groups who have reported both positive and negative outcomes (Henriksson C 2012; Szajewska and Chmielewska 2012). Breast-feeding at the time when gluten is
introduced is the most significant variable in reducing the risk. Timing of gluten introduction may also be a factor in the development of CD. There is evidence to support the possibility that avoiding introducing gluten before four months of age and later than seven months is a risk factor in the development of CD (Henriksson C 2012; Szajewska and Chmielewska 2012). However, Szajewska (2012) cited other studies that stated that breast-feeding, regardless of the infant’s age, is protective against the development of CD.

2.5.7 Caesarean section

The mode of delivery and associated alterations in the development of enteric homeostasis during the neonatal period may influence the risk of CD. In a retrospective multi-centre case-controlled study of 1950 children attending gastroenterology outpatient clinics, information about their birth methods, gestational ages, post-natal complications and breast-feeding was analysed. The data was compared to data from children visiting clinics not specialising in gastroenterology. The rate of C-section delivery of children with Crohn’s disease or ulcerative colitis was reported to be similar to that of control subjects, but a significantly enhanced likelihood of being born by C-section delivery was found in children with CD compared with control subjects (Decker, Engelmann et al. 2010). However, this study did not distinguish between elective and emergency surgery and risk of developing CD. This distinction is important when exploring a microbial hypothesis and the risk of CD development. Children who are born by elective C-section surgery will not have been exposed to the flora of the birth canal, whereas children born by emergency C-section will have most likely made contact with their mothers’ birth canals.

Interestingly, later studies by Marild et al. (2011) found a positive association with elective, but not emergency, C-section delivery and later CD development, suggesting that the bacterial flora of the newborn may play a role in the development of CD. However, the authors cautioned against altering delivery guidelines as the risk of CD was not influenced by caesarean delivery per se. In the same study, when assessing for other pregnancy exposures a 21% increased risk of developing CD was found for children born with a low birth weight for gestational age.

2.5.8 Intestinal microflora and dysbiosis in the pathogenesis of CD

The role of intestinal dysbiosis in the pathogenesis of CD has been the subject of a number of studies that are presented in Chapter 2.3. The role of the intestinal immune system is reviewed in chapter 2.2. The association between these two chapters, i.e. the intestinal microbiota and the intestinal immune system, is graphically presented in Figure 2.10 and is taken with
permission from Sanz and Palma (2011). A commentary for this Figure is found beneath the figure legend below.

The mucous layer covering the epithelial cells contains binding sites for indigenous commensal microbes and provides a protective ‘coating’ to prevent contact between enteric pathogens (Cinova, De Palma et al. 2011). Vecchi et al. (1989) detected a glycocalyx/mucous layer with a unique carbohydrate structure in the jejunal biopsies of patients with inactive CD that could modify the specificity of bacterial adhesion. More recently Forsberg et al. (2004) reported similar findings that the small intestinal mucosa of patients with CD were strongly stained with UEA lectin (major epitope β-(1,2)-fucose) but not with PNA lectin (major epitope β-gal (1-3) gal NAc), which was characteristic of healthy controls. Forsberg et al. (2004) proposed that these findings could support a hypothesis that a particular glycosylation pattern in predisposed individuals favours harmful bacterial adhesion, which contributes to CD pathogenesis. Sanz et al. (2011) suggested another possible explanation for Forsberg’s findings, proposing that modifications in the composition of the intestinal microbiota lead to alterations in the glycosylation pattern that subsequently reduce its capacity to defend the mucous layer against infections and CD. The hypothesis that bacteria alter glycosylation patterns that predispose patients to CD is still largely speculative. This speculation is based on the findings of several experimental models in animals that have proved that the microbiota and commensal bacterium Bacteroides thetaiotaomicron modify the fucosylation or galactosylation pattern of the glycoconjugates in the small intestinal epithelium (Umesaki, Okada et al. 1995; Bry and Falk 1996; Freitas, Cayuela et al. 2001; Freitas, Axelsson et al. 2002).

The expression of mucins is also thought to play a role in susceptibility to microbial infections, and specific animal studies have demonstrated the role of MUC2 in host protection against pathogenic infections and preservation of the commensal microbiota (Bergstrom, Kissoon-Singh et al. 2010). Bergstrom and Kissoon-Singh (2010) conducted an animal study and observed that mucin secretion was markedly increased during infection compared to uninfected controls, suggesting that the host secretes increased mucin to flush pathogens from the mucosal surface. However, the mucin-2-infected knock-out mice exhibited rapid weight loss and commensal bacteria depletion, and suffered up to 90% mortality. Forsberg et al. (2004) also reported that there were significantly higher mRNA levels of mucin-2 in active CD but that they returned to normal in treated CD. The increased expression of MUC2 mRNA in Forsberg’s study was related to goblet cell metaplasia induced by high IFN-γ production. Furthermore, as pointed out Sanz and Palmas’ (2011) review, this increased
expression of mucin 2 could additionally be in response to a higher load of potentially pathogenic bacteria detected in the microbiota of CD patients (Nadal, Donant et al. 2007a; Sánchez, Donat et al. 2010).
Figure 2.10 Schematic representation of the pathogenic mechanism underlying CD, and key points of possible interaction with the microbiota and bifidobacteria (taken with permission from Sanz et al. 2011)

(1) Expression of mucin 2 (MUC2) is significantly increased, probably in response to high IFN-γ production by intraepithelial lymphocytes and secondarily to the overgrowth of potentially pathogenic bacteria. (2) Expression of β-defensins HD-5 and HD-6 by Paneth cells is increased in active CD patients. (3) In active CD patients, the glycosylation pattern of the mucus layer is also modified in active and non-active CD and these aspects can also be modulated by the microbiota and predispose to CD. (4) Expression of TLR4 is increased in CD patients and TLR signalling involved in response to commensals and pathogens; (5) TLR4 signalling can lead to activate interferon regulatory factor 3 (IRF3) or IRF7, leading to the production of type 1 IFNs that stimulate IFN-γ, already over-produced in the aberrant response to gluten. (6) CD patients present increased intestinal permeability due to alterations in distribution and expression of tight junction and tight junction-associated proteins. In vitro and in vivo studies in germ-free rats suggest that specific bifidobacteria strains could play a protective role in CD pathogenesis by (6) increasing TJ expression of intestinal epithelial cells and reducing para-cellular permeability, thus preventing/limiting gliadin translocation to the lamina propria and consequent inflammatory response, (7) by regulating the inflammatory effects of the altered microbiota via production of inflammatory cytokines (IL-10) and reduction of IFN-γ by (8) contributing to hydrolysing gliadin peptides and thus reducing their toxicity on epithelial cells: by increasing the number of goblet cells producing mucus and enhancing the production of inhibitors of metalloproteases (TIMP-1), which protect against tissue damage (Sanz, Palma et al. 2011).
The effects of gliadin, IFN-γ and different bacterial strains on goblet cell and mucus secretion have also been evaluated in germ-free rat intestinal loop models (Cinova, De Palma et al. 2011). Gliadin fragments alone or together with pro-inflammatory cytokine IFN-γ significantly decreased the number of goblet cells and led to massive mucus secretion, resulting in extensive damage to the small intestinal mucosa (Cinova, De Palma et al. 2011). In addition, the presence of enterobacteria, isolated from CD patients and used as examples of potential (E.coli CBL2) and pathogenic (Shigella CBD8) toxin, aggravated the adverse effects of CD triggers, contributed to reducing even more goblet cells and induced massive mucus secretion (Cinova, De Palma et al. 2011). The constitutive expression of β-defensins HD-5 and HD-6 in Paneth cells is known to be exceptionally high in the human small intestine (Wehkamp, Chu et al. 2006), producing an antimicrobial effect by creating a hostile environment that prevents overgrowth of bacteria and infections (Salzman, Ghosh et al. 2003). Paneth cell defensins are secreted in response to bacterial antigens, including lipo-polysaccharide from Gram-negative bacteria and muramyl dipeptide from Gram-positive bacteria, possibly as a result of activation of TLR (Vaishnava, Behrendt et al. 2008). These antimicrobial peptides also seem to contribute to shaping the composition of microbial species in the small intestine (Salzman, Hung et al. 2010). A temporarily increased expression of β-defensins by Paneth cells has also been observed in active CD but which returned to normal in response to a GFD (Forsberg, Fahlgren et al. 2004). This finding suggested that increased expression of β-defensins is a secondary consequence of the inflammatory intestinal milieu characteristic of the disease linked to pathogenic bacterial overgrowth (Sanz, Palma et al. 2011). Conversely, IBD patients with affected small intestines have a lowered production of β-defensins by Paneth cells (Wehkamp and Stange 2010). This lowered production is thought to be the reason that entero-adherent E.coli colonise their mucosa.

β-Defensins are inducible antimicrobial peptides, mainly produced by epithelial cells, and play a crucial role in the innate immune system (Zhao, Wang et al. 1996). Reduced diversity of β-Defensins genes has been reported in CD (Fernandez-Jimenez, Castellanos-Rubio et al. 2010). The authors suggested that these results indicate that increased copy numbers of β-Defensins genes could afford protection from CD by preventing bacterial infiltration and preserving gut epithelial integrity (Fernandez-Jimenez, Castellanos-Rubio et al. 2010). However, further studies have not yet been undertaken to prove this hypothesis.

Dysfunction of the intercellular junctions between epithelial cells with alterations in distribution and expression of tight junction and tight junction proteins leads to increased intestinal permeability and is characteristic of CD (Drago, El Asmar et al. 2006). The toxicity of gliadin has been attributed to direct damage of tight junctions by decreasing the tight-
junction-associated protein ZO-1 and the tight junction protein occludin, thereby contributing to disruption of gut barrier function (Pizzuti, Bortolami et al. 2004; Drago, El Asmar et al. 2006; Elli, Roncoroni et al. 2011). Cinova et al. (2011) found that the specific combination of gliadin, IFN-γ with E.coli CBL2, reduced ZO-1 levels in biopsies of intestinal tissues. In addition, fragmentation of TJ proteins and consequently translocation of gliadin fragments into the lamina propria were induced in the presence of Shigella CBD8.

The important role of TLRs in immune function in discriminating between pathogens and harmless microbes is reviewed in chapter 2.2. The possible role of TLRs in CD has also been evaluated in recent studies. Szebeni et al. (2007) assessed the duodenal mucosa of children with treated and untreated CD compared with controls for expression of TLR2 and TLR4 mRNA. They found that children with both treated and untreated CD had higher expression of both TLR2 and TLR4 mRNA than the control group. Westerholm et al. (2010) also found an increase in TLR4-positive cells in the duodenal mucosa of active CD patients, although the differences observed by Szebeni et al. (2007) were not supported in regard to expression of TLR4 mRNA or TLR-2 mRNA. The immune phenotype of peripheral blood cells from children with CD before and after dietary intervention showed that both pre- and post-treatment groups had a higher prevalence of TLR 2 and TLR-4 positive DCs and monocytes compared with controls; moreover, TLR-2-expressing DCs and monocytes remained abnormal after adherence to a GFD (Cseh, Vásárhelyi et al. 2011). Associations between TLR2 and TLR4 gene polymorphisms and CD have not been detected so far (Santin, Castellanos-Rubio et al. 2007; Fernandez-Jimenez, Castellanos-Rubio et al. 2010). However, this data does not necessarily correlate with differences in expression. While there is not a consistent body of evidence to link TLRs and CD (Thomas, Sapone et al. 2006), Sanz et al. (2011) suggested it could be hypothesised that the increase in TLR2 and TLR4 expression in CD mucosa could amplify signalling through interactions with intestinal bacterial antigens and the innate immune system and contribute to the pathogenesis of CD.

“Signalling through TLR4 upon interaction with LPS from Gram-negative bacteria can activate interferon regulatory factor 3 (IFR3) or IRF7, among others, which leads to the production of type 1 IFNs, such as IFN-α. This is turn stimulates IFN-γ synthesis, which is over-produced in the aberrant response to gluten” (Lee and Kim 2007).

The potential activation of the characteristic inflammatory responses of CD by intestinal dysbiosis was investigated by Medina et al. (2008). They found that the faeces of CD patients with altered microbiota was associated with significant increases in TNF-α production and CD86 expression in peripheral blood mononuclear cells, while the reduction in IL-10
production was comparable with that of controls. These findings could be interpreted to suggest that the pro-inflammatory properties of the intestinal microbiota of CD patients, together with gluten peptides, could contribute to the Th1-type cytokine profile characteristic of CD (Sanz, Palma et al. 2011). Th1-type inflammatory cytokines IL-12 or IFN-γ have also been induced in vitro by enterobacteria isolated from CD patients (De Palma, Cinova et al. 2010). These authors concluded that interactions between the enterobacteria E.coli CBL2 and pathogenic Shigella CBD8 with gliadin and IFN-γ favoured the immune features of CD. Furthermore, intestinal bacteria could be additional factors regulating the ability of monocytes recruited to the mucosa to respond to gliadins and IFN-γ in CD patients, thereby influencing the course of the disease (De Palma, Cinova et al. 2010). Studies in rat intestinal loops have supported these findings by showing that inoculating E.coli CBL2 into the loops, together with gliadin and IFN-γ, decreased monocyte chemotactic protein-1 (MCP-1) and TIMP metallopeptidase inhibitor 1 (TIMP-1) release, and increased vascular endothelial growth factor (VEGF). In particular, a decrease in TIMP-1 could lead to an increase in activity of the matrix metalloproteinases involved in degrading extracellular matrix and tissue damage. Strikingly, in this same study enterobacteria Shigella CBD8 caused such a remarkable stimulation of cytokine production that the investigators were unable to quantify the data. While these rat studies would suggest that intestinal microbiota play a role in CD pathogenesis by activating aberrant immunologic responses, no such human studies have been conducted to date. However, retrospective and prospective studies indicate an association between gastroenteritis episodes and rotovirus-positive serology (Ivarsson, Hernell et al. 2003b; Stene, Honeyman et al. 2006).

Viral infection can cause a Th1-dominant immune response with type 1 IFN and inflammatory cytokine response (Huber and Farrar 2011). Theoretically this could increase the reaction to gluten peptides or favour their translocation and recognition by immune cells. Both human and mice studies have shown that IFN-γ influences the course of CD4 (+) and CD8 (+) T-cells during antigen recognition and serves as a counter-regulator of Th2 responses, which may promote autoimmunity. The potential role of viruses as a trigger of CD has attracted less research attention than the role of bacteria. The expression of TLRs 3 and 7-9 and retinoic acid-inducible gene -1-like receptors, which recognise viral nucleic acids and are responsible for virus-induced type 1 IFN production, have not been studied yet and therefore no clear association with CD pathogenesis can be made (Szebeni, Veres et al. 2007).

In summary, based on the studies presented above, it is feasible that microbes could interact with gluten peptides with common molecular targets of the innate immune system. Furthermore, the innate immune system activates similar pathogenic pathways that damage
the intestinal mucosal barrier, promoting inflammation. It is feasible that this inflammatory response influences the expression of the HLA DQ2 and DQ8 genes implicated in CD.

2.6 Current and future therapies for Coeliac Disease

As stated previously, the current standard practice and most effective therapy for patients with CD is adherence to a strict GFD. Dietary treatment may limit nutritional variety (Shepherd and Gibson 2006), is costly, often difficult to maintain outside the home and can affect various aspects of quality of life (Schuppan, Junker et al. 2009). Treatment should only begin after a complete diagnostic evaluation, including serology and biopsy. The GFD should result in a prompt and often dramatic improvement in the gastrointestinal and extra-intestinal atypical symptoms of CD (Murray, Watson et al. 2004; Rubio-Tapia, Rahim et al. 2010). Children generally have a more rapid response to treatment and are more likely to achieve full recovery of the villous architecture (van Koppen, Schweizer et al. 2009). However, in adults, clinical symptom improvements may take up to twelve months and with few attaining complete normalisation of villous architecture (Lanzini, Lanzarotto et al. 2009; Rubio-Tapia, Rahim et al. 2010).

Nevertheless, a GFD remains the mainstay of CD treatment. Stuckey et al. (2009b) found that dietary compliance was enhanced by educating patients about both CD and the GFD and through support from peers and professionals. It is recommended that the education and support should be provided by a CD specialist team including the general practitioner, dietician and gastroenterologist (Gibson, Shepherd et al. 2012). Annual follow-up assessments to monitor both GFD compliance and the nutritional adequacy of the GFD is recommended (Gibson, Shepherd et al. 2012). In addition, a review of CD patients should include the assessment of serological evidence of CD, biochemistry, nutritional deficiencies and the histological progress. A review of any complications and associated medical conditions is also advised (Stuckey, Lowdon et al. 2009a). In addition to health care professionals’ supervision and guidance, peer group support and education through the CD associations improve GFD knowledge and compliance (Aiello, Marshall et al. 2005; Lee, Ng et al. 2012a).

2.6.1 The gluten protein and peptides

Gluten is composed of the water soluble peptide called glutenin and the alcohol soluble peptide prolamines. The prolamines that trigger CD reactions and related health disorders are wheat (*triticum aestivum*), barley (*hordeum vulgare*), rye (*secale cereal*) and oats (*avena*
sativa) and any of their hybrid grains from the diet. Proteins in these grains, and peptides derived from these proteins during digestion, initiate the previously described pathophysiological process in genetically predisposed individuals. Avenein antibodies follow the same pattern as gliadin antibodies but with a lower magnitude (Hollén, Hogberg et al. 2003).

2.6.2 Gluten sources

There are a limited number of gluten-containing grains, however, they are widely used in food preparation. The high use of glutenous grains with a dominance of wheat in the Western diet makes it difficult but feasible for patients with CD to avoid them. Grain sources of gluten and safe non-gluten grain, seed, nut and legume flour alternatives are presented in Table 2.13. A comprehensive list of food sources are presented in Appendix A3.

Table 2.13 Gluten-containing grains and safe alternatives

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<th>Gluten-containing grains</th>
<th>Safe alternatives</th>
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<td>Wheat and wheat strains:</td>
<td>Grain flours</td>
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<td>• Durum</td>
<td>• Rice</td>
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<td>• Kamut</td>
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<td>• Spelt</td>
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<td>• Einkorn</td>
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<td>• Emmer</td>
<td>• Fava beans</td>
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<td>Barley</td>
<td>• Peanuts</td>
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<td>Rye</td>
<td>• Soya</td>
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<tr>
<td>Triticale (wheat/rye cross)</td>
<td>Tuber flours</td>
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<td>Oats</td>
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2.6.3 Adjunctive dietary therapy

As discussed in Chapter 2.5, some patients with CD continue to experience functional gastrointestinal symptoms despite adherence to a GFD (Gibson and Shepherd 2010). Shepherd et al. (2010) identified that this group of CD patients often poorly absorb short
chain carbohydrates known as FODMAPs (fermentable oligo-, di- and mono-saccharides and polyols), this includes fructans, lactose (in hypolactasic individuals) and galactans. Furthermore, in another study, 74% of IBS patients reported symptomatic improvement when adhering to a FODMAP free diet (Shepherd, Parker et al. 2008). While these findings are encouraging and useful for CD patients with persistent functional gastrointestinal symptoms, further dietary restrictions can be pose both practical and psychological challenges. Therefore, potential non-dietary treatments continue to attract research attention. Currently, any other issues associated with CD are treated symptomatically.

2.6.4 Future therapies

As discussed, there is an obvious appeal in developing new non-dietary therapies for the prevention, management or cure of CD. Based on the advances in understanding the pathogenesis of CD, as discussed in Chapter 2.5, a number of targeted non-dietary therapies have been devised, some of which are currently under phase one and phase two clinical trials. These novel therapies can be sub-divided into three categories (Schuppan, Junker et al. 2009):

1. Intra-luminal interventions, such as the modification of wheat, the pre-treatment of flour with microbial-derived enzymes and oral enzyme therapy;
2. Trans-epithelial interventions that aim to regulate intestinal permeability; and
3. Sub-epithelial interventions that aim to induce oral tolerance, including ‘vaccination, helminth and bacteriotherapy’.

2.6.4.1 Genetically modified wheat

Wheat has been a traditional element of human nutrition, but evolutionary studies have revealed that certain coeliac-toxic varieties have evolved since the beginning of wheat domestication and agriculture. Today’s most common wheat breeds began to be developed 9000 years ago. During domestication, hybridisation between two varieties, namely Turgidum and Aegilops, produced a cold hardy wheat species encoding the immunogenic α-gliadin sequences. The hybridisation of these wheat species is thought to have made a significant impact on the incidence of CD (Pinier, Fuhrmann et al. 2010).

Several groups have identified and successfully removed a number of immunogenic T-cell epitopes from the immunogenic α-gliadin sequences, with the objective of reducing the toxicity of wheat for patients with CD (Molberg, Uhlen et al. 2005; van Herpen, Goryunova et al. 2006; Vincentini, Maialetti et al. 2007; van den Broeck, van Herpen et al. 2009; Mitea 2011). This is achieved by either selecting wheat variants from already existing varieties or creating new wheat variants through genetic modification (Mitea, Salentijn et al. 2010; Mitea 2011). This forms an important step in the development of strategies to modify gluten genes.
in wheat so that it becomes safe for CD patients. Human trials are required to validate the efficacy and safety of including these designer grains in the diet of CD patients. In addition, the acceptance by patients with CD of genetically modified grains into their diets needs to be considered with care.

2.6.4.2 Pre-treatment of flours
Certain lactobacilli and fungal proteases added to sourdough for fermentation are able to proteolyze the proline/glutamine rich gluten peptides and thus decrease immuno-toxicity (Minervini, De Angelis et al. 2010). Pre-treated sourdough-hydrolysed wheat bread has been trialled in five CD patients compared to six CD patients consuming standard glutinous wheat bread for 60 days (Greco, Gobbetti et al. 2011). The results of this study suggested that sourdough bread pre-treated with fungal and lactobacillus proteases did not produce toxicity in CD patients, as evidenced by absences of clinical complaints, of increase in tissue transglutaminase antibodies and of small intestinal histopathological changes reported on biopsy. While these results are encouraging, the powering of this study was too low to draw a conclusion regarding safety or long-term efficacy of this intervention. Larger scale studies are warranted (Di Cagno, De Angelis et al. 2004).

Safety concerns regarding microbial modification of flours have been raised, where treatment of flour with microbial transglutaminase (a non-toxic lysine methyl ester) increased rather than decreased the stimulation of gliadin-specific T-cell lines (Cabrera-Chávez, Rouzaud-Sández et al. 2008). These findings highlighted the need to address the possibility of inadvertently generating new antigenic epitopes. Removal of coeliac-toxic epitopes from wheat while not developing new antigenic epitopes in the process is only one aspect of ‘safe’ flour development. Future studies also need to pay attention to non-coeliac-toxic products that are cost effective and of adequate nutritional value, in addition to being accepted by CD patients (Schuppan, Junker et al. 2009).

2.6.4.3 Oral enzyme therapy
Proteins reaching the intestinal lumen are digested by gastric pepsin and pancreatic proteases and are further degraded by brush border enzymes to yield single amino acids, di-peptides, or tri-peptides that are transported across the epithelial layer. However, the immuno-dominant gliadin peptides, including the α-gliadin 33mer, cause them to be highly resistant to human digestive proteases in all individuals. Several groups over the last decade have extracted enzymes from microorganisms that are known to cleave these immuno-dominant protein regions that are responsible for triggering CD (Cornell, MacRae et al. 2005; Marti, Molberg et al. 2005; Stepniak, Spaenij-Dekking et al. 2006; Mitea, Havenaar et al. 2008). The extraction
of prolyl endopeptidases (PEP) from the microorganisms *Flavobacterium meningosepticum*, *Sphingomonas capsulate*, and *Myxococcus xanthus* have been trialled in CD patients with some encouraging but inconclusive findings. PEP from *Aspergillus niger* has also been investigated due to its greater resilience in an acidic pH and has been found to have a higher specificity than the aforementioned microorganisms to inactivate gluten epitopes (Stepniak, Spaenij-Dekking et al. 2006). Therefore, a combination of endoprotease B2/PEP of germinating barley combined with *A. niger* PEP are currently in Phase 1 clinical studies. While enzyme therapy is promising it is thought it will be unable to sufficiently degrade immunogenic epitopes of an estimated daily gluten intake of $\geq 13$ g. However, it is hoped it will eliminate the detrimental effect of inadvertently ingesting a few grams of gluten by patients with high gluten sensitivity of refractory CD type 1 (Stepniak, Spaenij-Dekking et al. 2006). Enzyme therapy would be greatly welcomed by this group of CD patients whose quality of life is significantly affected by the social implications of dietary restrictions.

### 2.6.4.4 Intestinal permeability and zonulin

Inhibiting the influx of gluten peptides into the sub-epithelial lamina propria, where the destructive adaptive T-cell response to gluten is triggered and maintained, is an important focus of CD research. Some groups have proposed that this objective can be achieved by reducing intestinal permeability in CD patients (Fasano, Not et al. 2000; Kelly, Green et al. 2009; Fasano 2011). It is understood that *Vibrio cholerae* secretes the toxin zonula occludens toxin (ZOT) that opens the intestinal epithelial tight junctions, thereby increasing intestinal permeability (Lu, Wang et al. 2000; Uzzau, Lu et al. 2001). Furthermore, it is known that the injured epithelia of patients with CD release a paracrine protein product called zonulin that acts similarly to ZOT (Fasano, Not et al. 2000). Therefore, a zonulin antagonist (AT-1001) was developed and underwent a phase two dose escalation study (Kelly, Green et al. 2009). The outcome of this specific study was disappointing. The hypothesis that a significant decrease in the recovery of lactulose would be observed in those receiving the zonulin antagonist compared to those administered a placebo was not proven. The recovery of lactulose (a large sugar molecule) in urine is considered a reliable measure of the degree of intestinal permeability (Norman, Pirlich et al. 2012). However, this study did demonstrate an improvement in symptoms, a less pronounced antibody response and lower urinary nitrate excretion, suggesting that further trials are required to understand these findings more fully.

### 2.6.4.5 Oral glucocorticosteroids

Glucocorticosteroids have been prescribed cautiously in CD due to long-term or frequent use being associated with the development of chronic respiratory disease, diabetes and
osteoporosis (Pinheiro, Ciconelli et al. 2010), which CD patients are already at a much elevated risk of developing (Buysschaert 2003; Kurppa, Collin et al. 2010). Recently a topically active glucocorticosteroid, Budesonide, with low oral bioavailability has been trialled in patients with non-refractory and refractory type 1 and type II CD (Ciacci, Maiuri et al. 2009). The results were positive for both sub-groups of CD patients without the side-effects of bone demineralisation, diabetes or increases in infection. The benefit of this anti-inflammatory agent is that systemic exposure is negligible, so making the common side-effects of glucocorticosteroids also negligible. Currently budesonide targets gastrointestinal disorders of the lower intestine. It has been suggested that a new budesonide formulation that targets the small intestine be developed and assessed for safety and efficacy in patients with CD (Sollid and Khosla 2011).

2.6.4.6 Gluten vaccination to induce oral tolerance

Our understanding of the epitopes of pathogenic CD4\(^{+}\) T-cells is based primarily on responses shown by intestinal T-cells in vitro to hydrolysates or polypeptides of gluten, the causative antigen (Tye-Din, Stewart et al. 2010). A protease-resistant 33-amino acid peptide from wheat \(\alpha\)-gliadin has been identified as the immuno-dominant antigen in CD. However, less is known about the spectrum of T-cell epitopes in rye and barley or the hierarchy of immuno-dominance and consistency of recognition of T-cell epitopes in vivo. Therefore, Tye-Din et al. (2010) induced polyclonal gluten-specific T-cells in the peripheral blood of CD patients by feeding them cereal and performed a comprehensive analysis of responses to all CD toxic prolamins, a class of plant storage protein. The peptides that stimulated T-cells were the same among patients who ate the same cereal, but were different after wheat, barley and rye ingestion. A sequence from \(\omega\)-gliadin (wheat) and C-hordein (barley), but not \(\alpha\)-gliadin, was immuno-dominant regardless of the grain consumed. Furthermore, T-cells specific for just three peptides accounted for the majority of gluten-specific T-cells. These findings show that pathogenic T-cells in CD show limited diversity. These findings were promising for the possibility of developing peptide-based therapeutics for this HLA-restricted disease.

Based on this knowledge, it was argued that the most attractive and causal treatment would be the restoration of oral tolerance to ingested gluten. That this is feasible is suggested by the observations that a) only one of 30 carriers of the major predisposition for CD (i.e. HLA-DQ2 or HLA-DQ8) will develop the condition in their life-time (Anderson 2008), and b) 20% of 61 subjects whose CD was diagnosed in childhood and who remained on a GFD for several years did not develop CD despite having resumed, in adolescence, a normal diet containing gluten for an average of 10 years (Matysiak-Budnik, Malamut et al. 2007).
Induction of tolerance has been attempted by intranasal administration of gliadin peptides in gliadin-sensitised Balb/c or transgenic DQ8 mice, resulting in a decreased T-cell proliferative response to gliadin and a decrease in the production of inflammatory cytokines (Rossi, Maurano et al. 2005; Senger, Maurano et al. 2005; Ciccocioppo, Rossi et al. 2008). Another strategy used selected immunogenic peptides derived from α-gliadin, ω1-gliadin and hordein, that account for 60% of the overall gluten T-cell response, to immunise gliadin-specific TCR/DQ2 transgenic mice via subcutaneous injections. This gluten vaccination suppressed CD4+ cells in response to a gluten challenge (Keech, Dromey et al. 2009). A large human trial, a coordinated effort of scientists throughout the world, is currently under way at the Walter and Eliza Hall Institute Melbourne (Anderson and Tye-Din 2012).

2.6.4.7 Helminth therapy
An Australian group has conducted a phase one clinical trial in patients with CD using non-infectious larvae from Nector americanus, commonly known as the hookworm (Daveson, Jones et al. 2011). The study hoped to show a similar effect to that of Trichuris suis helminth therapy that had been trialled in IBD patients and demonstrated to successfully shift the pro-inflammatory Th1 T-cell response to a less aggressive Th2 or suppressive T-reg response (Summers, Elliott et al. 2003; Kradin, Badizadegan et al. 2009). However, the outcome in CD participants who were administered hookworm larvae, while shown to be safe, was that no obvious benefits to CD pathology were able to be demonstrated. The hookworm therapy induced duodenal eosinophilia, a transiently painful enteritis in 5/10 participants, and the subsequent wheat challenge resulted in deterioration in a number of parameters (Daveson, Jones et al. 2011).

2.6.4.8 Bacteriotherapy/probiotic therapy
Several groups have sought to identify characteristics of the intestinal milieu of patients with CD, with the ultimate goal of understanding the roles played by specific components of the gut microbiota in CD (Collado, Calabuig et al. 2007b; Ester, Inmaculada et al. 2008; Collado, Donat et al. 2009a; De Palma, Nadal et al. 2010; Laparra, Oliviares et al. 2012). The results of these studies are presented in Chapter 2.3. Briefly, the collective results of these findings demonstrated that compared to healthy paediatric controls, children with CD had lower diversity of Bifidobacteria sp. and higher counts of enterobacteria (Sanz, Palma et al. 2011). This bacterial distribution produced a similar cytokine profile, i.e. increased IFN-γ, TNF-α, and IL-12, to that known to be induced by gliadin in CD patients. These findings have led to in vitro investigations of possible benefits of potentially probiotic strains in this disorder.
Medina et al. (2008) found that *Bifidobacterium* genus *B. longum* CECT 7347 (IAT-ES1) and *B. bifidum* CECT 7365 (IATA-ES2) afford protection against the inflammatory response and mucosal damage caused by gliadin peptides both *in vitro* and in the intestinal loops of germ-free rats. The *in vitro* studies indicated that both *Bifidobacteria* strains could exert immuno-regulatory effects and counteract the inflammatory effects of the altered microbiota in CD patients by reducing inflammatory cytokines IFN-γ and TNF-α and increasing IL-10 in peripheral blood mononuclear cells *in vitro* (Medina, De Palma et al. 2008). In another rat model study *B. bifidum* CECT 7365 exerted protective effects by increasing the number of goblet cells in the small intestine, which were reduced in the presence of gliadin, IFN-γ, and enterobacteria (Cinova, De Palma et al. 2011). As well, adding *B. bifidum* CECT 765 with IFN-γ and gliadin up-regulated zonulin-1 (a protein that modulates the permeability of tight junctions) expression, which was down-regulated by CD triggers (Cinova, De Palma et al. 2011). More recently, Laparra et al. (2012) also demonstrated that *B. longum* reduced gliadin-induced Th1-type cytokines. *B. longum* also reversed the gliadin-induced tight junction protein ZO-1 expression. In relation to cytokine production *B. bifidum* CECT 7365 enhanced the production of chemotactic factors and inhibitors of metalloproteinases, which could contribute to gut mucosal protection.

Strains of different Lactobacillus species (*L. paracasei, L. fermentum* and *L. casei*) exerted inductive rather than suppressive effects on both innate and adaptive immunity in DQ8-transgenic mice, used as a model of intestinal gliadin hypersensitivity, characterised by a Th1-like phenotype (D’Arienzo, Maurano et al. 2009). In this context another study suggested that a *L. casei* strain could be exploited as a vaccine adjuvant to enhance both mucosal and systemic T-cell mediated responses to gluten (D’Arienzo, Maurano et al. 2008).

Hydrolysis of gliadin peptides is another potential effect exerted by *B. longum* CECT 7347 (Laparra and Sanz 2010). They showed that *B. longum* CECT 7347 contributed to hydrolysis of gliadin peptides during intestinal digestion by reducing the presence of toxic amino acid sequences and their adverse effects on Caco-2 cell cultures. The authors reported observing remarkable effects by *B. longum* CECT 7347 in reducing the cyto-toxicity of gliadin peptides and their ability to activate the NF-κB sub-units as well as TNF-α and II-1β production. A part of these findings were supported by an earlier study conducted by Lindfors et al. (2008) who demonstrated that *B. lactis*, but not *L. fermentum*, inhibited the gliadin-induced increase of epithelial permeability *in vitro*, modified the ZO-1 expression pattern and inhibited membrane ruffle formation induced by gliadins on Caco-2 cells. The results of these studies are encouraging for the development of probiotic strains aimed to reverse the inflammatory cytokine profile and intestinal permeability characteristic of CD. While a body
of in vitro and animal in vivo studies are convincing, to our knowledge no human clinical trials have yet been conducted.

This literature review was initiated due to a general interest in the association between the gastrointestinal microflora and the aberrations of immune function characterised by CD. The direction of the review explored whether there are predictable risk factors for CD and had alterations in gastrointestinal microflora been investigated in this population. In addition, the literature regarding probiotics interventions in gastrointestinal disorders including CD was explored.

At the conclusion of this review, three hypotheses were formed to investigate areas of the literature that had not been reported or were considered incomplete at this time. Therefore the following three hypotheses were formed and research projects devised:

- A survey: Individuals with CD would report a higher incidence of factors known to alter gastrointestinal microbiota during infancy and childhood including: C-section birth, low rates of breastfeeding, shorter duration of breastfeeding, feeding with cow’s milk in the first year of life, recurrent infection and frequent antibiotic use, compared to the general population. This hypothesis was based on the premise that these influences may alter gut-associated immune responses to gluten in individuals genetically predispose to CD.

- A comparative study: Australian adults with CD have a different microbiome from Australian adults without CD.

- A clinical trial: Incomplete resolution of symptoms in some individuals with CD, despite their adherence to a GFD, is attributed to aberrations in the intestinal microflora, and that manipulation of the gastrointestinal microflora with probiotics would result in symptom resolution and an improved quality of life.
3 A Survey Exploring the Incidence of Early Childhood Health Events and the Clinical and Social Management of Individuals with Coeliac Disease

3.1 Introduction

CD is a chronic enteropathy induced by dietary gluten in genetically predisposed individuals. The keystone of CD pathogenesis is an adaptive immune response orchestrated by the interplay between gluten and major histocompatibility complex (MHC) class II HLA-DQ2 and DQ8 molecules. This genetic predisposition, however, cannot be solely responsible for the disease aetiology, as the lack of complete genetic penetrance must be accounted for by additional factors that contribute to disease aetiology.

A more complex picture of the disease aetiology is currently emerging as risk factors are identified. The risk factors that have been associated with CD include caesarean birth (Decker, Engelmann et al. 2010), infant feeding practices (Szajewska and Chmielewska 2012), rotaviral infection in early childhood (Stene, Honeyman et al. 2006), and month of birth (Ivarsson, Hernell et al. 2003a).

Potential risk factors that have not been explored include pre-natal and peri-natal events that have been shown to contribute to establishing the intestinal microflora (Rigon, Vallone et al. 2012). To our knowledge, the factors that shape intestinal microbiota, such as frequency of maternal infection and maternal antibiotic use, cow’s milk in the first year of life, early childhood respiratory infection and exposure to antibiotics, have not been explored as potential risk factors in patients with CD.

3.2 Research aim

Our primary aim was to identify risk factors associated with the development of CD that are known to alter gastrointestinal microflora. Our additional aim was to gather information regarding the experience of medical, dietetic and social management of individuals with CD in Australia.
3.3 Hypothesis

It was hypothesised that individuals with CD would report a higher incidence of factors known to alter gastrointestinal microbiota during infancy and childhood, including C-section birth, low rates of breast-feeding, a shorter duration of breast-feeding, feeding with cow’s milk in the first year of life and recurrent infection and frequent antibiotic use, compared to the general population.

The hypotheses are based on the premise that these influences may alter gut-associated immune responses to gluten in individuals genetically predisposed to CD.

3.4 Materials and methods

3.4.1 Survey design

A comprehensive survey instrument was developed for online delivery using the professional version of the www.surveymonkey.com platform. The survey contained six sections including demographics, early childhood health, diagnostic journey, medical management, social implications and diet. The chart of symptoms and co-morbidities listed in the questionnaire was based on findings from the literature review (Lohi 2010). A pilot draft of the survey was administered to six staff members of the New South Wales Coeliac Association. Revisions were made accordingly and the survey questionnaire was considered validated and is presented in Appendix B1.

3.4.2 Participants

The NSW Coeliac Association is the leading representative organisation in the field of CD, educating and supporting health professionals and individuals diagnosed with CD and dermatitis herpetiformis in Australia. They agreed to distribute an email (Appendix B2) via their membership database which reached 4076 individuals. The email contained a link to the online survey. From 4076, a total of 1500 surveys were returned.

3.4.3 Survey participant procedure and response recording

The survey questionnaire was completed anonymously online by 1500 people. Completion of the survey was assumed as consent. Where family members were known to be under 18 years of age a parent or guardian was asked to complete the questionnaire on behalf of or with them. The survey was distributed on the 30\textsuperscript{th} January 2010 and closed on 1\textsuperscript{st} March 2010. Once participants had anonymously completed the survey questionnaire their answers were recorded and stored securely on a password-secure Survey Monkey database.
3.4.4 Statistical analysis
The responses recorded from completed surveys were exported from Survey Monkey to SPSS statistics software programme for analysis. Data was analysed using the PASW®Statistics GradPack. Descriptive statistics for the frequencies of demographic and health indices variables were conducted and reported as percentages. Analysis of demographic and health indices variables that may influence the degree of symptom improvement was conducted by cross-tabulations and chi-square analysis. Statistical significance was deemed to be at p≤0.05.

To analyse potential early childhood risk factors responses from participants who were 30 years of age or less were selected. This was due to a significant number of respondents in the older age groups not knowing their early childhood health history. In addition, the incidence for the proposed risk factors has increased in the last 30 years, which has coincided with a steady increase in the rate of CD diagnosis. Therefore, to answer our primary research question responses of participants from one to 30 years of age were analysed. The responses from this group were compared to the general population with a binomial test (where the general population data was available). To test the null hypothesis that CD patients were equally likely to have experienced certain early childhood health factors compared the general population, a binomial test was conducted. Significance was deemed to be at p≤0.05.

The ethics approval number for conducting this survey was granted by Southern Cross University Human Research Ethics Committee, approval number ECN-09-153 (Appendix B3).

3.5 Results of Survey

The results of the survey are presented throughout this chapter in Tables. Graphical representation of the data is presented in Appendix B4.

3.5.1 Demographics

All 1,500 participants returning the survey resided in the State of New South Wales. The response rate for the survey was 36%. Thirty surveys were not included in the analysis, as it was apparent the majority of questions were not understood. Therefore, 1470 formed the final data set. Where survey respondents were able to answer only some questions, the responses were included in the analysis and the number of responses stated in the appropriate column, which will vary due to this factor. The mean age of participants was 43.16 (±17.21) with a 3:1 distribution of females to males, as presented in Table 3.1. Distribution of genders and age group by decade is presented in Table 3.2.
Table 3.1 Gender and age of survey respondents

<table>
<thead>
<tr>
<th>Respondents n=1470</th>
<th>Male n (%)</th>
<th>Male Mean Age (SD)</th>
<th>Female n (%)</th>
<th>Female Mean Age (SD)</th>
<th>Age by Year Range of All Respondents</th>
<th>Mean Age of All Respondents (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>306 (20.8)</td>
<td>44.3 (19.5)</td>
<td>1164 (79.2)</td>
<td>43.1 (16.5)</td>
<td>2 to 82 years</td>
<td>43.1 (17.2)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Age groups by decade and gender

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Female n=1162 (%)</th>
<th>Male n=306 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 10 years</td>
<td>51 (4.4)</td>
<td>20 (6.5)</td>
</tr>
<tr>
<td>11 to 20 years</td>
<td>75 (6.4)</td>
<td>35 (11.4)</td>
</tr>
<tr>
<td>21 to 30 years</td>
<td>135 (11.6)</td>
<td>11 (3.6)</td>
</tr>
<tr>
<td>31 to 40 years</td>
<td>213 (18.3)</td>
<td>44 (14.4)</td>
</tr>
<tr>
<td>41 to 50 years</td>
<td>271 (23.3)</td>
<td>66 (21.6)</td>
</tr>
<tr>
<td>50 to 59 years</td>
<td>235 (20.2)</td>
<td>63 (20.6)</td>
</tr>
<tr>
<td>60 to 69 years</td>
<td>157 (13.5)</td>
<td>47 (15.4)</td>
</tr>
<tr>
<td>70 years and older</td>
<td>25 (2.1)</td>
<td>20 (6.5)</td>
</tr>
</tbody>
</table>

The results of descriptive statistics showed that 23.7% of participants had a relation with CD (Table 3.3). The family members reported and included in Table 3.3 were grandmother, grandfather, mother, father, son, daughter, brother, sister, aunty, uncle, niece and nephew.

Table 3.3 Number of family members with CD reported by survey respondents

<table>
<thead>
<tr>
<th>Number of Family Members</th>
<th>Number of Respondents n=558/1368 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 relative</td>
<td>348 (23.7)</td>
</tr>
<tr>
<td>2 relatives</td>
<td>132 (9)</td>
</tr>
<tr>
<td>3 relatives</td>
<td>39 (2.7)</td>
</tr>
<tr>
<td>4 relatives</td>
<td>21 (1.4)</td>
</tr>
<tr>
<td>5 or more relatives</td>
<td>18 (1.3)</td>
</tr>
</tbody>
</table>
Eighty-one percent of participants had lived in Australia all their lives, 10.6% had lived in Australia for more than 20 years and the remaining 8.3% had lived in Australia for less than 20 years (see Table 3.4). Diagnosis of CD had taken place in Australia for 97.6%, with 2.4% being diagnosed in the United Kingdom or New Zealand.

Table 3.4 Number of years resident in Australia

<table>
<thead>
<tr>
<th>Years Resident in Australia</th>
<th>n=1368 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2 years</td>
<td>12 (0.9)</td>
</tr>
<tr>
<td>3–10 years</td>
<td>49 (3.6)</td>
</tr>
<tr>
<td>11–20 years</td>
<td>53 (3.9)</td>
</tr>
<tr>
<td>21–30</td>
<td>40 (2.9)</td>
</tr>
<tr>
<td>31–40</td>
<td>105 (7.7)</td>
</tr>
<tr>
<td>All of their life</td>
<td>1109 (81)</td>
</tr>
</tbody>
</table>

A representative 1177/1470 respondents were categorised into decade of diagnosis showing that the majority of respondents had been diagnosed in the last 20 years (88.9%).

The month in which survey respondents were born was evenly distributed throughout the year for the total cohort (n=1470).

3.5.2 Early childhood health history and factors influencing gastrointestinal microflora

To investigate our hypothesis, responses regarding the incidence of early childhood health factors, including mothers’ peri-conceptual and pregnancy health, birth methods, infant feeding practices and early childhood infections were analysed. Data from respondents one to 30 years of age was chosen for our analysis of early childhood health factors. The results of this analysis commence in Table 3.5 and show that 9.3% of respondents’ mothers had an infection during pregnancy and 2.8% reported their mothers took antibiotics during pregnancy. There was no appropriate general population data to compare these two factors. Being born by a C-section delivery was reported in 17.6% of the CD respondents which was significantly lower (p=000) than that reported by Stavrou et al. (2011) for the general population (Table 3.5). As presented in Table 3.5 there was a highly significant difference (p=0.000) between the number of survey respondents who had been breastfed for any period of time (86.9%) and the general population (92%). The duration of breastfeeding is presented in Table 3.6.
Table 3.5 Incidence of early childhood factors that shape intestinal microflora in respondents 1 to 30 years of age

<table>
<thead>
<tr>
<th>Early Childhood Health Factor</th>
<th>Number of Valid Responses Out of 327</th>
<th>Yes n (%)</th>
<th>No n (%)</th>
<th>General Population n (%)</th>
<th>Binomial Test One-tailed Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal infection</td>
<td>205</td>
<td>19 (9.3)</td>
<td>186 (91.6)</td>
<td>NDA</td>
<td></td>
</tr>
<tr>
<td>Maternal antibiotic use</td>
<td>180</td>
<td>5 (2.8)</td>
<td>175 (97.2)</td>
<td>NDA</td>
<td></td>
</tr>
<tr>
<td>Caesarean birth</td>
<td>289</td>
<td>51 (17.6)</td>
<td>238 (82.4)</td>
<td>100 (30) (Stavrou, Ford et al. 2011)</td>
<td>p=0.107</td>
</tr>
<tr>
<td>Breast-fed for any period of time at all</td>
<td>289</td>
<td>251 (86.9)</td>
<td>38 (13.1)</td>
<td>3000 (92) (Baxter 2008)</td>
<td>p=0.000</td>
</tr>
</tbody>
</table>

The duration of breast-feeding periods reported by CD respondents are presented in Table 3.6, and show significantly shorter (p=0.000) breast-feeding periods than those reported by Baxter et al. (2008) for the general population.

Table 3.6 Duration of breast-feeding in respondents 1 to 30 years of age

<table>
<thead>
<tr>
<th>Duration of Breast-feeding</th>
<th>CD Survey Respondents n=207 (%)</th>
<th>General Population n=3000 (%) (Baxter 2008)</th>
<th>Binomial Test One-tailed Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast-fed &lt; 3 months</td>
<td>54 (23.8)</td>
<td>1680 (56)</td>
<td>p=0.000</td>
</tr>
<tr>
<td>Breast-fed &lt; 6 months</td>
<td>32 (14.1)</td>
<td>840 (28)</td>
<td>p=0.000</td>
</tr>
<tr>
<td>Breast-fed &lt; 12 months</td>
<td>79 (34.8)</td>
<td>840 (28)</td>
<td>p=0.000</td>
</tr>
<tr>
<td>Breast-fed &gt; 12 months</td>
<td>52 (22.9)</td>
<td>840 (28)</td>
<td>p=0.003</td>
</tr>
</tbody>
</table>

Tables 3.7 to 3.9 report the responses of survey participants to questions regarding antibiotic use in the first six years of life. Table 3.7 presents the number of participants reporting a history of antibiotic use in the first six years of life followed by the frequency of antibiotic use (Table 3.8), and the frequency of respiratory infections during respondents first six 6 years of...
life (Table 3.9). However, due to the lack of appropriate general population data we were unable to conduct a comparative analysis.

Table 3.7 History of antibiotic use during first 6 years of life in respondents 1 to 30 years of age

<table>
<thead>
<tr>
<th>History of Antibiotic Use in First 6 Years of Life</th>
<th>Number of Responses</th>
<th>Yes Antibiotic Use n (%)</th>
<th>No Antibiotic Use n (%)</th>
<th>General Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>168</td>
<td>108 (64.2)</td>
<td>60 (35.8)</td>
<td>NDA</td>
</tr>
</tbody>
</table>

Table 3.8 Frequency of antibiotic use by respondents 1 to 30 years of age during the first 6 years of life

<table>
<thead>
<tr>
<th>Frequency of Antibiotic Use</th>
<th>Number of Responses</th>
<th>Never n (%)</th>
<th>Once or Twice n (%)</th>
<th>Every Two Years n (%)</th>
<th>Yearly n (%)</th>
<th>More Than Once Year n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>258</td>
<td>24 (7.3)</td>
<td>94 (28.7)</td>
<td>30 (9.2)</td>
<td>52 (20.2)</td>
<td>58 (17.7)</td>
</tr>
</tbody>
</table>

Table 3.9 History of recurrent infection under 6 years of age in respondents 1 to 30 years of age

<table>
<thead>
<tr>
<th>Recurrent Respiratory Infections Under 6 Years Age</th>
<th>Number of Respondents</th>
<th>Yes Respiratory Infections n (%)</th>
<th>No Respiratory Infections n (%)</th>
<th>General Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>273</td>
<td>129 (47.2)</td>
<td>144 (52.8)</td>
<td>NDA</td>
</tr>
</tbody>
</table>

The results presented in Table 3.10 show that the number of respondents who were fed cow’s milk under the age of twelve months was significantly higher (p=0.05) than the number of infants fed cow’s milk in the general population, as reported by Binns et al. (2007).

Table 3.10 Milk introduced under 12 months of age in respondents 1 to 30 years old

<table>
<thead>
<tr>
<th>Type of Milk Given under 12 Months of Age</th>
<th>Breakdown of Milk Types n=124 (%)</th>
<th>General Population n=453 (%)</th>
<th>Binomial Test One-tailed Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow’s milk</td>
<td>102 (80)</td>
<td>178 (39.1)</td>
<td>(Binns, Graham et al. 2007)</td>
</tr>
<tr>
<td>Soy milk</td>
<td>18 (14.5)</td>
<td>7 (1.5)</td>
<td>(Binns, Graham et al. 2007)</td>
</tr>
<tr>
<td>Goat’s milk</td>
<td>6 (0.4)</td>
<td>NDA</td>
<td>NDA</td>
</tr>
</tbody>
</table>
3.5.3 Diagnostic journey

Responses from all age groups (n=1470) were included in the analysis of the diagnostic journey if the respondent had completed the question. Eighty percent of survey participants (n=1366/1470) had been diagnosed within the last decade. The time from experiencing symptoms until diagnosis was often delayed (mean 8.5 yrs; SD ± 17.2). Participants had been diagnosed by small bowel biopsy (92%) or by blood test (8%). Genetic testing had been undertaken in 13% of participants. Symptoms and co-morbidities reported at the time of diagnosis are presented in Table 3.11. This list was based on the symptoms and co-morbidities of CD reported in the literature (Lohi 2010).

Table 3.11 Prevalence of symptoms and co-morbidities at time of diagnosis of survey respondents

<table>
<thead>
<tr>
<th>Symptom at Diagnosis</th>
<th>n=1366 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatigue</td>
<td>885 (64.7)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>720 (52.7)</td>
</tr>
<tr>
<td>Bloating</td>
<td>690 (50.5)</td>
</tr>
<tr>
<td>Flatulence</td>
<td>594 (43.4)</td>
</tr>
<tr>
<td>Anaemia</td>
<td>574 (42.0)</td>
</tr>
<tr>
<td>Constipation</td>
<td>407 (29.7)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>375 (27.4)</td>
</tr>
<tr>
<td>Reflux and Indigestion</td>
<td>359 (26.3)</td>
</tr>
<tr>
<td>Headache</td>
<td>318 (23.2)</td>
</tr>
<tr>
<td>IBS</td>
<td>270 (20.0)</td>
</tr>
<tr>
<td>Weight gain</td>
<td>270 (20.0)</td>
</tr>
<tr>
<td>Anxiety</td>
<td>233 (17)</td>
</tr>
<tr>
<td>Depression</td>
<td>202 (14.8)</td>
</tr>
<tr>
<td>Chronic sinusitis</td>
<td>168 (12.2)</td>
</tr>
<tr>
<td>Migraine</td>
<td>151 (11)</td>
</tr>
<tr>
<td>Osteopaenia</td>
<td>129 (9.4)</td>
</tr>
<tr>
<td>Recurrent infection</td>
<td>114 (8.3)</td>
</tr>
<tr>
<td>Dermatitis herpetiformis</td>
<td>106 (7.7)</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>93 (6.8)</td>
</tr>
<tr>
<td>Autoimmune thyroid disease</td>
<td>65 (4.7)</td>
</tr>
<tr>
<td>Underactive thyroid</td>
<td>62 (4.5)</td>
</tr>
<tr>
<td>Colitis</td>
<td>50 (3.6)</td>
</tr>
<tr>
<td>Female infertility</td>
<td>46 (3.3)</td>
</tr>
<tr>
<td>Amenorrhoea</td>
<td>43 (3.1)</td>
</tr>
<tr>
<td>Recurrent Miscarriage</td>
<td>37 (2.7)</td>
</tr>
<tr>
<td>Delayed puberty</td>
<td>37 (2.7)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>37 (2.7)</td>
</tr>
<tr>
<td>Diabetes T1</td>
<td>35 (2.6)</td>
</tr>
<tr>
<td>Other symptoms</td>
<td>34 (2.4)</td>
</tr>
<tr>
<td>Overactive Thyroid</td>
<td>32 (2.3)</td>
</tr>
<tr>
<td>Asymptomatic incidental finding</td>
<td>28 (2.0)</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>8 (0.5)</td>
</tr>
<tr>
<td>Diabetes T2</td>
<td>8 (0.5)</td>
</tr>
<tr>
<td>Male infertility</td>
<td>7 (0.5)</td>
</tr>
<tr>
<td>Autism</td>
<td>1 (0.1)</td>
</tr>
</tbody>
</table>
The number of symptoms reported per survey participant is presented in Table 3.12, showing that 88.8% of survey respondents reported more than one symptom.

### Table 3.12 Number of symptoms or co-morbidities reported per survey respondent

<table>
<thead>
<tr>
<th>Number of Symptoms</th>
<th>n=1424 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>169 (11.2)</td>
</tr>
<tr>
<td>2</td>
<td>136 (9.6)</td>
</tr>
<tr>
<td>3</td>
<td>136 (9.6)</td>
</tr>
<tr>
<td>4</td>
<td>140 (9.8)</td>
</tr>
<tr>
<td>5</td>
<td>150 (10.5)</td>
</tr>
<tr>
<td>6</td>
<td>147 (10.3)</td>
</tr>
<tr>
<td>7</td>
<td>137 (10.3)</td>
</tr>
<tr>
<td>8</td>
<td>89 (6.3)</td>
</tr>
<tr>
<td>9</td>
<td>73 (5.1)</td>
</tr>
<tr>
<td>10</td>
<td>63 (4.4)</td>
</tr>
<tr>
<td>11 to 18 symptoms</td>
<td>184 (12.9)</td>
</tr>
</tbody>
</table>

#### 3.5.4 Symptom improvement after 12 months following a GFD and dietary compliance

We evaluated the incidence of symptom improvement after self-reported adherence to a GFD for twelve months or longer. Respondents who were diagnosed during or later than 2008 were excluded from the analysis to allow for the standard recovery time required to assess the efficacy of the GFD. This resulted in the analysis of 1054 respondents’ reports of symptom resolution since diagnosis and treatment. Complete symptom resolution was reported by 575 (53.6%), and 479 (44.7%) reported either none or only some improvement in their original presenting symptoms.

As presented in Table 3.13, there was no statistical difference between symptom improvement and the length of time since diagnosis and duration of treatment with a GFD for twelve months or longer (p>0.05). Table 3.13 presents the most prevalent symptoms reported by participants who reported that they had followed a GFD for twelve months or longer and the percentage of those symptoms within the total cohort. Dietary compliance was high with 90.1% reporting strict adherence to a GFD and 9.9% reporting dietary intake of gluten.
### Table 3.13 Symptom/condition reported as persistent 12 months after diagnosis and treatment with a GFD compared to the percentage of the total cohort (some participants reported more than one persistent symptom)

<table>
<thead>
<tr>
<th>Persistent Symptom or Condition</th>
<th>Respondents with persistent symptoms n=479 (%)</th>
<th>% of total cohort (of 1054 individuals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatigue</td>
<td>253 (52.8)</td>
<td>24</td>
</tr>
<tr>
<td>Flatulence and bloating</td>
<td>155 (32.4)</td>
<td>14.7</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>109 (22.8)</td>
<td>10.3</td>
</tr>
<tr>
<td>Constipation</td>
<td>69 (14.4)</td>
<td>6.5</td>
</tr>
<tr>
<td>Migraine and headaches</td>
<td>60 (12.5)</td>
<td>5.7</td>
</tr>
<tr>
<td>Reflux and indigestion</td>
<td>59 (12.3)</td>
<td>5.6</td>
</tr>
<tr>
<td>Anxiety and depression</td>
<td>47 (9.8)</td>
<td>4.5</td>
</tr>
</tbody>
</table>

### 3.5.5 Factors influencing persistent symptoms and symptom resolution

Cross-tabulations and chi-square analysis were conducted to explore the differences between degree of symptom resolution and the variables age, gender, family history of CD, month of birth, birth methods, breast-feeding, antibiotic use, time since diagnosis, symptom duration prior to diagnosis, medication use, complementary medicine use, food allergy other than gluten, and dietary compliance. Only the responses from participants diagnosed earlier than 2008 were included in the analysis (n=1054), to allow the effect of dietary treatment to be excluded. The p-values from the chi-square analysis are presented in Tables 3.14 to 3.20.

There were no differences between the responses of those individuals reporting only some degree of symptom resolution versus complete symptom resolution and the following variables: maternal antibiotic use (p=0.187), maternal infection (p=0.09), birth method (p=0.273), the month of participants’ births (p=0.442), being breast-fed (p=0.218), the duration of breast-feeding (p=0.880), having a family member with CD (p=0.703), antibiotic use under six years of age (p=0.702), frequency of antibiotic use under six years of age (p=1.00), receiving a dietician’s advice (p=0.442), dietary compliance (p=0.225), medication use (p=0.187).

As presented in Table 3.14 a significant difference between the males and females was observed, with a higher number of males (63%) reporting complete resolution of symptoms compared to females (52%) (p=0.02).
Table 3.14 Cross-tabulations with significant chi-square p-values for comparison of gender between those reporting complete symptom resolution and those reporting persistent symptoms (after 12 months adherence to a GFD)

<table>
<thead>
<tr>
<th>Degree of Symptom Improvement</th>
<th>Male n=223 (%)</th>
<th>Female n=831 (%)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some Improvement</td>
<td>83 (37.2)</td>
<td>396 (47.7)</td>
<td>p=0.003</td>
</tr>
<tr>
<td>Full resolution</td>
<td>140 (62.8)</td>
<td>435 (52.3)</td>
<td></td>
</tr>
</tbody>
</table>

As presented in Table 3.15, survey respondents reporting to have a diagnosed food allergy also reported a lower rate of complete symptom resolution than those without a known food allergy (p=0.000).

Table 3.15 Cross-tabulations with significant Chi-square p-values for comparison of food allergy incidence between those reporting complete symptom resolution and those reporting persistent symptoms after 12 months adherence to a GFD

<table>
<thead>
<tr>
<th>Degree of symptom improvement</th>
<th>Number of respondents</th>
<th>Known food allergy n (%)</th>
<th>No food allergy n (%)</th>
<th>Unsure n (%)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some improvement</td>
<td>467</td>
<td>140 (30)</td>
<td>210 (25)</td>
<td>117 (25.1)</td>
<td>p=0.000</td>
</tr>
<tr>
<td>Full resolution</td>
<td>564</td>
<td>116 (20.6)</td>
<td>402 (71.3)</td>
<td>46 (8.2)</td>
<td></td>
</tr>
</tbody>
</table>

As presented in Table 3.16, respondents who reported having recurrent respiratory infections in the first six years of life also reported a higher rate of partial symptom resolution compared to those who had complete resolution of symptoms (p=0.05).

Table 3.16 Cross-tabulations with significant Chi-square p-values for comparison of recurrent respiratory infections under 6 years of age between those reporting complete symptom resolution and those reporting persistent symptoms after 12 months adherence to a GFD

<table>
<thead>
<tr>
<th>Degree of Symptom Improvement</th>
<th>Number of Respondents n (%)</th>
<th>Infection under 6 Years of Age n (%)</th>
<th>No Infection under 6 Years of Age n (%)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some improvement</td>
<td>376</td>
<td>217 (57.7)</td>
<td>159 (42.3)</td>
<td>p=0.05</td>
</tr>
<tr>
<td>Full resolution</td>
<td>460</td>
<td>227 (49.3)</td>
<td>233 (50.7)</td>
<td></td>
</tr>
</tbody>
</table>
As presented in Table 3.17, the duration of symptoms reported by survey respondents leading up to the time of CD diagnosis was longer in those reporting persistent symptoms than in those with complete resolution (p=0.008).

Table 3.17 Cross-tabulations with significant Chi-square p-values for comparison of the duration of time a respondent experienced symptoms before diagnosis between those reporting complete symptom resolution and those reporting persistent symptoms after 12 months adherence to a GFD

<table>
<thead>
<tr>
<th>Degree of Symptom Improvement</th>
<th>Number of Respondents</th>
<th>Mean Duration of Symptoms Prior to Diagnosis Years (SD)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some improvement</td>
<td>581</td>
<td>9.9 (11.7)</td>
<td>p=0.008</td>
</tr>
<tr>
<td>Full resolution</td>
<td>634</td>
<td>7.7 (11.6)</td>
<td></td>
</tr>
</tbody>
</table>

The data collected regarding specific complementary medicines (CAM medications) participants took were difficult to code due the large variety of CAM medications reported, the lack of brand reporting and the broad variation in doses. The number of respondents out of 1470 survey respondents reporting the use of complementary medicine was 211 (14.4%). Among those reporting CAM use it was common for more than one type of CAM to be taken. The most frequently reported CAM were vitamins (multivitamins, B vitamin complex formulas and vitamin D), minerals (iron and calcium formulas), fish oils and probiotics. Respondents who reported taking CAM also reported a higher incidence of persistent symptoms compared to those with complete symptom resolution who reported less use of CAM (p=0.005).

Table 3.18 Cross-Tabulations with significant Chi-square p-values for comparison of the use of oral alternative and complementary medicines between those reporting complete symptom resolution and those reporting persistent symptoms after 12 months adherence to a GFD

<table>
<thead>
<tr>
<th>Degree of Symptom Improvement</th>
<th>Number of Respondents</th>
<th>Oral Complementary Medicine Usage n (%)</th>
<th>No Complementary Medicine Usage n (%)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some improvement</td>
<td>256</td>
<td>151 (59)</td>
<td>105 (41)</td>
<td>p=0.05</td>
</tr>
<tr>
<td>Full resolution</td>
<td>382</td>
<td>171 (44.8)</td>
<td>211(55.2)</td>
<td></td>
</tr>
</tbody>
</table>

As presented in Table 3.19, survey respondents under the age of 20 years reported a significantly higher rate of complete symptom resolution (p=0.03) and (p=0.05), than survey respondents in the older age groups.
Table 3.19 Symptom Improvement by age group

<table>
<thead>
<tr>
<th>Age Group</th>
<th>n</th>
<th>No Improvement n (%)</th>
<th>Some Improvement n (%)</th>
<th>Resolved n (%)</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–9 years</td>
<td>60</td>
<td>1 (1.6)</td>
<td>19 (31)</td>
<td>40 (66)</td>
<td>p=0.03</td>
</tr>
<tr>
<td>10–19 years</td>
<td>99</td>
<td>6 (6)</td>
<td>35 (35)</td>
<td>58 (58)</td>
<td>p=0.05</td>
</tr>
</tbody>
</table>

The results of descriptive statistics for medication use by survey respondents are presented in Table 3.20; showing the most frequently reported medicines taken by CD respondents. There was no statistically significant association between symptom resolution and proton pump inhibitors or any other medications listed.

Table 3.20 Medication use and symptom resolution

<table>
<thead>
<tr>
<th>Medication Name</th>
<th>Resolved Group n=682 (%)</th>
<th>Persistent Symptom Group n=651 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil medication</td>
<td>309 (45)</td>
<td>222 (34.1)</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>60 (8.79)</td>
<td>105 (16.1)</td>
</tr>
<tr>
<td>Oral contraceptive pill</td>
<td>53 (7.77)</td>
<td>66 (10.1)</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>45 (6.6)</td>
<td>71 (10.9)</td>
</tr>
<tr>
<td>Anti-depressants</td>
<td>45 (6.6)</td>
<td>67 (10.2)</td>
</tr>
<tr>
<td>Insulin</td>
<td>14 (2.0)</td>
<td>12 (1.8)</td>
</tr>
</tbody>
</table>

3.5.6 Experience of CD patients with Australian health care professionals

Survey participants had received dietary advice from gastroenterologists, general practitioners, dieticians and naturopaths. Table 3.21 presents descriptive statistics for the numbers of participants consulting health care professionals for professional advice, patients’ perception of health care professionals’ level of understanding of CD and reported usefulness of advice given. No data regarding the advice given by general practitioners and gastroenterologists was analysed as it was apparent the survey respondents had not understood the question.
Table 3.21 Australian health care professionals providing dietary advice to CD patients

<table>
<thead>
<tr>
<th>Professional Advice</th>
<th>Dietician n (%)</th>
<th>Naturopath n (%)</th>
<th>Gastro-enterologist n (%)</th>
<th>General Practitioner n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Offered dietary advice</td>
<td>1025 (69.7)</td>
<td>168 (11.4)</td>
<td>118 (8.9)</td>
<td>501 (34.1)</td>
</tr>
<tr>
<td>Gained a solid understanding of what they should eat</td>
<td>544 (53.3)</td>
<td>61 (36.3)</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Did not gain any further understanding of what they should eat</td>
<td>112 (10.9)</td>
<td>50 (29.7)</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Some of the dietary advice provided was helpful</td>
<td>364 (35.6)</td>
<td>57 (33.9)</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Health care professional was well informed about CD</td>
<td>704 (69.0)</td>
<td>97 (57.7)</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* The responses for these questions were not analysed as it apparent the survey respondents did not understand the question.

3.5.7 Non-adherence to a GFD

Adherence to a GFD was reported by the majority of survey participants (n=1181/1319, 89.5%). Intentional non-adherence to a GFD was reported by 138/1319 (10.5%). The reasons for non-adherence to the GFD are presented in Table 3.22. The highest reported reason for non-adherence to a GFD was a perception of it being a social inconvenience, closely followed by liking or craving glutenous foods and unavailability of gluten-free alternatives. Including gluten in the diet due to the apparent lack of health benefits from its exclusion was the least likely reason reported by participants reporting intentional gluten consumption. Adherence to a GFD was not significantly associated with symptom improvement.

Table 3.22 Reasons for not adhering to a GFD

<table>
<thead>
<tr>
<th>If you chose to eat glutenous foods what are your main reasons for doing so?</th>
<th>n=138 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I feel no health benefits from avoiding gluten</td>
<td>19 (13.7)</td>
</tr>
<tr>
<td>It is too hard to be gluten-free</td>
<td>58 (42.0)</td>
</tr>
<tr>
<td>I cannot find enough gluten-free alternatives</td>
<td>67 (48.5)</td>
</tr>
<tr>
<td>I crave them or simply like them</td>
<td>108 (78.2)</td>
</tr>
<tr>
<td>I eat them in company so I am not a social inconvenience</td>
<td>111 (80.4)</td>
</tr>
</tbody>
</table>

The common sources of gluten reported by those intentionally consuming gluten are presented in Table 3.23.
Table 3.23 Gluten-containing foods consumed by non-compliant participants

<table>
<thead>
<tr>
<th>If You Do Consume Glutinous Foods</th>
<th>Which Ones Do You Consume?</th>
<th>n=138 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confectionery</td>
<td>118 (85.5)</td>
<td></td>
</tr>
<tr>
<td>Sauces</td>
<td>86 (62.3)</td>
<td></td>
</tr>
<tr>
<td>Bread</td>
<td>51 (36.9)</td>
<td></td>
</tr>
<tr>
<td>Cake</td>
<td>46 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Beer</td>
<td>31 (22.4)</td>
<td></td>
</tr>
<tr>
<td>Pasta</td>
<td>17 (12.3)</td>
<td></td>
</tr>
<tr>
<td>Sweet Biscuits</td>
<td>1 (0.7)</td>
<td></td>
</tr>
</tbody>
</table>

3.5.8 Survey participants’ knowledge of gluten sources

Survey participants’ knowledge of gluten-free grain sources is presented in Table 3.24 below. Hybrid grains containing gluten were the least recognised as a source of gluten, with 29.2% of participants not electing spelt and 49.1% not electing triticale as sources of gluten. A comparison between knowledge of sources of glutinous grains and symptom improvement using cross-tabulations and chi-square analysis revealed no statistically significant relationship between them (p >0.05).

Table 3.24 Frequencies of knowledge of gluten-free grains

<table>
<thead>
<tr>
<th>Grains Thought to Be a Source of Gluten</th>
<th>Knew It Was a Source n (%)</th>
<th>Did not Know It Was a Source n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>1229 (95)</td>
<td>69 (5)</td>
</tr>
<tr>
<td>Rye</td>
<td>1251 (91.4)</td>
<td>117 (8.6)</td>
</tr>
<tr>
<td>Barley</td>
<td>1272 (93)</td>
<td>96 (7)</td>
</tr>
<tr>
<td>Oats</td>
<td>1282 (93)</td>
<td>86 (6.3)</td>
</tr>
<tr>
<td>Triticale</td>
<td>696 (50.9)</td>
<td>672 (49.1)</td>
</tr>
<tr>
<td>Spelt</td>
<td>968 (70.8)</td>
<td>400 (29.2)</td>
</tr>
</tbody>
</table>

3.5.9 Australian restaurants’ and food outlets’ knowledge of GFD

Statistical analysis of 1319 survey respondents regarding Australian restaurants and food outlets are presented in Tables 3.25 and 3.26, revealing that 77.6% (n=1024) of participants reported restaurant staff had some knowledge of GFDs, followed by 11.1% (n=146) reporting very knowledgeable restaurant staff and 8.5% (n=112) reporting no knowledge of GFDs among restaurant staff. The number of participants not attending restaurants was 2.8% (n=37). Most respondents found food outlets in general to be of some assistance, with 19.9% simply working out gluten-free options for themselves.
Table 3.25 Knowledge of gluten-free options by Australian restaurant staff, as reported by respondents

<table>
<thead>
<tr>
<th>Do You Find Restaurant Staff Knowledgeable about Gluten-free Food Options?</th>
<th>n=1319</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>146 (11.1)</td>
</tr>
<tr>
<td>No</td>
<td>112 (8.5%)</td>
</tr>
<tr>
<td>Some</td>
<td>1024 (77.6%)</td>
</tr>
<tr>
<td>Do not eat out</td>
<td>37 (2.8%)</td>
</tr>
</tbody>
</table>

Table 3.26 Assistance in food retail outlets

<table>
<thead>
<tr>
<th>Do You Find Food Outlets in General Cooperative about Informing You of Gluten-free Options?</th>
<th>n=1319 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>196 (14.8)</td>
</tr>
<tr>
<td>No</td>
<td>129 (9.7)</td>
</tr>
<tr>
<td>Some</td>
<td>731 (55.4)</td>
</tr>
<tr>
<td>I work it out for myself</td>
<td>263 (19.9)</td>
</tr>
</tbody>
</table>

Participants’ interest in receiving information about a clinical trial was high, with 50% requesting further information.

3.6 Discussion

3.6.1 Introduction

Our survey covered a number of demographic and health-related questions that were thought to be either related or potentially related to CD. Each category has been considered in varying depths in the published literature. The following discussion will address the major findings, specifically in relation to the categories of diagnostic journey, response to therapy and early childhood health influences, and examine how they compare to the findings of other groups studying potential risk factors in CD, or to general population data where available.

3.6.2 Diagnostic journey

The mean age at diagnosis was similar to reports from the United States (Lee, Ng et al. 2012b), the United Kingdom (Sanders, Hurlstone et al. 2001) and Europe (Lohi 2010), reflecting a greater number of individuals being diagnosed in adult life. The gender distribution of the survey respondents was 4:1 female to male which is higher than the estimated 3:1 ratio by a other surveys (Gray and Papanicolas 2010). The marked increase in
the number of individuals diagnosed in the last decade is also similar to reports from other nations (Lohi 2010; Violato, Gray et al. 2012). It is thought that this increased global prevalence has coincided with the development and adoption of more sensitive serological markers and an increased awareness of the clinical presentation of CD in most countries (Mustalahti, Catassi et al. 2010). Despite this increased awareness our findings demonstrate that an extended delay between presenting with symptoms and obtaining a formal diagnosis is common. The delay in receiving a CD diagnosis by Australians is longer compared to some other countries (Reilly and Green 2012; Violato, Gray et al. 2012) but very similar to other countries. This delay is possibly due to lack of knowledge regarding the ‘evolution’ of broader clinical manifestation in the adult population (Lohi 2010; Norstrom, Lindholm et al. 2011). Our findings regarding method of diagnosis are consistent with current standard practice in other countries (Lanzini, Lanzarotto et al. 2009).

The majority of participants in our study obtained their diagnoses of CD based on histological findings of a small bowel biopsy. Only a small number reported positive serology as the diagnostic endpoint. Our findings also suggest that coeliac genotyping plays only a small role in the diagnostic process. This is likely to be due to its poor predictive value for active disease and future disease development (Anderson 2008). However, its excellent negative predictive value is both highly sensitive and specific and is considered clinically helpful for those who are already following a GFD and those with a family history who want to exclude being at risk of developing CD (Anderson 2008). Our study has suggested that there is an ongoing need for educating Australian health care professionals about the broad clinical presentations of CD with a view to reducing the ongoing delay in diagnosis experienced by the majority of Australian adults.

3.6.3 The influence of family history

Our findings suggest that the numbers of individuals with a family history of CD is higher if both first and second degree relatives are included. Maki and Mustalahti (2003) reported an estimated 10% of CD patients will have a first degree relative with the condition. When we included a family history of second degree relatives the number reporting a known family member with CD increased to 23.7%. Furthermore, of those reporting a known relative with CD 38% reported having more than two relatives with the disease. While there are reports demonstrating an increased risk for distant relatives of families with two or more diagnosed CD members, having a more distant relative with CD has not been considered a risk factor (Book, Zone et al. 2003). Our findings have important clinical relevance for health care professionals taking a family history. We would conclude that due to the genetic inheritance
of this disorder a family history including second degree relatives may increase clinical evidential support for serological screening in both symptomatic and asymptomatic individuals.

3.6.4 Clinical presentation and symptom resolution after treatment

Our survey findings support the argument that the clinical presentation of CD has broadened. The ‘classical’ childhood diagnosis of failure to thrive, diarrhoea, vomiting and anaemia is less common in adults, and a greater number of individuals are diagnosed with CD during adulthood with mild or atypical gastrointestinal symptoms and/or extra-intestinal symptoms. While our data did not demonstrate this, it has been reported by others that there is a growing number of individuals who are asymptomatic at diagnosis (Nuti, Martini et al. 2001; Roma 2009). However, our findings are consistent with other groups, suggesting that in the last thirty years there has been a changing pattern in the clinical presentation of CD (Roma 2009). Lohi and Mustalahti (2007) attribute this to the common experience of a delayed diagnosis being associated with a broader clinical presentation in adulthood.

Our survey respondents reported an extensive list of symptoms and co-morbidities associated with CD at diagnosis. As reported in our results, fatigue, diarrhoea, bloating, flatulence, anaemia, headaches, anxiety, depression, reflux and constipation were most frequently reported at time of diagnosis. It is reasonable to expect that the malabsorption characteristic of CD will cause a number of macro- and micro-nutrient deficiencies, including iron deficiency anaemia, and as a consequence of these deficiencies and malabsorption patients will suffer from a broad clinical presentation. Conversely, it is also possible that some of the symptoms and co-morbidities listed could be distinct medical entities.

Interestingly, the most surprising finding from this survey was the high incidence of participants reporting only some symptom resolution after twelve months on a GFD. Green and Cellier (2007) suggested that up to 30% of CD individuals report having a poor response to dietary treatment; however, lack of dietary compliance is the most common reason for this poor response (Green and Cellier 2007). Adherence to a GFD is considered an effective treatment for symptom resolution and villous repair (McAllister and Kagnoff 2012). In regard to clinical improvements our findings would suggest otherwise. This is supported in part by other groups, who have found that regardless of clinical symptoms and patient perception of improvement it is rare for a patient with CD to have full recovery of the villous architecture following a GFD (Lanzini, Lanzarotto et al. 2009; Rubio-Tapia, Rahim et al. 2010). These investigators demonstrated that the degree of villous architecture improvement is not related to severity of symptoms. Furthermore, Selby and Painter (1999) reported that the persistent
mucosal abnormalities seen in patients with CD on a GFD are not due to the ingestion of trace amounts of gluten, suggesting co-existing food chemical sensitivities should be investigated. A limitation of our survey was not to have asked about follow-up small bowel biopsy results, as this would have assisted us in comparing symptom improvement ratings with follow-up histological findings. We do not know whether a dietary review and exclusion of other intestinal pathologies would have revealed the reason for such a significant number reporting only some improvement. In addition, the term ‘some improvement’ is subjective and our findings do not account for the individual variations within respondents’ reports. Should a survey such as this be re-administered, a Likert scale response rating within the categories of symptom resolution may reduce the ambiguity in interpretation.

Collectively, through both the work of others and these survey findings, it is evident that a significant proportion of people with CD report persistent symptoms and that villous atrophy is a poor indicator of symptom improvement and not necessarily indicative of poor dietary compliance.

3.6.5 Symptom resolution and demographic and health factors

The results of our survey indicated that a number of demographic and health factors were different among those reporting only some improvement. Our findings suggest that women are less likely to report complete symptom resolution than men; this is consistent with reports from other groups (Hallert, Midhagen et al. 2002; O'Leary, Wieneke et al. 2002). Recurrent respiratory infections in the first six years of life were reported at a higher rate in those reporting persistent symptoms compared to those reporting complete resolution (p=0.05). While a causative effect cannot be deduced from this finding a need for further attention to early childhood infection in the context of the intestinal microbiome hypothesis is suggested. Our findings support the assumption that a good response to dietary therapy would be more efficacious in younger patients who receive a prompt diagnosis than in older patients with a delayed diagnosis. Those under the age of 20 years reported a significantly higher rate of complete symptom resolution (p=0.05) than survey respondents in the older age groups. The importance of a prompt diagnosis to treatment outcome has been reported by others and our results further support this (Hershcovici, Leshno et al. 2010), indicating that the duration of symptoms reported by survey respondents before diagnosis was significantly longer in those reporting persistent symptoms (p=0.008).

Food allergies and food intolerances in addition to the aberrant immune response to gluten have been demonstrated by a number of groups. Gibson and Shepherd (2010) have shown that a significant number of patients with CD have functional gastrointestinal disorders.
in response to the ingestion of foods containing rapidly fermenting short chain carbohydrates. Faulkner-Hogg and Selby (1999) have also suggested that in addition to poor adherence to a GFD food chemical sensitivities are common in patients with CD. Survey respondents who reported having a known food allergy also reported a lower rate of complete symptom resolution than those without a known food allergy (p=0.000). A limitation of the survey was requesting an open-answered question about the identity of the food allergy or intolerance, and what symptoms respondents experienced. The responses were too long and numerous and difficult to categorise. However, those reporting a lactose intolerance and milk allergy were able to be categorised and reported frequently as food intolerance. Other investigators have reported that lactase deficiency is a common food intolerance due to a disaccharidase deficiency caused by damaged intestinal villi (Mones, Yankah et al. 2011).

Our survey indicated that patients with CD taking CAM were more likely to report only some symptom resolution compared to those who did not take CAM (p=0.005). Whether there is an association between these two factors is unclear. The CAM listed by respondents were primarily nutritional supplements rather than herbal or food supplements that are more likely to contain naturally occurring gluten. Nevertheless, patients with CD should be aware that some nutritional supplements may contain gluten or other excipients known to cause gastrointestinal symptoms. In addition, CD individuals with persistent symptoms are more likely to seek over the counter preparations including CAMs in an attempt to alleviate these symptoms or fill a perceived nutritional deficiency. Their physiological activity does not suggest that they are a cause of these symptoms.

It has been suggested that persistent symptoms after treatment with a GFD are seemingly distinct medical entities of IBS, migraine, depression and anxiety. Cady and Farmer (2012) have explored this relationship between migraine, IBS and CD and suggest that what appears to link these medical entities is a disease model of a genetically sensitive nervous system transformed into one that is hyper-vigilant, and that over time can often develop into disabling and pervasive disease. Other groups have suggested that while this sensitising response is generally considered to reside in the central nervous system it also resides in the enteric nervous system and is referred to as the ‘gut-brain connection’ (Li, Dowd et al. 2009; Cady, Farmer et al. 2012; Feng, La et al. 2012). It has been suggested that the enteric nervous system should be explored further to increase our understanding of mood disorders and a number of co-morbid medical diseases experienced by CD patients (Forsythe and Kunze; Forsythe, Sudo et al. 2010; Bercik, Collins et al. 2012; Dinan and Cryan 2012). While this may not entirely explain the high prevalence of symptoms among our CD cohort it is a supportive hypothesis in the context of this thesis. Furthermore, if a hyper-vigilant enteric
nervous system is a unifying link for the common co-morbidities and cluster of symptoms experienced by many patients with CD the question remains as to the trigger of this genetic hyper-vigilance. Our interest lies in the connection between the intestinal microbial environment and its influence on the enteric nervous system as a unifying link for those with incomplete resolution of symptoms.

3.6.6 Birth methods and early influences on the microbiome

It is plausible that perhaps caesarean section delivery has become a modern epigenetic factor contributing to disruptions in the processes of microbial inoculation and immune development. This process is thought to be influenced mainly by three early life events:

- A vaginal birth providing microbial inoculation during the birth process;
- Skin contact providing contact with the skin microbiome; and
- Ingestion of the breast milk microbiome.

These early life events are thought to be foundations for normal healthy immune function in infancy, childhood and adulthood (Cabrera-Rubio, Collado et al. 2012). Furthermore, deviations from these practices are associated with an increased risk of inflammatory and autoimmune diseases (Adlerberth 2008). There has been an increase in caesarean section births in the Australian general population over the last thirty years (Stavrou, Ford et al. 2011). This increase has coincided with a global increase in CD diagnosis in the last decade (Hurley 2012). This present survey did reveal an increased prevalence of caesarean births in survey respondents less than 30 years of age compared to older respondents; however, C-section birth rates for the total cohort were less than in the general population. Our hypothesis that caesarean section births would be more frequent among people with CD than among the general population was not supported by these survey results. Despite these findings other groups have been able to establish a link between caesarean birth and an increased predisposition towards the development of autoimmune disease, including CD (Wiklund, Andolf et al.; Decker, Engelmann et al. 2010; Cho and Norman 2012; Malmborg, Bahmanyar et al. 2012; Rautava, Luoto et al. 2012).

3.6.7 Breast-feeding and early influence on the gut microbiome

Significantly fewer survey respondents (p=0.000) were breast-fed in the first six months of life compared to the general population. In general, the CD cohort reported both a reduced frequency of ever being breast-fed and reported being breast-fed for a significantly shorter period than the general population (p=0.000).

A number of groups have established that the introduction of gluten before six months of age and after weaning is associated with an increased risk of developing CD (Henriksson C
The Australian guidelines for the introduction of solids to infants encourage introducing first foods by six months and continuing breast-feeding up to twelve months. The exact mechanism by which breast-feeding confers protection from CD has not been fully elucidated. Breast milk contains a number of immune complexes that act as immune modulators on the intestinal mucosa (Mosconi, Rekima et al. 2010), including protective secretory IgA, which is known to prevent gastrointestinal infections. Recurrent gastrointestinal infections have been identified as a risk factor for developing CD (Welander, Tjernberg et al. 2010). Part of the reason for a lowered incidence of CD in breast-fed infants is possibly the connection between breast milk, increased sIgA and a reduced risk of gastrointestinal infections. Sellitto and Bai (2012) caution against delaying the introduction of gluten in infants, suggesting the composition of the breast-fed infants’ microbiome assists in developing tolerance to gluten proteins. Furthermore, they dispute that a single organism is likely to cause aberrations in immune tolerance to gluten proteins and suggest that a distinct global composition is responsible for protecting the genetically predisposed from developing CD. However, other groups remain focussed on single species, reporting that infants who are breast-fed have higher numbers and greater diversity of Bifidobacter species (De Palma, Capilla et al. 2012). Furthermore, it has been suggested that Bifidobacter may confer protection against CD (Laparra, Olivares et al. 2012) and gastrointestinal infections (Fukuda, Toh et al. 2011). Extrapolation of our findings suggest that breast-feeding may confer protection during the introduction of gluten due to the lower number of survey respondents who reported being breast-fed at the time when, typically, gluten was introduced, compared to the number of babies breast-fed in the general population.

Obviously breast-feeding alone did not protect against CD development in the breast-fed respondents in this survey. Furthermore, there were no differences in the total cohort between the duration of breast-feeding and complete symptom resolution. Future studies are warranted to investigate the protective effects of the human milk microbiome.

While it was not statistically significant, we did observe that respondents less than 20 years of age reported a higher frequency of complete resolution and longer periods of breast-feeding than older respondents. This may be due simply to age and the fact that younger patients generally have a better treatment outcome because of a prompter diagnosis, resulting in less prolonged damage to the intestinal villi (Lanzini, Lanzarotto et al. 2009). In addition, the incidence and duration of breast-feeding in the general population has slowly increased in the last 20 years and these results may simply be indicative of a general population trend. Nevertheless, Javanparast (2012) recommended that breast-feeding should be encouraged as both a preventative strategy and to potentially enhance the dietary treatment outcomes of
people with CD. The results of this survey support the findings of others, suggesting that breast-feeding for less than six months may be a risk factor for CD.

**3.6.8 Cow's milk a possible risk factor for CD**

Cow’s milk consumption under twelve months of age has been suggested as a risk factor for the development of autoimmune disease, including CD (Hoffenberg and et al. 2005; Virta, Ashorn et al. 2013) and type I diabetes (Kolb and Pozzilli 1999; Ziegler A 2003; Binns, Graham et al. 2007; Chiu, Leslie et al. 2013). The number of survey respondents under the age of thirty years who reported being fed cow’s milk instead of breast milk was significantly higher than the general population (Binns, Graham et al. 2007). These figures did not take into consideration that autoimmune conditions in the general population have risen significantly (Shapira, Agmon-Levin et al. 2010) and that this rise coincides with an increase in feeding cow’s milk to children under twelve months of age instead of breast milk (Brock and Diggs 2013). However, in keeping with this survey’s hypothesis, Dahlquist et al.(2006) suggest that an overload of a combination of factors, including early exposure to cow’s milk protein, have contributed to the increase in autoimmune conditions.

Animal studies have suggested that bovine serum albumin peptide containing seventeen amino acids may be the reactive epitope involved in type-1 diabetes Karjalainen (2009). Antibodies to this peptide react with a beta-cell surface protein that may represent the target antigen for milk induced beta-cell-specific immunity.

Vaarala (2011) identified the milk proteins β-lactoglobulin, bovine serum albumin, and α-casein as inducers of humoral responses. They concluded that feeding infants with cow's milk-based formula induced systemic humoral and cellular responses to cow's milk proteins. T-cell response later declined, supporting the concept of oral tolerisation. Exposure to cow's milk proteins after the age of nine months resulted in depressed cellular and humoral responsiveness to these proteins. In relation to CD, it is plausible cow’s milk proteins may alter normal humoral and cellular responses that predispose HLA DQ2 and HLA DQ8 positive individuals to develop CD and that this may be age sensitive.

**3.6.9 Infection in childhood: early influences on the gastrointestinal microbiome**

Children with respiratory infections, including the common cold, sinusitis, ear, nose and throat infections and chest infections invariably swallow a significant amount of bacteria responsible for their infections, thus the bacteria end up as part of the intestinal microflora. It was disappointing not to find appropriate general population data to compare with the reported frequencies of respiratory tract infections under the age of six years. The lack of data...
is primarily due to most parents not taking children with mild respiratory tract infections to health care institutions where these data may be recorded. In addition, studies that recorded the frequencies of respiratory infection were conducted primarily in early pre-school day care centres where infection control was being assessed. Recurrent respiratory infections in early childhood have been associated with mental health issues (Liang and Chikritzhs) and the development of autoimmune conditions in both early life and adulthood (Brimberg, Benhar et al. 2012). We were unable to provide evidence of an increased frequency of early childhood respiratory infections in CD compared to the general population. However, the frequency of reported infection in CD respondents was reported so other groups can use this data in future investigations.

3.7 Conclusion

The hypothesis that health factors such as maternal infection, maternal antibiotic use, and early childhood infection and antibiotic use under six years of age would be more frequent in patients with CD was not proven. This was due to a lack of suitable data on some variables for the general population. However, our hypothesis that breast-feeding practices would be differ from the general population was shown. We did not find an increased frequency of caesarean section births in our total CD cohort compared to the general population. However, survey respondents born in the last 30 years had an increased frequency of C-section births compared to those in older age groups. While this increase is consistent with the trend in the general population it is important to note that it also coincides with an increased prevalence in CD.

Interestingly, the results relating to the clinical management of CD may be of greater significance. This study demonstrates that Australians present with a broad range of symptoms and experience considerable diagnostic delay, and that many continue to experience some degree of persistent symptoms despite self-reported adherence to a GFD. A significant number of patients with CD have a second degree relative affected by the disease, and this should be considered as a potential risk factor. Management of food allergies, assessment of CAM use and ruling out infectious triggers may assist in the management of persistent symptoms.

In conclusion, not breast-feeding or reducing its duration may be contributing risk factors in CD development. We recommend longitudinal studies that follow breast-feeding practices and CD development among genetically predisposed individuals. Based on these findings, education of primary health care providers about the broad clinical presentations of CD is warranted. In regard to the clinical management of CD patients with incomplete symptom
resolution, we suggest that a thorough clinical assessment of the factors discussed above be conducted. Furthermore, clinical studies focussing on exploring the intestinal microbiota in CD patients may provide some insight into the high incidence of persistent symptoms in this population.
4 The Microbiome Study

4.1 Introduction

The gastrointestinal microbiome of children with CD and without CD has been the subject of a number of studies (Sanz, Palma et al. 2011). This data identifies significant differences in the numbers and diversity of gastrointestinal bacterial species in the microbiota of children with CD compared to healthy children. To date, however, there have been no studies that have assessed differences in the gastrointestinal microbiota of adult CD patients reporting persistent symptoms compared to non-coeliac controls. Furthermore, the assessment of parasites and yeasts has not been conducted in either children or adults with CD.

If there were specific differences identified in the adult microbiome of CD participants with persistent symptoms, this information would provide information for further research that explores the treatments of intestinal microorganisms in this sub-group of CD patients. In addition, should significant differences in the intestinal microbiome be identified in this sub-group of CD patients, it could provide important information for future studies that investigate the microbiome in the general CD population. Therefore, a research plan was devised to investigate the microbiome of Australian adults with CD reporting persistent symptoms despite adherence to a strict GFD.

4.1.1 The research question

Do Australian adults with CD who report mild to moderate persistent symptoms despite adhering to a GFD have a different microbiome to Australian adults without CD?

4.1.2 Hypothesis

It was hypothesised that a sub-group of Australian adults with CD reporting mild to moderate symptoms despite a GFD will have differences in their gastrointestinal microbiome compared to non-CD Australians with mild to moderate gastrointestinal symptoms.

4.1.3 Null hypothesis

The null hypothesis was that the distribution of the microorganisms measured would be the same for both CD and control groups.
4.2 Materials and methods

4.2.1 Study design

The microbiome study and the clinical trial (CT) presented in the following Chapter were undertaken simultaneously. The microbiome was a sub-study nested within the CT.

This study was designed as a retrospective case-controlled study with two arms: people with symptomatic CD despite a GFD and the test results of people without CD with similar gastrointestinal symptoms.

4.2.2 Primary outcome measure

The primary outcome measures were quantitative bacterial counts from a single faecal specimen, in addition to semi-quantitative measures of yeasts and the detection of parasites if present.

4.2.3 Assessment tools

The primary outcome measure was calculated using counts obtained from the microbial faecal analysis. A microbial ecology sample report is presented in Appendix C1. The microbial population was measured using polymerase chain reaction amplification of the DNA of each organism reported and the laboratory method is described below in 4.2.3.1.

4.2.3.1 Laboratory assessment: faecal molecular and microscopic analysis

Specimen Requirements: faecal specimen samples were collected in 50 ml conical tubes containing Formalin, Culture and Sensitivity media and Nucleic Acid Extraction buffer. The specimen collection instructions followed by the participants are presented in Appendix C2. The participant refrigerated the faecal samples immediately after collection. The specimens were collected from the participant by a biological courier and sent to the study site at Diagnostic Insight. After the study coordinator checked the specimens for temperature and time stability they were shipped to Metametrix Laboratory on ice. Faecal specimens were processed between one and three days of receipt for DNA extraction and sensitivity assays, and microscopy was performed on the tubes containing formalin. The stool specimens were stored at 4°C following processing and discarded 30 days from the accessioning date.

Nucleic acid extraction from faecal specimens: stool samples were mixed thoroughly before removal of faecal material for processing. The specimens were weighed and the pH of the faecal material was measured and recorded. Genomic DNA was extracted from a 250 µL
aliquot of the faecal specimen contained in NA buffer. Zirconia bead-beating was used to efficiently lyse microbial cells (Lantz, Matsson et al. 1997; Yu and Morrison 2004; Persson, de Boer et al. 2011). The extracted DNA samples were stored at -20°C following extraction.

**PCR amplification of bacteria, fungi and parasites in stool samples:** Genova Diagnostics Clinical Laboratory used a PCR buffer mix described by Saiki et. al (1988). PCR reactions were performed in 50 µL reaction volumes using 25 mM MgCl₂, 100 µM DNTP, 1% PVP, 0.1-10 pMol primer, 2.5U/µL of *Taq* polymerase and 3 µL of extracted genomic DNA. PCR reactions for the amplification of *Streptococcus* and *Enterococcus* species included 22.5 mM Tetramethyl ammonium chloride. Cycling conditions for PCR amplification in an Applied Biosystems 9700 Thermo-cycler were as follows: 95°C for five minutes; 35 cycles of 95°C for 30 seconds, 37°C to 50°C (dependent upon the target) for 30 seconds, and 72°C for 30 seconds; and extension of 72°C for seven minutes. PCR amplicons were evaluated by both fluorescent dye SYBR® Green post amplification and Agarose gel electrophoresis (1.5%) stained with ethidium bromide, and visualized under UV illumination. SYBR® Green I is an exceptionally sensitive nucleic acid-binding dye that has bright fluorescence when bound to dsDNA (double stranded DNA), making it ideal for detecting dsDNA using standard UV trans-illuminators (Skeidsvoll and Ueland 1995). The PCR-amplified product (20 µl) containing SYBR ® green bound to the product was mixed with an equal volume of 8M urea, incubated at 50°C for five minutes before detection of the fluorescence signal. The resulting SYBR® green data was used to evaluate whether the specimen was positive or negative for the target organism(s).

**Determination of opportunistic and predominant bacteria in the gut microbiota:** PCR primers targeting the 23S rDNA and 16S rDNA were used to amplify PCR products used in a downstream hybridisation assay. The hybridisation probes were species-, group- and genus-specific for monitoring major bacterial species in human faecal samples. The quantitative amounts of predominant and opportunistic bacteria are reported in CFU/gram of faeces. The opportunistic bacteria targeted were as follows: *Achromobacter/Alcaligenes* sp., *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., *Enterobacter* sp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morganii*, *Pseudomonas* sp., *Salmonella* sp. and *Staphylococcus aureus*. Predominant bacteria targeted include: *Bacteroides* sp., *Clostridia* sp., *Prevotella* sp., *Fusobacteria* sp., *Streptomyces* sp., *Mycoplasma* sp., *Lactobacillus* sp., *Bifidobacter* sp. and *Escherichia coli*.

The hybridisation assay used plates that were prepared by coating with an Avidin Biotin-binding protein-coating buffer solution (the hybridisation solution). Following the 16S and 23S PCR reaction a 1:100 dilution of the PCR product was added to the hybridisation plates.
The plates were then incubated, washed with DI water and blocked with biotin. A 1:100 dilution of the hybridisation probes were added to their corresponding columns on the plate and incubated for one hour at 58°C. The 23S hybridisation probes bound to the 23S PCR product targeting the 23S rDNA and the 16S hybridisation probes bound to the PCR product targeting the 16S rDNA and were incubated. After stringency washes 100 µl of the signalling compound was added to each well and incubated for 15 minutes. Following a final wash the commercially available substrate solution was added to the wells and allowed to proceed for 20 minutes. The colorimetric reaction was stopped by adding 100 µl of Stop Solution and the plates were read at 450 nm using Gen5 software and Synergy four Bitek plate reader.

_Determination of bacterial pathogens in the gut microbiota:_ primer pairs that specifically target the bacterial pathogens _Helicobacter sp., Campylobacter sp., Clostridium difficile_ and _E. Coli H1:O157_ were used to quantify the amount of bacteria in CFU/gram of faeces. Additional primer pairs were developed to specifically target the _Enterococcus_ species _Enterococcus faecalis, Enterococcus faecium, Enterococcus durans_ and _Enterococcus hirae_, and _Streptococcus_ species _Streptococcus pneumoniae, Streptococcus mutans, Streptococcus sanguinis, Streptococcus sobrinus, Streptococcus oralis, Streptococcus gordonii_ and _Streptococcus agalactiae_.

_Determination of drug-resistant genes (DRG) in the gut microbiota:_ primer pairs that specifically target the DRG analytes _aacA-aphd., PBP1a, parE, vanB, vanC-I_ and _mecA_ were used to determine the presence or absence of the DRG in the faecal sample.

_Determination of fungi in the gut microbiota:_ PCR primers were used to amplify a product from genomic DNA extracted from faecal material that was used as a template for the amplification of _Saccharomyces sp., Candida albicans, Geotrichum sp. and Rhodotorula sp._ The fungi species-specific primer pairs bind internally to the universal amplified region. A 1:100 dilution of the fungi universal product was prepared in water and three µL of the diluted product were used as template for the subsequent fungal species-specific PCR reactions.

_Determination of parasitic protozoans and helminths in the gut microbiota:_ primer pairs were developed to target the parasitic protozoans _Blastocystis hominis, Cryptosporidium sp., Entamoeba sp., Entamoeba hystolitica, Enatmoeba coli, Dientamoeba fragilis, Endolimax nana, Trichomonas sp. and Giardia intestinalis_. Primer pairs were developed to target the parasitic worms _Ascaris lumbricoides, Enterobius vermicularis, Necator americanus, Strongyloides sp, Taenia solium, Trichuris trichuria, Shistosoma mansoni_ and _Clonorchis sinensis._
Bacterial counts were reported as colony-forming units per gram of faeces (CFU/g). One CFU is equivalent to one microorganism. Each genome detected represents one cell, or one CFU. Results for bacteria are expressed in scientific notation, e.g. Bacteroides sp. may be reported as 2.57 +E7 CFU/gram, which is read as 25 million (or 2.57 x 10^7) colony-forming units per gram of faeces.

The exponent is kept constant for each classification of bacteria. The exponent for predominant bacteria is 1.0E + 007 (10^7 or 10,000,000), and for opportunistic and pathogenic bacteria 1.0E + 005 (10^5 or 100,000).

Yeast counts are reported as +1, +2, +3 or +4 indicating >100, >1000, >10,000 or 100,000 parts per gram (pg) of DNA per gram (g) of faeces respectively.

Parasites were reported as detected or not detected.

Drug-resistant genes were reported as positive or negative.

The testing was undertaken at Metametrix™ Laboratory, Duluth, Atlanta, Georgia USA.

4.2.4 Study population

A targeted population of patients with CD who were still experiencing some degree of persistent symptoms despite twelve months’ adherence to a strict GFD was recruited. The control group was recruited from doctors’ medical records; they were serologically negative for CD and had already undertaken the same DNA faecal analysis within the last two years that was undertaken by the CD arm. The clinical characterisation of both groups is described in 4.3.1.1 and 4.3.1.2.

4.2.5 Ethics

The study was approved by the Human Research Ethics Committee of Southern Cross University (ethics approval number ECN-12-021) (Appendix C3). The research was conducted in compliance with Good Clinical Practices (GCP) and in accordance with the guidelines of the Australian National Health and Medical Research Council and the Declaration of Helsinki (as revised in 2004). The trial was registered with the Australian and New Zealand Clinical Trials Register (ACTRN12610000630011).

4.2.5.1 Recruitment

The CD group was recruited through a cohort of survey respondents sent via the NSW CD Association email database. The survey recruitment procedure is outlined in Chapter 3 and the email is presented in Appendix C4.
4.2.5.2 Sample size
We recruited 45 participants in the CD arm. A group of 27 control participants’ faecal microbial analysis results formed the data for the control group.

A sample size calculation was undertaken by the methodologist and statistician at Southern Cross University. The sample size was estimated using PASS 2008™ sample size software. Data from a pilot study that had demonstrated larger counts of Gram-positive bacteria in CD participants compared to controls was used in the calculation. The pilot study data used for the calculation was the mean of the Gram-negative to Gram-positive bacteria (CD group, n=30) and the mean of the laboratory data (healthy people n=117).

It was estimated that sample sizes of 19 per group would have 80% power (1-β) to detect differences between groups in a mean change of 2.5 with standard deviations of the changes of 3.0 in each group at α = 0.05 (1-tailed). This would occur if there was a mean increase of 2.5 from 2.0 to 4.5 in the treatment group and no change in the placebo group.

4.2.6 Inclusion/Exclusion criteria
This thesis used the Australian Coeliac Association’s (ACA) diagnostic criteria/definition for CD which was considered up-to-date at the time the thesis was conducted. The updated version of the 1990 ESPGHAN criteria for CD which were under revision at the time were published in 2012 (JPGN 2012;54:136–160) and demonstrates that the choice of the ACA criteria was appropriate.

4.2.6.1 Inclusion criteria for CD group
- CD confirmed by small bowel biopsy more than twelve months before enrolling in the study
- Followed a GFD for at least twelve months
- Followed by normalisation of tTg and/or endomysial antibodies and/or evidence of partial or complete villous architecture repair.
- Aged between 18 and 70 years of age
- Experiencing persistent gastrointestinal symptoms

4.2.6.2 Exclusion criteria for CD group
As the CD arm of this comparative study would go on to be participants in the clinical trial phase of this project detailed exclusion criteria were required:
- Pregnant women
- People under the age of 18

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• People with cancer or who are HIV-positive
• People who had been diagnosed with CD in the past twelve months
• People who consumed a gluten-containing diet
• People with gastrointestinal pathology such as cancer, Crohn’s disease or ulcerative colitis
• People with short bowel syndrome
• People who had had recent oral or bowel surgery
• People who were on chemotherapy or radiotherapy
• People using non-steroidal or steroidal anti-inflammatory drugs and/or antibiotics in the four weeks before the start of the trial
• Participants with serum urea, electrolytes and creatinine greater than two times the upper limit of normal at baseline
• Participants with liver function tests greater than three times the upper limit of normal at baseline
• Participants who were unwilling to comply with the study protocol
• Participants with any other condition which, in the opinion of the investigators, could compromise the study
• People with active alcohol and/or illicit drug dependence

4.2.6.3 Inclusion criteria for the control group
• Men and women aged 18-75 years without CD
• Individuals who had undertaken DNA faecal analysis in the previous two years
• Individuals who had tested serologically negative to tissue transglutaminase (tTg) and anti-gliadin IgA in the previous twelve months
• Individuals with normal blood sIgA

4.2.6.4 Exclusion criteria for the control group
• Individuals who failed to meet any of the inclusion criteria outlined above in 4.2.6.3.

4.2.7 Study environment
The study venues for the participants with CD were Diagnostic Insight Pathology consultation rooms in St Leonards, Sydney, and the SCU Health Clinic at Southern Cross University, Lismore. The control group participants’ data were extracted from records at a medical practice in Manly, NSW, Australia.
4.2.8 Study procedures

4.2.8.1 Study procedure for CD group

The first step in the recruitment process was a screening interview by telephone to ensure that CD participants met the inclusion and exclusion criteria for the study. Participants meeting the criteria were invited to attend an assessment session for a clinical trial (see Chapter 5). Part of this assessment session included instructions for faecal specimen collection and submission. The information obtained from the baseline faecal test would form the data for the CD arm of this comparative study. All faecal results were forwarded, with the participants’ permission, to their nominated doctor with a covering letter and information about the study.

4.2.8.2 Study procedure for the control group

The first step of the recruitment process of the control group was approaching a doctor who had previously requested that patients undertake molecular faecal microbial testing with Diagnostic Insight Pty Ltd. Diagnostic Insight Pty Ltd requested the doctors’ permission for the study coordinator, who was also an employee of Diagnostic Insight, to examine patient result records at his practice, but not clinical progress notes. Written permission from the doctor was obtained. The study coordinator was granted access for eight hours to the electronic records of results at the doctor’s practice. The study coordinator compared the last 50 faecal microbial analysis results held by Diagnostic Insight with records of CD serology held by the doctor. The first 50 individuals with faecal microbial results who were also proven to be serologically negative to CD and over the age of 18 were contacted by mail. A letter providing information and requesting permission to use their test result data (Appendix C5), accompanied by a consent form (Appendix C6), was sent to the 50 individuals meeting the study criteria. Twenty-seven individuals returned a signed consent form and these individuals’ faecal microbial results formed the non-CD control data group.

4.2.9 Statistical analysis

The statistical packages used were SPSS PASW®Statistics GradPack 18 and version 20 SPSS. Significance was assumed if p<0.05.

Bacteria and phylla classes were measured as quantitative counts; yeasts were measured on a semi-quantitative count scale and parasites were measured as detected or not detected. Normality was assessed for the predominant bacterial quantification count scores and logarithmic transformations were performed where applicable, as count data can often be skewed. Independent t-tests to compare the CD and non-CD groups were conducted on
transformed or raw scores. Non-parametric Mann-Whitney U-tests on raw scores were also conducted to compare CD and non-CD groups.

Due to the absence in some cases of count scores for pathogenic and opportunistic bacteria, i.e. where the count scores were below the laboratory reporting level, cross-tabulations and chi-square analysis were conducted to ascertain whether there were any differences between the two groups. The number and percentage for each microorganism was recorded. Chi-square analysis was conducted to ascertain the statistical differences between groups. Significance was assumed at p≤0.05.

Parasite detection data were analysed by conducting exact chi-square tests on the two-way contingency Tables (CD/non-CD and detected/not detected).

4.3 Results

The results of the microbiome study are presented in Tables throughout this chapter. Graphical representation of the data is presented in Appendix C7.

4.3.1 Demographics

A total of 37 females and eight males (mean age 47.3 years) residing in New South Wales, Australia, were allocated to the CD arm of this study. The control data in this trial was obtained from patients residing in NSW Australia test records. A total of 20 females’ and 7 males’ (mean age 44.5 years) faecal microbial ecology profile results and Coeliac serology formed the final control data set.

4.3.1.1 Clinical characterisation of CD group with persistent symptoms

The thesis used the Australian Coeliac Association’s (ACA) diagnostic criteria/definition for CD which was considered up-to-date at the time the thesis was conducted. The updated version of the 1990 ESPGHAN criteria for CD which were under revision at the time were published in 2012 (JPGN 2012;54: 136–160) and demonstrates that the choice of the ACA criteria was appropriate.

All CD participants reported being troubled by gastrointestinal symptoms despite adherence to a GFD. We classified these symptoms with scores obtained from a baseline CDSQOL. As presented in Appendix D1 the CDSQOL consisted of 4 sub-categories including emotion (including fatigue), worries, social and gastrointestinal symptoms (urgency to defecate, loose bowel motions, abdominal cramping, bloating or flatulence, incomplete bowel evacuation, repeated belching, nausea or retching). The gastrointestinal symptoms were classified by responses to 7 questions with a 7 point Likert-scale response. The total gastrointestinal
symptom sub-scale scores could range from 0-49. Lower scores are associated with more severe persistent symptoms (0-21) and are more likely to be associated with CD activity and/or other pathology. Scores of 22 to 35 were considered mild to moderate and scores 42 to 49 were considered normal. As presented in section 5.3.7.1, Table 5.27, the gastrointestinal symptom scores were mild to moderate in all cases except two cases who scored higher than 42 but were lower in the symptom of fatigue, therefore warranting their inclusion. In addition, the comprehensive clinical assessment at baseline included questions that would identify red flags, i.e. family history of GI cancer, unexplained weight loss, blood in the stool or black stools (see Appendix D10). All participants were questioned about whether they had consulted their doctor and/or gastroenterologist about their gastrointestinal symptoms. Fatigue was also considered a persistent symptom. As identified in the survey (Table 3.11), fatigue is both a common presenting symptom in CD and also a frequently reported persistent symptom (Table 3.13 page 130). Fatigue scores at baseline were mild to moderate.

4.3.1.2 Clinical characteristics of non-CD control group
The control data in this trial was obtained from patients residing in NSW Australia test records. A single medical practitioner held this data. The clinical reason for the GP ordering the microbial ecology profile was on the basis of suspected Irritable Bowel Syndrome, to explore parasitosis, bacterial infection and to determine if there was significant dysbiosis as the cause of the patients’ symptoms. In addition, all of the control group participants had negative Coeliac serology and normal sIgA levels.

4.3.2 Primary outcome measures
The primary measures were for bacteria, yeast and parasites. Colour codes are used throughout the results section to assist in differentiating between microorganism categories. Table 4.1 presents the colours used throughout this Chapter.

Table 4.1 Colour codes for Tables in this Chapter

<table>
<thead>
<tr>
<th>CD Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predominant bacteria CD group</td>
<td>Predominant bacteria control group</td>
</tr>
<tr>
<td>Opportunistic bacteria CD group</td>
<td>Opportunistic bacteria control group</td>
</tr>
<tr>
<td>Pathogenic Bacteria CD group</td>
<td>Pathogenic bacteria control group</td>
</tr>
<tr>
<td>Yeasts CD group</td>
<td>Yeast control group</td>
</tr>
<tr>
<td>Parasites CD group</td>
<td>Parasites control group</td>
</tr>
</tbody>
</table>
The descriptive statistics including estimated means, standard deviations (SD), minimum (min) and maximum (max) values of the predominant bacteria counts and phyla classes are presented for the CD group and the non-CD group (control group) and Tables 4.2 and 4.3.

Table 4.2 Predominant bacteria: descriptive statistics for predominant bacteria measures in CD and Control Groups (measures are reported for instance as $4.1 \times 10^7$ CFU/g of faeces)

<table>
<thead>
<tr>
<th>Predominant Bacteria Measures</th>
<th>CD Group (n=45)</th>
<th>Control Group (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>4.0</td>
<td>2.49</td>
</tr>
<tr>
<td>Clostridia sp.</td>
<td>4.1</td>
<td>1.88</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>3.7</td>
<td>2.22</td>
</tr>
<tr>
<td>Fusobacteria sp.</td>
<td>4.4</td>
<td>3.26</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>3.5</td>
<td>1.75</td>
</tr>
<tr>
<td>Mycoplasma sp.</td>
<td>3.3</td>
<td>1.53</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>5.4</td>
<td>4.84</td>
</tr>
<tr>
<td>Bifidobacteria sp.</td>
<td>5.2</td>
<td>2.65</td>
</tr>
<tr>
<td>Ecoli sp.</td>
<td>3.5</td>
<td>1.69</td>
</tr>
<tr>
<td>Predominant Bacteria Total counts</td>
<td>37.4</td>
<td>9.78</td>
</tr>
</tbody>
</table>

Table 4.3 Phyla classes: descriptive statistics for total bacteria phylum measures in CD and Control Groups (measures are reported for instance as $4.1 \times 10^7$ CFU/g of faeces)

<table>
<thead>
<tr>
<th>Bacterial Phyla Measures</th>
<th>CD Group n=45</th>
<th>Control Group n=27</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>67.80</td>
<td>9.139</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>32.20</td>
<td>9.139</td>
</tr>
</tbody>
</table>

Table 4.4 and Table 4.5 present the number, percentage distribution and the p-value results of chi-square analysis for opportunistic and pathogenic bacteria respectively. Opportunistic bacterial organisms assessed for but not detected included *Staphylococcus* sp. and *Klebsiella pneumonae.*
Table 4.4 Opportunistic bacteria: results of Chi-square analysis, number and percentage distribution and p-values for comparison between detection rates of opportunistic bacteria in CD and Control Group (the cut off level for reporting the detection of opportunistic bacteria was $10^5$ CFU/g of faeces)

<table>
<thead>
<tr>
<th>Opportunistic Bacterial Species</th>
<th>CD Group: n=45</th>
<th>Control Group: n=27</th>
<th>Chi-square p-values Fisher’s Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opportunistic bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Detected</td>
<td>Detected</td>
<td>Not detected</td>
<td>Detected</td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>38 (84.4)</td>
<td>7 (15.6)</td>
<td>25 (92.6)</td>
<td>2 (7.4)</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44 (97.8)</td>
<td>1 (2.2)</td>
<td>27 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44 (97.8)</td>
<td>1 (2.2)</td>
<td>27 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Achromobacter sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44 (97.8)</td>
<td>1 (2.2)</td>
<td>27 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 (95.6)</td>
<td>2 (4.4)</td>
<td>27 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43 (95.6)</td>
<td>2 (4.4)</td>
<td>27 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Citrobacter sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44 (97.8)</td>
<td>1 (2.2)</td>
<td>26 (96.3)</td>
<td>1 (3.7)</td>
</tr>
</tbody>
</table>

Table 4.5 Pathogenic bacteria: results of Chi-square analysis, number and percentage Distribution and p-values for comparison between detection rates of pathogenic bacteria in CD and Control Group (the cut-off level for reporting the detection of pathogenic bacteria was $10^5$ CFU/g of faeces)

<table>
<thead>
<tr>
<th>Pathogenic Bacterial Species</th>
<th>CD Group n=45</th>
<th>Control Group n=27</th>
<th>Chi-square p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Detected</td>
<td>Detected</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>H. Pylori</td>
<td>28 (62)</td>
<td>17 (38)</td>
<td>20 (74.1)</td>
</tr>
<tr>
<td>EHE coli</td>
<td>41 (91)</td>
<td>4 (9)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Campylobacter sp.</td>
<td>45 (100)</td>
<td>0 (0)</td>
<td>26 (96.3)</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>45 (100)</td>
<td>0 (0)</td>
<td>26 (96.6)</td>
</tr>
</tbody>
</table>

Table 4.6 presents the number, percentage distribution and p-values of chi-square analysis for mycology results. The detection of Saccharomyces sp. and Candida sp. were significantly
higher in the CD group compared to the control group. Where an organism was not reported as present in any of the participants in one or both groups the statistical analysis was unable to provide the test statistic, and these cases were reported as UAC (unable to calculate).

Table 4.6 Mycology: results of Chi-square analysis, number and percentage distribution and p-values for comparison of detection of yeasts/fungi between CD Group and Control Group (the cut-off level for reporting the detection of yeast/fungi was > 10,000 parts per gram of DNA per gram of faeces)

<table>
<thead>
<tr>
<th>Yeast/Fungi species</th>
<th>CD Group n=45</th>
<th>Control Group n=27</th>
<th>Chi-square p-value Fisher’s Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast taxonomy unavailable</td>
<td>Not detected n (%)</td>
<td>Detected n (%)</td>
<td>Not detected n (%)</td>
</tr>
<tr>
<td>Candida sp.</td>
<td>27 (60)</td>
<td>18 (40)</td>
<td>16 (59.3)</td>
</tr>
<tr>
<td>Saccharomyces sp.</td>
<td>30 (66.6)</td>
<td>15 (33.3)</td>
<td>27 (100)</td>
</tr>
</tbody>
</table>

Parasitology results are presented in Table 4.7 showing the number, percentage distribution and p-values of chi-square analysis. Non-human parasites with an unknown taxonomy were detected at a significantly higher rate in the CD group compared to the control group. Parasites that were assessed for but not detected in either group on either measurement occasion included Entaeomba histolytica, Entaeomba sp., Cryptosporidium sp., Endolimax nana, Giardia sp., Trichomonas hominis, Ascaris lumbricoides (Round worm), Clonorchis sinensis (Chinese liver fluke worm), Schistosoma mansoni, Strongyloides sp., and Taenia solium (Tape worm).

Independent samples t-tests were conducted to compare the microbiota measures of the control group and the CD group. The results are presented in Table 4.8 and show that the microorganisms identified as being significantly higher were Clostridia sp. (p=0.002), Prevotella sp. (p=0.002), Bifidobacteria sp. (p=0.03), total predominant bacteria counts (p=0.02), Candida sp. (p=0.00), and Saccharomyces spp (p=0.02). Before the independent t-test analysis, a log transformation of data was conducted using PASW ® statistics programme due to the inherently skewed nature of some predominant bacteria and mycology measures detected during the preliminary descriptive analysis.

As presented in Table 4.9 the results of the non-parametric Mann-Whitney U-test employed to test the null hypothesis showed there were significantly higher numbers of five
gastrointestinal microbiota species for the CD group compared to the control group. These results suggest that there are significant differences in the counts of certain predominant/commensal bacteria and yeasts between participants with CD and the control group.

Table 4.7 Parasitology: results of Chi-square analysis, number and percentage distribution and p-values for comparison between detection rates of parasites in CD and Control Group (parasite DNA was reported simply as detected or not detected)

<table>
<thead>
<tr>
<th>Parasite Name</th>
<th>CD Group n=45</th>
<th>Control Group n=27</th>
<th>Fisher’s Exact Test (Chi-square p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Detected n (%)</td>
<td>Detected n (%)</td>
<td>Not Detected n (%)</td>
</tr>
<tr>
<td>General Parasite incidence</td>
<td>4 (8.9)</td>
<td>41 (91)</td>
<td>5 (21%)</td>
</tr>
<tr>
<td>Parasite taxonomy unavailable</td>
<td>5 (11.1)</td>
<td>40 (88.9)</td>
<td>7 (28.6)</td>
</tr>
<tr>
<td>Blastocystis hominis</td>
<td>37 (82.2)</td>
<td>8 (17.8)</td>
<td>22 (82.2)</td>
</tr>
<tr>
<td>Dientoemba fragilis</td>
<td>43 (95.6)</td>
<td>2 (4.4)</td>
<td>23 (85.7)</td>
</tr>
<tr>
<td>Nector americanus</td>
<td>42 (93.3)</td>
<td>3 (6.7)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Trichuris trichuria</td>
<td>43 (95.6)</td>
<td>2 (4.4)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>Enterobius vermicularis</td>
<td>41 (91.1)</td>
<td>4 (8.9)</td>
<td>23 (85.7)</td>
</tr>
</tbody>
</table>
Table 4.8 Results of independent \( t \)-tests for statistically significant differences in microbial measures between the CD and Control Group

<table>
<thead>
<tr>
<th>Microbial Measure</th>
<th>( t )</th>
<th>df</th>
<th>Sig. 2-tailed ( t )-test</th>
<th>Mean Difference</th>
<th>SE Difference</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridia sp.</td>
<td>2.75</td>
<td>71</td>
<td>0.007</td>
<td>1.18</td>
<td>0.428</td>
<td>0.325</td>
<td>2.03</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>3.61</td>
<td>57.86</td>
<td>0.001</td>
<td>1.30</td>
<td>0.359</td>
<td>0.582</td>
<td>2.02</td>
</tr>
<tr>
<td>Bifidobacter sp.</td>
<td>2.12</td>
<td>71</td>
<td>0.037</td>
<td>.108</td>
<td>0.050</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>Total Predominant Bacteria counts</td>
<td>2.93</td>
<td>44.84</td>
<td>0.005</td>
<td>7.11</td>
<td>2.419</td>
<td>2.23</td>
<td>11.98</td>
</tr>
<tr>
<td>Candida sp.</td>
<td>4.69</td>
<td>44.00</td>
<td>0.000</td>
<td>0.66</td>
<td>0.142</td>
<td>0.38</td>
<td>0.95</td>
</tr>
<tr>
<td>Saccharomyces sp.</td>
<td>2.44</td>
<td>70.69</td>
<td>0.017</td>
<td>0.45</td>
<td>0.185</td>
<td>0.08</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Table 4.9 Results of non-parametric Mann-Whitney U-Test: significant differences in microbial measures between CD and Control Group to test the Null Hypothesis

<table>
<thead>
<tr>
<th>Null Hypothesis: That the Distribution of the Measure is Same across Categories of the Group for the Following Microbiota</th>
<th>Significance ( p \leq 0.05 ) to Reject the Null Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridia sp.</td>
<td>0.002</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>0.009</td>
</tr>
<tr>
<td>Bifidobacteria sp.</td>
<td>0.022</td>
</tr>
<tr>
<td>Total Predominant bacteria</td>
<td>0.003</td>
</tr>
<tr>
<td>Candida sp.</td>
<td>0.001</td>
</tr>
<tr>
<td>Saccharomyces sp.</td>
<td>0.030</td>
</tr>
</tbody>
</table>

4.4 Discussion

4.4.1 Major findings of the study and their importance

This study suggests that a sub-group of adults with CD have a different gastrointestinal microbiome from that of individuals without CD. In the present study, adults with CD had statistically significant higher counts of the gastrointestinal microbial species *Clostridia*,...
Prevotella, Bifidobacteria, Candida and Saccharomyces, in addition to higher total counts of predominant bacteria than in the control group. This is the first time such differences have been demonstrated in an Australian adult CD population. The identification of these microbial differences provides important information for the focus of larger scale studies in the future. In addition, these results, if supported by future studies, could aid in the development of appropriate antimicrobial or probiotic intervention. Such interventions, if proven efficacious and safe, may serve to be either an adjuvant or as an alternative intervention to the current dietary treatment model in people with CD, potentially significantly improving their quality of life.

4.4.2 Comparison to similar studies and possible explanation of findings

4.4.2.1 Significant differences in predominant bacteria

There are a number of considerations when making comparisons of our study to publications investigating the gastrointestinal microbiota of people with CD. Currently the literature is not limited to the same molecular techniques and methodologies. As discussed in Chapter 2.3 there is no global gold standard for the molecular assessment of the intestinal microbiota. Each technique has its strengths and limitations that may result in publication bias.

However, our findings that higher levels of faecal Prevotella sp. and Clostridia sp. in this sub-group of adult patients with CD replicates in part the findings of Ou et al. (2009), who found Clostridium sp., Prevotella sp. and Actinomyces sp. to be the main components of small intestinal microbiota of children during the Swedish CD epidemic of 2004-2007. We did not measure Actinomyces sp. so the differences in the findings may simply be due to omission rather than a discrepancy.

Results in comparison studies by other groups in paediatric populations differ from those we found in adults. Bifidobacter sp. counts have been consistently reported to be lower among children with CD compared to non-CD children (Collado, Calabuig et al. 2007b; Nadal, Donant et al. 2007b; Collado, Donat et al. 2009a; De Palma, Nadal et al. 2010). However, our findings suggest that Bifidobacter is higher among adult CD patients than among the control group. This has been supported by the assessment by Sánchez et al. (2010) of the duodenal biopsies of children with CD. Bifidobacter sp. is thought to be the dominant bacteria of the healthy infant gastrointestinal tract. Bifidobacter sp. confers a number of health benefits in an infant and it may be expected that to see lowered numbers in a diseased intestine, such as that of CD patients. However, this does not explain why higher levels may be associated with adult CD, as our findings suggest. Larger studies assessing sub-species of Bifidobacter sp. in adult CD patients may provide a better understanding of the significance of this finding.
We found no difference in the total number of *Bacteroides* sp. between the CD sub-group and control group in our study. Differences in diversity and species composition of the genus *Bacteroides* have been reported by other groups (Sánchez, Donat et al. 2010). The different findings reported for total counts of the *Bacteroides* sp. maybe due to the fact that we did not sub-speciate *Bacteroides* sp; therefore we could speculate that, while the total species count was not significantly different between our two groups, sub-speciation may have yielded results similar to those of Sánchez’s group.

### 4.4.2.2 The potential significance of Candida sp. in CD

The very significant differences found between the incidence of faecal *Candida* sp. in the CD sub-group and its absence in the control group (p=0.000) raise two critical considerations. First, is *Candida* sp. a potential trigger of CD in the genetically predisposed individual? Such a proposition could be supported by Niuwenhuizen et al. (2003) who hypothesised that *C. albicans* is a trigger in the onset of CD. These authors hypothesised that the virulence factor of *C. albicans*-hyphal wall protein 1 (HWP1) contains amino acid sequences that are identical or highly homologous to known CD-related α-gliadin and γ-gliadin T-cell epitopes. The HWP1 is used by *C. albicans* to adhere to the intestinal epithelium. It is thought that tTg and endomysium components link to the yeast and act as an adjuvant that activates the immune system to fight the HWP1 and gluten, thereby forming autoimmune antibodies against tTg and endomysium, resulting in the characteristic villous atrophy of CD.

Other research groups have identified at least three species of *Candida* sp. that produce proteases that can degrade IgA1, IgA2 and sIgA. Interestingly, Cataldo et al. (1998a) reported that of 2098 patients with CD 2.6% had a sIgA deficiency, representing a 10 to 16-fold increase over that of sIgA deficiency in the general population. Importantly, the sIgA deficiency results in a false negative in CD serological markers. Furthermore, it is well established that sIgA deficiency may cause a predisposition to autoimmune diseases and to recurrent infections (Cataldo, Marino et al. 1998a). We did not measure sIgA in our study as it was not a diagnostic screening model. However, our finding of a higher prevalence of elevated *Candida* sp. in the CD group compared to controls raises the question: can *Candida* sp. act as an immunosuppressant through its ability to cause a sIgA deficiency, thus perpetuating its ability to colonise the intestine and trigger autoimmune responses such as CD in the genetically predisposed? Further research is required to investigate specifically the relationship and mechanisms between *Candida* sp. and sIgA in patients with CD.

The second consideration is that while *Candida* sp may or may not be a microbial environmental trigger for CD it could simply be associated with the persistent IBS-like
symptoms in CD patients adhering to a GFD. The microorganism *Candida* produces alcohol (Howard 1993) and contains glycoproteins that have the potential to stimulate mast cells to release histamine and inflammatory series 2 prostaglandins (PGE2), resulting in inflammation that has been related to the IBS-like symptoms that were reported by our CD cohort (Nosál, Novotný et al. 1974; Kanda, Tani et al. 2002).

The most common cause of opportunistic colonisation by *C. albicans* is antibiotic use. Our CD cohort reported a history of recurrent antibiotic use as children, i.e. > once per year in the first twelve years of life; however, we were unable to find suitable general population data to compare this to. Furthermore, we did not gather data regarding the specific proximity of antibiotic use and CD onset due to the difficulty of determining the duration of CD before diagnosis. Highly refined sugars and refined white flour products eaten on a regular basis have been suggested to encourage the growth of yeasts, including *Candida albicans*; however, there are conflicting results regarding this (Weig, Werner et al. 1999; Santelmann and Howard 2005). Analysis of the CD group’s diet diaries did demonstrate regular refined and white gluten-free flour intake at every meal, but we did not have access to information on dietary intake in our control group to allow for a statistically meaningful comparison. In addition, we did not obtain pre-diagnosis diet histories of the CD group, which may have provided more relevant support for the argument that diet influences the counts and opportunistic growth of *Candida* sp., thereby providing a microbial ‘assassin’ in HLA DQ2 and DQ8 genetically predisposed individuals. Well controlled dietary and mycology studies in individuals genetically predisposed to CD would be an important addition to future research.

### 4.4.2.3 The potential significance of *Saccharomyces* sp. in CD

To our knowledge it has not been reported that patients with CD have a significantly higher incidence of *Saccharomyces* sp. counts in their faeces than non-CD individuals. However, Mallant-Hent et al. (2006) found that up to 43% of patients with CD have anti-*Saccharomyces cerevisiae* antibodies (ASCAs) at diagnosis and that these antibodies disappear during treatment with a GFD. The disappearance of ASCAs after treatment was found to be more pronounced in children than in adults. It is suggested that ASCAs are more likely to persist in treated adult CD patients due to the more profound damage of the intestinal wall (as a consequence of their delayed diagnosis); therefore, resolution of intestinal permeability is slower (Mallant-Hent, Mary et al. 2006; Pérez-Torrado, Llopis et al. 2012). Our study findings would suggest otherwise: that treated adult CD patients with persistent symptoms have higher indigenous counts of *Saccharomyces* sp. and the presence of ASCAs may not be secondary to intestinal permeability, but rather an infection which may be the cause of this
persistent intestinal permeability. Furthermore, *Saccharomyces boulardii* is commonly prescribed as a probiotic supplement for individuals with gastrointestinal symptoms such as diarrhoea and has attracted research attention by many groups for its therapeutic potential (Hempel S and et al. 2012). In the light of these findings, supplementing individuals who have CD with *Saccharomyces* sp. may be a cause for concern. To our knowledge there have not been specific trials of the effects of *Saccharomyces* supplementation in patients with CD. Our finding may have important implications for clinical management, and the role of *Saccharomyces* sp. needs to be investigated in more detail.

### 4.4.2.4 Differences in opportunistic and pathogenic bacteria levels

A previous study has reported increased incidence of virulent *E.coli* strains and *Staphylococcus* sp. in patients with CD compared to controls. However, these were normalised after treatment with a GFD (Collado, Donat et al. 2009b). We did not find any significant differences between the numbers of pathogenic or opportunistic bacteria in the CD group compared to controls, possibly due to the CD group being on a GFD for more than twelve months at time of testing. This suggests that perhaps these organisms are simply opportunistic organisms in the untreated CD intestine rather than playing an aetiological role.

### 4.4.2.5 Summary of this comparative study’s findings with other groups’ findings

A number of studies reported that intestinal dysbiosis is associated with CD, findings that our study partially corroborates (Sanz, Palma et al. 2011). Some of the alterations in the microbiota are thought to be secondary opportunistic consequences of a damaged mucosa due to a normalisation of these alterations after adherence to a GFD. However, other alterations, such as the increased numbers of *Bacteroides* sp., *Clostridia* sp., *Prevotella* sp. and *Bifidobacteria* sp., have been associated with the disease regardless of symptoms and inflammation, suggesting a more significant role in the aetiology of CD. Our findings of the increased numbers of the yeasts *Candida* sp. and *Saccharomyces* sp. in patients with persistent symptoms of CD have not been reported before. We encourage other groups to explore the significance of these organisms in both this sub-group of CD patients but also their potential role in the aetiology of the disease.

Whilst this study cannot establish with certainty whether the differences in intestinal microflora observed in this study are a consequence or a cause of CD. The dietary impact of a GFD on the intestinal microbiome is not well understood and needs to be considered. Well controlled studies to elucidate whether there is indeed a difference between those including gluten in the diet and treated CD patients who are on a GFD are warranted.
4.4.3 The limitations of comparisons with other study findings

There are a number of points to consider when making comparisons of our study to publications investigating the gastrointestinal microbiota of CD. Firstly, we studied a sub-population of CD patients with persistent symptoms and not a paediatric population, which is a frequent focus of the literature. While studies of children over two years may be comparable to our results, it is thought that two categories of infants – healthy infants of less than twelve months and those of twelve months to two years – will both have a different gastrointestinal microbial composition to that of adults (Sellitto, Bai et al. 2012). Secondly, the molecular assessment of the gastrointestinal microbial ecology through faecal analysis has been found to be comparable to duodenal biopsies in CD (Collado, Donat et al. 2009a) and in IBS (Kerckhoffs, Ben-Amor et al. 2011). However, because the small intestine and the colon harbour different numbers of microbiota actual counts should not be compared. Thirdly, the predominant/commensal bacteria Bacteroides sp., Clostridia sp., Streptomyces sp., Fusobacteria sp. Prevotella sp., Mycoplasma sp. Bifidobacter sp., Lactobacillus sp and yeast levels were measured at a genus level, thereby including multiple species within the reported counts. Therefore we cannot compare our findings with others in regard to the specific composition within a genus. Fourthly, when considering the results of this study with the results of studies conducted in other countries it is important to consider that the intake of dietary sources of prebiotics may result in different microbial counts.

Lastly, the control group in this study is assumed not to be a homogeneous healthy control group. Due to the ethical and financial limitations of the study there was limited information gathered regarding the control group, other than results demonstrating they were serologically negative to CD, residing in NSW, Australia, between the ages of 18 and 75 years and had undertaken a faecal investigation for reasons other than the management of CD. Therefore, it is possible that other gastrointestinal pathologies were present in the control group that could influence both the microbial counts and species present.

4.4.4 Clinical relevance and recommendations for further research

CD patients who present to their health care professionals with persistent symptoms despite adherence to a GFD are often faced with further invasive and non-invasive investigations and are subject to a dietary audit. If these investigations yield no explanation for their symptoms they are sent home with a range of pharmaceuticals that may provide symptomatic relief. Our research indicates that gastrointestinal dysbiosis is prevalent in this population and faecal assessment may provide important clinical information in this sub-group of CD patients.
To extrapolate these findings further, where alterations in the faecal microbial ecology are identified the implementation of appropriate antimicrobial or bacteriotherapy may be indicated. Further studies are required to demonstrate that treatment of such findings is meaningful both clinically and microbiologically.

4.5 Conclusion

This study suggests that a sub-group of Australian CD patients who report persistent symptoms have differences in their gastrointestinal microbiome compared to Australians without CD. If this present study’s result were replicated in another cohort of individuals with CD, then we may have identified important microbial targets that could be involved in the development of symptoms despite adherence to a GFD and should also be explored in the aetiology of CD.
5 A Randomised Controlled Trial Exploring the Role of Intestinal Microbiota in the Symptom Scores and Quality of Life of Individuals with Coeliac Disease

5.1 Introduction

The survey of individuals with CD demonstrated that a significant number continue to experience some degree of persistent gastrointestinal symptoms despite adherence to a strict GFD diet for twelve months or longer (Chapter 3). Other groups have demonstrated that persistent symptoms are associated with a reduced QOL in patients with CD (Hallert and Lohiniemi 1999; Gray and Papanicolas 2010). A plausible hypothesis for these continued symptoms is that they are due to altered microflora in individuals with CD. In a study of the gastrointestinal microbiome we demonstrated that there were significant differences in the numbers of bacterial and fungal species between the adults with CD and those without CD (Chapter 4).

Therefore, a research plan was devised to investigate whether supplementation with a combination of probiotic species could alter the gastrointestinal microbiota and improve the symptom scores and QOL in individuals with CD.

5.2 Materials and methods

5.2.1 Hypothesis

We hypothesised that the incomplete resolution of symptoms in some individuals with CD, despite their adhering to a GFD, is attributed to aberrations in the intestinal microflora, and that manipulation of the gastrointestinal microflora by taking a multi-species Gram-positive probiotic supplement would result in symptom resolution and an improved quality of life.

5.2.2 Study aim

The aim of the study was to assess the gastrointestinal microbiota of adults with CD and the effects of a multi-strain probiotic supplement (VSL# 3™) on the composition of their gastrointestinal microbiota, symptom scores and quality of life in a rigorous controlled clinical study.
5.2.3 Study design

This multi-centre study was a randomised, double-blinded and placebo-controlled trial with staggered enrolment over a six week period. After a two to four week baseline assessment and lead-in period participants were enrolled for a twelve-week intervention and assessment stage, followed by a final assessment.

Study treatment arms were:

- Arm 1: a combined multi-species probiotic powdered formula (VSL#3™)
- Arm 2: placebo containing the same excipient base as the active treatment

The microbiome study and the clinical trial (CT) presented in the previous Chapter were undertaken simultaneously. The microbiome was a sub-study nested within the CT. As they were done at the same time there was obviously no way to select a probiotic mix for the CT study based on the findings of the microbiome study.

5.2.3.1 Study population

The study sought a targeted population of people with CD who were still experiencing persistent gastrointestinal symptoms despite reporting adherence to a strict GFD for the previous twelve months and who were willing to complete a series of questionnaires and pathology investigations. All participants had undertaken small bowel biopsy confirmation of CD and reported normalisation of serology for CD following twelve months adherence to a gluten free diet except in one case. Surrogate nutritional markers for disease activity included haemoglobin, red cell count, mean cell volume and red cell diameter width. These functional markers of anaemia and iron, B12 and folate deficiency were within normal limits in all CD participants.

5.2.3.2 Inclusion criteria

- Individuals who had CD confirmed by small bowel biopsy more than twelve months before enrolling in the study
- Individuals who had followed a GFD for at least twelve months
- Followed by normalisation of tTg and/or endomysial antibodies and/or evidence of partial or complete villous architecture repair.
- Individuals who were between 18 and 70 years of age.
5.2.3.3 Exclusion criteria

- Pregnant women
- Individuals under the age of 18
- Individuals who had cancer or who were HIV-positive
- Individuals who had been diagnosed with CD in the preceding 12 months
- Individuals who consumed a diet containing gluten
- Individuals with gastrointestinal pathology such as cancer, Crohn’s disease or ulcerative colitis
- Individuals with short bowel syndrome
- Individuals who had had recent oral or bowel surgery
- Individuals who were being treated with chemotherapy or radiotherapy
- Individuals who were using or had used non-steroidal or steroidal anti-inflammatory drugs and/or antibiotics in the four weeks before the start of the trial
- Individuals with serum urea, electrolytes or creatinine values greater than twice the upper limit of normal at baseline
- Individuals with liver function tests greater than three times the upper limit of normal at baseline
- Individuals who were unwilling to comply with the study protocol
- Individuals with any other condition which, in the opinion of the investigators, could compromise the study
- Individuals with active alcohol and or illicit drug dependence.

5.2.3.4 Randomisation

Participants were randomly allocated into a treatment or a placebo group. The randomisation schedule was prepared by an external researcher who had not participated in devising the study methodology. This researcher used a computer-generated blocked random sequence. The results of the randomisation were not revealed to the trial researchers and the data base was locked until analysis of the study was complete. Blinding was undertaken to ensure that at no stage did either the participants or the study group know to which treatment a participant had been randomly allocated. The preparations were distributed in numerical order, matching the participants’ enrolment number with the number on the intervention label.
5.2.3.5 Blinding
The intervention sachets were packaged identically; however, when two batches of the left-over sachets were returned and opened at the end of the study it became apparent that there were two visibly different contents. Considering the study participants did not know each other the chance of them discovering this difference was extremely small.

5.2.3.6 Sample size
A power calculation was undertaken using data from a pilot study that had demonstrated larger counts of Gram-positive bacteria in CD participants than in controls. The sample size was estimated using PASS 2008 (Dattalo 2008). The data used for the calculation were the mean of the Gram-negative to Gram-positive bacteria from the pilot study (CD group, n=30) and the mean of the laboratory data of healthy people (control group, n=117).

It was estimated that sample sizes of 19 per group would have 80% power (1-beta) to detect differences between groups in a mean change of 2.5 with standard deviations of the changes of 3.0 in each group at \( p=0.05 \) (1-tailed). This would occur if there was a mean increase of 2.5 from 2.0 to 4.5 in the treatment group and no change in the placebo group. Forty-five participants were recruited to accommodate for a possible 20% drop-out rate resulting from withdrawals and exclusions due to failure to meet study criteria, adverse events and non-compliance.

5.2.3.7 Duration
The duration of the study was 16 weeks. The participants enrolled in the study at different times over a six month period. Their involvement after screening ranged from two to 16 weeks, due to one participant’s withdrawing after the first two weeks following an exacerbation of their gastrointestinal symptoms.

5.2.3.8 Adverse events
To ensure that symptoms measured at week four, eight or twelve were not caused by ill health, accident or extreme stress, participants were required to immediately report to the study coordinator any events, medication additions or changes. Details of ill health events and medication changes were recorded and discussed by telephone and at the final interview. Serious ill health and severe stress, e.g. hospitalisation, were grounds for withdrawal from the trial and reported as serious adverse events.

5.2.4 Primary outcome measure
The primary outcome measure was a comparison between the measures of a number of gastrointestinal microbiota, including predominant, pathogenic and opportunistic bacteria.
(CFU per gram of faeces), yeasts (parts per gram of faeces), and detection or non-detection of parasites. A sample report is presented in Appendix C1.

5.2.5 Secondary outcome measures

The secondary outcome measures included:
- QOL of life and symptom score parameters;
- urinary metabolomics; and
- faecal lactoferrin;

5.2.5.1 The CD-specific quality of life and symptom score questionnaire

The validated CD symptom score and quality of life (CDSQOL) questionnaire (Hauser, Gold et al. 2007) instrument is presented in Appendix D1 and consisted of twenty-eight questions divided into four sub-scales with seven possible Likert scale responses pertaining to:
- emotion;
- social;
- worry; and
- gastrointestinal.

The CDSQOL individual symptom sub-scale and total scores for each participant were calculated as a secondary outcome measure.

5.2.5.2 Urinary metabolomics

Urinary organic acids that are products of dietary, bacterial, protozoal or fungal metabolism in the luminal gut were measured as µg/ML at baseline and again at week twelve in both treatment arms (placebo and probiotic-supplemented group). A sample report is presented in Appendix D2.

5.2.5.3 Faecal lactoferrin

Faecal lactoferrin levels were measured at baseline and again at week twelve after probiotic or placebo supplementation.

5.2.6 Assessment tools

A faecal molecular and microscopic analysis as described Chapter 4 was conducted. The analysis was undertaken at Metametrix™ Laboratory (Atlanta, GA, USA).

5.2.6.1 Urinary metabolomic analysis

A published methodology was employed for the assessment of urinary organic acids (Crow, Bishop et al. 2008) at Metametrix™ Laboratory (Atlanta, GA, USA) and is described here.
**Instrumentation:** The chromatographic system consisted of a Waters (Milford, MA, USA) 2695 high performance liquid chromatograph featuring a refrigerated auto-sampling compartment and column heater. This system was connected to a Waters Quattro-micro tandem mass spectrometer equipped with an electrospray ionization source. Data was collected and processed using MassLynx v4.1.

**Reagents and supplies:** formic acid, ACS-grade, was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA) and high performance liquid chromatography-grade acetonitrile was obtained from EMD™ Chemicals Inc. (Darmstadt, Germany). The deuterated isotopes of p-hydroxyphenyllactic acid and hippuric acid were purchased from Content Delivery Network (CDN) Isotopes™ (Quebec, Canada) and deuterated benzoic acid from Cambridge Isotopes (Andover, MA, USA). Formic acid ammonium salt p-hydroxyphenylacetate, p-hydroxybenzoic acid, 3,4 di-hydroxyphenylprorionic acid, phenylacetate, phenylprorionic acid, indican and hippurate were obtained from Sigma Chemical (St Louis, MO, USA).

**Preparation of solutions:** the internal standard was prepared in a urine matrix. In a 200 mL volumetric flask d5-benzoic acid (d5-BA, 2.5 mg), d4-p-hydroxybenzoic acid (d4-p-OH BA, 5.0 mg) and d2-hippuric acid (d2-HIP, 125 mg) were dissolved in pooled urine and stored at 4°C for up to one month without any degradation. A standard stock solution with a concentration five times greater than the highest calibrator was prepared by dissolving the reported amount of aromatic acids with a 95:5 water–acetonitrile solution in a 200 ml volumetric flask. Working solutions of the calibrators were prepared by serial dilution with deionized water. All standard solutions were stored at 4°C for up to one month.

**Sample collection and preparation:** participant preparation and sample collection as per laboratory instructions are presented in Appendix C2. Urine samples were collected in tubes containing thymol as a preservative and stored at −20°C until time for analysis. Samples were prepared by addition of 350 μL of either calibrator or urine to 650 μL of internal standard matrix solution in an autosampler vial.

**Chromatographic separation:** separation was performed on a Phenomenex (Torrance, CA, USA) Synergi Polar-RP 4 μm 80 Å, 2.0. 50 mm analytical column maintained at 40°C throughout the analysis. The mobile phase consisted of 10 mM ammonium formate adjusted to a pH of 3.5 with formic acid (MPA) and acetonitrile with 0.15% v/v formic acid (MPB). Samples were injected (15 μL) on column with initial conditions set to 95% MPA and 5% MPB at a flow rate of 0.4 mL/min. The concentration of MPB was increased by the use of Water’s concave gradient 7 to 50% over four minutes, followed by a one minute hold. The flow rate was increased to 0.5 mL/min at five minutes and held until the start of the next
injection cycle. The mobile phase composition returned to the original conditions at six minutes. The total run time, including column reequilibration, was eight minutes.

**Evaluation of matrix effects:** Matrix effects were evaluated using a simple matrix matching experiment (Bishop *et al.*, 2007; Norton *et al.*, 2007). Specifically, evaluation was performed by comparing the slopes of calibration curves representing standard solutions prepared in solvent (without matrix) and prepared in urine (with matrix). For both calibration curves linear plots were made for the area under the curve versus the standard concentration. The slopes for each curve, with and without matrix, were compared using Student’s *t*-test to determine the effects of the matrix on analyte sensitivity.

**Mass spectrometry conditions:** Detection of all analytes was carried out in negative electrospray ionization mode. Mass spectrometer conditions were obtained by a direct infusion of individual aromatic acid standard solutions in line with the high performance liquid chromatography (HPLC) set to initial mobile phase conditions. The desolvation and nebulizer nitrogen gases were maintained at 650 and 50 L/h, respectively. The capillary voltage was set to 2.0 kV, with source and desolvation temperatures at 140 and 350°C, respectively. The cone and collision settings were established individually for each analyte and internal standard for multiple reaction monitoring detection was conducted. The dwell time for each mass was 0.1 seconds, and each mass was collected at unit mass resolution.

**Measurement of urinary creatinine:** Urinary creatinine concentration was measured on a Cobas Mira Plus using a creatinine assay kit purchased from Roche (Quebec, Canada), following a modified picric acid method (Slot, 1965).

### 5.2.6.2 Faecal lactoferrin

On receipt of faecal samples at Metametrix™ Laboratory, and before DNA-PCR analysis, faecal samples were assessed specifically for lactoferrin. The methodology employed is now proprietary to Genova Diagnostic Laboratories, (Ashville, USA).

### 5.2.6.3 Blood analysis

Standard safety blood measures (Full Blood Count, Liver Function Test, Electrolytes, Urea and Creatinine) were conducted. All blood samples were drawn and analysed by accredited Sonic Health Care Laboratories in Sydney (Douglas Hanly Moir), Woolongong (Southern IML Pathology) and the North Coast of New South Wales (Sullivan & Nicolaides). All laboratories were fully accredited with the National Association of Testing Authorities (NATA) and the Royal College of Pathologists of Australasia (RCPA).
5.2.6.4 Health-related quality of life: CDSQOL

The assessment of HRQOL is an important outcome measure in clinical studies in gastroenterology (Hauser 2007). HRQOL refers to the patients’ subjective assessment of their physical, mental and social dimensions of wellbeing and functioning. The disease-specific HRQOL questionnaire for CD employed in this study was developed using a methodological framework following pre-defined criteria of testing theory and standards for validating health measures (Hauser, Gold et al. 2007).

The CDSQOL scores were calculated using scores obtained from participants’ self-reporting. (The CDSQOL and interpretative guidelines are presented in Appendix D1). Participants were asked to score the twenty-eight questions on a scale of 1-7 at baseline and at weeks four, eight, twelve and 16 of the study period. The questions were categorised into four sub-scales (emotion, worry, social and gastrointestinal symptoms). Each of the four sub-scales’ total scores were then calculated to answer specific areas related to symptoms and quality of life. The total sum of all four sub-scales was calculated at baseline and at week twelve.

5.2.6.5 Adherence to a GFD: the three-day diet diary

Strict adherence to a GFD is associated with a resolution in symptoms by the majority of patients with CD (Faulkner-Hogg, Selby et al. 1999). Therefore, evaluation of GFD adherence was important to rule out non-compliance or accidental dietary ingestion of gluten as the cause of the study participants’ persistent symptoms.

Current serological tests have a high sensitivity and specificity for the diagnosis of CD and are employed to monitor response to and adherence to a GFD. However, a number of studies have concluded that serology cannot replace the sensitivity and specificity of an expert nutritionist’s assessment (Leffler, Edwards George et al. 2007; Leffler, Dennis et al. 2009).

Therefore, a nutritionist’s assessment utilising a three-day diet diary instrument was employed (Appendix D3). Participants were asked to list what they had consumed, including foods, fluids and medications and their brand names, how they had prepared or purchased them, i.e. at home or at a food outlet, over a three-day period at baseline, and during week six and week twelve. The diet diary was then analysed for sources of gluten, in conjunction with an in-depth nutritionist consultation (conducted by the study coordinator). The nutritionist’s general rating score is outlined in Table 5.1. The instrument was used as a monitoring and assessment tool to determine either inclusion in or exclusion from the trial. If gluten was reported at less than an excellent level (Table 5.1) at baseline the participant was not included in the trial. If foods containing gluten were reported in the diet diary at weeks six and 12 this
was simply recorded and rated as in the scale below, but participants in this category were not excluded as the diaries were not sighted until the final interview.

Table 5.1 Dietary compliance rating score (Faulkner-Hogg, Selby et al. 1999)

<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Excellent</td>
<td>Participant eats gluten less than three times per year</td>
</tr>
<tr>
<td>2. Good</td>
<td>Participant eats gluten once per month</td>
</tr>
<tr>
<td>3. Fair</td>
<td>Participant eats gluten two to three times per month</td>
</tr>
<tr>
<td>4. Poor</td>
<td>Participant eats gluten once or twice per week</td>
</tr>
<tr>
<td>5. Very Poor</td>
<td>Participant eats gluten more than twice per week</td>
</tr>
<tr>
<td>6. Poorest</td>
<td>Participant does not eat a GFD</td>
</tr>
</tbody>
</table>

5.2.6.6 Study formulation
The study formula and placebos were manufactured and supplied by VSL Pharmaceuticals Inc., Rome. The sachets were labelled according to the randomisation schedule and delivered to the study site in Sydney.

5.2.6.7 Active formulation
The study preparation VSL#3™ was a proprietary blend of probiotic bacteria containing 450 billion viable lyophilised bacteria *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaris*. The preparation was manufactured by VSL Pharma (USA) and is not listed on the Australian Register of Therapeutic Goods (TGA). Therefore notification and subsequent approval was obtained from the TGA for the use of a non-registered product (TGA file no. 2010/019542) and is presented in Appendix D4.

5.2.6.8 Placebo
A placebo was made to match the active formulation. It was made up with the same excipient base, i.e. maltose derived from corn, and was identical in size, weight and packaging to the active medicine.

5.2.7 Ethics
The study was approved by the Human Research Ethics Committee of Southern Cross University (ethics approval number ECN-12-021) (Appendix D5). The research was conducted in compliance with GCP and in accordance with the guidelines of the Australian National Health and Medical Research Council and the Declaration of Helsinki (as revised in
2004). The trial was registered with the Australian and New Zealand Clinical Trials Register (ACTRN12610000630011) (Appendix D6).

5.2.8 Study procedures

5.2.8.1 Recruitment
Participants for the clinical trial were recruited through the New South Wales Coeliac Association email data base. The online study survey invited respondents who experienced persistent symptoms despite a GFD to contact the study coordinator if they were interested in receiving information about a clinical trial.

Interested individuals who had read the information sheet provided (Appendix D7) contacted the clinical trial coordinator to participate in a phone screening interview (Appendix D8). Eligible participants were provided with a comprehensive verbal overview of the study requirements and invited to attend an initial visit and assessment.

5.2.8.2 Study environment
The study venues were Diagnostic Insight Pathology consultation rooms in St Leonards, Sydney, and the Southern Cross University Health Clinic at Lismore.

The study procedures for the clinical trial are presented in Table 5.2.

5.2.8.3 Phase one of clinical interview
The aims and protocols were explained to the participants. Participants were then invited to ask any questions before signing the informed consent form (Appendix D9). After signed consent was obtained participants commenced the processes involved in the clinical trial.

The study coordinator took a comprehensive medical history and systems review (Appendix D10). A physical examination of the abdomen was conducted and blood pressure, heart rate, weight, and waist and hip circumference were measured and recorded. Participants were requested to complete a baseline CDSQOL (Appendix D1).
Table 5.2 Schedule of Events

<table>
<thead>
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<th>Week</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>8</th>
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<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Phone /email contact/monitoring</td>
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<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
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</tr>
<tr>
<td>Inclusion/exclusion</td>
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<td></td>
<td></td>
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<tr>
<td>Informed consent</td>
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<tr>
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<td>CD-specific questionnaire</td>
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<td>Three-day dietary recall diary</td>
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<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concomitant medication use</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Record study drug use</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Record adverse events</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

5.2.8.4 Phase two of clinical trial interview

After the assessment session participants were guided through the procedure required for having a blood sample taken and the instructions for faecal and urine specimen collection and submission. The participants left the study site with a plastic-sleeved folder containing

- a dated schedule of activities to be completed through the clinical trial;
- a clinical trial wallet card (Appendix D11);
- clinical trial information sheet;
- blood test request forms for blood collection at baseline and week twelve (Appendix D12);
- CDSQOL questionnaires for weeks four, eight, twelve and 16;
- diet diaries to be completed in weeks six and twelve; and
- instructions and courier slips for collection and return of faecal and urine kit (Appendix D13)
Participants were also given other items necessary for the clinical trial:

- two boxes of ninety sachets each containing either placebo or VSL# 3 probiotic (to be commenced after blood test results were assessed as being within normal limits, and urine and faecal samples were submitted);
- faecal collection kits for baseline and week twelve specimen collections; and
- urine collection kits for baseline and week twelve specimen collections.

Once the initial blood sample had been taken participants were instructed to wait to hear from the study coordinator about their results before commencing the next stage.

The blood test reports were reviewed on receipt by the study coordinator to check for violation of the study exclusion criteria. This was followed by the study coordinator contacting the participant by phone. The participants were instructed to use the kits provided at the initial visit and to submit both a faecal and urine specimen that had been passed on the same day. The specimens were collected on the same day by an authorised biological specimens courier and returned immediately to the study site for checking. The urine was frozen and the faeces were refrigerated for overnight shipment on ice to Metametrix™ Laboratories in (Atlanta, GA, USA).

5.2.8.5 Safety measures
Hanly Moir Pathology analysed the blood samples taken from participants in Sydney. Sullivan & Nicolaides Pathology analysed the samples taken from participants in Lismore and the Northern NSW region. Southern IML Pathology analysed the blood samples taken from participants in the southern NSW region. Standardised measures were employed so that results were comparable. Where blood test results were outside the parameters of the reference range participants were contacted by phone and referred to their doctor. A letter was sent to the relevant medical practitioner with the permission of the participant. Case-by-case decisions were made by the research team regarding a participant’s continued involvement if any blood parameters were outside the reference range but were lower than those stated in exclusion criteria.

5.2.9 Intervention stage
The intervention stage consisted of twelve weeks of self-administration of either the probiotic VSL#3™ or the placebo.

5.2.9.1 Allocation to treatment
Participants were randomly allocated to the probiotic or the placebo group according to the randomisation schedule provided by an independent researcher. Participants were given with
the appropriate intervention, which was supplied by Orphan-Sigma Proprietary Ltd. Twenty-three received the probiotic and twenty-two the placebo sachets.

5.2.9.2 Dispensing of study medication
The medication was supplied in two labelled cardboard boxes containing ninety airtight sealed sachets and labelled according to the Therapeutic Goods Administration (TGA) GCP guidelines. This was a sufficient supply for twelve with an extra twelve sachets.

5.2.9.3 Administration and dosage
The active and placebo groups were instructed to mix one sachet into water or juice with both morning and evening meals (twice daily). The sachets were not to be mixed with hot water or carbonated drinks (Appendix D14).

5.2.9.4 Contact with participants at weeks four, six, eight, twelve and 16
Throughout the intervention stage the study coordinator contacted the participants by email or phone to remind them to complete the CDSQOL questionnaire or diet diary at weeks four, eight and twelve. Study participants then had an opportunity to notify the study coordinator of any changes to concomitant medication. Participants were also invited to contact the researcher at any time throughout the trial if they had any concerns regarding the trial or their health.

5.2.9.5 Phase one of final interview and clinical assessment
Participants attended a final interview and assessment at the end of week 12. They were instructed to have a blood sample taken the day before the interview and to bring a urine and faecal specimen in the kits provided at the initial interview. The remaining study medicine was returned and counted. Completed diet diaries and CDSQOL questionnaires were retained for analysis. The study coordinator re-administered the systems review and measured and recorded blood pressure, heart rate, weight, and waist and hip circumference. Participants were invited to ask any questions or remark on any aspect of their experience as a participant in the clinical trial.

Participants were informed that they would be contacted in due course regarding the outcome of their week twelve blood, urine and faecal tests. With their permission a copy of results would be sent to their doctors with an accompanying letter explaining their involvement in the trial.

5.2.9.6 Compliance
All remaining sachets were returned to the study site at the final interview and counted; the study coordinator maintained an inventory of the sachets received from the manufacturer and
those dispensed to each participant (180 sachets per participant). It was assumed that any sachets not returned had been taken. Full compliance was equivalent to one sachet taken twice daily for twelve weeks, i.e. 168 sachets. Compliance was recorded simply as doses missed out of a total of 168 sachets.

5.2.10 Statistical analysis

The statistical packages used were SPSS PASW®Statistics GradPack 18 and version 20 SPSS. Significance was assumed if $p \leq 0.05$.

Basic descriptive statistics were conducted to describe the characteristics of the sample population and check the variables for any violation of the assumptions underlying the statistical techniques planned to answer the research questions. Demographic and health indices data were analysed using independent $t$-tests, ANOVA, and chi-square in order to determine the homogeneity of the allocation to the groups.

The primary outcome was the existence of changes in faecal microorganisms from baseline to week twelve for the two groups. Analysis of quantitative counts of bacteria and phyla classes was conducted with a two-way repeated measures ANOVA, with the repeated time factor baseline and week twelve, and between subject treatment groups (probiotic or placebo), where the interaction of time and group effect was the main interest. Analysis of scale count data for mycology and binary data for parasites was conducted with a Generalised Estimated Equation with a repeated time factor baseline and week twelve, and between subject treatment groups (probiotic or placebo), where the interaction of time and group was the main effect.

Secondary endpoints were the changes in CDSQOL scores, urinary organic acid, complete blood counts, and blood urea, creatinine and electrolyte levels compared to changes in gastrointestinal microflora. A two-way repeated measure of ANOVA was employed here too. A repeated measures model was used with four time points (baseline and weeks four, eight and twelve) for the treatment groups (probiotic or placebo), and time by treatment interaction for:

- The CDSQOL sub-scale and total scores, where the question of difference between the groups and time was determined by the time by treatment interaction in all cases; and
- Where the question of difference was between the symptom scores/quality of life and microflora as the interaction effect, and the symptom scores/quality of life and organic acids as the interaction effect.
5.3 Results

The results of the Clinical Trial are presented in Tables throughout this chapter. Graphical representation of the data is presented in Appendix D17.

5.3.1 Demographics

Seventy-five people were screened by phone, resulting in 45 interviews and subsequent enrolments. A total of thirty-seven females and eight males (mean age 47.3 years) residing in NSW, Australia, were enrolled in the study at baseline.

5.3.1.1 Clinical characterisation of persistent symptoms

All CD participants reported being troubled by gastrointestinal symptoms despite adherence to a GFD. The assessment and classification of the symptoms reported by the CD participants is described in section 4.3.1.1.

5.3.2 Withdrawals

A total of three participants withdrew in the intervention stage of the clinical trial. One participant failed to comply with the study protocol by not completing the questionnaires and not taking the clinical study medicine. One participant was withdrawn due to mild adverse effects (constipation, bloating and flatulence). One participant withdrew due to a cycling accident and hospitalisation. The randomisation code for this participant was broken revealing their allocation to the placebo arm of the trial. The event was reported to TGA and was considered to be unlikely to be associated with the trial medicine (Appendix D15).

5.3.2.1 Demographics (after intervention stage withdrawals)

Forty-two participants aged between 18 and 74 years (mean age 47.5 years) completed the study: 21 in the active group aged between 18 and 74 years (mean age 47.1 years), with a mean body mass index (BMI) of 23.64 kg/m², and twenty-one in the placebo group aged between 23 and 66 years (mean age 47.5 years), with a mean BMI of 23.2 kg/m².

To see if there were any differences between demographic and health indices at baseline between the active group and the placebo group, frequency descriptive statistics, ANOVA and independent samples t-tests were conducted. The results are presented in Table 5.3. The two groups did not differ significantly (p≥0.05) in any of the comparisons.
Table 5.3 Comparison of demographic and health indices

<table>
<thead>
<tr>
<th>Demographic and Health Indices</th>
<th>Placebo (n=21)</th>
<th>Probiotic (n=21)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age mean (±SD)</td>
<td>47.5 (±12.87)</td>
<td>47.1 (±16.06)</td>
<td>0.924</td>
</tr>
<tr>
<td>Gender: males</td>
<td>4 (18)</td>
<td>3 (14)</td>
<td>0.946</td>
</tr>
<tr>
<td>Gender: females</td>
<td>17 (77)</td>
<td>18 (85)</td>
<td>0.946</td>
</tr>
<tr>
<td>Proton pump inhibitor use</td>
<td>4 (18)</td>
<td>4 (19)</td>
<td>0.271</td>
</tr>
<tr>
<td>Anti-depressant use</td>
<td>4 (18)</td>
<td>3 (14)</td>
<td>0.644</td>
</tr>
<tr>
<td>Anti-inflammatory use</td>
<td>2 (9)</td>
<td>2 (9)</td>
<td>0.964</td>
</tr>
<tr>
<td>No family history of CD</td>
<td>13 (59)</td>
<td>16 (76)</td>
<td>0.474</td>
</tr>
<tr>
<td>Family history of CD</td>
<td>9 (40)</td>
<td>7 (33)</td>
<td>0.474</td>
</tr>
<tr>
<td>No improvement of villous architecture &gt; 12 months of GFD</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>0.975</td>
</tr>
<tr>
<td>Partial improvement of villous architecture after &gt;12 months of GFD</td>
<td>8 (36)</td>
<td>12 (57)</td>
<td>0.975</td>
</tr>
<tr>
<td>Full villous architecture restoration after &gt;12 months of GFD</td>
<td>5 (22)</td>
<td>5 (23)</td>
<td>0.975</td>
</tr>
<tr>
<td>No follow-up biopsy conducted</td>
<td>5 (22)</td>
<td>5 (23)</td>
<td>0.975</td>
</tr>
<tr>
<td>Had a tonsillectomy</td>
<td>11 (50)</td>
<td>12 (57)</td>
<td>0.843</td>
</tr>
<tr>
<td>Breast-fed for 6–12 months</td>
<td>8 (36)</td>
<td>8 (38)</td>
<td>0.887</td>
</tr>
<tr>
<td>&gt; 3 course of antibiotics in first 12 months of life</td>
<td>13 (59)</td>
<td>13 (61)</td>
<td>0.975</td>
</tr>
<tr>
<td>&gt; 3 respiratory infections in first 12 months of life</td>
<td>13 (59)</td>
<td>15 (71)</td>
<td>0.975</td>
</tr>
<tr>
<td>Known food intolerance other than gluten</td>
<td>11 (50)</td>
<td>12 (57)</td>
<td>0.843</td>
</tr>
<tr>
<td>No known food intolerance other than gluten</td>
<td>11 (50)</td>
<td>11 (52)</td>
<td>0.887</td>
</tr>
</tbody>
</table>

* Statistical significance between groups set at p≤0.05
5.3.3 Compliance with medication use

The study coordinator maintained an inventory record of the sachets received and dispensed throughout the study. It was assumed that sachets not returned had been taken. Non-compliance was recorded as doses missed. In addition, participants were asked to record any missed doses. The mean number of doses missed in the total group was eight, equivalent to four days. The results of an independent \( t \)-test showed there was no significant difference \( (p=0.488) \) between the two groups in number of doses missed. Good compliance was considered to be ingestion of greater than 80–85\% of the study medication. Compliance in this study was 95.2\%.

5.3.4 The primary outcome measure: faecal microbiology

5.3.4.1 Differences between active and placebo groups in faecal predominant bacteria at baseline and week 12

Descriptive statistics for the predominant bacterial outcome measures and phyla classes by treatment arm and by measurement occasion (baseline and week 12) are presented in Tables 5.4 and 5.5 below. At baseline only *Streptomyces* sp. \( (p=0.058) \) differed between the two groups, with the active group having 39 million CFU \( (3.99 \times 10^7, \text{SD} \pm 1.98) \) per gram of faeces and the placebo group 30 million \( (3.00 \times 10^7, \text{SD} \pm 1.34) \). At week 12 only, *Mycoplasma* sp. \( (p=0.026) \) differed between the two groups, with the active group having 43 million CFU per gram of faeces \( (4.36 \times 10^7, \text{SD} \pm 2.46) \) and the placebo group 29 million CFU \( (2.99 \times 10^7, \text{SD} \pm 1.13) \).
Table 5.4 Descriptive statistics for the levels of predominant bacteria and Phyla classes between groups

| Predominant bacterial species measures | Week | Probiotic Group | | Placebo Group | |
|--------------------------------------|------|----------------||----------------|----------------|
|                                      | n    | Mean | SD  | Min  | Max  | n    | Mean | SD  | Min  | Max  |
| *Bacteroides* sp.                    | 0    | 23   | 4.1 | 2.72 | 1.7  | 11.9 | 22   | 4.0 | 2.29 | 1.5  | 8.0  |
|                                      | 12   | 21   | 5.6 | 5.39 | 1.9  | 24.0 | 21   | 4.6 | 2.57 | 1.7  | 12.4 |
| *Clostridia* sp.                     | 0    | 23   | 4.4 | 2.09 | 1.8  | 9.1  | 22   | 3.8 | 1.62 | 1.9  | 7.4  |
|                                      | 12   | 21   | 4.4 | 2.93 | 1.6  | 10.7 | 21   | 4.6 | 2.42 | 1.4  | 9.9  |
| *Prevotella* sp.                     | 0    | 23   | 4.2 | 2.59 | 1.5  | 13.6 | 22   | 3.2 | 1.67 | 1.6  | 7.4  |
|                                      | 12   | 21   | 3.3 | 1.73 | 1.7  | 8.5  | 21   | 2.9 | 1.33 | 1.7  | 5.8  |
| *Fusobacteria* sp.                   | 0    | 23   | 4.5 | 3.51 | 1.9  | 13.2 | 22   | 4.3 | 3.06 | 1.7  | 13.4 |
|                                      | 12   | 21   | 4.9 | 7.18 | 1.4  | 34.8 | 21   | 3.8 | 2.84 | 1.3  | 10.2 |
| *Streptomyces* sp.                   | 0    | 23   | 3.9 | 1.98 | 1.7  | 9.9  | 22   | 3.0 | 1.34 | 1.6  | 6.7  |
|                                      | 12   | 21   | 4.1 | 2.15 | 1.5  | 9.2  | 21   | 3.2 | 2.04 | 1.7  | 9.8  |
| *Mycoplasma* sp.                     | 0    | 23   | 3.3 | 1.34 | 1.6  | 6.4  | 22   | 3.4 | 1.73 | 1.7  | 8.3  |
|                                      | 12   | 21   | 4.3 | 2.46 | 1.9  | 9.8  | 21   | 2.9 | 1.13 | 1.6  | 5.4  |
| *Lactobacillus* sp.                  | 0    | 23   | 4.3 | 2.30 | 1.8  | 11.3 | 22   | 6.5 | 6.41 | 1.8  | 29.8 |
|                                      | 12   | 21   | 4.6 | 3.48 | 1.5  | 17.3 | 21   | 6.2 | 6.63 | 2.1  | 32.0 |
| *Bifidobacteria* sp.                 | 0    | 23   | 5.2 | 2.91 | 2.2  | 11.8 | 22   | 5.1 | 2.41 | 1.8  | 9.5  |
|                                      | 12   | 21   | 4.8 | 3.22 | 1.9  | 15.2 | 21   | 3.4 | 1.45 | 1.5  | 7.6  |
| *Escherichia coli* sp.               | 0    | 23   | 3.7 | 1.92 | 1.6  | 8.3  | 22   | 3.3 | 1.44 | 1.6  | 6.4  |
|                                      | 12   | 21   | 5.0 | 2.86 | 1.5  | 11.7 | 21   | 4.0 | 2.36 | 1.7  | 10.2 |
| Predominant bacterial total counts   | 0    | 23   | 38.06 | 8.95 | 23.3 | 58.7 | 22   | 36.8 | 10.75 | 23.6 | 75.3 |
|                                      | 12   | 21   | 41.34 | 15.35 | 22.3 | 73.4 | 21   | 36.0 | 12.18 | 24.5 | 68.0 |
Table 5.5 Descriptive statistics for Probiotic and Placebo groups in measures of Phyla classes at baseline and week 12

<table>
<thead>
<tr>
<th>Phyla class measures</th>
<th>Week</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>0</td>
<td>23</td>
<td>65.7</td>
<td>8.60</td>
<td>50</td>
<td>78</td>
<td>22</td>
<td>69.9</td>
<td>9.40</td>
<td>54</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21</td>
<td>67.0</td>
<td>11.84</td>
<td>31</td>
<td>82</td>
<td>21</td>
<td>68.5</td>
<td>9.04</td>
<td>41</td>
<td>86</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>0</td>
<td>23</td>
<td>34.2</td>
<td>8.60</td>
<td>22</td>
<td>50</td>
<td>22</td>
<td>30.0</td>
<td>9.40</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21</td>
<td>33.0</td>
<td>11.84</td>
<td>18</td>
<td>69</td>
<td>21</td>
<td>46.1</td>
<td>2.57</td>
<td>1.7</td>
<td>12.4</td>
</tr>
</tbody>
</table>

5.3.4.2 Primary outcome measure: comparison between groups by treatment arm and measurement occasion for opportunistic and pathogenic bacteria

Cross-tabulations were conducted for the presence or absence of opportunistic and pathogenic bacteria to see if there were differences between the groups by treatment arm (probiotic or placebo) and measurement occasion (baseline and week 12). Table 5.6 presents the results of cross-tabulations for opportunistic bacteria. Table 5.7 presents the results of cross-tabulations for pathogenic bacteria. The results show that there were no statistical differences at baseline or week 12 between the groups for the presence or absence of any opportunistic or pathogenic bacteria (p>0.05). If an organism was not present in any participants on one or both treatment arms statistical analysis failed to provide the test statistic and is reported as unable to calculate (UAC). It is thought that opportunistic and pathogenic organisms have a greater clinical and biological impact on gastrointestinal ecology when counts exceeding 1.0E + 005 (100,000) CFU per gram of faeces are detected. Therefore organisms in these two categories are reported as present when >100,000 CFU/g faeces were detected. In the present study an exception to this was for the opportunistic organisms Streptococcus sp. and Enterococcus sp., as the laboratory was unable to count the number of CFU but instead reported whether the DNA of Streptococcus species or of Enterococcus species were present or not in the faecal samples. Therefore the results for the Streptococcus sp. and Enterococcus species cannot be interpreted with any biological or clinical significance. For this reason these two organisms are reported in the grey shade code in Table 5.6.
Table 5.6 Results of cross-tabulations between groups and measurement occasions for presence or absence of opportunistic bacteria

<table>
<thead>
<tr>
<th>Bacteria Species</th>
<th>Week</th>
<th>Probiotic</th>
<th>Placebo</th>
<th>Chi-square Analysis p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not Detected</td>
<td>Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td><strong>Streptococcus sp.</strong></td>
<td>0</td>
<td>1 (4.5)</td>
<td>21 (95.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0 (0)</td>
<td>21 (100)</td>
<td>1 (5.3)</td>
</tr>
<tr>
<td><strong>Enterococcus sp.</strong></td>
<td>0</td>
<td>4 (19)</td>
<td>17 (81)</td>
<td>3 (15)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>9 (47.4)</td>
<td>10 (52.6)</td>
<td>9 (50)</td>
</tr>
<tr>
<td><strong>Aeromonas sp.</strong></td>
<td>0</td>
<td>23 (100)</td>
<td>0 (0)</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20 (95.2)</td>
<td>1 (4.8)</td>
<td>21 (100)</td>
</tr>
<tr>
<td><strong>Klebsiella oxytoca</strong></td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>22 (100)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td><strong>Achromobacter sp.</strong></td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>22 (100)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>19 (90.5)</td>
</tr>
<tr>
<td><strong>Bacillus sp.</strong></td>
<td>0</td>
<td>23 (100)</td>
<td>0 (0)</td>
<td>20 (90.9)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td><strong>Morganella morganii</strong></td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20 (95.2)</td>
<td>1 (4.8)</td>
<td>21 (100)</td>
</tr>
<tr>
<td><strong>Citrobacter sp.</strong></td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>22 (100)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td><strong>Total opportunistic bacteria detected</strong></td>
<td>0</td>
<td>20 (87)</td>
<td>3 (13)</td>
<td>18 (81.8)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18 (85.7)</td>
<td>3 (13.3)</td>
<td>19 (90.5)</td>
</tr>
</tbody>
</table>

UAC denotes ‘unable to calculate’ due to organisms not being present in any participants in one or both treatment arms

---

Table 5.7 Results of Cross-tabulations between groups and measurement occasion for the presence or absence of pathogenic bacteria

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Week</th>
<th>Probiotic</th>
<th>Placebo</th>
<th>Chi-square Analysis p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not Detected</td>
<td>Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td><strong>H. pylori</strong></td>
<td>0</td>
<td>16 (69.6)</td>
<td>7 (31.4)</td>
<td>12 (54.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16 (76.2)</td>
<td>5 (32.8)</td>
<td>15 (71.4)</td>
</tr>
<tr>
<td><strong>Escherichia haemorrhagic coli</strong></td>
<td>0</td>
<td>20 (87.0)</td>
<td>3 (13)</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18 (85.7)</td>
<td>3 (13.3)</td>
<td>21(100)</td>
</tr>
</tbody>
</table>
5.3.4.3 Primary outcome measure: comparison between groups by treatment arm and measurement occasion for mycology

The categorical data and scale counts reported by the laboratory for mycology measures (0+ or 2+) was cross-tabulated to see if there was any difference between groups and measurement occasion. Results of cross-tabulations are presented in Table 5.8. Where some fungal organisms were not present in any participants on one or both treatment arms, statistical analysis failed to provide the test statistic and these organisms are reported as UAC. The value set by the laboratory for reporting the presence of a fungal organism was 1000 per gram DNA/g of faeces. The results showed that the probiotic group had a significantly higher prevalence of *Saccharomyces* sp. at baseline (p=0.02) than the placebo group.

Table 5.8 Results of cross-tabulations for the presence or absence of yeasts and fungi between groups and measurement occasion

<table>
<thead>
<tr>
<th>Yeast/Fungi Species</th>
<th>Week</th>
<th>Probiotic Group</th>
<th>Placebo Group</th>
<th>Chi-square Fisher’s Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Not Detected n (%)</td>
<td>Detected n (%)</td>
<td>Not Detected n (%)</td>
</tr>
<tr>
<td><em>Candida</em> sp.</td>
<td>0</td>
<td>18 (78.3)</td>
<td>5 (21.7)</td>
<td>12 (54.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17 (81)</td>
<td>4 (19)</td>
<td>13 (61.9)</td>
</tr>
<tr>
<td><em>Saccharomyces</em> sp.</td>
<td>0</td>
<td>19 (82.6)</td>
<td>4 (17.4)</td>
<td>11 (50)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17 (81)</td>
<td>4 (19)</td>
<td>14 (66.7)</td>
</tr>
</tbody>
</table>

5.3.4.4 Primary outcome measure: comparison between groups by treatment arm and measurement occasion for parasitology

The binary data reported by the laboratory for parasites, i.e. detected or not detected, was cross-tabulated to see if there was a difference between groups in the detection or non-detection of both individual and total parasites between groups and measurement occasion. There were no significant differences (p>0.05) between groups at baseline or week 12 for any of the parasites measured. Tables 5.9 and 5.10 presents the results of cross-tabulations for parasitology.
Table 5.9 Results of cross-tabulations for the detection or non-detection of parasites between groups and measurement occasion

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Week</th>
<th>Probiotic Group</th>
<th>Placebo Group</th>
<th>Chi-square Fisher Exact Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Detected n (%)</td>
<td>Detected n (%)</td>
<td>Not Detected n (%)</td>
</tr>
<tr>
<td>General parasite</td>
<td>0</td>
<td>2 (8.7)</td>
<td>21 (91.3)</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5 (23.8)</td>
<td>16 (76.2)</td>
<td>6 (28.6)</td>
</tr>
<tr>
<td>Parasite unknown taxonomy</td>
<td>0</td>
<td>3 (13)</td>
<td>20 (87)</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7 (33.3)</td>
<td>14 (66.6)</td>
<td>8 (38.1)</td>
</tr>
<tr>
<td>Blastocystis hominis</td>
<td>0</td>
<td>20 (87)</td>
<td>3 (13)</td>
<td>17 (77.3)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20 (95.2)</td>
<td>1 (4.8)</td>
<td>17 (81)</td>
</tr>
<tr>
<td>Dientoemba sp</td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td>Necator americanus (Hookworm)</td>
<td>0</td>
<td>21 (91.3)</td>
<td>2 (8.7)</td>
<td>21 (95.5)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>19 (90.5)</td>
<td>2 (9.5)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td>Trichuris sp. (Whipworm)</td>
<td>0</td>
<td>21 (91.3)</td>
<td>2 (8.7)</td>
<td>22 (100)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>19 (90.5)</td>
<td>2 (9.5)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Enterobius vermicularis (Pinworm)</td>
<td>0</td>
<td>22 (95.7)</td>
<td>1 (4.3)</td>
<td>19 (86.4)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>21 / 100</td>
<td>0 / 0</td>
<td>19 / 90.5</td>
</tr>
</tbody>
</table>

Table 5.10 Gastrointestinal organisms measured but below the detection limit in both groups, and not reported on either measurement occasion (weeks 1 and 12)

<table>
<thead>
<tr>
<th>Pathogenic Bacteria Campylobacter sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenic Bacteria Clostridium difficile</td>
</tr>
<tr>
<td>Opportunistic Bacteria Enterobacter sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Pseudomonas sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Salmonella sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Vibrio sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Yersinia sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Staphylococcus sp.</td>
</tr>
<tr>
<td>Opportunistic Bacteria Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Yeast Geotrichum sp.</td>
</tr>
<tr>
<td>Yeast Rhodotorula sp.</td>
</tr>
<tr>
<td>Yeast Microsporidia</td>
</tr>
<tr>
<td>Parasite Entamoeba sp.</td>
</tr>
<tr>
<td>Parasite Giardia sp.</td>
</tr>
<tr>
<td>Parasite Giardia lamblia</td>
</tr>
<tr>
<td>Parasite Trichomonas hominis</td>
</tr>
</tbody>
</table>
5.3.5 Repeated measures analysis of variance (ANOVA) of predominant bacteria, phyla classes and lactoferrin

Some variables were not normally distributed and therefore did not meet the assumptions of parametric statistical tests. If skewness was deemed to be non-normal, i.e. greater or less than zero (MacGillivray 1986), transformation of data was conducted. Where indicated, a logarithmic transformation \( \log_{10}(x) \) was conducted before an ANOVA.

The repeated measure ANOVA was conducted over two measurement times, baseline and week twelve. The effects of time, treatment and time interaction were fitted to each of the measures. Baseline and week 12 measurements for all predominant bacteria, phyla classes and the inflammatory marker lactoferrin were used in the repeated measures ANOVA. The \( p \)-values of the effects on predominant bacteria are presented in Table 5.11. There were no statistically significant changes for the principal comparison of interest, which was the time by treatment interaction \( (p \geq 0.05) \), for any of the predominant bacterial species, phyla classes or lactoferrin. A significant change in Streptomyces sp. \( (p=0.02) \) was observed with the treatment effect. The bacterial species Bifidobacter \( (p=0.001) \) and Escherichia coli \( (p=0.005) \) both showed a significant change over time. The \( p \)-values for phyla classes are presented in Table 5.12. There were no significant changes in phyla class for the effects over time, by treatment or time by treatment. The \( p \)-values for lactoferrin are presented in Table 5.13. No significant changes were observed for lactoferrin. While the \( p \)-value for significance in this study was set at \( p<0.05 \), a lower \( p \)-value may have more accurately reflected clinical and biological significance due the multiple comparisons run in this logistics regressions model.

Table 5.11 Results of repeated ANOVA: \( p \)-values for comparison of time, treatment and time by treatment effects on predominant bacterial organisms (\( \log_{10} \) transformed data)

<table>
<thead>
<tr>
<th>Predominant bacteria</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides sp.</td>
<td>0.686</td>
<td>0.198</td>
<td>0.992</td>
</tr>
<tr>
<td>Clostridia sp.</td>
<td>0.831</td>
<td>0.589</td>
<td>0.162</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>0.279</td>
<td>0.8</td>
<td>0.279</td>
</tr>
<tr>
<td>Fusobacteria sp.</td>
<td>0.772</td>
<td>0.270</td>
<td>0.806</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>0.022</td>
<td>0.794</td>
<td>0.990</td>
</tr>
<tr>
<td>Mycoplasma sp.</td>
<td>0.097</td>
<td>0.719</td>
<td>0.106</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>0.239</td>
<td>0.880</td>
<td>0.656</td>
</tr>
<tr>
<td>Bifidobacteria sp.</td>
<td>0.245</td>
<td>0.001</td>
<td>0.137</td>
</tr>
<tr>
<td>Escherichia coli sp.</td>
<td>0.235</td>
<td>0.051</td>
<td>0.516</td>
</tr>
<tr>
<td>Total predominant bacterial sp.</td>
<td>0.252</td>
<td>0.877</td>
<td>0.458</td>
</tr>
</tbody>
</table>
Table 5.12 Results of repeated measures ANOVA: p-values for comparison of time, treatment and time by treatment effects for Phyla classification

<table>
<thead>
<tr>
<th>Phyla class</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyla Firmicutes</td>
<td>0.971</td>
<td>0.372</td>
<td>0.175</td>
</tr>
<tr>
<td>Phyla Bacteroidetes</td>
<td>0.971</td>
<td>0.372</td>
<td>0.175</td>
</tr>
</tbody>
</table>

Table 5.13 Results of repeated measures ANOVA: p-values for comparison of time, treatment and time by treatment effects for the inflammatory marker lactoferrin

<table>
<thead>
<tr>
<th>Lactoferrin</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>lactoferrin</td>
<td>0.069</td>
<td>0.269</td>
<td>0.256</td>
</tr>
</tbody>
</table>

5.3.5.1 Results of Bonferonni-adjusted pair-wise comparisons for the significant time response

The significant changes observed in the repeated measures ANOVA were analysed for inflation problems by conducting a Bonferonni-adjusted pair-wise comparison. Estimated means and results of Bonferonni-adjusted pair-wise comparisons are presented in Table 5.14 for the significant time response of the predominant bacteria Bifidobacteria sp. and Escherichia coli.

Table 5.14 P-values for results of Bonferonni-adjusted pair-wise comparisons of predominant bacteria measures that changed significantly with time

<table>
<thead>
<tr>
<th>Predominant Bacteria</th>
<th>Week</th>
<th>Mean</th>
<th>SD</th>
<th>Lower CI (95%)</th>
<th>Upper CI (95%)</th>
<th>Multiple Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacteria sp.</td>
<td>1</td>
<td>5.40</td>
<td>0.412</td>
<td>0.443</td>
<td>2.109</td>
<td>1 vs 12 p=0.004</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4.12</td>
<td>0.386</td>
<td>3.340</td>
<td>4.904</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
<td>3.662</td>
<td>0.265</td>
<td>3.129</td>
<td>4.195</td>
<td>1 vs 12 p=0.036</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4.551</td>
<td>0.405</td>
<td>3.713</td>
<td>5.349</td>
<td></td>
</tr>
</tbody>
</table>

5.3.6 Generalised estimated equations analysis for changes in pathogenic and opportunistic bacteria, mycology, parasites and DRG

Repeated ANOVA could not be conducted for measures of pathogenic and opportunistic bacteria, yeasts and fungi and parasites due to the nature of the presence/absence of data. Therefore, Generalised Estimated Equations (GEE) for a binomial outcome, using a log link, were conducted where the effects of time, response to treatment, and the interaction between time and treatment were assessed. The results showed a change in *H. pylori* over time to be close to reaching significance (p<0.08). There was no significant effect for the interaction between time and treatment for any of the opportunistic or pathogenic microorganisms.
measured between the probiotic and placebo group. The results of the GEE analysis of pathogenic and opportunistic bacteria are presented in Tables 5.15 and 5.16. Where an organism was not reported as present in any of the participants in one or both treatment arms the statistical analysis was unable to provide the test statistic and these cases are reported as UAC.

Table 5.15 Results of generalised estimated equations: p-values for comparison of time, treatment and time by treatment effects on pathogenic bacteria

<table>
<thead>
<tr>
<th>Pathogenic Bacteria</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicobacter Pylori</td>
<td>0.42</td>
<td>0.08</td>
<td>0.59</td>
</tr>
<tr>
<td>Enterohaemorrhagic E.coli</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
</tbody>
</table>

UAC denotes ‘unable to calculate’ due to organisms not being present in any participants in one or both treatment arms

Table 5.16 Results of generalised estimating equations: p-values for comparison of time, treatment and time by treatment effects on opportunistic bacteria

<table>
<thead>
<tr>
<th>Opportunistic Bacteria</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total opportunistic Bacteria</td>
<td>0.95</td>
<td>0.65</td>
<td>0.52</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>0.98</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Staphylococcus sp.</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Achromobacter sp.</td>
<td>0.97</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
</tbody>
</table>

UAC denotes ‘unable to calculate’ due to organisms not being present in any participants in one or both treatment arms

Repeated ANOVA could not be conducted for mycology data due to the scale counts 1+ to 4+ reported by the laboratory. Therefore GEEs were conducted and the results showed Saccharomyces sp. counts reduced significantly with treatment (p=0.04). No significant change was seen for time or the principal comparison of interest, the time by treatment interaction. The results for mycology are presented in Table 5.17.
Table 5.17 Results of generalised estimated equations: p-values for comparison of time, treatment and time by treatment effects for mycology

<table>
<thead>
<tr>
<th>Mycology</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida sp.</td>
<td>0.06</td>
<td>0.53</td>
<td>0.88</td>
</tr>
<tr>
<td>Saccharomyces sp.</td>
<td>0.04</td>
<td>0.41</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Parasitology data was reported as detected or not detected and, due to the binomial nature of the data statistical analysis was conducted with GEEs. The results showed the general parasite incidence (p=0.002) and parasites with an unknown taxonomy (p=0.01) reduced significantly over time (p<0.016) in both groups. No significant change was seen for the principal comparison of interest, the time by treatment interaction between groups. The results for parasitology are presented in Table 5.18.

Table 5.18 P-values for comparison of time, treatment and time by treatment effects for parasites

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Treatment</th>
<th>Time</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Parasite DNA detected with known taxonomy</td>
<td>0.830</td>
<td>0.016</td>
<td>0.841</td>
</tr>
<tr>
<td>Blastocystis hominis</td>
<td>0.211</td>
<td>0.137</td>
<td>0.343</td>
</tr>
<tr>
<td>Dientoemba sp.</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Parasite taxonomy unavailable</td>
<td>0.892</td>
<td>0.002</td>
<td>0.514</td>
</tr>
<tr>
<td>Trichuris trichuria</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Enterobius vermicularis</td>
<td>UAC</td>
<td>UAC</td>
<td>UAC</td>
</tr>
<tr>
<td>Nector americanus</td>
<td>0.583</td>
<td>0.094</td>
<td>0.565</td>
</tr>
</tbody>
</table>

UAC denotes “unable to calculate” due to organisms not being present in any participants in one or both treatment arms

5.3.7 Secondary outcome measure: symptom scores and quality of life

Table 5.19 presents the descriptive statistics for the total scores CDSQOL by treatment arm and measurement occasion (baseline, weeks four, eight and twelve). Tables 5.20 to 5.23 present the descriptive statistics for each sub-scale of the CDSQOL. Figure 5.7 graphically summarises Tables 5.19 to 5.23 showing a slight improvement in both groups for total CDSQOL and sub-scales over the study period.
<table>
<thead>
<tr>
<th>Table 5.19 Comparison of total CDSQOL scores</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Scores for CDSQOL</strong></td>
</tr>
<tr>
<td><strong>Week</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5.20 Comparison of Emotion CDSQOL sub-scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDSQOL Sub-scale Scores for Emotion</strong></td>
</tr>
<tr>
<td><strong>Week</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5.21 Comparison of Social CDSQOL sub-scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDSQOL Sub-scale Scores for Social</strong></td>
</tr>
<tr>
<td><strong>Week</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5.22 Comparison of Worries CDSQOL sub-scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDSQOL sub-scale scores for Worries</strong></td>
</tr>
<tr>
<td><strong>Week</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5.23 Comparison of Gastrointestinal CDSQOL sub-scale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CDSQOL Sub-scale Scores for Gastrointestinal</strong></td>
</tr>
<tr>
<td><strong>Week</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>
Repeated measures ANOVA

Repeated measures ANOVA was conducted over four measurement times: baseline, week four, eight and twelve, with the effects of time, time by treatment interaction and treatment as the main effect being fitted to each of the sub-scales measures and to total CDSQOL measures. The significant values (p-values) of these effects from the repeated measures analyses are presented in Table 5.24. None of the time by treatment effects, the principal comparison interest, was significant, nor was any treatment main effect significant (p>0.05). However, the outcome measures of Emotion, Worries, Gastrointestinal and Total CDSQOL showed a significant change over time (p<0.05).
Table 5.24 P-values for the comparison of time, treatment and time by treatment effects for CDSQOL sub-scales

<table>
<thead>
<tr>
<th>CDSQOL</th>
<th>Time</th>
<th>Treatment</th>
<th>Time by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotion</td>
<td>0.02</td>
<td>0.26</td>
<td>0.49</td>
</tr>
<tr>
<td>Worries</td>
<td>0.04</td>
<td>0.22</td>
<td>0.60</td>
</tr>
<tr>
<td>Social</td>
<td>0.39</td>
<td>0.25</td>
<td>0.81</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>0.04</td>
<td>0.62</td>
<td>0.34</td>
</tr>
<tr>
<td>Total CDSQOL</td>
<td>0.02</td>
<td>0.35</td>
<td>0.89</td>
</tr>
</tbody>
</table>

5.3.7.1 The significant time response for CDSQOL measures: Bonferonni-adjusted pair-wise comparisons

Estimated means and results of Bonferonni-adjusted pair-wise comparisons are presented in Table 5.25 for the significant time response for Emotion, Table 5.26 for Worries, Table 5.27 and for Gastrointestinal, Table 5.28 for the significant time effect on total CDSQOL scores.

As shown by Tables 5.25 to 5.28 all CDSQOL sub-scales (except Social) and total CDSQOL improved over time with no difference observed between the active and placebo groups.

For these CDSQOL outcomes the significant time effect resulted in a significant difference between baseline and week 12 for the measures Emotion, Worries, Gastrointestinal and Total CDSQOL measures, with all significantly increasing over time (p=0.025, p=0.046, p=0.045 and p=0.023, respectively). A significant time effect between weeks four and twelve resulted in Emotion and Total CDSQOL measures increasing significantly (p=0.009, p=0.01, respectively). A significant time effect resulting in differences between weeks eight and 12, significantly increasing Emotion, Gastrointestinal and CDSQOL measures (p=0.019, p=0.051, p=0.019, respectively).

Table 5.25 The significant time effects for Emotion CDSQOL sub-scale

<table>
<thead>
<tr>
<th>Measure</th>
<th>Week</th>
<th>Mean</th>
<th>SD</th>
<th>Lower CI (95%)</th>
<th>Upper CI (95%)</th>
<th>Multiple Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotion</td>
<td>0</td>
<td>32.9</td>
<td>0.99</td>
<td>30.9</td>
<td>34.8</td>
<td>1 vs 12 p=0.02</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>32.7</td>
<td>1.12</td>
<td>30.4</td>
<td>35.0</td>
<td>4 vs 12 p=0.00</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>33.5</td>
<td>1.21</td>
<td>31.0</td>
<td>35.9</td>
<td>8 vs 12 p=0.01</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>35.1</td>
<td>1.04</td>
<td>33.0</td>
<td>37.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.26 The significant time effects for Worries CDSQOL sub-scale

<table>
<thead>
<tr>
<th>Measure</th>
<th>Week</th>
<th>Mean</th>
<th>SD</th>
<th>Lower CI (95%)</th>
<th>Upper CI (95%)</th>
<th>Multiple Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>39.8</td>
<td>0.79</td>
<td>37.2</td>
<td>40.0</td>
<td>1 vs 12 p=0.006</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>41.0</td>
<td>0.89</td>
<td>39.2</td>
<td>42.8</td>
<td>1 vs 8 p=0.006</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>41.7</td>
<td>1.00</td>
<td>39.2</td>
<td>43.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>42.2</td>
<td>1.00</td>
<td>40.0</td>
<td>44.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.27 The significant time effects for Gastrointestinal CDSQOL sub-scale

<table>
<thead>
<tr>
<th>Measure</th>
<th>Week</th>
<th>Mean</th>
<th>SD</th>
<th>Lower CI (95%)</th>
<th>Upper CI (95%)</th>
<th>Multiple Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastro-</td>
<td>0</td>
<td>33.0</td>
<td>0.88</td>
<td>31.2</td>
<td>34.8</td>
<td>1 vs 12 p=0.05</td>
</tr>
<tr>
<td>intestinal</td>
<td>4</td>
<td>34.7</td>
<td>1.00</td>
<td>36.7</td>
<td>36.7</td>
<td>4 vs 8 p=0.02</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>35.4</td>
<td>0.99</td>
<td>37.4</td>
<td>37.4</td>
<td>8 vs 12 p=0.05</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>36.1</td>
<td>0.98</td>
<td>38.1</td>
<td>38.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.28 The significant time effects for total CDSQOL scores

<table>
<thead>
<tr>
<th>Measure</th>
<th>Week</th>
<th>Mean</th>
<th>SD</th>
<th>Lower CI (95%)</th>
<th>Upper CI (95%)</th>
<th>Multiple Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0</td>
<td>147</td>
<td>3.31</td>
<td>141</td>
<td>154</td>
<td>1 vs 12 p=0.025</td>
</tr>
<tr>
<td>Scores for</td>
<td>4</td>
<td>150</td>
<td>3.12</td>
<td>144</td>
<td>156</td>
<td>4 vs 12 p=0.009</td>
</tr>
<tr>
<td>CDSQOL</td>
<td>8</td>
<td>153</td>
<td>3.34</td>
<td>146</td>
<td>159</td>
<td>8 vs 12 p=0.019</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>156</td>
<td>3.21</td>
<td>150</td>
<td>163</td>
<td></td>
</tr>
</tbody>
</table>

5.3.8 Secondary outcome measure: urinary organic acids

Urinary organic acids were measured and reported as being within the laboratory reference range or above the upper limit of the reference range. The numbers, percentages and p-values for normal and high abnormal results for individual organic acid measures by treatment arm and measurement occasion are presented in Table 5.29. A significant difference for D-lactate measures (p=0.004) was found between groups at week 12. In addition, on review of individual cases three out of the five abnormal cases at baseline were still abnormal for D-lactate at week twelve in the placebo group.
Table 5.29 Comparison of Urinary Organic Acids levels between groups and measurement occasion

<table>
<thead>
<tr>
<th>Organic Acid</th>
<th>Ref Range µ/mg Creatinine</th>
<th>Week</th>
<th>Probiotic Group</th>
<th>Placebo Group</th>
<th>Chi-square p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal Range n (%)</td>
<td>Above Normal Range n (%)</td>
<td>Normal Range n (%)</td>
</tr>
<tr>
<td>Benzoate</td>
<td>&lt; 2.1</td>
<td>0</td>
<td>17 (77.3)</td>
<td>5 (22.7)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>19 (90.5)</td>
<td>2 (9.5)</td>
<td>18 (85.7)</td>
</tr>
<tr>
<td>Hippurate</td>
<td>&lt; 667</td>
<td>0</td>
<td>16 (72.7)</td>
<td>6 (27.3)</td>
<td>14 (66.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>13 (61.9)</td>
<td>8 (38.1)</td>
<td>9 (42.9)</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>&lt; 0.14</td>
<td>0</td>
<td>22 (100)</td>
<td>0 (0)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>22 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Phenylpropionate</td>
<td>&lt; 0.06</td>
<td>0</td>
<td>22 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>22 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>&lt; 2.2</td>
<td>0</td>
<td>20 (90.9)</td>
<td>2 (9.1)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>20 (95.2)</td>
<td>1 (4.8)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>p-Hydroxyphenylacetate</td>
<td>&lt; 24</td>
<td>0</td>
<td>18 (81)</td>
<td>4 (18.2)</td>
<td>18 (85.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>19 (90.5)</td>
<td>2 (9.5)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td>Indican</td>
<td>&lt; 64</td>
<td>0</td>
<td>20 (90.9)</td>
<td>2 (9.1)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>20 (95.2)</td>
<td>1 (4.8)</td>
<td>20 (95.2)</td>
</tr>
<tr>
<td>Tricarballylate</td>
<td>&lt; 1.18</td>
<td>0</td>
<td>20 (90.9)</td>
<td>2 (9.1)</td>
<td>17 (81)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>19 (90.5)</td>
<td>2 (9.5)</td>
<td>19 (90)</td>
</tr>
<tr>
<td>D-Lactate</td>
<td>&lt; 2.5</td>
<td>0</td>
<td>18 (81.8)</td>
<td>4 (18.2)</td>
<td>16 (76.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>14 (66.7)</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylproprionate</td>
<td>&lt; 0.12</td>
<td>0</td>
<td>21 (95.5)</td>
<td>1 (4.5)</td>
<td>15 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>21 (100)</td>
<td>0 (0)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>D-Arabinitol</td>
<td>&lt; 53</td>
<td>0</td>
<td>16 (72)</td>
<td>6 (27)</td>
<td>19 (90.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>18 (85.7)</td>
<td>3 (14.3)</td>
<td>19 (90.5)</td>
</tr>
</tbody>
</table>

5.3.9 Blood safety measures

Repeated measures ANOVA were used to determine any difference in blood safety measures with time (baseline and week 12) and treatment (probiotic or placebo) and are presented in Table 5.30.
Table 5.30 Comparison of blood safety measures by treatment arm and measurement occasion

| Measure                  | Week | Probiotic | | | | Placebo | | | |
|--------------------------|------|-----------|--------|--------|--------|---------|--------|--------|--------|--------|--------|--------|
|                          | n    | Mean      | SD     | Min    | Max    | n       | Mean   | SD     | Min    | Max    | n       | Mean   |
| Sodium                   | 23   | 137.13    | 2.17   | 132.27 | 141.16 | 22      | 137.25 | 2.55   | 133.16 | 144.34 | 21      | 138.19 |
| Urea                     | 23   | 5.30      | 1.33   | 4.87   | 6.61   | 22      | 4.60   | 0.84   | 4.23   | 5.00   | 21      | 4.38   |
| Creatinine               | 23   | 67.70     | 10.50  | 50.84  | 89.93  | 22      | 67.97  | 9.47   | 55.91  | 95.95  | 21      | 66.82  |
| Bilirubin                | 23   | 8.83      | 6.11   | 3.32   | 16.16  | 22      | 11.50  | 10.00  | 3.48   | 16.42  | 21      | 11.70  |
| Alkaline phosphate       | 23   | 64.70     | 18.00  | 34.00  | 109.00 | 22      | 68.41  | 21.53  | 33.13  | 133.00 | 21      | 67.80  |
| Gamma GT                 | 23   | 21.10     | 11.60  | 8.47   | 32.14  | 22      | 19.55  | 13.30  | 8.64   | 29.64  | 21      | 19.56  |
| Lactate dehydrogenase    | 23   | 168.00    | 29.20  | 121.03 | 231.23 | 22      | 165.32 | 32.25  | 7.38   | 221.22 | 20      | 174.37 |
| Aspartate aminotransferase| 23   | 21.00     | 5.30   | 10.32  | 32.04  | 22      | 19.30  | 3.31   | 14.25  | 22.05  | 21      | 17.65  |
| Alanine transaminase     | 23   | 19.70     | 8.60   | 9.45   | 32.14  | 22      | 17.50  | 6.73   | 3.33   | 37.73  | 21      | 11.70  |
| Total protein            | 23   | 68.20     | 4.46   | 62.78  | 47.02  | 22      | 69.50  | 3.67   | 63.77  | 77.02  | 21      | 69.50  |
| Albumin                  | 23   | 43.70     | 2.36   | 39.48  | 48.12  | 22      | 42.90  | 4.34   | 27.47  | 45.74  | 21      | 44.10  |
| Globulin                 | 23   | 24.70     | 3.50   | 19.33  | 32.14  | 22      | 25.90  | 3.51   | 22.35  | 34.75  | 21      | 26.10  |
| Haemoglobin              | 23   | 133.00    | 7.40   | 119.14 | 147.22 | 22      | 137.17 | 11.73  | 113.16 | 159.35 | 21      | 138.13 |
| Red cell count           | 23   | 4.40      | 0.35   | 3.70   | 5.24   | 22      | 4.56   | 0.37   | 3.75   | 5.25   | 21      | 4.56   |
| Haematocrit              | 23   | 0.40      | 0.02   | 0.36   | 0.46   | 22      | 0.41   | 0.03   | 0.35   | 0.48   | 21      | 0.42   |
| Mean cell volume         | 23   | 91.90     | 3.61   | 83.98  | 98.92  | 21      | 90.80  | 3.57   | 87.98  | 101.00 | 20      | 91.60  |
| Mean cell haemoglobin    | 23   | 30.10     | 1.48   | 27.14  | 32.20  | 17      | 30.20  | 1.46   | 28.50  | 33.30  | 16      | 29.90  |
| Mean Cell Haemoglobin concentration | 23   | 3.29      | 8.44   | 309.34 | 341.17 | 17      | 332.00 | 7.91   | 323.00 | 347.17 | 16      | 329.00 |
| Red cell diameter width  | 23   | 13.40     | 0.67   | 12.40  | 14.70  | 17      | 13.00  | 0.77   | 11.90  | 15.30  | 15      | 13.00  |
| White cell count         | 23   | 12.30     | 0.54   | 12.50  | 14.30  | 15      | 13.00  | 0.73   | 11.50  | 14.80  | 15      | 13.00  |
| Neutrophils              | 23   | 3.53      | 0.93   | 2.06   | 5.50   | 22      | 3.56   | 1.16   | 2.04   | 7.20   | 21      | 3.94   |
| Lymphocytes              | 23   | 2.08      | 0.49   | 1.30   | 3.01   | 22      | 2.28   | 0.70   | 1.08   | 4.02   | 21      | 2.24   |
| Monocytes                | 23   | 0.51      | 0.16   | 0.24   | 0.84   | 22      | 0.49   | 0.13   | 0.30   | 0.74   | 21      | 0.52   |

191
Table 5.30 (continued)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>23</th>
<th>0.15</th>
<th>0.06</th>
<th>0.01</th>
<th>0.29</th>
<th>22</th>
<th>0.23</th>
<th>0.19</th>
<th>0.06</th>
<th>0.79</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>12</td>
<td>20</td>
<td>0.15</td>
<td>0.08</td>
<td>0.04</td>
<td>0.37</td>
<td>21</td>
<td>0.20</td>
<td>0.14</td>
<td>0.03</td>
<td>0.54</td>
</tr>
<tr>
<td>Basophils</td>
<td>1</td>
<td>23</td>
<td>0.03</td>
<td>0.02</td>
<td>0.00</td>
<td>0.10</td>
<td>22</td>
<td>0.03</td>
<td>0.01</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20</td>
<td>0.03</td>
<td>0.02</td>
<td>0.00</td>
<td>0.10</td>
<td>21</td>
<td>0.03</td>
<td>0.01</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td>Platelets</td>
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<td>269</td>
<td>49.3</td>
<td>169</td>
<td>369</td>
<td>22</td>
<td>256</td>
<td>61.2</td>
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<td>462</td>
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<td>12</td>
<td>20</td>
<td>279</td>
<td>40.36</td>
<td>208</td>
<td>350</td>
<td>21</td>
<td>265</td>
<td>73.5</td>
<td>195</td>
<td>516</td>
</tr>
</tbody>
</table>

Repeated measures ANOVA for blood safety parameters

To establish the safety of the probiotic compared to the placebo repeated measures ANOVA was conducted over two measurement times, baseline and week 12. The effects of time, time by treatment interaction and treatment main effect were fitted to each of the blood safety measures.

The significance values (p-values) of these effects from the repeated measures analysis are presented in Table 5.31. The effects of time were statistically significant for sodium, ALT, globulin, total protein, red cell count and haematocrit. However, these were not clinically significant when participants were further assessed. The increase in blood urea levels increased significantly with treatment and also with the time by treatment interaction.

However, on further analysis for outliers the changes in urea were skewed by one participant’s urea result being elevated at baseline and week 12; therefore the overall increase in blood urea levels in the probiotic group was unlikely to be a group effect.

The results of Bonferonni-adjusted pair-wise comparisons for the significant time by treatment effects did not yield statistical significance.

5.3.10 Adverse events

The probiotic VSL#3™ was generally well tolerated, with some bloating reported during the first week of supplementation that settled with time. Interestingly, participants on the placebo maltose were as likely to report bloating as those on the active arm. This is discussed in 5.4.12. Three participants on the placebo arm experienced adverse events during the trial period that were considered very unlikely to be related to the clinical trial. Notification of the events was filed with the Therapeutic Goods Administration and copies of these notifications are presented in the Appendix D15.
Table 5.31 Comparison for time, treatment and time by treatment effects for blood safety measures

<table>
<thead>
<tr>
<th>Measure</th>
<th>Time</th>
<th>Time by treatment</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>0.002</td>
<td>0.234</td>
<td>0.949</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.366</td>
<td>0.366</td>
<td>0.999</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.722</td>
<td>0.068</td>
<td>0.975</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.777</td>
<td>0.211</td>
<td>0.857</td>
</tr>
<tr>
<td>Urea</td>
<td>0.234</td>
<td><strong>0.011</strong></td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.497</td>
<td>0.865</td>
<td>0.527</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.812</td>
<td>0.817</td>
<td>0.148</td>
</tr>
<tr>
<td>Alkaline phosphate</td>
<td>0.146</td>
<td>0.387</td>
<td>0.984</td>
</tr>
<tr>
<td>Gamma GT</td>
<td>0.288</td>
<td>0.885</td>
<td>0.626</td>
</tr>
<tr>
<td>LDH</td>
<td>0.255</td>
<td>0.730</td>
<td>0.555</td>
</tr>
<tr>
<td>AST</td>
<td>0.096</td>
<td>0.628</td>
<td>0.171</td>
</tr>
<tr>
<td>ALT</td>
<td><strong>0.013</strong></td>
<td>0.865</td>
<td>0.376</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.021</td>
<td>0.538</td>
<td>0.733</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.097</td>
<td>0.243</td>
<td>0.844</td>
</tr>
<tr>
<td>Globulin</td>
<td><strong>0.038</strong></td>
<td>0.260</td>
<td>0.934</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>0.277</td>
<td>0.778</td>
<td>0.140</td>
</tr>
<tr>
<td>Red cell count</td>
<td>0.095</td>
<td>0.979</td>
<td>0.103</td>
</tr>
<tr>
<td>Haematocrit</td>
<td><strong>0.031</strong></td>
<td>0.950</td>
<td>0.121</td>
</tr>
<tr>
<td>Mean cell volume</td>
<td>0.707</td>
<td>0.822</td>
<td>0.269</td>
</tr>
<tr>
<td>Mean cell haemoglobin</td>
<td>0.150</td>
<td><strong>0.059</strong></td>
<td>0.724</td>
</tr>
<tr>
<td>Mean cell haemoglobin concentration</td>
<td>0.587</td>
<td>0.131</td>
<td>0.547</td>
</tr>
<tr>
<td>Red cell diameter width</td>
<td>0.192</td>
<td>0.650</td>
<td>0.263</td>
</tr>
<tr>
<td>White cell count</td>
<td>0.346</td>
<td>0.4831</td>
<td>0.422</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.153</td>
<td>0.496</td>
<td>0.583</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.313</td>
<td>0.717</td>
<td>0.458</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.684</td>
<td>0.465</td>
<td>0.703</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.980</td>
<td>0.799</td>
<td>0.179</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.504</td>
<td>0.307</td>
<td>0.855</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.199</td>
<td>0.778</td>
<td>0.367</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.269</td>
<td>0.256</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Significant p-values are bolded

5.4 Discussion

This present study demonstrated that the administration of the Gram-positive probiotic VSL#3™ was not superior to placebo in improving the CDSQOL scores. Furthermore, the intervention did not result in a significant change in the counts of the microorganisms measured. The reduction in urinary D-lactate measures in response to VSL#3™ suggested a change in gastrointestinal microbial metabolism as a secondary outcome but this change was not related to improved symptom scores or quality of life.
This is the first human study to have measured the microbiological and clinical effects of a multiple species gram-positive probiotic VSL#3™ in individuals with CD. The results differed significantly from the positive outcomes reported by others studying the clinical and biological effects of VSL#3 in gastrointestinal pathologies other than CD (Bibiloni, Fedorak et al. 2005; Chapman, Plosker et al. 2007; Huynh, de Bruyn et al. 2009; Appleyard, Cruz et al. 2011; Park, Perez et al. 2011; Dai, Guandalini et al. 2012; Dai, Zhao et al. 2012).

The present study’s findings have important implications for individuals with CD as the results suggest that administering gram-positive probiotic bacteria is unlikely to be of any clinical benefit to this population. Importantly, this outcome also raises a number of issues related to the design and methodology employed in this study, which are addressed below.

5.4.1 Study homogeneity

The homogeneity of a sample population affects the accuracy of the results of a comparison between interventions. This requirement must be balanced by another constraint: results obtained from a too narrowly defined sample cannot be broadly generalised. Homogeneity is achieved by having a rigorous inclusion and exclusion criteria and was included in this study design.

The literature suggests the reasons to be considered for persistent CD symptoms other than poor adherence to a GFD, which was controlled for in this study (Faulkner-Hogg 1999) include:

a. Small intestinal bacterial overgrowth (Tursi, Brandimarte et al. 2003).
b. Lactose intolerance (Green and Cellier 2007).
c. Pancreatic insufficiency (Green and Cellier 2007).
d. Poorly absorbed short chain carbohydrates (Gibson and Shepherd 2010).
e. Intestinal permeability (Anonymous 2008).

All of these conditions have individually been reported in the literature to potentially benefit from probiotic intervention. Therefore, we hypothesised that these subtypes of CD patients with persistent symptoms may benefit from a global treatment approach.

5.4.2 Faecal specimens

The results of this study raise the issue of whether the choice of specimen for assessment of the microbial community was appropriate to measure our primary endpoints. Choice of specimen is a source of ongoing discussion, with several authors arguing against the

Faecal samples were chosen as the preferred specimen for analysis in this study for four reasons. Firstly, a similar microbial composition (with some distinctions) can be found at each anatomical site throughout the GIT, with the bacterial species of the intestinal tract incrementally and exponentially increasing from its proximal to its distal regions. It has been suggested that perhaps the facultatively anaerobic bacteria detected in the faeces represent much larger populations inhabiting the proximal colon (Marteau et al. 2001). Therefore, while the microbial measurements of faecal specimens may not be a reflection of the actual measures of microbes in the proximal small intestinal counts they are likely to be an accurate reflection of the indigenous and pathogenic inhabitants of the microbial community, and this is what we wanted to know.

Secondly, the non-invasive nature of the faecal specimen collection attracted a greater number of willing individuals and therefore enabled a higher-powered study than if we had chosen an endoscopic procedure that required catharsis as preparation and sedation for the sampling. It is reasonable to argue that the patho-physiological site of CD is the proximal small intestine (the duodenum); therefore a specimen from this via an endoscopy would yield an accurate measure of its microbial composition. However, the catharsis required in preparation for the procedure of endoscopy will alter the microbial composition of a duodenal aspirate retrieved, thereby yielding a potential misrepresentation of microflora present (Tannock and Famularo 2006). Furthermore, intestinal biopsies taken from living persons may not yield satisfactory results because the biopsies are only a minimal part of the total intestinal wall and the number of people sampled must be large enough to generate reliable results (Zoetendal, von Wright et al. 2002).

Thirdly, material rarely completely fills the lumen of the duodenum. It is usually present for a relatively short period of time and invariably represents transient organisms derived from the oral cavity and the upper respiratory tract as well as from food (Ouwehand 2010). These organisms are not usually regarded as being members of the indigenous microbiota. In saying this, it is important to note that the microflora of the proximal colon is more metabolically active than the microflora of the distal colon (Lord and Bralley 2008); therefore urinary organic acid metabolites of microbial metabolism were measured to assess the metabolic activity of the proximal intestine. Importantly, the colonic content (faeces) is thought to be a reflection of the terminal ileum’s or transitional colon’s indigenous microflora (Lord and Bralley 2008). The influence of microorganisms that inhabit the intestinal ecosystem of individuals with CD was the primary outcome measure of this study. For the
aforementioned reasons faecal samples in conjunction with urinary metabolomics were thought to provide the best information to answer the study question.

Lastly, several groups have reported the results of both molecular analysis of faecal samples and duodenal aspirates that they obtained from the same CD individuals, and to some degree they report comparable microbial compositions from the two anatomical sites (Nadal, Donat et al. 2007; Sanz 2007; Collado, Donat et al. 2009b; Kerckhoffs, Ben-Amor et al. 2011; Sanz, Palma et al. 2011). Their findings support the current knowledge that the bacterial species of the intestinal tract incrementally and exponentially increase and that a similar microbial composition can be found at each anatomical site, with some distinctions throughout the gastrointestinal tract.

In summary, faecal samples were used as the preferred specimen as they were minimally invasive and for the reasons stated above were considered to be representative of the composition of the gastrointestinal microbiome, which was the primary outcome measure of the present study.

5.4.3 Transportation of specimens

A factor that may possibly have compromised the results of this study is transportation. A valid criticism of studies undertaking faecal analysis is the potential breach of either time and/or temperature stability requirements due to issues involved in the transportation of faecal specimens. These breaches can negatively influence test reliability. The majority of concern regarding transportation is focussed on culture-dependent analysis that requires that a specimen must be collected in a vial containing nutrient broth to maintain microbial viability. The nutrient-broth allows continued growth of, and a significant change in, the balance of microbes present, since many aerobes will grow at the expense of anaerobes. In the present study DNA analysis eliminated this problem by placing specimens in vials of fixative for transport. This immediately kills all organisms, freezing the exact balance at the time of collection. With DNA-based methods that detect genes of the microbiota, living specimens are not necessary and the laboratory measures described in the methodology section overcome the concerns of determining whether a detected organism was alive or dead at the time of passing the faecal specimen. Although it was possible that the transportation of specimens could have compromised the results of this study it was highly unlikely due to the molecular technique the study employed.
5.4.4 Molecular assessment methodology

In order to rationally use probiotics as therapeutic agents, an analytical method that provides an accurate assessment of the microbial composition of the gastrointestinal tract is important. The present study employed PCR primers targeting the 23S rDNA and 16S rDNA to amplify PCR products used in a downstream hybridisation assay. PCR detects non-viable organisms that are not retrievable by other methods and allows a more rapid detection of the DNA of organisms that grow slowly, such as mycobacteria and fungi, and due to DNA PCRs analysis being regarded by some as the gold standard of molecular techniques (Ouwehand 2010).

The term ‘molecular technique’ also refers to a number of other techniques based on 16S ribosomal DNA genes. Molecular techniques include florescent in situ hybridisation, denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis. These techniques have been reported to yield limited results due to low sensitivities (Ouwehand 2010).

Due to the capacity of DNA-PCR analysis this study was able to contribute to the existing body of knowledge that has explored the microbial ecology of individuals with CD. These groups (Collado, Calabuig et al. 2007a; Nadal, Donat et al. 2007; Yolanda Sanz 2007) used a variety of molecular techniques and their findings in regard to the bacterial populations in CD cohorts were similar to this present study. This further supports that this study’s employment of real-time PCR and molecular techniques based on 16S ribosomal RNA (rRNA) gene analysis resulted in comparable data in the area of bacteriology. However, to our knowledge molecular assessment of yeasts and fungi and parasites in faecal samples of individuals with CD has not been conducted. By employing DNA-PCR as the molecular technique, the baseline faecal results of this present study were able to be compared to the results of bacteriology reported by other groups employing molecular techniques. In addition, the analysis of both baseline and follow-up specimens were conducted as one batch to improve scientific rigor and reduce any risk of bias. A limitation of this present study was that the laboratory assessment of faecal specimens did not include counts of Streptococcus sp. and Enterococcus sp. as the laboratory had not established algorithms for the quantitative computation of these microorganisms. This limitation resulted in not reporting the recoverable numbers of Streptococcus sp. which is a constituent of VSL#3™.

Another consideration of these findings was that it is possible that the probiotic species would not survive the physiological environment of the upper gastrointestinal tract (Bezkorovainy 2001; Gaudier, Michel et al. 2005), and this is discussed in detail in section below 5.4.11.
5.4.5 Distinguishing between live and dead cells

While molecular technology is considered to be a method of analysis with high sensitivity and specificity (Ouwehand 2010), concerns were raised over whether the formalin fixative specimens were placed in would result in an inability to distinguish between live and dead microbial cells on analysis. As described in Chapter 2.3, the laboratory technicians stained the PCR amplicons with ethidium bromide and thus rendered them visible under UV illumination as a way of differentiating between live and dead microbial cells (Rudi, Moen et al. 2005).

5.4.6 Shipment and storage of VSL#3™

A possible reason for a decrease in Bifidobacteria sp. and for no change in Lactobacillus sp after 12 weeks of oral administration observed in the active group was the viability of these organisms in the VSL#3™ formulation. Viability of organisms can be compromised by storage conditions. In response to these findings we undertook a quality assurance process. Firstly, we verified that our randomisation coding was correct. Secondly, we employed the services of an independent laboratory to measure the numbers of CFU from a sample of the active probiotic and from a placebo sample. We undertook this process to a) verify the strength of the medicine was consistent with the manufacturer’s claims; and b) check that shipment temperature from the manufacturer in Italy to our study site in Sydney had been adequate to preserve the original strength reported at time of manufacturing. The laboratory’s report demonstrated that the probiotic bacteria remained viable and that the strength was consistent with manufacturer’s claims, and that our storage system had maintained that strength. The independent laboratory report is presented in Appendix D16. Lastly, we verified that the study participants had adhered to the instructions to keep their study medicine refrigerated. We concluded that shipment and storage had not affected the quality of the trial medicine, therefore we do not attribute our study outcome to violation of these factors.

Conversely, our concerns regarding the number of live viable probiotic cells may have been allayed had we considered the possibility that dead probiotic cells may also exert a biological effect. Adams et al. (2010) suggested that many of the effects obtained from viable cells of probiotics are also obtained from populations of dead cells. The probiotic paradox is that both live and dead cells in probiotic products can generate beneficial biological responses due to a dual action. The live probiotic cells influence both the gastrointestinal microflora and the immune response while the components of the dead cells exert an anti-inflammatory effect in the gastrointestinal tract (Adams 2010). However, the comparison between the effects of live and dead cells were not considered in the design of this trial.
This study maintained the current standard practice for management of probiotic supplements, and the negative outcome was unrelated to the viability of the organisms administered. However, our understanding that probiotics require refrigeration to maintain viability was challenged by this trial. Refrigeration requirements should be reviewed when designing research that aims to modulate the gastrointestinal immune function and reduce gastrointestinal inflammation, as is the case in CD research.

5.4.7 Effects of dose

The outcome of this study raised a question regarding adequacy of dosing of VSL#3™. Our choice of dosage was based on a considered review of the literature in which probiotics had been used for various conditions (Chapter 2.4). When Hoyveda et al. (2009) conducted a systematic review and meta-analysis of fourteen randomised trials they suggested that due to the tendency for negative outcome trials not to be published their meta-analysis may have over-represented positive outcome studies. Furthermore, their analysis suggested little consensus regarding duration of therapy, organisms and strengths of probiotics used (Hoveyda, Heneghan et al. 2009), reiterating the difficulty researchers face in establishing an efficacious dose. We were unable to find any specific publications to refer to in regard to dose and duration of therapy of probiotics in individuals with CD and we therefore consider the present trial pioneering.

Interestingly, since the design and completion of the present clinical trial, Ritchie et al. (2012) completed a meta-analysis reporting changes across all diseases and probiotics species. Significant efficacy was observed for three doses: $1 \times 10^{10}$ CFU/day, $5 \times 10^{6}$, $10^{7}$, $10^{8}$ CFU/day and $1 \times 10^{9}$ CFU/day. One dose ($1 \times 10^{11}$, $10^{12}$ CFU/day) did not have significant efficacy. The dose of two sachets of $45 \times 10^{10}$ per day used in the present clinical trial was considerably higher than in any trials included in Ritchie et al’s (2012) systematic review. The possibility of a negative dose effect should be considered when discussing this trial’s result, with one consideration being the dose was too high for this CD population. Conversely, based on the existing literature that focussed specifically on VSL#3™, higher doses may have produced a more positive clinical and microbiological outcome. VSL#3™ is arguably the best researched multi-species probiotic in relation to IBD, pouchitis and IBS. Since the completion of our clinical trial several other trials have reported significant improvements in a number of parameters, including symptom scores and quality of life in individuals taking VSL#3™ for other gastrointestinal pathologies as a result of higher doses of VSL#3™ (Tursi, Brandimarte et al. 2003; Sood, Midha et al. 2009; Guandalini, Magazzù et al. 2010; Turcotte and Huynh 2011). Other study endpoints measured by these groups included improvements in markers of
intestinal permeability (Mennigen and Bruewer 2009; Dai, Guandalini et al. 2012) and reductions in inflammatory cytokines (Huynh, de Bruyn et al. 2009; Mastrangeli, Corinti et al. 2009). These studies always used up to four times the dose employed in the present study. However, one animal study did demonstrate that administering an equivalent human daily dose of 6g VSL#3™ (450 CFU) to rats with induced colitis resulted in improved microbial diversity of the luminal contents and improved histopathology of colonic mucosa (Uronis, Arthur et al. 2011). This dose was supportive of the decision to administer 900 CFU of VSL#3™ per day. While clinical symptom scores in response to VSL#3™ have been assessed, to our knowledge no human studies have assessed a broad microbial composition of faeces in humans in response to dosage of VSL#3™. The dose of 450 CFU twice daily implemented in the present study was lower than in other human studies reporting positive clinical outcomes for other gastrointestinal disorders.

Based on past and current knowledge it is possible that the dosage employed in this study was too low and resulted in both a negative clinical and microbiological outcome. It is important to consider that should future studies administer higher doses of VSL#3™ similar to that employed in the IBD trials with positive outcomes then the economic feasibility for people with CD maintaining such a therapy becomes questionable.

5.4.8 The effect of duration of therapy

The length of time over which an intervention is administered can affect the outcome of a clinical trial. At the time of designing this study there was a limited amount of literature regarding the differential efficacy of therapy periods for probiotics. Since then there has been support for the possibility that duration of probiotic therapy that exceeds nine weeks has an increased likelihood of resulting in a significant clinical positive outcome. In Ritchie et al’s (2012) meta-analysis significant efficacy was reported for length of treatments of one to two weeks, three to four weeks and five to eight weeks, with treatments of nine to 24 weeks having significantly higher efficacy than those of three to four weeks. We considered that twelve weeks would be an adequate duration of therapy and within the parameters reported in the literature. We do not consider that duration of therapy was a negative influencing factor in the outcome of this clinical trial.

5.4.9 The biological and clinical rationale for choosing VSL#3

Prior to commencing the research with this study design, a careful review of the potential consequences of supplementing probiotic bacteria, including bifidobacteria in individuals where counts were on the higher ends of the laboratory reference range, was considered. It
was concluded that in the event higher numbers of bifidobacteria were identified the intervention would not result in toxicity. Importantly, the potential biological and clinical benefits of supplementing high doses of bifidobacteria were considered to be appropriate to trial in CD patients. The biological mechanisms are presented in sections 2.4 to 3.5. (Pages 77 to 87) and discuss in detail the known mechanisms of probiotic bacteria, including bifidobacteria and VSL#3. These mechanisms of action are appropriately matched to the pathophysiology of CD reviewed in section 2.5 and are graphically presented in Figure 2.10 (page 101).

Clinically, due to positive outcome studies that have administered VSL#3™ in a number of gastrointestinal disorders, it was decided that using a multi-species could be efficacious. This assumption has been further supported recently by Ritchie et al. (2012) who concluded that there was no significant difference in efficacy outcome between single and multiple species probiotics. However, it has been suggested that the viability of organisms, when administered together, may offset each other and become ineffective. Perhaps this is a plausible explanation for the insignificant increase in the recoverable numbers of *Bifidobacteria and Lactobacillus* species in this clinical trial.

**5.4.10 Compliance**

Compliance was documented as high. However, due to the study participants being ‘outpatients’ we were unable to strictly control compliance. Current standard practice of measuring outpatient compliance based on the amount of medicine returned was applied. This procedure relies on participants honestly reporting their compliance.

**5.4.11 Timing of administration**

Our finding that there was no significant increase in the numbers of *Lactobacillus* sp or *Bifidobacteria* sp. recovered in the faeces raises a number of questions. Firstly, are these organisms viable once inside the human body? One possible explanation is that our method of administering the probiotic formula was questionable. The manufacturers of probiotics provide conflicting information on the form and timing of administration of probiotic bacteria. It has been suggested that probiotics should be administered in enteric coated capsules and taken away from meal times to circumvent the defence systems of gastric acid and digestive secretions stimulated by the ingestion of food. It is thought that this timing of administration will result in viable counts of bacteria making their way to the small and large intestine, where they exert their beneficial effects (Stummer, Salar-Bezhadi et al. 2010).

However, this does not take into consideration traditional methods of administering lactic acid
bacteria, i.e. in a complete food form as part of a meal e.g. yoghurt and sauerkraut (Metchnikoff 1908).

Based on this later concept of food acting as a buffering agent, we instructed our participants to take a sachet of VSL#3™ lyophilised granules stirred into cooled food or fluid at meal times. Since the present clinical trial was completed, Tompkins et al. (2011) have examined the effects of time with respect to mealtime and of the buffering capacity of food on the survival of probiotic microbes during gastrointestinal transit. They concluded that, ideally, non-enteric coated bacterial probiotic products should be taken with or just before a meal, and that the meal should contain some fats (Tompkins, Mainville et al. 2011). In response to this finding we conducted a retrospective analysis of the food diaries of the active arms. Our analysis revealed that gluten-free cereal and milk constituted the most common breakfast; however, due to lack of detail regarding brand names and quantities we were unable to conduct an analysis of the fat content of the milk or cereal to compare our findings with Tompkin et al’s work. The second VSL#3™ dose was taken with the evening meal, but the variations both among participants’ and within individuals’ diets made it impossible to draw a meaningful conclusion regarding fat content.

5.4.12 The placebo effect

The placebo effect may account for a number of microbiological, symptom score and quality of life measure changes observed in this study. Other groups who have integrated a placebo arm into the design of their studies have also reported psychological and biological effects observed in the placebo groups (de la Fuente-Fernández, Ruth et al. 2001). Expectation and conditioning theory are the explanatory models used to describe the placebo mechanism (Meissner, Kohls et al. 2011). The placebo phenomenon makes it difficult to evaluate new treatments, and a number of arguments against attributing any therapeutic benefits to a placebo intervention have been proposed. Reasons that have been put forward against the placebo effect that may relate to the improvement observed in the present study include spontaneous improvement, fluctuation of symptoms, regression to the mean, unreported additional treatment, answers of politeness, experimental subordination, conditioned answers and neurotic or psychosomatic phenomena (Kienle and Kiene 1997). Interestingly, the possible neurotic and psychosomatic influences were highlighted in this study by three participants. A middle-aged male reported a resolution of 25 years of diarrhoea after two doses of the placebo, and a young adult female participant reported relief from a chronic anxiety disorder and a cessation of her weekly unexplained vomiting episodes after seven days on the placebo. The benefits were sustained in both cases for the full 12 week duration.
In a third case, a possible nocebo effect was experienced by a middle-aged female participant allocated to the placebo arm of the trial who requested information regarding the possible side-effects of the study medication on enrolling into the study. One week after commencing the placebo this participant reported nausea and bloating that subsided when she halved the amount taken. It should be acknowledged that the third case may have been an example of an adverse reaction to the placebo substance maltose, which raises the next critical point in this discussion.

5.4.13 Possible placebo interference?

The results of the present study raised concerns about the inertia of maltose as a placebo. While double-blinded, RCTs are still considered the gold standard of clinical research (Meissner, Kohls et al. 2011), what constitutes a true placebo is an ongoing point of debate, and it is not known beyond reasonable doubt whether all substances that are considered inert will be inert in all cases (Golomb 2010). Due to the potential of a placebo to influence trial outcomes others have suggested mandatory reporting of placebo ingredients (Golomb 2010). However, placebos are seldom described in randomised controlled trials, thus making it challenging for the present study to draw inferences from existing publications that deal with the specific use of maltose as a placebo. Therefore, the argument herein remains largely speculative.

The marginal overall improvement in symptom scores and quality of life were reported by both the placebo and the active group. In addition, the measures of a number of microbiological markers changed over time in both groups. Both groups received maltose, either as 6 g in the placebo arm or 2.4 g maltose as an excipient in the active arm. We propose two possible theories as to the potential interference of the placebo in the present study. Firstly, it is important to consider that the main energy source for the intestinal milieu is food. This energy source enables a complex cycle of cross-feeding, growth and metabolic activity. Secondly, maltose is technically a food source, and theoretically any microbiological changes it may have caused could have masqueraded as a time effect in both groups, rather than a time by treatment effect. Secondly, maltose requires the disaccharidase enzyme maltase to break down the two glucose molecules for absorption into blood as glucose. At the time of designing this study it was well established that a significant percentage of individuals with CD frequently have a transient disaccharidase lactase deficiency; however, less was known regarding other disaccharidase deficiencies in this population. Since this time, Mones et al. (2011) have demonstrated that children with CD with intact villi have significant disaccharidase deficiencies, including a maltase deficiency. In the present study,
administering maltose to individuals with the small intestinal pathology CD, who have an increased prevalence of maltase deficiency, may have skewed our microbiological and symptom score outcomes. While a number of positive outcome RCTs have been published using VSL#3™ and maltose as placebo, it is important to note that these studies were of patient populations with large intestinal disorders, who have not been reported as having a higher prevalence of dissacharidase deficiencies (Sood, Midha et al.; Mimura, Rizzello et al. 2004; Miele, Pascarella et al. 2009). The work of Mones et al. (2011) provides a plausible reason for placebo interference and that maltose may cause gastrointestinal symptoms in individuals with CD.

5.4.14 Significant time by treatment effect on urinary D-lactate

This study demonstrated that urinary D-lactate levels reduced significantly over time in response to treatment. The reduction in urinary D-lactate was not associated with a reduction in any specific microorganisms measured in the faecal analysis, suggesting that it may be associated with a decrease in a D-lactate-producing organism not measured in the study. Other possible reasons for the observed reduction in D-lactate levels are overall metabolic stabilisation of the intestinal milieu, improvement in intestinal permeability, and improved glucose control (Mencarelli, Cipriani et al. 2012; Scheijen, Hanssen et al. 2012). Glucose control was not this study’s focus, nor was it measured in the study, and none of the participants reported having diabetes. Considering that D-lactate is a biomarker of microbial metabolism the results are perplexing. Some understanding may be gained from two concepts in the following discussion.

Firstly, a number of microorganisms are capable of producing D-lactate. Paradoxically, all three bacterial species of VSL#3™ (Streptococcus, Bifidobacteria and Lactobacillus species) are major producers of lactic acid (Masood, Qadir et al. 2011), yet their administration for twelve weeks appears to be associated with a significant reduction in D-lactate. Lactate accumulates only when there is fast fermentation. If substrates are fermented slowly D-lactate is converted into other SCFAs, primarily propionate and butyrate, and does not accumulate (Rehman, Heinsen et al. 2012). This study did not measure changes to faecal SCFAs. However, we speculate that the reduction in D-lactate observed was due to a competitive inhibition and/or antimicrobial effect on an unmeasured D-lactate-producing pathogenic organism by VSL#3™. Through the eradication of a D-lactate-producing microorganism an intestinal milieu that fermented substrates more slowly was promoted, so converting D-lactate to more beneficial SCFAs. We propose that VSL#3™ changed the
metabolism of the fermentation rate of the intestinal milieu but we are unable to attribute that change to a specific organism nor prove it.

Secondly, the breakdown of the mucosal barrier potentially leads to translocation of the microbiota and/or their toxic products, including D-lactate (Derikx 2010). D-lactate has been proposed as a promising new marker of intestinal permeability (Derikx 2010). Reduction in urinary D-lactate has been associated with improved integrity of the tight junctions in the intestinal mucosa. Furthermore, administration of VSL#3™ and other mixed species probiotics by other groups has resulted in improved integrity of the intestinal mucosal barrier (Madsen, Cornish et al. 2001; Rosenfeldt, Benfeldt et al. 2004; Mennigen and Bruewer 2009). We speculate that the significant reduction in urinary D-lactate observed in the VSL#3™ group may be associated with an improvement in the intestinal mucosal integrity, unrelated to the microbial measures counted in this study.

We have proposed potential placebo interference by the placebo maltose due to a possible disaccharidase deficiency in the CD participants. However, it is important to consider that the significant urinary D-lactate reduction observed in the VSL#3™ group may be attributed to an improvement in disaccharidase activity, specifically of lactose. Other groups have demonstrated that, due to their microbial beta-galactosidase activity, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. hydrolyse lactose during transit through the small intestine (Marteau and Rambaud 1993; de Vrese, Stegelmann et al. 2001; Schrezenmeir and de Vrese 2001). Improved lactose digestion is associated with a reduction in the urinary metabolite D-lactate (Jiang and Savaiano 1997). In the present study only a modest improvement in symptoms was observed in both groups, suggesting that the association between improved lactose digestion and reduction in urinary D-lactate is not strong.

It is unclear exactly why a significant reduction in urinary D-lactate in the VSL#3™ supplemented group occurred in this study. Nevertheless, based on the proposed mechanism of the action of probiotics discussed in Chapter 2.4, we have suggested a number of possible reasons for reductions in urinary D-lactate:

- microbial interference by an undetermined microorganism;
- metabolic stabilisation of the intestinal milieu resulting in a reduction in D-lactate production;
- improvement in the intestinal mucosal epithelium with improved tight gap junction integrity, preventing microbial metabolites from entering into the blood and urine; and
- improved lactose digestion promoted by proteolytic enzymes produced from probiotic bacteria.
5.4.15 Time effect: ‘The tincture of Time’

The effect of time was significant in a number of outcome measures, including specific microbial markers, urinary organic acids and certain quality of life measures.

5.4.15.1 The time effect: *Helicobacter pylori*

Our findings that some gastrointestinal pathogens may clear from the intestine over time are not new (Ouwehand 2010). *H. pylori* is the most successful human pathogen, infecting an estimated 50% of the global population (Al-Ahmad, Körschner et al. 2012; Malfertheiner, Megraud et al. 2012). *H. pylori* is rightly classified as a formidable pathogen and is the first bacterium to be classified as a carcinogen by the World Health Organisation: it infects up to half the world’s population, although disease is the exception rather than the rule (Amieva and El–Omar 2008). We observed that faecal *H. pylori* nearly significantly decreased over time across the entire group, suggesting that perhaps some participants may have had a transient infection. Transient *H. pylori* infection with spontaneous resolution, without intervention, has been discussed by others (Malfertheiner, Megraud et al. 2012). It has been suggested that a lower incidence of *H. pylori* detection in follow-up faecal samples may be due to the presence of inhibitory molecules from other organisms that can prevent amplification of the target DNA (Horemans, Deschacht et al. 2011). However, this could not have happened in our study, as the study design specified that all samples were analysed in one batch with the same microbiologist and methodology. Despite the high sensitivity and specificity of PCR, the interpretation and significance of a reduction in counts of *H. pylori* DNA in the participants’ faeces is still unclear. Cover et al. (2009) suggested that despite *H. pylori* genetic sequences being identified and verified in oral and colonic contents it is not clear whether these organisms are transient or residential. In a clinical setting, further information regarding the significance of the faecal detection of *H. pylori* would be obtained from the results of a urea breath test and faecal antigens (Malfertheiner, Megraud et al. 2012). Developing a diagnostic process to discern whether this organism is transient, pathogenic, resident or commensal would allow clinicians to make case-by-case decisions that could prevent the need for composite and multiple courses of antibiotics. Further research investigating the association between the findings of faecal DNA detection of *H. pylori*, faecal antigens, urea breath tests and clinical presentation are warranted.

5.4.15.2 ‘Naïve reductionism’: reduction in *Bifidobacteria* over time

A significant reduction in faecal counts of *Bifidobacteria* species was observed in both groups over time. Interestingly, other researchers have demonstrated that probiotic supplementation results in recovery of increased numbers of probiotic bacteria, including *Bifidobacteria* sp., in
faecal specimens (Saxelin, Lassig et al. 2010; Martarelli, Verdenelli et al. 2011; Simakachorn, Bibiloni et al. 2011; Baffoni, Gaggia et al. 2012). Potential explanations for our findings that pertain to the mode of delivery, dosage, and timing of administration, compliance and placebo/excipient interference are discussed in 5.4.11. In addition to these points, it is worth briefly considering the terms ‘naïve reductionism’ and systems biology, to further extract some meaning from this finding. ‘Naïve reductionism’ is a term used to describe the limitations of a purely reductionist approach applied to understanding single components of biological systems (Strange 2005). The view that understanding the smaller sub-units would make it easier to understand the whole is standard research practice and one that clearly has great value in science (Romero, Espinoza et al. 2006). However, Strange et al. (2005) have proposed that ‘naïve reductionism’, defined as the belief that reductionism alone will lead to complete understanding of the living organisms, is not tenable, and that a systems approach that considers time, space and context is required to understand complex ecosystems such as the microbiome.

The reductionist interpretation of this study is that administration of *Bifidobacteria* sp. did not result in increased numbers being recovered in the faeces of the active compared to the placebo group and is not associated with improved symptom scores and quality of life. Further research questions have been raised by the reductionist approach employed in this study, i.e. measures of other gastrointestinal microorganisms and microbial urinary metabolites, and regarding the relationship between other members of the microbiome and their behaviours, i.e. metabolomic parameters. These questions may be better answered by employing a systems approach of investigation.

Moving away from the context of probiotic intervention and turning our attention to the relationship of organisms in the gastrointestinal tract, the present study found that while *Bifidobacteria* sp. decreased with time indigenous *Escherichia coli* species increased, and *H. pylori* and parasite incidence decreased, accompanied by decreases in the bacterial metabolites D-lactate and p-hydroxybenzoate. Of interest is the potential of cross-feeding networks of bacteria such as *Bifidobacteria* to promote *Escherichia coli* growth. We propose that the indigenous bacteria of the intestinal tract that promote growth of other predominant species may be associated with reductions over time in potential pathogenic organisms, including *H. pylori* and parasites, as observed in this study.

**5.4.15.3 *Escherichia coli*: A significant increase in *Escherichia coli* species with time**

This study observed significant increases in the measures of commensal *Escherichia coli* species with time. We have proposed a potential relationship between increased counts of
*Escherichia coli* and a decrease in potential pathogens that are likely to be associated with natural changes in microbial ecology over time rather than with VSL#3™. The antagonism exerted by a number of non-pathogenic *Escherichia coli* strains against pathogenic intestinal bacteria is well established (Nissle 1951; Altenhoefer, Oswald et al. 2004; Grozdanov, Raasch et al. 2004; Kruis, Frič et al. 2004; Duncker, Lorentz et al. 2006; Do, Baird et al. 2010; Matthes, Krummenerl et al. 2010). How indigenous microorganisms function in humans is still a largely unanswered question in many cases, but progress is continually being made in recognising the dynamic interactions between the microbes within the gastrointestinal tract and their hosts (Tannock and Famularo 2006; Versalovic, Wilson et al. 2009). The availability of indigenous microbial species such as *Bifidobacteria* to promote the growth of more potent effector organisms such as *Escherichia coli* species warrants exploration.

### 5.4.15.4 The time effect: transient parasites

Our findings suggest that the number of participants with parasites detected at baseline significantly reduced with time and that this reduction was not associated with treatment. Furthermore, this detection did not relate to a significant improvement in symptom scores and quality of life, or any other microbiological parameters measured. We propose that this observation may be related to simple detection and not to quantification of parasites. The molecular technique employed is sufficiently sensitive to detect as few as five cells per gram of faeces, a 5000-fold increase over microscopy in sensitivity of parasite detection (Forbes 1998; Ghosh 2000; Verweij 2004). While this degree of sensitivity is impressive, detection of just five cells of a parasite will result in a parasite being reported as detected. It is well documented that quantity of parasites is thought to directly correlate with degree of pathogenesis and severity of clinical symptoms (Bogitsh 2013). Therefore, we suggest that the reduction in total parasite incidence over time observed in this study was not related to clinical improvements, because the sensitivity of molecular detection employed reported parasites that were at levels incapable of eliciting a symptomatic response in the host.

### 5.4.15.5 Parasite incidence in CD

Interpretation of the frequency of parasite detection in this study is made from two perspectives: firstly, in the context of the sensitivity of molecular technology and secondly, in the relationship between parasites and autoimmunity.

The ‘high’ incidence of parasites reported in this study may be misleading due to the sensitivity of the molecular methods, and may lead to unnecessary treatments. Comparatively, our cohort of forty-five individuals had a similar incidence of parasites detected compared to 5347 consecutive faecal samples submitted to Metametrix™ Laboratory for reasons other
than and including CD (Metametrix™ Laboratory, Georgia, Atlanta, 2010). Many organisms that enter the human gastrointestinal tract are transient rather than colonising, playing an important immuno-modulatory role as they pass through the digestive tract. Transient parasite infections stimulate a TH2 response in the intestinal mucosa, thereby playing an important role in a number of immuno-dysregulation disorders, including autoimmune disease (Weinstock and Elliott 2009). Immuno-modulatory mechanisms have attracted the attention of a number of groups, and are summarised in a review of helminth therapy by Reddy and Fried et al. (2009), who concluded that treatment with Trichuris suis and hookworm larvae appear to be well tolerated and safe at therapeutic doses. More specifically, ongoing research focussing on helminth therapy in individuals with CD is taking place (McSorley, Gaze et al. 2011). We propose that routine anti-parasitic eradication protocols commonly administered to children and their families should be reviewed in individuals genetically predisposed to autoimmune conditions.

5.4.15.6 The time effect of symptom scores and quality of life
The finding of this study suggests that VSL#3™ was not superior to placebo in improving symptom scores and quality of life in individuals with CD. To our knowledge there is currently no published literature on the impact of probiotics on persistent symptoms and quality of life scores in the CD population to compare our findings to. Nevertheless, there is a considerable body of literature reporting both positive and negative outcomes in regard to symptoms and quality of life from the use of various probiotic formulas, including VSL#3™ in other gastrointestinal pathologies other than CD (Moayyedi, Vakil et al. 2003; Hoveyda, Heneghan et al. 2009; Moayyedi, Ford et al. 2010b; Korpela and Niittynen 2012).
5.5 Conclusion

In conclusion, VSL#3™ did not improve the symptom scores or quality of life of individuals with CD, or significantly change their gastrointestinal microbial counts. Our findings would suggest that the gram-positive multi-species formula VSL#3™ is unlikely to be of clinical benefit in individuals with CD. Further research is required to elucidate whether there is any clinical significance to the reductions observed in the urinary microbial metabolite D-lactate found by this study.
6  General Discussion and Conclusions

In this thesis the role of intestinal microbiota in people with CD and their clinical management was investigated. The focus of this Chapter is to discuss the important findings of this research and its implications for people with CD. Suggestions for future research will be proposed. Issues relating to the limitations of the survey, the microbiome study and the RCT have been discussed in Chapters 3 to 5.

6.1 Risk Factors

The paradoxical situation that medicine often finds itself in is that the ‘cure’ is often found in prevention of an illness. Bearing this in mind this research started by exploring risk factors. One of the research questions proposed and explored by survey was: are there early childhood risk factors associated with CD?

Our results supported and expanded upon the findings of a range of other researchers. We have provided further evidence that reduced periods of breast-feeding is a potential risk factor for CD (Ivarsson, Persson et al. 2000; Silano, Agostoni et al. 2010; Szajewska and Chmielewska 2012). The components of breast milk play a critical role in the development of the immune system (Field 2005) and have been shown to provide antimicrobial, anti-inflammatory and immuno-modulatory activity (Goldman 1993; Walker 2010). However, not all individuals who are breast-fed for the first year of life or while gluten is being introduced into the diet are protected from developing CD, suggesting this is only one of the risk factors involved. In addition, there have been no longitudinal follow-up studies that have assessed breast-feeding practices in the first year of life in the CD genetically predisposed to their fourth to sixth decade of life, where an increase in diagnosis of CD has become more common (Green, Stavropoulos et al. 2001b). A suggestion for further research regarding the efficacy of breast-feeding as a preventative for CD is conducting a longitudinal follow-up study following a cohort of genetically predisposed individuals to CD (defined by a buccal swab at birth). Recording the individual’s breast-feeding practice in the first year and screening for CD each decade until the end of the fifth decade. In addition, such a study, should record how the child was born i.e. whether by a C-section or vaginal birth. The efficacy of breast-feeding practices to prevent CD could potentially be increased by other early life events that impact the intestinal microbiota and subsequent immune function.

In addition to breast-feeding, being born vaginally, being born at home, minimising antimicrobial therapy as an infant, family hygiene practices and being part of a larger family
are associated with an individual acquiring a more diverse intestinal microbial composition and having less infections (Adlerberth et al 2008).

Interestingly, the increase in diagnosis of CD (Cabrera-Rubio, Collado et al. 2012) and other autoimmune diseases (Vehik and Dabelea 2012) in the last decade does coincide with an increase in the rate of C-section births globally, including Australia (Stavrou, Ford et al. 2011). While the findings of this study were unable to support an association between caesarean section birth and CD, it is important to remember other groups have demonstrated a significant risk for the development of autoimmune disease, including CD, and being born by caesarean section (Mårild, Stephansson et al. 2011; Phillips, Gill et al. 2012).

As discussed in Chapter 3, a significant limitation of this study was the lack of comparative data from a general state population for the variables related to maternal infection and antibiotic use, and early childhood and antibiotic. The association between frequent early childhood infection and subsequent antibiotic use in other gastrointestinal pathologies, including IBD, has been suggested by others (Hviid, Svanström et al. 2011; Virta, Auvinen et al. 2012). Considering that IBD and CD share some common immunological features it is important that both infection and antibiotic use in CD genetically predisposed individuals during childhood be investigated.

This present study was unable to establish a clear association; however, the survey respondents in this present study that reported experiencing some degree of persistent symptoms also reported a higher frequency of respiratory infections during early childhood than those with complete symptom resolution. This finding could provide a plausible reason to investigate the intestinal microflora as a cause of persistent symptoms a significant number of CD patients. Early childhood infection in people with CD could have interfered with the normal development of the intestinal microbiome, resulting in reduced microbial diversity and increased numbers of opportunistic and pathogenic bacteria, as described by De Palma et al. (2010) in paediatric CD patients. Secondly, it is plausible that CD patients reporting persistent symptoms have a distinct medical entity, namely a post-infectious IBS (Barbara, Cremon et al. 2009).

The significantly higher incidence of cow’s milk consumption in the first year of life reported by survey respondents compared to the general population is an important finding in this study with potentially important clinical implications for infants at genetic risk of CD. Cow’s milk consumption in the first year of life has been shown to be an immunological trigger in Type-1 diabetes and other autoimmune diseases (Kolb and Pozzilli 1999; Wasmuth and Kolb 2000; Binns, Graham et al. 2007; Megeid, Bakeit et al. 2011; Chiu, Leslie et al. 2013). Others have demonstrated that cow’s milk protein is not a risk factor for
autoimmunity, including multiple sclerosis (Ramagopalan, Dyment et al. 2010) and Type-1 diabetes (Theberge 2010). Nevertheless, as discussed in Chapter 2.1, oral tolerance depends on immunological homeostasis and normal maturation of the gut. These factors are influenced by growth factors and cytokines from breast milk, normal bacterial colonisation, infection and diet. It is thought that cow’s milk proteins may provide mimicry epitopes relevant to autoimmunity, as well as destabilizing oral tolerance mechanisms by biologically active peptides (Wasmuth and Kolb 2000). A recent review by Vaarala et al. (2011) suggested that dietary intervention studies in infants at genetic risk of Type-1 diabetes showed that early diet could modulate the development of beta-cell autoimmunity in humans and that by weaning to hydrolysed casein formula decreased the risk of beta-cell autoimmunity by age ten. If these results could be replicated in a cohort of infants at genetic risk of CD this could result in a non-invasive preventative intervention.

6.2 The Microbiome

The results of the comparative microbiome study (Chapter 4) suggested that significant differences exist in a number of microbial measures between adults with CD who have persistent gastrointestinal symptoms compared to adults without CD. This study cannot establish with certainty whether the differences in intestinal microflora observed in this study are a consequence or a cause of CD. The dietary impact of a GFD on the intestinal microbiome is not well understood and needs to be considered when interpreting the results of this study. Well controlled studies to elucidate whether there is indeed a difference between those including gluten in the diet and treated CD patients who are on a GFD are required to answer this important question.

While the participants in this study did not fit the category of being immuno-compromised, the higher number of CD patients with yeasts detected may provide some insight into the cause of the persistent symptoms reported. Considering the participants in this study were adults, it is expected that the inflammatory response in the intestinal epithelium will take longer to normalise than in children (Wahab, Meijer et al. 2002). If inflammation of the small intestine persists, the normal defences in the intestinal epithelium may be less competent in circumventing a range of opportunistic organisms (Ashida, Ogawa et al. 2012), including the yeasts as found in this study. The presence of these opportunistic organisms could potentially promote inflammation (Packey and Sartor 2009), leading to slower symptom resolution and villous repair. The higher counts of individual and total predominant indigenous bacteria present in the CD group may indicate that the aberrations of immune
function characteristic of CD may be in response to indigenous intestinal microflora that have colonised in higher counts than in people without CD.

The results showed that CD participants had significantly higher counts of the opportunistic yeasts *Candida* and *Saccharomyces* species compared to the control group was of specific interest and is of potential clinical significance. Higher counts of *Candida* sp. are typically associated with the microbial ecology of the mucosal membranes of immunocompromised patients (Ouwehand 2010; Schuster, Meibohm et al. 2012). The definition and significance of higher counts of *Candida* sp. in the gastrointestinal tract is less understood but counts that are considered higher than the expected normal indigenous numbers have been associated with antibiotic therapy (Kennedy and Volz 1985; Noverr, Noggle et al. 2004). An inclusion criterion of the clinical trial was that participants had not taken antibiotics in the previous four weeks prior to the baseline faecal test. This is considered the average period of time for the gastrointestinal microbiota to return to its normal profile after antibiotics. Therefore, it is plausible that the higher detection rate of *Candida* sp. in the CD group was related to a long-term residual colonisation from a lifetime history of antibiotic use. From an aetiological perspective, it has been proposed that *Candida albicans* is a good candidate for an infectious aetiology for Crohn’s disease (Colombel, Poulain et al. 2008), and more pertinent to this discussion *Candida albicans* has been implicated as a potential trigger of CD (Nieuwenhuizen, Pieters et al. 2003).

The possible clinical implication arising from these results for CD patients presenting with persistent symptoms of unknown aetiology would indicate that assessment of gastrointestinal candidiasis should be considered. Where indicated, an individualised prescription of an appropriate anti-fungal treatment may be recommended.

From a research perspective, a large scale clinical intervention study that first defines gastrointestinal candidiasis, assesses for its existence in CD patients presenting with persistent symptoms and provides individualised anti-fungal treatment based on treatment sensitivity results would demonstrate the extent that candidiasis plays in symptom formation.

### 6.3 Probiotic Interventions

Clinical studies with a negative outcome are often not published in the literature due to commercial reasons and perhaps the researchers perceiving there is little to report. However, the outcome of the clinical trial described in this thesis, while negative, does have important implications for patients with CD. We consider that had VSL#3™ been an appropriate intervention for patients with persistent GI symptoms despite adherence to a GFD, we would
have expected some statistically significant differences in the microbiome profiles, symptom scores and quality of life to have been demonstrated. While there are some limitations (discussed in Chapter 5) that need to be considered, we concluded that at this time probiotic therapy with VSL#3™ is not effective in symptom control in individuals with CD who remain symptomatic while adhering to a GFD.

Based on the identification of increased opportunistic organisms in the CD cohort, future studies that assess the potential efficacy of antimicrobial and/or immune modulating substances may provide a more positive clinical outcome.

6.4 The clinical management of CD

There were a number of key findings with implications for the future clinical management of people with CD. These findings include:

- CD survey respondents reported a significant delay in diagnosis.
- CD survey respondents reported a broad number of clinical symptoms at diagnosis.
- Inclusion of 2nd degree relatives resulted in 23.7% reporting a relative with CD.
- Nearly half of CD respondents who reported adhering to a GFD continue to experience some degree of persistent symptoms.
- Food allergies, infection, gender and a delayed diagnosis were more likely in CD participants reporting persistent symptoms.

Due to the wording of the survey question: “How long had you been experiencing the symptoms of CD prior to diagnosis?” we cannot attribute the lengthy delays in diagnosis entirely to the inadequacies of the health care profession. It is possible that as the clinical presentation of CD in mid-life can simply be vague gastrointestinal symptoms and fatigue (Ludvigsson and Green 2011) for which people do not promptly seek medical advice, resulting in a delay in a diagnosis of CD. Nevertheless, the survey results in this study supported the findings of others who reported that the diagnosis of CD is frequently delayed (Green, Stavropoulos et al. 2001a; Hershcovici, Leshno et al. 2010). Patients can present with a broad range of gastrointestinal and extra-intestinal symptoms, making the diagnostic process challenging with numerous options to consider, thereby resulting in other conditions being excluded prior to a diagnosis of CD (Mitchison, Record et al. 1989; Preussner 1998; Lohi 2010; Ludvigsson and Green 2011). Directions for future research should consider implementation and assessment of public awareness programmes effect on patterns of CD diagnosis. Investigating the effect of public awareness programmes could be enhanced by collaborating with existing organisations such as the Australian Coeliac Association. In
addition, promoting awareness of the clinical presentations of CD to health care professionals is essential. Such education could be included in professional continuing education programmes.

Lastly, population screening for CD has been a contentious topic, especially in childhood where it is possible to find a transient rise in tTg as immune tolerance is developing (Tye-Din 2012). Others argue the financial and ethical implications of screening the population for CD due to not fully understanding the effects of CD on people without symptoms or complications (Evans, McAllister et al. 2009). While these arguments are valid, it is undebatable that the impact of undiagnosed CD has potential to cause serious health complications, to reduce one’s quality of life and to be a burden to the health care system. Furthermore, delayed diagnosis can result in slower recovery and symptom resolution. The findings of this survey regarding a delay in diagnosis of over eight years prompts further consideration of investigating the cost-benefit to the Australian health care system to fund a screening programme for the detection of CD in people during the fourth and fifth decade of life. In addition, the results of this study should prompt health care professionals to consider taking a history of CD in 2nd degree relatives into account.

The finding that the GFD results in only partial symptom resolution in a significant number of patients with CD (Green and Cellier 2007) was supported by the results of this study. The higher incidence of those reporting food intolerances and allergies among those with persistent symptoms further supports the research by Gibson and Shepherd (2012) who found that CD patients with persistent symptoms benefit from the exclusion of some fermentable carbohydrates. The clinical implications of these findings are important and should prompt health care professionals who have patients with CD to exclude food intolerance as a cause of persistent symptoms.

Our finding that those who had infections in the first six years of life were more likely to report experiencing only some symptom resolution is of potential clinical significance. While we were unable to make firm conclusions about early childhood infection from this observation it may complement the findings of the microbiome study and the recommendations for exploring altered intestinal micobiota.

In conclusion, the results of this research suggest that a reduced duration of breast-feeding and the ingestion of cow’s milk in the first twelve months of life may increase the risk of developing CD. The higher counts of predominant bacteria and gastrointestinal yeasts may be secondary microbial aberrations in response to the damaged intestinal mucosa, or alternatively these organisms may play a role in the aetiology of CD, warranting further research. The persistent symptoms reported by patients with CD could be associated with
early childhood infections and food intolerances, and these should be considered clinically. Probiotic intervention with VSL#3™ did not alter the intestinal microbiome of individuals with CD, nor did it improve their symptom scores and quality of life.

This research has both supported and contributed to the body of knowledge in the field of CD as pertains to disease risk factors, microbial influences and its clinical management.
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Appendix A

A1 Literature Review: The GALT

Protective mucous secretions

The mucous layer that covers the entire intestinal epithelium plays an important role in the innate defence system. It consists mainly of water and mucins (principally MUC2) with high oligosaccharide content (>80%), which polymerise to form a viscous gel (Wilson 2008). Other mucins produced by the intestinal mucosa remain anchored to the surface of the intestinal epithelial cells. The high carbohydrate content of the mucins endows the gel with lectin-binding properties, which enable it to bind and trap both commensal and pathogenic bacteria, thereby preventing their access to the underlying epithelium. In addition to the mucins, the mucous layer contains various effector molecules of the innate and acquired host defence systems. Wilson states that the main functions of the mucous layers are to (a) protect the layer under the epithelium from microbial colonisation; (b) provide lubrication to facilitate movement of the luminal contents; (c) protect the underlying epithelium (mainly in the stomach and duodenum) from acid and digestive enzymes; and (d) protect the mucosa from the shearing forces generated by material moving through the GIT (Wilson 2008). In addition, the intestinal mucosa provides a range of molecules with antimicrobial activities. These molecules include lysozymes, lactoferrin, lactoperoxidase, secretory phospholipase A2, bacterial permeability-inducing protein, collectins, adreno-medulin, histone H1, regenerating gene gamma (Reg IIIγ) and several antimicrobial peptides (Mayer 2003). An alteration in mucus production can affect the ability of the mucosa to provide protection against luminal bacteria, thus permitting access to the intestinal epithelium (Cobrin and Abreu 2005). An alteration to the mucosal composition in conjunction with the presence of luminal bacteria that have increased adherence properties has been observed in inflammatory bowel disease (Cobrin and Abreu 2005). Therefore mucus secretions play a critical role in protection of the intestinal epithelium and sub-mucosal tissues.

The intestinal epithelium

While we understand the basic cellular anatomy of the intestinal epithelium (IE) and the fact it is involved in a complex array of physiological functions, it is still considered a vast, dynamic and a largely unknown frontier. While the IE is frequently referred to throughout this review, a specific description of this tissue is critical in appreciating the pathogenesis of CD. The intestinal epithelium is a single cell layer that constitutes the largest and most important
barrier against the external environment (Ashida, Ogawa et al. 2012). The IE cells consist of intraepithelial cells such as absorptive enterocytes, dendritic cells, M-cells, goblet cells and paneth cells. The collective function of these cells is to act as a selectively permeable barrier, permitting the absorption of nutrients, electrolytes and water while maintaining effective defence against intra-luminal toxins, antigens and enteric microflora (Ashida, Ogawa et al. 2012). The epithelium maintains its selective barrier through the formation of complex protein networks that mechanically link adjacent cells and seal the intercellular space. The protein networks connecting epithelial cells form three adhesive complexes: desmosomes, adherence junctions and tight junctions (Groschwitz and Hogan 2009).

The lamina propria lies directly beneath the intestinal epithelium and is richly populated with macrophages, dendritic cells, B-cells and T-cells (Harvey 1989). The lymphocyte population in the gastrointestinal tract represents an estimated 70% of the total lymphocyte count in the entire body, highlighting the importance of the role of adaptive immunity in the human gastrointestinal tract (Tomas 2009). Macrophages and dendritic cells participate in antigen presentation to B- and T-cells (CD-4 and CD-8) that form the basis for tolerance and adaptive immunity (Wells, Rossi et al. 2011). The efficacy of the IE as a permeable barrier between the intestinal luminal contents and the immune cells that lay beneath the lamina propria is integral in maintaining immune tolerance to commensal organisms and activation of acute immune responses to pathogenic organisms.

**Macromolecular transport through the enterocyte and immune activation**

Macromolecules can be transported through the enterocyte (trans-cellular transport) or between adjacent epithelial cells (para-cellular transport) (Snoeck, Goddeeris et al. 2005). The mucus layer covering the epithelium in conjunction with the microvilli on the apical surface commonly called the brush border of the enterocytes, contains specialised glycoprotein’s (Frey, Giannasca et al. 1996). This specialised surface proteins called the glycolax prevents direct contact between the antigen and the enterocytes (Frey, Giannasca et al. 1996). The mechanisms by which pathogens or their secretions gain access to the cells of the intestinal epithelium ultimately determine the outcome of the immune response (Berg 1985; Atkins and Furuta 2010). Likewise, the immune response to the presence of pathogens by intestinal epithelium is dependent on the pathway by which the pathogen gained access (Duchmann, Neurath et al. 1997; Vora, Youdim et al. 2004). Once transport of a pathogenic organism is detected by enterocytes in the sub-mucosa of the intestinal epithelium, neutrophils infiltrate and initiate an adaptive immune response (Strobel and Mowat 2006; Gaboriau-Routhiau,
Rakotobe et al. 2009), demonstrating the important role that enterocytes play in surveillance, transport and initiation of immune responses (Snoeck, Goddeeris et al. 2005).

**Enterocytes as antigen-presenting cells**

A number of researchers have demonstrated that enterocytes act as antigen-presenting cells (APCs), due to their ability to ‘internalise’ and process antigens (Kaiserlian, Vidal et al. 1989; Martín-Villa, Ferre-López et al. 1997; 2005). Enterocytes are classified as non-professional APCs but many have the characteristics required to be professional APCs, i.e., they can express both major histocompatibility class I (MHC-I) and MHC-II complexes required for T-cell proliferation (Rescigno 2010). While it has been suggested that enterocytes have the ability to process and present suitable antigens to primed CD4+ Th-cells, it is acknowledged that differences exist in class II molecules compared to those expressed on professional APCs (Rescigno 2010). It appears that the anatomical location of the expression of MHC-II molecules will affect their function (Shanahan 2002). Shanahan suggests that MHC-II molecules are expressed on apical surfaces of enterocytes rather than on baso-lateral membrane during inflammation and this may not function in normal lympho-epithelial interactions. The expression of MHC-II molecules on the baso-lateral membrane during inflammation has been reported by Snoeck et al (2005) who demonstrated that in the absence of inflammation, enterocytes do not express co-stimulatory molecules, so antigen presentation may result in either anergy or tolerance. In addition, Snoeck et al (2005) observed that leaky tight junctions allow the entry of antigens to the baso-lateral surface of the enterocytes. When antigens are processed apically a tolerogenic response is promoted. However, when antigens are processed on the baso-lateral surface of the enterocyte they become immunogenic. The consequences of leaky tight junctions are discussed further in Section 2.4.

**The role of microfold cells in intestinal immunity**

While it is apparent that enterocytes play a critical role in intestinal immune function, they are only part of the orchestra, including T-cells, B-cells and antigen presenting cells. The microfold cells (M-cells) were previously called lympho-epithelial cells (Mahajan, Deshmukh et al. 2012). Mahajan et al (2012) describe these specialised cells found in the follicle-associated epithelium of Peyer's patches of intestinal mucosa as having a characteristic apical surface, a disorganised brush border of short irregular microvilli. This is in contrast to the characteristically highly uniformed densely packed microvilli of the enterocyte. Another differentiating characteristic is that M-cells lack surface glycoprotein’s found on the enterocyte (Corr, Gahan et al. 2008). Unlike enterocytes, M-cells do not play a
role in digestion and absorption of nutrients and this is attributed to the morphological
differences between them (Mahajan, Deshmukh et al. 2012). The M-cell pocket contains B-
lymphocytes and T-lymphocytes, macrophages and dendritic cells (Snoeck, Goddeeris et al.
2005).

The M-cells are said to be gatekeepers to the mucosal immune system. The exposed nature of
the apical surface of the M-cell indicates that the primary function of M-cells is trans-
epithelial transport. M-cells transport substances from the intestinal lumen across the
epithelial barrier and antigens to the underlying lymphoid tissue for processing and initiation
of immune responses (Corr, Gahan et al. 2008). This transport process occurs via trans-
cellular endocytosis of the substance at the apical membrane, followed by transport of the
substance via an endocytic vesicle to the endosomal compartment, and finally, exocytosis at
the baso-lateral membrane. M-cell mediated translocation is very efficient and rapid. The
mechanisms by which M-cells take up microorganisms and molecules vary according to the
characteristics and nature of the material and the presence or absence of an M-cell specific
receptor (Corr, Gahan et al. 2008).

M-cells are the main sites for the continuous sampling and transport of antigens from the
intestinal lumen to mucosal lymphoid tissues. However, M-cells are also employed by viruses
and bacteria as a route of entry to underlying host tissues (Goitsuka, Chen et al. 2007).
Salmonella (Bäumler, Tsolis et al. 1996), Yersinia sp. (Wiedemann, Linder et al. 2001),
Vibrio cholerae (Blanco and DiRita 2006), Escherichia coli (Mahajan, Deshmukh et al. 2012)
and HIV virus have all demonstrated ability to translocate M-cells.

In addition, M-cells are also thought to play a role in the transport of prions, the proteinaceous
infectious particles thought to cause disease (Foster and Macpherson 2010). After oral uptake
prions may penetrate the intestinal mucosa through M-cells and reach Peyer’s patches and the
enteric nervous system (Foster and Macpherson 2010). It has been shown that follicular
dendritic cells are important for development of prion pathogenesis and subsequent infection
of the neural system and that Peyer’s patches support the role of M-cells in prion
transportation (Baird, Campion et al. 2004).

While inferior in numbers to enterocytes M-cells have characteristics that provide them with
an important role in antigen sampling, bacterial translocation, initiation of mucosal immune
responses and disease causation. The role of M-cells in CD pathogenesis is discussed in
Section 5.
Dendritic cells

Dendritic cells were first described by Ralph Steinman and colleagues in the mid-1970s as a rare subset of accessory cells, which are characterised by their rare stellate cytoplasmatic protrusions (Steinman and Cohn 2007). It is this morphology that led to their name (dendron is Greek for ‘tree-like’). Soon after this description was made it was observed that dendritic cells have unrivalled properties to stimulate unique T-cells (Steinman and Cohn 1974). Dendritic cells are found in the lamina propria of the small and large intestine and in the GALT, which includes isolated lymphoid follicles, Peyer's patches (PPs) and mesenteric lymph nodes (Jaensson, Uronen-Hansson et al. 2008).

Characteristics of Peyer’s patches dendritic cells

Peyer’s patches are highly organised lymphoid tissues that are the major portal of entry of bacteria. As discussed in Section 2.2.9, M-cells, the highly specialised epithelial cells found in the follicle-associated epithelium, lack an organised brush border and are only slightly covered in mucus, and are highly susceptible to bacterial invasion (Atkins and Furuta 2010). It has been demonstrated by the dissection of mouse Peyer’s patches that dendritic cells are found in the sub-epithelial dome located under the follicle-associated epithelium, where they can capture antigens and bacteria after their transcytosis across M-cells, and that dendritic cells are also located in the inter-follicular T-cell regions (IFR), where they present antigens to T-cells (Sato, Hashiguchi et al. 2003).

Peyer’s patches are the site for antigen capture and presentation to T-cells and induction of T-cell-dependent Immunoglobulin (Ig)-A responses. Hence it is not surprising that dendritic cells within Peyer’s patches can be distinguished from the expression of the chemokine receptors CX3CR1, CCR6, and CCR7, which impose their distribution. CX3CR1 dendritic cells are in close contact with the follicle-associated epithelium and take up antigens after M-cell transcytosis; however, they do not initiate T-cell immunity (Salazar-Gonzalez, Srinivasan et al. 2007). Those expressing CCR6 are just underneath, in the sub-epithelial dome, are ready to migrate to the follicle-associated epithelium in response to Salmonella infection and contribute to antigen specific T-helper cell activation (Salazar-Gonzalez, Srinivasan et al. 2007). CCR7 dendritic cells are found in T-cell areas. These cells are likely to drive from the other CX3CR1 or CCR6 subsets after chemokine receptor switching that allows them to migrate into the inter-follicular T-cell regions (Iwasaki and Kelsall 2000). Dendritic cells in the Peyer’s patches’ can also be distinguished on the basis of the expression of CD11b and CD8 that characterises dendritic cells with a Th2 or Th1 polarising ability respectively.
A population of CD103 dendritic cells is also present in Peyer’s patches’ and is expanded in response to probiotics, but its localisation and function are not yet clear (Fink and Frøkiær 2008). Rescigno et al (2010) who have contributed significantly in the last decade to the characterisation and function of Peyer’s patches’ dendritic cell subsets, acknowledge that further characterisation is still required.

**The role of dendritic cells in intestinal immunity**

Dendritic cells have the ability to migrate through the intestinal epithelium. Their main role is unceasing surveillance for antigens and activating T-cells from the secondary lymphoid tissue (Kelsall, Biron et al. 2002).

Dendritic cells isolated from intestinal tissues are endowed with unique mucosal functions that are specific for dendritic cell subsets (Fagarasan 2010). Coombes et al (2007) proposed that CD103 dendritic cells isolated either from the lamina propria or from the mesenteric lymph nodes can promote the conversion of Foxp3 T-cells. This activity relies on retinoic acid and transforming growth factor β-1. As retinoic acid inhibits the development of interleukin-17 (IL-17) producing T-cells (Mucida, Park et al. 2007) it is unlikely that the same population can polarise both T-reg- and Th17-cells. CD103 dendritic cells express the enzyme indoleamine 2,3 dioxygenase (IDO), that is required for their tolerogenic functions (Matteoli, Mazzini et al. 2010). IDO participates in tryptophan catabolism and its immuno-suppressive effects are linked to either the reduction of local tryptophan concentration or to the production of immuno-modulatory tryptophan metabolites, like kynurenin (Torres, López-Casado et al. 2007). IDO expression in CD103 dendritic cells inhibits Th17 development and reduces T-cell proliferation, and probably allows for the up-regulation of Foxp3 by TGFβ1 (Matteoli, Mazzini et al. 2010).

Undoubtedly it has been established that dendritic cells pathogen interactions are complex and diverse and that dendritic cells function may be modulated by microbial stimulation (Ju, Clark et al. 2009). Dendritic cells can induce tolerance under appropriate physiological conditions and can the ability to be reactive to pro-inflammatory stimuli, allowing T-cell primed inflammatory responses to pathogens and food antigens. Dendritic cells can also down-regulate T-cell activation and induce tolerance (Brix, Lund et al. 2010).

**Dendritic cell sampling**

Over the past few years one of the most important concepts to have emerged in the area of gastrointestinal tract immunology has come from the work of Rescigno et al (Rescigno 2010)
who observed the ability of dendritic cells to internalise bacteria and shuttle them across the epithelial barrier (Rescigno 2010). This sophisticated mechanism of sampling supplements work investigating M-cell mediated transport of particulate antigens and bacteria (Corr, Gahan et al. 2008). The dendritic cells are capable of sensing changes in the luminal environment via dendrites insinuated through tight junctions between adjacent enterocytes and projecting into the intestinal lumen (Foti and Ricciardi-Castagnoli 2005). Rescigno et al (2010) was able to show that immature dendritic cells with genes encoding the adhesion and tight junction proteins E-cadherin, occludin and zonulin-1 (Zo-1), were up-regulated and able to migrate between the intestinal epithelial cells, by opening tight junctions and capturing the antigens of both pathogenic and potentially pathogenic bacteria. It has been controversial to claim that dendritic cells’ primary role is to directly sample luminal contents despite evidence to the contrary (Cobrin and Abreu 2005). The controversy arises from the dual ability of dendritic cells. First, dendritic cells express E-cadherin, occludin and zonulin that preserve the integrity of the intestinal barrier and avoid exposure to potentially harmful agents, and secondly they can produce pro-inflammatory cytokines that can preferentially differentiate T-cells to T-helper-1 type effectors cells (Cobrin and Abreu 2005).

Stagg et al (2003) demonstrated that dendritic cell sampling can occur indirectly via the M-cells in the follicular-associated epithelium and this was confirmed more recently by Rescigno et al (2010). It is thought that appropriately stimulated follicular-associated epithelium secretes CCL-20, the chemokine responsible for chemotactic migration of dendritic cells into Peyer’s patches. CCL-20 binds the chemokine receptor CCR-6, expressed by immature dendritic cells, naïve B-cells and memory T-cells (Iwasaki and Kelsall 2000). Interestingly, dendritic cells were absent from the sub-epithelial region of Peyer’s patches in CCR-6-deficient mice, impairing immune response to pathogens, although the size of Peyer’s patches and the distribution of B- and T-cells remained normal. Stagg et al (2003) found that expression of CCL-20 is up-regulated in epithelial villi of the small intestine in response to a flagellin challenge. They concluded that this response is probably instrumental in recruiting dendritic cells into the intestinal epithelium but that pathways other than M-cells or Peyer’s patches are essential for generating dendritic cell mediated inflammatory responses.

The origin and development of dendritic cells

Nakamura et al (2010) have made significant contributions to understanding how dendritic cells develop. The sub-type of ‘freshly’ made mature dendritic cells direct what type of T-cell will develop out of the undifferentiated T-cells. This is achieved by selective expression of
polarising molecules such as interleukin (IL)-12, IL-18, IL-23, IL27, interferon-γ (IFN-γ) and 1-CAM-1 (Nakamura, Otani et al. 2010). For example, IL-23 acts primarily on effector T-cells, prolonging and sustaining their IFN-γ production, while IL-27 influences naïve Th-cells, and is crucial in the initial and early synthesis of IFN-γ, either alone or in synergy with IL-12 (Nakamura, Otani et al. 2010). Interestingly, the type of pathogen interacting with immature dendritic cells determines subsequent Th-cell polarising capacity (Ng, Benjamin et al. 2011). Dendritic cells promoting a Th1 response develop after exposure of immature dendritic cells to pathogenic bacteria or dietary antigen. Non-pathogenic bacteria also contribute to the class of gut-associated and systemic effector Th-cell response by influencing the polarising capacity of Th-cells (Didierlaurent, Sirard et al. 2002). It is thought that the predominant commensal microflora affect dendritic cells by influencing their expression levels of T-cell polarising signals via toll-like receptors. This was described by Ospelt et al (2010) who reviewed a number of signalling pathways, including how commensal microflora affects dendritic cells by influencing their expression levels of Th-cell polarising signals via toll-like receptors. Schnare and Barton (2001) examined IL-10 and IL-12 production, and the T-cell polarising potential of dendritic cells after stimulation by three microbial toll like receptor activators and found dramatically different profiles of IL-10 and IL-12 production in dendritic cells for each stimulus. This supports the work of other groups who found that microbe-specific information sensed by dendritic cells through different toll-like receptors is linked to differential Th-priming through the release of dendritic cell derived cytokines (Rakoff-Nahoum and Medzhitov 2008). The ability of dendritic cells to launch a qualitatively different immune response depends on the dendritic cell subset involved, the nature of the pathogen recognition receptors and the nature of the antigens (Rakoff-Nahoum and Medzhitov 2008). Iwasaki et al (2000) identified distinct subsets of dendritic cells that present different types of antigens. Rakoff-Nahoum and Medzhitov (2008) suggest that some dendritic cell populations might initiate different arms of adaptive immunity, with certain dendritic cells specialising in stimulating cytotoxic T-cell CD-8 responses, whereas others stimulate CD4 + T-cells. Consistent with research by a number of other groups (Kelsall, Biron et al. 2002; Drakes, Blanchard et al. 2004; Hart, Lammers et al. 2004; Karlsson, Larsson et al. 2004; Nakamura, Otani et al. 2010), there is accumulating evidence that dendritic cells contribute to the differentiation of suppressive T-cells (T-regulatory cells) (Colonna, Pulendran et al. 2006).
The relationship between intestinal epithelial and dendritic cells

The conditioning of the dendritic cells by intestinal epithelial cells to mediate a Th2 or Th3 response is altered during infection (Miossec, Korn et al. 2009). It was thought that continuous cytokine expression rendered dendritic cells incapable of stimulating a Th1-mediated response (Rescigno 2010). However, during infection activated dendritic cells migrate to regional lymph nodes and induce a Th1-mediated inflammatory state (Martín-Fontecha, Lanzavecchia et al. 2009).

Other groups have supported this suggestion by demonstrating that the dendrites of dendritic cells only extend into the terminal ileum in healthy individuals, but extend throughout the apical surfaces of the villi in the small intestine in infected individuals (Germain and Margulies 1993; Reinecker and Podolsky 1995). This observation was repeated more recently by Rescigno et al (2010) further supporting the role of dendritic cells as luminal antigen samplers. There is also evidence to support the antigen presenting cell role of dendritic cells during infection in the dome region of Peyer’s patches, where dendritic cells have been found taking up viral antigens from dying intestinal epithelial cells and inducing Th1 activation (Kelsall, Biron et al. 2002; Kelsall, Leon et al. 2005).

Interferon activates dendritic cells during infection

It is well established that type 1 interferon pro-inflammatory cytokines activate dendritic cells (Brix, Lund et al. 2010). This occurs when interferon binds to a common cell-surface receptor (Brix, Lund et al. 2010). Mouse model studies have demonstrated that type 1 interferon was induced in the presence of bacterial and viral infection but not in low and normal levels of bacteria. The type 1 interferon’s were also elicited by lipo-polysaccharides, bacterial DNA and double stranded RNA, further suggesting infectious triggers. The exposure of type 1 interferon to dendritic cells enhances their expression and hence their ability to stimulate T-cells (Huber and Farrar 2011). The capacity of type-1 interferon to activate dendritic cells is paradoxical, as these cells also produce type-1 interferon’s in response to infection (Huber and Farrar 2011). Interferons are secreted by dendritic cell precursors in response to pathogen-associated signals. Thus dendritic cell-secreted type-1 interferons can act in an autocrine manner, promoting survival of dendritic cell precursors and stimulation expression of type-1 interferon induced genes in activated dendritic cells.
The interplay between the luminal contents including commensal bacterial, pathogenic bacteria and dendritic cells and intestinal epithelial cells is critical for dendritic cell function in both health and infection (Colonna, Pulendran et al. 2006).

**Interleukins’ effect on para-cellular permeability**

Interleukins (IL) are a large family of cytokines, and several have been studied for effects on para-cellular permeability in vitro. These include IL-1, 2, 4, 6, 8, 10 and 13, all of which have been found to have a variety of effects on epithelial and endothelial para-cellular permeability that is presented in Table A1.

**Growth Factors**

The potential role that growth factors play in para-cellular permeability is presented in Table A2. Growth factors have a variety of effects on para-cellular permeability either by increasing or decreasing permeability, depending on the cell environment. Transforming growth factor beta (TGF-β) is a multi-functional cytokine that has been shown to enhance epithelial barrier properties in vitro (Howe, Gauldie et al. 2002; Howe, Reardon et al. 2005).

**Toll-like receptors function**

Innate immunity was long thought to be a non-specific response based upon engulfment and digestion of microorganisms by macrophages. Over the last decade it has become apparent that a family of non-clonal, germ-line-encoded pattern-recognition receptors (PRRs), namely the TLRs, provide the innate immune system with considerable specificity for a vast range of microbial pathogens; this offers the host an immediate antimicrobial response system (Testro and Visvanathan 2009). The innate immune response is a critical prerequisite for triggering adaptive immune responses (Schnare, Barton et al. 2001). Toll-like receptors are type 1 trans-membrane proteins consisting of a cytoplasmic and an extracellular domain. A total of 13 mammalian TLRs have been described, with ten of these are expressed in humans with each carrying a distinct ability to recognise invariant microbial structures. They are collectively referred to as pathogen-associated molecular patterns (PAMPs) (Hopkins and Sriskandar 2005).

Many bacterial, viral, fungal and protozoal components are capable of stimulating innate immune responses mediated by TLRs and are presented in Table A3. TLRs are expressed in most tissue, with differential expression of TLRs being particularly marked in the gastrointestinal epithelium. This expression of TLRs in the intestinal epithelium has important
consequences for both innate defence and microbial pathogenesis (Testro and Visvanathan 2009).

Table A1 Permeability modification by interleukins and growth factors

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Permeability</th>
<th>Cells</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>Human umbilical vascular endothelial cells (HUVECs)</td>
<td>Unknown (Marcus, Wyble et al. 1996)</td>
</tr>
<tr>
<td>IL-1</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>Caco-2</td>
<td>NF-κB Decrease in occludin (Al-Sadi and Ma 2007)</td>
</tr>
<tr>
<td>IL-1</td>
<td>Increase in trans-epithelial resistance</td>
<td>Astrocytes</td>
<td>Increase claudin 1, Decrease in occludin (Duffy, John et al. 2000)</td>
</tr>
<tr>
<td>IL-1</td>
<td>Increase in trans-epithelial resistance</td>
<td>PAE</td>
<td>Unknown (Coyne, Vanhook et al. 2002)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>Human lung epithelial cells</td>
<td>Decrease in Zo-1, occludin (Ahdieh, Vandenbos et al. 2001)</td>
</tr>
<tr>
<td>IL-4</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>T84 human colonic epithelial cells</td>
<td>Unknown (Colgan, Resnick et al. 1994) Increase in claudin 2 (Wisner, Harris et al. 2008)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increase in trans-epithelial resistance</td>
<td>Intestine</td>
<td>Decrease in zonulin 1 (Yang, Han et al. 2003)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increase in small molecular flux</td>
<td>Human umbilical vascular endothelial cells</td>
<td>Actin restructuring , Zonulin-1, protein kinase Z (alpha or beta) (Desai, Faubion et al. 2007)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Decrease in trans-epithelial resistance</td>
<td>Liver</td>
<td>Increase in ZO-1, claudin 1 (Mazzon, Puzzolo et al. 2002)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Decrease in trans-epithelial resistance and small molecule flux</td>
<td>T84 human colonic cells</td>
<td>Antagonises IFN-γ (Madsen, Lewis et al. 1997)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Decrease in trans-epithelial resistance and small molecule flux</td>
<td>Human umbilical vascular endothelial cells</td>
<td>Antagonises IFN-γ and increases occludin (Oshima, Laroux et al. 2001; Fukuda, Toh et al. 2011)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>T84 human colonic cells</td>
<td>Increase in claudin 2 (Prasad, Mingrino et al. 2005)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Increase in trans-epithelial resistance and small molecule flux</td>
<td>Calu-3</td>
<td>Decrease in zonulin, occludin (Ahdieh, Vandenbos et al. 2001)</td>
</tr>
</tbody>
</table>
### Table A2 Role of growth factors in para-cellular permeability

<table>
<thead>
<tr>
<th>Transforming Growth Factor β (TGF-β)</th>
<th>Increase in trans-epithelial resistance</th>
<th>Uterine epithelial cells</th>
<th>Unknown (Grant-Tschudy and Wira 2005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Decrease trans-epithelial resistance</td>
<td>T84 colonic cells</td>
<td>Increase in claudin 2, extracellular signal-regulated kinases, and mitogen-activated protein kinase (Howe, Gauldie et al. 2002; Howe, Reardon et al. 2005)</td>
</tr>
<tr>
<td>Hepatocyte growth factor/scatter factor (HGF/SF)</td>
<td>Increase in trans-epithelial resistance</td>
<td>cerebrovascular</td>
<td>Decrease in occludin and decrease in zonulin (Date, Takagi et al. 2006)</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>Decrease in small molecule flux</td>
<td>Uterine epithelial cells</td>
<td>Unknown (Grant-Tschudy and Wira 2005)</td>
</tr>
<tr>
<td>Heparin binding epidermal growth factor (HB-EGF)</td>
<td>Decrease in small molecule flux</td>
<td>Madine-Darby canine kidney cells</td>
<td>Decrease in claudin 2 (Harhaj, Barber et al. 2002)</td>
</tr>
<tr>
<td>Platelet derived growth factor (PDGF)</td>
<td>Increase in trans-epithelial resistance</td>
<td>Madine-Darby canine kidney cells</td>
<td>Tight junction structure, (Harhaj, Barber et al. 2002)</td>
</tr>
</tbody>
</table>

### Table A3 Role of TLRs in detecting microorganisms

<table>
<thead>
<tr>
<th>Micro organism component</th>
<th>Detecting Toll-like Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipo-polysaccharide (LPS)</td>
<td>TLR4 (found to be elevated in CD)</td>
</tr>
<tr>
<td>Lipoteichoid acid (fungal component)</td>
<td>TLR2 (found to be elevated in CD)</td>
</tr>
<tr>
<td>Bacterial lipopeptide (gram-positive and mycobacterial component)</td>
<td>TLR2 (found to be elevated in CD)</td>
</tr>
<tr>
<td>Monomerc flagellin (flagellin component)</td>
<td>TLR5</td>
</tr>
<tr>
<td>Lipoteichoic acid (the unmethylated CpG DNA of bacteria and viruses)</td>
<td>TLR9</td>
</tr>
<tr>
<td>Double stranded RNA</td>
<td>TLR7</td>
</tr>
</tbody>
</table>
The distribution of TLRs on the gastrointestinal tract epithelium is highly sophisticated in order to avoid over-stimulation by the commensal microorganisms in certain locations. Therefore different anatomical compartments of the GI tract exhibit specific TLR expression patterns (Ortega-Cava, Ishihara et al. 2003). In addition to their ability to locate certain anatomical regions of the GI tract TLRs can also respond in a dynamic manner to exposure to environmental stress, microbial pathogens or host mediators such as cytokines (Ortega-Cava, Ishihara et al. 2003). TLRs compartmentalise not only throughout the gastrointestinal tract but also cellularly. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface of immune cells, while TLRs 3, 7, 8 and 9 are expressed intra-cellularly. This cellular compartmentalisation of TLRs further circumvents the potential for erroneous stimulation of the innate immune system by host antigens.

TLRs signal through one of two distinct pathways, the myeloid differentiation factor 88 (MyD88)-dependent and the MyD88-independent, resulting in the induction of pro-inflammatory cytokine genes and type 1 interferon’s respectively. TLR4 is able to signal through both pathways (O’Neill, Fitzgerald et al. 2003). Toll-like receptor signalling pathways are graphically presented in Figure A1.

Figure A1 Toll-like receptor signalling pathways (Akira, Uematsu et al. 2006)
The regulation of the TLR signalling pathways is closely regulated to avoid damage to the host from an excessive or prolonged inflammatory response and in order to be permissive for the commensal indigenous microbiota (Akira, Uematsu et al. 2006). This is achieved at several sites along the signalling cascade with intra-cellular negative regulation being mediated by protein phosphorylation, degradation and interaction with inhibitory adaptor molecules, or sequestration (Hopkins and Sriskandan 2005). TLR signalling may serve as a protective function in the intestinal epithelium by inducing defensin 2 and limiting pathogenic infection, or preventing commensal bacteria from breaching the intestinal epithelial barrier (Wilson 2008). The function of TLRs in linking innate and adaptive immunity is graphically presented in Figure A2. In addition to this response TLR-activated APCs enhance expression of co-stimulatory surface molecules, such as CD80 and CD86, and activate T-cells, together with antigen presentation. CD4+ T-cells can differentiate into either T-helper (Th)1 cells of Th2 cells: T-helper 1 cells produce IFN, mediating antibacterial and antiviral immunity, whereas Th2 cells secrete IL-4 and IL-12; these are involved in immunity against helminths and in allergic reactions. TLR9 signalling activated by bacterial CpG DNA has the strongest activity to induce Th1 responses and as such forms the basis of clinical trials in the management of asthma and allergic conditions (Fonseca and Kline 2009).

**The role of nucleotide oligomerisation domain proteins**

The nucleotide oligomerisation domain protein-like receptors (NLRs) are a specialised group of intra-cellular receptors that represent a key component of the host innate immune system. Since the discovery of the first NLR a decade ago numerous groups have worked to gain a better understanding of the mechanism by which these receptors recognise microbes and other antigens and how they activate inflammatory signalling pathways (Rietdijk, Burwell et al. 2008; Chen, Shaw et al. 2009). In addition to their primary role in defending the host against invading pathogens NLDs can regulate nuclear factor-kappa B (NF-kB) signalling, interluekin-1-β production, and cell death, indicating that they are critical to the pathogenesis of a variety of inflammatory human diseases (Chen, Shaw et al. 2009).
Figure A2: The role of the Toll-like receptors in linking innate and adaptive immunity (Akira, Uematsu et al. 2006). Through a number of pathways, the innate immune system provides immediate protection to the host, mediated by the release of antimicrobial peptides, cytokines, chemokines, adhesion molecules and acute phase proteins. The resultant innate immune system causes activation of inflammatory cells at the site of infection.

NOD1 and NOD2 proteins contribute to regulation of pro-inflammatory pathways via NF kappa B induced bacterial ligands (Rietdijk, Burwell et al. 2008). NF-kappa B transcription factor also regulates the expression of antimicrobial agents, cytokines and chemokines (Ashall, Horton et al. 2009). NOD1 appears to be primarily involved in sensing gram-negative bacterial pathogens. NOD2 senses the specific MDF motif that is found in a broader range of bacteria, with some overlap with those recognised by NOD1 (Chen, Shaw et al. 2009). Studies have suggested that the intra-cellular sensors NOD1 and NOD2 play a critical role in host defence when Toll like receptor signalling is reduced, such as within the intestine due to low expression levels of toll-like receptors or after induction of tolerisation by exposure to toll like receptor ligands (van Meurs, Shapiro et al. 2010).
Nucleotide oligomerisation domain protein is critical in protecting against intestinal bacterial infections, via macrophages or paneth cells. In the presence of muramyl dipeptide (MDP), NOD2 induces NF kappa B activation and the production of pro-inflammatory mediators. The expression of NOD2 takes place primarily in dendritic cells and intestinal epithelial cells, with greater expression in the intestinal crypts than in the intestinal villi (Santaolalla, Fukata et al. 2011). NODs are also expressed in monocytes, macrophages, B-and T-cells, and Paneth cells. The pro-inflammatory mediators IFN-γ and TNF secreted by Th1 cells up-regulate expression of NOD2 by intestinal epithelial cells (Brand 2009). NOD2 variants are categorised according to their ability to activate NF-kappa B in response to PGN, suggesting that without adequate NF-kappa B activation translocated bacteria are not effectively eliminated, resulting in antigen presenting cell activation and induction of T-cell proliferation by secretion of Th-1 stimulating cytokines (IL-12, IL-23 or IL-18), thus causing inflammation (Brand 2009). Brand and colleagues made an association between mutations in NOD2 and susceptibility to intestinal inflammation leading to inflammatory bowel disease (Brand 2009).

![Figure A3](image)

Figure A3 TLRs are expressed on the cell surface or in endosomal compartments while NLR are expressed in the cytoplasm. Both families of receptors recognise microbial products and “danger signals” released by dead cells and activate signalling pathways that initiate the inflammatory response and regulate development of adaptive immunity (Ting, Duncan et al. 2010).

The role of NOD like protein receptors in coeliac disease

It has been suggested that excessive IL-1β and IL-18 production due to a mutation in NOD like protein receptor 3(NLPR3), could contribute to the generation of gliadin reactive T-cells (Shaw, McDermott et al. 2011). Pontillo et al (2010) observed that patients with CD and Type 1 diabetes had a significantly lower occurrence of NLRP3 SNP than healthy children. They acknowledged that it was too early to know whether NLRP3 was involved in the predisposition or progression of CD. However, they go on to hypothesise that the downstream
production by NLRP3 inflammasome, activation of IL-1beta and control of the IL-23 response to gliadin affect Th17-cell differentiation and survival. Additionally, the receptor for IL-18 protein is a susceptibility locus for CD and IL-18 is believed to play a role in the pathogenesis of the disease by inducing Th1 responses.

The role of B-cells in humoral immunity

The cytokines produced from antigen presenting cells, B-cells and T-cells influences the activity of adaptive immunity. Immature B and T-cells are located in distinct areas of the intestinal mucosa. B-cells participate in humoral immune responses, whereas T-cells are involved in both cellular and humoral responses (Brandtzaeg, Carlsen et al. 2006). The antibodies produced by B-cells are released into interstitial and vascular tissues, forming antibody-antigen complexes that trigger granulocytes to liberate inflammatory mediators that are specific to and destroy particular antigen (Brandtzaeg, Carlsen et al. 2006). The antibodies produced include Immunoglobulin M (IgM), immunoglobulin A (IgA), Immunoglobulin G (IgG), Immunoglobulin E (IgE) and Immunoglobulin D (IgD). Each of the immunoglobulins plays a specific role in immunity. Naïve B-cells, i.e. B-cells that have not yet been introduced to a pathogen, can be activated either with the assistance of T-cells or independently. Naïve B-cells are activated by T-cells when active macrophages attach part of the pathogen's proteins to a major histocompatibility class II protein (MHC-II). The MHC-II protein moves outside the cell-membrane, where the epitope on the antigen is recognised by a T-cell. If there is a match between the B-cell and T-cell structures, the T-cell will activate the B-cell to produce antibodies against the antigen by using its B-cell receptor.

In response to T-cell-dependent antigens isotypes can switch to IgG, IgA or IgE and generate memory cells in the Peyer’s patches under the influence of TGF, IL-10 and signals generated from dendritic cells and T-cells. This process is not reversible once switching has occurred, i.e., IgG, IgA or IgE cannot switch back to IgM or IgD (Harriman, Volk et al. 1993). There is a number of factors that influence whether isotypes switch, including the route of antigen presentation and the type of cells and cytokines present combined with the genetic predisposition of the host (Lemanske, Atkins et al. 1983).

All humoral responses, including IgE-mediated responses, are modulated by Th2-CD4+ cells (Weigmann, Schughart et al. 2012). Th2-CD4+ responses are characterised by the secretion of Interleukin-4 (IL-4) by APCs. The consequence to the host of a local IgE-mediated response is due to IgE mounting the mast cells in the GALT and eliciting an immediate
hypothesusensitiv response. The IgE-mediated response is classically characterised by inflammation and associated swelling (Weigmann, Schughart et al. 2012).

Inflammation and damage can also be caused by skewed Th1 responses to non-IgE-mediated responses, including IgG (Brenchley and Douek 2012). This is due to IgGs' active Fc-receptors (FcRs) that bind to a pathogens and protect against the pathogens active toxicity action by activating complement that neutralises the toxin. However, this process results in an IgG delayed type sensitivity response that triggers inflammation and damages the intestinal mucosa (Albanesi and Daëron 2012). Conversely, mucosal IgA responses do not cause inflammatory damage to the intestine, as IgA do not have an active fragment crystallisation receptor, therefore it cannot mount a pro-inflammatory response (Albanesi and Daëron 2012).

The role of T-cells in intestinal immunity

T-cells play a critical role in intestinal adaptive immune function. T-cell recruitment from the thymus gland is initiated through toll like receptor signalling in dendritic cells (Blander, Torchinsky et al. 2012). As depicted in Figure A4, toll-like receptors activate dendritic cells to produce distinct patterns of cytokines that direct the immature T-cells in Th-cell subsets (Th0, Th1, Th2 or T-regulatory cells).

The differential development of T-cell subsets determines the outcome of physiological and pathological immune responses (Nakamura, Otani et al. 2010). Huse et al (2006) identified two distinct pathways used by T-cells for secretion of cytokines and this was later confirmed by Maldonado et al (2009). They both found that the cytokines IL-2 and IFN-γ were secreted into the synapse, whereas TNF-α and the chemokine CCL3 were released multi-directionally. Moreover, each secretion pathway was associated with distinct trafficking proteins, indicating that they are molecularly distinct processes. The authors concluded that T-helper-cells release some cytokines into the immunological synapse to impart specific communication and others to promote inflammation and to establish chemokine gradients (Huse, Lillemeier et al. 2006; Dethlefsen, Huse et al. 2008; Maldonado, Soriano et al. 2009).
Figure A4 Functional development and activity of Th-cell subsets. Activation of dendritic cells follow interaction of pattern-recognition receptors such as C-type lectin receptors (CLR) or TLR with molecules on the surface of microorganisms. Antigen presentation to naïve T-cells results in the development of Th1, Th2 or Th17 cells, depending on the cytokine milieu. (Costa, Mattana et al. 2010).

T-cell subsets and the role of T-helper 1 cells in intestinal immunity

As presented in Table A4, the cytokines interleukin 12 (IL-12) and IL-4 are dominant factors in driving the development of Th1 and Th2-cells respectively, through specific signalling pathways (Costa, Mattana et al. 2010). Earlier this decade it was demonstrated that T-helper-cell-specific transcription factors exist that determine the commitment of Th1- and Th2-cells for the production of distinct profiles of cytokines (Anne and Arai 2000). In addition to the expression of distinct cytokine genes and transcription factors, the molecular basis for commitment to a Th1 or Th2 phenotype can probably be explained by multiple mechanisms, including differential cytokine signalling, exclusive cytokine receptor expression, differential transcription of transcription factors and/or differential chromatin remodelling of Th1- and Th2-specific genes (Anne and Arai 2000; Maldonado, Soriano et al. 2009).

The polarisation of the naïve T-cells towards Th1-type differentiation occurs through the expression of the transcription factor T-beta (Feleszko, Jaworska et al. 2006). Th1-cell production is induced by type 1 IFNs in concert with the signalling component of the IL-12
receptor (IL-12R) (Feleszko, Jaworska et al. 2006). Activation by type 1 IFNs of NK and
cytotoxic T-cells occurs in response to viral infections and to double-stranded RNA.
However, it is also thought that there are other non-viral induced cytokines that can trigger the
expression of IL-12R (Maldonado, Soriano et al. 2009). It has been proposed that the
interaction between T-cells and dendritic cells may trigger the production of type-1 IFNs
required for Th1-cell development (Feleszko, Jaworska et al. 2006).

**Th2 T-cells in intestinal immunity**

The signature Th2 cytokines include IL-4, IL-5, IL-10 and IL-13 (Costa, Mattana et al. 2010).
Th2 cells provide cytokines required for immunity to extracellular pathogens. The
proliferation of B-cells is reliant on the presence of IL-4. In addition IL-4 is required for the
immunoglobulin class' switching to IgG and IgE (Harriman, Volk et al. 1993; Albanesi and
Daëron 2012). Th2 cytokines (IL-4, IL-9, IL-10 and IL-13) are critical in the generation of
protective immunity against helminths (Turner, Faulkner et al. 2003).

**Th1/Th2 balance**

As previously stated T-lymphocytes and their cytokines play a key role in orchestrating a
specific mucosal immune response (Mayer 2003). In particular, the signature cytokines of
distinct T-cell subsets and the transcriptional regulation of T-cell differentiation appear to be
of fundamental importance in mucosal immunity (Harvey 1989; Mayer 2003). However,
uncontrolled mucosal T-cell responses may lead to immunologic diseases such as allergy,
Polarisation of T-helper subsets is required for specific biological processes such as
pregnancy. A full-term pregnancy relies on a Th2-dominant environment to prevent rejection
of the foetus (Makhseed, Raghupathy et al. 1999). In utero a foetus uses humoral immunity to
defend itself but has no cellular immunity. At birth it down-regulates humoral immunity and
up-regulates Th1 production to develop a reciprocal relationship between Th1 and Th2
immunity. If a humoral predominance remains pro-allergy and pro-inflammatory tendencies
exist (Straub, Buttgereit et al. 2005). The amelioration of pro-inflammatory states such as
arthritis and autoimmune diseases have been observed in pregnant women since the early
1900s (Nelson, Hughes et al. 1993), but it is only relatively recently that the Th2 dominance
mechanism has been elucidated (Straub, Buttgereit et al. 2005). Intestinal microbiota play a
critical role in the Th1/Th2 balance (Shanahan 2002) and are discussed in Chapter 3.
T-regulatory cells

Maintenance of immunological tolerance relies on T-regulatory (T-reg) cells, formerly known as T-suppressor cells (Nakamura, Otani et al. 2010). The main role of T-reg cells is to promote tolerance to self-antigens and abrogate autoimmune disease, thus preventing excessive reactions. The T-reg cells CD4, CD25 and Foxp3 are involved in terminating immune response after the successful eradication of an antigen or microorganism has been completed (Nakamura, Otani et al. 2010). Two major classes of CD+4 regulatory T-cells have been described, including the naturally occurring T-reg cells and the adaptive T-reg cells (Saurer and Mueller 2009). Naturally occurring T-reg cells (also known as CD4+CD25+ T-reg cells) originate in the thymus, whereas T-reg cells are produced during normal immune responses (Nakamura, Otani et al. 2010). The major difference between suppressor or natural T-reg cells and T-cells is that T-reg cells always suppress the immune system, whereas effector T-cells usually begin with immune-promoting cytokines and then switch to inhibitory cytokines later in their response (Nakamura, Otani et al. 2010). Regulatory and Th3-cells both produce the cytokines TGF-β and IL-10, which are inhibitory to helper cells. TGF-β suppresses the activity of most of the immune system, but there is evidence that TGF-β may not suppress activated Th2-cells as effectively as it does naïve cells. Irrespective of this, TGF-β is not typically considered a Th2-cytokine. As mentioned above, T-reg cells were formerly known as suppressor T-cells, but such terms as 'regulatory' or 'suppression' become ambiguous after the discovery that helper CD4+T-cells also regulate and suppress their own responses outside dedicated T-cells. The characterisation of another novel T-helper sub-type, T-helper-17 cells (Th17), has called into question the basic Th1/Th2 model. These IL-17-producing cells were initially described as a pathogenic population implicated in autoimmunity but are now thought to have their own distinct effector and regulatory functions (Miossec, Korn et al. 2009; Nakamura, Otani et al. 2010; Blander, Torchinsky et al. 2012).
Table A4 The properties of Th1- and Th2-cells (Huse, Lillemoier et al. 2006)

<table>
<thead>
<tr>
<th></th>
<th>Th1 helper cells</th>
<th>Th2 helper cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main cell partner type</strong></td>
<td>Macrophage</td>
<td>B-cells</td>
</tr>
<tr>
<td><strong>Cytokines produced</strong></td>
<td>INF-γ and TNF alpha (IL-2 was classically associated with Th1-cells, but this association may be misleading as IL-2 is produced by all helper T-cells early in their activation).</td>
<td>IL-4, IL-5, IL-6, IL-10 and IL-13</td>
</tr>
<tr>
<td><strong>Immune stimulation promoted</strong></td>
<td>Cellular immune system. Maximises the killing efficacy of the macrophages and promotes the proliferation of cytotoxic CD8+ T-cells.</td>
<td>Humoral immune system. Stimulates B-cells into proliferation to induce B-cell antibody class switching and to increase antibody production.</td>
</tr>
<tr>
<td><strong>Other functions</strong></td>
<td>The Type 1 cytokine IFN-γ increases the production of IL-12 by dendritic cells and macrophages and, via positive feedback, IL-12 stimulates the production of IFN-γ in helper T-cells, thereby promoting the Th1 profile. IFN-γ also inhibits the production of cytokines such as IL-4, an important cytokine associated with the Type 2 response, and thus it also acts to preserve its own response.</td>
<td>The Type 2 response promotes its own profile using two different cytokines. IL-4 acts on helper T-cells to promote the production of Th2-cytokines (including itself; it is auto-regulatory), while IL-10 inhibits a variety of cytokines including IL-2 and IFN-γ in helper T-cells and IL-12 in macrophages. The combined action of these two cytokines suggests that once the T-cell has decided to produce these cytokines that decision is preserved (and also encourages other T-cells to do the same).</td>
</tr>
</tbody>
</table>

**The role of toll-like receptors in immune tolerance**

The role of TLRs in gastroinestinal immunity has been discussed in 2.2.5. However, for the purposes of this review the specific role of TLRs in tolerance of self will be reviewed here. The TLRs recognise the PAMPs of commensal and pathogenic bacteria carried on dendritic cells, but remain in the lamina propria and are not found any deeper in the intestinal epithelium (Scott, Manunta et al. 2005; Rakoff-Nahoum and Medzhitov 2008). TLR expression is regulated to minimise a pro-inflammatory response to commensal bacteria while permitting the induction of signals that establish homeostasis and tolerance (O’Neill, Fitzgerald et al. 2003; Hopkins and Sriskandan 2005). An appropriate amount of TLR signalling improves the intestinal barrier function by tightening the apical junctions and triggering intestinal epithelial cell proliferation that prevents entry of luminal bacteria. In the absence of TLR signalling, expression of cytokines decreases, neutrophil recruitment diminishes and subsequent translocation of bacteria to mesenteric lymph nodes occurs, all of
which favour tolerance (Cobrin and Abreu 2005; Lee and Mazmanian 2010). Via TLR responses to commensal bacteria, dendritic cells contribute to tolerance by the selective induction of IgA secretion by B-cells (Lee and Mazmanian 2010). It has been demonstrated that prolonged exposure of the intraepithelial cells to lipo-polysaccharide substances may lead to tolerance (Shirai, Hashimoto et al. 2004; Brix, Lund et al. 2010). Induction of Th1-cell responses is initiated by bacterial motifs in the DNA and LPS of commensal bacteria, allowing them to be recognised by TLRs that trigger the release of Th1 cytokines IFN, IL-12 and IL-18 (Feleszko, Jaworska et al. 2006). Conversely, inflammation of the intestine involves the expression of other TLRs and subsequent release of inflammatory cytokines (Bruewer, Luegering et al. 2003).

**The role of nuclear factor-kappaB in intestinal immunity**

Nuclear factor kappa B (NF-κB) transcription factors are critical regulators of immunity, stress responses, apoptosis and differentiation. A variety of stimuli including infections, oxidants and antigens, coalesce on NF-κB activation, which can in turn mediate varied transcriptional programmes (Spehlmann and Eckmann 2009). Consequently, NF-κB dependent transcription is not only tightly controlled by positive and negative regulatory mechanisms but also closely coordinated with other signalling pathways. This intricate crosstalk is crucial to shaping the diverse biological functions of NF-κB into cell type and context-specific responses (Oeckinghaus, Hayden et al. 2011).

In the intestine commensal bacteria interact directly with intestinal epithelial cells and attenuate the synthesis of NF-κB elicited via a diverse set of stimuli (Oeckinghaus, Hayden et al. 2011). NF-κB forms the basis of disease-specific inflammatory processes through the coordinated activation of inflammatory genes. Both IL1 and TNF alpha activate and are activated by NF-κB in a positive regulatory loop which increase and maintains local inflammatory responses (Kayama and Takeda 2012). The activation leads to the coordinated expression of genes encoding proteins involved in mediator synthesis and the further amplification and perpetuation of the inflammatory response (Oeckinghaus, Hayden et al. 2011).

The functions of NF-κB in normal homoeostasis and specific disease processes in the intestinal tract have been discussed extensively (Spehlmann and Eckmann 2009). Inflammatory bowel diseases and experimental intestinal inflammation are characterised by NF-κB activation and increased expression of NF-kappa B target genes. Conversely, NF-κB inhibition protects against chronic intestinal inflammation and necrotizing enterocolitis.
(Pasparakis 2009; Petrof, Claud et al. 2009). In addition, it has been found that NF-κB not only has pro-inflammatory but also tissue-protective functions (Spehlmann and Eckmann 2009). Deficiency in TLR-5, a strong activator of NF-κB, results in spontaneous colitis and exacerbates mucosal inflammatory responses to *Salmonella* infections (Eckmann and Neish 2011). Conversely, TLR-5 stimulation confers radioprotection in the intestine (Garrett, Gordon et al. 2010). NF-κB has multiple, often opposing, functions in the intestine. The anti-apoptotic action of NF-κB in intestinal epithelial cells dominates tissue responses to many acute inflammatory and injurious challenges, whereas pro-inflammatory and cell survival functions of NF-κB in macrophages and T-cells govern chronic intestinal inflammation (Spehlmann and Eckmann 2009).
A2 The intestinal microbiome

Urinary organic acids

**Dietary influences on bacterial metabolites in urine**

Interpretation of urinary organic acid levels needs to be in the context of certain dietary compounds consumed by the patient. Dietary polyphenols that are not transported from the gut lumen into the enterocytes can be used by gut microbes for growth (Lord 2008). Possibly the best researched metabolites of bacterial metabolism are phenolic compounds. Dietary polyphenols have been shown to be one of the dominant substrates for yielding phenolic compounds, whereas dietary simple sugars lead to generation of non-phenolic hydrocarbon products. Many polyphenolic structures are found in foods, although it has been suggested that only a small number of phenolic compounds are formed in healthy individuals (Rechner, Smith et al. 2004). Therefore variation in food consumption between individuals may have only small effects on the potential for generating phenolic products. Lord (2008) suggests that the greater factor in the production of phenolic compounds is the species of intestinal microbiota present and their metabolic activity.

**Benzoate and Hippurate**

Van der Heiden et al (1971) were one of the earlier groups to identify high urinary benzoate in patients with intestinal bacterial overgrowth from various origins. They identified that many patients with intestinal bacterial overgrowth resulting from cystic fibrosis or unclassified enteritis, CD or short bowel syndrome had elevated benzoate along with varying degrees of elevated phenylacetate, p-hydroxybenzoate and p-hydroxyphenylacetate, and thought that these products were likely to be derived from unabsorbed tyrosine or phenylalanine from poor digestion of dietary protein. However, later reports cited by (Lord 2008) suggested that bacterial catabolism of dietary polyphenols may be the predominant origin of benzoate, which normally conjugates with glycine in the liver to form hippurate (Goodwin, Ruthven et al. 1994). As demonstrated by Scalbert et al (2002) dietary polyphenols generally persist in the lower small intestine because they are resistant to degradation by digestive fluids. Table A5 presents a summary of possibly patterns observed in the measures of urinary benzoate and hippurate (Lord 2008).
Table A5 Interpretation of urinary benzoate and hippurate abnormality patterns (Lord 2008)

<table>
<thead>
<tr>
<th>Benzoate</th>
<th>Hippurate</th>
<th>Other Bacterial Markers</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Low</td>
<td>No elevations</td>
<td>Low intake of benzoate and precursors, plus normal or low dietary polyphenol conversion by intestinal microbes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple elevations</td>
<td>Low intake of benzoate and precursors with intestinal microbial overgrowth of species that do not metabolize dietary polyphenols (very rare)</td>
</tr>
<tr>
<td>High</td>
<td>Low</td>
<td>No elevations</td>
<td>Glycine conjugation deficit (possibly genetic polymorphic phenotype if hippurate is very low); dietary benzoate or precursor intake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple elevations</td>
<td>Glycine conjugation deficit; presume benzoate is at least partially from intestinal microbial action on dietary polyphenols</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>No elevations</td>
<td>Normal hippurate production via active glycine conjugation; No indication of microbial overgrowth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple elevations</td>
<td>Normal hippurate production via active glycine conjugation; Presume hippurate is at least partially derived from intestinal microbial action on dietary polyphenols</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>No elevations</td>
<td>Very high dietary benzoate or precursor intake with partial conversion to hippurate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple elevations</td>
<td>Very high benzoate load, some, or all, of which is contributed by intestinal microbial action on dietary polyphenols</td>
</tr>
</tbody>
</table>

In addition to dietary intake of polyphenols, elevations in urinary benzoate and hippurate may be clinically significant in two areas: detoxification and dysbiois. Urinary benzoate has been linked to gastrointestinal disorders associated with intestinal bacterial overgrowth (van der Heiden, Wauteks et al. 1971). In a group of patients with CD urinary benzoic acid was increased in 9 out of ten untreated cases and interestingly, in six out of eleven patients given a gluten-free diet (van der Heiden, Wauteks et al. 1971). The significance of urinary benzoate as a dysbiosis indicator is strengthened when elevations are accompanied by elevations in urinary phenylacetate, p-hydroxybenzoate and p-hydroxyphenylacetate (Goodwin, Ruthven et al. 1994). Catabolism of dietary polyphenols by bacteria in the small intestine is thought to be the predominant origin of urinary benzoate, which is normally conjugated with glycine in the liver to form hippurate (Lord and Bralley 2008). Therefore elevations in benzoate and low or undetectable levels of urinary hippurate may suggest a deficit in glycine conjugation, preventing the conversion of benzoate to hippurate. The clinical significance of elevated benzoate and hippurate levels should be interpreted in the context of other urinary metabolites.
Phenylacetate and hydroxyphenylacetate

Intestinal bacterial metabolism on dietary polyphenols may also result in the appearance of phenylacetic acid (PAA) in the urine. However, urinary PAA is also the product of unidentified, specific strains of bacteria (Chalmers, Valman et al. 1979). Urinary phenylacetate may be elevated in small intestinal bacterial overgrowth (SIBO) (Chalmers, Valman et al. 1979).

Enteric bacteria that possess L-ammonia acid decarboxylase produce hydroxyphenylacetic (HPA) acid from dietary tyrosine. Increased excretion has been demonstrated in both children and adults with small bowel disease or bacterial overgrowth syndrome (Chalmers, Valman et al. 1979). Chalmers et al (1979) evaluated measurement of 4- hydroxyphenylacetic acid as a potential screening method for small bowel disease and bacterial overgrowth syndrome in 360 unselected acutely ill infants and children compared with 150 healthy infants and 48 healthy children (Chalmers, Valman et al. 1979). No false negative results and only 2% false positives were observed. Among the ten patients whose urinary excretion was abnormal were patients with Giardia lambia infestation, ileal resection with blind loop, and other diseases of the small intestine associated with bacterial overgrowth. It was concluded that measurement of 4- hydroxyphenylacetic acid excretion is a useful screening for such diseases (Chalmers, Valman et al. 1979).

Hydroxyphenylpropionate

Dietary intake of polyphenols such as caffeic acid from coffee, catechins from green tea and the proanthocyanidins found in grapes can increase the excretion of hydroxyphenylpropionate (HPPA) in the urine of healthy patients. High levels of HPAA may also indicate increased bacterial metabolism of dietary polyphenols (Chalmers, Valman et al. 1979). A dietary loading of catechins and caffeic acid resulted in a significant increase in HPPA excretion in rat urine to 200 mcg/24 hours. However, when a combination of the antibiotics sulphathiozide and auromycin was administered the level of HPPA dropped to 10 µg/mL (Griffiths 1964). These results suggest that dietary polyphenol intake and the individual's microbial mass play a role in the variations observed in HPPA excretion. Furthermore, Khan et al (2002) demonstrated that the urinary metabolites p-hydroxybenzoate, p-hydroxyphenylacetate, phenylpropionate, phenyllactate and phenylpyruvate are specifically products of bacterial, not protozoal, action on HPPA.
Phenylacetate (PAA)

Scalbert et al (2002) found that excretion of phenylacetate (PAA) is markedly increased after the gastrointestinal tracts of germ-free rats are inoculated with faecal microorganisms, suggesting it is microbial in origin. Lord and Bralley (2008) suggest that PAA should not appear at more than background concentrations in urine. However, it is acknowledged that PAA is a trace product of endogenous phenylalanine catabolism that can accumulate in the phenylalaninemic state found in PKU.

Phenylproprionate (PPA)

Phenylproprionate (PPA) is also produced by anaerobic gut microbiota (Lambert and Moss 1980). As cited by Lord and Bralley (2008), Bhala found that of 67 bacterial and five yeast isolates that were examined only the three isolates of *Clostridium sporogenes* and one of *Clostridium difficile* produced PPA. They went on to investigate whether antibiotics to treat *Clostridium* species would result in reduction of PPA. The results suggested that elevated PPA is associated with increased levels of PPA-producing clostridium species. Furthermore they cautioned the traditional interpretation of the marker as indicative of medium chain acyl coenzyme A dehydrogenase deficiency (MCAD) alone.

Indican

Indican is a product of bacterial action on the amino acid tryptophan. An elevated level of urinary indican has been demonstrated as a possible marker of SIBO (Powell-Jackson, Maudgal et al. 1979). Patients with small bowel disease are at a greater risk of SIBO. In a systematic review of the diagnosis of small intestinal bowel overgrowth urinary indican was considered to be a valid indicator of SIBO (Khoshini, Dai et al. 2008). As with the interpretation of all urinary organic acids, dietary factors must be considered. Impaired protein digestion may be a cause of high excretion of urinary indican by increasing the available tryptophan for bacterial action. Therefore in the absence of any other urinary metabolites of bacterial metabolism, the clinician should consider protein digestion capacity. To this end, urinary indican has been used in conjunction with plasma amino acid levels to assess enteric protein loss in patients with cirrhosis of the liver and malabsorption of protein in the elderly (Mayer and Beeken 1975).
Tricarballylate

Tricarballylate is a product of aerobic bacterial metabolism with a significant mineral chelating capacity. Tricarballylate-producing bacteria that are overgrown are principally related to nutrient deficiencies of magnesium, zinc and calcium (Schwartz, Topley et al. 1988). In ruminant studies, wheat grass feed fermented to form high levels of tricarballylate, resulting in loss of appetite, tetanic convulsions and death (Russell and Forsberg 1986). Therefore a laboratory finding of high urinary tricarballylate indicates the need to assess the patient for mineral deficiencies (Lord and Bralley 2008).

D-Lactate

In mammals, D-lactate is normally produced in the fermentative organs such as the rumen, caecum and colon of the gastrointestinal tract, mainly by commensal organisms including lactobacilli and bifidobacteria (Ewaschuk, Naylor et al. 2005). D-lactic acidosis has been described as turning sugar into acid in the gastrointestinal tract and has been defined as metabolic acidosis accompanied by an increase in serum D-lactate of 3 mmol/L. D-lactate production, accumulation and acidosis are caused by excessive gastrointestinal fermentation of carbohydrate by lactobacilli or by endogenous production from ingested ethylene glycol, and the subsequent inability of the body to adequately clear the D-lactate (Petersen 2005). Severe metabolic D-lactic acidosis is generally rare in humans, but patients who have undergone re-sectioning of the bowel due to morbid obesity, congenital defects, necrotizing entero-colitis, mid-gut volvulus, gangrene and trauma are at greatest risk (Petersen 2005). The shorter bowel results in impaired digestion of protein, fat, carbohydrate, vitamins, fluids, electrolytes and minerals. D-lactic acidosis is associated with neurological dysfunction presenting as confusion, ataxia and slurred speech in association with a high anion gap metabolic acidosis. It is important to note that D-lactate is not the only organic acid produced from simple carbohydrates. Carbohydrates can also produce the organic acids p-hydroxybenzoate and tricarballylate discussed above, but they are not absorbed at rates high enough to cause such dramatic physiological effects as D-lactate (Uribarri, Oh et al. 1998).

An elevation in urinary D-lactate may predict bacterial overgrowth due to carbohydrate malabsorption, ischaemic bowel, and specific types of pancreatic insufficiency, acute appendicitis and occurs as a result of certain gastrointestinal surgical procedures. Clinical management depends on the patient’s age and clinical status. Cessation of lactobacillus supplements and restriction of carbohydrates to starve the lactic acid-producing bacteria is indicated. Severe acute care cases may require the addition of specific antibiotic, bicarbonate...
and rehydration therapy (Bongaerts, Bakkeren et al. 2000). See Table A6 for a list of the lactate isomers produced by individual species of Lactobacillus species.

**Table A6 Lactate isomers produced by individual species of Lactobacillus species (Lord and Bralley 2008)**

<table>
<thead>
<tr>
<th>Producers of Only d(-)-Lactate</th>
<th>Producers of Racemate dL-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus delbrueckii subsp. delbrueckii</td>
<td>Lactobacillus acidophilus</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii subsp. lactis</td>
<td>Lactobacillus amylovorus</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii subsp. bulgaricus</td>
<td>Lactobacillus aviarius subsp. aviarius</td>
</tr>
<tr>
<td>Lactobacillus jensenii</td>
<td>Lactobacillus brevis</td>
</tr>
<tr>
<td>Lactobacillus vitulinus</td>
<td>Lactobacillus buchnari</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus crispatus</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus curvanus</td>
</tr>
<tr>
<td><strong>Producers of Only l(+-)-Lactate</strong></td>
<td>Lactobacillus formentum</td>
</tr>
<tr>
<td>Lactobacillus agilis</td>
<td>Lactobacillus gasseri</td>
</tr>
<tr>
<td>Lactobacillus amylophilus</td>
<td>Lactobacillus gaminis</td>
</tr>
<tr>
<td>Lactobacillus animalis</td>
<td>Lactobacillus hamsteri</td>
</tr>
<tr>
<td>Lactobacillus bavariicus</td>
<td>Lactobacillus helviticus</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>Lactobacillus homohiochii</td>
</tr>
<tr>
<td>Lactobacillus mali</td>
<td>Lactobacillus pentosus</td>
</tr>
<tr>
<td>Lactobacillus maltaromicus</td>
<td>Lactobacillus plantarum</td>
</tr>
<tr>
<td>Lactobacillus murinus</td>
<td>Lactobacillus reuteri</td>
</tr>
<tr>
<td>Lactobacillus paracasei subsp. paracasei</td>
<td>Lactobacillus sake</td>
</tr>
<tr>
<td>Lactobacillus paracasei subsp. tolerans</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus ruminis</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus salivarius</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus sharpeae</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus rhamnosus</td>
<td></td>
</tr>
</tbody>
</table>
gluten-free diet
clinical information for practitioners and patients

Gluten is a protein found in all forms of wheat (including durum, semolina, and spelt), rye, barley and related grain hybrids such as triticale and kamut. It is present in smaller amounts in oats and is undisclosed in an endless variety of processed foods.

Coeliac Disease and Gluten Sensitivity

Coeliac Disease (CD) is also known as gluten-induced enteropathy or sprue. It is an autoimmune disease that leads to a permanent intestinal intolerance to gluten. The gluten causes inflammation of the intestinal wall and a flattening of the villi, finger-like projections that line the inside of the bowel. When these villi atrophy (flatten) the surface area for absorption is greatly reduced resulting in deficiencies of a number of nutrients. The overall prevalence of CD is estimated as 1 in approximately 130, however if you have a family history of CD your risk is increased to approximately 1 in 10.

Screening tests for CD include an AGA test or an EMA test. AGA measures the level of Anti Gladin Antibodies (AGA) present in the blood and requires that the person tested include gluten in the diet for a positive result in CD. The EMA (Endomysial Antibody) test looks at the genetic marker associated with CD, which is independent of gluten inclusion in the diet. Both tests are simple screening tests and can produce false negative or false positive results; it is therefore recommended that a bowel biopsy be done if symptoms and screening tests suggest CD.

Gluten Sensitivity is a condition that involves a degree of gluten intolerance without the presence of immune markers associated with CD. The treatment approach is similar however, since removal of gluten often results in clinical improvement and symptom relief. After a period of avoidance, small amounts of gluten-containing foods may be well tolerated. Guidance will be provided by your healthcare practitioners.

Symptoms of Coeliac Disease

Although there may be no symptoms of CD, the most common ones involve the digestive system and include abdominal bloating or pain, diarrhoea, constipation, flatulence, heartburn, or nausea with or without vomiting. Other possible symptoms include sinusitis, asthma, skin disorders including eczema and dermatitis herpetiformis, fatigue, bone, joint and muscle pains, mouth ulcers, loss of tooth enamel, mood and behavioural problems, poor growth or development in children, weight loss, hair loss and menstrual problems. CD is associated with a higher risk of osteoporosis, iron deficiency anaemia, menstrual problems including amenorrhoea, miscarriage and infertility, and thyroid or other autoimmune diseases.

Helpful hints
- Read all food labels carefully.
- The Australian food standards code requires that foods labelled as ‘gluten free’ must not contain any detectable gluten. Food labelled as ‘low gluten’ must contain less than 0.02% gluten. Look for this symbol when shopping for gluten-free foods in Australia.
- Lactose intolerance is a common accompanying problem to CD. Your practitioner may recommend the removal of dairy products as part of your treatment.
- Avoid cross contamination in the kitchen by developing gluten-free kitchen habits, storage plans and procedures for mixing, cooking and baking.
- Gluten-free breads taste better toasted and should be stored in the fridge or freezer.
- When eating out select food without crumbing, ‘creaming’, coatings, gravies and sauces. Ideally, call ahead to notify the chef of your dietary requirements.
- Obtain your fibre from brown rice, buckwheat, unpeeled potatoes, fresh and dried beans & legumes, fresh fruit & vegetables (see Nutrimedicine Dietary Fibre Information Sheet for more detail)
- Nutritional deficiencies are common, particularly of iron, zinc, vitamins B2, folate and B12. (See Nutrimedicine Nutrient Food List for the best sources of these nutrients)
- Avoid skipping meals, eat slowly and chew all food thoroughly. Enjoy your food!
- Plan your meals and carry snacks with you so you are prepared for all eventualities.

(c) Nutrimedicine 2004
## gluten-free diet

**clinical information for practitioners and patients**

### WHAT TO INCLUDE

<table>
<thead>
<tr>
<th>Grains /Flours /Roots /Tubers and Legumes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grains</strong>: Buckwheat, brown rice, basmati rice, wild rice, maize (corn), quinoa, amaranth, millet, sorghum. (Some CD patients may have a secondary sensitivity to the grains quinoa, amaranth, buckwheat &amp; millet)</td>
</tr>
<tr>
<td><strong>Roots and Tubers</strong>: potato, tapioca, arrowroot, sweet potato, parsnip, jerusalem artichoke</td>
</tr>
<tr>
<td><strong>Legumes</strong>: Beans, soy, lentils, peanut, pea, chickpea.</td>
</tr>
<tr>
<td><strong>Flours</strong>: Any flours made from the above sources, chickpea flour.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Breads and Cereals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breads</strong>: Gluten free breads based on buckwheat, corn, rice, chickpea flour and/or soya flour.</td>
</tr>
<tr>
<td><strong>Cereals</strong>: Gluten free muesli, homemade muesli made from a combination of: Brown rice flakes, millet flakes, organic cornflakes, puffed corn, puffed rice, soy bran, soy grits, raw nuts &amp; seeds, shredded coconut.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pastas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buckwheat noodles, rice noodles, vegetable, corn, spinach or quinoa pasta.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crackers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice cakes, corn cakes, gluten free products.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Snacks and Desserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popcorn, dried fruit (limit), fresh fruit, carrot, sesame snacks, fruit and nut bars, gluten free biscuits or other snack.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock, seasonings and thickeners</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouillon stock powder, sesame salt, tamari (check label), mustard seeds, fresh dried herbs and spices, potato flour, apple cider vinegar, authentic balsamic vinegar, wine vinegar, maize corn flour, soy flour, arrowroot, kuzu &amp; agar-agar.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Beverages</th>
</tr>
</thead>
<tbody>
<tr>
<td>White wine, light rum, gin, tequila without dyes, potato vodka, Teas, coffee, soft drinks, mineral water, fresh fruit and vegetable juices.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other sources of gluten</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some medications use gluten as a binder. Contact your doctor or pharmacist for more information. If ingredients are not itemised, check with the manufacturer of the product or with your state Coeliac Society.</td>
</tr>
</tbody>
</table>

### WHAT TO AVOID

<table>
<thead>
<tr>
<th>Grains /Flours /Roots /Tubers and Legumes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grains</strong>: Wheat (including, durum, semolina, triticale), rye, barley, bulgur, couscous and possibly oats. (Spelt and kamut are ancient grains but may be well tolerated by people with gluten sensitivity or wheat intolerance.)</td>
</tr>
<tr>
<td><strong>Roots and Tubers</strong>: French fries (check labels)</td>
</tr>
<tr>
<td><strong>Legumes</strong>: Baked Beans unless gluten-free.</td>
</tr>
<tr>
<td><strong>Flours</strong>: Wheat flour, wholemeal flour, bakers flour, semolina, barley, rye (avoid battered or crumbed food).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Breads and Cereals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breads</strong>: Wheat breads - wholegrain or white, rye bread, oat bread, barley bread, burritos, pumpernickel bread. (Spelt may be suitable for some people with wheat intolerance)</td>
</tr>
<tr>
<td><strong>Cereals</strong>: Commercial cereals (rice bubbles, weetbix, wheat containing muesli, coco pops etc) wheat germ, wheat bran, porridge oats, oat bran, oat germ. Any cereal containing malt.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pastas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durum wheat pasta (spaghetti, macaroni etc), egg noodles, holkey noodles, barley pasta, spelt pasta.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crackers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat crackers, bran biscuits, ryvita, kavli, oatcakes.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Snacks and Desserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial biscuits, cookies, cakes, scones, pastries, liquorice, some lollies and ice creams, some commercial fruit pies, flavoured or frozen yoghurts, processed cheeses &amp; creams.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stock, seasonings and thickeners</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt, malt vinegar, Vegemite, wheat starch, modified starch, mustard pickles, soy sauce, gravy mixes and seasoning 'nubs'; Hydrolysed vegetable protein (HVP), texturized vegetable protein (TVP); Some binders, fillers, excipients, extenders etc (it is best to contact the manufacturer or state Coeliac Society)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Beverages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beers, ale and lager, cereal and malted beverages, malted or flavoured milk drinks, instant tea, coffee substitutes.</td>
</tr>
</tbody>
</table>

---

Adult Coeliacs, parents of Coeliac children and those with Dermatitis Herpetiformis have formed Coeliac Societies in all Australian States. These Societies provide information on the disease, gluten free diet, ingredients, where to buy, cooking and recipes, overseas travel, education material, etc. If you would like to become a member or would like more information, please contact your State Society: www.coeliac.org.au
Appendix B

B1. Coeliac Survey

1. Coeliac Survey Information and Background

You are invited to participate in an on-line survey that has kindly been sent to you by the New South Wales Coeliac Association email database.

This survey is part of a research project being conducted by Southern Cross University PhD candidate Joanna Harnett under the supervision of Dr Tin Gruner and Professor Stephen Myers.

Name of the Project:
"Does intestinal microflora play a role in the life quality and symptom scores of people with Coeliac Disease?"

Background:
This survey is part of a larger study exploring gastrointestinal bacteria in people with Coeliac Disease. The survey is the first stage of this research and is designed to gather information to try and understand the possible risk factors, social difficulties and health problems related to Coeliac Disease. Eight thousand people with Coeliac Disease from New South Wales have been invited to take part in this survey. It is hoped that by gathering your answers we can gain a better understanding about the issues facing people with Coeliac Disease and promote this knowledge to health professionals and the food industry. In addition, we are gathering specific information regarding gastrointestinal bacteria to assist in the second stage of our research, to be conducted in 2010.

Important Information
- This survey has been approved by Southern Cross University Human Research Ethics Committee. (Approval number will be advised.)
- Your participation is voluntary and anonymous.
- Return of the survey implies your consent.
- Your answers will remain strictly anonymous.
- There is a section at the end of the survey, where you can choose to have the results of the survey emailed to you.
- There is a section at the end of the survey, where you can indicate whether you would like to receive further information about the Coeliac Clinical Trial to be conducted in 2010.

Your contact details will remain strictly confidential and will not be used for any other purpose.

Any inquiries regarding this study should be directed to the:
Principal Researcher
Joanna Harnett
Mona Vale Surgery
22/12-14 Warrilla St,
Mona Vale, NSW 2103
Phone: 0429 707 740 or 02 9999 0630
e-mail: joanna.harnett@cptusnet.com.au

Principal Supervisor
Dr Tin Gruner
School of Health & Human Sciences
Southern Cross University
P.O Box 157
Lismore, NSW 2480
Phone: 02 6620 3349
e-mail: tin.gruner@scu.edu.au

If you have any complaints about this research project, you may contact, in writing, the following:
Ethics Complaints Officer
Human Research Ethics Committee
P.O Box 157
Lismore NSW 2480
sue.kelly@scu.edu.au
All complaints will be treated in confidence and investigated fully. You will be informed of the outcome.
2. Personal Information

* 1. What is your age (in years)?

* 2. What is your gender?
   - Male
   - Female

* 3. What is the postcode of your suburb?
<table>
<thead>
<tr>
<th>Question</th>
<th>Answer Options</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. What is the month of your birth (e.g. April)?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>2. What Country were you born in?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>3. How long have you lived in Australia?</strong></td>
<td>- 0-2 years</td>
</tr>
<tr>
<td></td>
<td>- 3-10 years</td>
</tr>
<tr>
<td></td>
<td>- 11-20 years</td>
</tr>
<tr>
<td></td>
<td>- All my life</td>
</tr>
<tr>
<td><strong>4. Were you diagnosed with CD in Australia?</strong></td>
<td>- Yes</td>
</tr>
<tr>
<td></td>
<td>- No</td>
</tr>
<tr>
<td></td>
<td>- Can't remember</td>
</tr>
<tr>
<td><strong>5. If you were diagnosed with CD in another country, please name the country.</strong></td>
<td></td>
</tr>
</tbody>
</table>
4. Early Childhood Health History

* 1. Was your mother taking antibiotics or chronic pain relief around the time of your conception?
  - Yes
  - No
  - Don't know

* 2. Did your mother have any type of infection during pregnancy?
  - Yes
  - No
  - Don't know

* 3. How were you born?
  - Vaginal
  - Caesarean
  - Don't know

* 4. Were you breastfed?
  - Yes
  - No
  - Don't Know

5. If you were breastfed, approximately how long were you breastfed for?
  - Don't know
  - Less than 3 months
  - Less than 6 months
  - Less than 12 months
  - Longer than 12 months

6. If you were bottle fed, what type of formula were you given? (You can tick more than one answer.)

- Cows milk formula
- Soya formula
- Goats milk formula
- Cows milk lactose free formula
- Don't know
7. Did you have recurrent respiratory infections under six years of age (e.g. tonsillitis, ear infections, bronchitis, common colds)?
   - Yes
   - No
   - Don't know

8. If you had recurrent infections were you prescribed antibiotics under six years of age?
   - Yes
   - No
   - Don’t Know

9. How frequently did you take antibiotics in the first six years of your life?
   - Never
   - Once or twice
   - About once every 2 years
   - Yearly
   - More than once a year

10. Was there any concerns regarding your height and or weight during the first 12 years of life?
    - Yes
    - No
    - Don’t know
5. Diagnostic History

These questions seek to understand the 'diagnostic journey' of patients diagnosed with CD.

* **1. Please state the approximate year of diagnosis of CD.**

* **2. Was CD diagnosed by a blood test?**
  - Yes
  - No
  - Can't remember

* **3. Did you have a small bowel biopsy to confirm the diagnosis of CD?**
  - Yes
  - No

* **4. Did you have genetic tests to see if you carry the gene for CD?**
  - Yes
  - No
  - Unsure

* **5. Please indicate whether certain members of your family have Coeliac Disease**
  - Grandmother
  - Grandfather
  - Mother
  - Father
  - Sister
  - Brother
  - Aunt
  - Uncle
  - Cousin
  - niece
  - Nephew
  - None of my relatives have Coeliac Disease
6. What were the symptoms or other illnesses you were experiencing at the time you were diagnosed with CD? You can tick as many boxes as apply to you.

- Anemia
- Anxiety
- Asperger's
- Autism
- Autoimmune thyroid disease
- Bloating
- Chronic sinusitis
- Cystitis
- Constipation
- Crohn's disease
- Delayed Puberty
- Dental cavities
- Dental enamel defects
- Depression
- Dermatitis Herpetiformis
- Diabetes Type 1
- Diabetes Type 2
- Diarrhoea
- Epilepsy
- Fatigue
- Female Infertility
- Fluoride
- Headaches
- Irritable bowel syndrome
- Lack of menstruation (amenorrhoea)
- Male infertility
- Migraine
- Multiple Sclerosis
- None of the symptoms listed
- Osteopenia (early thinning of the bones)
- Osteoporosis
- Other (specify below)
- Overactive thyroid function
- Recurrent infections
- Recurrent miscarriage
- Reflux/digestion/burning
- Rheumatoid arthritis
- Underactive thyroid function
- Weight gain
- Weight loss

Other (please specify)

7. Approximately how long (months, years) had you been experiencing the symptoms of CD before you were diagnosed?

8. Have the symptoms you had prior to diagnosis improved since following a gluten free diet?
   - No improvement
   - Some improvement
   - Completely resolved

Please list symptoms or illnesses that persist while you follow a gluten free diet
6. Medication

* 1. Please list any medications you are currently taking.

* 2. Please list any supplements such as vitamins, minerals, herbs, homeopathics etc you are currently taking.

* 3. Do you have any allergies to any medicine or supplements?
  - Yes
  - No

4. If you do have allergies please list the medication(s) you are allergic to and describe your reaction.

* 5. Do you have any food intolerances or allergy other than gluten?
  - Yes
  - No
  - Unsure

6. If you have food intolerances or allergy to food substances please list the offending foods and describe your symptoms when you consume them.

* 7. In your adult life, how often have you taken antibiotics?
  - Never
  - Every 5-10 years
  - Every 2-5 years
  - Every year
  - More than once a year
## 7. Section Four: Social Implications and Diet

The purpose of this section is to understand the personal and social implications of being prescribed a gluten free diet. Answering these questions will provide important information for the food industry and health care professionals.

**1. Did your Gastroenterologist provide dietary advice?**
- [ ] Yes
- [ ] No

**2. If your gastroenterologist provided dietary advice was it helpful?**
- [ ] Yes
- [ ] No
- [ ] Some of it

**3. Did your Doctor offer dietary advice?**
- [ ] Yes
- [ ] No

**4. If your doctor provided dietary advice was it helpful?**
- [ ] Yes
- [ ] No
- [ ] Some of it

**5. Were you referred to a dietitian or nutritionist for dietary advice?**
- [ ] Yes
- [ ] No

**6. If you answered yes to the question above, did your nutritionist or dietitian appear well informed about CD?**
- [ ] Yes
- [ ] No
- [ ] Somewhat
7. Was the nutritionist’s or dietitian’s dietary advice helpful?
   ☐ I gained a solid understanding of what I should eat
   ☐ I didn’t gain a greater understanding about what I should eat
   ☐ Some of the information was helpful

* 8. Have you consulted a Naturopath for dietary advice?
   ☐ Yes
   ☐ No

9. If you did consult a Naturopath did they appear well informed about Coeliac Disease?
   ☐ Yes
   ☐ No
   ☐ Somewhat

10. If you did consult a Naturopath, was their advice helpful?
    ☐ I gained a solid understanding about what I should eat
    ☐ I didn’t gain any further understanding of what I should eat
    ☐ Some of the information was helpful

* 11. Do you comply with a strict gluten free diet?
    ☐ Yes
    ☐ No

12. If you do consume glutenous foods which ones do you consume?
    ☐ Bread
    ☐ Cakes
    ☐ Sweet biscuits
    ☐ Savory cracker breads
    ☐ Sauces
    ☐ Pasta
    ☐ Confectionery
    ☐ Beer
    ☐ Other (please specify)
17. Do you have any insights or comments that you think may be relevant to the question "What influences whether a person develops CD or not?"
### 8. Interested in receiving information about a Clinical Trial?

An opportunity exists for you to receive further information regarding a clinical trial. We are looking for participants who still experience digestive or other symptoms of Coeliac Disease despite following a gluten free diet.
9. Coeliac Disease Clinical Trial

1. I would like further information on the clinical trial.
   - Yes
   - No

2. I would like the results of this survey sent to me.
   - Yes
   - No

If you have answered YES to any of the above please email joanna.harnett@spielusnet.com.au and we will forward you your request as soon as possible.
Your confidentiality is assured. Your email address will be used for no other purpose than to send you the information requested.
10. Conclusion

Thank you very much for taking the time to complete this survey. Your contribution is extremely valuable for both the continued education of health care professionals and the food industry in understanding your needs. We look forward to hearing from you if you would like information on the clinical trial.
B2 Email to Survey Participants

From: Penny Dellisperger [penny.dellisperger@coeliacsociety.com.au]
Sent: Friday, 29 January 2010 3:33 PM
To: Penny Dellisperger
Subject: Research Opportunity

Dear Member,

The Coeliac Society of NSW Inc supports research on coeliac disease that follows the National Health and Medical Research Council's Human Research Ethics Guidelines. A team at Southern Cross University is currently undertaking such research (Approval number ECN-09-153).

You are invited to participate in an on-line survey that may also lead to you being invited to participate in a clinical trial. Your participation is entirely voluntary. If you are interested in participating in an on-line survey as part of this research project please click on the following link:

http://www.surveymonkey.com/s/FL+ETJ

This email has been sent in accordance with The Society’s privacy policy. No member details were provided to any third party.

Kind Regards

Penny Dellisperger
Administration Manager
Accredited Practising Dietitian
The Coeliac Society of NSW Inc
PO Box 271 Warringah NSW 2076
Suite 1, 41 - 45 Pacific Hwy, Waitara NSW 2077
Phone 02 9487 5088
Fax 02 9487 5177
penny.dellisperger@coeliacsociety.com.au
www.coeliacsociety.com.au

COELIAC AWARENESS WEEK
13-20 MARCH
Is your child out of sorts?
Thank you for your responses regarding the survey for your research project.

The Chair of the HREC has now approved this survey component and you may commence this aspect of your research.

Please note that the standard conditions of approval apply.

**Standard Conditions** in accordance with the National Statement on Ethical Conduct in Human Research (National Statement) (NS).

1. **Monitoring**

   **NS 5.5.1 – 5.5.10**

   Responsibility for ensuring that research is reliably monitored lies with the institution under which the research is conducted. Mechanisms for monitoring can include:

   (a) reports from researchers;
   (b) reports from independent agencies (such as a data and safety monitoring board);
   (c) review of adverse event reports;
(d) random inspections of research sites, data, or consent documentation; and
(e) interviews with research participants or other forms of feedback from them.

The following should be noted:

(a) All ethics approvals are valid for **12 months** unless specified otherwise. If research is continuing after 12 months, then the ethics approval MUST be renewed. Complete the Annual Report/Renewal form and send to the Secretary of the HREC.

(b) **NS 5.5.5**
   Generally, the researcher/s **provide a report every 12 months** on the progress to date or outcome in the case of completed research specifically including:
   - The maintenance and security of the records.
   - Compliance with the approved proposal
   - Compliance with any conditions of approval.
   - Any changes of protocol to the research.

   Note: Compliance to the reporting is **mandatory** to the approval of this research.

(c) Specifically, that the researchers **report immediately** and notify the HREC, in writing, for approval of **any change in protocol**. **NS 5.5.3**

(d) That a report is sent to HREC when the **project has been completed**.

(e) That the researchers **report immediately any circumstance** that might affect ethical acceptance of the research protocol. **NS 5.5.3**

(f) That the researchers **report immediately any serious adverse events/effects** on participants. **NS 5.5.3**

2. **Research conducted overseas**
   **NS 4.8.1 – 4.8.21**
   That, if research is conducted in a country other than Australia, all research protocols for that country are followed ethically and with appropriate cultural sensitivity.

3. **Complaints**
   **NS 5.6.1 – 5.6.7**
   Institutions may receive complaints about researchers or the conduct of research, or about the conduct of a Human Research Ethics Committee (HREC) or other review body.
   Complaints may be made by participants, researchers, staff of institutions, or others. All complaints should be handled promptly and sensitively.
Complaints about the ethical conduct of this research should be addressed in writing to the following:

Ethics Complaints Officer
HREC
Southern Cross University
PO Box 157
Lismore, NSW, 2480
Email: sue.kelly@scu.edu.au

All complaints are investigated fully and according to due process under the National Statement on Ethical Conduct in Human Research and this University. Any complaint you make will be treated in confidence and you will be informed of the outcome.

All participants in research conducted by Southern Cross University should be advised of the above procedure and be given a copy of the contact details for the Complaints Officer. They should also be aware of the ethics approval number issued by the Human Research Ethics Committee.

Sue Kelly
Secretary HREC
Ph: +61 +2 6626 9139
sue.kelly@scu.edu.au

Professor Bill Boyd
Chair, HREC
Ph: (02) 6620 3569
william.boyd@scu.edu.au
B4 Figure representation of survey results

Figure B.1 Gender distribution of survey respondents

Figure B.2 Distribution of survey respondents by age in decades
Figure B.3 Number of known family members with CD

Figure B.4 Month of birth (n=1470)
Figure B.5 Incidence of early childhood factors that shape intestinal microflora in respondents 1 to 30 years of age

Figure B.6 Duration of breast-feeding in respondents 1 to 30 years of age
Figure B.7 Milk introduced under 12 months of age in respondents 1 to 30 years old

Figure B.8 Prevalence of symptoms and co-morbidities at time of diagnosis of survey respondents
Figure B.9 Number of symptoms or co-morbidities reported per survey respondent

Figure B.10 Symptom/condition reported as persistent 12 months after diagnosis and treatment with a GFD compared to the percentage of the total cohort (some participants reported more than one persistent symptom)
Figure B.11 Symptom improvement by age group
Appendix C

C1 Microbial Ecology Sample Report

2105 Microbial Ecology Profile

<table>
<thead>
<tr>
<th>Prevalent Bacteria</th>
<th>Percentile Ranking by Quintile</th>
<th>95% Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>Oblique anerobes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>4.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Clostridia sp.</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Fusobacteria sp.</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Mycoplasma sp.</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Facultative anerobes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>2.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Bifidobacter sp.</td>
<td>15.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Obligate aerobes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>20.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Opportunistic Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yersinia sp.</td>
<td>2.8E+00</td>
<td>4</td>
</tr>
</tbody>
</table>

Units and Reference Ranges

Organisms are detected by DNA analysis. One colony-forming unit (CFU) is equivalent to one bacterium. Each genus detected represents one cell, or one CFU. Results are expressed in scientific notation, so an organism reported as 2.5 E7 CFU/gm is read as 25 million colony forming units per gram of feces. The cutoff value for significant growth is 0.001. New methods provide for full quantitative analysis.

Prevalent Bacteria play major roles in health. They indicate colonization resistance against potentially pathogenic organisms, aid in digestion and absorption, produce vitamins and SCFAs, and stimulate the GI immune system. DNA probes allow detection of multiple species (sp.) within a genus, so the genera that are reported cover many species.

Opportunistic Bacteria may cause symptoms and be associated with disease. They can affect growth and absorption, nutrient production, pH and immune state. Antibiotic sensitivity tests will be performed on all opportunistic bacteria found although clinical history is usually considered to determine treatment since the organisms are not generally considered to be...
### 2105 Microbial Ecology Profile

#### Pathogenic Bacteria
- **Helicobacter pylori**: 3.2E+005 H <= 1.0E+005
- **Clostridium difficile**: <0.01 <= 1.0E+005
- **E.H.E. coli**: <0.01 <= 1.0E+005
- **Campylobacter sp.**: <0.01 <= 1.0E+005

#### Yeast/Fungi
- **Rhodotorula sp.**: +1 => 100 pg DNA/g specimen

#### Parasites
- **Blastocystis hominis**: Positive
- **Trichuris sp.**: Positive

#### Adiposity Index
- **Firmicutes**: 41 <= 80
- **Bacteroidetes**: 59 >= 20

#### Drug Resistance Genes
- **aacA, aohD**: Neg
- **mecA**: Neg
- **grrB, ParE**: Neg
- **PBP1a, 2B**: Neg
- **vanA, B, and C**: Neg

---

**Accession Number:** A1003240302  
**Reference Number:**  
**Patient:** Sample Report  
**Age:** 48  
**Sex:** Female  
**Date of Birth:** 02/05/1962  
**Date Collected:** 3/23/10  
**Date Received:** 3/24/10  
**Report Date:** 3/25/10  
**Telephone:** (770) 446-5483  
**Fax:** (770) 441-2237  
**Reprinted:** 4/27/10  

**Methodology:** DNA Analysis, GCMS, Microscopic, Colorimetric, Automated Chemistry, EJSA

**Yeast/Fungi**  
Yeast overgrowth has been linked to many chronic conditions, in part because of antigenic responses in some patients to even low rates of yeast growth. Potential symptoms include diarrhea, headache, bloating, atopic dermatitis and fatigue. Positives are reported as +1, +2, +3 or +4 indicating >100, >1000, >10000 or >100000 pg DNA/g.

**Parasites**  
Parasite infections are a major cause of non-viral diarrhea. Symptoms may include constipation, gas, bloating, increased allergy response, colitis, nausea and distention.

The **Adiposity Index** is derived by using D1 probes that detect multiple genera of the phyla Firmicutes and Bacteroides. Abnormalities of these phyla may be associated with increased caloric extraction from food.
2150 Sensitivity - Bacteria

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>Sensitive</th>
<th>Resistant</th>
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</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td></td>
<td>R</td>
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<tr>
<td>Amoxicillin</td>
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<td>R</td>
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<tr>
<td>Cefuroxime</td>
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<td>Ciprofloxacin</td>
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<td>Clindamycin</td>
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<td>Erythromycin</td>
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<td>Levofloxacin</td>
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<td>Polymyxin Clavula</td>
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<tr>
<td>Rifaxin</td>
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<td>Sulfamethoxazole</td>
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<tr>
<td>Tebravacin</td>
<td>S</td>
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<tr>
<td>Trimethoprim-Sulfa</td>
<td>S</td>
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</table>

<table>
<thead>
<tr>
<th>Botanicals</th>
<th>Sensitive</th>
<th>Resistant</th>
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</thead>
<tbody>
<tr>
<td>5-Hydroxy-1,4-naphthoquinone</td>
<td>S</td>
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<tr>
<td>Black Walnut</td>
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<tr>
<td>Allin</td>
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<td>Aechmenin</td>
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<td>Artemisia Womwood</td>
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<tr>
<td>Berberine Goldenseal</td>
<td>S</td>
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<tr>
<td>Caprylic acid</td>
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<td>R</td>
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<tr>
<td>Ocatonic acid</td>
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<td>Carvacrol</td>
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<td>Oregano</td>
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<tr>
<td>Oleuropein Olive Leaf</td>
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<tr>
<td>Quinic Acid</td>
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<td>R</td>
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<tr>
<td>Cat's Claw</td>
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<tr>
<td>Thymol</td>
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<tr>
<td>Oil of Thyme</td>
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<tr>
<td>Undecylic acid</td>
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<tr>
<td>Undecylic acid acid</td>
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<td>R</td>
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</tbody>
</table>

Bacterial growth suppression is measured in a liquid growth medium where fungal growth is suppressed and specific antibiotic agents are introduced before incubation. In contrast to the old isolation and culture techniques, such universal culturing more closely approximates the actions of antibacterial agents in the complex milieu of the colon.

Agents marked as "Sensitive" cause effective bacterial growth suppression. Those antibacterial agents are candidates for suppressing the growth of bacteria in the patient's colon. The results apply to all organisms reported under "Opportunistic Bacteria".

Agents indicated as "Resistant" have low effectiveness. If all tested agents are resistant, synergistic mixtures of antibacterial agents may be effective. Agents indicated as "Resistant" have low effectiveness. If all tested agents are resistant, synergistic mixtures of antibacterial agents may be effective.

For Botanical sensitivity testing the active ingredients are tested and an example of the available source is shown.

Sensitivities are not performed on "Pathogens" or "Parasites" because they do not grow in culture under normal laboratory conditions. Standard protocols are generally used for treatment of pathogens and parasites.
GI Effects SM Stool Profiles

Specimen Collection Instructions

This specimen collection kit can be used for the following test(s):
2100 GI Function Profile - Stool
2105 Microbial Ecology Profile - Stool
2110 Mycology Profile - Stool
2115 Parasitology Profile - Stool
2120 Chemistry Profile - Stool

IMPORTANT:
All patient specimens require two unique identifiers
(patient’s name and date of birth), as well as date of collection.
Patient’s first and last name, date of birth, gender, and date of collection must be
recorded on the Test Requisition Form as well as all tube(s) and/or vial(s), using a
permanent marker, or the test may not be processed.

Specimen
Stool, 3 vials (filled per instructions), refrigerated

Collection Materials
- Collection container
- Orange cap C&S vial
- Pink cap 10% Formalin vial
- White cap nucleic acid vial
- 2 Disposable gloves
- Black disposable bag

Shipping Materials*
- Absorbent pad
- Personal Health Assessment Form
- Test Requisition Form
- Biohazard bag with side pocket
- Specimen collection kit box
- FedEx® Clinical Lab Pak and Billable Stamp

*International shipping may vary, please see shipping instructions for more details.
Please read all instructions carefully before beginning.

Patient Preparation

- It is best to ship your specimen within 48 hours of collection. Please refer to the enclosed shipping instructions before you collect to determine what days you can ship your specimen.
- Refrain from taking digestive enzymes, antacids, and aspirin for two days prior to specimen collection, unless otherwise instructed by your healthcare provider.
- Finish taking antifungal or antibiotic medications and wait three days before beginning collection.
- Never discontinue prescription medications without consulting your healthcare provider first.

CAUTION: FLUID IN VIALS. KEEP OUT OF REACH OF CHILDREN.
- Use caution when opening the vials. At high altitudes, vials may be under pressure. Cover vial cap with a cloth and remove cap slowly.
- Avoid contact with the skin and eyes. For eye contact, flush with water thoroughly for 15 minutes. For skin contact, wash thoroughly with soap and water. For accidental ingestion, contact your local poison control center immediately.

Stool Collection

1. Write patient’s first and last name, date of birth, gender and date of collection on the Test Requisition Form (located in the pouch on top of the Specimen Collection Kit Box), as well as on all 3 vials, using a permanent marker.
   - IMPORTANT: To ensure accurate test results you MUST provide the requested information.
2. Put on the disposable gloves.
3. Collect your stool specimen using the enclosed collection container. DO NOT contaminate the specimen with either urine or water from the toilet.
4. Record stool consistency on the tube labels using a permanent marker. On the Test Requisition Form write the actual consistency of your stool specimen on the “stool consistency” line.

   Hard/constipated  Formed/normal  Loose stool  Watery/diarrhea
5. **Remove** the cap from the **orange** cap vial and, using the attached spoon, **transfer** stool specimen into the vial. **Take** multiple portions from different areas of the collection container. **Fill** the vial to the “fill line”. **DO NOT OVERFILL.**
   **Screw** the cap on tightly.

6. **Remove** the cap from the **pink** cap vial and, using the attached spoon, **transfer** stool specimen into the vial. **Take** multiple portions from different areas of the collection container. **Fill** the vial to the “fill line”. **DO NOT OVERFILL.**
   **Screw** the cap on tightly.

7. **Remove** the cap from the **white** cap vial and, using the attached spoon, **transfer** stool specimen into the vial. **Take** multiple portions from different areas of the collection container. **Fill** the vial to the “fill line”. **DO NOT OVERFILL.**
   **Screw** the cap on tightly.

8. **Shake** all vials vigorously for approximately 30 seconds to mix the stool specimen with the preservative in the vial.

9. **Dispose** of the remaining specimen and the collection container appropriately using the black disposable bag.

10. **Refrigerate** all vials until ready to ship.

---

**Specimen Preparation**

1. **Place** the refrigerated stool specimens and absorbent pad into the biohazard bag.

2. **Staple** payment to the bottom right-hand corner of the completed Test Requisition Form. **Fill out** the Personal Health Assessment Form. **Fold** and **place** them in the side pocket of the biohazard bag.

3. **Seal** the biohazard bag; **Place** it into the specimen collection kit box and close the box.
C3 Ethics Approval for RCT

HUMAN RESEARCH ETHICS COMMITTEE (HREC)

NOTIFICATION

To: Dr Tini Gruner/Joanna Harnett
    School of Health and Human Sciences
    tini.gruner@scu.edu.au,joanna.harnett@optusnet.com.au

    cc stephen.myers@scu.edu.au,joan.oconnor@scu.edu.au

From: Secretary, Human Research Ethics Committee
    Division of Research, R. Block

Date: 2 February 2010

Project: Does intestinal microflora play a role in the life quality and symptom scores of people with Coeliac Disease?

Clinical Trial Approval Number ECN-10-008

Thank you for your responses regarding the clinical trial aspect for the above research.

On behalf of the HREC, The Chair has now approved the clinical trial subject to the following conditions. This executive approval will be ratified by the full HREC at the 1 March 2010 meeting.

Special Conditions
1. Provision of all clinical trial documentation, as discussed with the researcher, supervisor and Dr Joan O’Connor, to the HREC, before commencement of the trial. The researchers have indicated that the documentation will be available by mid-February 2010.

2. It is noted that the researchers will provide a six monthly progress report to the HREC. The first report would be available at approx. August 2010.

3. It is noted that Professor Stephen Myers, co-supervisor, will be monitoring the trial with visits to Sydney and phone contact. Please ensure that the HREC is aware of these visits and when they have occurred and that all clinical trial
protocols are being followed.

4. Dissemination of the results from the clinical trial – it is noted that the researchers have clarified how the results will be disseminated and that this protocol is to be adhered to.

Please note that the standard conditions of approval also apply.

**Standard Conditions** in accordance with the National Statement on Ethical Conduct in Human Research (National Statement) (*NS*).

1. **Monitoring**

   **NS 5.5.1 – 5.5.10**

   Responsibility for ensuring that research is reliably monitored lies with the institution under which the research is conducted. Mechanisms for monitoring can include:

   (a) reports from researchers;
   (b) reports from independent agencies (such as a data and safety monitoring board);
   (c) review of adverse event reports;
   (d) random inspections of research sites, data, or consent documentation; and
   (e) interviews with research participants or other forms of feedback from them.

   The following should be noted:

   (c) All ethics approvals are valid for **12 months** unless specified otherwise. If research is continuing after 12 months, then the ethics approval MUST be renewed. Complete the Annual Report/Renewal form and send to the Secretary of the HREC.

   (d) **NS 5.5.5**

   The researcher/s have agreed to **provide a report in 6 months** on the progress of the clinical trial to date.

   - The maintenance and security of the records.
   - Compliance with the approved proposal.
   - Compliance with any conditions of approval.
   - Any changes of protocol to the research.
   - Any adverse events.

   **Note:** Compliance to the reporting is **mandatory** to the approval of this research.

   (c) Specifically, that the researchers **report immediately** and notify the HREC, in writing, for approval of **any change in protocol. NS 5.5.3**
(d) That a report is sent to HREC when the project has been completed.

(e) That the researchers report immediately any circumstance that might affect ethical acceptance of the research protocol. NS 5.5.3

(g) That the researchers report immediately any serious adverse events/effects on participants. NS 5.5.3

4. Research conducted overseas  
NS 4.8.1 – 4.8.21  
That, if research is conducted in a country other than Australia, all research protocols for that country are followed ethically and with appropriate cultural sensitivity.

5. Complaints  
NS 5.6.1 – 5.6.7  
Institutions may receive complaints about researchers or the conduct of research, or about the conduct of a Human Research Ethics Committee (HREC) or other review body.

Complaints may be made by participants, researchers, staff of institutions, or others. All complaints should be handled promptly and sensitively.

*Complaints about the ethical conduct of this research should be addressed in writing to the following:*

Ethics Complaints Officer  
HREC  
Southern Cross University  
PO Box 157  
Lismore, NSW, 2480  
Email: ethics.lismore@scu.edu.au  
sue.kelly@scu.edu.au

*All complaints are investigated fully and according to due process under the National Statement on Ethical Conduct in Human Research and this University. Any complaint you make will be treated in confidence and you will be informed of the outcome.*
All participants in research conducted by Southern Cross University should be advised of the above procedure and be given a copy of the contact details for the Complaints Officer. They should also be aware of the ethics approval number issued by the Human Research Ethics Committee.

Sue Kelly
Secretary HREC and Lismore HRESC
Ph: +61 +2 6626 9139
sue.kelly@scu.edu.au

Professor Bill Boyd
Chair, HREC
Ph: (02) 6620 3569
william.boyd@scu.edu.au
C4 Email Request for CT

From: Penny Dellesperger [penny.dellesperger@coeliacsociety.com.au]
Sent: Friday, 11 March 2011 9:57 AM
To: Penny Dellesperger
Cc: Joanna Harnett
Subject: Research Opportunity

Dear Member,

We are emailing you on behalf of a PhD student conducting ethics approved research involving people with coeliac disease. No member details have been provided to any third party. Please contact Joanna Harnett on the details below if you would like more information about the study.

Dear NSW Coeliac Society Member

Southern Cross University is conducting a clinical trial in Sydney. Some members have already registered, however more participants are required.

Participants will be:

(a) over the 18 years of age
(b) diagnosed by a small bowel biopsy
(c) following a gluten free diet for 12 months or longer
(d) experiencing ongoing gastrointestinal symptoms

If you would like to receive information regarding the background and requirements of the study please email joanna.harnett@optusnet.com.au or call 02 9966 9990

Kind regards

Penny Dellesperger
Administration Manager
Accredited Practising Dietitian
The Coeliac Society of NSW Inc
PO Box 271 Wahroonga NSW 2076
Suite 1, 41 - 45
Pacific Hwy, Waitara NSW 2077
Phone 02 9487 5068
Fax 02 9487 5177
penny.dellesperger@coeliacsociety.com.au
www.coeliacsociety.com.au
School of Health & Human Sciences  
Clinical Trial NON-COELIAC Control Group Information Sheet

“Does intestinal microflora play a role in the life quality and symptom scores of people with coeliac disease?”

We have asked Dr Varipatis to send this letter to patients that he has ascertained do not have coeliac disease, but have undertaken the same faecal tests that have been performed on patients with coeliac disease in a clinical trial.

Southern Cross University (SCU) invites you to participate in a study investigating the effects of a natural product on the ‘bugs in the gut’ and ongoing symptoms which some people with coeliac disease experience. Coeliac disease is an autoimmune condition that requires those afflicted with it to adhere to a strict gluten-free diet for life. The researchers believe that people with coeliac disease have different ‘bugs’ in their intestines (gut) to people without coeliac disease. To prove this the researchers need to compare non-coeliac individuals’ results with people who do have coeliac disease. Once the researchers can establish there is a difference they will give the participants with coeliac disease a natural product that is thought to improve the intestinal ‘bugs’ and therefore improve the health and quality of life of people with coeliac disease. The study is being conducted by PhD candidate Joanna Harnett and supervised by Dr Tini Gruner and Professor Stephen Myers.

You have been contacted directly by Dr Varipatis and your consent is required prior to the use of any results for this research project. Your name and any identifiable personal information would be removed by the study coordinator prior to any research analysis being conducted. You will not have to do anything except tick the appropriate boxes on the attached consent form, sign it and give it back to your doctor. Please retain the information sheet. Any inquiries regarding this study should be directed to

Principal Researcher:  
Joanna Harnett  
Phone: 0429 707 740  
email: joanna.harnett@optusnet.com.au

Principal Supervisor:  
Dr Tini Gruner  
School of Health & Human Sciences  
Southern Cross University  
PO Box 157  
Lismore, NSW 2480  
Phone: 02 6620 3349  
email: tini.gruner@scu.edu.au

Complaints about the ethical conduct of this research should be addressed in writing to the following:
Ethics Complaints Officer
Human Research Ethics Committee
Southern Cross University
PO Box 157
Lismore, NSW 2480
email: sue.kelly@scu.edu.au
School of Health & Human Sciences

“Does intestinal microflora play a role in the life quality and symptom scores of people with coeliac disease?”

Please tick the box that applies, sign, date and return in the envelope provided.

I agree to take part in the SCU research project specified in the attached information sheet.
Yes ☐ No ☐

I understand that my participation is voluntary.
Yes ☐ No ☐

I agree to have my faeces and urine results used for control data in the research project.
Yes ☐ No ☐

I understand that only the authorised study coordinator will see my original results prior to removal of any identifiable information.
Yes ☐ No ☐

I have been provided with information at my level of comprehension about the purpose, methods, demands, risks, inconveniences and possible outcomes of this research.
Yes ☐ No ☐

I understand this information.
Yes ☐ No ☐

I understand that any information that may identify me will be de-identified at the time of analysis of any data. Therefore, I, or any information I have provided, cannot be linked to my person (Privacy Act 1988).
Yes ☐ No ☐

I understand that all information gathered in this research is confidential. It is kept securely and confidentially for 20 years at the University.
Yes ☐ No ☐

I am aware that I can contact the Principal Supervisor or Supervisor at any time, their contact details appear on the information sheet.
Yes □ No □

I understand that the ethical aspects of this research have been approved by the SCU Human Research Ethics Committee.
Yes □ No □

If I have concerns about the ethical conduct of this research, I understand that I can contact the SCU Ethics Complaints Officer; contact details appear on the accompanying information sheet.

I would like a copy of the research outcome
Yes □ No □

If yes please provide a suitable mailing address

Email__________________________________________________________
Mail __________________________________________________________

Participant’s name:______________________________________________

Participant’s signature:__________________________________________

Date:__________________________________________________________
C7 Figure representation of The Microbiome Study

Figure C.1 Predominant bacteria: descriptive statistics for predominant bacteria measures in CD and Control Groups (measures are reported for instance as $4.1 \times 10^7$ CFU/g of faeces)

Figure C.2 Opportunistic bacteria: percentage distribution and p-values for comparison between detection rates of opportunistic bacteria in CD and control group (the cut-off level for reporting the detection of opportunistic bacteria was $10^5$ CFU/g of faeces)
Figure C.3 Pathogenic bacteria percentage distribution and p-values for comparison between detection rates in CD and control group (the cut-off level for reporting the detection of pathogenic bacteria was $10^5$ CFU/g of faeces)

Figure C.4 Yeast and fungi percentage distribution and p-values for comparison of detection between CD group and control group (the cut-off level for reporting the detection of yeast/fungi was > 10,000 parts per gram of DNA per gram of faeces)
Figure C.5 Parasites percentage distribution and p-values for comparison between detection rates in CD and control group (parasite DNA was reported simply as detected or not detected)
Appendix D

D1 CDSQOL Questionnaire

The Celiac Disease Questionnaire CDQ – Health-related quality of life index for adult patients with celiac disease

This questionnaire has been developed to find out how you have been feeling during the last two weeks. You will be asked about symptoms related to your celiac disease, your general wellbeing and your mood. The questionnaire includes 28 questions. Each question offers seven possible answers ranked (1) to (7). Please read each question carefully and tick the answer that best describes how you felt during the past two weeks. If an item does not apply to you (eg. sexual activity), please leave the question unanswered.

1. How many times during the past two weeks was your life affected by a sudden urge to visit a bathroom for a bowel movement?

   1 All of the time
   2 Most of the time
   3 A good bit of the time
   4 Some of the time
   5 A little of the time
   6 Hardly any of the time
   7 None of the time

2. How often during the last two weeks did you feel physically exhausted or fatigued?

   1 All of the time
   2 Most of the time
   3 A good bit of the time
   4 Some of the time
   5 A little of the time
   6 Hardly any of the time
   7 None of the time
3. How often during the last two weeks have you felt frustrated, impatient or restless?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

4. How many times during the last two weeks did you refuse or avoid an invitation for dinner with friends or relatives due to your celiac disease?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

5. How often during the last two weeks have your bowel movements been loose?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time
6. How much intellectual energy did you have during the last two weeks?

1. No energy at all
2. Very little energy
3. Little energy
4. Some energy
5. A moderate amount of energy
6. Lots of energy
7. I was full of energy

7. How many times during the last two weeks were you concerned that your children could inherit or may have inherited your celiac disease?

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

8. How many times during the last two weeks have you been troubled by cramps in your abdomen?

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time
9. Did you encounter any difficulties with recreational activities or sports due to your celiac disease during the last two weeks?

1. Extreme difficulties, no activities possible
2. Very considerable difficulties
3. Considerable difficulties
4. Some difficulties
5. Minor difficulties
6. Hardly any difficulties
7. No difficulties, celiac disease did not affect my recreational activities or sports

10. How often during the last two weeks did you feel depressed or discouraged?

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time

11. How many times during the last two weeks did you suffer from bloating or flatulence?

1. All of the time
2. Most of the time
3. A good bit of the time
4. Some of the time
5. A little of the time
6. Hardly any of the time
7. None of the time
12. People with celiac disease often have worries and fears related to their disease. How many times during the last two weeks did you worry about or were afraid of getting cancer as a result of your celiac disease?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

13. How many times during the last two weeks were you affected by a feeling of incomplete bowel evacuation?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

14. How often during the last two weeks have you felt relaxed and free of tension?

1 None of the time
2 Hardly any of the time
3 A little of the time
4 Some of the time
5 A good bit of the time
6 Most of the time
15. How many times during the last two weeks did you feel isolated from or excluded by others due to your celiac disease?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

16. How much of the time during the last two weeks have you felt tearful or upset?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

17. How many times during the last two weeks did you suffer from repeated belching?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

18. **To what extent did your celiac disease restrict your sexual activity during the last two weeks?**

1 No sex due to celiac disease
2 Considerable restraint due to celiac disease
3 Moderate restraint due to celiac disease
4 Some restraint due to celiac disease
5 Little restraint due to celiac disease
6 Almost no restraint due to celiac disease
7 No restraint due to celiac disease

19. **How many times during the last two weeks did you suffer from nausea or retching?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

20. **How many times during the last two weeks did you feel that important people such as members of your family or friends showed a lack of understanding for your celiac disease?**

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
21. How satisfied, happy or pleased have you been with your personal life you during the last two weeks?

1 Very unsatisfied, mostly unhappy
2 Generally unsatisfied, unhappy
3 Somewhat unsatisfied, unhappy
4 Generally satisfied, pleased
5 Most of the time satisfied, happy
6 Most of the time very satisfied, happy
7 Very satisfied, could not be happier or more pleased

22. How many times during the last two weeks did you feel that colleagues or superiors showed a lack of understanding for your celiac disease?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

23. How many times during the last two weeks did you feel limited in your professional training or career by your celiac disease?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

24. How many times during the last two weeks did you feel burdened by the expenses and time required obtaining gluten-free food?

Modified: How many times during the last two weeks did you feel burdened by difficulties obtaining gluten-free food?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

25. How many times during the last two weeks did you feel burdened by problems with your health or pension insurance provider regarding meeting the costs of gluten-free food or other celiac therapies?

Modified: How many times during the last two weeks did you feel burdened by problems regarding financing (e.g. costs, prescription, reimbursement) gluten-free food or other celiac therapies?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time
26. How many times during the last two weeks did you experience lack of expertise regarding celiac disease from your doctors?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

27. How many times during the last two weeks did you worry that your celiac disease was diagnosed too late?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
7 None of the time

28. How many times during the last two weeks did you suffer from fear of medical examinations in relation to your celiac disease, e.g. blood withdrawal or enteroscopy?

1 All of the time
2 Most of the time
3 A good bit of the time
4 Some of the time
5 A little of the time
6 Hardly any of the time
CDQ- realization and interpretation

Instructions to the investigator and the patient

The CDQ is presented to the patient so that he can find the instructions on how to answer the questions at the beginning of the questionnaire. It takes about 10 minutes to answer the questions. The patient should be instructed to answer the questions by himself and not ask any other persons such as partners. If the patient has completed the CDQ, the investigator should control that all questions are answered and ask if the patient has any questions regarding the CDQ and its interpretation.
Interpretation of the CDQ

The sub-scale scores result from an addition of the items of the respective sub-scale. The following sheet can be used to calculate the scores:

**Emotion (E)**

Item 2 +3 + 6 + 10 + 14 + 16 + 21 = E

**Social (S)**

Item 4 + 9 + 15 + 18 + 20 + 22 + 23 = S

**Worries (Wo)**

Item 7 + 12 + 24 + 25 + 26 + 27 + 28 = Wo

**Gastrointestinal (GI)**

Item 1 + 5 + 8 + 11 + 13 + 17 + 19 = GI

**Total (To)**

E + S + Wo + GI = To

Test interpretation

The sub-scale scores range between 0-49 in each sub-scale. The total score ranges between 0 – 196. High scores are indicative of a high health-related quality of life, low scores indicate a reduced health-related quality of life. Up to 1 missing item of each sub-scale can be substituted by the individual median of the other items of the sub-scale. If more than 1 item of one scale is not answered the scores cannot be used for scientific studies.

Gender differences (women with celiac disease as well as women in the general population
report lower HRQOL than men; Häuser et al. 2007) should be considered. In case of group comparisons and different sex-ratios compared to the German sample (Häuser 2007) the scores should be adjusted by multiple regression analysis. If multiple measurements (such as in intervention studies) are performed, differences of \( \geq 12 \) within the total score and \( \geq 3 \) within each subscore can be regarded to be a minimum important clinical difference for intra-individual comparisons. For comparisons of groups, changes of \( \geq \frac{1}{2} \) standard deviation can be regarded to be a minimum important clinical difference (Norman 2003).

**Copyright**

The use of the CDQ for clinical studies without license fees is possible after written confirmation of the authors of the CDQ. Requests should be send to whaeuser@klinikum-saarbruecken.de

**Literature**

Norman GR, Sloan JA, Wyrwich KW. Interpretation of changes in health-related quality of life: the remarkable universality of half a standard deviation. Med Care 2003; 41:582-92

## D2 Urinary Organic Acid Sample Report

### Metametrix Clinical Laboratory

**Address:** 3425 Corporate Way, Duluth, GA 30096  
**Phone:** 707.446.5483 **Fax:** 707.441.2237

### Sample Report

**Accession Number:** A0907210002  
**Patient:** Sample Report  
**Sex:** Male  
**Age:** 41  
**Date of Birth:** 02/05/1968  
**Date Collected:** 7/30/09  
**Date Received:** 7/21/09  
**Rept Date:** 7/21/09  
**Telephone:** (770) 446-4583  
**Fax:** (770) 441-2237  
**Repted:** 9/4/09

### Organix™ Dysbiosis Profile

**Methodology:** LIT/Temporal Mass Spectrometry, Colorometric

**Ranges are for ages 13 and over**

<table>
<thead>
<tr>
<th>Component</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>95% Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial - general</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Benzoate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;= 9.3</td>
</tr>
<tr>
<td>2 HIPPURATE</td>
<td>1.07</td>
<td>H</td>
<td>0.54</td>
<td></td>
<td></td>
<td>&lt;= 1.150</td>
</tr>
<tr>
<td>3 Phenylacetaate</td>
<td>0.03</td>
<td></td>
<td>0.04</td>
<td></td>
<td></td>
<td>&lt;= 0.15</td>
</tr>
<tr>
<td>4 PHENYLPROPIONATE</td>
<td>&lt;DL*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;= 0.4</td>
</tr>
<tr>
<td>5 p-Hydroxybenzacetate</td>
<td>0.97</td>
<td></td>
<td>0.90</td>
<td></td>
<td></td>
<td>&lt;= 2.08</td>
</tr>
<tr>
<td>6 p-Hydroxyphenylacetate</td>
<td>35 H</td>
<td></td>
<td>35 H</td>
<td></td>
<td></td>
<td>&lt;= 34</td>
</tr>
<tr>
<td>7 Indican</td>
<td>4</td>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td>&lt;= 74</td>
</tr>
<tr>
<td>8 Tricarballylic</td>
<td>0.75 H</td>
<td></td>
<td>0.73</td>
<td></td>
<td></td>
<td>&lt;= 1.41</td>
</tr>
<tr>
<td><strong>L. acidophilus / general bacterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 D-Lactate</td>
<td>1.3</td>
<td></td>
<td>2.3</td>
<td></td>
<td></td>
<td>&lt;= 7.0</td>
</tr>
<tr>
<td><strong>Clostridial species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 3,4-Dihydroxyphenypropionate</td>
<td>&lt;DL*</td>
<td></td>
<td>0.12</td>
<td></td>
<td></td>
<td>&lt;= 0.12</td>
</tr>
<tr>
<td><strong>Yeast / Fungal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 D-Arabinol</td>
<td>71 H</td>
<td></td>
<td>36</td>
<td></td>
<td></td>
<td>&lt;= 73</td>
</tr>
</tbody>
</table>

*Creatinine = 235 mg/dL*

*<DL = less than detection limit*

These test results are not for the diagnosis of disease. They are intended to provide nutritional guidelines to qualified healthcare professionals with full knowledge of patient history and concerns to assist in their design of an appropriate healthcare program.
## D3 Daily Diet Diary for Clinical Trial

<table>
<thead>
<tr>
<th>DAY:</th>
<th>DIET DIARY</th>
<th>BASELINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meal</td>
<td>Foods consumed – please include quantity (e.g. 2 slices of bread)</td>
<td>Preparation (e.g. fried boiled) Where eaten? (e.g. home, work, Restaurant) Medication (Name and brand) Fluids consumed (include approx quantity) Sauces and condiments (please include brands) Foods avoided other than gluten</td>
</tr>
<tr>
<td>Pre-breakfast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakfast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morning Tea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lunch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Afternoon Tea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-dinner</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinner</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CTN Scheme (Drugs): Acknowledgement of New Trial

Your notification to conduct a clinical trial under the Clinical Trial Notification (CTN) Scheme, pursuant to Schedule 5A of Regulation 12 of the Therapeutics Goods Regulations, has been received by the Office of Scientific Evaluation (OSE).

Trial Number: 2010/0628
Protocol Number: 61042013

Drug(s):

<table>
<thead>
<tr>
<th>Drug Active Name</th>
<th>Trade Name</th>
<th>Code Name</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium breve</td>
<td>Pharma VSL#3</td>
<td>N/A</td>
<td>46 billion CFUs</td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td>Pharma VSL#3</td>
<td>N/A</td>
<td>46 billion CFUs</td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td>Pharma VSL#3</td>
<td>N/A</td>
<td>46 billion CFUs</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>VSL#3</td>
<td>N/A</td>
<td>1 billion CFUs</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii ssp. bulgaricus</td>
<td>VSL#3</td>
<td>N/A</td>
<td>1 billion CFUs</td>
</tr>
<tr>
<td>Lactobacillus paracasei</td>
<td>VSL#3</td>
<td>N/A</td>
<td>1 billion CFUs</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>VSL#3</td>
<td>N/A</td>
<td>1 billion CFUs</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>VSL#3</td>
<td>N/A</td>
<td>300 billion CFUs</td>
</tr>
</tbody>
</table>

It is noted that:

i. the approval of the goods for this trial was given in accordance with Item 3 of Schedule 5A of the Therapeutic Goods Regulations by the body or organisation conducting the trial at each additional site.

ii. the representative of the Ethics Committee for each additional site has certified that the Committee is constituted and operates in accordance with the NHMRC “National Statement on Ethical Conduct in Human Research” has considered this clinical trial, and has provided advice to the body or organisation conducting the trial.
CTN Scheme (Drugs): Clinical Trial Site List

This document lists all sites acknowledged to date for the following clinical trial:

Sponsor: 26183 - Centre for Phytochemistry & Pharmacology at SCU
Protocol Number: 01042013
Trial Number: 2010/0628

<table>
<thead>
<tr>
<th>Date Acknowledged</th>
<th>Site Name</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 December 2010</td>
<td>Diagnostic Insight</td>
<td>NSW</td>
</tr>
</tbody>
</table>
D5 Ethics Approval for Clinical Trial

HUMAN RESEARCH ETHICS COMMITTEE (HREC)

NOTIFICATION

To: Dr Tini Gruner/Joanna Harnett  
School of Health and Human Sciences  
tini.gruner@scu.edu.au,joanna.harnett@optusnet.com.au  
cc stephen.myers@scu.edu.au,joan.oconnor@scu.edu.au

From: Secretary, Human Research Ethics Committee  
Division of Research, R. Block

Date: 2 February 2010

Project: Does intestinal microflora play a role in the life quality and symptom scores of people with Coeliac Disease?

Clinical Trial Approval Number ECN-10-008

Thank you for your responses regarding the clinical trial aspect for the above research.

On behalf of the HREC, The Chair has now approved the clinical trial subject to the following conditions. This executive approval will be ratified by the full HREC at the 1 March 2010 meeting.

Special Conditions

5. Provision of all clinical trial documentation, as discussed with the researcher, supervisor and Dr Joan O’Connor, to the HREC, before commencement of the trial. The researchers have indicated that the documentation will be available by mid-February 2010.

6. It is noted that the researchers will provide a six monthly progress report to the HREC. The first report would be available at approx. August 2010.

7. It is noted that Professor Stephen Myers, co-supervisor, will be monitoring the trial with visits to Sydney and phone contact. Please ensure that the HREC is aware of these visits and when they have occurred and that all clinical trial protocols are being followed.

8. Dissemination of the results from the clinical trial – it is noted that the researchers have clarified how the results will be disseminated and that this protocol is to be adhered to.

Please note that the standard conditions of approval also apply.
Standard Conditions in accordance with the National Statement on Ethical Conduct in Human Research (National Statement) (NS).

1. **Monitoring**

   **NS 5.5.1 – 5.5.10**

   Responsibility for ensuring that research is reliably monitored lies with the institution under which the research is conducted. Mechanisms for monitoring can include:

   (a) reports from researchers;
   (b) reports from independent agencies (such as a data and safety monitoring board);
   (c) review of adverse event reports;
   (d) random inspections of research sites, data, or consent documentation; and
   (e) interviews with research participants or other forms of feedback from them.

   The following should be noted:

   (e) All ethics approvals are valid for **12 months** unless specified otherwise. If research is continuing after 12 months, then the ethics approval MUST be renewed. Complete the Annual Report/Renewal form and send to the Secretary of the HREC.

   (f) **NS 5.5.5**
   The researcher/s have agreed to **provide a report in 6 months** on the progress of the clinical trial to date.
   - The maintenance and security of the records.
   - Compliance with the approved proposal.
   - Compliance with any conditions of approval.
   - Any changes of protocol to the research.
   - Any adverse events.

   Note: Compliance to the reporting is **mandatory** to the approval of this research.

   (c) Specifically, that the researchers report immediately and notify the HREC, in writing, for approval of **any change in protocol. NS 5.5.3**

   (d) That a report is sent to HREC when the **project has been completed**.

   (e) That the researchers report immediately any circumstance that might affect ethical acceptance of the research protocol. **NS 5.5.3**

   (h) That the researchers report immediately any serious adverse events/effects on participants. **NS 5.5.3**

2. **Research conducted overseas**

   **NS 4.8.1 – 4.8.21**

   That, if research is conducted in a country other than Australia, all research protocols for that country are followed ethically and with appropriate cultural sensitivity.

3. **Complaints**

   **NS 5.6.1 – 5.6.7**

   Institutions may receive complaints about researchers or the conduct of research, or about the conduct of a Human Research Ethics Committee (HREC) or other review body.
Complaints may be made by participants, researchers, staff of institutions, or others. All complaints should be handled promptly and sensitively.

*Complaints about the ethical conduct of this research should be addressed in writing to the following:*

Ethics Complaints Officer  
HREC  
Southern Cross University  
PO Box 157  
Lismore, NSW, 2480  
Email: ethics.lismore@scu.edu.au  
sue.kelly@scu.edu.au

All complaints are investigated fully and according to due process under the National Statement on Ethical Conduct in Human Research and this University. Any complaint you make will be treated in confidence and you will be informed of the outcome.

All participants in research conducted by Southern Cross University should be advised of the above procedure and be given a copy of the contact details for the Complaints Officer. They should also be aware of the ethics approval number issued by the Human Research Ethics Committee.

Sue Kelly  
Secretary HREC and Lismore HRESC  
Ph: +61 +2 6626 9139  
sue.kelly@scu.edu.au

Professor Bill Boyd  
Chair, HREC  
Ph: (02) 6620 3569  
william.boyd@scu.edu.au
Dear Joanna,

Re: Intestinal microbiota in Coeliac disease (CD) compared to non-coeliac patients using Deoxyribonucleic Acid (DNA) methods of analysis, followed by a double-blind placebo controlled study assessing the microbiological and quality of life improvements after supplementation with a specific probiotic supplement.

Thank you for submitting the above trial for inclusion in the Australian New Zealand Clinical Trials Registry (ANZCTR).

Your trial has now been successfully registered and allocated the ACTRN: ACTRN1261000630011


**Date submitted:** 25/07/2010 4:22:20 PM  
**Date registered:** 2/08/2010 12:34:57 PM  
**Registered by:** Joanna Harnett

If you have already obtained Ethics approval for your trial, could you please send the ANZCTR a copy of at least one Ethics Committee approval letter? A copy of the letter can be sent to info@actr.org.au (by email) or (61 2) 9565 1863, attention to ANZCTR (by fax).

Please be reminded that the quality and accuracy of the trial information submitted for registration is the responsibility of the trial’s Primary Sponsor or their representative (the Registrant). The ANZCTR allows you to update trial data, but please note that the original data lodged at the time of trial registration and the tracked history of any changes made will remain publicly available.


If you have any enquiries please send a message to info@actr.org.au or telephone +61 2 9562 5333.

Kind regards,
ANZCTR Staff
T: +61 2 9562 5333  
F: +61 2 9565 1863  
E: info@actr.org.au  
W: [www.ANZCTR.org.au](http://www.ANZCTR.org.au)
School of Health & Human Sciences
Clinical Trial Information Sheet

“How does intestinal microflora play a role in the life quality and symptom scores of people with Coeliac disease?”

Southern Cross University invites you to participate in a study investigating the effects of a natural product on the gastrointestinal bacteria and persistent symptoms experienced by some people with Coeliac disease. During the study you will take either an active medication or placebo. (A placebo medication is one that looks the same as the active treatment, but does not contain the active substance). You will undertake several tests and complete several questionnaires during the study. These will give us information on your progress.

The study is being conducted by an accredited Nutritionist, Naturopath and PhD candidate, Joanna Harnett and supervised by Dr Tini Gruner and Professor Stephen Myers. The pathology tests will be analysed by accredited pathology laboratories. The medication will be supplied by a registered pharmaceutical company.

During the trial you will need to

- Continue any prescription medicines you have been prescribed by your doctor.
- Stop taking any herbal or probiotic medicines for the trial period of 16 weeks.
- Attend an interview at week one and week twelve of the trial. The interview will be conducted by Joanna Harnett and take approximately 1 to 1 ½ hours of your time. These interviews will also include a clinical assessment (including a physical examination) and health questionnaires.
- Have a blood sample taken to measure full blood count, liver function and serum electrolytes at the start and the end of the trial by a qualified blood collector.
- Provide faecal samples with the collection kits provided at the beginning and end of the trial.
- Provide urine samples with the collection kits provided at the beginning and end of the trial.
- Complete a questionnaire on your health-related quality of life and your symptoms every 4 weeks throughout the trial period.
- Take the medication we supply in water or juice with morning and evening meals for 12 weeks.

During the study we will:

Ring you at a convenient time to arrange an initial interview and to book two follow-up appointments.

- Contact you to collect your answers from your questionnaires on the 4th, 8th and 16th week.
- Keep all your information confidential in secure locked filing cabinets and analyse your data according to your study number, not your name.
- With your permission forward your pathology, urine and faecal sample results to your doctor.
- If you do not have a doctor, we can organise for you to see a doctor that you nominate, if you so wish.
- Let you know the study outcomes after the results are analysed.
• You can withdraw from the study at any time. If you decide to withdraw from the study all of your data would be destroyed if you ask us to do this.

• Fully disclose information that could affect your personal safety, including if you have any reaction to the medication. Detailed information regarding the medication is available on request.

Are there any risks involved in the study?

• Personnel engaged in blood collection are qualified blood collectors who will withdraw about 8 ml of blood. Occasionally, when blood is extracted, patients experience minor bruising around the vein. There is also a slight risk of infection related to the use of needles. The method of blood collection will be carried out according to the ‘Universal Precautions’ guidelines set out in the NHMRC publication ‘Infection Control in the Health Care Setting’.

• You will receive two collection kits for your faecal samples. The kits contain tools that minimise any contact with the faeces. To further minimise any risk of infection it is important you wash your hands thoroughly with soap after you have collected your faecal samples.

The treatment to be used in this study is considered non-toxic and generally safe to use. However, it is possible for an individual to have an adverse reaction to any supplement or medication; these non-specific reactions may include nausea, headache, loose stools and bloating. In the unlikely event you experience a severe adverse reaction, discontinue the medication and seek appropriate medical attention immediately. In addition, it is important you report any reactions to Joanna on 0429 707 740 within 24 hours.

At any time throughout the trial you will be able to contact the researchers with any concerns or questions.

Your participation is entirely voluntary and you may withdraw from the study at any time without reason and without penalty.

Any inquiries regarding this study should be directed to

**Principal Researcher:**
Joanna Harnett
02 9966 9990
0429 707 740
e-mail: joanna.harnett@optusnet.com.au

**Principal Supervisor:**
Dr Tini Gruner
School of Health & Human Sciences
Southern Cross University
PO Box 157
Lismore, NSW 2480
Phone: 02 6620 3349
e-mail: tini.gruner@scu.edu.au

Complaints about the ethical conduct of this research should be addressed in writing to the following:

**Ethics Complaints Officer**
Human Research Ethics Committee
Southern Cross University
PO Box 157
Lismore, NSW 2480
e-mail: sue.kelly@scu.edu.au
D8 Phone Screening Sheet

INITIAL PHONE SCREENING SHEET

<table>
<thead>
<tr>
<th>Personal Details</th>
<th>Medications</th>
<th>Time taken</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phone:</td>
<td>Mobile:</td>
<td></td>
</tr>
<tr>
<td>Email:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age:</td>
<td>DOB:</td>
<td></td>
</tr>
<tr>
<td>GP Name:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We will collect blood twice during the study is this ok with you? Yes No

You will be required to provide a faecal sample to the laboratory is this ok with you? Yes No

Inclusion criteria:

Do you have small bowel biopsy confirmed CD? Yes No

What was the approximate date of your diagnosis? Yes No

Are you 18 years or older? Yes No

Have you been following a strict GFD for greater than 12 months? Yes No

Are you still troubled by gastrointestinal symptoms e.g. burping, bloating, flatulence, urgency to defecate, incomplete defecation, sense of fullness? Yes No

Do you believe your quality of life is affected by CD? Yes No

Are you willing to be involved in a clinical trial for 16 weeks? Yes No

Exclusion criteria:

Are you pregnant? Yes No

Do you have cancer? Yes No

Do you have HIV? Yes No

Are you taking antibiotics? Yes No
<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you taken antibiotics, anti-inflammatory, pain relief or the OCP in the last month?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any problems with your gastrointestinal tract other than CD?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have short bowel syndrome?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you had any recent oral or bowel surgery?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you on chemotherapy?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have an alcohol or illicit drug dependence?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you willing to have a physical examination performed?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you willing to be given an active or placebo medication to take twice daily for 12 weeks?</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Do you have anaphylaxis to dairy products?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Participant included:**

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appointment made</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Email information</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post information</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
School of Health & Human Sciences

“Does intestinal microflora play a role in the life quality and symptom scores of people with Coeliac disease?”

NOTE: This consent form will remain with the Southern Cross University researcher for her records.

Please tick the box that applies, sign and date and give to the researcher

I agree to take part in the Southern Cross University research project specified above.
Yes ☐ No ☐

I agree to provide faeces, urine and blood samples on two separate occasions.
Yes ☐ No ☐

I understand that I may be given either an active medication or placebo in the trial.
Yes ☐ No ☐

I have been provided with information at my level of comprehension about the purpose, methods, demands, risks, inconveniences and possible outcomes of this research.
Yes ☐ No ☐

I understand this information.
Yes ☐ No ☐

I agree to complete questionnaires asking me about my health.
Yes ☐ No ☐

I understand that my participation is voluntary.
Yes ☐ No ☐

I understand that I can choose not to participate in part or all of this research at any time, without consequence.
Yes ☐ No ☐
I understand that any information that may identify me will be de-identified at the time of analysis of any data. Therefore, I, or any information I have provided, cannot be linked to my person (Privacy Act 1988).
Yes ☐ No ☐

I understand that all information gathered in this research is confidential. It is kept securely and confidentially for 20 years at the University.
Yes ☐ No ☐

I am aware that I can contact the principal researcher or supervisor at any time with any queries.
Yes ☐ No ☐

I understand that the ethical aspects of this research have been approved by the SCU Human Research Ethics Committee.
Yes ☐ No ☐

Participant’s name: ________________________________________________________________

Participant’s signature: ____________________________________________________________

Date: __________________________________________________________________________

Witness’s name: _________________________________________________________________

Witness’s signature: ______________________________________________________________

Date: __________________________________________________________________________
D10 Systems Review and History

**Case Report Form**
Coeliac Disease Microbiota and Quality of Life

Principal Investigator: Dr Tini Gruner

### Current Pharmaceutical and Complementary Medicines

<table>
<thead>
<tr>
<th>Name</th>
<th>Route</th>
<th>Daily Dosage</th>
<th>Reason for use</th>
<th>Date From</th>
<th>Date To</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

### FAMILY HISTORY

<table>
<thead>
<tr>
<th>Paternal</th>
<th>Maternal</th>
<th>Siblings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>Mother</td>
<td>GM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>GM</td>
<td></td>
</tr>
</tbody>
</table>

Uncles, aunts, cousins

Uncles, aunts, cousins

Other
# SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

<table>
<thead>
<tr>
<th>General</th>
<th>Feeling unwell</th>
<th>1: occasionally/in the past</th>
<th>2: frequently</th>
<th>3: (almost) constantly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lack of energy</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Weakness</td>
<td></td>
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<tr>
<td></td>
<td>Fatigue</td>
<td></td>
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<tr>
<td></td>
<td>Unexplained fever</td>
<td></td>
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<tr>
<td></td>
<td>Weight gain</td>
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<td></td>
<td>Weight loss</td>
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<tr>
<td></td>
<td>Worse in hot conditions</td>
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<td></td>
<td>Worse in cold conditions</td>
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<tr>
<td></td>
<td>Worse in damp conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

## Gastro-intestinal

<table>
<thead>
<tr>
<th>Changes in appetite</th>
<th>Loss of taste/smell</th>
<th>Difficulty swallowing</th>
<th>Heartburn</th>
<th>Reflux</th>
<th>Nausea</th>
<th>Vomiting</th>
<th>Bad breath</th>
<th>Coated tongue</th>
<th>Belching</th>
<th>Bloating</th>
<th>Flatulence</th>
<th>Fullness after meals</th>
<th>Food cravings (please list):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>
SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

Respiratory /12
☐ Shortness of breath
☐ Cough
☐ Wheezing/asthma
☐ Expectoration

Cardiovascular /36
☐ Racing heart beat
☐ Irregular heart beat
☐ High blood pressure
☐ High cholesterol
☐ Chest pains
☐ Angina
☐ Varicose veins
☐ Swollen ankles
☐ Cold hands and feet
☐ Bruising easily
☐ Blood clots
☐ Anaemia

Musculo-skeletal /27
☐ Sore back
☐ Sore neck
☐ Sore/stiff joints
☐ Soreness/stiffness/feeling of bruising
☐ Cramps
☐ Bone pain
☐ Bad posture
☐ Injuries
☐ Deformities

Genito-urinary /39
☐ Frequent urination
☐ Urination at night
☐ Pain on urination
☐ Blood in urine
☐ Incontinence
☐ Incomplete emptying
☐ Infections/Cystitis
☐ Discharge
☐ Sores
☐ Herpes
☐ Sexually transmitted disease
☐ Loss of libido

Study Coordinator_________________________ Date__________Page 3
Infertility

SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

Male reproduction /24
- Prostate problems
- Hesitant flow
- Diminished flow
- Dribbling
- Pain in testicles
- Swollen testicles
- Inability to achieve erection
- Inability to maintain erection

Endocrine /18
- Excessive thirst
- Excessive hunger
- Needing meals on time
- Excessive sweating
- Feeling sluggish
- Dizziness on rising

Immunity /36
- Frequent colds
- Frequent influenza
- Frequent infections
- Slow wound healing
- Autoimmune disorders
- Viral infections (e.g. Ross River, Barmah Forest, Cytomegalovirus)
- Swollen lymph nodes
  - Neck
  - Under arms
  - In groin
- Allergies general
  - Hayfever
  - Hives
  - Eczema
- Allergies to environment
  - Pollen
  - House dust
  - Dust mite
  - Animal fur/feathers
  - Other ........................................
SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

☐ Food allergies/sensitivities/intolerances
  ☐ Dairy
  ☐ Wheat
  ☐ Gluten
  ☐ Fish
  ☐ Shellfish
  ☐ Eggs
  ☐ Pork
  ☐ Chicken
  ☐ Citrus
  ☐ Other fruit
  ☐ Vegetables
  ☐ Nuts (tree)
  ☐ Peanuts
  ☐ Soy

☐ Allergies to drugs (please list)

................................................................................................................................................

☐ Specific allergies/sensitivities/ intolerances not listed above:
................................................................................................................................................

................................................................................................................................................

Female reproduction /42

☐ Pain during menstruation
☐ Heavy menstrual flow
☐ Scanty menstrual flow
☐ Irregular menses
☐ Premenstrual syndrome
☐ Bleeding between periods
☐ Hot flushes
☐ Night sweats
☐ Sore breasts
☐ Lumps in breast
☐ Pain during intercourse
☐ Oral contraceptive pill use
☐ Intra-uterine device (IUD) use
☐ Absence of menstruation

Skin /30

☐ Rash
☐ Lumps
☐ Sores
☐ Eczema/dermatitis
☐ Acne
☐ Psoriasis
☐ Itching
☐ Dryness

Study Coordinator__________________________  Date_____________Page 5
<table>
<thead>
<tr>
<th>Participant’s initials</th>
<th>Allocation No.</th>
<th>I</th>
<th>M</th>
<th>Protocol No. CD 29</th>
</tr>
</thead>
</table>

**SYSTEMS REVIEW and PHYSICAL EXAM BASELINE**

**Hair**
- [ ] Falling out
- [ ] Breaking/splitting
- [ ] Oily
- [ ] Dry
- [ ] Lustreless
- [ ] Excessive hair growth

**Nails**
- [ ] Soft
- [ ] Brittle
- [ ] Splitting

**Eyes**
- [ ] Wearing glasses/contact lenses
- [ ] Floaters
- [ ] Blurred/double vision
- [ ] Narrowed visual field
- [ ] Itchy
- [ ] Redness/inflammation
- [ ] Tearing
- [ ] Dark circles under eyes

**Ears**
- [ ] Hearing loss
- [ ] Earaches
- [ ] Infections
- [ ] Discharge
- [ ] Tinnitus (ringing in the ears)

**Nose**
- [ ] Colds
- [ ] Congestion
- [ ] Discharge
- [ ] Hay fever
- [ ] Post-nasal drip
- [ ] Nosebleeds

**Throat/mouth**
- [ ] Sore throat
- [ ] Dry mouth
- [ ] Hoarseness
- [ ] Tonsillitis
- [ ] Sore tongue
- [ ] Cold sores
- [ ] Bleeding gums

Study Coordinator_________________________ Date_________ Page 6
SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

Throat and Mouth cont
- Receding gums
- Mouth ulcers
- Infected teeth
- Missing teeth
- Unattended cavities
- Mercury fillings
- Root canal fillings
- Dentures

Neurological /42
- Dizziness
- Tremors
- Numbness
- Tingling/crawling sensation
- Pins and needles
- Shooting or radiating pain
- Weakness
- Restless legs
- Sensitivity to light
- Sensitivity to noise
- Unsteady gait
- Headaches
- Migraines
- Seizures/fits

Sleep /15
- Difficulty falling asleep
- Difficulty staying asleep
- Unrefreshed sleep/tired on waking
- Unable to remember dreams
- Nightmares/disturbing dreams

Psychological /42
- Depression
- Nervousness
- Excessive worry
- Anxiety
- Feeling overwhelmed/unable to cope
- Suicidal thoughts
- Lack of self esteem
- Irritability
- Mood swings
- Recurring thoughts
- Poor memory
- Poor concentration
- Confusion/mental fog
- Other (please list):
SYSTEMS REVIEW and PHYSICAL EXAM BASELINE

Toxic exposure /27

☐ Paint/paint fumes
☐ Agricultural chemical
☐ Laboratory chemicals (esp. organic solvents)
☐ Petrol fumes
☐ Cigarette smoke
☐ Corrosive agents
☐ Gas
☐ Metals
☐ Other (please list):

....................................................

....................................................

Total: /633
<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
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</thead>
<tbody>
<tr>
<td>Heart attack</td>
<td></td>
<td>Parkinson’s disease</td>
<td>Stroke</td>
<td></td>
<td>Dementia</td>
</tr>
<tr>
<td>Diabetes mellitus:</td>
<td></td>
<td>Multiple sclerosis</td>
<td>Type I</td>
<td></td>
<td>Chronic Fatigue Syndrome</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td>Fibromyalgia</td>
<td>Gestational</td>
<td></td>
<td>Glandular fever</td>
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<td>Thyroid disease</td>
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<td>Crohn’s disease</td>
<td>Kidney disease</td>
<td></td>
<td>Ulcerative colitis</td>
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<tr>
<td>Hepatitis</td>
<td></td>
<td>Coeliac disease</td>
<td>Glaucoma</td>
<td></td>
<td>Gall stones</td>
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<tr>
<td>Goitre</td>
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<td>Haemachromatosis</td>
<td>Osteoporosis</td>
<td></td>
<td>Blood transfusions</td>
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<tr>
<td>Osteoarthritis</td>
<td></td>
<td>Genetic abnormalities</td>
<td>Gout</td>
<td></td>
<td>Gum disease</td>
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<tr>
<td>Broken Bones</td>
<td></td>
<td>Root canal fillings</td>
<td>Epilepsy</td>
<td></td>
<td>Cancer</td>
</tr>
<tr>
<td>Nervous breakdown</td>
<td></td>
<td>Vaccinations for Childhood illnesses</td>
<td>Mental disorder</td>
<td></td>
<td>HIV exposure</td>
</tr>
<tr>
<td>Attempted suicide</td>
<td></td>
<td>Travel</td>
<td>Lupus erythematosus</td>
<td></td>
<td>Work related</td>
</tr>
<tr>
<td>Autoimmune disorders</td>
<td></td>
<td>Influenza</td>
<td>Rheumatoid arthritis</td>
<td></td>
<td>Others (list below)</td>
</tr>
</tbody>
</table>

Do you have or have you had any of the following?
Physical Examination Record Baseline Date: ________________
D11 Wallet Card

THE STUDY IS CoRDINATED BY
JoANNA HarneTT
MOBILE: 0429 797 740

THE STUDY IS SuPervised BY
Dr TiNi GroNER
CONTACT: (02) 9620 3349

I Am cuRRently pArticiPATING IN A CLINICAL TRIAL INVOLVING
PROBIOTIC MEDICATION
APPROVED BY SOUTHERN CROSS UNIVERSITY HuMAN RESEARCH ETHICS
COMMITTEE
ETHICS NUMBER UCN-10.01
D12 Blood Test Request Form

Patient Details
Surname: ____________________________ Given Name: ____________________________
Date of Birth: ___/___/____ Sex: Male [ ] Female [ ]
Address: ____________________________________________________
Your Reference: ____________________________________________ (optional)
(DATA ENTER on line 18)

Doctor: H19452-Y
Ms Joanna Harnett
Diagnostic Insight
Suite 101A/54-56 Pacific Hwy
St Leonards NSW 2065

Billing: CDM
Orphan
Attn: Ms Jennifer Goss
300 Frankston-Dandenong Rd
Dandenong VIC 3175

Tests Requested
[ ] FBC Full Blood Count [ ] COL1 Collection Fee
[ ] LFT Liver Function Tests
[ ] UEC Urea/Electrolytes/Great

Clinical Notes
Fasting: Yes [ ] No [ ] hours ________ Doctor signature NOT required

Collection Centre Use
Collection Centre: ____________________________ Collector Initials: ____________________________
Date of Collection: ___/___/____ Time of Collection: ______:____ AM / PM

Laboratory Use

September 2017
D13 Shipment Instructions for Biological Specimens

**DISPATCH INSTRUCTIONS**

- **DISPATCH** your specimen the same day of collection. **Monday through Thursday only.**
  - DO NOT **DISPATCH** SPECIMEN ON A DAY PRIOR TO A NEW SOUTH WALES HOLIDAY OR AUSTRALIAN NATIONAL HOLIDAY.
- If specimen is collected on Friday, **STORE** as specified in Specimen Collection Instructions and dispatch on Monday.
- **STORING** your specimen(s) **AS OUTLINED IN THE INSTRUCTIONS** is vital.

1. **PACK your specimen for DISPATCH**
   - PLACE collected specimen vials/tubes on frozen gel pack (if required), wrap in bubble wrap and place into the biohazard bag.
   - SEAL the biohazard bag
   - COMPLETE the Test Requisition Form (TRF) with Payment details and place TRF in side pocket of biohazard bag.
   - Ensure that the Date of Collection is filled in on the form.
   - PLACE the biohazard bag with its contents into specimen collection kit box
   - SEAL the specimen collection kit box with tape.

**DO NOT USE ANY OTHER BOX OTHER THAN THE SPECIMEN KIT BOX SUPPLIED BY METAMATRIX.**

2. **COMPLETE the Toll Priority consignment note**
   - FILL IN your name and address in the "FROM" section of the consignment note and **SIGN** and **DATE** the bottom of the consignment note.

This is an **OVERNIGHT SERVICE**, (not same day delivery) and the **WEIGHT CATEGORY** for this kit is **1kg only** (as already marked on the Consignment Note)

**DO NOT ALTER THE DETAILS ALREADY MARKED ON THE CONSIGNMENT NOTE.**

Additional charges incurred due to incorrect submission (up to $200) will be charged back to you.

- FEEL off the backing of the consignment note and **SECURE** consignment note on top of specimen collection kit.
- RETAIN the customers copy for your record of the dispatch of the Specimen Collection Kit.

3. **Call TOLL PRIORITY to pick up your specimen for delivery to Diagnostic Insight**

   **Note:** Call must be made before 12pm to ensure same day pick up.

   Pick UP is between 12pm to 5pm Monday to Thursday.

   The sender or a responsible person **MUST** be present for courier pick up

   - PHONE 13 15 31 TOLL PRIORITY and follow the prompts to book a pickup.
   - **QUOTE** the following information:
     a. CUSTOMER ACCOUNT NUMBER: 320947.
     b. PICK UP FROM – your address
     c. TYPE OF PACKAGE – Diagnostic specimen in a BOX (BIOLOGICAL SUBSTANCE CATEGORY B)
     d. WEIGHT – 1kg
     e. TYPE OF SERVICE: Overnight Service
     f. RECEIVERS ADDRESS –Diagnostic Insight address preprinted on consignment note

For further queries on specimen collection please contact Diagnostic Insight on 1300 764 054
<table>
<thead>
<tr>
<th>PARTICIPANT NO: 10001</th>
<th>PARTICIPANT NO: 10002</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLINICAL TRIAL STOCK</strong></td>
<td><strong>CLINICAL TRIAL STOCK</strong></td>
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<tr>
<td>90 sachets</td>
<td>90 sachets</td>
</tr>
<tr>
<td><strong>ACTIVE OR PLACEBO MEDICATION</strong></td>
<td><strong>ACTIVE OR PLACEBO MEDICATION</strong></td>
</tr>
<tr>
<td>DOSAGE: Mix one sachet into water or juice with both morning and evening meals (twice daily)</td>
<td>DOSAGE: Mix one sachet into water or juice with both morning and evening meals (twice daily)</td>
</tr>
</tbody>
</table>
Dear Ethics Committee/Therapeutic Drugs Administration and Sigma Orphan

Re: Clinical Trial - Does intestinal microflora play a role in symptom scores and life quality of patients with Coeliac disease? HREC ECN10-08

Clinical Trial Notification Number: 2010/0628

Protocol number: 01042013

Active medication: Bifidobacterium breve 46 billion CFU
Bifidobacterium infantis 46 billion CFU
Bifidobacterium longum 46 billion CFU
Lactobacillus acidophilus 1 billion CFU
Lactobacillus delbruecki 1 billion CFU
Lactobacillus paracasei 1 billion CFU
Lactobacillus plantarum 1 billion CFU
Streptococcus thermophilus 300 billion CFU

Trade name: VSL#3™

Participant 10028 a 51 year old female experienced an allergic reaction resulting in angioedema at week 6 of the clinical trial. She consulted her General Practitioner who decided her condition was unlikely to be related to the study medicine and prescribed her prednisone. Her condition has now persisted for 6 weeks. She continued to take the trial medicine. She did not report this incident until yesterday at her final week 12 interview. I have discussed this condition with my co-supervisor Professor Stephen Myers and my supervisor Dr Tini Gruner and we have rated this event as severe but probably not related to the trial medication. The envelope containing her allocation number was broken and she was on the placebo.

Yours Sincerely

Joanna Harnett (Clinical Trial Co-ordinator)

Clinical Trial site: Suite 101/454-456 Pacific Highway St Leonards 2065

00 612 9966 9994 (m) 00612 0429 707 740

Joanna.harnett@optusnet.com.au
Dear Ethics Committee/Therapeutic Drugs Administration and Sigma Orphan

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Lactobacillus paracasei 1 billion CFU
Lactobacillus plantarum 1 billion CFU
Streptococcus thermophillus 300 billion CFU

Trade name: VSL#3™

Participant 10026 a 31 year old female who while travelling in Europe had an accident resulting in a broken thumb and wrist. Also whilst travelling she was hospitalised for pneumonia. I have discussed this event with my co-supervisor Professor Stephen Myers and we have rated this event as severe but probably not related to the trial medication. The participant delayed reporting the event and remained on the clinical trial medication for the duration of the trial. The envelope containing her allocation number was broken and she was on the placebo.

Yours Sincerely

Joanna Harnett

Clinical Trial study coordinator
Dear Ethics Committee/Therapeutic Drugs Administration and Sigma Orphan

Re: Clinical Trial - Does intestinal microflora play a role in symptom scores and life quality of patients with Coeliac disease? HREC ECN10-08

Clinical Trial Notification Number: 2010/0628

Protocol number: 01042013

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- Bifidobacterium infantis 46 billion CFU
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- Lactobacillus acidophilus 1 billion CFU
- Lactobacillus delbruecki 1 billion CFU
- Lactobacillus paracasei 1 billion CFU
- Lactobacillus plantarum 1 billion CFU
- Streptococcus thermophillus 300 billion CFU

Trade name: VSL#3™

Participant 10021 a 61 year old female completed taking a 12 week course of clinical trial medicine. Nineteen days after completing the medication she was admitted to hospital for scheduled surgery to remove two neuromas on her right foot. Ten days following the surgery she was diagnosed with a DVT in both legs. This diagnosis was followed by a pulmonary embolism. She is currently under the care of her general practitioner and haematologist. I have discussed this case with my supervisor Dr Tini Gruner and co-supervisor Professor Stephen Myers and we have rated this event as severe but probably not related to the trial medication. The envelope containing her allocation number was broken and she was on the placebo.

Yours Sincerely

Joanna Harnett

PhD Scholar Southern Cross University Australia

MHSc, BHS, Accredited Nutritionist NSA

Clinical Trial site: Suite 101/454-456 Pacific Highway St Leonards 2065

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D16 Certificate of Analysis of Probiotic

Attention: Dr Tini Gruner
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CERTIFICATE OF ANALYSIS

Report No: S 451278613 sn
Refer to S 451278611
Date Received: 21 June 2011
Report Date: 15 July 2011
Date Tested: 1 July 2011
Arrival Temp: 20°C

RESULTS

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Bifidobacterium Count Method: ISO29981 CFU per g</th>
<th>Total Lactic Organism Count Method: M56 CFU per g</th>
<th>Streptococcus thermophilus Method/Media Used: M17 Oxid CM785</th>
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</thead>
<tbody>
<tr>
<td>Sample 1 – Active</td>
<td>50,000,000,000</td>
<td>53,000,000,000</td>
<td>730,000,000</td>
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<tr>
<td>Sample 2 – Control place (6)</td>
<td>&lt;1,000,000</td>
<td>&lt;1,000,000</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Notes: ‘<’ indicates Less than

SELINA BEGUM MApSci., MAIFST
CONSULTANT MICROBIOLOGIST

TGA Licence No. 152612
D17 Figure representation of Clinical Trial results

Figure D.1 Predominant bacteria measures between the Probiotic and Placebo groups at baseline and week 12

Figure D.2 Measures of Phyla classes at baseline and week 12 in the Probiotic and Placebo groups
Figure D.3 Detection rate of opportunistic bacteria at baseline and week 12 between Probiotic and Placebo groups

Figure D.4 Detection rate of pathogenic bacteria at baseline and week 12 between Probiotic and Placebo groups
Figure D.5 Detection rate of yeasts and fungi at baseline and week 12 between Probiotic and Placebo groups

Figure D.6 Detection rate of parasites at baseline and week 12 between Probiotic and Placebo groups

In this study we investigated whether probiotics *Lactobacillus fermentum* or *Bifidobacterium lactis* can inhibit the toxic effects of gliadin in intestinal cell culture conditions. The ability of live probiotics to inhibit peptic-tryptic digested gliadin-induced damage to human colon cells Caco-2 was evaluated by measuring epithelial permeability by transepithelial resistance, actin cytoskeleton arrangements by the extent of membrane ruffling and expression of tight junctional protein ZO-1. *B. lactis* inhibited the gliadin-induced increase dose-dependently in epithelial permeability, higher concentrations completely abolishing the gliadin-induced decrease in transepithelial resistance. The same bacterial strain also inhibited the formation of membrane ruffles in Caco-2 cells induced by gliadin administration.


**BACKGROUND AND AIM:** Functional gastrointestinal symptoms are common and their management is often a difficult clinical problem. The link between food intake and symptom induction is recognized. This review aims to describe the evidence base for restricting rapidly fermentable, short-chain carbohydrates (FODMAPs) in controlling such symptoms. **METHODS:** The nature of FODMAPs, their mode of action in symptom induction, results of clinical trials and the implementation of the diet are described. **RESULTS:** FODMAPs are widespread in the diet and comprise a monosaccharide (fructose), a disaccharide (lactose), oligosaccharides (fructans and galactans), and polyols. Their ingestion increases delivery of readily fermentable substrate and water to the distal small intestine and proximal colon, which are likely to induce luminal distension and induction of functional gut symptoms. The restriction of their intake globally (as opposed to individually) reduces functional gut symptoms, an effect that is durable and can be reversed by their reintroduction into the diet (as shown by a randomized placebo-controlled trial). The diet has a high compliance rate. However it requires expert delivery by a dietitian trained in the diet. Breath hydrogen tests are useful to identify individuals who can completely absorb a load of fructose and lactose so that dietary restriction can be less stringent. **CONCLUSIONS:** The low FODMAP diet provides an effective approach to the management of patients with functional gut symptoms. The evidence base is now sufficiently strong to recommend its widespread application.


Purpose of review: Several studies indicate that factors affecting the gut are capable of modulating the development of autoimmune diabetes. This review discusses the recent research on these mechanisms, which may reveal novel pathogenic pathways and new possibilities for prevention of type 1 diabetes (T1D).

Recent findings: The role of the gut as a regulator of T1D is mainly based on animal studies in which changes affecting the gut immune system have been shown to modulate the immune-mediated destruction of insulin-producing beta-cells. Dietary interventions, alterations in the intestinal microbiota and exposure to enteral pathogens regulate the development of autoimmune diabetes in animal models. In several studies, it has been demonstrated that these modulations affect the gut barrier mechanisms and intestinal immunity. Also, in humans, increased gut permeability and intestinal inflammation are associated with T1D. A recent report of dietary intervention study in infants at genetic risk of T1D showed that early diet could modulate the development of beta-cell autoimmunity in humans; weaning to hydrolyzed casein formula decreased the risk of beta-cell autoimmunity by age 10.

Summary: The gut modulation affecting permeability, inflammation and microbiota is evidently associated with the regulation of the inflammation leading to beta-cell destruction. Although the mechanisms of action are not fully understood, the recent research points out the lines of approach for the prevention of T1D.