VARIATION IN POPULATIONS OF ENTERAL MICROFLORA IN PEOPLE WITH COELIAC DISEASE FOLLOWING THE IMPLEMENTATION OF A GLUTEN FREE DIET

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ABSTRACT

Coeliac disease (CD) is a disorder resulting from interactions between diet, genome and immunity. This research seeks to further our understanding of the pathology of CD in regard to its secondary effects on the diversity of enteral microflora via changes in immune tolerance. It proposes that enteral mucosal pro-inflammatory change in CD is associated with a decrease in microbial diversity whilst remission from inflammation may result in an increase in enteral microbial diversity that could contribute to the restoration of tolerance. The first study analyses whether remission from active CD is associated with change in generic enteral microbial diversity by assessing people at diagnosis and following their response to gluten exclusion. A comparison is made to people without CD consuming a 'normal diet'. DGGE profiling of faecal microflora in subjects with CD at diagnosis (confirmed by serology and by duodenal biopsy) and over three consecutive months on a gluten-free diet (GFD) was performed and profiles were compared with those of age and gender matched control subjects taken at monthly intervals. Diversity of faecal microflora (measured as Simpsons Index) was significantly lower in people with CD than in control subjects. It was possible to distinguish the profiles of coeliac subjects at diagnosis from those obtained after three months on a GFD but it was not possible to distinguish between the samples from control subjects taken at monthly intervals. The profiles of CD subjects after three months on a GFD were more dissimilar to those of the control subjects than those obtained prior to dietary treatment, chiefly on the basis of three bands that were not found in the faeces of any control subjects.

The second study analyses dietary intake to determine if a lack of nutrients at diagnosis (before institution of a GFD) and at monthly intervals for three consecutive months post diagnosis (on a GFD) exists, as it is known that CD is associated with nutrient deficiencies resulting from malabsorption due to intestinal inflammation and damage. Subjects completed a customised food questionnaire at each sampling period. Dietary intake was analysed using Foodworks Professional 2007. Significant differences were identified in gluten, starch and carbohydrate intake but not in other macronutrients. Contrary to established literature, these analyses identified few significant differences in micronutrient intake within coeliac subjects over time, however, significant differences were found in iron and sodium.

Keywords: Coeliac disease, enteral intestinal microflora, gluten avoidance, DGGE, nutrient aberrations

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A glossary of key terms is included as Appendix A to ensure clarity of terminology used throughout this paper. Definitions of words ***italicised*** and ***bolded*** can be found in the glossary.

List of Abbreviations used

ADCC	antibody cell-mediated cytotoxicity
AGA	antigliadin antibodies
AMPs	antimicrobial peptides
AN-PEP	prolyl-endopeptidase from *Aspergillus niger*
APCs	antigen presenting cells
BCR	B-cell receptor
BIR	baculovirus inhibitor repeat
BMD	bone mineral density
CAMs	cell adhesion molecules
CARD	caspase-activating and recruitment domain
CCL	chemokine
CCR	chemokine receptor
CD	coeliac disease
cDNA-AFLP	cDNA amplified restriction fragment length polymorphism
CLA	conjugated linoleic acids
CTLA-4	cytotoxic T-lymphocyte-associated antigen-4
DCs	dendritic cells
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribose nucleic acid
DS	Down syndrome
ECM	extracellular matrix
ELISA	enzyme loinked immunosorbent assay
EMA	endomysial antibody
FAE	follicle associated epithelium
FCM	flow cytometry
FCM-FISH	combination of FISH with flow cytometry detection
FFQ	food frequency questionnaire
Fiaf	fasting-induced adipocyte factor
FISH	fluorescent *in situ* hybridisation
FOS	fructo-oligosaccharides
FSANZ	Food Standards Australia New Zealand
FVL	factor V Leiden
GALT	gut associated lymphoid tissue
GDP-fucose	nucleotide-activated fucose
GF	gluten-free
GFD	gluten-free diet
GI	gastrointestinal tract
GLN	glutamine
GOS	galacto-oligosaccharides
GP	guinea pig
HLA	human leukocyte antigen
HR	human recombinant
HU	human unmbilical cord
IBD	inflammatory bowel disease

IBS	irritable bowel syndrome
ICAM	intercellular cell adhesion molecule
IDA	iron deficiency anaemia
IE	intestinal epithelium
IEC	intestinal epithelial cells
IELs	intraepithelial lymphocytes
IFN	interferon
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IKK	IκB kinase
IL	interleukin
LA	linoleic acid
LPL	lipoprotein lipase
LPS	lipopolysaccharides
LRRs	leucine rich repeats
M-CELL	microfold cell
MDP	muramyl dipeptide
ME	monkey eosophagus
MHC	major histocompatibility class molecule
MLN	mesenteric lymph nodes
MMPs	metalloproteinases
MOH	Ministry of Health
mRNA	messenger ribosenucleic acid
MYO9B	myosin IXB gene
NBS	intermediary nucleotide-bonding site
NF-κB	nuclear factor kappa B
NHL	non Hodgkins lymphoma
NK T-cells	natural killer T-cells
NKG2D	innate immune receptor
NODs	nucleotide-binding oligomerisation domain proteins
NPV	negaitve predictive value
NSPs	non-starch polysaccharides
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PGN	peptidoglycans
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
PPAR-γ	peroxisome-proliferator-activated receptor gamma
PPM	parts per million
PRRs	pathogen recognition receptors
PPV	positive predictive value
PRO	proline

qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SCFAs	short chain fatty acids
SDD	selective digestive tract decontamination
SER	serine
sIgA	secretory immunoglobulin A
SIR	standardised incidence ratio
SMR	standardised mortality ratio
STAT6	signal transducer and activator of transcription-6
TCR	T-cell receptor
TGF	transforming growth factor
TGGE	temperature gradient gel electrophoresis
TIR	toll/interleukin receptor with an intracellular tail containing a conserved region
TIRAP	TIR adaptor protein
TLRs	toll-like receptor
TNF	tumour necrosis factor
TPOA	thyro-peroxidase antibody
t-RFLP	terminal restriction fragment length polymorphism
TS	Turner syndrome
tTg	tissue transglutaminase
TTGE	temporal temperature gradient electrophoresis
WS	Williams syndrome
ZO-1	zonulin-1

Abbreviations used in clinical study

CSI	Coeliac subject I
CST	Coeliac subject T
CSU	Coeliac subject U
CSW	Coeliac subject W
CSZ	Coeliac subject Z
CSAA	Coeliac subject AA
CSAB	Coeliac subject AB
CSAC	Coeliac subject AC
CSCGA	control subject A
CSCGB	control subject B
CSCGC	control subject C
CSCGD	control subject D
CSCGE	control subject E
CSCGF	control subject F
CSCGG	control subject G
CSCGH	control subject H

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Chapter One: INTRODUCTION

1.1 Overview

Coeliac disease (CD) is a diet-related disorder resulting from a number of interactions between diet, genome and immunity. As the aetiology of CD is multi-faceted, this thesis extensively reviews the systems that participate in these interactions, namely gut-associated immunity (in gut-associated lymphoid tissue-GALT), the microbial ecosystem of the gut, the diet and the genome. The epidemiology and pathogenesis of CD and existing or possible therapies will also be reviewed.

The core mechanism in the pathogenesis of CD is the inappropriate immune response to dietary gluten. This is characterised by changes in the humoral and cellular immune systems in the intestinal mucosa. However, the precise details of the processes underlying the genetic and immune mechanisms have not yet been completely elucidated.

The research conducted as part of this thesis seeks to further our understanding of the pathology of CD in regard to its secondary effects on the diversity of enteral microflora via changes in immune tolerance. Appropriate immune tolerance results from cross-talk between the cells of the GALT and elements of commensal enteral microflora residing in the gut lumen or attached to various cellular elements of the gut mucosa (Mowat *et al.,* 1997). It is known that immune tolerance is influenced by the prevailing cytokine environment within the cellular and paracellular elements of GALT. However, the extent to which pro-inflammatory conditions, such as CD, abrogate tolerance to common enteral microflora remains unknown (Kelly *et al*., 2005a;Cunningham-Rundles 1998). Tolerance of commensal enteral microflora is important in general health. It is increasingly recognised that a number of commensal microflora exert beneficial influences on elements concerned with gene regulation and immune status. Beneficial microflora may also contribute to the exclusion of pathogen and to the suppression of inappropriate responses to normal dietary elements (such as allergies)(Kelly *et al*., 2005b).

This research proposes that enteral mucosal pro-inflammatory change in CD is likely to be associated with a decrease in enteral microbial diversity. Conversely,

the remission of pro-inflammatory change may be accompanied by an increase in the diversity of enteral microflora that may help restore tolerance. The testing of this hypothesis may enable a more complete understanding of the role of tolerance and microflora in the pathogenesis of CD.

1.2 What is coeliac disease?

CD (also known as gluten-sensitive enteropathy or coeliac sprue) is a chronic autoimmune disorder with genetic, environmental and immunological components. It has been defined as a *" disease in which there is an abnormality of the small intestinal mucosa, made manifest by contact with the gluten fraction of wheat and related cereals "* (Howdle 2003).

It is the most common human food-induced enteropathy caused by an uncontrolled autoimmune response in which the partial digestion of storage proteins found in wheat, rye and barley (collectively referred to as 'gluten'), trigger a sequence of immune related reactions in genetically predisposed people that ultimately lead to intestinal damage (Srinivasan *et al*., 2006; Hamer 2005; Zarkadas *et al*., 2005; Spaenij-Dekking *et al*., 2004). Similar components in oats have also been implicated in the pathogenesis of CD, however the gluten-like portion of oats contain significantly lower percentages of proline residues which is thought to make them less likely to trigger an immune response (Vader *et al*., 2002a). The omission of dietary gluten generally leads to the disappearance of symptoms and recovery of the small intestine (Spaenji-Dekking *et al*., 2004). Subsequent consumption of gluten will, however, inevitably lead to a recurrence of pathology (Hamer 2005; Mowat 2003b). If left untreated, CD is associated with increasing malabsorption, an increased incidence of associated immune pathologies and an increase in general morbidity and mortality (Shan *et al*., 2002). The mechanisms underlying CD will be explained in later chapters.

1.3 History of Coeliac disease

CD was first described in 100 AD by an ancient Greek physician, Aretaeus who suggested the disorder was an affliction of the gut, but it took centuries to confirm his ideas. Another seventeen hundred years passed before a link was made between the ingestion of particular cereals and the development of gastrointestinal (GI) symptoms typical of CD (Hadjivassiliou *et al*., 2004; Fasano 2001; Paveley 1988). In 1888, the "coeliac syndrome" was described by Dr Samuel Jones Gee who was of the opinion that restriction of certain foods was a suitable treatment for this disorder. Since then efforts have been made to find an alternative treatment for CD, with a variety of therapies having been tried (Guandalini 2008; Williams 1997; Paveley 1988; Weijers *et al*., 1965; Gee 1888).

The same disease was documented by Dr Herter, an American physician in 1908, and CD was subsequently termed 'Gee-Herters disease' for a period (in Auricchio *et al*., 1996). Interestingly, in the context of this thesis, Herter suggested that CD resulted from inflammation of the intestine caused by an overgrowth of particular intestinal flora during breastfeeding, notably *bacillus bifidus* and *bacillus infantilis*. He observed that dietary proteins were well tolerated, fats moderately so and that carbohydrates exacerbated the condition (Paveley 1988).

In 1918, it was suggested that bread was poorly absorbed by people with CD, but the reasons for this were not defined (Still 1918). The consumption of wheat was subsequently found to exacerbate CD, but there was a general unwillingness to implicate such a widely used foodstuff as the prime agent in the pathogenesis of this disease (Paveley 1988; Weijers *et al*., 1965). Dietary elimination of wheat emerged as a treatment for CD in the late 1920s and early 1930s, and subsequently became the mainstay of therapy (Paveley 1988). The treatment of CD by Haas in 1924 with a 'banana diet' was successful, as it excluded bread, crackers, potatoes and cereals. Haas subsequently became convinced of a relationship between CD and the consumption of dietary carbohydrates. A fruit and vegetable based diet devised by Fanconi (in Auricchio *et al*., 1996), again proved successful in the treatment of CD, as it incidently excluded all cereal-based foods. It was not until 1938, that a correlation between steatorrhoea (fatty stools) in people

with CD and the nature of dietary carbohydrates was established (Weijers *et al*., 1965).

In 1930, Dicke, a Dutch paediatrican, identified a relationship between a disorder in children typified by malabsorption and a rash, and the consumption of wheat. He subsequently carried out extensive studies giving wheat-free diets to people with CD. Dicke's hypothesis was confirmed when the rationing of cereal grains during the war years contributed to the improvement in the clinical status of his patients (Paveley, 1988). Dicke later determined that wheat and rye flour, but not wheat starch were responsible for the steatorrhoea. He went on to show that the toxic moiety in wheat flour was alcohol soluble and subsequently identified this as gliadin (Auricchio *et al*., 1996). By 1949, Sheldon had shown that steatorrhoea was reduced when the patient was maintained on a diet that excluded wheat flour, but the general unwillingness of the medical profession to implicate wheat remained (in Weijers *et al*., 1965).

It was not until biopsy of the intestinal mucosa in living subjects was possible in the 1940s that further breakthroughs were made (Wood *et al*., 1949; Kenamore 1940). Schein (1947) described crypt hyperplasia, villous atrophy and increased intraepithelial lymphocytes in the mucosa of the small intestine in people with CD and popularised the use of jejunal biopsies in confirming a diagnosis of CD. Further explanation of the histopathology of CD was provided when Paulley *et al*., (1954) found chronic inflammatory cell infiltrate and broad, short villi in the submucosa of full thickness small intestinal biopsy taken at laparotomy (in Quinn *et al*., 2007). Subsequently more sophisticated biopsy methods were developed (Crosby 1957; Shiner *et al*., 1956) which further confirmed the gut as the principal site of pathology in CD.

At this point CD was considered to only affect the gut. Any association of CD with pathology in other organs was attributed to secondary effects of vitamin deficiencies induced by malabsorption. While it was thought there could be an immunological component to CD, no objective evidence (e.g. the demonstration of antibodies to gluten) was found to support this (Auricchio *et al*., 1996). However, in 1961, Taylor demonstrated the existence of antibodies to gluten (anti-gliadin antibodies) in the

blood of people with CD and an immunological aetiology for CD was accepted by the medical community (Taylor *et al*., 1961).

Rubin *et al*., (1962) confirmed that an inappropriate response to dietary gluten led to the generation of mucosal abnormalities by showing that consumption of gluten induced the characteristic histological changes in the mucosa of the small intestine. A 'diagnostic criteria' for CD was established by the European Society for Paediatric Gastroenterology and Nutrition in 1969 (in Auricchio *et al*., 1996). Three intestinal biopsies were recommended; the first taken at the time of presentation, the second following the implementation of a gluten-free diet (GFD) and the third following re-challenge with gluten. This procedure became the standard diagnostic protocol for a number of years (Auricchio *et al*., 1996). It was subsequently found that dermatitis herpetiformis (DH) had a similar pathogenesis to CD and responded to exclusion of gluten from the diet. This initiated a shift away from the concept that CD was exclusively a disorder of the gut (Marks *et al*., 1970).

The link between CD and the genetic elements of immune tolerance became clearer with the rise of genetic technology. This led to gluten sensitivity being described as 'a state of heightened immunological responsiveness in genetically susceptible people' (Quinn *et al*., 2007; Marsh 1995). It has since become evident that CD results from an inappropriate T-cell mediated immune response to degradation products of dietary gluten (Fasano 2001). Incumbent in this definition, is the idea that whilst the intestinal epithelium (with its intact intercellular tight junctions) acts as a barrier to the passage of gluten, significant amounts of gluten are absorbed across the intestinal mucosa through transcellular and paracellular pathways even in people who do not have CD. Transport of gluten, via the paracellular route, leads in healthy people, to immune tolerance. In people with a genetic predisposition to CD, larger quantities of gluten are absorbed and the integrity of the tight junctions is compromised (Fasano 2001). The inappropriate up-regulation of zonulin, a tight junction protein, was identified as being responsible for the laxity of tight junctions associated with CD (Wang *et al*., 2000).

The introduction, in the 1980's and 1990's, of sensitive, specific and non-invasive screening tests, such as the measurement of serum antibodies to gliadin, and to endomysium, has further improved the diagnosis of CD. The ease of these tests

has made population screening a viable undertaking. Hence, the extensive epidemiological studies that have furthered our understanding of the genetic components of this disease discussed in the next chapter.

Chapter Two: THE EPIDEMIOLOGY OF COELIAC DISEASE

2.1 Statistical measures used in the epidemiology of CD

The epidemiological study of CD is made complex by the differing sensitivities, specificities and predictive value of the various diagnostic tests.

2.1.1 Sensitivity

Sensitivity measures how well a binary classification test correctly identifies a condition. The results of screening tests are compared to an absolute (Gold Standard). The sensitivity of a test is the proportion of all true disease cases detected in those members of the population who have the disease. Sensitivity can be calculated as;

$$\text{Sensitivity} = \frac{\text{no. of true positives identified}}{\text{no. of true positives} + \text{no. of false negatives}}$$

High sensitivity (i.e. a high proportion of all cases being detected) is required when early diagnosis and treatment are beneficial. A test with a sensitivity of 100% indicates that the test is positive in all people who have the disease. Sensitivity is not the same as the positive predictive value which is a statement about the proportion of actual positives in the population being tested (Sokal *et al*., 1995).

2.1.2 Specificity

Specificity is a statistical measure of how well a binary classification test correctly identifies the negative cases (i.e. those without the disease). Specificity assesses the probability that the test is `negative' in people who do not have the disease. Thus the specificity is the proportion of true negatives in all negative cases.
It is defined as:

$$\text{Specificity} = \frac{\text{no. of true negatives identified}}{\text{no. of true negatives} + \text{no. of false positives}}$$

High specificity is important when the treatment or diagnosis may be harmful to the patient. A specificity of 100% means that the test recognises all healthy people as healthy, i.e. a test with a high specificity has a low Type-I error rate. Specificity is

sometimes confused with the positive predictive value, which refers to the fraction of returned positives that are true positives (Sokal *et al.*, 1995).

2.1.3 Predictive values

Predictive values are used to evaluate the usefulness of a diagnostic test. The predictive value can be positive or negative.

The **positive predictive value** (PPV) is the proportion of patients with positive test results who have been correctly diagnosed. Hence the PPV can be used to indicate the probability that in the case of a positive test, the patient actually has the specified disease. However there may be more than one cause for a disease (this is not the case with CD) and any single potential cause may not always result in the overt disease seen in a patient. The PPV is considered the physician's gold standard, as it reflects the probability that a positive test reflects the underlying condition being tested for (Sokal *et al.*, 1995).

The **Positive Predictive Value** is defined as;

$$PPV = \frac{\text{no. of true positives}}{\text{no. of true positives + no. of false positives}}$$

or, alternatively,

$$PPV = \frac{\text{sensitivity)(prevalence)}}{\text{(sensitivity)(prevalence)+ (1-specificity)(1-prevalence)}}$$

The **negative predictive value** is the proportion of patients with negative test results who are correctly excluded from the diagnosis (Sokal *et al.*, 1995).

The **Negative Predictive Value** is defined as;

$$NPV = \frac{\text{no. of true negatives}}{\text{no. of true negatives + no. of false negatives}}$$

or, alternatively,

$$NPV = \frac{\text{specificity x (1-prevalence)}}{\text{specificity x (1-prevalence)+ (1-sensitivity) x prevalence}}$$

2.2 Tests to determine the presence of CD

The awareness of CD has increased in the past fifteen years due to significant progress in the development of reliable serological screening tests. At first glance, the determination of the sensitivity and specificity of the various diagnostic modalities for CD seems straight-forward however this is not always the case. Numerous studies assessing the diagnostic characteristics of serological tests using a variety of different laboratory methods are remarkably heterogeneous on a number of levels (i.e. whether defined by biopsy, by serology, the extent of intestinal villous atrophy, or the type of antibody kits used) (Rostom *et al*., 2007; Fasano *et al*., 2001). The presence of anti-endomysial serum antibodies is used for primary diagnosis of CD as auto-antibodies to the endomysium have the highest specificity for CD (Bai *et al*., 2005). A less reliable serological test that detects antibodies to tTG is cheaper and is often used for epidemiological studies (Bai *et al*., 2005; Zarkadas *et al*., 2005).

2.2.1 The use of anti-gliadin antibody assays (IgA-AGA and IgG-AGA)

Purified gliadin is readily available for use as the antigen for enyzme-linked immunosorbent assay (ELISA) tests to detect serum anti-gliadin antibodies which are frequently elevated in untreated CD. Anti-gliadin assays based on IgA and IgG-antibodies have been used for a number of years as a diagnostic tool. Variation in the prevalence of CD in populations worldwide (obtained using IgA and IgG-based assays) has been attributed to the different specificities of these tests (Kumar 2006). Both IgA-AGA and IgG-AGA tests demonstrate poorer sensitivity and specificity than other tests, however IgA-AGAs are considered superior to those based on IgG because they have a higher sensitivity and specificity (Bai *et al*., 2005; Zarkadas *et al*., 2005).

2.2.1.1 IgA-AGA

Rostom *et al*., (2007) showed that IgA-AGA tests had varying degrees of accuracy in the diagnosis of CD in both children and adults. In a series of studies (see Tables 2.1, 2.2, 2.3 in Appendix B) the sensitivity for IgA-AGA in children ranged from 22.2-100 %, while the specificity ranged from 51-100 %. In adult populations, the sensitivity for IgA-AGA ranged from 30.8-100% and the specificity from 45-100% (see Table 2.2 in Appendix B). The PPV for IgA-AGA in children showed

huge variability ranging from 0-95.7 while the NPV ranged from 36.3-100. In adults the PPV ranged from 22.2-100 and the NPV from 42.7-100.

2.2.1.2 IgG-AGA

In the same series of studies for IgA-AGA, the sensitivity for IgG-AGA in children ranged from 33.3-100 %, whereas the specificity ranged from 36-97.8 % and in adult populations the sensitivity for IgG-AGA ranged from 17-100% however, there was less variation in the reported specificities with a range of 69.7- 97% (see Table 2.1 in Appendix B). The PPV for IgG-AGA in children was between 31.2-100 and the NPV between 0-100. In studies assessing adult populations, the PPV ranged between 14-87.6 and the NPV from 67.6-100.

So while these IgA and IgG-AGAs are low cost and easily administered across large groups within individual populations, they generally have a lower sensitivity and specificity than other tests, and have a poor PPV in the general population, so are no longer routinely recommended (Bai *et al*., 2005).

2.2.2 The use of the IgA-endomysial antibody test (IgA-EMA)

IgA-endomysial antibodies bind to the endomysium producing characteristic staining thatis detected by indirect immuno-fluorescence using commercial sections of monkey oesophagus (ME) or human umbilical cord (HU). The result is reported as positive or negative, as even low titres of serum IgA-EMAs are specific for CD (Bai *et al*., 2005). One of the difficulties in comparing sensitivities and specificities obtained using this testing method is that the commercial kits containing ME or HU give different results. However, the sensitivity and specificity of EMA for most studies analysed by Rostom *et al*., (2007) (see Tables 2.4, 2.5 and 2.6 in Appendix B) are quite high (over 92% for sensitivity and close to 100% for specificity), except for a couple of rogue results (Iltanen *et al*., 1999; Russo *et al*., 1999), as are their PPVs and NPVs. However, it should be noted that the reported diagnostic parameters were taken from studies where the prevalence of CD was, for the most part, much higher than that seen in usual clinical practice. The PPVs reported for these tests will not be as high as that reported when these tests are used to screen the general population (Rostom *et al*., 2007).

2.2.2.1 IgG-EMA

IgG-EMA is also used to diagnose CD. Rostom *et al*., (2007) found the sensitivity of IgG-EMA varied in all age groups from 94-100% whereas specificity was between 98-100%.

These EMA tests are cheap, easily administered, non-invasive and highly efficient for routine laboratory screening, but they should not replace intestinal biopsy for population screening as their PPVs are lower than the tTG antibody tests.

2.2.3 The use of anti-tissue transglutaminase antibodies (IgA-tTg or IgG-tTg)

The finding that tTg is the main autoantigen of anti-endomysium enabled the discovery of anti-tTg antibodies and subsequent identification of the tTg enzyme as the target of EMAs. This has improved diagnostic testing as anti-tTg antibodies exhibit high sensitivity and specificity for the diagnosis of CD (Sblattero *et al*., 2000; Dietrich *et al*., 1997). Many studies have assessed the sensitivity, specificity, PPVs and NPV of diagnostic tests using IgA-tTg-GP and IgA-tTg-HR in both adults and children (see Tables 2.7 and 2.8 in Appendix B). ELISA tests for IgA anti-tTg are now widely available, less observer dependent, easier to perform and less costly than the immuno-fluorescence assay used to detect IgA-EMAs. The diagnostic accuracy of IgA anti-tTg immunoassays has further improved by using human-tTg (human recombinant-HR) rather than the non-human tTg (usually guinea pig-GP) preparations used in earlier immunoassays (Cranney *et al*., 2006; Bai *et al*., 2005).

From the studies reviewed, it is clear that the sensitivity of the IgA-tTg test using IgA-tTg-HR is higher than that found using IgA-tTg-GP, as is the specificity (see Table 2.8 in Appendix B). However, the accuracy of all these tests varies with the laboratory used and with the degree of villous atrophy (Wolters *et al*., 2002; Hansson *et al*., 2000).

2.2.4 The use of the gene (histocompatibility) test

Since it was discovered that CD (Sollid *et al*., 1989) is closely associated with the human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 genes, genetic testing has been used to exclude the presence of, or susceptibility to CD however, the presence of HLA-DQ2 or HLA-DQ8 is not helpful as a positive predictor of CD (Anderson 2006). The HLA-DQ2 or HLA-DQ8 gene is seen in 99.6% of people with CD and these genes are thought to be conditional for its development (van

Heel *et al*., 2006; Anderson 2005). The specificity of this test can be quite low, making its PPV relatively low also, as people can have these genes but not actually develop CD (Rostom *et al*., 2007). Irrespective of this, HLA DQ2/DQ8 testing appears is a useful adjunct in the diagnosis of CD, as the test has a sensitivity greater than 90-95% (Rostom *et al*., 2007).

Several studies (Rostom *et al*., 2007) indicate that trends for the presence of HLA-DQ2 are similar in different population groups worldwide, despite there being differing percentages of the HLA-DQ2 and DQ8 genes in countries due to different racial mixing within these populations (i.e. a different gene pool) (see Table 2.9 in Appendix B).

In both a Finnish study (Iltanen *et al*., 1999) and an Italian study (Sacchetti *et al*., 1998), the sensitivity was quite high, but the specificity was lower. This suggests that a negative DQ2 test result rather than a positive one probably provides the greatest diagnostic information.

The frequency of HLA-DQ8 in Western European populations with CD varies from approximately 2.7-6%. The frequency is slightly higher in studies from Italy, the United Kingdom (UK) and France (5.6%-8%). The frequency of HLA-DQ8 in an American serology screening study (Fasano *et al*., 2003) was 22%, which is considerably higher than the European studies (Rostom *et al*., 2007). Rostom *et al*., (2007) found the sensitivity and specificity of having either HLA-DQ2 or DQ8 was close to 100% in Western populations. Probably the most accurate information about the sensitivity and specificity of the HLA gene test comes from a case-control study (Balas *et al*., 1999) which compared people with biopsy-proven CD to healthy controls. Thus the HLA gene test is a powerful tool for excluding a diagnosis of CD. It is minimally invasive and 5-10 times cheaper than gastroscopy and duodenal biopsy (Anderson 2005). HLA genetic testing at birth is being mooted as a means of mass population screening to identify a genetic predisposition for CD before it clinically presents and causes health issues (Anderson 2006).

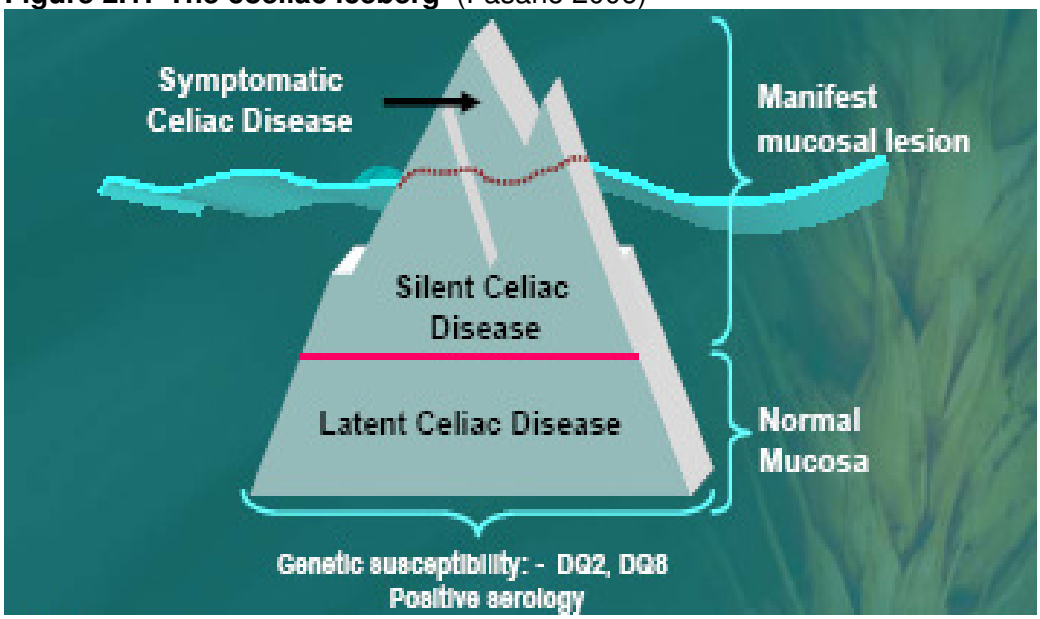
Note: It has recently been suggested that the HLA-DQ2/DQ8 genes may not be the true risk-genes associated with CD. Evidence suggests that genes other than HLA-DQ2 or HLA- DQ8 may be associated with CD (Rostom *et al*., 2007). This is an area of ongoing research which could greatly impact on the epidemiology of CD.

2.3 Prevalence and /or incidence of CD

Historically, CD was considered a rare condition with an estimated prevalence of 1:2000. This is no longer an accurate assessment (Beattie 2006). The number of cases being diagnosed continues to increase worldwide but its true prevalence is often difficult to estimate because of outdated testing methods and failure to test for CD where appropriate (Bai 2005; Zarkadas *et al*., 2005; Hadjivassiliou *et al*., 2004). Statistics show the diagnosis rate of adult CD rose dramatically between 1997-2003 (in most countries where data is available to monitor trends) due to targeted larger scale screening programmes, increasing recognition of presenting symptoms by clinicians and greater accuracy of diagnostic testing (van Heel *et al*., 2006; Murray *et al*., 2003; Collin *et al*., 1997). CD is now commonly found in European populations living in the United States (US), Europe, Australia, New Zealand, North Africa, the Middle East, India and South Asia (van Heel *et al*., 2006; Zarkadas *et al*., 2005).

The epidemiology of CD has distinct “iceberg” characteristics with many cases remaining unidentified when screening tests are unavailable or screening is not performed. This ‘coeliac iceberg’ theory, proposed by Logan (1992) (graphically depicted in Figure 2.1) has been confirmed by large scale screening surveys (Bai *et al*., 2005). The total size of the ‘coeliac iceberg’ is similar globally however the waterline may differ between continents (Bai *et al*., 2005). For example, in Europe and the US, prevalence is comparable across all population groups, but the iceberg is more submerged in the US with fewer cases being diagnosed than in Europe (Bai *et al*., 2005).

Figure 2.1: '**The coeliac iceberg**' (Fasano 2006)



Numerous studies (Berti *et al*., 2000a; Gandolfi *et al*., 2000a; Hill *et al*., 2000; Shahbazkhani *et al*., 2000; Yachha *et al*., 2000; Berti *et al*., 1999; Catassi *et al*., 1999; Not *et al*., 1998; Fasano 1996; Hill *et al*., 1995; Talley *et al*., 1994; Catassi *et al*., 1994; Rossi *et al*., 1993) conclude a similar prevalence rate worldwide. Rostom *et al*., (2007) show the prevalence by serology in unselected populations of North America and Western Europe ranges from 1:658-1:37 and the prevalence by biopsy from 1:658-1:53. Serological prevalence data from other studies (Fasano *et al*., 2003; Hill *et al*., 2002; Volta *et al*., 2001; Catassi *et al*., 2000; Green *et al*., 2000; Riestra *et al*., 2000; Korponay-Szabo *et al*., 1999; Trevisiol *et al*., 1999; Not *et al*., 1998; Pittschieler *et al*., 1996; Catassi *et al*., 1995; Mazzetti *et al*., 1992) found a prevalence range of 1:320-1:85. Green *et al*., (2001) found a prevalence of 1:200 in patients undergoing upper endoscopy. More recently, Anderson (2005) and Bai *et al*., (2005) estimated the serological prevalence of CD worldwide as 1:100. Fasano *et al*., (2003) determined the serological prevalence of CD as; 1:10 for 'at risk'-first degree relatives (i.e. parent, child, sibling); 1:39 at risk-second degree relatives; 1:56 symptomatic patients; and 1:133 for groups 'not at risk', for a predominantly Caucasian population (94%), however other ethnic groups were included (3% black; 1.5% Hispanic; 1% Asian; 0.5% other).

Based on comprehensive prevalence data from the US and Europe, the ratio of diagnosed to undiagnosed CD is between 1:5-1:13 (Mustalahti *et al*., 2004; Fasano

2003; Maki *et al*., 2003; Murray *et al*., 2003; Carlsson *et al*., 2001;Fasano *et al*., 2001). Recently, the ratio of clinically diagnosed to undiagnosed cases (from prevalence data) in the United Kingdom(UK) was identified as 1:8 (van Heel *et al*., 2006). Unfortunately, data has not been collected in New Zealand other than that gathered by clinicians or hospitals from people presenting with other GI disorders (personal communication with epidemiologist, Ministry of Health, 2006), however the ratio of diagnosed to undiagnosed people is believed to be about 1:10 (Anderson 2005).

The actual frequency of diagnosis for CD between 2001-2006 in the UK, was reported as only 0.25 per 1000 people, which is considerably lower than expected from prevalence data, indicating that CD remains under-diagnosed (Ravikumara *et al*., 2006). Such under-diagnosis has important implications for the health of populations worldwide as early diagnosis allows for dietary avoidance of gluten which mostly results in remission from disease symptoms and avoidance of major systemic complications (Zarkadas *et al*., 2005; Ravikumara *et al*., 2006).
Incidence or prevalence of CD between different ethnic groups is further complicated by differing availability of diagnostic tests and the per capita consumption of cereals (i.e. whether there is sufficient consumption of gluten to precipitate CD in people who are predisposed) (Bai *et al*., 2005).

Ultimately, the prevalence of CD depends on the prevalence of predisposing genes prerequisite for its development (i.e. the HLA-DQ2 gene, and to a lesser extent the DQ8 gene). Given that it is neither feasible nor practical to perform histocompatibility tests on significant proportions of lower socio-economic populations, the extent of variation in predisposing genes between populations is not known (Howdle 2003). Populations exist who do not possess either genotype (HLA-DQ2 or DQ8) and are thus not expected to develop CD. This may explain the rarity of CD in people of Negro, Japanese and Chinese descent (Bai *et al*., 2005).

The ‘iceberg theory’ is further complicated by the fact that a number of subjects with predisposing genotypes do not develop CD due to no environmental trigger or some other as yet, unidentified factor that distinguishes normal individuals from potential coeliac patients (Howdle 2003).

2.3.1 Gender differences in incidence

Many intestinal diseases exhibit gender differences in clinical presentation, diagnosis and in treatment responses (Addesa *et al*., 2004). However, the full extent of gender differences with CD is unknown, irrespective of how the disease state is identified (i.e. positive serology or clinical manifestations) (Addesa *et al*., 2004). Research (van Heel *et al*., 2006; Bai *et al*., 2005; Zarkadas *et al*., 2005) shows a female to male ratio for adults diagnosed with CD of 2.3 :1, however this sex difference is less apparent in people diagnosed as children where the ratio is 1:1. Females are the dominant demographic and gender-specific health issues related to CD have been identified. The menstrual cycle, fertility and pregnancy can all be adversely affected. These are secondary complications that are not genetically predetermined (Bai *et al*., 2005; Adessa *et al*., 2004; Eliakima *et al*., 2001; Green *et al*., 2001; Gasbarrini *et al*., 2000; Malnick *et al*., 1998; Ciacci *et al*., 1996; Sher *et al*., 1994; Ferguson *et al*., 1992; Maki *et al*., 1988; Stenhammar *et al*., 1986; Farthing *et al*., 1983).

Some authors suggest an immune response during pregnancy may exacerbate CD. This is feasible given that CD is an inflammatory autoimmune disease and some aspects of immune function are naturally suppressed during pregnancy to prevent miscarriage (Morein *et al*., 2007). The Th_1/Th_2 cytokine balance is important in maintaining pregnancy with the production of Th_2 type cytokines favouring maintenance and the production of Th_1 type cytokines promoting foetal rejection (Koch *et al*., 2007; Zenclussen *et al*., 2007; McCracken *et al*., 2003). The Th_1/Th_2 balance is also vital in maintaining tolerance and preventing allergic reactions and autoimmune diseases so it would follow that immune activity could exacerbate CD if gluten is ingested.

Few studies have focused specifically on the male demographic for CD however men with untreated CD have higher rates of infertility, hypogonadism, semen quality abnormalities (motility and morphology) and sexual dysfunction than the general population (McClure 2005).

2.3.2 Age of onset

Traditionally, CD was considered a paediatric disorder, however over the past 20 years, the age at presentation and presenting features have changed considerably

(Bai *et al*., 2005; Hawkes *et al*., 2000; Collin *et al*., 1997). This is largely explained by a change in practice patterns where physicians are more aware of the condition and its atypical manifestations or associated conditions, as well as a result of improved serological testing and an increased incidence over this time (Bingley *et al*., 2004; Sanders *et al*., 2002; Hawkes *et al*., 2000; Tuthill 1999; Jenkins *et al*., 1998; Collin *et al*., 1997; Sategna-Guidetti *et al*., 1994; Corazza *et al*., 1993).

The age at diagnosis has increased over the past 25 years in most populations (Mäki *et al*., 1988; Pare *et al*., 1988; Logan *et al*., 1983; Swinson *et al*., 1980) which has facilitated changes in dietary habits in some populations based on the hypothesis that delayed exposure to gluten would prevent the onset of CD. This resulted in a decreased incidence in the infant population, but a corresponding increase in atypical cases, with the onset of symptoms occurring in adolescents and adults (Fasano 2001). Ruoff (1996) found that presentation peaks around 40 years in females compared to 60-70 years in males and that 27% of people were diagnosed for the first time over 60 years. Older people are more likely to manifest symptoms of the underlying malabsorptive process, (i.e dyspnea and fatigue due to anaemia from low folate levels, bone pain and compression fractures, or osteomalacia and osteopenia). It was also found that people diagnosed over 50 years have a greater chance (1:10) of developing lymphoma.

2.3.3 Delay in diagnosis

CD is often misdiagnosed as irritable bowel syndrome (IBS), chronic fatigue, or fibromyalgia because it is associated with a plethora of non-specific symptoms which means the true diagnosis is frequently delayed between 4-11 years (Zarkadas *et al*., 2005; Farrell *et al*., 2002; Green *et al*., 2001). Such delay dramatically increases the risk of developing autoimmune disorders, neurological problems, osteoporosis and cancer (Ventura *et al*., 1999).

Logan *et al*., (1989) reported the all-cause mortality rate increased in people diagnosed as adults, but did not increase in those diagnosed as children. The standardised mortality ratio (SMR) for people diagnosed between 18-29 years was slightly lower than those diagnosed later in life. If diagnosed at 30 years, the mortality ratio was 2.5 compared with 2.4 for 49 years and 1.9 for those diagnosed

over 50 years. More recently, Corrao *et al*., (2001) found the longer the untreated symptomatic period, the greater the mortality rate.

Askling *et al*., (2000) found an increased risk of non-Hodgkins lymphoma (NHL) in CD diagnosed in adulthood compared to those diagnosed as children. Thus early diagnosis is important, as it reduces or prevents severe complications (Rampertab *et al*., 2006). Catassi *et al*., 2007 recently screened (using serological testing) all subjects belonging to known "at-risk" groups and found more than a 40-fold increase in diagnosis rates. This active case-finding strategy in a primary care setting was effective for improving the diagnostic rate and reducing complications and mortality.

2.3.4 Typical Presentation of CD

Historically, CD typically presented with symptoms of weight loss and diarrhoea, however many other symptoms are indicative of CD. Fasano *et al*., (2003) found that only 35% of people newly diagnosed with CD had chronic diarrhoea, dispelling the myth that diarrhoea is a major presenting feature. This was confirmed by Hernandez *et al*., (2006) who established that less than 50% of cases now present with diarrhoea. Other presentations commonly include iron-deficiency anaemia, osteoporosis, dermatitis herpetiformis and neurologic disorders (mainly peripheral neuropathy and ataxia). Arthritis is common in CD (when systematically sought) and autoimmune diseases occur 3-10 times more frequently than in the general population (Hernandez *et al*., 2006; Bai *et al*., 2005).

2.3.5 Change in typical presentation of CD

There has been a noticeable shift away from predominantly GI manifestations to one of increased non-GI and asymptomatic cases identified through screening. In fact, many subjects diagnosed via screening are asymptomatic and totally unaware of their condition (Kumar 2006; Bai *et al*., 2005; Fasano *et al*., 2003; Berti *et al*., 2000; Fasano 2001; Mäki *et al*., 1988). Ravikumara *et al*., (2006) established that GI manifestations were twice as prevalent in the UK between 1983-89 as they were between 1999-2004.

2.3.6 Prevalence of CD in people with iron deficiency anaemia (IDA)

Research shows an increased prevalence of CD in people with IDA (Annibale *et al*., 2003; Oxentenko *et al*., 2002; Howard *et al*., 2002; Ransford *et al*., 2002; Van Mook *et al*., 2001; Unsworth *et al*., 2000; Dickey *et al*., 1997; Ackerman *et al*., 1996; Corazza *et al*., 1995; Kepczyk *et al*., 1995; McIntyre *et al*., 1993). Annibale *et al*., (2001) evaluated the impact of a GFD on anaemia and iron deficiency in newly diagnosed cases of CD in adults with IDA between 1994-1997and found 77% with total villous atrophy and 23% with subtotal villous atrophy. Six months establishing a GFD, 77% had recovered from IDA but only 27% had increased iron stores as defined by normal ferritin levels. At 12-months post-GFD, 94% recovered from IDA and 50% reversed from iron deficiency which indicates that recovery from IDA occurs within the first 6-12 months, but reversal from iron deficiency only occurs in 50% of cases (predominantly pre-menopausal women). Long-term follow-up of ferritin results and small intestinal biopsies in subjects with CD is needed to determine if iron deficiency resolves completely. Overall, in people with asymptomatic IDA assessed by serology or biopsy, the prevalence of CD was between 2.3%-6%. Therefore, there is a case to be made that people with IDA, (particularly those without a clearly identifiable cause) should be evaluated for CD as part of their medical investigation (Catassi *et al*., 2007).

2.3.7 Prevalence of CD in people with low bone mineral density, fractures, osteopenia or osteoporosis

Prevalence of CD in people with osteoporosis is likely higher than that in the general population (between 0.9%-3%) (Catassi *et al*., 2007). Bone mineral density (BMD) is consistently lower in people with untreated CD compared with controls (Catassi *et al*., 2007) and the prevalence of osteopenia or osteoporosis in newly diagnosed CD varies. Satgena-Guidetta *et al*., (2000) found 34% of subjects had normal BMD, while 40% had osteopenia and 26% had osteoporosis. Valdimarsson *et al*., (1996b) found the prevalence of severe osteopenia ranged from 22% at the forearm through 15% at the spine to 9% at the femur whereas the prevalence of mild osteopenia remained close to 24% at all sites. No difference was found in spine BMD between people with malabsorption, compared to those without malabsorption. Valdimarsson *et al*., (1996b) also found that 27% of subjects with CD had secondary hyperparathyroidism, (excessive amounts of parathyroid hormone-PTH), which disrupts the regulation of calcium resulting in leaching of

calcium from the bones, hypercalcaemia and increased calcium excretion in urine. After 1 year on a GFD, prevalence of severe osteopenia decreased from 23%-14%.

2.3.8 Co-existence of CD with other disorders

CD likely co-exists with other 'autoimmune' disorders, possibly due to genetic predisposition as the ability to develop an autoimmune disease is determined by a dominant genetic trait (Fasano 2006; Ventura *et al*., 1999). These genetic traits may present within the same family as different autoimmune diseases (Fasano 2006; Ventura *et al*., 1999). The overall incidence of autoimmune diseases in the US general population is 3.5%. Ventura *et al*., (1999) found people with CD had a greater chance of developing an autoimmune disorder and that the prevalence of autoimmune disorders in CD increased with increasing age at diagnosis (see Table 2.10 in Appendix B).

Disorders closely associated with CD include thyroid disease, asthma, pulmonary diseases, rheumatoid arthritis (RA), serum IgA deficiency, glomerulonephritis, IgA nephropathy, vasculitis, ulcerative colitis, Crohn's disease, liver disorders (i.e. primary biliary cirrhosis or autoimmune chronic active hepatitis), pernicious anaemia, polymyositis, sarcoidosis, dermatitis herpetiformis, type-1 diabetes, systemic lupus erythematosus (SLE), Sjogren's syndrome, scleroderma, Grave's disease, Addison disease, and Myasthenia gravis. CD is also commonly found in people with Turner's, Down and Williams syndromes (Fasano 2006; Ventura *et al*., 1999; Jones *et al*., 1995). These CD-associated autoimmune disorders can be either organ-specific, in which the autoantibodies are specifically directed against antigens localised in a particular organ and are often detected in circulation (e.g. Hashimoto's thyroiditis and type-1 diabetes), or non-organ-specific autoimmune disorders characterised by the presence of autoantibodies directed against ubiquitous antigens (e.g. SLE, RA, Sjögren's syndrome and scleroderma) (Fasano 2006). Only those considered directly relevant to the present study will be discussed.

2.3.8.1 Prevalence of CD with Type-1 Diabetes

The incidence of CD associated with type-1 diabetes is high with prevalence rates varying greatly (Bai *et al*., 2005; Cerutti *et al*., 2004; Rewers *et al*., 2004; Ashabani *et al*., 2003). Collin *et al*., (1994) found a highly significant correlation between

endocrine disorders in CD patients compared with controls in a 10-year, age-matched study concluding that people with CD have a significantly higher prevalence of type-1 diabetes. For example, type-1 diabetes is known to affect 3 million people in the US and 6% (180,000) of these people also have biopsy proven CD. CD is known to have a prevalence of around 1.0 % (1:100) in the general Western population (Anderson 2005) whilst the prevalence rates of CD in children with type-1 diabetes are estimated to range between 1.7-12%. Screening studies show prevalence among adults with type-1 diabetes to be similar, between 1.3-6.4%, which is much higher than the prevalence in the general population (Schwarzenberg *et al*., 2002).

Rashid *et al*., (2003) found 8% of Canadian children with CD had been previously diagnosed with type-1 diabetes. More recent studies (Tanure *et al*., 2006; Mahmud *et al*., 2005) show a similar incidence of CD in people with type-1 diabetes. This co-morbidity suggests possible genetic polymorphisms may dictate the risk of CD in people with type-1 diabetes (Fasano 2006; Zarkadas *et al*., 2005; Rewers *et al*., 2004). To test this hypothesis, Sumnik *et al*., (2006) investigated whether the susceptibility to CD in diabetic children is modified by positivity for HLA-DQB1*02-DQA1*05 and DQB1*0302-DQA1*03, and by alleles of single nucleotide polymorphisms within the genes encoding several cytokines. They compared genotypic data of children with diabetes and CD with control children who only had diabetes. The best-fit model showed the risk of CD is increased by the presence of HLA-DQB1*02-DQA1*05 and also independently by tumor necrosis factor, whereas interleukin-1α showed a weak negative association. These results indicate the risk of CD in children with type-1 diabetes is significantly modified by both the presence of HLA-DQB1*02-DQA1*05 and by a variant of another gene within the major histocompatibility complex (MHC) (i.e. tumor necrosis factor) (Fasano 2006).

Very recently, Rostom *et al*., (2007) reviewed data relating to CD and diabetes. Their findings are shown in Table 2.11 (in Appendix B). These studies show great variability in the prevalence of CD associated with type-1 diabetes, both by serology and biopsy.

Chapter Three: GUT IMMUNITY, INFLAMMATION AND TOLERANCE

3.1 The Function of Gut-Associated Lymphoid Tissue (GALT)

The functions of GALT include the mediation of inflammation and tolerance in the GI tract and associated structures. The specialised cells, tissues and signalling systems of GALT together detect, protect from and remember harmful substances and organisms whilst identifying and tolerating harmless ones. Tlaskalova-Hogenova *et al*., (2002) outline characteristic features of mucosal immunity that distinguish it from systemic immunity including mechanisms of innate defense, populations of unique lymphocytes, the 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 tolerance and systemic immunity towards inflammation (Mayer 2003).

The gut acts as a defence system in three ways. Firstly, ***intestinal epithelial cells*** (IECs) (also called ***enterocytes***) in the mucosa protect against entry of harmful substances by secreting mucins and defensins (Perdue 1999). Secondly, gut cells capable of generating an autogenous innate immune response trigger the production of antibodies that bind, deactivate and eliminate antigen (Guarner *et al*., 2003). Thirdly, IECs are able to communicate with ***commensal microflora*** to co-ordinate an immune response. Hence, commensal bacteria may aid in digestion, protect against invading bacteria by generating an adverse environment for pathogens and compete for nutrients, or receptor sites on the intestinal wall (Wilson 2005; Guarner *et al*., 2003).

Immune responses broadly divide into innate and adaptive systems, with adaptive immunity being characterised by specificity and memory whilst innate immunity is less specific, with no development of memory for previously encountered antigen (Wilson 2005). Innate immune mechanisms discriminate between pathogen and self-tissue then destroy harmful substances. The activation of innate immunity is often a prerequisite for triggering adaptive immunity (Akira *et al*., 2001).

The surface of the intestinal mucosa consists of a single layer of enterocytes, which separate the intestinal lumen from underlying cells, making it vulnerable to breach by pathogen (Neutra *et al*., 2001), however the threat of pathogenic breach is met by a highly sophisticated system (Mowat 2003; Shanahan 2000). On occasion, GALT responds to harmless proteins, activating beneficial immune mechanisms inappropriately. This forms the basis of food hypersensitivities (allergies), and autoimmune disorders (such as CD) which are detrimental to health.

3.1.1 The interaction between commensal microflora and GALT

The gut is normally colonised by both pathogenic and beneficial micro-organisms with each eliciting different immune responses. A layer of thick intestinal mucous and the secretion of strong antimicrobial agents limits direct interaction of enteral microflora with cells of the intestinal mucosa (Kelly *et al*., 2005). Pathogenic bacteria may penetrate these elements and interact with cellular elements of the mucosa and induce an inflammatory response (Wilson 2005). Commensal microflora form a protective layer against pathogen, (the 'mucosal barrier effect') (Thompson *et al*., 2005) and interact with the mucosa to a limited extent via lympho-epithelial and bacterial cross-talk which induces and maintains immune tolerance (Isolauri *et al*., 2001).

The precise molecular mechanisms underlying bacterial signaling and tolerance are not fully understood. Immune tolerance of commensal microflora permits colonisation of the intestine. Thus some non-pathogenic microflora may develop a symbiotic relationship with the host and enhance resistance to colonisation by pathogens (Perdue 1999). Recently, active chemical dialogue between commensal microflora and mucosal immune cells (via ***toll-like receptors (TLRs)*** and ***nucleotide-binding oligomerisation domain proteins (NODs***) (which is thought to underpin this symbiotic relationship) has been described (Kelly *et al*., 2005).

3.1.2 Immune responses to commensal bacteria

Commensal bacteria initiate and regulate the level of intestinal inflammation and tolerance (Haller 2006). Some induce immune tolerance by triggering an increase in lymphocyte cells associated with Peyer's patches and subsequent synthesis of immunoglobulin A (IgA) (Moreau *et al*., 2000). However, in the absence of normal enteral microflora, appropriate populations of B and T-lymphocytes do not locate in

the lamina propria and IgAs are not secreted (Mowat 2003b). Failure to establish a bias towards tolerance in GALT may lead to persistent inflammation (Haller 2006). Other structural and functional modifications occur in the gut wall in the absence of diverse populations of microflora including decreases in epithelial turnover, decreased motility, reduced smooth muscle function, decreased vascularisation and diminished development of GALT (Mowat 2003b).

Some species within the 'normal population' of enteral microflora initiate adaptive immunity, via their influence on ***antigen-presenting cells (APCs)*** in the submucosa. Reis e Sousa *et al*., 1999 identified typical strains of commensal microflora (i.e. gram-positive *Lactobacillus plantarum* and *Bifidobacterium adolescentis* or gram-negative *Escherichia coli* and *Veillonella parvula*), which differentially stimulate APCs. Other enteral commensal bacteria programme IECs to down-regulate epithelial pro-inflammatory responses by interfering with nuclear factor-kappa B (NF-κB) signaling and genetic readout (Neish *et al*., 2000).

3.1.3 Bacterial Motifs

Innate immune mechanisms rely on recognition of common molecular motifs of micro-organisms, using ***pattern-recognition receptors (PRRs***) expressed on surfaces of effector cells. These ***pathogen-associated molecular patterns (PAMPs)*** include peptidoglycans (PGN), lipopolysaccharides (LPS), lipoproteins, lipoteichoic acids, mannans and bacterial DNA. Most bacteria contain teichoic acids, but LPS are commonly associated with gram-negative bacteria. Since different classes of pathogen express distinct PAMPs, motif recognition identifies potentially threatening micro-organisms and provides signals that prime an innate response (Cobrin *et al*., 2005; Didierlaurent *et al*., 2002).

It is not known why commensal bacteria do not trigger pro-inflammatory responses in a similar manner to pathogens, it may be that PAMPs are not so evident in commensal bacteria, or are more diluted in the intestinal lumen (Mowat 2003b). Alternatively, IECs may be less responsive and not produce the same density of PRRs or active transductional complexes when not inflamed (Neish *et al*., 2000). IEC unresponsiveness to LPS could be caused by the lack of epithelial membrane-linked CD_{14} and other molecules required for LPS recognition, or by low levels of toll-like receptor-4 (TLR-4) (Neish *et al*., 2000).

3.1.4 The role of mucins and defensins in epithelial barrier function and gut immunity

The surface of the GI tract is covered by mucous secretions which protect underlying cells from digestive enzymes, acidity, abrasion and pathogenic bacteria as well as providing a basis for non-pathogenic adhesion (des Rieux *et al*., 2006; Suskovic *et al*., 2001). Bacterial cell wall mannans (fucosylated oligosaccharides) may directly interact with mucins, however their role in immune function is described elsewhere as it has not been directly linked to pro-inflammatory changes in the immune system (Morrow *et al*., 2005; Newburg *et al*., 2005; Nanthakumar *et al*., 2002).

The mucous layer consists of ***mucins, lysozyme*** and ***defensins*****,** secreted by IECs, which contribute substantially to host protection and are, in many cases, responsive to alterations in intestinal microflora (IM) (Moal *et al*., 2006).

The range of glycoconjugates (mucins) present in the intestinal lumen or on the surface of the mucosa, are collectively called the '***carbohydrate repertoire'*** and are recognised by specific structures such as lectin-like bacterial adhesins. This carbohydrate repertoire is genetically directed via the presence or absence of specific enzymes (Lenoir-Wijnkoop *et al*., 2003). Alteration in mucous production affects entry of luminal bacteria into the epithelium whilst changes in the composition of mucous, or changes in bacterial properties that increase the ability of bacteria to adhere, may be the underlying cause of inflammatory bowel disease, although this is not yet fully understood (Cobrin *et al*., 2005). (Bacterial adhesion will be discussed in section 4.1.3).

3.1.5 The structure and function of the intestinal epithelium

The intestinal epithelium (IE) consists of IECs (i.e. ***absorptive enterocytes, dendritic cells (DCs), M-cells, goblet cells,*** and ***paneth cells***). The lamina propria lies directly beneath the IE and is richly populated with macrophages, DCs, B-cells and T-cells. Both macrophages and DCs participate in antigen presentation to B and T-cells ($CD\text{-}_4$ and $CD\text{-}_8$) that form the basis of tolerance and adaptive immunity (Abreu *et al*., 2005; Wijnkoop *et al*., 2003). The importance of adaptive immunity is highlighted by the size of the lymphocyte population present in the gut, estimated to be about 70% of total lymphocyte numbers in the human body (Feleszko *et al*., 2006).

While the primary function of the IE is the absorption of nutrients, it is an effective barrier between the intestinal lumen and underlying lamina propria immune cells. Immune components maintain a state of 'controlled tolerance 'in the absence of pathogen, however pathogenic bacteria trigger the recruitment of acute inflammatory cells to both the IE and lamina propria (Vora *et al*., 2004) . The IE participates in innate immune responses to pathogen through the expression of TLRs that recognise PAMPs associated with micro-organisms and are pathogen-specific.

This interaction with PAMPs activates the ***nuclear-factor kappa-B (***NF-κB) pathway and the secretion of inflammatory cytokines (Vora *et al*., 2004). This TLR signaling may serve a protective function in the IE by inducing β-defensin-2 and limiting pathogenic infection or preventing commensal bacteria from breaching the epithelial barrier (Vora *et al*., 2004).

3.1.6 Antigen uptake and transport by the intestinal epithelium

Antigens are transported from the gut lumen to underlying cells of the mucosa via enterocytes (transcellular transport), via M-cells or between epithelial cells to the DCs (i.e. paracellular route) (Snoeck *et al*., 2005). The uptake of antigen across the IE is controlled by the filtering action of the mucous layer (Snoeck *et al*., 2005) and by the brush border of enterocytes (i.e. the apical plasma membrane). The tips of the rigid, tightly packed microvilli on the apical surface of enterocytes bear glycoproteins which form the glycocalyx, a specialised structure preventing direct interaction of pathogens with enterocytes (Shanahan 2000). The mechanisms and pathways by which pathogen or their secretions gain access to cells of the IE, ultimately determines the outcome of a response (Vora *et al*., 2004; Shanahan 2000).

When viable pathogenic organisms are detected in the sub-mucosa of the IE, enterocytes alert the immune system of a mucosal barrier breach, triggering infiltration of the mucosa with ***neutrophils*****,** and the subsequent initiation of an adaptive immune response (Mowat 2003).

3.1.7 The role of enterocytes in immune responses

3.1.7.1 Antigen Presentation

Discussion continues about the role of enterocytes as ***antigen presenting cells*** (APCs). On occasion, enterocytes have been shown to act as APCs by internalising and processing antigens (Snoeck *et al*., 2005; Hershberg *et al*., 1998; Strobel *et al*., 1998). Enterocytes are classified as non-professional APCs, but may have properties characteristic of professional APCs. Non-professional APCs would not normally express both MHC-II and I complexes required for T-cell proliferation in response to antigen, however MHC-II complexes have been found on enteroctyes (Snoeck *et al*., 2005; Hershberg *et al*., 1998; Strobel *et al*., 1998). Hence enterocytes may process and present suitable antigens to primed $CD4^{+}$Th-cells. However, it is acknowledged that differences exist in the class-II molecules expressed on the enterocyte surface and those expressed on professional APCs (Snoeck *et al*., 2005).

Shanahan (2000) suggests that MHC-II molecules are expressed on apical surfaces of enterocytes rather than the baso-lateral membrane, and thus may not function in normal lympho-epithelial interactions. The expression of MHC-II molecules have been reported on the baso-lateral membrane during inflammation (Snoeck *et al*., 2005). Snoeck *et al*., (2005) suggest in the absence of inflammation, enterocytes do not express co-stimulatory molecules (i.e. CD-28), so antigen presentation results in '***anergy'*** and ***'tolerance'***. Furthermore, they observed that antigens not normally eliciting a response, or promoting a tolerogenic response when processed apically, become immunogenic after gaining access to the baso-lateral surface of enterocytes through leaky ***tight junctions*** (TJs) (Snoeck *et al*., 2005). (Tight junction configuration will be discussed in a section 3.1.9).

3.1.7.2 *Immune Activation*

Enterocytes are considered key regulators of the pro-inflammatory immunologic state (Snoeck *et al*., 2005; Van Niel *et al*., 2001; Shanahan 2000; Hershberg *et al*., 1998). Shanahan (2000) proposes that the luminally expressed MHC-II molecules act as sensory receptors to partially digested bacterial peptides and are utilised during up-regulation of expression on enterocytes during inflammatory disorders, such as CD. Under inflammatory conditions, enterocytes are known to secrete ***exosomes*** that express MHC-II-peptide complexes, via the baso-lateral membrane

(Van Niel *et al*., 2001). Hence these exosomes transmit information to non-adjacent T-cells. This would partly explain how epithelial cells can present peptides to CD_4T-cells separated from enterocytes by a basement membrane, which prevents direct interaction.

Hershberg *et al*., (1998) suggests the presence of IFN-γ causes enterocytes to process and present antigen to primed T-cells. Others remain skeptical. Mayer (1998) questions whether specific T-cell interactions required for primary MHC-II-mediated immune responses can be generated by enterocytes. Snoeck *et al*., (2005) proposes that incomplete T-cell activation by enterocytes is involved in the down-regulation of T-cells, pointing out that enterocytes targeted by MHC-class-I restricted virus-specific cytotoxic T-cells fail to prime an antiviral response. While enterocytes may induce cytotoxic (CD_8) T-cell responses by expressing class-1b molecules, the functional role of class 1b molecules, ***in vivo***, (under physiological or pathological conditions in the gut) remains unknown. Based on research presented, it is likely that enterocytes act as APCs capable of processing and presenting antigen under inflammatory conditions, but further investigation is needed to confirm the role of enterocytes as APCs under normal physiological conditions**.**

3.1.7.3 The mode of action of M-cells

Intestinal M-cells reside in the ***follicle-associated epithelium*** (FAE) on the surface of Peyer's patches in organised GALT and singly throughout the mucosa. M-cells usually develop from undifferentiated dome-associated crypt cells, but under appropriate antigenic stimulation, M-cells also originate from enterocytes (Clark *et al*., 2003). The numbers of M-cells present is often dependent on the microbial content of the lumen. For example, under experimental conditions, the presence of either *Salmonella enterica* (Gebert *et al*., 1999) or *Streptococcus pneumoniae* (Borghesi *et al*., 1999) caused an increase in M-cell numbers.

M-cells bind invasive pathogens to transport them across the IE but do not process antigen themselves, or express MHC-II molecules. Instead M-cells transfer intact antigen directly to professional APCs in the epithelium or underlying region by assimilating material transported by M-cells which is then taken up by macrophages, B-cells and DCs that subsequently present them to T-cells

(Didierlaurent *et al*., 2002; Shanahan 2000). M-cells (like enterocytes) internalise and trancytose a wide variety of substances and may represent a portal in the IE (Clark *et al*., 2003), as they lack the apical membrane-associated mucin found on enterocytes. M-cells have a micro-folded apical membrane which may make them vulnerable to attack by invasive pathogens. The mechanisms whereby some pathogenic micro-organisms specifically target M-cells are only just being elucidated (Krahenbuhl *et al*., 2000). Pathogen access to M-cells may be facilitated by a decreased abundance of ***secretory immunoglobulin A*** (sIgA) at the epithelial surface (Clark *et al*., 2003). Access of pathogens to the M-cell apical membrane may be facilitated by an irregular brush border and thinner glycocalyceal layer than those found on enterocytes (Clark *et al*., 2003). This confirms the importance of the glycocalyx in preventing direct interaction of pathogens with the apical membrane glycolipids of IECs (Krahenbuhl *et al*., 2000; Mantis *et al*., 2000; Neutra *et al*., 1999).

Krahenbuhl *et al*., (2000) noted that pathogenic micro-organisms target specific receptor proteins (such as integrins) located in or attached to either the M-cell apical membrane, or the closely associated glycocalyx. Whilst these receptors appear identical to those exhibited by enterocytes, the marked M-cell trophism exhibited by some micro-organisms suggests otherwise. The fact that a number of cell-surface markers distinguish M-cells from enterocytes supports this (Clark *et al*., 2003). The role of M-cell surface markers in microbial infection has not yet been fully elucidated, with the exception of *Yersinia* interaction with M-cell-surface β1-proteins (integrins) (Clark *et al*., 2003). Further studies to investigate the role of M-cell surface markers in M-cell/microbial interactions, and to identify the receptors responsible for M-cell targeting of specific microbial species are needed.

3.1.8 The role of dendritic cells (DC) in intestinal immunity

Recent advances show that DC-pathogen interactions are complex and diverse, and that DC function may be modulated by microbial stimuli. Mowat *et al*., (2003) describe DCs as 'gatekeepers' of the intestinal immune system, capable of inducing tolerance under appropriate physiological conditions, but reactive to pro-inflammatory stimuli, allowing T-cell primed inflammatory responses when necessary. They are potent APCs that activate resting T-cells and can regulate the

pattern of cytokine response in T-cells. DCs also have the ability to down-regulate T-cell activation and induce tolerance (Montoya *et al*., 2002).

They are found in organised lymphoid tissue associated with the IE (Peyer's patches) and interspersed throughout the epithelium and lamina propria. DCs can migrate through the mature IE, allowing continuous detection of incoming antigen and prompt activation of T-cells in secondary lymphoid organs (Sallusto *et al*., 1999).

3.1.8.1 Direct sampling

DCs sense changes in the luminal environment via ***dendrites*** insinuated through tight junctions between adjacent enterocytes thus projecting into the intestinal lumen (Roux *et al*., 2003; Nagler- Anderson *et al*., 2001). ***In vitro,*** immature DCs with genes encoding adhesion and tight junction proteins (E-cadherin, occludin, and ZO-1) were up-regulated and found to migrate between IECs, opening tight junctions and capturing antigen (Rescigno *et al*., 2001). Rescigno *et al*., (2001) showed that the presence of *S. typhimurium* and non-pathogenic *E. coli* (potentially pathogenic bacteria) stimulated the recruitment of DCs into the IE, with subsequent pathogen sampling by these cells. This is consistent with earlier work (Vazquez-Torres *et al*., 1999) which demonstrated that orally administered *S. typhimurium* appeared promptly in DCs.

The idea that luminal contents are directly sampled by DCs remains controversial, despite evidence that DCs in underlying lamina propria effectively open tight junctions between adjacent IECs and sample antigen directly (Cobrin *et al*., 2005). DCs also appear to express tight junction proteins that preserve the integrity of the intestinal barrier and avoid exposure to potentially harmful agents or the production of pro-inflammatory cytokines (TNF-α and IL- β) and preferential differentiation of T-cells to T-helper-1 type effector cells (Th_1) (Cobrin *et al*., 2005).

3.1.8.2 Indirect sampling

DC sampling also occurs via M-cells in the FAE of Peyer's patches, and by similar lymphoid-associated tissue in the large intestine (Stagg *et al*., 2004). Appropriately stimulated FAE secretes CCL-20, the chemokine responsible for chemotactic migration of DCs into Peyer's patches. CCL-20 binds the chemokine receptor CCR-6, expressed by immature DCs, naïve B-cells, and memory T-cells (Iwasaki *et*

al., 2000; Tanaka *et al*., 1999). DCs were absent from the sub-epithelial region of Peyer's patches in CCR-6 deficient mice, impairing immune responses to pathogens, although the size of Peyer's patches and the distribution of B and T-cells remained normal (Varona *et al*., 2001). Cook *et al*., (2000) found the expression of CCL-20 is up-regulated in epithelial villi of the small intestine in response to challenge with flagellin. They concluded that this response is probably instrumental in recruiting DCs into the IE. However, other pathways of antigen sampling by DCs must exist, as neither M-cells nor Peyer's patches are essential for the generation of DC-mediated inflammatory responses (Stagg *et al*., 2004).

3.1.8.3 Ontogenesis of DC sampling

The population characteristics of newly mature DCs determine whether undifferentiated Th-cells develop into Th_1, Th_2 cells, or cells of mixed phenotype. This is achieved by selective expression of polarising molecules such as IL-12, IL-18, IL-23, IL-27, IFN-α and I-CAM-1(Smits *et al*., 2004; Lenoir-Wijnkoop *et al*., 2003). For example, IL-23 acts primarily on effector T-cells, prolonging and sustaining their IFN-γ production while IL-27 influences naive Th-cells, and is crucial in the initial and early synthesis of IFN-γ, either alone or in synergy with IL-12 (Smits *et al*., 2004).

The type of pathogen interacting with immature DCs determines subsequent Th-cell polarising capacity (Morrow *et al*., 2005). DCs promoting Th_1 responses develop after exposure of immature DCs to pathogenic bacteria or dietary antigen. Non-pathogenic bacteria also contribute to the class of gut-associated and systemic effector Th-cell responses by influencing the polarising capacity of Th-cells (Didierlaurant *et al*., 2002). For example, Qi *et al*., (2003) showed that commensal microflora affect DCs by influencing their expression levels of Th-cell polarising signals via TLRs. They examined IL-10 and IL-12 production, and T-cell polarising potential of DCs after stimulation by three microbial TLR activators- (LPS, PGN and zymosan) and found dramatically different profiles of IL-10 and IL-12 production in DCs for each stimuli.

This supports previous research by Pulendran *et al*., (2001) which found that microbe-specific information sensed by DCs, through different TLRs, is linked to differential Th-priming through the release of DC-derived cytokines.

The ability of DCs to launch qualitatively different immune responses depends on the DC subset involved, the nature of the PRRs (TLRs and NODs) and the nature of the antigens. Medzhitov (2001) identified distinct subsets of DCs that present different antigens finding specific DCs present pathogen-associated antigen whereas others continuously present self-antigen in the steady state. Medzhitov (2001) also suggests that some DC populations might initiate different arms of adaptive immunity, with certain DCs specialising in stimulating cytotoxic T-cell CD^{+}_{8} responses, whereas others stimulate CD_{4}^{+}T-cells. This is consistent with research by Villadangos *et al*., (2005), Mowat *et al*., (2003), Nussenzweig (1997) and Shortman *et al*., (1997). There is also accumulating evidence that DCs contribute to the differentiation of suppressive T-cells (T-regs) (Colonna *et al*., 2006).

3.1.8.4 Dendritic cell response to intestinal epithelial cells (IEC)

DCs are conditioned by IECs to mediate Th_2 or Th_3 responses, but not to mediate Th_1 responses. Rescigno *et al*., (2001) showed this is achieved by continuous cytokine expression which renders them incapable of driving Th_1 mediated responses. However, this interaction is altered by infection, with activated DCs then extending their dendrites throughout the small intestine, with activated lamina DCs migrating to regional lymph nodes to induce Th_1 mediated inflammation (Medzhitov 2001). This concurs with research (Reinecker *et al*., 1995; Germain *et al*., 1993) demonstrating that in healthy subjects, dendrites only extend into the lumen of the terminal ileum, but in infected subjects dendrites extend throughout the small intestine. Dendritic extensions in infected subjects were most abundant at the top of villi indicating their involvement in luminal antigen sampling. DCs have been shown to enter the dome-region of Peyer's patches during infection, indicating that they may be important for the presentation of bacterial antigen to T-cells in this site. Germain *et al*., (1993) demonstrated that in viral infection, DCs in the dome-region take up viral antigens from dying IEC and induce Th_1 activation (Kelsall *et al*., 2002).

3.1.8.5 Interferon activation of DCs during infection

Pro-inflammatory cytokines known to activate DCs are type-I interferons (type-I IFNs). These include proteins encoded by closely related and linked genes; the major species are IFN-α and IFN-β. All type-I IFNs exert their DC activity by binding to a common cell-surface receptor (Lelouard *et al*., 2001). Montoya *et al* ., (2002)

determined experimentally that type-I IFNs are expressed at low levels in normal or germ-free mice, but are induced to high levels by viral or bacterial infection. Type-I IFNs were also elicited by chemical signatures of infectious agents, such as LPS, bacterial DNA, and double-stranded RNA.

Further evidence that type-I IFNs activate DCs comes from studies on DCs generated *in vitro* from peripheral blood or bone marrow precursor cells (Lelouard *et al* ., 2001). Generally in these models, the addition of type-I IFNs enhanced the expression of co-stimulatory molecules by DCs and their ability to stimulate T-cells. Other researchers report that type-I IFNs have the opposite effect (McRae *et al*., 2000). The ability of type-I IFNs to activate DCs is interesting given that these cells also produce type-I IFNs in response to infection. Type-I IFNs are secreted by DC precursors, in response to pathogen-associated signals. Hence DC-secreted type-I IFNs can act in an autocrine manner, promoting survival of DC precursors and simulating expression of type-I IFN-induced genes in activated DCs (Montoya *et al* ., 2002). Thus cross-talk between commensal bacteria, pathogenic bacteria, DCs and IECs is critical for DC function in both health and infection (Colonna *et al*., 2006).

3.1.9 Tight Junction Configuration

All IECs are linked by TJs that seal the apical poles of enterocytes. These highly regulated regions maintain the barrier function of the IE and prevent or reduce the lateral diffusion of glycolipids and proteins between apical and baso-lateral domains of plasma membranes (Snoeck *et al*., 2005). The interplay of TJs is influenced by events occurring in the IE, lamina propria and lumen and has the potential to open and transfer antigenic material (Raimondi *et al*., 2004; Perdue 1999). Small molecules are readily transported via the paracellular route, whilst TJs restrict the transport of larger molecules (Snoeck *et al*., 2005). The strict control of TJ permeability generally prevents entry of antigenic molecules into underlying tissue (Van Niel *et al*., 2002).

The baso-lateral surface of an IEC comprises;

(a) a lateral sub-domain linked to adjacent cells by adhesion molecules, adherens junctions, desmosomes, gap junctions and interdigitations

(b) a basal sub-domain which interacts with the extracellular matrix and basement membrane Snoeck *et al*., 2005).

While the structural organisation of all epithelial-cell junction complexes appear to be similar, (i.e they contain TJs, adherens junctions and desmosomes) TJs between M-cells differ structurally from those of other cell types having greater depth and different morphology (Clark *et al*., 2003). Thus the adherens junctions of M-cells in Peyer's patches can be differentiated from those of enterocytes by their enhanced expression of β-catenin, α-actinin, polymerised actin and in some cases, cadherin (Clark *et al*., 2003). M-cells may require a more rigid cytoskeleton and more stable intercellular adhesion to cope with increased interactions with and transcytosis of enteral bacteria.

3.1.10 Changes in tight junction configuration

The presence of either interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-4 (IL-4) or interleukin-13 (IL-13) increases the permeability of TJs (Clark *et al*., 2003). Further, any alteration in TJ configuration causes intercellular leakage, and increases transit of antigen to the intestinal immune system (Heyman 2001). Thus an increase in intestinal permeability is associated with increased inflammation, epithelial dysfunction and alteration in antigen processing (Heyman 2001).

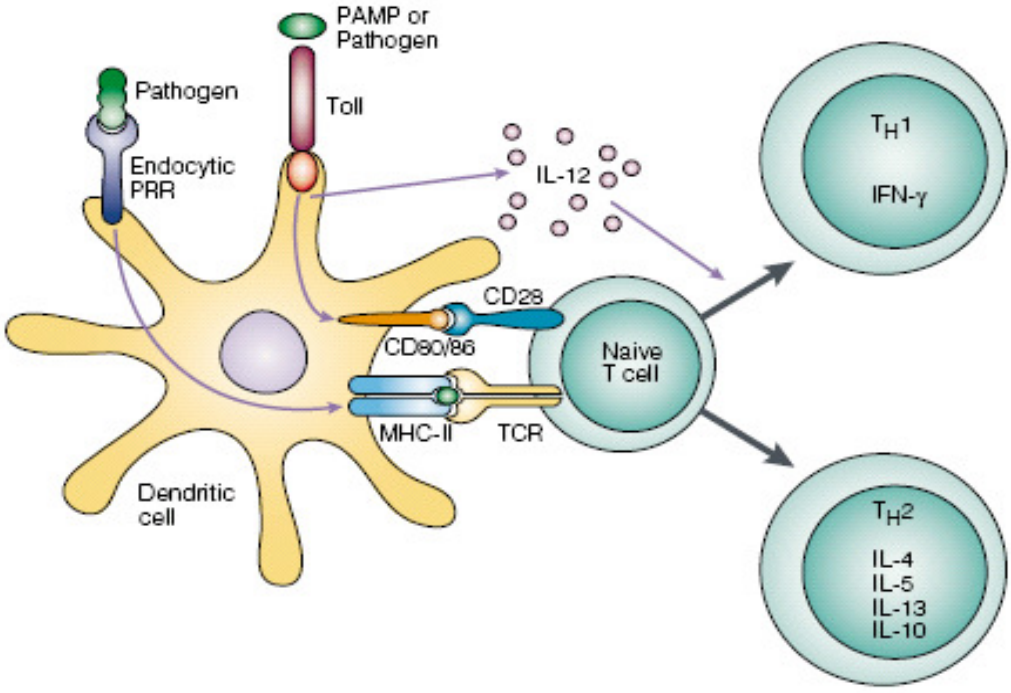
The quantity of antigen absorbed may influence the outcome of antigen presentation to underlying T-cells, directing the immune response either toward tolerance or inflammation. In the context of an inflammatory environment, local DCs switch from a tolerogenic state to an immunogenic state. In Crohn's disease, for example, abnormal TJ configuration and enhanced intestinal permeability promote pro-inflammatory change (Heyman 2001). Mucosal cytokine profiles in CD are similarly associated with the up-regulation of pro-inflammatory cytokines (mainly IFN-γ), whilst the concurrent induction of nitric-oxide synthase reduces barrier integrity (Snoeck *et al*., 2005).

Conversely in the context of pro-tolerant conditions, a number of cytokines act to decrease permeability. TGF-β enhances the integrity of TJs thereby improving epithelial barrier function, while IL-10 down-regulates epithelial permeability by reducing production of pro-inflammatory cytokines, or possibly by acting directly on the epithelium. Intestinal epithelial permeability may also be improved by growth factors such as insulin-like growth factor (Snoeck *et al*., 2005).

3.1.11 The Morphology and Function of TLRs

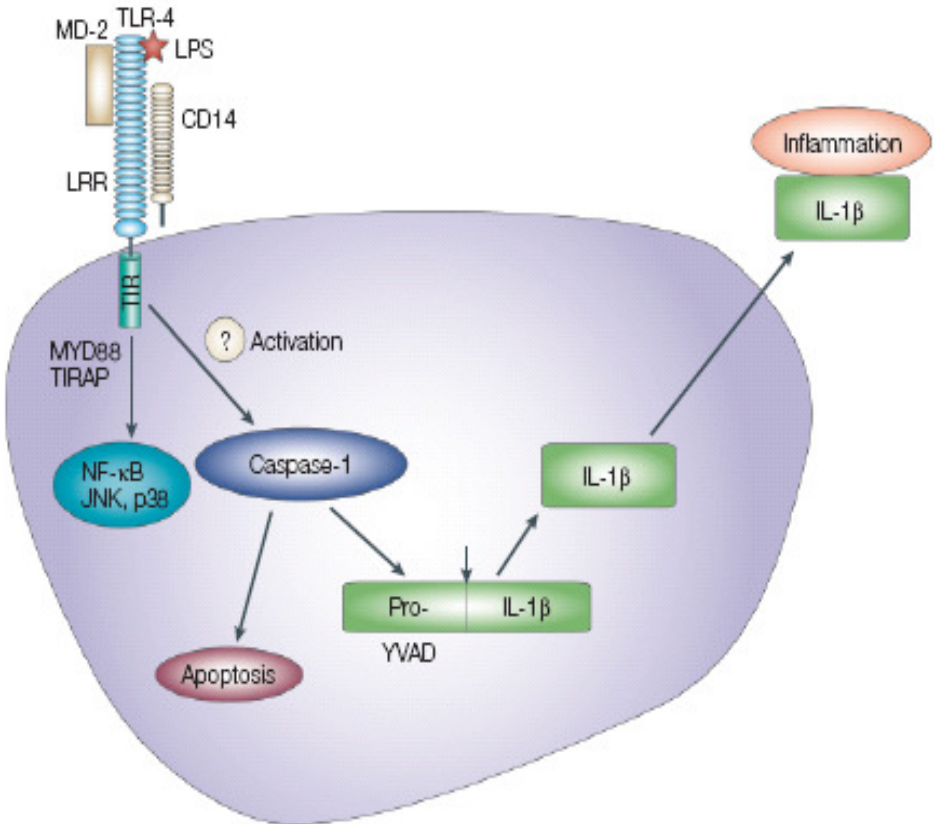
TLRs are type-1-transmembrane proteins with an extracellular leucine-rich domain that participates in ligand recognition, and an intracellular tail containing a conserved region (TIR domain). TLR signaling is integral to innate immune defense. TLRs are expressed by macrophages, DCs, neutrophils and IECs (Abreu *et al*., 2005). TLRs responding to PAMPs initiate antimicrobial peptide expression, barrier fortification and proliferation of epithelial cells (Abreu *et al*., 2005). TLRs also link with adaptive immunity and play a significant role in responses to non-infectious agents (Feleszko *et al*., 2006; Goldstein *et al*., 2004 (see Figure 3.1 and 3.2).

Figure 3.1: The role of TLRs in adaptive immunity (Tschopp *et al*., 2003).



The recognition of PAMPs by TLRs expressed on APCs up-regulates cell-surface expression of co-stimulatory (CD80 and 86) and MHC-II molecules. TLRs also induce expression of IL-12, or chemokines and their receptors, and trigger other events associated with DC maturation. Induction of CD80/86 on APCs by TLRs causes the activation of T-cells specific for pathogen that trigger TLR signaling. IL-12 induced by TLRs also contributes to the differentiation of activated T-cells into Th1-cells. It is unknown whether TLRs have a role in inducing Th 2 responses.

Figure 3.2: The recognition of PAMPs by TLRs (Tschopp *et al.*, 2003).



Recognition of LPS by TLR-4 is aided by CD14 and MD-2. TLR-4 contains leucine rich repeats (LRRs) and toll/interleukin-1 receptor (TIR) domain, and transfers signals through MYD88 and TIR adaptor protein (TIRAP) which causes activation of a terminal kinase (JNK) and NF-κB signaling pathways. Caspase-1 is activated by an unknown mechanism, which cleaves to an inactive IL-1 precursor thereby liberating the active form of IL-1β. Processing of IL-1β is associated with its release into the extracellular space, where it triggers inflammation.

Activation of TLRs causes recruitment of ***MyD88*** and its associated serine kinase (IRAK), to propagate a pro-inflammatory signal (Feleszko *et al.*, 2006; Scott *et al.*, 2005; Goldstein *et al.*, 2004). Differing subsets of DCs (or their precursors) express different subsets of TLRs allowing them to induce specific patterns of immune response to different pathogen. This is called ligand specificity and is unchangeable. Specific TLRs respond to LPS, lipopeptides, lipoarabinomannan, flagellin from bacterial flagella, double stranded RNA of viruses, or the unmethylated CpG motifs of bacterial and viral DNA, and certain other RNA and DNA (Goldstein *et al.*, 2004).

Hence;

(a) TLR-4 recognises LPS

(b) TLR-2 recognises fungal, gram-positive, and mycobacterial components

(c) TLR-6, in combination with TLR-2, recognises zymosan and PGN.

(d) TLR-9 recognises bacterially-derived CpG DNA motifs

(e) TLR-5 recognises flagellin, the subunit of *Salmonella* flagella identified as the virulence factor triggering a pro-inflammatory IEC response (Tschopp *et al*., 2003).

3.1.12 The role of nucelotide oligomerisation domain (NOD) proteins

Nucleotide-binding site and leucine-rich repeat proteins (NBS-LRR) including ***nucleotide oligomerisation domain*** (***NOD***)$_1$/***caspase-activating and recruitment domain*** (***CARD***) 4 and ***NOD$_2$*** constitute distinct sets of PRRs involved in innate immune responses against pathogen (Abreu *et al*., 2005;Tschopp *et al*., 2003). The discovery of NOD proteins has increased our understanding of the complex ways TLR signaling can be muted in the presence of PAMPs from micro-organisms (Cobrin *et al*., 2005; Didierlaurent *et al*., 2002).

Twenty NOD proteins that act as cytoplasmic sensors of bacterial components and allow the regulation of inflammatory processes and apoptosis have been identified (Abreu *et al*., 2005).

NOD proteins are characterised by 3 structural domains;

(a) a C terminal leucine-rich repeat (LRR) domain (that senses bacterial motifs)

(b) an intermediary nucleotide-bonding site (NBS)

(c) a caspase-activating and recruitment domain (CARD), or baculovirus inhibitor of apoptosis protein repeat (BIR) domain (Abreu *et al*., 2005).

NOD_1 and NOD_2 proteins contribute to regulation of pro-inflammatory pathways via NF-κB-induced bacterial ligands (Chamaillard *et al*., 2003). The PRRs containing LRRs probably provide the link between recognition of PAMPs and signal transduction that activates NF-κB (Haller 2006). As both TLR and NOD proteins target NF-κB transcription factor through protein-protein interaction, this proposal appears credible. This same essential NF-κB transcription factor regulates the expression of antimicrobial agents, cytokines and chemokines and will be discussed separately.

The NOD_1 specific ligand is only present in bacterial PGN of gram-negative organisms (Cobrin *et al*., 2005) whereas NOD_2 is thought to participate in both innate and adaptive immunity (Kobayashi *et al*., 2005; Pauleau *et al*., 2003). NOD_2 is critical in protecting against intestinal bacterial infections, via macrophages or paneth cells (Kobayashi *et al*., 2005). In the presence of ***muramyl dipeptide*** (MDP), NOD_2 induces NF-κB activation and the production of pro-inflammatory mediators. NOD_2 expression mainly occurs in DCs and IECs, with greater expression in crypts than in villi (Yuan *et al*., 2004). NOD_2 is also expressed in monocytes, macrophages, B and T-cells, and paneth cells.

Cobrin *et al*., (2005) and Gutierrez *et al*., (2002) note that, IFN-γ and TNF-α, (pro-inflammatory mediators secreted by Th_1-cells) up-regulate expression of NOD_2 by IECs. NOD_2 variants are categorised according to their ability to activate NF-κB in response to PGN, suggesting that without adequate NF-κB activation, translocated bacteria are not effectively eliminated, resulting in APC activation and induction of T-cell proliferation by secretion of Th 1-stimulating cytokines (IL-12, IL-23 or IL-18) causing inflammation (Chamaillard *et al*., 2003).

An association between mutations in NOD_2, (which render the molecule insensitive to MDP and unable to induce NF-κB activation when stimulated) and susceptibility to intestinal inflammation, leading to inflammatory bowel disease has been described (Chamaillard *et al*., 2003).

Another distinct group of PRRs recently isolated are ***NALPs***, which are emerging as significant in immune interactions relating to cytokine expression and inflammation (Petrilli *et al*., 2005; Tschopp *et al*., 2003), however they will not be discussed here.

3.1.13 The role of dedicated immune cells (B and T-cells) in gut immunity

Adaptive immunity is influenced by the action of APCs, B-cells, the production of T-cell subsets and cytokines induced by these cells. Immature B and T-cells are distributed in distinct zones in the intestinal mucosa.

3.1.13.1 B-cells

B-cells participate in humoral immune responses, whereas T-cells are involved in both cellular and humoral responses (Janeway *et al*., 2005). B-cells primarily release antibodies into interstitial and vascular tissues, forming antibody-antigen complexes that trigger granulocytes to liberate inflammatory mediators that are specific to and destroy particular antigen (Janeway *et al*., 2005). Five isotypes of antibody exist, immunoglobulin IgM, IgA, IgG, IgE and IgD, each having a specific role in immunity. The function of each type of immunoglobulin is explained in the glossary in Appendix A.

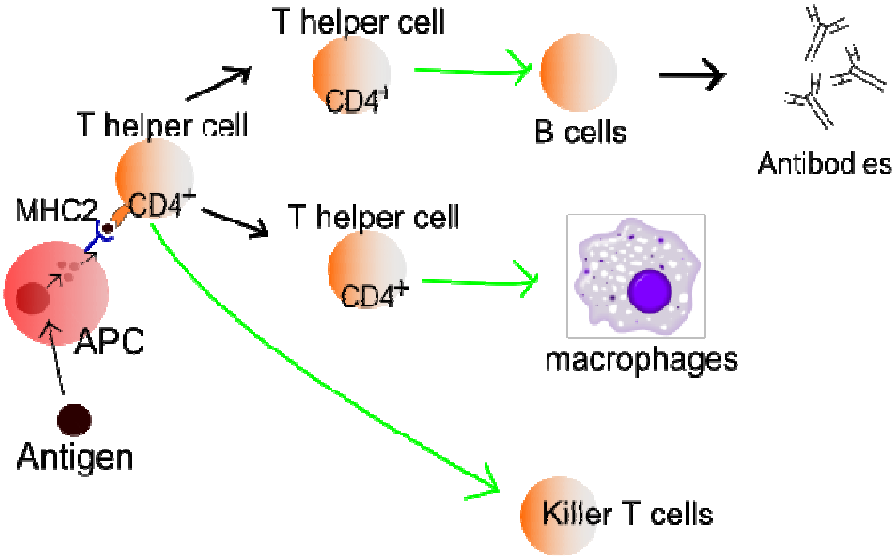
3.1.13.1.1 Activation of B-cells

B-cells require the help of T-cells to trigger the production of antibodies. B-cells not previously exposed to antigen, (known as naive B-cells) are activated in a T-cell dependent or independent manner (Metcalfe *et al*., 2003).

3.1.13.1.2 T-cell dependent activation of B-cells

On ingestion of a pathogen, a macrophage attaches part of the pathogen's proteins to a class-II MHC (major histocompatibility class) protein. This complex moves outside of the cell membrane, where the epitope on the antigen is recognised by a T-cell. If the B-cell and T-cell structures match, the T-cell will activate the B-cell to produce large quantities of antibodies against the antigen, using its B-cell receptor (BCR). Since, many clones of B-memory/naive cells can recognise the same antigen, the response is ***polyclonal*** (Parham 2005). Figure 3.3 shows APCs presenting antigen on their class-II MHC molecules (MHC2) and helper T-cells recognition of these by expression of a CD4 co-receptor (CD4+). The activation of a resting helper T-cell causes the release of cytokines and other stimulatory signals that stimulate the activity of macrophages, killer T-cells and B-cells, the latter producing antibodies (Rang 2003).

Figure 3.3: Function of T helper cells



Isotype switching to IgG, IgA or IgE and memory cell generation occur in response to T-cell dependent antigens. Once switching has occurred, that particular B-cell can not synthesise the earlier isotypes (i.e. IgM or IgD) (Mowat *et al.*, 2003). B-cells undergo immunoglobulin (Ig) class switching in Peyer's patches, under the influence of transforming growth factor (TGF)-β, IL-10 and cellular signals delivered by DCs and T-cells (Mowat *et al.*, 2003; Shanahan 2000). Isotype switching depends on the route of antigen presentation, the presence of Th-cells, mast cells, eosinophils and basophils, the cytokine milieu in the tissue and the genetic susceptibility of the individual (Metcalfe *et al.*, 2003).

3.1.13.1.3 Secretion of IgE mounted on mast cells

An IgE-mediated response is modulated by Th_2-CD_4^+ cells (as are all humoral responses). These Th_2-CD_4^+ responses are defined by the secretion of IL-4 by APCs, and probably IL-13 which have a functional relationship. The receptors for both these cytokines contain the same α-chain and transduce their signals via ***STAT6***. Local production of IgE mounted on mast cells in the gut induces an immediate-hypersensitivity reaction characterised by inflammation (Kaminogawa 1996).

3.1.13.1.4 Secretion of IgM and IgG

Non-IgE mediated reactions are triggered by inappropriate Th_1-responses that cause inflammation and damage. Differences exist between the actions of IgG and

IgA. Mucosal sIgA smothers epitopes without mounting a pro-inflammatory response so the mucosa remains intact. This is because IgA does not have an active ***FC portion*** which up-regulates a pro-inflammatory response. In contrast, IgG binds pathogen and protects against their action by complement activation, opsonisation and neutralisation of their toxins. IgG promotes a delayed-type sensitivity response that triggers inflammation causing damage in the intestinal mucosa due to the presence of active FC portions (Alpan *et al*., 2001).

3.1.13.2 T-cells

T-cell activity is vital to adaptive immune function in the gut. T-cell recruitment to the intestine, initiated through TLR signaling in DCs has been demonstrated on numerous occasions (Johansson-Lindbom *et al*., 2003; Papadakis *et al*., 2003; Stagg *et al*., 2002; Papadakis *et al*., 2001). TLR signaling activates DCs to produce distinct patterns of cytokines that direct immature T-cells into Th-cell subsets (Th_0, Th_1, Th_2 or T regs), through secretion of cytokines. The differential development of T-cell subsets determines the outcome of physiological and pathological immune responses. Recent research (Huse *et al*., 2006) identifies two distinct pathways used by T-cells for secretion of cytokines. It identifies that Th-cells release some cytokines through an ***immunological synapse*** toward APCs that impart specific communication (i.e. IL-2 and IFN-γ) whereas TNF and the chemokine CCL3, are released multi-directionally to establish chemokine gradients or promote inflammation.

A full description of each different T-cell subsets (i.e. γ/δ T-cells, Th_0 T-cells, Th_1/Th_2 T-cells (helper T-cells) can be found in the glossary in Appendix A).

(i) Th_1 T-cells

The polarisation of naive T-cells towards Th_1-type differentiation occurs through expression of the transcription factor ***T-bet*** (Feleszko *et al*., 2006). Type-1 IFNs also induce Th_1-cell production, which is partly mediated by inducing IL-12Rβ, the signaling component of the IL-12 receptor (IL-12R) (Feleszko *et al*., 2006; Foster *et al*., 2000). IL-12Rβ is necessary for Th_1-cell development and the expression of IL-12R, is in turn, induced by type-1IFN. Type-1 IFNs activate NK and cytotoxic T-cells and are induced by viral infection, or by exposure to double-stranded RNA. However, in the absence of viral infection, the origin of the type-1 IFN required for the induction of IL-12Rβ is not clear. It also appears other unidentified cytokines

may stimulate expression of IL-12Rβ. Alternatively DCs, may secrete type-1IFNs, as cells with similar morphology to DCs can produce IFN-α in response to viral infection. Further, both Feleszko *et al*., (2006) and Foster *et al*., (2000), have identified a specific subset of DCs capable of releasing large amounts of IFN-α when infected by viruses. Foster *et al*., (2000) suggest that the source of the IFN-α required for Th_1-cell development, may actually be the DC. They speculate that in the absence of viral infection, the interaction between T-cells and DCs may trigger the production of type-1 IFNs.

(ii) Th_2 T-cells

Th_2-cells co-ordinate humoral immunity. The signature Th_2 cytokines are IL-4, IL-5 and IL-10. The main role of Th_2-cells is to provide cytokines required for immunity to extracellular pathogens. IL-4 is essential for the activiation and proliferation of B-cells and is needed for immunoglobulin class switching to IgG and IgE (Davies *et al*., 1999). The interactions between cytokines from Th_1/Th_2 T-cells can be complicated. For example, the Th_2 cytokine IL-10 inhibits cytokine production of both Th_1 and Th_2 subsets. Whilst IL-10 suppresses the proliferation and cytokine production of all T-cells and the activity of macrophages, it continues to stimulate plasma cells, ensuring that antibody production continues. As such, IL-10 is not believed to truly promote a Th_2 response, but acts to prevent over-stimulation of helper T-cells, while still maximising the production of antibodies (Davies *et al*., 1999).

(iii) Th_1/Th_2 balance

While it is commonly accepted that uncontrolled Th_1 and Th_2 responses trigger chronic inflammation and allergies, it also appears the Th_1/Th_2 balance determines the onset and outcome of such disorders (Lenoir-Wijnkoop *et al*., 2003; Akira *et al*., 2001).

To avoid rejection, a foetus is maintained in a Th_2 context during pregnancy. 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 Th_1 production to develop a reciprocal relationship between Th_2 and Th_1 immunity. If a humoral predominance remains pro-allergy and pro-inflammatory tendencies exist. It is thought that gut microflora up-regulate cellular and down-regulate humoral responses. It has also been found that bacteria themselves can trigger (via TLR-4)

DC populations that are pro-tolerance rather than pro-inflammatory (Lenoir-Wijnkoop *et al*., 2003).

(iv) T_{reg} cells (Regulatory T-cells)

T_{reg} cells are crucial for the maintenance of immunological tolerance. Their major role is to terminate T-cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T-cells. Two major classes of CD4+ regulatory T-cells have been described, including the naturally occurring T_{reg} cells and the adaptive T_{reg} cells. Naturally occurring T_{reg} cells (also known as $CD4^+CD25^+$ T_{reg} cells) arise in the thymus, whereas the adaptive T_{reg} cells (also known as Tr_1 cells or Th_3 cells) originate during a normal immune response (Jiang *et al*., 2004). The major difference with 'suppressor' (or 'natural regulatory') T-cells is that they always suppress the immune system, while effector T-cells usually begin with immune-promoting cytokines and then switch to inhibitory cytokines later in their response. The latter is a feature of Th_3-cells, which transform into a suppressor subset after their initial activation and cytokine production (Jiang *et al*., 2004). Regulatory T-cells and Th_3-cells both produce TGF-β and IL-10, cytokines which are inhibitory to helper T-cells. TGF-β suppresses the activity of most of the immune system however there is evidence that TGF-β may not suppress activated Th_2-cells as effectively as it suppresses naive cells. Irrespective of this, TGF-β is not typically considered a Th_2 cytokine. Note; T-regs were formerly known as suppressor T-cells, however terminology such as 'regulatory' or 'suppression' become ambiguous after the discovery that helper $CD4^+$ T-cells also regulate (and suppress) their own responses outside of dedicated suppressor T-cells (Jiang *et al*., 2004). The characterisation of another novel T-helper subtype, T-helper-17 cells (Th17) has called into question the basic Th_1/Th_2 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 (Harrington *et al*., 2005). Further research is needed in this area. The function of Th_1 and Th_2 T-cells are summarised in Table 3.1.

Table 3.1: The functions of Th_1 and Th_2 T-cells (from Davies *et al.*, 1999)

	Type 1/ Th_1	**Type 2/ Th_2**
Main partner- cell type	Macrophage	B-cell
Cytokines produced	INF-γ and TNF-β. (IL-2 was classically associated with Th_1 cells, but this association may be misleading as IL-2 is produced by all helper T-cells early in their activation.)	IL-4, IL-5, IL-6, IL-10, IL-13
Immune stimulation promoted	Cellular immune system. Maximises the killing efficacy of the macrophages and in the proliferation of cytotoxic $CD8^+$ T-cells.	Humoral immune system. Stimulates B-cells into proliferation, to induce B-cell antibody class switiching, and to increase antibody production
Other functions	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 Th_1 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.	The Type 2 response promotes its own profile using two different cytokines. IL-4 acts on helper T cells to promote the production of Th_2 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 dendritic cells and 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).

3.1.14 The action and up-regulation of PPAR-γ and its role in gut immunity

The nuclear hormone receptor, peroxisome-proliferator-activated receptor gamma (PPAR-γ), is involved in cellular differentiation, apoptosis, lipid metabolism and anti-inflammatory responses and is expressed in high levels in the IE. Clark *et al.*, (2000) found that PPAR-γ is involved in the innate activity of macrophages while Gilroy *et al.*, (1999) demonstrated PPAR-γ expression in T-cells, finding two ligands

for PPAR-γ that mediate a direct inhibitory role on T-cell responses via the inhibition of IL-2 secretion. Szatmari *et al*., (2004) observed how PPAR-γ activity triggers the development of immature DCs to activate natural killer T-cells (NK T-cells). They demonstrated that PPAR-γ regulates expression of the CD_1 gene family encoding a glycoprotein responsible for the presentation of self and foreign lipids to T-cells. Furthermore, they found CD_1 was indispensable for the generation of NK T-cells. Szatmari *et al*., (2004) further determined that PPAR-γ is immediately up-regulated following induction of monocyte-derived DC differentiation which prevented autoimmune reactivity. This research highlights how extracellular signals influence differentiation and gene expression and indicates that modulating CD_1 expression and NK T-cell activation could be an entry point for intervention into autoimmune disorders, such as CD.

More recently, Haller (2006) showed that the PPAR-γ specific ligand, 15 deoxy-prostaglandin J2, triggered protein phosphatase activity in IECs which directly reversed *Bacteroides vulgatus*-mediated ***RelA*** phosphorylation, resulting in inhibition of NF-κB-dependent gene expression. This research and subsequent documentation of T-cell inhibition by PPAR-γ greatly expands the role and significance of PPARs in immuno-regulation and may be of benefit for future investigation of CD (Haller 2006).

3.2 The Mechanisms of Immune Tolerance

Immune tolerance allows the immune system to suppress inappropriate responses to innocuous antigen (Wiedermann 2003). Induction (and maintenance) of tolerance is crucial to immune function and is influenced by host age, antigen dose and the timing of postnatal feeding of the host, the structure of antigen and the composition of the ingested antigen. The integrity of the epithelial barrier and the presence of certain commensal microflora also contribute (Brandtzaeg 2002). Immune tolerance is selective. Under normal circumstances, immune cells ignore 'self' molecules, but respond aggressively to 'foreign' molecules. The intestinal immune system mostly tolerates commensal bacteria, (as measured by T-cell proliferation, Th_1-cytokine or IgG secretion) (Cobrin *et al*., 2002).

While the exact sites or mechanisms of immune tolerance remain unclear, it is known that tolerance is induced by cellular cross-talk between immune cells resident in GALT.

It has been established that 'unresponsiveness' can occur by the elimination of T-cells through;

(a) anergy
(b) clonal deletion
(c) interaction with suppressor T-cells (T-regs) or mediators (Kaminogawa 2000), such as TGF-β and IL-10 produced by T-cells, by IECs (Xian *et al*., 1999), lamina propria stromal cells (Fagarasan 2001) and Peyer's patch DCs (Iwasaki 2000).

It is also known that while not naturally tolerant, DCs remain '***quiescent***' and capable of presenting antigen and inducing tolerance. Incomplete signaling (i.e. B-cell activation without CD4+T-cell activation) results in anergy (Mowat 2005). DCs (and macrophages) use PRRs to recognise PAMPs to facilitate the development and maintenance of tolerance, in response to commensal bacteria. This tolerant response is characterised by the development of T-regs and the secretion of IgA.

The molecular mechanisms underlying the suppressive action of T-regs are not clear however, it is known that T-regs;

(a) do not proliferate in response to antigen binding
(b) prevent the activation of Th_1 and Th_2 $CD4^+$ and $CD8^+$ cytotoxic T-cells
(c) express ***cytotoxic T-lymphocyte-associated protein-4*** (CTLA-4) (Coenen *et al*., 2006).

To avoid suppression of appropriate inflammatory responses to pathogen, DCs can generate IL-6 (and additional factors) that counter the suppressive effect of CTLA-4 and programme type-1 regulatory cells (Tr_1) or Th_3-cells to secrete IL -10, or high levels of TGF-β respectively (Feleszko *et al*., 2006; Mizoguchi *et al*., 2000), both of which induce Th_2-cell differentiation and promote Ig isotype switching in Peyer's patches and in the lamina propria. They also down-regulate Th_1-responses (Izcue *et al*., 2006).

It remains unclear whether intraepithelial lymphocytes (IELs) participate in immune tolerance. Technically, IELs should migrate out of the IE and reach systemic lymphoid tissue to exert a suppressive function. Evidence suggests that IELs migrate in and out of the IE *in vitro*, but whether they migrate back into the lamina propria and return to circulation *in vivo* is unknown (Shaw *et al*., 1998). ***Exosomes*** released by IELs may participate in the induction of immune tolerance (Denzer *et al*., 2000)as research shows exosomes loaded with food antigen are released basolaterally by IELs and taken up by DCs before being transported to draining lymph nodes where they induce tolerance in the absence of co-stimulatory molecules at their surfaces (Diderlaurent *et al*., 2002). However, further research is required to establish the place of exosomes in immune tolerance and the mechanisms driving this immunological state.

3.2.1 The role of TLRs in immune tolerance

Both commensal and pathogenic bacteria gain access to GALT, because TLRs recognise their PAMPs. However, DCs carrying commensal bacteria are not found any deeper in the IE, (i.e. they remain in the lamina propria) (Scott *et al*., 2005; Roux *et al*., 2003). Research indicates responsiveness to a given PAMP is directly linked to the level of expression of its relevant TLR (Stagg *et al*., 2004). TLR expression is regulated to minimise a pro-inflammatory response to commensal bacteria while permitting the induction of signals that establish homeostasis and tolerance (Cobrin *et al*., 2005; Karlsson *et al*., 2004). Appropriate TLR signaling improves barrier function by tightening apical junctions, and triggering IEC proliferation that prevents entry of luminal bacteria. In the absence of TLR signaling, expression of cytokines decreases, neutrophil recruitment diminishes and subsequent translocation of bacteria to mesenteric lymph nodes occurs, all of which favour tolerance (Cobrin *et al*., 2005). DCs may participate in tolerance through the selective induction of IgA secretion by B-cells (plasma cells), again via TLR signaling in response to commensal bacteria (Abreu *et al*., 2005; Uhlig *et al*., 2003). Didierlaurent *et al*., (2002) found that prolonged exposure of IECs to LPS leads to tolerance. Commensal bacteria induce Th_1-cell responses using CpG-motifs in the DNA and LPS of their cell membrane (discussed in detail in the chapter on microbiology of the gut). Both these components are recognised by TLRs (TLR-9 and 4 respectively) and trigger the release of the Th_1-associated cytokines, IFN's, IL-12 and 18 (Feleszko *et al*., 2006). Also, IECs are poorly responsive to LPS due

to low levels of MD-2 in a normal IE and a muted TLR-2 response which favours tolerance. The situation in an inflamed intestine involves the expression of different TLRs and release of different cytokines (Didierlaurent *et al*., 2002).

3.2.2. The role of NF-κB in gut immunity

The activation of NF-κB is a cellular response to cytokines or the presence of bacterial infection. Understanding of the mechanisms involved improved with the discovery that TLR stimulation activates NF-κB. Commensal bacteria interact directly with IECs and attenuate the synthesis of NF-κB elicited via a diverse set of stimuli (Kelly *et al*., 2004; Neish *et al*., 2000).

NF-κB forms the basis of disease specific inflammatory processes through the coordinated activation of inflammatory genes. It is known that products of genes regulated by NF-κB increases the activation of NF-κB, hence amplifying the pro-inflammatory response. Both IL-1β and TNF-α activate and are activated by NF-κB in a positive regulatory-loop which increases and maintains local inflammatory responses (Barnes *et* al., 1997). Production of NF-κB can be activated by viral infection, by oxidants and by antigens, all factors that increase an inflammatory response. This activation leads to the coordinated expression of genes encoding proteins involved in mediator synthesis and the further amplification and perpetuation of the inflammatory response (Barnes *et al*., 1997). NF-κB-induced gene expression operates in combination with the action of activator-protein-1 and IL-6 (Kelly *et al*., 2005). NF-κB regulates the expression of genes encoding pro-inflammatory cytokines and chemokines that generate mediators of inflammation, immune receptors and adhesion molecules (e.g intercellular adhesion molecule-1, vascular-cell adhesion molecule-1 and E-selectin); all key activators in the initial recruitment of cells to inflammatory sites (Barnes *et al*., 1997).

The activated form of NF-κB comprises two proteins, a p65 (***RelA***) subunit and a p50 subunit. In most cell types, nuclear p50-RelA is seen only transiently and does not contribute to the constitutive NF-κB activity in mature B-cells, which consist mainly of c-Rel or RelB (Bargou *et al*., 1997). Unlike RelA, ***RelB***, and ***c-Rel, p50*** and ***p52*** do not contain trans-activation domains in their C-termini. Nevertheless, research (Li *et al*., 2002) shows that these two NF-κB members are critical in modulating the specificity of NF-κB function. The activity of DNA-binding NF-κB

molecules is tightly controlled by accessory proteins called ***I-kappaB (IκB)*** subunits. For a description of IκB-proteins see the glossary in Appendix A.

Interference with the activity of NF-κB may suppress toxic shock, graft-versus-host reactions, acute inflammatory reactions, acute phase response, and radiation damage (Shehata 2005). The inhibition of NF-κB activation by antioxidants and specific protease inhibitors may provide a pharmacological basis for interfering with these acute processes (Kelly *et al*., 2005; Bauerle *et al*., 1994). NF-κB is therefore an obvious target for new types of anti-inflammatory therapies (Uhlig *et al*., 2003). It is also possible to block the action of NF-κB with naturally occurring inhibitors however it may be unwise to block the activation of NF-κB for prolonged lengths of time due to its critical role in immune responses and other defensive mechanisms (Izcue *et al*., 2006).

3.3 The Mechanisms of Intestinal Inflammation

Inflammation is a localised or general defensive response to a pathogenic agent which leads to its elimination. This finely tuned process is regulated in magnitude, space and in a limited time period by balancing pro-inflammatory and anti-inflammatory signals (Brissoni 2005). The onset of a pro-inflammatory response is triggered by the release of mediators, in response to injury or antigen. While inflammation is essential for host defence, excessive or unregulated inflammation is harmful, as is especially the case with CD (Zhou *et al*., 2004). The IE constantly interprets information via host-derived signals and bacteria to maintain homeostasis (Haller 2006). In turn, homeostasis or the development of chronic intestinal inflammation is determined by the presence or absence of control mechanisms that terminate immune responses (Abreu *et al*., 2005). An inflammatory response orchestrates the activation of both innate and adaptive immunity through the release of cytokines (Brissoni 2005; Zhou *et al*., 2004).

In 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 (Cobrin *et al*., 2005). However, changes in this tightly regulated system may lead to chronic inflammation and functional disturbances of the GI tract.

The initiation of inflammation can be induced by antigen specific recognition (i.e. antibody and TCR), by non-specific recognition by phagocytes or the complement pathways. Inflammation induces blood vessel dilation, increased blood flow and permeability, increased expression of adhesion molecules, exudation of fluids and extravasations of inflammatory cells (neutrophils, macrophages, mast cells) activated Th-cells, cytotoxic T-cells, memory T and B-cells to tissues (Brissoni 2005; Murray 2005; Metcalfe *et al*., 2003). Activation of inflammation at a molecular level is triggered by TNF-α, IL-6, and IL-1β which stimulate endothelial cells to up-regulate receptors for immune cells (Tschopp *et al*., 2003).

3.3.1 Innate mechanisms of inflammation

Innate immune responses and inflammation are interwoven. Such a response is immediate (between 4-96 hours) and uses a range of preformed non-specific and transiently synthesised effector molecules and phagocytes (Brissoni 2005). If the innate response is overwhelmed, a delayed antigen specific humoral and cellular response is triggered (i.e. an adaptive response).

3.3.2 Generation of inflammation by the adaptive immune system

An adaptive response is based on the recognition of antigen presented by APCs. PRR signaling in response to the PAMPs of microorganisms activates immune mechanisms (Haller 2006), with the resulting release of TLRs and NODs. This triggers a signaling cascade which alerts and protects the host. However, the unbalanced activation of these signaling pathways can turn a physiological response into a pathological situation resulting in inflammation (especially in genetically predisposed people such as with CD) (Reis e Sousa *et al*., 1999). Haller (2006) proposes that host–derived negative regulators cross-talk to TLR and NOD signaling cascades and shape the magnitude and duration of inflammatory processes.

IL-12 is crucial in driving intestinal inflammation when stimulated by bacterial PGN (Murray 2005) while LPS is known to induce cytokine secretion which initiates an inflammatory response (Eun *et al*., 2006). Bamias *et al* ., (2006) describe a reduction in intestinal inflammation in Crohn's disease which correlates with a decrease of IL-12, IFN-γ and TNF present in lamina propria cells, indicating a role in the pathogenesis of intestinal inflammation. Gorelik *et al*., (2000) found the

abrogation of TGF-β signaling in T-cells is instrumental in inflammatory responses and has a dual role as a potent suppressor of T and B-cell responses while promoting the production of IgA. Mowat (2003) 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 Uhlig *et al*., (2006) describe the action of effector cytokines TNF-α and IFN-γ, IL-12 and IL-23 as essential in inflammatory processes. T-regs are crucial to the preservation of immune homeostasis because they prevent the uncontrolled expansion of effector T-cells after exposure to bacterial antigen (Bamias *et al.,* 2006). These researchers document changes in T-reg levels during inflammation, suggesting that decreasing numbers of T-regs may increase intestinal inflammation. Eun *et al*., (2006) identify that TLR-4, becomes activated after damage occurring during intestinal inflammation, thus triggering NF-κB activation.

It can be seen then, that the breakdown of intestinal immune regulation ultimately results in inflammation which causes damage and loss of barrier integrity. An antigen induces inflammation so microflora become more aberrant, which leads to more inflammation that locally affects intestinal barrier function, resulting in greater mucosal permeability, which means new antigen are absorbed, inducing further inflammation. All this makes the mucosa vulnerable to infection, inflammation and intestinal disease (Metcalfe *et al*., 2003).

Chapter Four: MICROBIOLOGY OF THE GUT

4.1 The Microbial Environment of the Gut

The microbial environment of the intestine constitutes a complex ecosystem that influences the overall health of the host via its effects on local immune status. Gut microflora play a role in maintaining host health through interactions with cells of the mucosa, through interactions with the intestinal immune system (GALT) and interactions with dietary components (Tuohy *et al*., 2006).

The GI tract is colonised by a range of commensal microflora. As the anatomy, physiology and flow characteristics differ along the length of the GI tract, microbial communities will also vary along its length (Tuohy *et al*., 2006; Wilson 2005). Hence each region of the gut provides a different set of environmental conditions, capable of supporting different species of microflora. For example, more than 200 species reside in the mouth e.g. *streptococci, actinomyces, veillonella and spirochaetes* spp (Wilson 2005), while around 128 species reside in the stomach, including *Helicobacter* spp. The small intestine contains lower numbers of bacteria mainly *lactobacilli* and *enterococcus*, while the large intestine harbours a greater range including *bacteroides, bifidobacterium* and *escherichia coli* (*E.coli*) (Wilson 2005b). Research suggests that the relationship between beneficial gut microflora and humans is not merely commensal, it is in fact, advantageous to both the bacteria and the host (Sears 2005).

Enteral commensal microflora are thought to perform a number of useful actions. They ferment unused energy substrates, increase intestinal absorption of water and repress the growth of harmful micro-organisms by competing for nutrients and adhesion sites (competitive exclusion) (Wynne *et al*., 2004; Guarner *et al*., 2003). They also regulate the development of the gut, stimulate the growth of intestinal cells, produce vitamins for the host (such as biotin and vitamin K), produce hormones that direct the host to store fats and programme the immune system to regulate inflammatory responses, so it only responds to pathogens, but can still defend against disease and infection (Sears 2005: Steinhoff 2005; Guarner *et al*., 2003). Intestinal Miicroflora (IM) aid in the apoptosis of potentially harmful cells (Gibson 2004) and may influence the risk of coronary heart disease by modulating serum lipid concentrations, as circulating levels of HDL and LDL cholesterol appear

to be influenced by the population profile and metabolic activity of these microflora (Bird *et al*., 2000).

It is estimated that 10^{14} micro-organisms reside in the gut (Sears 2005: Steinhoff 2005; Björkstén *et al*., 2001). While mostly bacterial species, fungi and protozoa also make up a percentage, but little is known about their activities (Guarner *et al*., 2003). Currently known genera of commensal fungi include *candida, saccharomyces, aspergillus* and *penicillium* (Sears 2005). Between 300 (Guarner *et al*., 2003) and 1000 (Steinhoff 2005: Gibson 2004) different bacterial species inhabit the gut, with most published estimates lying between 400 and 500 (Sears 2005). However, it is probable that the bulk (99%) of the bacterial numbers come from about 30 or 40 species (Beaugerie *et al*., 2004). The genera, *bacteroides, bifidobacterium, eubacterium, clostridium, peptococcus, peptostreptococcus* and *ruminococcus* tend to predominate whereas *escherichia, enterobacter, enterococcus, klebsiella, lactobacillus* and *proteus* are less numerous. (Beaugerie *et al*., 2004: Guarner *et al*., 2003; Vedantam *et al*., 2003).

4.1.1 Specific types of microorganisms present in the gut

Members of the enteral bacterial community have adapted to survive in close association with and to withstand the defence mechanisms of the host (Bibiloni *et al*., 2004). ***Autochthonous*** (adherent) species as well as ***allochthonous*** (species that occupy the luminal space) are found simultaneously. The characteristics of microflora in a particular gut segment vary with age, diet, transit time, pH of the gut and the availability of oxygen within the intestinal environment (Dethlefsen *et al*., 2006). Some of these factors are general factors (age, diet, transit time) that influence the intestinal environment whilst others are region specific (pH, and oxygen availability) (Dethlefsen *et al*., 2006).

There is also evidence that microbial colonisation progresses sequentially through a series of stages, starting with aerobes and facultative anaerobes. As these initial micro-organisms consume oxygen, they create environmental niches more readily colonised by anaerobes (Bry *et al*., 1996). Over 99% of enteral bacteria in the lumen of the GI tract in adults are anaerobes, with studies indicating that anaerobic bacteria outnumber aerobic bacteria by a factor of 100-1000 (Sears 2005;

Beaugerie *et al*., 2004; Guarner *et al*., 2003). *Bacteroides. bifidobacteria, eubacteria* and *E. coli* are all anaerobic organisms, however only *E. coli* is facultative while the others are obligate anaerobes. *Bacteroides* constitute about 30% of enteral bacteria suggesting this species is well adapted for the role (Sears 2005). *Bacteroides thetaiotaomicron* (one of the most abundant species in the lower intestinal tract) is known to have potentially beneficial properties. This is despite the majority of *bacteroides* species having high pathogenic potential (Thompson-Chagoyan *et al*., 2005).

E. coli are estimated to comprise less than 1% of the total number of organisms in the adult intestine, and while 16 species of lactobacillus inhabit the gut, they too contribute less than 1% of total enteral bacteria (Bry 1996). Some lactobacilli bind to intestinal mucous and polymers associated with the surface of enterocytes, others do not. *Lactobacillus acidophilus, L. salivarius, L. ruminis* (previously identified as *catenbacterium catenaforme*) *L. gasseri* and *L. crispatus* all adhere (autochthonous) to the intestinal mucosa whereas *L. paracaseii, L. rhamnosus, L. delbruekii, L. brevis, L. johnsonii, L. plantarum,* and *L. fermentum* are allochthonous strains. Other species regularly found in faecal samples include *L. sakei, L. curvatus, leuconstoc mesenteroides, leuconstoc argentinum, pedioccus pentosaceus* and *pedioccus acidilactici* (Wilson 2005; Bry 1996). The genera of microflora most frequently present in the GI tract of humans, their characteristics and optimum conditions for growth are outlined in Table 4.1 (in Appendix C), while the predominant genera of microflora found in human faeces are shown in Table 4.2 (in Appendix C) and the most frequently isolated species of microflora in human faeces are identified in Table 4.3 (in Appendix C). It is difficult to predict the composition of the intestinal microbial community from faeces because the significantly different physiology along the length of the gut mean that those species numerically predominant in the latter part would be inaccurately represented (Roos *et al*., 2002). There is also interplay between genetics of the host, the type of food eaten and chance in determining the composition of gut microflora (Dethlefsen *et al*., 2006; Tannock *et al*., 2000).

4.1.2 Localisation of intestinal microflora (IM)

Greater numbers of microflora reside in the large intestine than in the small intestine (Kelly *et al*., 2005b; Borriello 2002). Even within the large intestine itself, microbial species vary between proximal and distal areas and differences occur

between luminal and mucosal-associated populations. Regions of inflamed mucosa occurring in inflammatory disorders of the gut (such as CD) are likely to have differing microflora from non-inflamed regions thus the level of inflammation may influence the location of microbial species (Lenoir- Wijnkoop *et al*., 2003).

4.1.2.1 Impact of oxygen availability in the lumen and the mucosa

The precise location of microflora in the gut is partially determined by the level of oxygen present in specific areas resulting in differing regions of the intestine supporting different species. The anoxic conditions in the lumen support anaerobic species, whereas the greater availability of oxygen at the mucosal surface allows aerobic species to survive (Vedantam *et al*., 2003). Samples obtained (by surgery from the jejunum and ileum or by colonoscopy) show significant differences in mucosal and luminal microflora (Peach *et al*., 1982). Peach *et al*., (1982) found that obligate and facultative anaerobes were equally represented in mucosal samples, whereas facultative anaerobes outnumbered obligate anaerobes in samples taken from the lumen of the small intestine.

Bird *et al*., (2000) found that bacterial numbers, (especially obligate anaerobes) increase along the length of the small intestine attaining a density of between 10^6-10^8 cells/ml in the distal ileum, however rapid peristalsis and the bactericidal actions of gastric secretions limit colonisation in the upper small intestine to usually less than 10^5 cells/ml of digesta.

4.1.2.2 Influence of intestinal pH

The pH of the intestinal environment influences where particular bacterial species can survive. Individual species of microflora tolerate a specific pH range. While the initial pH of the digestive juices is dictated by the host, the activities of the microbes colonising the area has a profound effect on the final pH of digesta (Wilson 2005a). The secretion of bicarbonate by the mucosa also contributes to this change in pH (Wilson 2005a). Bacteria are broadly classified as acidophiles, neutrophiles or alkaliphiles depending on the pH range over which they grow and their optimum pH for growth. Acidophiles tolerate a pH of less than 5.5, neutrophiles grow best between pH range 5.4-8.0 and alkaliphiles tolerate a pH of around 8.0 (Wilson 2005a). The lumen pH gradually increases along the small intestine from 5.7-6.4 in the duodenum to between 5.9-6.8 in the jejunum and 7.3-7.7 in the ileum (Wilson 2005b). The mean pH of the proximal small intestine is 6.6, whereas the mean pH

in the terminal ileum is 7.5 (Cleusix *et al*., 2007). The pH of the large intestine also increases along its length. The pH of the caecum is approximately 5.7 and remains low in the ascending colon at pH 5.6, but increases to around 6.6 in the descending colon due to the formation of ammonia from microbial fermentation of amino acids (Wilson 2005b). Bacterial growth is generally most rapid in the caecum and ascending colon where the pH is low, but it is slower in the descending colon, where the pH is closer to neutral (Guarner *et al*., 2003). Although the monocultural growth of a particular microbe is limited to a certain pH range, when the organism is part of a mixed species community such as occurs in the gut, the range of pH over which it can grow is extended considerably (Cleusix *et al*., 2007). Table 4.4 (see Appendix C) shows the pH of different regions of the GI tract and the predominant microflora colonising each site.

The pH of the intestinal environment influences both commensal and pathogenic microflora equally. *Lactobacillus reuteri,* a beneficial commensal bacteria, successfully transits the low pH of the stomach and colonises the intestine allochthonously (Cleusix *et al*., 2007). *L. reuteri* produces ***reuterin***, which provides an ecological competitive advantage to *L. reuteri* and is involved in colonisation resistance against potentially pathogenic bacteria especially *E coli* (Cleusix *et al*., 2007). The promotion of short chain fatty acid (SCFA) production through the provision of fermentable carbohydrates can augment the lowering of the pH of the large intestine (Gibson 2004; Bird *et al*., 2000).

4.1.2.3 Impact of nutrient availability on location of microflora

Microflora present in specific areas of the gut is partially influenced by the availability of nutrients within the region. For example, gram-positive commensal species predominate in the proximal small intestine which is generally where carbohydrate digestion occurs (Beaugerie *et al*., 2004: Gibson 2004; Guarner *et al*., 2003). This agrees with the finding that gram-positive microflora, notably *bifidobacteria* exist in higher numbers where increased amounts of fermentable carbohydrates are available (Borriello *et al*., 2002; Riordan *et al*., 2001). Similarly, the majority of microflora in the colon where some proteins, amino acids, non-digestible fibre and starch are fermented and absorbed are gram-negative species (Gibson 2004; Guarner *et al*., 2003).

A wide range of bacterial interactions occur in the intestine which enable microflora to collaborate and degrade complex dietary and metabolic substrates (e.g. glycoproteins and plant polymers), while others result in a particular species dominating a habitat. Most people's diets are so varied that the GI tract regularly receives a diverse range of nutrients for microflora (Wilson 2005b). Some are relatively simple (such as monosaccharides, amino acids or fatty acids) that are used directly as sources of energy, carbon or nitrogen, but other macromolecules are more problematic. Some micro-organisms do not secrete hydrolases and must rely on the activities of other species to provide assimilable degradation products (Wilson 2005b). For example, mucin is so complex that very few individual species can degrade it fully therefore microbes must consort in a sequential manner to achieve this. The levels of enzymes involved in the degradation of insoluble plant cell wall components are higher in some adherent bacterial species suggesting they are important in the digestion of complex carbohydrates (Wilson 2005b). The role of IM in the degradation of dietary components will be explained under utilisation of nutrients.

4.1.3 The adhesion of autochthonous bacterial species

The nature of the glycocalyceal structure (i.e. the 'carbohydrate repertoire'), influences microbial adhesion. Bacterial adhesins link specific carbohydrate configurations on the cell glycocalyx (Kelly *et al*., 2005; Lenoir-Wijnkoop *et al*., 2003). The 'carbohydrate repertoire' while genetically determined may subsequently be modified by the process of bacterial adhesion. Hence, initial bacteria adhering to fucosylated residues may promote the formation of more fucosylated residues which means that the evolution of enteral microbial communities is recursive (Lenoir-Wijnkoop *et al*., 2003). Although pathogenic bacteria initially bind to the same glycocalyceal moieties as commensal bacteria, they subsequently bind directly to ***cell adhesion molecules*** (CAMs) by mimicking host-cell receptors or ligands, or by producing their own receptor (Wilson 2002). These CAMs are located on the cell surface and bind with other cells or with the extracellular matrix (ECM) by cellular adhesion. Most cell adhesion molecules are ***integrins, cadherins, IgCAM's*** and ***selectins*** (Wein *et al*., 1995).

IM are implicated in the pathogenesis of chronic inflammatory diseases of the intestine (Swidsinski *et al*., 2005; Bullock *et al*., 2004; Sartor *et al*., 2004; Cummings *et al*., 2003). Past research (Swidsinski *et al*., 2005; Bullock *et al*., 2004; Sartor *et al*., 2004; Cummings *et al*., 2003) links cellular components of gram-negative bacteria (such as flagellins and lipopolysaccharides) to systemic inflammatory changes in the pathogenesis of Crohn's disease, ulcerative colitis and more recently of CD (Nadal *et al*., 2007). The changes detected in the overall composition of IM in people with CD could be either a consequence or a cause of the disease. Ludvig *et al*., (2004) identified pathogenic bacteria adhering to the IE of people with CD, but not to that of healthy controls, which potentially implicates bacterial adhesion and colonisation in the pathogenesis of CD. Alternatively, colonisation by gram-negative bacteria in genetically predisposed people could contribute to the abrogation of tolerance to gluten (Nadal *et al*., 2007). An increase in intestinal permeability caused by a damaged mucosa and the presence of necrotic material could create conditions favouring colonisation with gram-negative bacteria and the progression of inflammatory change (Nadal *et al*., 2007). Furthermore, the composition of the glycocalyx and mucous layer is different in people with CD. Both Forsberg *et al*., (2004) and Ludvig *et al*., (2004) propose that altered ***glycosylation*** could favour glycocalyceal bacterial adhesion and may be a primary factor predisposing people to CD.

4.1.4 Bacterial communication

Bacterial cells communicate with each other and with host cells. In fact, it has been suggested that bacteria are 'multi-lingual', having both a species specific 'language' and a species non-specific language (Kelly *et al*., 2005b). Bacteria are thus able to assess their own population numbers and the population density of other bacterial species in biofilm communities then induce and express additional genes to maintain their competitive advantage (Tannock in Fuller *et al*., 2003). The control of gene expression in response to cell density is termed "Quorum sensing", and is used by both gram-positive and gram-negative bacteria to regulate their physiological functions (Wilson 2005a; Lenoir-Wijnkoop *et al*., 2003). The most studied quorum sensing systems involve N-acylhomoserine lactone signals in gram-negative bacteria. Once a critical concentration of density-dependent signal molecules has been exceeded, gene transcription is activated. Quorum sensing is important in microbial colonisation of the intestine due to the large numbers of

microflora present and the degree of ecological competition that exists (Lenoir-Wijnkoop *et al*., 2003). The active dialogue between intestinal bacteria and the host is called cross-talk (Lenoir-Wijnkoop *et al*., 2003). This cross-talk affects immunological tolerance and homeostasis within the gut and explains some of the differential host responses to commensal and pathogenic bacteria.

4.1.5 Bacterial crosstalk

Commensal bacteria modulate intestinal glycosylation in the host by signals that interfere with cellular glycosyl-transferase activity or expression which, in turn, modifies the carbohydrate repertoire in favour of providing additional adhesion sites for bacteria e.g. fucosylation induced by *bacteroides thetaiotaomicron* (Lenoir-Wijnkoop *et al*., 2003).

4.1.5.1 Microbial communication and interaction in the intestinal tract

Bacteria not only communicate with the host, they also communicate with each other via cross-talk and quorum sensing. Their interactions can have both positive and negative effects (Lenoir-Wijnkoop *et al*., 2003; Tannock in Fuller *et al*., 2003). Positive interactions include quorum sensing, oxygen utilisation by anaerobes/facultative anaerobes, degradation of polysaccharides, degradation of proteins, degradation of mucins and other glycoproteins, excretion of metabolic end products (e.g. lactate, ethanol, hydrogen, ammonia) (Wilson 2005b). Table 4.5 shows the positive interactions between members of the IM (see Appendix C).

Microbes can have detrimental effects on other IM in order to maintain their competitive advantage in four main ways;

(1) by consuming an essential nutrient;
(2) by occupying sites for adhesion
(3) by creating an environment unsuitable for growth;
(4) by producing antimicrobial agents, bacteriocins and bacteriophages that interfere in the metabolic processes (Wilson 2005b).

4.2 The establishment of commensal intestinal microflora

The commensal intestinal microbial community of an individual remains remarkably stable, although some fluctuation occurs over time and with diet (Dethlefsen *et al*., 2006; Kelly *et al*., 2005b; Guarner *et al*., 2003). Other factors such as host genotype, order of colonisation and microbial interactions also contribute to generate a diverse individualised IM.

4.2.1 The changing profile of intestinal microflora on ageing

The composition and diversity of bacterial communities changes with age. Breast-fed infants normally harbour larger numbers of facultative anaerobes (*enterococci, enterobacteria*) and *bifidobacteria* than adults but as they grow this changes, due in part to a change in diet and consequent increase in the quantity and diversity of short chain fatty acids (SCFAs) generated by saccharolytic species (Wilson 2005b; Tannock in Fuller *et al*., 2003).

4.2.1.1 Intestinal microflora in infancy

The GI tract is sterile at birth and colonised thereafter by bacteria from the maternal faeces and the local environment (Bettelheim *et al*., 1974). Infants born by caesarian section are exposed to microflora on maternal skin (Schwiertz *et al*., 2003). Initial colonisation of the infant gut is dominated by *bacillus, pseudomonas* and *micrococcus* as well as *lactobacilli, streptococci* and *bifidobacteria* with bacterial numbers reaching 10^8–10^{10} /g faeces within a few days, but diversity remains low (Schwiertz *et al*., 2003; Mackie *et al*., 1999). During the first week of life, these initial bacteria create an environment with a reduced level of oxygen favourable to the subsequent bacterial succession of strict anaerobic species, predominantly *bifidobacterium, bacteroides, clostridium*, and *ruminococcus* (Borrelli 2002; Favier *et al*., 2002).

Commensal microflora in the gut of breast-fed infants become dominated by *bifidobacteria,* with these bacteria forming up to 91% of the total bacterial community possibly due to bifidobacterial growth factors in breast milk (Coppa *et al*., 2004). In contrast, the microflora of formula-fed infants is more diverse with 75% *bifidobacteria* and the presence of *enterobacteria, enterococci, bacteroides* and *clostridia* species (Fanaro *et al*., 2003; Harmsen *et al*., 2000; Tannock 2003).

Following the introduction of solid food and weaning, the IM of both breast-fed and formula-fed infants becomes similar. By the second year of life, the faecal microflora resembles that of adults. Once a climax microflora is achieved the same pattern of bacterial species is generally maintained throughout life (Zoetendal *et al*., 2001). By adulthood there are core intestinal species that are persistent and common (Lenoir- Wijnkoop *et al*., 2003) Table 4.6 shows the dominant and minor genera of the climax community (see Appendix C).

With the initiation of bacterial colonisation in infancy, significant changes occur in components of the immune system such as the expansion and differentiation of specific cells involved in mucosal immunity (Kelly *et al*., 2005b). Individual species of commensal bacteria differ in their ability to both promote the development of GALT and to maintain its function, however the molecular basis for this remains unknown (Kelly *et al*., 2005b). Normal commensal microflora maintain immune homeostasis in both the developing infant and adult gut, and the consequences of early post-natal programming persist throughout life and significantly impact on whole body health (Kelly *et al*., 2005b). The hygiene hypothesis which links reduced early exposure to important gut bacteria with the rising incidence of allergies and autoimmune diseases may be involved in this process (Noverr *et al*., 2005; Noverr *et al*., 2004).

4.2.1.2 Intestinal microflora in adults and the elderly

The controlling effects of secretions and motility within the gut established in infancy are fully operational throughout childhood and adulthood, but lose potency in old age (Goldin 2003). Studies show that changes in gut microflora occur in the seventh through the ninth decade of life (Goldin 2003; Hebuterne 2003). Changes in the composition of microflora with age include a decrease in numbers of *veillonella* and *bifidobacteria*, and an increase in the proportion of *clostridia*, *lactobacilli*, and *enterobacteria* in faecal microflora (Wilson 2005a). Table 4.7 compares the microbial counts per gram of faeces for selected bacteria in both adults and the elderly (see Appendix C). Several markers exist which indicate dysfunction of the immune response in the elderly (i.e. immuno-senescence), including decreased range of antibody production, decreased numbers of circulating lymphocytes, impaired T-cell proliferation, impaired phagocytosis and microbial killing by lymphoctyes (Wilson 2005a). However, the precise contribution of these changes to alterations in the composition of IM is not known. Malnutrition

in the elderly also affects immune function and the composition of intestinal secretions, which ultimately impacts on the composition of IM (Wilson 2005a). Table 4.8 shows the factors contributing to alterations in IM in the elderly (see Appendix C).

4.3 Stability of the intestinal microflora

Some species of IM are more susceptible to change than others. In the past, intestinal microbial communities were considered stable when temporal variability was smaller than inter-individual differences (Vanhoutte *et al*., 2004 Zoetendal *et al*., 2001; Franks *et al*., 1998; Zoetendal *et al*., 1998). More recent studies using culture or molecular techniques with greater phylogenetic resolution, found that variation exists in the abundance and identity in some species of microflora while others remain very stable (Dethlefsen *et al*., 2006).

4.3.1 Fluctuations in stability of intestinal microflora

Dietary change is one of the most commonly cited factors associated with fluctuations in the stability of IM. Some diets promote the growth of beneficial microflora while others promote the growth of potentially pathogenic microflora whose microbial activity is detrimental to the host (Hawrelak *et al*., 2004; Smith *et al*., 1997; Linder 1991). A raw vegetable based diet, the consumption of yoghurt and foods with prebiotic activity are linked with increased growth of commensal microflora, whilst diets high in dietary sulphates are associated with an increase in potentially pathogenic microflora (i.e. sulphate-reducing bacteria) (Hawrelak *et al*., 2004).

Dietary sources of sulphates include foods high in animal protein (meat, milk, cheese, eggs) and cruciferous vegetables as well as foods containing preservatives and alcoholic beverages (Hawrelak *et al*., 2004). These bacteria (e.g. *Desulfovibrio*) reduce sulphite and sulphate to sulphide producing potentially toxic hydrogen sulphide that can damage the intestinal mucosa by inhibiting the oxidation of butyric acid, the primary fuel for enterocytes. This can cause an increase in intestinal permeability presumably due to the breakdown of mucin through cleavage of disulphide bonds (Cummings *et al*., 2003; Roediger *et al*., 1997; Levitt *et al*., 1995).

Sulphate-reducing bacteria directly compete with methanogenic bacteria for vital substrates (i.e hydrogen and acetate), thus altering the balance of microbial species within the intestinal environment (Hawrelak *et al*., 2004). It is not that the typical diet of New Zealanders is inherently pathogenic but certain foods consumed have the potential to alter the intestinal milieu and the balance of microflora. There are, of course, positive effects of eating animal protein and cruciferous vegetables especially those associated with eating foods from the allium genus (i.e. onion, leeks and garlic) (Ferary *et al*., 1998).

However, it is impossible to accurately predict how dietary changes will impact on the diversity and abundance of particular microbial species as diet-driven changes in microbial populations is dependent on knowledge of existing community composition and dynamics (Dethlefsen *et al*., 2006; Hawrelak *et al*., 2004). It is questioned whether sampling microbial populations over several weeks or months, (or at most a year), adequately protrays the variability within the intestinal microbial population and whether this information accurately describes community stability (Dethlefsen *et al*., 2006).

4.3.1.1 Intestinal dysbiosis

When populations of commensal microflora co-exist, they exert an inhibitory and controlling influence on each other to maintain intestinal microbial stability and prevent the overgrowth of pathogenic microflora (Hawrelak *et al*., 2004). However, if these populations become *dysbiotic*, this increases the risk of colonisation by pathogenic microflora and development of chronic and inflammatory disease (Hawrelak *et al*., 2004). Changing numbers and species of IM may reduce the fermentation of non-digestible carbohydrates and metabolisation of enteral bile acids, possibly resulting in diarrhoea (Hawrelak *et al*., 2004). Increased levels of undigested carbohydrates result in osmotic disturbances, or it could be that the lack of SCFAs produced by gut microflora causes the diarrhoea (Hawrelak *et al*., 2004). The use of broad-spectrum antibiotics changes the profile of gut microflora by reducing numbers of commensal bacteria and disrupting their ability to inhibit the growth of pathogenic species such as *clostridum difficile* and *salmonella kedougou* (Beaugerie *et al*., 2004: Carman *et al*., 2004; Guarner *et al*., 2003). Intestinal dysbiosis also occurs in severe illness, due to a combination of antibiotic use, ischemia of the gut, failure to eat, and immune compromise. All of which may occur

in people with advanced CD. The negative effects associated with dysbiosis have led to increased interest in the use of selective digestive-tract decontamination (SDD), a treatment which eliminates pathogenic bacteria while allowing the re-establishment of commensal microflora to restore the ecological balance within the intestine (Knight *et al*., 2003).

4.4 The effects of host genotype on intestinal microflora

The mucosal intestinal environment is expected to be more alike in genetically related people (Biblioni *et al*., 2004). PCR-DGGE methods show that host genotype partially influences the composition of the enteral microbial community. Faecal samples from people of differing degrees of relatedness, varying from monozygotic twins to marital partners were compared and the DGGE profiles analysed (Biblioni *et al*., 2004). A high degree of similarity exists in the range of microbial species between monozygotic twins whereas the level of similarity between dizygotic twins is similar to that of co-habiting couples, aunts and uncles, nephews and nieces, parents and children, brothers and sisters (Biblioni *et al*., 2004; Zoetendal *et al*., 2001).

Similarities exist between the products of enteral microbial communities in genetically related hosts. Toivanen *et al*., (2001) found the fatty acid profiles of faecal microflora in mice showed greater variability in the MHC-encoded genes, indicating that a genetic aspect is involved. It is already known that host genotype is related to the severity of colonisation of *helicobacter pylori* and that certain strains of this organism are only found in people of Asian descent (Letley *et al*., 2003). There are positive associations between gastric colonisation by *H. pylori* and cytokine genotypes (polymorphisms in promoter regions of the IL-10 and IL-14 genes) (Michetti *et al*., 2003; Zavros *et al*., 2003; Bodger *et al*., 2001; Heneghan *et al*., 2000) and blood group antigens (Lewis-b antigen) and a negative association with an HLA allele (Class II DR-DQ)) (Michetti *et al*., 2003; Zavros *et al*., 2003; Bodger *et al*., 2001;Heneghan *et al*., 2000).

4.5 Diet and intestinal microflora

People used to think that changing the proportions of meat, fat, carbohydrate and fibre in the diet would significantly alter the range of commensal microbial species

within the gut, however, research shows only low levels of change (Hawrelak *et al*., 2004; Lenior-Wijnkoop *et al*., 2003). Comparative studies of people eating radically different diets found selective changes in the number and diversity of IM rather than a general shift in microflora (Dethlefsen *et al*., 2006; Goldin 2003). Some studies have examined the variation of particular species of microflora in adults with diet (Mueller *et a*l., 2006; Hayashi *et al*., 2002; Gibson 1998; Finegold *et al*., 1977). An early study comparing Japanese and Western diets found only moderate effects involving a few specific genera (Finegold *et al*., 1974). Subjects consuming a Japanese diet had higher faecal counts of *streptococcus faecalis, eubacterium lentum* and *ebacterium contortium* but lower counts of *bacteroides* species.

Newer techniques for analysing IM (i.e. PCR-DGGE, TGGE) have revealed that whilst their composition is relatively stable, diet does trigger alterations (Dethlefsen *et al*., 2006; Favier *et al*., 2002; Harmsen 2000). The effects of various diets on IM are summarised in Table 4.9 (see Appendix C).

4.5.1 Bacterial breakdown of indigestible nutrients

The intestine efficiently digests and absorbs nutrients, with more than 95% of nutrients that enter the small intestine being utilised however, the quality and quantity of nutrients available is ultimately determined by the type of diet a person consumes, which in turn influences the composition of IM (Tuohy *et al*., 2006; Wilson 2005b). Carbohydrates (saccharides) are present in the diet as monosaccharides, disaccharides, polysaccharides and oligosaccharides. Simple sugars are digested by disaccharidases and other hydrolases in the small intestine and their component monosaccharides are absorbed across the IE (Flint 2004; Hill *et al*., 1990).

Complex carbohydrates are dietary fibre (non-starch polysaccharides- NSPs) and starch. Soluble fibre includes inulin, fructans, xanthan gum, guar gum, cellulose, hemicellulose and insoluble dietary fibre is lignin (Wilson 2005b). Most non-digestible carbohydrates (i.e. resistant starch, NSPs, fibre of plant origin and non-digestible oligosaccharides) and resistant protein are partially digested in the small intestine, before passing into the colon where they are fermented by IM (Sears 2005; Beaugerie *et al*., 2004; Gibson 2004; Guarner *et al*., 2003). The majority of NSPs are degraded by fermentation in the proximal colon while resistant proteins

are broken down in the proximal and distal colon. A high dietary fibre intake increases the amount of complex carbohydrate reaching the small intestine, however humans only possess saccharolytic enzymes to digest starch and its breakdown products and are unable to digest α 1-4 or β-glycan linkages which typify NSPs. Thus all other NSPs and oligosaccharides enter the colon where they undergo bacterial fermentation (Bird *et al*., 2000). The presence of these resistant carbohydrates drives bacterial fermentation in the gut, as dietary carbohydrates are the principle substrates for bacterial growth (Tuohy *et al*., 2006).

4.5.1.1 Fermentation of Carbohydrates

The rate at which different carbohydrates are degraded by gut microflora varies greatly. Transit time slows when diets are high in simple sugars which increases fermentative bacterial activity although the mechanism underlying this change in transit time is not fully understood (Lewis *et al*., 1999). Some species of microflora use bile acids as a substrate so a high sugar intake may cause alterations in species by increasing bile output. Hence increase in bile production results in increased production of these bacterial species giving them a competitive advantage (Hudson *et al*., 1995). Some sugars (e.g. raffinose) are selectively fermented (being mainly digested by *bifidobacteria* and *lactobacilli*) while others (e.g. lactose) support the growth of a wide range of IM (Hudson *et al*., 1995). Flint (2004) found an increase in fermentable carbohydrates led to an overall increase in microflora present in the intestinal ecosystem, while Borriello (2002) observed an increase only in *bifidobacteria*. Similarly, non-digestible oligosaccharides reaching the large intestine display differing fermentabilities and different bacterial species show different glycosidic preferences (Tuohy *et al*., 2006). *Bifidobacteria* may preferentially ferment fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and the polysaccharide inulin (Bäckhed *et al*., 2005).

Saccharolytic bacterial species within the IM include *bacteroides, bifidobacterium, ruminococcus, peptostreptococcus* and clostridia species (e.g. *roseburia intestinalis*). *Bacteroides* are versatile members of the dominant microflora, possessing an array of polysaccharide hydrolysing enzymes (Bäckhed *et al*., 2005; Xu *et al*., 2003; Schell *et al*., 2002). For example, *bacteroides thetaiotaomicron* hydrolyses dietary and host-derived polysaccharides, as it induces the fucosylation

of glycoconjugates produced by enterocytes (Bry *et al*., 1996a). *B. thetaiotaomicron* cells utilise L-fucose as an energy source and control the availability of this growth substrate in the intestine.

Bifidobacteria are more efficient at utilising shorter oligosaccharides (Bäckhed *et al*., 2005). As with *B. thetaiotaomicron*, *Bifidobacterium longum* contains genes encoding proteins specialised at catabolising of a variety of oligosaccharides, glycoproteins and glycoconjugates (Bibiloni *et al*., 2004). *B. thetaiotaomicron* and *B. longum* base their ecological competitiveness on the utilisation of complex nutrients using well-regulated pathways to conserve energy and assure high proliferation rates in the gut (Bibiloni *et al*., 2004).

Lactobacilli also have complex nutrient requirements (amino acids, peptides, vitamins, salts, fatty acid esters and fermentable carbohydrates), so *lactobacilli* are only abundant in areas where these nutrients are readily available (Bibiloni *et al*., 2004). As easily accessible nutrients are usually in short supply in the colon (having already been absorbed in the small intestine), *lactobacilli* make only a minor contribution to total faecal microflora (Bibiloni *et al*., 2004).

4.5.1.2 Short chain fatty acid production

Intestinal bacteria produce short chain fatty acids (SCFAs) from carbohydrates providing a major source of usable energy and nutrients for use by host cells (Beaugerie *et al*., 2004; Gibson 2004). The SCFAs produced during saccharolytic fermentation are acetic acid, propionic acid, and butyric acid, along with lactic acid, hydrogen, methane and carbon dioxide (Beaugerie *et al*., 2004; Gibson 2004). Acetic acid is absorbed by muscles, propionic acid is utilised by the liver to produce ATP, and butyric acid is used by IECs to produce adenosine triphosphate (ATP) (Gibson 2004). SCFAs increase growth of IECs, control their proliferation and differentiation and stimulate the growth of GALT (Guarner *et al*., 2003 (Tuohy *et al*., 2006). Bacterial cells use SCFAs and influence the growth of IECs by altering the expression of cell surface proteins such as sodium/glucose transporters (Sears 2005). In addition, changes they make to cells may prevent injury to the intestinal mucosa (Keeley 2004). Table 4.10 lists all of the SCFAs, other organic substrates and their location in the human intestine (see Appendix C).

4.5.1.3 Fermentation of protein and fat

Bacteria ferment protein and amino acids in the intestine. The quantity of protein reaching the large intestine is governed by dietary protein intake and its digestibility in the small intestine (Tuohy *et al*., 2006). Increasing amounts of protein in the diet increases proteolytic digestion. Certain microflora (*bacteroides, eubacterium, peptococcus* and *clostridia*) produce proteolytic enzymes and ferment undigested protein to produce SCFAs, branched-chain fatty acids (isovalerate, isobutyrate and 2-methylbutyrate) which are beneficial. Increased levels of protein reaching the small intestine corresponds with increased numbers of facultative anaerobes (*streptococci* and *coliforms*) and *lactobacilli,* but little change in numbers of obligate anaerobes. Table 4.11 shows how diet influences composition of microflora in the large intestine although it is questioned whether the comparisons are valid given that most diets are mixed (see Appendix C).

Diets high in animal protein have distinct effects on IM when compared to diets high in overall protein (Wilson 2005b). While not appearing to dramatically alter the composition of microflora (compared to control diets), diets high in animal protein increase the activity of bacterial enzymes (beta-glucuronidase, azoreductase, nitroreductase and 7-alpha-hydroxysteroid dehyroxylase) resulting in increased amounts of harmful metabolites into the large intestines (i.e ammonia, amines, phenols, sulphide and indoles) (Tuohy *et al*., 2006; Hawrelak *et al*., 2004; Roediger *et al*., 1997; Smith *et al*., 1997). Ammonia alters morphology and intermediate metabolism, increases DNA synthesis and reduces the lifespan of mucosal cells (Macfarlane *et al*., 1995). The level of harmful compounds is reduced by consuming diets higher in fibre or indigestible starch and lower in animal protein, both of which reduce intestinal pH (Hawrelak *et al*., 2004; Guarner *et al*., 2003).
Proteins can be modified during processing to contain biologically active components that alter bacterial populations within the large intestine. When absorbed, these may interact with disease mechanisms by inducing inflammatory responses, however little is known about their action at this stage (Tuohy *et al*., 2006).

High dietary fat intake corresponds to increased numbers of obligate anaerobes (*bacteroides* and *clostriduim*), but little change to facultative anaerobes (Wilson 2005b). Fernandez *et al*., (1985) examined enteral microflora of people with

malabsorption, on diets low in fat and protein, then compared results to a control diet. Results showed little effect on overall numbers of microflora, but diversity changed considerably. *Bifdobacteria* disappeared completely and only 2 out of 11people had *veillonella* species on a low protein diet. *Lactobacilli* were recovered maximally on a high protein diet (8 out of 10) and a high fat diet (4 out of 9).
Evidence suggests that certain bacterial species enhance the absorption and storage of the lipids they produce, they assist in absorption of vitamin K and that SCFAs produced by bacterial fermentation aid calcium, magnesium and iron absorption (Sears 2005; Guarner *et al*., 2003).

4.6 Reducing the growth of pathogenic micro-organisms

Commensal microflora prevent colonisation of the intestine with pathogenic bacteria by "competitive exclusion" (Thompson *et al*., 2005). This is where two species can not occupy the same ecological niche and under such conditions one species will dominate whilst the other is excluded (Starr 2005). This is the desired outcome in the gut, where it is advantageous for commensal microflora to flourish at the expense of pathogenic species. Growth of pathogenic bacteria (i.e *clostridium difficile*) is restricted by competition from beneficial microflora for nutrients and adhesion sites (Guarner *et al*., 2003). Commensal microflora are more acclimatised to this ecological niche and thus more successful in competition. Commensals utilise nutrients efficiently, competitively limiting availability of nutrients to pathogenic bacteria as well as producing bacteriocins that kill pathogenic micro-organisms. Bacteriocin levels can be closely regulated by enzymes produced by the host (Guarner *et al*., 2003).

4.7 The role of intestinal microflora in immunity

IM continuously and dynamically affect both gut and systemic immune activity. They promote early development of the intestinal mucosal immune system (both in terms of its physical components and its function) and continue to exert an influence throughout life (Steinhoff 2005; Shanahan 2002). From birth bacteria colonise the GI tract with the first bacteria influencing immune responses, making it more favourable to their own survival and less hospitable to competing species. Thus these initial colonisers determine the lifelong composition of IM (Sears 2005; Guarner *et al*., 2003). Hundreds of bacterial species co-exist in intimate association

with the host without their antigenic load stimulating a marked inflammatory response in GALT as the immune system effectively differentiates between innocuous and pathogenic micro-organisms
(Sears 2005).

Investigations to resolve how the mucosal system differentiates commensal microflora from pathogenic species identify involvement of TLRs, mechanisms of immune tolerance and the production of secretory IgA molecules that opsonise commensal microflora (Bibiloni *et al*., 2004). IM stimulate GALT to produce antibodies to pathogenic bacteria enabling the immune system to recognise and destroy pathogenic species, while leaving beneficial species alone. In contrast, the presence of pathogenic bacteria results in stimulation of both innate and adaptive immune mechanisms (Guarner *et al*., 2003; Medzhitov 2001) (refer Chapter 3; Gut immunity, inflammation and tolerance).

The immunological environment of the host provides a selective force for the evolution of a beneficial relationship by selection of genetic variants less antigenically foreign to the host thus favouring establishment of certain bacterial strains as members of the commensal enteral microflora (Biblioni *et al*., 2004). It is unclear whether a genetic predisposition of the host to tolerate certain kinds of bacteria over others exists, although allochthonous bacteria elicit a greater immune response than most autochthonous species (Biblioni *et al*., 2004).
Some microflora (e.g. *bacteroides fragilis*) modulate their antigenicity by producing polysaccharides and regulating their expression by reversible inversion of DNA which is thought to assist *bacteroides* in maintaining residence in the gut despite constant immune pressure from the host. Polysaccharides with similar properties have also been identified in *B. Thetaiotaomicron*) (Bibioni *et al*., 2004; Xu *et al*., 2003). *Bacteroides* change their surface receptors to mimic those of host cells to avoid an immune response. Bacteria with neutral and harmful effects on the host also use these strategies however the immune system has adapted to this activity to prevent overgrowth of pathogenic species (Sears 2005; Steinhoff 2005; Keely 2004; Guarner *et al*., 2003). Further study aimed at recognising the molecular mechanisms of persistence in the gut is needed to increase understanding of gut homeostasis, the degree to which the host develops immunological tolerance to

microflora and the extent to which bacteria adapt to conditions provided by the immune system (*Jewell 2005*).

4.8 The role of intestinal microflora in disease

Many species of IM have pathogenic properties as well as beneficial ones. They produce toxins and carcinogens and are implicated in multi-system organ failure, sepsis, colon cancer and inflammatory bowel disease (IBD) (Guarner *et al*., 2003). A major factor in health is the balance of bacterial numbers within the intestinal environment. An overgrowth of pathogenic species or low numbers of commensal species can potentially be detrimental to the host. However, it is not yet known whether a shift in IM drives the inflammatory process or if changes in microflora are a consequence of the inflamed mucosa itself (Lenoir- Wijnkoop *et al*., 2003). Some genera of IM (*bacteroides* and *clostridium*) are associated with increased tumour growth rates, while other genera (*lactobacillus* and *bifidobacteria*) prevent tumour formation (Guarner *et al*., 2003).

4.8.1 Intestinal microflora and inflammatory bowel disease (IBD)

IBD is likely caused by a reduction in immune tolerance and subsequent over-reaction of the immune system to pathogenic or commensal bacteria. It may be triggered by all species or specific species of IM (Hugot 2004; Wynne *et al*., 2004). Both Ulcerative colitis and Crohn's disease (the two predominant types of IBD) have genetic components, but are not inherited in a Mendelian fashion. Rather they are due to complex factors rather than solely to the presence (or absence) of a particular gene (Hugot 2004). However, neither genetics nor bacterial colonisation alone, are sufficient to cause the disease (Hugot 2004).

The inflammation characterising IBD results from increased permeability of the intestinal mucosa, which allows bacteria to translocate causing an immune reaction leading to prolonged inflammation. Abnormal tight junctions are also present in people with IBD (Steinhoff 2005; Suenaert *et al*., 2002). While CD is not traditionally considered an IBD, it has characteristics that typify IBD. Both children and adults with CD have increased intestinal permeability that is only partially corrected by a GFD (Cummins *et al*., 2001). Intestinal tissues of people with CD show increased zonulin expression and gliadin modulates tight junctions via a

zonulin dependent pathway. Thus in genetically predisposed people gluten may 'open the gates' to intestinal inflammation which can alter the profile of IM or allow translocation triggering intestinal inflammation similar to that associated with IBD (Clemente *et al*., 2003).

Both Guarner *et al*., (2003) and Elliot *et al*., (2000) have identified that lack of breast-feeding, high standard of hygiene in childhood ***('the hygiene hypothesis')***, lack of exposure to helminthic parasites, and consumption of large amounts of sucrose and animal fat may contribute to the development of intestinal inflammatory disorders and to allergic reactions. Breast milk contains soluble PPRs that recognise microbial constituents in the gut and regulate activation of the innate immune system in a way that dampens immuno-pathology (LeBouder *et al*., 2006; LeBouder *et al*., 2003; Labeta *et al*., 2000). Breast-milk-derived soluble receptors are also vital in linking fatty acids with the innate immune system, further strengthening the beneficial host-microbe cross-talk for inflammation control (Laitinen *et al*., 2006).

4.9 The role of intestinal microflora in preventing allergy

Infants and young children who have or later develop allergies possess different IM from those without allergies (Björkstén *et al*., 2001). It is also known that ***atopic*** children have a higher chance of harbouring harmful species (i.e *clostridium difficile* and *staphylococcus aureus*) but have a lower prevalence of *bacteroides* and *bifidobacteria* (Björkstén *et al*., 2001). Since commensal microflora stimulate the immune system to respond appropriately to antigen, a lack of exposure to these commensal bacteria in early life may lead to an inadequately programmed immune system which over-reacts to antigen (Björkstén *et al*., 2001). This relates to the balance between Th_1/Th_2 immune responses established in infancy. While it is known that uncontrolled Th_1 and Th_2 responses trigger chronic inflammation and allergies, the balance determines the onset and outcome of such disorders (Lenoir-Wijnkoop *et al*., 2003; Akira *et al*., 2001) (refer Chapter 3; Gut immunity, inflammation and tolerance). Conversely, the differences in IM could be the result, not the cause, of the allergies (Björkstén *et al*., 2001).

4.10 The role of commensal enteral microflora in preventing or reducing intestinal inflammation

Commensal microflora help to maintain immune homeostasis within the gut (Kelly *et al*., 2004). Specific strains of commensal microflora (*bacteroides* and *roseburia*) and the use of probiotic organisms can reduce intestinal inflammation and normalise mucosal dysfunction (Kelly *et al*., 2005; Sartor *et al*., 2004).

4.10.1 The role of *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*

Bacteroides species thrive in the intestinal environment using complex systems that sense and adapt to nutrient availability (Wexler 2007). While they generally maintain a beneficial relationship with the host (when they remain in the gut) and influence the host immune system, *Bacteroides* species are significant clinical pathogens (Wexler 2007).

The ability of the gut to tolerate large numbers of potentially pro-inflammatory flagellated commensal microflora is partly explained by the anti-inflammatory effects of commensal microflora (i.e *Bacteroides thetaiotaomicron*) which restrict the signaling induced by both flagellin protein and flagellated pathogens (Kelly *et al*., 2004). The anti-inflammatory activity of *B. thetaiotaomicron* operates at the level of the nucleus, promoting the export of transcriptionally active RelA but leaving TLR-mediated signalling unaffected (Kelly *et al*., 2005).

A healthy gut normally maintains a physiological level of inflammation in response to commensal bacteria, however, the presence of a threshold number of pathogenic bacteria activates NF-κB, inducing pro-inflammatory gene expression, which triggers both innate and adaptive immune mechanisms to destroy pathogenic microorganisms (Moal *et al*., 2006; Kelly *et al*., 2004). Kelly *et al*., (2004) found that *B. thetaiotaomicron* also acts on NF-κB but that its mode of action is distinct from that produced by pathogenic species. *B. thetaiotaomicron* attenuates pro-inflammatory cytokine expression by promoting nuclear export of the NF-κB subunit Rel A through a PPAR-γ dependent pathway. PPAR-γ, in complex with nuclear RelA also undergoes cytoplasmic redistribution in response to *B. thetaiotaomicron.* A decrease in PPAR-γ abolishes both the nuclear export of RelA and the anti-inflammatory activity of *B. thetaiotaomicron.* Kelly *et al*., (2004) observed

differential regulation of the biochemical or biological properties of PPAR- γ by *B. thetaiotaomicron,* but this did not appear to attenuate the IL-α or IL- β-mediated induction of IL-8.

The breakdown of tolerance to commensal bacteria and dietary antigen causes inflammation and ultimately allergic disorders and diseases, so this PPAR-γ dependent anti-inflammatory mechanism defines new cellular targets for therapeutic drug design and interventions for the treatment of such chronic inflammatory conditions (Izcue *et al*., 2006).

Experimentally, Kelly *et al*., (2004) found that *B. thetaiotaomicron* triggers PPAR-γ mediated nuclear export of transcriptionally active RelA that directly abolished *salmonella enteriditis*-induced inflammatory effects in IECs which suggests that commensal bacteria provide protective negative-feedback signals to the IE and are critically important in maintaining intestinal homeostasis.

4.10.1.1 Interaction of Bacteroides species and the intestinal epithelium

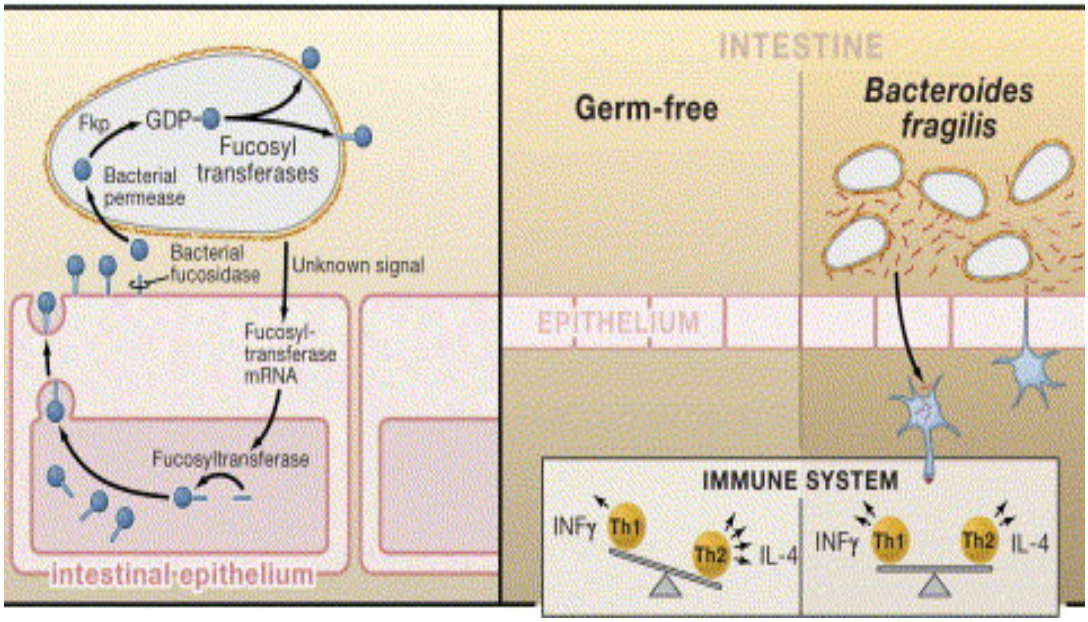
Studies using gnotobiotic mice (i.e. mice that are raised germ-free and then infected with specific bacteria) show that *B. thetaiotaomicron* modulates expression of genes involved in several important intestinal functions including nutrient absorption and mucosal barrier fortification (Hooper *et al*., 2001a: Hooper *et al*., 2001b). It was found that *B. thetaiotaomicron* cross-talks with host cells and competing microbial species then adapts its own metabolism to the environment and influences the host at the expression level of fuco-transferase (Lenoir-Wijnkopp *et al*., 2003). (Even though *B. thetaiotaomicron* does not appear to adhere to the IE, it is still capable of signaling the host to up-regulate such gene expression) (Lenoir-Wijnkoop *et al*., 2003).

A fucose-utilising operon was identified in *B. thetaiotaomicron* which contains genes for enzymes that digest fucose once it has been assimilated by IECs and for sensors that regulate these enzymes. When no fucose is available the enzymes that digest it are not produced, meaning that a signal is generated to induce the production of fucose. Fucose is an essential metabolite required by the body for optimal function of intercellular communication and it is now known that fucose glycoconjugates (glycoproteins and glycolipids) are crucial for eliminating or reversing disease processes (e.g. cancer and inflammation) and it may normalise

immune function (Comstock *et al*., 2006). This could be beneficial for reducing the intestinal damage associated with inflammatory disorders of the gut such as CD.

Bacteroides fragilis is also known to function in a similar manner to *B. thetaiotaomicron* (Bry *et al*., 1996) (see Figure 4.1). This increased gene expression leads to an up-regulation of fucosylated molecules on the epithelium. *Bacteroides* organisms synthesise multiple fucosidases that cleave terminal fucose residues, which allows fucose to be internalised by the bacteria through the action of a fucose permease. Once inside the bacterial cell, fucose can either be catabolised for energy or converted to the nucleotide-activated form (GDP-fucose) by the action of the bifunctional bacterial enzyme Fkp (Coyne *et al*., 2005). Activation of fucose to GDP-fucose ensures that host-derived fucose will be incorporated into capsular bacterial polysaccharides or bacterial glycoproteins. This entire fucosylation process results in a co-ordination of the surface architectures of bacterium and host and probably has significant consequences for the success of *Bacteroides* in the gut.

Figure 4.1: Interaction of *Bacteroides species* and the intestinal epithelium (Comstock *et al*., 2006).



Bacteroides fragilis colonises the GI tract and secretes its capsular polysaccharides into the intestinal lumen where the polysaccharide is endocytosed by DCs that carry it to the mesenteric lymph nodes. $CD4^+$ T-cells are stimulated via presentation of polysaccharides by the MHC-II pathway and results in the production of Th_1 cytokines such as INF-γ, with associated down-regulation of the Th_2 cytokine IL-4 (Comstock *et al*., 2006).

4.10.2 The role of *Roseburia intestinalis*

Roseburia is a bacterial species belonging to the clostridial genus. *Roseburia intestinalis* is a gram-positive, strictly anaerobic, commensal bacteria that promotes anti-inflammatory action within the intestinal environment. This species of microflora are among the most active in metabolising linoleic acid (LA) (18:2) in the colon via conjugated linoleic acids (CLA) to vaccenic acid (18:1) and then to stearic acid (18:0) (Devillard *et al*., 2007). Some strains of *lactobacillus*, *propionibacterium*, and *bifidobacterium* can generate CLA, but the numbers of *lactobacilli*, *propionibacteria*, and *bifidobacteria* are low, (less than 5% of the total microbiota) so their contribution to LA metabolism is not significant (Rosberg-Cody *et al*., 2004; Alonso *et al*., 2003; Coakley *et al*., 2003; Rainio *et al*., 2001; Lin *et al*., 1999; Jiang *et al*., 1998). Devillard *et al*., (2007) found that *Roseburia* spp. are probably the most important organisms involved in metabolising CLA. Given the greater abundance of *clostridium*-like bacteria in the IM, it may be deduced that LA metabolism by this major group is quantitatively important (Eckburg *et al*., 2005). Both animal studies and clinical trials found that CLA (from bacterial sources) is useful in reducing inflammation within the gut (Mensink 2005; Pariza 2004; Belury *et al*., 2002). While the uptake of CLA formed in the intestine is minor, significant local effects on gut tissue have been observed (Kemp *et al*., 2003). It is now well established that CLA have anti-proliferative and anti-inflammatory effects on ***colonocytes*** (Kemp *et al*., 2003), so provision of CLA in the intestinal lumen could be considered beneficial, particularly for control of inflammatory bowel diseases (such as CD, Crohn's disease and ulcerative colitis) (Greicius *et al*., 2004).

Chapter Five: PATHOGENESIS OF COELIAC DISEASE

5.1 Overview of the pathogenesis of CD

The pathogenesis of CD involves interactions between environmental, genetic and immunological factors which trigger immunologically-mediated inflammation in the small intestine and subsequent impaired absorptive function. The key steps underlying the intestinal inflammatory response in CD include: a direct response of the epithelium (via the innate immune system) to deamidated proteins in gluten, the modification of gluten proteins by tissue transglutaminase (tTg),- the autoantigen, the role of HLA-DQ2/DQ8 genes in presenting deamidated proteins to activated CD_4^+T-cells and the identification of key toxic protein sequences in wheat, rye and barley (Quinn 2007). However, these factors do not work in isolation and are all intricately connected.

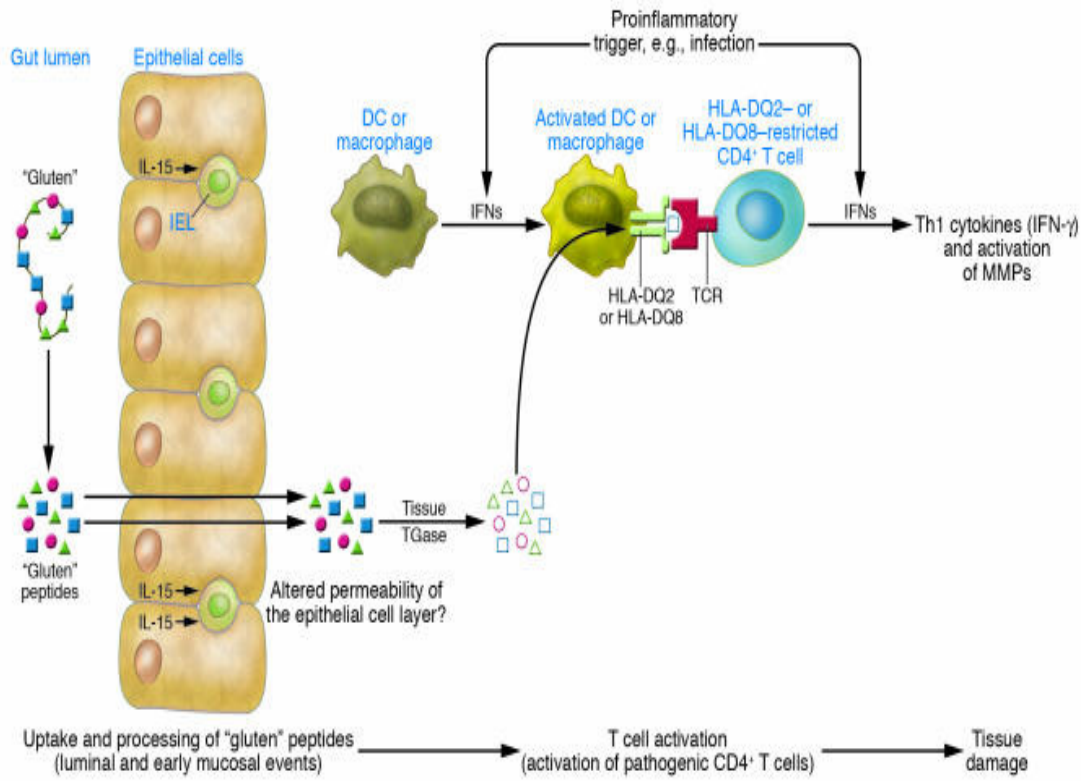
5.2 The development pathway of CD

The pathogenesis of CD essentially occurs in three major steps: luminal and early mucosal events; the activation of pathogenic $CD4^+$ T-cells; and the subsequent events leading to tissue damage.

During the luminal and early mucosal events, key features include the ingestion of 'gluten' by a genetically susceptible person. In people not genetically susceptible, the deamidated proteins are treated as innocuous by the surveillance mechansims of the immune system (Koning *et al*., 2005) (see Figure 5.1).

When 'gluten' is not fully digested, a number of large undigested gluten fragments (gliadin and glutenin) are liberated. Those components surviving digestion and resistant to processing by luminal and brush border enzymes are transported across the epithelial barrier (as polypeptides) to the lamina propria. Here they encounter tTg and APCs that express HLA-DQ2 or HLA-DQ8 heterodimers that are capable of binding proline-rich peptides containing negatively-charged glutamic acid residues resulting from glutamine deamination by tTg. (tTg is mainly located extracellularly in the sub-epithelial region of the intestinal mucosa, but is also found in the brush border (Arentz-Hansen *et al*., 2000).

Figure 5.1: The development pathway of CD (Kagnoff 2007)



This conversion of glutamine to glutamic acid is probably the crucial toxic mechanism in CD (Metcalf 2003). In a further series of events, APCs present these peptides to HLA-DQ2 and HLA-DQ8–restricted populations of $CD4^+$ T- helper cells which are activated to release mediators that ultimately lead to tissue damage (Koning *et al*., 2005).

The activation of $CD4^+$T-cells produces signals that trigger the production of IgA antibodies, anti-tTg-antibodies and lymphocytes which initiates a cascade of reactions that destroys the villi of the IE reducing its ability to absorb nutrients leading to the myriad of symptoms associated with CD (Kagnoff 2007).

There are still many unknowns including the mechanism by which 'gluten' peptides cross the epithelial-cell barrier, the role of innate immunity and IELs in both the early and the late phases of CD pathogenesis, the role of IL-15 and type I-IFNs in disease pathogenesis, the underlying basis for the release of tTg that leads to deamination of gluten peptides, and the sequence of, and relationship between, $CD4^+$ T-cell responses and the responses of the IEL population (Kagnoff 2007).

5.3 Environmental factors associated with CD

The key environmental factor triggering CD is ingestion of proteins found in some dietary cereal grains (i.e. wheat, rye, barley and possibly oats). Collectively, these disease activating proteins (called 'gluten') are responsible for inducing an inflammatory response in the mucosa of the small intestine, which results in the villous atrophy, crypt hyperplasia and lymphocytic infiltration characteristic of CD (Alaedini *et al*., 2005).

5.3.1 The composition of gluten

Cereals are polyploid (i.e. have multiples of the basic number of chromosomes) in nature. When coupled with the high levels of allelic variation in the gluten genes, this results in a high level of genetic and phenotypic variation (Van Heel *et al*., 2006). Although, the precise number of individual proteins in gluten has not yet been fully determined, over 50 have been isolated by electrophoresis (Ciclitira *et al*., 2005; Dewar *et al*., 2004). The protein components of gluten are the major grain storage elements for amino acids within the grain. They are deposited in the developing starchy endosperm and are ultimately used for germination in the grain (Shewry *et al*., 1995).

Gluten proteins synthesised in the rough endoplasmic reticulum are secreted into the lumen of the seed, via a standard signal peptide-mediated mechanism, then subsequently deposited in protein bodies however the mechanism of protein body formation remains unclear (Shewry *et al*., 1995). When the endosperm is milled and the flour mixed with water and kneaded, the storage proteins form an interconnected network called 'gluten'. The structure and properties of gluten are, therefore, partly determined by molecular interactions established in the developing grain. The nature of these interactions must be understood if the properties of gluten are to be manipulated to reduce antigenicity for people with CD (Shewry *et al*., 1995).

Technically, 'gluten' is applied specifically to the prolamin proteins (gliadins) and glutelin proteins (glutenins) in wheat. However, the term 'gluten' is also used in reference to prolamin and glutelin proteins in other cereal grains (Kagnoff 2005). While all cereal grains contain prolamin and glutelin proteins with similar amino acid sequences to 'gluten', these protein components vary between different grains. Not

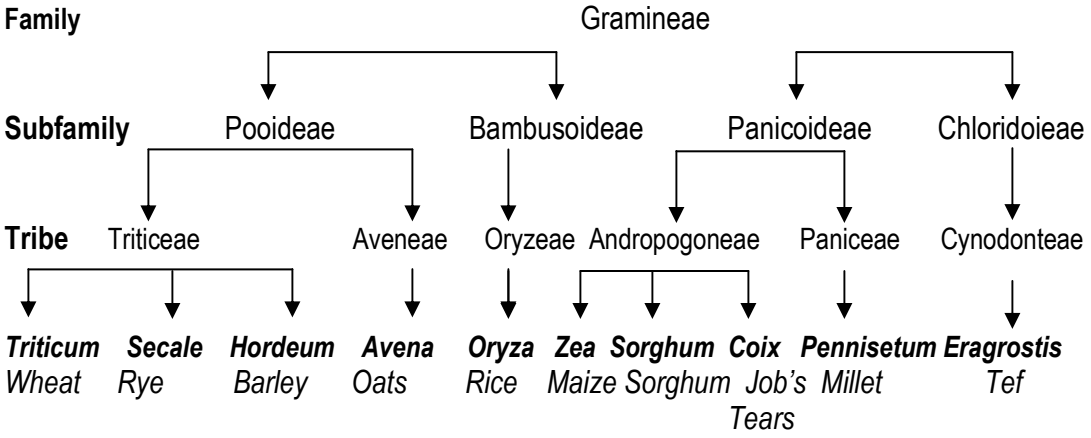
all such amino acid sequences evoke an abnormal immune response in genetically predisposed people (Kasarda, 2003). The term 'gluten' will be used generically in this thesis to describe a combination of prolamin and glutelin proteins found in cereal grains.

Wheat proteins and storage proteins of most other cereal grains are insoluble in water but soluble in alcohol (Hamer 2005; Shewry *et al*., 1995). The gliadins are readily soluble in alcohol due to their monomer configuration. These monomers lack cysteine and have only intra-chain disulphide bonds (Hamer 2005). Gliadins generally comprise about 50% of wheat proteins with the remainder being non-gliadin proteins such as globulins, glutenins and albumins. Proteins deposited in a single wheat variety may contain up to 45 different gliadins. Thus their complexity makes them difficult to investigate (Hamer 2005).

By contrast, glutenins are insoluble in alcohol and comprise high molecular-weight polymers stabilised by inter-chain disulphide bonds. Glutenins are largely responsible for the elasticity of gluten whilst gliadin accounts for its viscosity (Hamer 2005; Shewry *et al*., 1995). The species of grains capable of inducing gluten sensitivity (e.g. durum wheat, spelt, kamut, barley, rye, and triticale) are closely related taxonomically, whereas oats are more distantly related to wheat, rye and barley (Kagnoff 2005; Kasarda, 2004).

The toxicity of oats (*Avena*) is still under debate. The amino acid sequences *Pro-Ser-Gln-Gln-*and *Gln-Gln-Gln-Pro* common in toxic gliadin peptides can be isolated from tryptic digests of wheat, rye, barley and oats (Shewry *et al*., 1995). Thus, it is generally recommended that oats are avoided initially on adoption of a GFD, but may be introduced if purity can be assured and no adverse reaction is noted (Hamer 2005; Kagnoff 2005; Arentz-Hansen, 2004; Lundin *et al*., 2003b). Rice, maize, sorghum, millet, amaranth, buckwheat, corn, Indian rice grass, Job's tears, quinoa, ragi, and teff (or tef) are more distantly related to wheat and are considered 'safe' in CD (Kasarda, 2004b; Kupper, 2004; Kasarda, 2001) (see Figure 5.2).

Figure 5.2: Taxonomy of dietary grains (Kagnoff 2007)



Gluten proteins are unusual in their high proline and glutamine content, comprising ~ 15 and ~35% of residues respectively (Dewar *et al*., 2004). The very high glutamine and proline content of gliadins and glutenins contribute to the pathogenesis of CD.

Proline residues confer resistance to proteolysis by digestive enzymes and influence the selective targeting of glutamine residues by tTG. When a bulky hydrophobic amino acid (i.e. tryptophan) is followed by proline, it may block the activity of intestinal peptidases e.g. pepsin and chymotrypsin. Hence, the presence of proline residues in a protein structure may lead to the generation of oligopeptide fragments. This occurs during the digestive process in all people not just those susceptible to CD (Dewar *et al*., 2004).

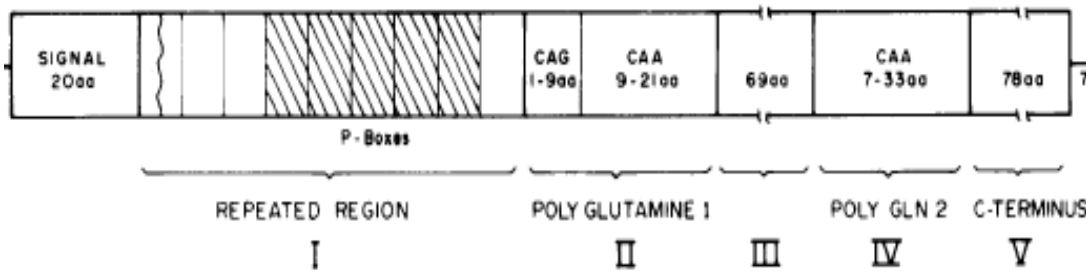
Glutamine contains an additional γ-carboxyamide group and can be deamidated to glutamic acid, converting a neutral molecule to a negative one (Dewar *et al*., 2004). The deamidated peptides show an affinity for HLA-DQ2, a molecule known to preferentially bind peptides containing negatively charged residues (Arentz-Hansen *et al*., 2000). The relatively large gluten oligopeptides (high in proline and glutamine) generated in this process are resistant to degradation by all gastric, pancreatic and intestinal proteases in the brush border. These oligopeptides transit to the submucosa where they initiate a T-cell response (Van Heel *et al*., 2006; Kagnoff 2005).

5.3.2 The structure of gluten proteins and antigenic peptides

Gluten sensitivity is directed against gliadins which are classified as either α, β, γ, and ω-gliadins. α, β, and γ- gliadins are structurally related, but the ω-gliadins are structurally different, comprising a single domain made up almost entirely of a single repeat motif (Ciclitira *et al*., 2005; Dewar *et al*., 2004).

α/β-gliadins consist of a 5 domain structure, containing an N-terminal repetitive region (domain 1), a poly-glutamine region domain 2, a central 'unique' domain (domain 3), a second shorter glutamine-rich domain (domain 4) and a 'unique' C terminal (domain V) (see Figure 5.3). Domain-1 of alpha-type gliadins is involved in activating CD (Ferranti *et al*., 2007; Hamer 2005; Skerritt *et al*., 1992).

Figure 5.3: General structure of α/β-gliadin genes.



(Domain 1) (Domain 2) (Domain 3) (Domain 4)(Domain 5)

The signal sequence precedes five regions in the mature polypeptide defined by analysis of the DNA sequences. The first region (I) consists of a series of nine, typically dodecapeptide repeats. Five of these repeats (crosshatched) are closely related to each other. Two polyglutamine stretches (II) and (IV) separate two regions of non repeated sequence (V)

Early studies implicated only the α-gliadins in CD (Kendall *et al*., 1972), however later studies implicate both β and γ, but not ω-gliadins (Ensari *et al*., 1998). More recently all gliadin fractions have been implicated, with decreasing antigenicity from α to ω-gliadins (Aleanzi *et al*., 2001; Osman *et al*., 2000).

Despite the diversity of gluten epitopes, relatively few epitopes account for most of the interactions with $CD4^+$T-cells in CD (Shan *et al*., 2002). A unique 33-mer peptide fragment of α-gliadin is particularly important in initiating immune response to gluten, as it contains a number of protease resistant epitopes. An immuno-dominant T-cell epitope within the 33-mer gliadin fragments has been identified in the peripheral blood and small intestinal T-cells of people with CD. Deamidation of

glutamine at position 65 of this T-cell epitope is crucial for $CD4^+$T-cell recognition and activation (Dewar *et al*., 2004). Very recently, a further 19-mer peptide and a 25-mer peptide have also been linked to immune responses in CD (Ferranti *et al*., 2007).

The optimal requirement for MHC binding and T-cell stimulation is a peptide of 10-15 amino acids. The peptic-tryptic or chymotryptic digests of gluten contain many such peptides (Fraser 2004; Hausch *et al*., 2002; Shan *et al*., 2002). The gluten epitopes involved in the pathogenesis of CD are shown in Table 5.1 in Appendix D. Vader *et al*., (2002a) found that in children with CD, half the T-cells do not react with immuno-dominant epitopes. Several minor epitopes have been characterised within both gliadins and glutenins, which also stimulate gluten specific T-cell lines and some T-cells show cross-reactivity between different epitopes. The disparate results between adults and children with CD could be due to the fact that in early stages of CD T-cell reactivity evolves from a diverse array of native gluten peptides, and converges towards more immuno-dominant epitopes as the disease progresses (Vader *et al*., 2002a).

These considerations all apply to the association of oligopeptide epitopes with T-cells as associations with B-cell epitopes have been less well investigated (Osman *et al*., 2000).

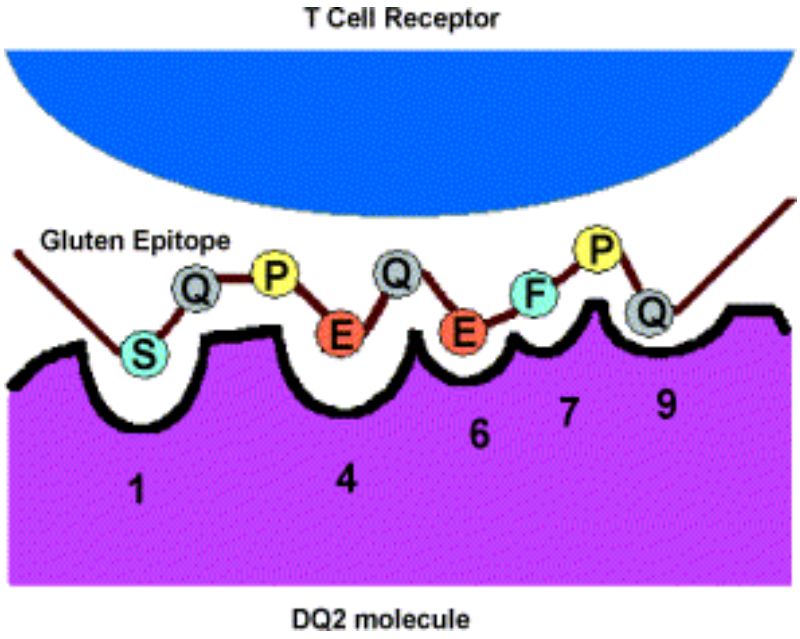
5.3.3 The exclusive association of gluten peptides with HLA-DQ2 and DQ8

The exclusive association of HLA-DQ2/DQ8 with CD suggests a pivotal interaction between gluten peptides and these class-II MHC molecules. The peptide binding properties of DQ2 have been extensively studied, whereas HLA-DQ8 is less studied (Wolters *et al*., 2008; Kagnoff 2007; van Heel *et al*., 2006; Dewar *et al*., 2004; Arentz-Hansen *et al*., 2000; Arentz-Hansen *et al*., 2002). A different set of epitopes have been identified for DQ8, including an immuno-dominant peptide, but as yet no explanation as to how comparable susceptibility to CD is conferred by HLA DQ8 has been elucidated (Dewar *et al*., 2004).

DQ2 shows preferential binding of peptides containing negatively-charged amino acids. While gluten contains few negatively-charged residues, glutamine residues

do when deamidated to glutamic acid. The allergenicity of gliadin epitopes is confirmed by Ellis *et al.*, (2003), who found key residues are required at specific positions (1, 4, 6, 7 and 9) to anchor these peptides in the HLA-binding groove (Figure 5.4). It was subsequently found that because negatively-charged residues are specifically preferred in positions 4, 6 and 7 of the antigen-binding groove of HLA-DQ2, deamidated gliadin elicits a strong T-cell response (Wolters *et al.*, 2008; Kagnoff 2007; van Heel *et al.*, 2006).

Figure 5.4: Diagram of the interaction between the DQ2 molecule peptide-binding groove and an epitope from gliadin.

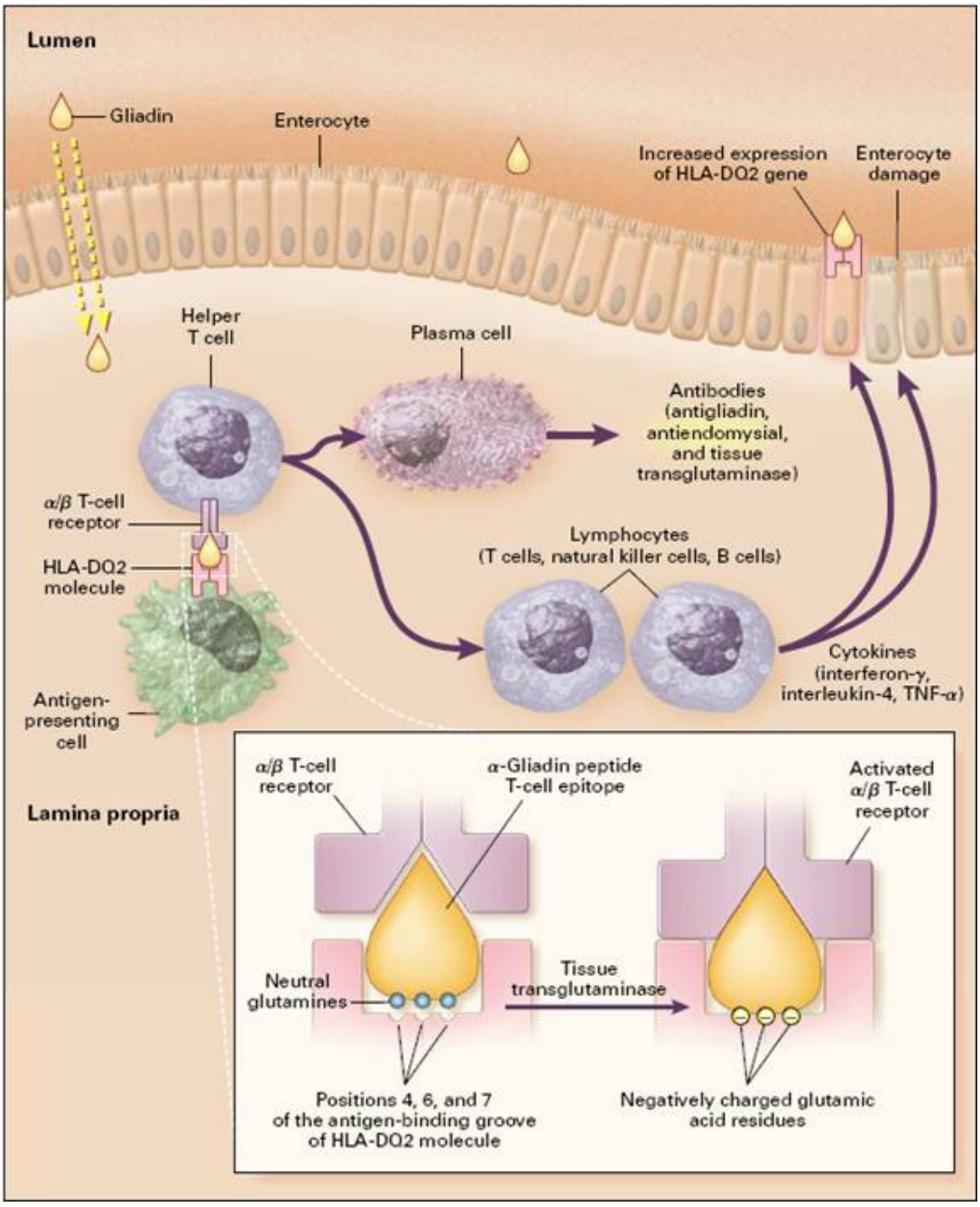


Key anchor points are at positions 1, 4, 6, 7 and 9. Negative charge is preferred at positions 4, 6 and 7. E represents deamidated residues with production of glutamic acid.

Q and P denote glutamine and proline, respectively (Dewar *et al.*, 2004).

CD is limited to people with HLA-DQ2 or DQ8 because their peptide binding groove has several "pockets" that favour negatively-charged residues and HLA-DQ2 prefers to bind to peptides with a left handed poly-proline II configuration, which is characteristic of gluten peptides (Kagnoff 2007). Any alteration of positioning within the groove leads to reduced reactivity, suggesting that T-cell receptor interaction is critically sensitive to amino acid structure. Large gliadin peptides that contain multiple HLA-DQ binding epitopes have greater T-cell stimulatory activity than smaller peptides containing a single HLA-DQ2 binding sequence.

Figure 5.5: A diagram of gliadin-HLA interactions underpinning the pathogenesis of CD (Wolters *et al.*, 2008).



The gliadin reactive CD_4^+T-cell response contributes to damage of the intestinal mucosa culminating in villous atrophy and crypt hyperplasia (Rizzello *et al.*, 2007).

5.3.4 Other environmental considerations

While it is clear that ingesting gluten is the key environmental factor involved in the presentation of CD, this is not always a sufficient cause. Hence in people with genetic susceptibility, other environmental and inherited factors may come into play (Kagnoff 2005; Forsberg *et al*., 2004). Epidemiological and clinical evidence suggests that factors influencing the composition of gut microflora could also act as triggers, or predispose people to CD. Hence milk feeding practices, early microbial infection and the use of antibiotics all have the potential to alter glycosylation in the glycocalyx or mucous layer of the IE (Ivarsson *et al*., 2003; Ivarsson *et al*., 2002), which could facilitate pathogenic bacterial adhesion. Forsberg *et al*., (2004) found increased pathogenic bacterial penetration and adhesion, in people with both active and treated CD however, it is not possible to conclusively state that altered glycosylation and bacterial adhesion are predisposing factors in the development of CD.

5.4 Genetic factors associated with CD

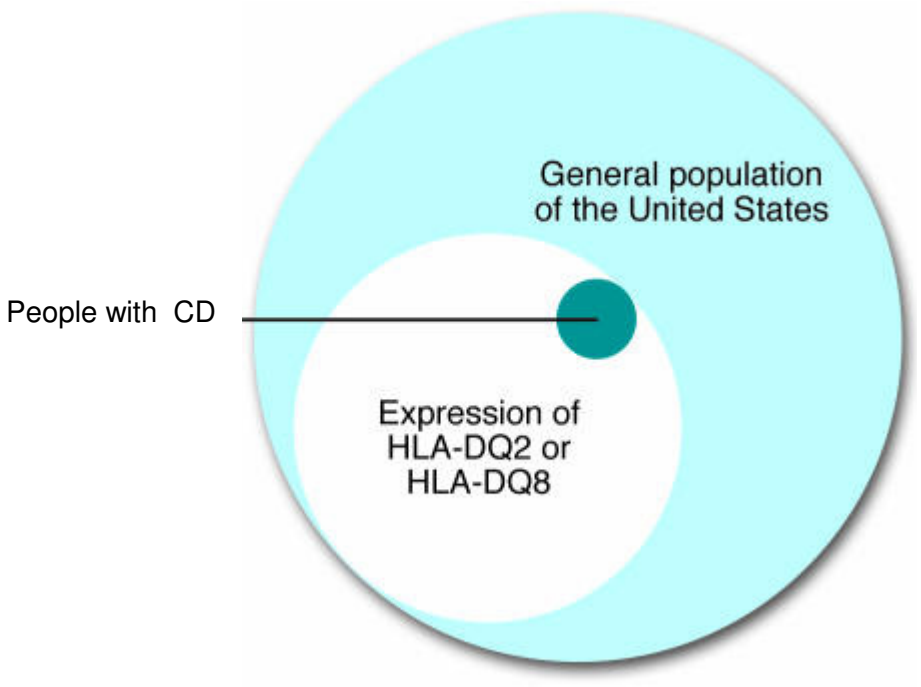
5.4.1 Genetic susceptibility/ predisposition

Genetics clearly contribute to the pathogenesis of CD. There is a 5-15% prevalence of multiple cases of CD within affected families (i.e. ~10% of first degree relatives have CD, a risk 20-30 times greater than in the general population) and a concordance with monozygotic twins greater than 75%, but only an 11% concordance with dizygotic twins (Wolters *et al*., 2008; Kagnoff 2005; Howdle 2003) (see Chapter Two).

Accumulating evidence suggests that CD is a polygenic disorder involving both HLA and non-HLA genes, but susceptibility is specifically associated with HLA-DQ2/DQ8 (Troncone *et al*., 2008; Kagnoff 2005). HLA-DQ2 is expressed in 90-95% of people with CD whilst HLA-DQ8 is found in most of the remaining 5-10% (Kagnoff 2005). In one study 90% of participants possessed genetic variants encoding for HLA-DQ2 and 6% had HLA-DQ8 without DQ-2 (Karell *et al*., 2003). Approximately 25-30% of people in the general population carry the DQ2 or DQ8 gene but only a small proportion of people who express this molecule will develop CD (Troncone *et al*., 2008; van Heel *et al*., 2006). It is estimated that HLA genes (DQ2 and DQ8) account for ~ 40% of the heritability of CD (Louka *et al*., 2003) which confirms that

possession of HLA-DQ2 or DQ8 is necessary, but not sufficient for the development of CD (van Heel *et al.*, 2006).

Figure 5.6: Distribution of HLA-DQ2 and HLA-DQ8 in the general population and in CD (in United States) (Kagnoff 2007)



This Venn diagram shows the distribution of HLA-DQ2 and DQ8 in the general population in the US, and its expression within that population. Only a small subset of people expressing the HLA-DQ2 or DQ8 gene will develop CD.

Interestingly, and consistent with the known DQ genetics of disease susceptibility, (as discussed in Chapter 2) CD is rare in populations where the disease associated DQ alleles are also rare (e.g. Japan) (Kagnoff 2005).

5.4.2 Gene and HLA Nomenclature

The genetics underlying CD are complex and the nomenclature requires a little explanation. A gene is the segment of DNA that contributes to phenotype or function. In the absence of demonstrated function, a gene is characterised by its sequence, transcription or homology. The word "***locus***" is not a synonym for gene but refers to a map position. In the context of gene nomenclature "chromosome region" is a genomic region associated with a particular syndrome or phenotype, particularly when there is a possibility of several genes within it being involved in

the phenotype (Wain *et al*., 2002). This naming system is distinct from HLA nomenclature.

HLA is the name for the genetic system on human chromosome number 6, responsible for the presentation of foreign peptides to the immune system. Several protocols exist for an HLA antigen description e.g as HLA-DR3, HLA-DR17, HLA-DRB1*03 or HLA-DRB1*0301. These could all refer to the same antigen (ASEATTA 2003). The second part (DR) denotes the specific locus. There are 6 loci; A, B, C, DR, DQ and DP. The HLA-A, B and C loci produce antigens that normally present peptides of viral origin and are expressed on all nucleated cells. The HLA-DR, DQ and DP loci produce antigens that normally present peptides broken-down from bacterial or other proteins engulfed by the cell during immune surveillance. They are only expressed on cells actively involved in an immune response (e.g. B-cells and activated T-cells). The HLA-DR, DQ and DP antigens are termed Class-II. There are other Class-I loci besides A, B and C and other Class-II loci besides DR, DQ and DP. However, these loci are not normally tested for and their significance is not entirely clear (ASEATTA 2003).

The third part of the desription; the number (e.g. 3, 17, 03, 0301), refers to the actual antigen at the locus. This difference results in a specific type HLA-DR molecule. These various HLA-DR molecules are given individual names (i.e HLA-DR17) however the method of writing the antigen based on using antibodies that react to the antigens is outdated. DNA can now be observed directly so accuracy is much greater allowing for identification of more variation between the different antigens. A new designation system was devised to accommodate this (ASEATTA 2003).

The term HLA-DR3 is the name for a specific group of antigens and it is the broadest description of the antigen. The DR3 group divides into HLA-DR17 and HLA-DR18 by serology. At the DNA level, the DR locus is called DRB1 (because there are others termed A and B2, B3, etc) and 03 for the general antigen or 01 for the specific variant of 03. So, the new designation for HLA-DR17 is HLA-DRB1*0301. This is similar for other antigens, at either HLA Class-II or Class-I. e.g. HLA-B60 becomes (HLA-B*4001) (ASEATTA 2003).

5.4.3 Identification of genetic susceptibility genes in CD

Two complementary approaches are used to find genetic susceptibility genes in CD.

(1) genetic linkage studies

(2) genetic association studies.

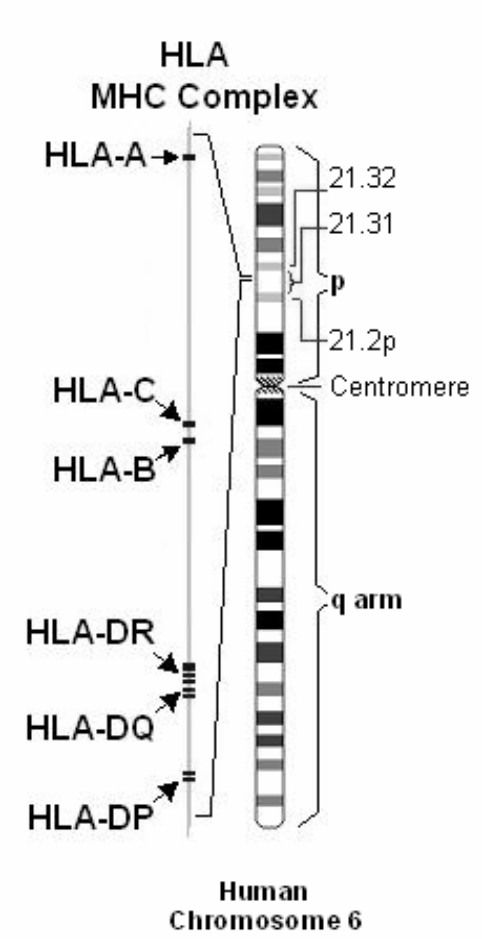
Genetic linkage studies use familial clustering (affected sibling pairs) to identify chromosomal regions shared between the affected siblings above the mean of what is statistically expected. Linkage regions usually encompass 10–100 genes (Wolters *et al*., 2008).

Once linkage is identified, a genetic association study is performed to identify the specific disease gene from the candidate gene locus. Candidate gene association studies search for differences in frequencies of genetic variants in people with CD compared to control individuals. Such association studies either focus on positional candidate genes from a linkage region, or on functional candidate genes selected from hypothesised disease pathology. More recently, it has become feasible to perform genome-wide association studies (Wolters *et al*., 2008).

Such genetic linkage analyses have identified susceptibility loci on various chromosomes (such as 2, 5, 6, 9, 15, and 19), revealing the complexity of CD (Wolters *et al*., 2008). It is already known these areas code for a number of genes implicated in other immune-mediated diseases (Wolters *et al*., 2008). In people with CD, particular interest has focused on chromosome regions 2q, 5q, 11q and 15q, especially in a locus at 2q33 (Dewar *et al*., 2004). However, the principal determinants of genetic susceptibility are the highly variable HLA-class-II DQ genes located in the MHC on chromosome 6 (Figure 5.7) (Troncone *et al*., 2008). (The terms HLA and MHC are sometimes used interchangeably).

The HLA chromosome region 5q located on the long arm of chromosome 5 and 11q is another potentially interesting region for a susceptibility factor in CD (Quinn *et al*., 2007), along with a significant linkage at chromosome 19p13.1. This chromosome is already known to have significant linkage to IBD (*IBD6*) (van Belzen *et al*., 2003).

Figure 5.7: The HLA/MHC complex on human chromosome 6



5.4.4 Genetic (Allelic) structure

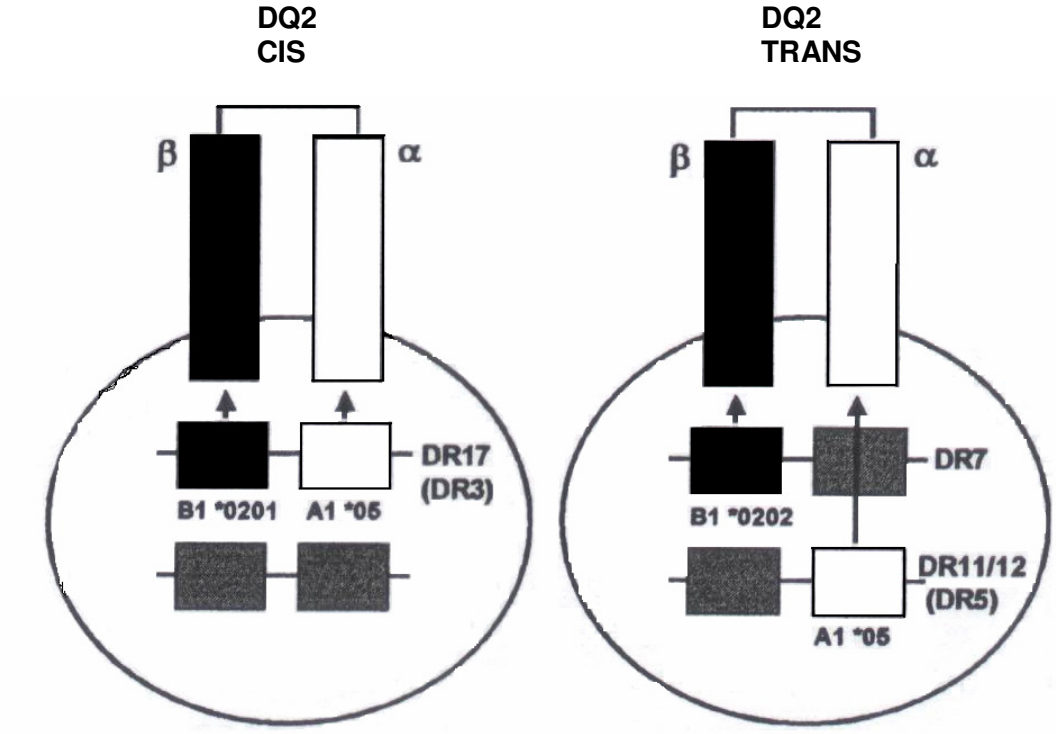
The HLA-DQ2 heterodimer encodes in several different ways, leading to variation in the proportion of HLA-DQ2 molecules present (van Heel *et al*., 2005).

The DQ2 heterodimer is formed by a β-chain encoded by the allele DQB1*02 (either DQB1*0201 or *0202) and an α-chain encoded by the allele DQA1*05 whereas the HLA-DQ8 associated heterodimer is formed by a β-chain and α-chain encoded by DQB1*302 and DQA1*03 respectively (Troncone *et al*., 2008; Kagnoff 2005).

DQ2 susceptibility alleles are inherited either in *cis* or *trans* configuration with one DQ allele coming from a chromosome of each parent (see Figure 5.8) (Kagnoff 2005). People with the DR17 (previously called DR3) haplotype carry alleles in *cis* configuration whilst those who are heterozygous for DR11/12 (formerly DR5) and

DR7 carry these alleles in *trans*. In both cases, the relevant APCs of these people express the disease related DQ2 heterodimer (Kagnoff 2005).

Figure 5.8: The two ways of inheriting the DQ2 heterodimer associated with CD



In people homozygous for DR17, all of the DQ molecules are DQ2. In those heterozygous for DR17, around 50% of the DQ molecules are DQ2, whilst in those heterozygous for DR5/7 or DR17/other, around 25% of the DQ molecules are DQ2 (Kagnoff 2005).

CD is more prevalent in people where 50-100% of DQ molecules are DQ2 than in those who have 25% of the DQ molecules as DQ2 (Kagnoff 2005). So although only about 2 % of the population is homozygous for DR17, this group may account for as many as 25% of all people with CD (Kagnoff 2005). Individuals who are homozygous for DQB1*02 appear at highest risk of developing CD (Wolters *et al.,* 2008). Nevertheless, once CD develops the clinical course of the disease is similar in all cases, where the DQ molecules are DQ2. CD is also found associated with DR53 (Wolters *et al*., 2008).

5.4.5 Other genes associated with CD

Further association analyses show an association between CD and the myosin IXB gene (*MYO9B)* (Wolters *et al*., 2008). Interestingly, *MYO9B* is a good candidate

gene for CD, because of its function. It encodes an unconventional myosin molecule that has a role in actin remodeling of epithelial enterocytes. This genetic variant might lead to an impaired intestinal barrier, thus allowing the passage of immunogenic gluten peptides which could be a factor involved in the early mucosal events preceding the inflammatory response in CD (Wolters *et al*., 2008).

A strong association to *MYO9B* is reported in people with refractory CD type-II (a severe form of CD). In this group, the enteropathy persists, despite adherence to a GFD or it recurs after an initially good response to the diet. Refractory CD is characterised by the presence of aberrant IELs in the mucosa of the small intestine (Wolters *et al*., 2008). *MYO9B* might also promote susceptibility to other intestinal inflammatory diseases, although the precise mechanism of how gene variants in *MYO9B* lead to altered gut function is unclear (Wolters *et al*., 2008). *MYO9B* has recently been associated with ulcerative colitis (van Bodegraven *et al*., 2006) and with Crohn's disease (Rioux *et al*., 2000).

The cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)-a negative co-stimulatory molecule of the T-cell response was pinpointed as a candidate gene in CD, before the era of genome-wide linkage studies (Wolters *et al*., 2008). Although no evidence for a single mutation in *CTLA4* specific to CD has been found, a strong association is indicated at the haplotype level (Wolters *et al*., 2008). Other genes like tTG, FAS, MMP-1 and 3, TCR αβγδ, IL12β, CD28, CD80, CD86, KIR, LILR, STAT 1, PGPEP1, IRF1, DPPIV, TGM2, NOS2, or IFN-γ have also been linked with CD, however studies have not confirmed this association (Wolters *et al*., 2008; van Heel *et al*., 2005).

Recently, a genetic association between Factor V Leiden (FVL) and CD was identified (Mari *et al*., 2006). A family was described in which these two diseases segregated in all cases as no sibling was affected by only one of the diseases, suggesting that the genetic mutation responsible for the development of CD in this family occurs in a gene very close to *FVL* on chromosome 1q. Further assessment of this association is needed (Mari *et al*., 2006).

5.5 Immunological factors associated with CD

5.5.1 CD as an autoimmune disorder

CD typifies how genetics interact with an environmental trigger to initiate allergic disease (Papadopoulos *et al*., 2001). The time and amount of gluten ingestion in infancy is emerging as a risk factor for the development of CD and other autoimmune diseases, i.e. type-1 diabetes mellitus (Troncone *et al*., 2008). In fact, CD is associated with concomitant autoimmune disease, 5–10 times more frequently than in the general population (Troncone *et al*., 2008). The association of CD with other autoimmune conditions results from a common genotype i.e. HLA (HLA-DQ2/8) found in people with other autoimmune diseases (van heel *et al*., 2007; Monsuur *et al*., 2005).

CD is autoimmune because of the highly disease-specific autoantibodies to gliadin and tTg in both serum and intestinal mucosa (Sollid *et al*., 2005b; Zarkadas 2005). Gluten avoidance prevents mucosal damage, however the permanent intolerance to gluten indicates the development of immunological memory (Howdle 2003).

5.5.2 Pro-inflammatory immune mechanisms of CD

Whilst both humoral and cell-mediated mechanisms are involved in the development of CD, the relative significance of each is unknown (Howdle 2003). Once gliadin oligopeptides enter the lamina propria after changes in intercellular tight junctions and increased intestinal permeability, plasma cell numbers increase leading to production of antibodies to gliadin and tTg. The resulting gliaden-tTg complex initiates the immune reaction (Alaedini *et al*., 2005; Forsberg *et al*., 2004Howdle 2003). An increase in T-cells (mainly $CD4^+$) in the lamina propria triggers production of pro-inflammatory cytokines and CD8 cells in the mucosa (Howdle 2003).

5.5.2.1 Humoral responses

CD is a non-IgE mediated, type IV-delayed hypersensitivity reaction, however it involves the production of immunogloblins including IgE, in response to the presence of gliadin oligopeptides (Howdle 2003; Lundin 2003). Secretion of IgE is commonly seen in allergies and data suggests that cross-linking of bound IgE on B-cells controls antigen presentation, allowing for uptake of antigen that direct the

immune response towards a B-cell response. Conversely, IgG directs towards a response by macrophages and monocytes (Howdle 2003).
Past studies show an increase in the concentration of antibody (IgM, IgA IgG and IgE) producing cells during untreated CD when compared with controls (Lundin *et al*., 2003). Anti-gliadin and anti-tTg autoantibodies are detected in both treated and untreated CD, suggesting a role for antibodies and immune complexes in the pathogenesis of CD.

However, in cases of gluten removal from the diet, a decrease in mucosal damage is seen with persisting autoantibodies. Even small amounts of gluten and possible cross-reacting food antigens cause increases in antibody concentration by generating a locally-produced acute response. Some mucosal damage may be caused by a possible immunopathogenic role of IgM by fixing complement. Little evidence exists to suggest this persists or culminates in the mucosal damage seen in untreated CD (Lundin *et al*., 2003). In treated CD, IgM plasma cells persist even when the jejunal mucosa has normalised, whereas IgA persistence is only found in people with minor histological damage. The causative nature of such an increase in antibody production is uncertain (Lundin *et al*., 2003).

5.5.2.2 Cellular responses

In active CD, the lamina propria expands in volume, due to recruitment of distinct populations of T-cells, plasma cells and dendritic macrophages that express HLA molecules, ICAM-1 and CD25 (IL-2 receptor α-chain)-an infiltrate indicative of a T-cell mediated immune response (Dewar *et al*., 2004) (see Chapter Three).

T-cells classify as $CD4^+$ and $CD8^+$ T-cells according to their surface molecules and function. $CD8^+$T-cells down-regulate the immune system whilst $CD4^+$ T-cells (helper cells) divide into two types, based on their pattern of cytokine secretion: Th_1 $CD4^+$ T-cells release interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α as cytokines and Th_2 $CD4^+$ T-cells secrete IL-4, IL-5, IL-6, and IL-10 (Spiekermann *et al*., 2001).
Th_1 $CD4^+$ T-cells mediate cellular immune responses, whereas Th_2 $CD4^{+T}$-cells are more critical to humoral immunity. Activated Th_1 or Th_2 cells help other cells either through direct cell-cell interaction or through secretion of cytokines. Th_2 cells

facilitate the proliferation of B-cells and their differentiation into antibody-secreting plasma cells (Spiekermann *et al*., 2001).
$CD4^+$T-cell activation only follows presentation of gliadin by HLA-DQ2 molecules on B-cells, indicating both humoral and cellular involvement in CD (Lundin *et al*., 2003; Przemioslo *et al*., 1995).

Most T-cells are $CD8^+$T-cells which possess α/β TCRs expressed by NK cells, (i.e. CD94) suggesting they may be cytotoxic (Jabri *et al*., 2000). Russell *et al*., (1993) found perforin within membrane-bound granules, thus providing circumstantial evidence that T-cells (especially the γ/δ subset) possess cytolytic characteristics. This could account for the mucosal damage seen in CD, although the exact mechanism is not clear (Meresse *et al*., 2006). A number of studies show an increase in T-cells, especially γ/δ types; specifically an increase in the CD3+CD8-CD4-, TCR γ/δ $^+$T-cell population (Kagnoff 2007). Activation of this double negative population of T-cells ($CD8^-CD4^-$) leads to cell damage, since this prompts the release of IFN-γ, which triggers alterations in epithelial-cell permeability thus exacerbating the disease (Kagnoff 2007).

IFN-γ also increases HLA-DQ expression, permitting presentation to $CD4^+$T-cells to further activate T-cells and macrophages, again possibly contributing to mucosal damage (Halstensen *et al*., 1990b). In the lamina propria, the up-regulation of IL-15 expression by epithelial cells and DCs contributes to altered signaling properties of CD 8^+T-cell populations (Kagnoff 2007). Unlike the $CD8^+$ T-cells, the double-negative population ($CD8^-CD4^-$) does not regress on gluten withdrawal. This population may actually be part of innate immunity rather than acquired immune mechanisms, as they do not require HLA for antigen recognition, and they recognise stress proteins i.e. ***MICA*** and ***MICB*** expressed on epithelial cells (Meresse *et al*., 2006).

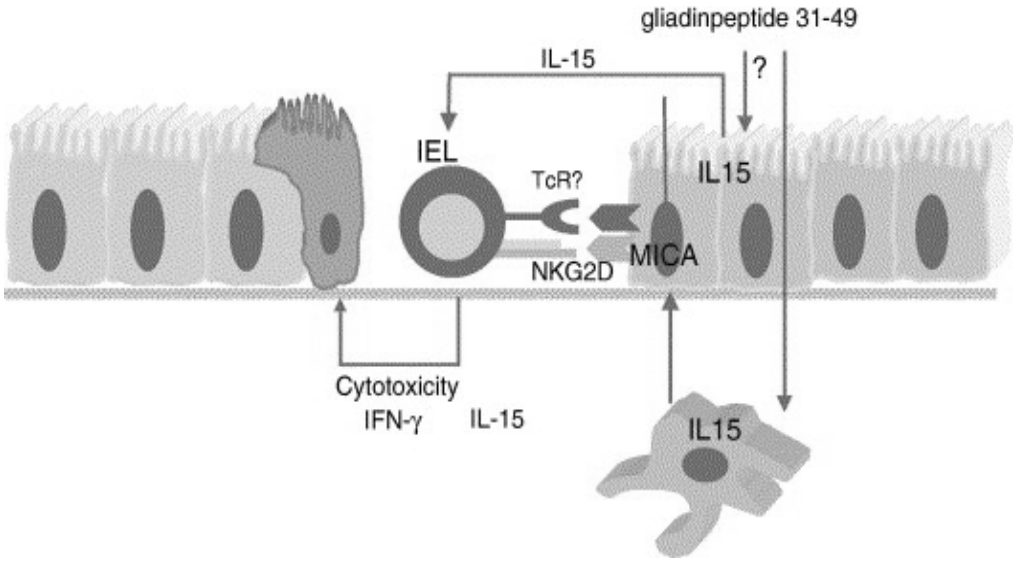
It is not only the presence of the various TCR subsets that conveys differing functions, but also the differing effects of CD45 isoforms. CD45 is a tyrosine phosphatase, with the differing isoforms dephosphorylating different substances. $CD45RO^+$cells may possess a primed antigen memory function and they show increased expression of adhesion molecules and more abundant secretion of IFN-γ. These $CD45RO^+$ T-cells produce IL-2, IL-4 and IFN-γ, and provide help for B-cells

(Halstensen *et al*., 1990). Halstensen *et al*., (1990) found that gluten exposure caused an increase in the number of CD45RA cells indicating the existence of a population of $CD45RO^{+}$ or memory T-cells, particularly on TCR γ/δ+ T-cells. In general, CD can be conferred by genetic susceptibility with ongoing exposure activating and reactivating primed memory CD45RO+ γ/δ+ T-cells (Kerttula 1998).

5.5.3 Enterocyte processing of gliadin epitopes

Initially gluten was thought to be directly toxic to enterocytes. In fact, several recent reports suggest that an early event in CD pathogenesis may be the direct effect of gluten peptides on the IE (e.g the α-gliadin, p 31-43 (LGQQQPFPPQQPY) which induces changes associated with CD within 4 hours of ingestion). This response is too rapid for a purely T-cell mediated response and precedes lymphocyte infiltration (Kagnoff 2005; Koning 2005; Hue *et al*., 2004). Maiuri *et al*., (2003) showed that this α-gliadin peptide induces secretion of IL-15 (a key cytokine involved in T-cell activation) by enterocytes.

Figure 5.9: Mechanisms leading to the activation of T-cells by IL-15 in CD



Peptide 31-49, common to the N-termini of α-gliadins, induces production of the cytokine IL-15 in IECs and macrophages, via as yet unknown mechanisms. IL-15 stimulates the cytotoxic properties of T-cells and their expression of NKG2D and induces the expression of MICA, the epithelial ligand of NKG2D. Binding of NKG2D to MIC triggers the cytotoxicity of T-cells against IECs. In IL-15 stimulated T-cells, (as in active and refractory CD), MIC-NKG2D-mediated cytotoxicity of T-cells occurs independently of a specific signal given via the TCR. The contribution of additional signals from the TCR and/or other innate receptors is not excluded (Koning *et al*., 2005).

IL-15 also induces expression of a stress molecule MICA on enterocytes and up-regulates ***NKG2D*** receptors on T-cells rendering them susceptible to γ/δ^{+} T-cell NK-like activity confirming the earlier observations described above. The interaction between enterocyte MICA and lymphocyte NKG2D kills enterocytes, and is the likely mechanism of villous atrophy in CD (Allez *et al*., 2007; Koning 2005; Hue *et al*., 2004) (Figure 5.10).

5.5.4 Failure of oral tolerance and hypersensitivity in CD

Oral tolerance allows the immune system to suppress responses to prevent over-reactivity (Barbeau *et al*., 2007; Wiedermann 2003). Tolerance is induced by cellular cross-talk between immune cells resident in GALT (see Chapter Three). While tolerance is usually a robust adaptive immune mechanism, failure to induce long-lasting tolerance forms the basis of food hypersensitivity and intolerances (Wiedermann 2003; Buttriss 2002). This is the case with CD where abrogation of tolerance to gliadin peptides underlies its pathogenesis (Mowat 2003b; Eigenmann *et al*., 1999).

Kaufmann (1996) believes the population of γ/δ^{+}T-cells in the intestine specifically cancel out oral tolerance, thereby facilitating the development of CD. This is consistent with earlier research reporting that transfer of antigen-specific CD3^{+}CD4^{-}CD8^{-} T-cells from nude BALB/c mice into normal BALB/c mice, resulted in abrogation of oral tolerance (Fujihashi *et al*., 1989). A follow-up study found two γ/δ T-cell receptor positive (γ/δ TCR^{+}) subsets involved in abrogation of oral tolerance, a double negative CD3^{+}CD4^{-}CD8^{-} and a CD8^{+} CD3^{+}CD4^{-}CD8^{+} subset (Fujihashi *et al*., 1992).

5.5.4.1 Effect of increased intestinal permeability

Intracellular tight junctions in the intestinal epithelium open when zonulin is secreted into the intestinal lumen. This step is critical in the pathogenesis of CD, as it allows gliadin oligopeptides to reach the lamina propria (Barbeau *et al*., 2007). Zonulin concentration increases when high numbers of pathogenic bacteria are present in the gut (Drago *et al*., 2006). Opening of the tight junctions triggers an influx of water into the intestine which removes bacterial pathogens. Evidence (Barbeau *et al*., 2007; Drago *et al*., 2006; Clemente *et al*., 2003) suggests that gliadin proteins and peptides also activate zonulin-dependent intracellular signaling pathways that open

tight junctions and increase intestinal permeability, which likely facilitates delivery of gliadin to the lamina propria via the paracellular route.

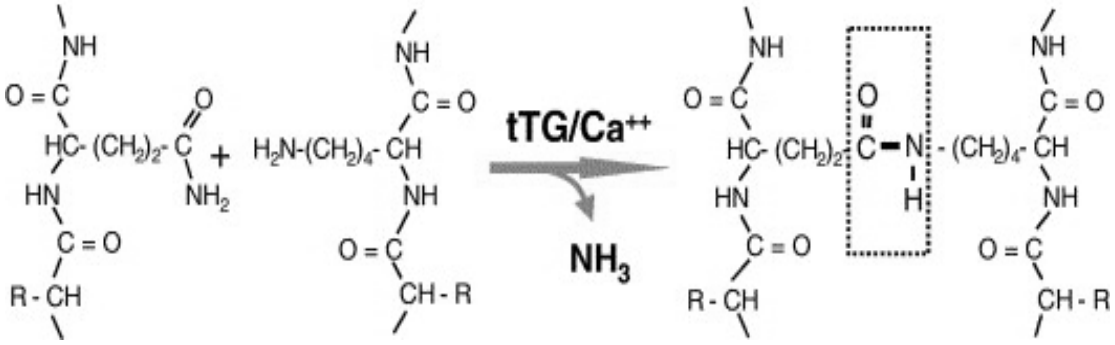
5.6 The role of tissue transglutaminase in CD

tTg is a widely distributed enzyme that can cross-link proteins (Fleckenstein *et al*., 2002; Molberg *et al*., 2000). The cross-linking activity of tTg is involved in wound healing, the formation of cell envelopes in apoptosis, and the stabilsation of the extracellular matrix (Bateman *et al*., 2004; Reif *et al*., 2004; Mowat 2003). Cross-linking and deamidation of tTg is dependent on having a low pH, an abundance of glutamine residues and a low availability of protein-bound lysine (Koning *et al*., 2005). The most established property of tTg (and other transglutaminases) is to catalyse a Ca^{2+}-dependent cross-linking of a specific glutamine residue in the substrate protein to a primary amine (Molberg *et al*., 2000).

5.6.1 Enhancement of gliadin antigenicity

tTg is critical to the pathogenesis of CD as it augments the antigenic epitopes present in α- gliadin prior to their recognition by T-cells. It catalyses an acyl transfer between the γ-carboxamide group of glutamine and the α-amino group of lysine or a soluble primary amine. This leads to the formation of an irreversible, highly resistant iso-peptidyl bond between glutamine and lysine residues of corresponding proteins which produces gliadin-gliadin or gliadin-tTg complexes, thus creating antigenic epitopes that initiate an immune response in genetically susceptible people (see Figure 5.10) (Koning *et al*., 2005;Ciccocioppo *et al*., 2003).

Figure 5.10: Cross-linking of proteins by tTg

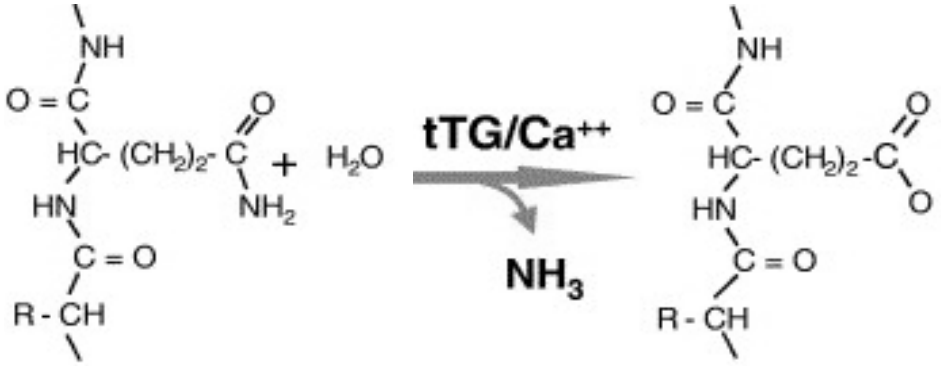


Ciccocioppo *et al*., (2003) identified *in vitro*, that tTg permits or enhances T-cell responses to gliadin epitopes. As prolamins are high in glutamine residues they make excellent substrates for tTg, although only particular glutamine residues are targeted. These findings confirm research by Dieterich *et al*., (1997b) which found that tTg-gliadin complexes form *in vitro.* They argued that these complexes contain 'neo-epitopes' that trigger mucosal T-cells and degrade tolerance. They also suggested that production of the anti-tTg-IgA antibodies are dependent on activated T-cell help to facilitate the isotype switching of the autoreactive B-cells.

5.6.2 Formation of tTg gliadin complexes

In the absence of a suitable lysine residue and at relatively low pH (<7) an alternative reaction occurs. Deamidation of glutamine with water, (catalysed by tTg) forms glutamic acid and ammonia (Koning *et al*., 2005; Mowat 2003).

Figure 5.11: Deamidation of protein by tTG



This tTg generated deamidation leads to the additional formation of hydrogen bonds within or between proteins (Bateman *et al*., 2004). Again the reaction is calcium-dependent (see Figure 5.11). Deamidation results in the generation of gluten peptides with the negatively-charged residue glutamic acid which facilitates gluten peptide binding to HLA-DQ2 and HLA-DQ8 (Koning *et al*., 2005).

Identification of tTg as the target of CD-specific IgA anti-endomysial antibodies cemented the idea that these large complexes are critical to the pathogenesis of CD (Dieterich *et al.* 1997). While formation of tTg-gliadin complexes stimulate gluten-specific T-cells to induce anti-tTg antibody production, it is likely that T and

B-cells recognise different parts of this antigen complex, with T-cells reacting to the smaller gliadin peptides and B-cells responding to the larger 76 kD tTg enzyme, analogous to a hapten carrier (Koning *et al*., 2005).

The triggering of pathogenic immune responses relates to the deamidating activity of tTg. Deamidation only procedes when tTg activity is high, when there is an excess of substrate and when the level of acceptor amines is low. Such prerequisites occur during episodes of intestinal inflammation. Inflammation induces tTg activity, disturbs epithelial integrity and increases the need for exogenous and endogenous amines, in that cross-linking of amines by tTg is critical for normal repair functions of the intestinal mucosa (Wang *et al*., 1992). The amine content in a normal diet is not high and a rate-limiting enzyme restricts synthesis of amines. Thus, any inflammation may cause a local depletion of amines concurrently with an increased leakage of gluten proteins across the epithelial barrier.

There is also the possibility that the deamidating activity identified in CD is not actually mediated by tTg, but by some other as yet unidentified enzyme. No other human enzyme that catalyses deamidation of specific protein-bound glutamine residues has been identified, but it should be remembered that bacterial-Tg, which could be present in the gut lumen, preferentially deamidates certain subtrates, instead of cross-linking them (Molberg *et al*., 2000).

Chapter Six: TREATMENT OF COELIAC DISEASE

The only current treatment for CD is the total exclusion of gluten containing foods from the diet of affected people. A recent review identified major issues relating to the treatment of CD that are not yet being addressed and urgently need to be investigated (Troncone *et al*., 2008). These are outlined in Table 6.1.

Table 6.1: Treatment of CD – major issues in need of investigation
(Troncone *et al*., 2008)

Decision on treatment criteria	Long-term health risks of silent and potential CD and the impact of early diagnosis Natural development of permanent or transitory gluten tolerance in CD cases
Improvement in health care and quality of life	Nutritional consequences of the GFD and advantages of a better dietary support Food labelling, availability of gluten-free foods and awareness of the disease
Development of safe and new foods	Oats toxicity Threshold of tolerance to gluten Genomics and proteomics of different wheat cultivars and implementation of traditional or biotechnologically modified gluten-free cereal variants
Exploring treatment alternatives	Enzyme supplements therapy Re-establishment of the intestinal barrier against gluten entry Blocking of gliadin presentation by HLA blockers and tTG inhibitors Cytokines and anti-cytokines such as IL10, anti-IFNγ and anti-IL15 Re-establishment of tolerance (modified gluten peptides, nasal tolerance) Development of a relevant animal model Explore the cost-effectiveness of alternative treatments Assess the demand from patient support group of the nature of alternative treatment to GFD Strategies to stimulate collaborative studies and alliances between industry and researchers from in and outside Europe

Research into addressing some of these issues is underway and will be outlined at the end of this section.

6.1 The gluten-free diet

The mainstay of treatment for CD is strict lifelong adherence to a gluten-free diet (GFD) in which people avoid food products containing wheat, rye or barley. Ideally, this would eliminate any prolamin or similar protein capable of stimulating an adverse T-cell response (Schwarzenberg *et al*., 2002). Unfortunately, there is no way to directly determine the capacity of individual grains to elicit a response and current recommendations rely on *in vivo* testing. As a result, controversy exists regarding the most appropriate diet for people with CD.

Although research shows that oats are generally well tolerated, a number of people remain sensitive to them and the presence of oat-specific intestinal T-cells has been demonstrated (Thompson 2003). There is no consensus as to whether oats present a hazard for all people with CD. Several studies show that most coeliacs tolerate moderate amounts (e.g., 50-70 grams daily) of oats (Janatuinen *et al*., 2002; Janatuinen *et al*., 2000; Janatuinen *et al*., 1995). However, more recent studies investigating the effects of daily consumption of 50 grams of oats, suggest that oat proteins can elicit symptoms in some coeliacs (Arentz-Hansen *et al*., 2004; Lundin *et al*., 2003). The oats used in these studies were tested for purity and did not contain any gluten proteins from wheat, rye or barley.

Of more concern, is the issue of contamination of oats with other gluten-containing cereal grains during growth, harvest or packaging of commercially available oat-based products. The recommendation remains to avoid oats along with other gluten containing foods and to replace removed grains with rice, corn, quinoa, buckwheat or amaranth. Maintaining a strictly GFD is not easy given that wheat or wheat-derived products are so widely used in the food industry worldwide (Alaedini *et al*., 2005).

6.2 What are gluten-free foods?

The Codex Alimentarius Commission of the World Health Organisation and the Food and Agriculture Organisation of the United Nations provides the only *international* gluten-free food standards for manufacturers. The Codex Alimentarius defines gluten-free foods as those with a gluten content below 200 parts/million (i.e.

200mg/100g food) which is equivalent to 100ppm of gliadins (Spaenij-Dekking *et al*., 2004). (Further explained in section 6.3).

The standards for gluten-free foods as defined by the commission allow small amounts of prolamin in foods that are designated gluten-free (Spaenij-Dekking *et al*., 2004). In Europe, the UK and Scandinavia, ingestion of wheat starch that has been rendered gluten-free is permitted. European Codex Alimentarius-quality wheat starch has also been used in some European countries for several decades and is considered safe (Kaukinen *et al*., 1999b). However, the US and Canada have adopted a strict gluten-free regimen and do not recommend the use of wheat starch that contains even small amounts of toxic prolamins until further studies confirm its safety (Thompson 2001). People with CD travelling abroad should be aware of this discrepancy.

Analytical information is not available on the actual amount of gluten proteins in different grain-derived food ingredients or finished foods. For single ingredient foods made from wheat, rye, barley, triticale or oats, the simple presence of "protein" in that food may be used as an indicator that gluten proteins are present (Gendel *et al*., 2005). The major source of composition data for foods in the U.S is the USDA *National Nutrient Database for Standard Reference, Release 17* (USDA 2004), which includes hundreds of food items containing gluten-based cereals as an ingredient (Gendel *et al*., 2005). Wheat, in particular, is used to manufacture a wide range of food ingredients and finished foods. Rye, barley, triticale, and oats are used to make substantially fewer food products (Gendel *et al*., 2005).

New Zealand has a similar database which lists foods manufactured in New Zealand or Australia that contain gluten from any source. It is the *Manufactured Food Database* online @ www.mfd.co.nz.

Using US data, Koehler (2005) estimated the average amount of total grain and individual types of grain available for consumption per person and the total exposure to gluten-forming proteins that would result from this grain consumption. The estimated mean daily consumption rate was approximately 250 grams (g) grain per capita. Wheat provided 180 of the 187 grams/ person/ day of grains that are of

concern for people with CD. While this information is not yet available for the New Zealand population, results are assumed to be similar.

6.3 Gluten-Free Labeling

Although a GFD is considered the only effective treatment for people with CD, it has been recognised that it is difficult, if not impossible, to maintain a diet that is completely devoid of gluten (Collin *et al*., 2004). Therefore, several attempts have been made to define gluten-free in regulatory contexts. Efforts by the Codex Alimentarius (FAO/WHO 1998) to define a standard for 'gluten-free' date back to 1981. At that time, due to the lack of sensitive, specific analytical methods, a threshold value of 0.05 g nitrogen per 100 g dry matter was set for wheat starch, on the assumption that wheat protein would be the only source of nitrogen in starch (Codex Standard 118-1981). The Codex Committee on Nutrition and Foods for Special Dietary Uses is developing a revised standard. The current draft proposal would define three categories of gluten-free foods: naturally "gluten free" (≤ 20 ppm of gluten), products rendered gluten-free by processing (≤ 200 ppm), and any mixture of the two (≤ 200 ppm) (Spaenij-Dekking *et al*., 2004; Gendel *et al*., 2005).

Food Standards Australia New Zealand (FSANZ) defines gluten to mean "*the main protein in wheat, rye, oats, barley, triticale and spelt relevant to the medical conditions, coeliac disease and dermatitis hepetiformis "* (FSANZ 2002).

FSANZ recognises two classes of foods (a) gluten-free foods i.e. no detectable gluten, and (b) low gluten. In New Zealand, the low gluten standard has always been recommended as a safe and appropriate treatment for the vast majority of people with CD.

The current FSANZ Food Standards Code: *Standard 1.2.8 Claims in relation to gluten content of food* includes a claim relating to low gluten. *"A claim to the effect that a food has a low gluten content must not be made in relation to a food unless the food contains no more than 20mg per 100g of food "* (FSANZ 2002).

For a food to be called 'gluten-free' and be included in the MFD as gluten-free, it must follow the current FSANZ Food Standards Code: *Standard 1.2.8 Claims in relation to gluten content of food*.

"A claim to the effect that a food is gluten free must not be made in relation to a food unless the food contains no: (a) detectable gluten; and (b) no – (i) oats or their products; or (ii) cereals containing gluten that have been malted, or their products." (FSANZ 2002).

6.4 Measuring Gluten in Food

Currently, commercial immunology-based ELISA test kits for the detection of gluten in foods are manufactured by Immunotech (Czech Republic), Ingenasa (Spain), Morinaga (Japan), Diffchamb (Sweden), Neogen Corporation (U.S.), R-Biopharm (Germany), and Tepnel BioSystems (U.K.). All of these detect prolamins, the proteins in soluble aqueous-alcohol extracts from cereals. However, none detect all proteins associated with CD. Five of the assays have undergone multi-laboratory validation studies (Akiyama *et al*., 2004; Gabrovská *et al*., 2004; Immer *et al*., 2003; Skerritt *et al*., 1991). Each study employs different target levels and matrices. TheTepnel kit was validated by the Association of Official Analytical Chemists (AOAC) at 100 ppm (Skerritt *et al*., 1991). All ELISA kits rely on the preparation of an aqueous-alcohol extracts as analytical samples. Four manufacturers include the use of reducing-denaturing conditions for the analysis of baked goods.

During the 25th Codex Committee on Nutrition and Foods for Special Dietary Uses in 2003, the R5-Mende zELISA method, which involves the use of reducing-denaturing conditions, was forwarded to the Codex Committee on Methods of Analysis and Sampling for endorsement (Codex Alimentarius Commision, 2003). These ELISA test kits cross react to differing degrees, with prolamins from wheat, rye, and barley. No test kits cross-reacted with protein extracts from oats (Abouzied, 2004; Brewer *et al*., 2004; Gabrovská *et al*., 2004; Nonaka, 2004). As such, ELISA test kits do not provide protection for people with CD who are sensitive to oats (Arentz-Hansen *et al*., 2004; Peraaho *et al*., 2004; Storsrud *et al*., 2003; Lundin *et al*., 2003). Proficiency testing studies conducted by the Food Analysis Performance Assessment Scheme (FAPAS®) found variability between the prolamin ELISA test kits (FAPAS Series 27 Round 05, Report No. 2705, 2003), indicating that further validation studies need to be carried out under comparable conditions. In addition to ELISA test kits, two of the manufactures, Tepnel

BioSystems and R-Biopharm, market lateral flow devices for the detection of gluten. To date, neither have been validated (Gendel *et al*., 2005).

Currently, no correlative information on the efficacy of using these tests to predict or help prevent adverse effects in people with CD exists (Gendel *et al*., 2005). Refinements in techniques for measuring gluten in food, using monoclonal antibodies to the toxic epitopes, will assist appropriate labelling of foodstuffs and reduce inadvertent ingestion (Dewar *et al*., 2004).

6.5 What foods are permitted on a gluten-free diet?

A GFD means avoiding foods containing wheat (including spelt, triticale, and kamut), rye, and barley or products made from these. Despite these restrictions, a GFD can include a variety of gluten-free breads and pastas such as those made from potato, rice, soy, amaranth, quinoa, buckwheat, corn or bean flour.

Gluten-free products are increasingly available from regular stores. Checking labels for "gluten free" is important, since many corn and rice products are produced in factories that also manufacture wheat products. Hidden sources of gluten include additives (i.e modified food starches, preservatives, and stabilisers). Wheat and wheat products are commonly used as thickeners, stabilisers, texture and flavour enhancers in foods. "Plain" meat, fish, rice, fruits, and vegetables do not contain gluten, so people on a GFD can freely eat these foods. Examples of foods that are safe to eat and those that are not recommended are provided in Table 6.2 and Table 6.3 in Appendix E.

Since gluten is also used as an additive in unexpected products (i.e medications), it is important to read all labels. If ingredients are not listed on the product label, the manufacturer should provide the list upon request.

In severely symptomatic people, treatment may include supplementation to correct nutrient deficiencies (especially iron, folate, calcium, and vitamins A, D, E, K, C and other water soluble B vitamins) (Howdle 2003). Constipation is often a problem on a GFD, reflecting the omission of wholegrain cereals from the diet. Rice bran, soya bran, psyllium or lignin can reduce the effects of a low fibre intake (Murray *et al*.,

2004). Treatment of CD with a strict GFD usually results in complete clinical and histological recovery within 6-12 months (Srinivasan *et al*., 2006).

6.6 Finding an alternative to the gluten-free diet

At present, dietary restriction (i.e. a life-long GFD) is the only viable treatment for CD, but it has a number of drawbacks. It is inconvenient, expensive and some gluten-free products are relatively unpalatable, which may cause poor compliance, or inadvertent consumption of gluten. In addition, some people do not respond rapidly (or completely) to dietary restriction and require treatment with immuno-suppressive agents (Dewar *et al*., 2004; Corrao *et al*., 2001). Currently, a number of other options are being explored.

A number of new therapies are being investigated in an attempt to find a 'cure' for CD. One such emerging therapy is the "coeliac vaccine", currently being researched and trialed in Australia (Anderson 2006). This research focuses on production of an immune modifying vaccine that reverses immune intolerance to gluten. The identification of key dietary peptides in wheat, rye and barley presented by T-cells is crucial to the success of immunologically-based therapies aimed at inducing tolerance, as it is for any attempts at genetic modification of grains in order to render them non-toxic (van Heel *et al*., 2006).

This Australian research has so far generated a comprehensive "map" of the toxic peptides within wheat, rye, barley and oats. It has found that toxic peptides differ significantly after gluten ingestion depending on whether the person has HLA-DQ2 CD or DQ8 CD. HLA-DQ2 CD behaves as a completely different disease from HLA-DQ8 CD which has major implications for the design of an effective vaccine, as both people with HLA-DQ2 and DQ8 will need to be studied. While knowledge of the mechanisms of DQ2 CD is quite well advanced, more research is needed into DQ8 CD (van Heel *et al*., 2006; Tye Din 2005).

Other novel therapies are currently being investigated as possibilities for treating CD. It has been known for some time that one of the key T-cell stimulatory peptides in CD is resistant to breakdown by intestinal proteases. As far back as the late 1950's, the use of non-human proteases for the detoxification of gluten was proposed as a possible means of treating CD, but this was not clinically

investigated until 1976 (Messer *et al*., 1976). Shan *et al*., (2005) described how bacterial prolyl-endopeptidases degrade the 33-mer T-cell stimulatory peptide in CD which sparked renewed interest in trying to digest these peptides to eliminate their toxicity. Subsequent research found that very high concentrations of prolyl-endopeptidases reduce the quantity of immuno-stimulatory gliadin peptides (both innate and T-cell activating) reaching the mucosa (Matysiak-Budnik 2005).

Recently, a new prolyl-endopeptidase from *Aspergillus niger* (AN-PEP) was found to efficiently degrade gluten *in vitro* (Stepniak *et al*., 2006). Perhaps more significantly, this can be cheaply and efficiently produced to food grade quality. This therapy is awaiting *in vivo* trials, as no animal model for CD is available (Stepniak *et al*., 2006). Methods for screening of peptides in food to guarantee food safety are already developed and are currently undergoing refinement for viability in a commercial environment (Spaenij-Dekking *et al*., 2004).

Certain ancient varieties of wheat have fewer toxic T-cell sequences than modern wheat varieties so research has begun into whether it is possible to generate wheat and other cereals with reduced or absent immunogenicity by selective breeding or genetic modification (Molberg *et al*., 2005). However, given the complexity of wheat genetics and scattered nature of toxic epitopes throughout the gluten genome, this is unlikely to be achieved by conventional breeding techniques. Concerns also exist about the safety and ethics of genetic modification (Spaenij-Dekking *et al*., 2005; Dewar *et al*., 2004). While this option is currently being explored, it is not yet possible to breed commercially usable crops with the necessary baking properties and still overcome all T-cell stimulatory sequences, including glutenins, or a product suitable for people with DQ8 CD (van Heel *et al*., 2006).

Other strategies in the early stages of experimental investigation for the treatment of CD include;

(a) blocking tissue transglutaminase (tTG) mediated deamidation of gluten peptides
(b) blocking zonulin mediated increases in intestinal permeability
(c) blocking HLA-DQ2 peptide interactions
(d) blocking MICA-NKG2D interactions (Allez *et al*., 2007).

6.6.1 Blocking of gliadin presentation by HLA blockers and tTG inhibitors

Prevention or blocking of tTg activity has been suggested, however, this enzyme has a diverse biological role and even local inhibition may create unpredicted adverse affects. Furthermore, the discovery of tTg-independent epitopes makes this an unlikely avenue (Troncone *et al*., 2008; Dewar *et al*., 2004). On-going interest in the use of altered peptide ligands in medicine continues. By making specific point alterations in the peptide sequence of antigens, HLA binding affinity can be retained, but T-cell responses to that peptide may be qualitatively altered and down-regulated. Treatment with an appropriate synthetic antigen may then modulate the immune response favorably (Troncone *et al*., 2008; Dewar *et al*., 2004; Brocke *et al*., 1996; Sloan-Lancaster *et al*., 1996).

6.6.2 Re-establishment of tolerance

Restoring immunological tolerance to gluten would represent the ideal cure for CD. Much interest exists in the concept of oral tolerance in immune-mediated disease, whereby an oral antigen is administered to de-sensitise auto-reactivity against self-antigens (Troncone *et al*., 2008). In animal models, these strategies have prevented the development of some autoimmune diseases, but none have modulated established disease (Troncone *et al*., 2008). The timing and nature of antigenic exposure, together with the immunological status of the host, appear critical in influencing whether sensitisation or tolerance develops.

Continued research into CD disease may help with understanding of the mechanisms that result in loss of tolerance and allow identification of people who have the potential to develop the condition. This could allow the design a regimen of gluten epitope exposure that does not result in CD. In established disease, successful immunotherapy is likely to prove more difficult. However, if gluten-specific T-cells could be inactivated or deleted, tolerance to gluten should be restored. Theoretically, such immuno-modulation could provide a selective, tailored treatment of CD, although this is likely to be many years in the future (Troncone *et al*., 2008; Dewar *et al*., 2004).

Recent studies (De Angelis *et al*., 2006; Di Cagno *et al*., 2005) found that wheat and rye based products can be made tolerable to people with CD using selected

sourdough lactobacilli and slow fermentation processes in place of the bakers yeast and fast processes currently used. Rizzello *et al*., (2007), successfully produced a sourdough wheat bread made using bacteria to degrade gluten, which did not trigger an immune response when tested on people with CD. The bread has similar baking and storage qualities to standard bread. These findings are encouraging and further research is underway, including a long term *in vivo* challenge of people with CD. Food processing by selected sourdough lactobacilli and fungal proteases may be the efficient approach chosen in the future to eliminate the problem of gluten toxicity for people with CD. Despite the exciting advances that have been made in our understanding of the pathogenesis of CD and the potential development of novel therapies, a strict GFD remains the only safe and effective treatment for CD.

Chapter Seven: THE FAECAL STUDY

CD is an immune-mediated enteropathy with a complex aetiology characterised by chronic inflammation in the intestinal mucosa of the small intestine. Intestinal microflora (IM) contribute to other chronic inflammatory disorders (i.e. Crohn's disease, ulcerative colitis and IBS), but their role in CD remains undetermined (Collado *et al*., 2007). A key to understanding the role of IM in the pathogenesis of CD is an accurate knowledge of the microbial species inhabiting the gut and how these are influenced by inflammation.

7.1 Aims and rationale of the faecal study

7.1.1 Aims

The aim of the faecal study is to compare the composition of faecal microflora in adults with CD in the inflammatory phase of the disease and during resolution from inflammation after the institution of a GFD, in order to determine whether a reduction in inflammation is associated with a corresponding change in the diversity of generic enteral microbiota. A comparison with healthy control subjects was included to define whether any stochastic changes in microbial species are inherent or whether specific microbial changes are associated with CD. More specifically, it is proposed that enteral mucosal inflammatory change in CD will be associated with a decrease in microbial diversity, whereas remission from inflammation will be accompanied by an increase in enteral microbial diversity.

7.1.2 Rationale

Most information on the composition and metabolic activity of the IM in people with CD comes from analyses of faecal samples (Nadal *et al*., 2007) hence the reason faecal sampling was selected for this study. Strong interest continues as to whether enteral microbial species are actually precipitating factors in CD. This topical and relevant sphere of research will become increasingly important in the future as the incidence of CD continues to rise. Early research (Corazza *et al*., 1987) identified bacteria as a precipitating factor in CD, and more recently a high prevalence of bacterial overgrowth in adults with CD was found (even after gluten withdrawal) (Tursi *et al*., 2003). Forsberg *et al*., (2004) identified specific rod-shaped bacteria attached to the IE in children with CD (both active CD and in remission) that were not present in controls, again indicating an association.

The fact that different microbial communities exist in people with CD compared to 'normal' subjects is already supported by differences in the pattern of faecal SCFA production in active CD (Tjellstrom *et al*., 2005). This altered pattern of some SCFAs in CD (at presentation and during treatment with a GFD) indicates a new aspect of CD that is not affected by diet, the presence of inflammation or the autoimmune status of the person (Tjellstrom *et al*., 2005). This enhances the idea that bacteria are involved in the pathogenesis of CD.

7.2 Possible Methodologies for the Faceal study

A number of methods could have been used to investigate the microflora present in the faecal samples for this study, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or fluorescent in-situ hybridization (FISH). Some methods of microbial analysis such as cultivation, DGGE, and TGGE inventorise microflora (i.e. define all microbial species present in a faecal sample), while others identify or quantify different species present (e.g. DGGE, TGGE and real time polymerase-chain reactions-PCR). Rapid profiling procedures (i.e DGGE, TGGE) allow for analysis of multiple samples, but their community fingerprints do not directly translate into taxonomic information (Obsorn *et al*., 2000). Active or inactive methods such as reverse transcription PCR (rtPCR) exist, as do those that determine where bacteria adhere (such as FISH, confocal microscopy, flow cytometry) and those that identify the variance of particular species (such as tRLP) (Lentle 2008).

An ideal methodology allows investigation of IM for both its qualitative and its quantitative composition. This depends on a number of factors, including the detection limits, the degree of reproducibility of a particular method and the specific biological question to be addressed (e.g. determination of the overall diversity of a particular environment, identification of the predominant members of a community, monitoring population dynamics, etc)(Bibiloni *et al*., 2004; Osborn *et al*., 2000).

Investigations of microflora in inflammatory diseases of the gut such as CD are, however, hampered by the difficulty of culturing IM in the laboratory. Large proportions of bacterial cells within intestinal samples are not culturable either because they are anaerobes or because of the complex nutritional interactions between inhabitants of bacterial communities which can not be replicated outside of

the body (Sears 2005: Beaugerie *et al*., 2004). Even using sophisticated bacteriological methods, it is estimated that only about 58-60% of bacteria is culturable (Tannock 2003; Lenoir-Wijnkoop *et al*., 2003). However, new technologies for isolating and identifying genetic material that detect both culturable and non-culturable bacterial species, make it possible to identify previously unknown species thus permitting comprehensive comparisons of IM in health and disease (Nordgård *et al*., 2005; Stebbings *et al*., 2002).

Early research (Woese 1987) found small ribosomal subunits of RNA containing regions of highly conserved nucleotide sequences interspersed with hypervariable regions ('V' regions) which contained the signatures of phylogenetic groups (Collins *et al*., 2002; Stebbings *et al*., 2002). Consequently, IM can now be accurately identified by extraction of DNA from a pure culture of a bacterial isolate, followed by PCR amplification of the 16S r RNA gene using universal primers that target conserved bacterial sequences and determine their nucleotide base sequence (Tannock 2003; Stebbings *et al*., 2002). Such a technique is denaturing gradient gel electrophoresis (DGGE).

The main uses, advantages and disadvantages of each method that could potentially have been applied to this research are only briefly outlined here as they are summarised in Table 7.1 in Appendix F.

7.2.1 Denaturing gradient gel electrophoresis (DGGE)

In DGGE, fragments of 16S rRNA are amplified by PCR using primers that anneal the conserved regions spanning the selected 'V' regions. The double-stranded 16S fragments migrate through a polyacrylamide gel containing a gradient of urea and formamide until they partially denature in the chemical conditions (Bibiloni *et al*., 2004;Tannock 2003). One PCR primer has a GC-rich 5' end clamp that prevents complete denaturation of the DNA fragments during gradient gel electrophoresis which radically slows migration through the gel.

The 16 s sequences of different bacterial species vary and chemical stability is also different, therefore the 16s fragments from different species can be differentiated by electrophoresis (Bibiloni *et al*., 2004;Tannock 2003). Studies using PCR/DGGE reveal that each person has an individualised bacterial community in their faeces that provides a unique genetic 'fingerprint'. These

unique profiles are conserved over time and have a high degree of stability (Bibiloni *et al*., 2004;Tannock 2003).

While DGGE produces more accurate and reliable results than earlier techniques, it still has a number of inherent limitations. Chemical gradients (such as those used in DGGE) are not readily reproducible, they are difficult to establish and often do not completely resolve ***heteroduplexes*** (Westberg *et al*., 2001). Nevertheless, DGGE remains a useful tool for the analysis of faecal microbial communities and is the method of choice for this research as PCR/ DGGE analysis successfully detects changes in the composition of IM. However results do vary depending on whether generic or species-specific primers are used (Walter *et al*., 2000). Recent investigation of monthly faecal samples using PCR/DGGE in combination with specific primers for lactic acid bacteria revealed large fluctuations in species, which contrasts to PCR/DGGE profiles generated with universal bacterial PCR primer which found the composition of microflora very stable. *Lactobacillus ruminis* was the dominant species over several months of testing with *L. salivarus, L. acidophilus, L crispatus* and *L gasseri* regularly detected (Biblioni *et al*., 2004).

DGGE is the preferred method of analysis for this research because it is reliable, sensitive to variations in DNA sequence, because it allows for simultaneous analysis of multiple samples, because it is a useful method for monitoring shifts in microbial community structure over time, and because it has been successfully used in recent similar research. A universal primer will be used.

7.2.2 Temperature gradient gel electrophoresis (TGGE)

TGGE is one of a family of electrophoretic methods for separation of nucleic acids (like DNA or RNA) that rely on temperature dependent changes in structure. Since a gradient of denaturant and a gradient of temperature are linearly related, DGGE and TGGE are, from a theoretical standpoint, almost identical (Panga *et al*., 2005; Collins *et al*., 2002). (See the glossary in appendix A for a full description).

7.2.3 Temporal temperature gradient electrophoresis (TTGE)

Temporal temperature gradients are used for electrophoretic separations where the spatial temperature gradient is replaced with a temporal temperature

gradient (Wiese *et al*., 1995; Yoshino *et al*., 1991). (See the glossary in appendix A for a full description).

7.2.4 Polymerase Chain Reaction (PCR)

PCR involves the use of a pair of primers, each about 20 nucleotides in length that are complementary to a defined sequence on each of the two strands of the DNA (Hunter 2006). These primers are extended by a DNA polymerase so a copy is made of the designated sequence. After making this copy, the same primers are used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis. This leads to logarithmic amplification (Hunter 2006).

When used in conjunction with DGGE, short sequences of 16s rDNA can also be amplified rather than the whole gene being sequenced which allows for a rapid identification of microbial species (Tannock 2003). For example, the variable (V2-V3) region of the 16S r RNA gene can be amplified from pure cultures of *Lactobacillus* isolates. The PCR amplicons, when examined by DGGE allow for the identification of many lactobacillus species because their V2-V3 sequences have characteristic migration properties in the electrophoretic gel. This method is also appropriate for the identification of *Bacteroides* and *Bifidobacterium* species so group specific PCR primers add a new dimension to this method of microbial analysis (Tannock 2003). PCR, does have some limitations. While culture bias is removed, another type of bias is introduced because PCR is known to amplify DNA sequences from mixed populations of microbial species with differing efficiency (See the glossary in appendix A for a full description).

7.2.5 Reverse transcription polymerase chain reaction (RT- PCR)

RT-PCR is used to amplify a defined piece of RNA. The RNA strand is first reverse transcribed into its DNA complement followed by amplification of the resulting DNA using PCR as outlined above. This can either be a 1 or 2 step process (Hunter 2006). (See the glossary in appendix A for a full description).

7.2.6 Real-time polymerase chain reaction

Real time PCR, also called quantitative real time polymerase chain reaction (qPCR), or kinetic polymerase chain reaction, is used to amplify and

simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalised to DNA input or additional normalising genes) of a specific sequence in a DNA sample (Nailis *et al*., 2006). (See the glossary in appendix A for a full descripton). Real-time PCR shows promise for analysis of bacterial populations, and could have been successfully applied to this research however, it was beyond the level of expertise of the principal researcher so was not chosen.

7.2.7 Fluorescent in-situ hybridisation (FISH)

Molecular approaches based on nucleic acid sequence similarities (i.e. FISH, DGGE and 16S rDNA sequencing), partly overcome the limitations of plate culture methods, and are particularly useful for the detection of uncultivable bacteria (Harmsen *et al*., 2000b; Harmsen *et al*., 2000c). FISH can be combined with flow cytometry which has the advantage of counting each cell as a single event and at least four different probes can be used simultaneously on a single sample. This process can be automated so large amounts of data can be captured for analysis (Collins *et al*., 2002). It can also be combined with confocal laser microscopy using a semi-automated protocol to measure the concentration of bacteria (in cells per volume) in environmental samples (Daims *et al*., 2001). (See the glossary in appendix A for a full description).

FISH has been successfully applied in recent research (Collado *et al*., 2007) which looked at differences in faecal microflora between infants with CD and healthy controls. They found greater diversity in microbial species in children with CD compared to controls subjects. Levels of *Bacteroides, Clostridium* and *Staphylococcus* were significantly higher in faecal samples from subjects with CD, than control subjects when analysed by culture methods. Numbers of *Bacteroides-Prevotella ,Clostridium histolyticum, Eubacterium rectale-C. coccoides, Atopobium and* sulfate-reducing bacterial groups were significantly higher in CD when analysed by FISH, whereas numbers of *bifidobacterium* were slightly higher in the control group. This research notes this is the first identification of specific bacterial groups responsible for alterations in IM in children with active CD (Collado *et al*., 2007). This bacterial pattern in CD is consistent with epidemiological data and involves bacterial species linked to

other chronic inflammatory disorders of the gut such as Nadal *et al*., (2007) who found an overgrowth of certain bacterial populations, combined with the absence of specific protective commensal microflora contributed to the impairment of intestinal homeostasis in people with IBD.

FISH could have been used in this study rather than DGGE however, again, it was not chosen as it is beyond the level of expertise of the principal researcher.

7.2.8 Flow cytometry

The combination of FISH with flow cytometry detection (FCM-FISH) appears to be the best high throughput method for analysis of microbial communities in the GI tract (Vaahtovuo *et al*., 2005; Rigottier-Gois *et al*., 2003a; Rigottier-Gois *et al*.,2003b). Flow cytometry (FCM) discriminates between populations within complex bacterial communities based on fluorescence and size differences among the cells. In conjunction with a sorting unit, defined FCM populations can be physically separated and then subjected to further taxonomic analysis. However, the combined methods are challenging to use, hence the reason this method was not selected (Collado *et al*., 2007b).

7.2.9 cDNA-Amplified restriction fragment length polymorphism (cDNA-AFLP)

This RNA fingerprinting technique is used to identify dominant transcripts expressed by IM. It enables the functional analysis of complex microbial ecosystems and is believed to be able to detect more subtle variation in diversity than 16S r RNA gene targeted PCR -DGGE profiles (Booijink *et al*., 2006).

7.2.10 Terminal restriction fragment length polymorphism (t-RFLP)

t-RFLP can assess subtle genetic differences between strains as well as providing insight into the structure and function of microbial communities. t-RFLP has both high sensitivity and throughput making it ideal for comparative analysis (Marsh 1999). This method could have been successfully applied to this research however, it was not chosen for the same reasons previously outlined.

7.3 Justification for choice of methodology used in faecal study

After extensive investigation of possible methodologies for this project PCR/DGGE was specifically selected because;

(a) this gel-based technique allows for the rapid comparison of 16S rRNA from the different faecal samples collected over a four-month time period (Lenoir-Wijnkoop *et al*., 2003)

(b) the Institute of Food, Nutrition and Human Health at Massey University has facilities and technical expertise available for this investigation to be performed using PCR/DGGE.

(c) previous research relating to CD and other inflammatory disorders of the intestine have used PCR/DGGE to investigate microbial diversity in faecal samples with excellent results

(d) It is a non-invasive method that allows investigation of changes in intestinal communities and over time in people with CD.

It is acknowledged however, that no technique is perfect. There are inherent problems with all techniques and DGGE is no exception. DGGE-gels are only semi-quantitative and damage can occur to the DNA during extraction and processing (i.e. bands from broken DNA will be different from bands extracted from unbroken DNA). Despite this DGGE is an effective technique for this research.

7.3.1 The recent use of PCR/ DGGE in research

Different 16S rRNA-based techniques are appropriate for different investigations including identifying microbial species or phylotypes, quantifying microbial taxa or making broad comparisons of microbial communities (Dethlefsen *et al*., 2006). DGGE can separate nucleotide sequences that differ by as little as one nucleotide (Martinez-Medina *et al*., 2006) so it is ideal here where we were seeking to identify differences in microbial diversity within the same subject over time, under altered dietary conditions, and between the healthy control group and the coeliac group.

The DGGE profile of the faecal community in adults remains relatively constant over long periods of time, but the individual profiles of different people can vary greatly (Tannock 2003). Previous research found the diversity and stability of bacterial communities in the intestine is easily and reliably analysed using PCR-

DGGE on 16S rRNA (Zoetendal *et al*., 2001; Vaughan *et al*., 2000; Muyzer *et al*., 1998). Later research using DGGE to analyse faecal communities in Crohn's disease and ulcerative colitis found people with these disorders harbour a different type and diversity of IM (Bibiloni *et al*., 2006; Martinez-Medina *et al*., 2006; Scanlan *et al*., 2006). Scanlan *et al*., (2006) found significant variation in diversity of lactic acid bacteria between those with Crohn's disease and the healthy control group whilst Martinez-Medina *et al*., (2006) found people with Crohn's had greater variability in microflora than the healthy control group using PCR-DGGE. Similarly, Bibiloni *et al*., (2006) used PCR/DGGE to analyse the microflora of people with newly diagnosed, with untreated Crohn's disease and with ulcerative colitis (UC). Samples from inflamed and non-inflamed subjects showed no variation, but greater variability was found in people with UC than Crohn's with *Bacteroides* being more prevalent in Crohn's than UC. Bullock *et al*., (2004) found greater variability in microbial species in UC.

Sanz *et al*., (2007) used DGGE analysis of PCR amplicons to identify microflora present in faecal samples from children with CD, and compared these with samples taken from a matched control group of children without CD. The DGGE profiles obtained using universal primers were complex and unique for each individual. The profiles of healthy children showed lower diversity than those with CD. The DGGE profiles of healthy subjects showed between one and four bands with *lactobacillus caseii* appearing as dominant compared to DGGE profiles of coeliac children where one to six *lactobacillius* group specific bands and species other than *Lactobacillus* were dominant. *Lactobacillus curvatus, Leuconostoc mesenteroides and Leuconostoc carnosum* were significantly higher in coeliac patients than in controls. Most lactic acid bacterial species detected in coeliac patients were allochthonous species whereas in control samples, *Lactobacillus gasseri* and *Lactobacillus casei* group were dominant (Sanz *et al*., 2007).

7.4 Subject recruitment

16 people were recruited: 8 with active coeliac disease (age range; 24 - 68 yrs; gender; 3 male, 5 female) and 8 (age and gender matched) non-coeliac control subjects.

The coeliac group were recruited (over one year) through local gastroenterologists as they presented with symptoms indicative of CD. The diagnosis of CD was confirmed by positive serology and duodenal biopsy. Only those people with confirmed CD were included in the study.

The non-coeliac control group was a 'sample of convenience' chosen from a local school and church community. An invitation was extended to participate in the study if people had no known food intolerances, GI allergies, history of abdominal discomfort, coeliac or other inflammatory bowel disease and were of the same age and gender as each person in the CD group. The study protocol was approved by the Massey University Ethics Committee; approval number 06/08. Written informed consent was gained from all participants (A copy of the consent form is included in Appendix O).

7.5 Sampling procedures

Faecal samples were collected from all subjects at monthly intervals over four successive months. Subjects immediately chilled their samples using saline-based freezer packs, then couriered their samples to the principal researcher who stored these in a sealed environment at -18°C pending transport to Massey University and where they were stored at -80°C.

For the coeliac group, the initial sample was collected at diagnosis (0 months), prior to the implementation of a GFD (during active mucosal inflammation). Further samples were collected at 1, 2 and 3 months following the implementation of a GFD (during resolution from inflammation). For the control group, monthly samples where collected over four consecutive months.

A custom-designed questionnaire was completed monthly by all participants prior to the collection of each faecal sample. This questionnaire incorporated a 3-day dietary recall, a food frequency questionnaire, a description of CD-related symptoms (Bai *et al*., 2005), questions relating to frequency of defaecation, to the nature of faeces produced and the consumption of any medication. This was designed to exclude anyone who had consumed antibiotics, or in the case of the coeliac group, people who did not adhere to the GFD during the course of the study (see Appendix N for a copy of the questionnaire).

7.6 Sample treatment and extraction of DNA

7.6.1 DNA extraction

Bacterial DNA was extracted from 200mg of wet faecal material using QIA amp® DNA stool minikit (Biolab-Cat 3 51504). Isolated DNA was cleaned using a PowerClean ™ DNA Clean-Up kit (MoBio Laboratories Inc., USA- Cat # 12877-50). The cleaned DNA was stored at -40℃ pending PCR.

7.6.2 PCR mixture and incubation conditions

Amplification of 16s ribosomal DNA was carried out in a thermal cycler (Bio Rad Cat # 170-872 using a Universal Primer (U 968-GC F, L1401-r). The reaction mixture (50µl) contained 0.5 µM of each primer (U 968-GC F with a GC-clamp and L1401 R). 25 µl of 2x Go Taq Master mix (Promega Corporation, USA-Cat M7132) and 5 µl of DNA template. A universal primer was chosen on the basis that DGGE profiles obtained with universal primers are known to be stable (Bibioni *et al*., 2004).

Amplification routine was initial denaturation at 95℃ for 15 minutes followed by 30 cycles each of for 30 seconds at 94℃, 60 seconds at 57℃ then 30 seconds at 72℃ and finally 72℃ for 7 minutes. The PCR products thus obtained were frozen at -20℃ pending DGGE. This procedure was carried out by the specialist genomic technician at the Institute of Food, Nutrition and Human Health at Massey University.

7.7 Denaturing gradient gel electrophoresis (DGGE) of microbial DNA

PCR products were checked for purity by absorbance ratios at 260nm vs 280nm (Stanford 2002) and concentration determined using a nanodrop spectrophotometer (IMPLEN). The volume of the PCR product from each sample that was applied to each DGGE lane was adjusted according to concentration of DNA so that 200 microgram (µg) was loaded. A ladder of eight known bacterial species which included two species of *Lactobacilli* was run with each gel to form a basis for standardisation of RF values.

DGGE gels comprised 6% polyacrylamide in 1.75X TAE buffer with a urea and formamide denaturing gradient (55%-22%) prepared using a Hoefer gradient mixer

primed with 6.0 M urea and formamide. Electrophoresis was performed in 1.75X TAE at 130 V at 60℃ for 5 hours using a DCode System for DGGE (Bio-Rad). The gel was subsequently stained with SYBR safe nucleic acid gel stain (1:10,000 molecular probes) then scanned and photographed on GelDoc (Bio-Rad) system for analysis in Phoretics @ (Total Lab systems) software.

7.8 Analysis of DGGE gels

DGGE profiles of samples obtained from a single subject at monthly sampling times were compared in order to evaluate the temporal stability of the predominant faecal bacterial populations. The Gaussian volumes of the constituent amplicon peaks in the densiometric profiles of the DGGE gels above a rolling ball baseline were determined for each sample by profile deconvolution in the PHORETICS suite. Amplicon peaks with a total surface area of less than 1% of total were excluded from the similarity analysis.

7.9 Statistics

7.9.1 Species diversity

The ecological diversity of faecal microflora (i.e. the number of different classes (richness) and the relative distribution of individual species within each class) was assessed using Simpsons Index of Diversity, a means of quantifying information content in tables of richness and distribution (Begon *et al*., 1990; Washington 1984). Simpsons Index (D) was devised to measure diversity of ecosystems by quantifying the probability that two specimens picked at random from a community of species would be from different species. Hence if the proportion of individuals in a particular species is represented in the community by p_i, the probability of picking two members of this species is $[p_i]$ $[p_i]$ or $[p_i]^2$. If the probabilities of all of the 'I' species in a community are summed, the probability of picking two different species *(D)* can be calculated. Hence diversity is measured as the probability of picking two different species (Begon *et al*., 1990; Washington 1984).

$$D = 1-\sum_{i=1}^{s} (p_i)^2$$

Another way of representing this is; $SI = 1 - \sum (n/N)^2$ where n is the area of a particular band and N is the sum of areas of all the bands in the sample.

7.9.2 Differences between allochthonous and autochthonous species

When the composition of a community or series of communities varies over time in a manner that affects, it is possible to assess the range of species that are affected by this variation in a similar manner. Hence,

$$V = 1-\sum_{i=1}^{s} (dp_i)^2$$

where dp_i is the difference in the relative proportion of the i th species between two samples. If there is no difference then V =1-0 = 1, as the sum of dpi^2 increases. In which case, V is the probability that more than one species will be affected. Thus, for example, were variation to occur only in the i th species then $dp_i \rightarrow 1$ and V→0. Hence provided two equally representative samples are obtained, (i.e. that population size is effectively infinite), V increases with the number of species whose relative proportions change over time. In the event of the two treatments affecting the relative proportion of allochthonous and autochthonous populations differently, then this may be reflected in the presence of a significant interaction term between treatment and time in a comparison by repeated measures ANOVA.

To assess whether variation between successive samples resulted from changes in autochthonous or allochthonous species in this study, an analysis based on change in the diversity of species over successive samples was conducted. It was reasoned that in earlier samples change was more likely to result from effects on allochthonous species but in later samples change was more likely to result from effects on autochthonous species. Hence variation in species within subject between successive samples was quantified by computing a modified Simpsons index based on differences between the number of individuals within a species computed from differences in areas under the densiometric curve of the initial and subsequent DGGE gel profiles calculated as the Gaussian volume above a rolling baseline in PHORETICS @ suite as indicated above. Thus any differences in consecutive total areas under the densiometric curve of a particular species was included, as was the area from any species gained or lost.

This is relevant in the context of commensal enteral microflora as it permits assessment of overall stability of the various autochthonous communities

(Dethlefsen *et al*., 2006). There is likely some contribution from day-to-day variation in allochthonous species with diet but whether this variation overshadows that arising from autochthonous communities remains to be seen (Dethlefsen *et al*., 2006).

Simpsons index scores were determined for samples taken from people with CD and from control subjects. Data was normalised using a Johnson algorhythm based on arcsine transformation and analysed by one-way repeated measures ANOVA in SYSTAT (Wilkinson 1990).

7.9.3 Similarity of DGGE profiles within and between subjects

Simpsons Index of Diversity (Begon *et al*., 1990; Washington 1984) does not utilise all information available on DGGE gels as it does not consider band position on the denaturation profile (i.e. species identity). However if one species band replaces another band of identical volume then the index of diversity will not change. Hence Simpsons Index does not provide an assessment of relatedness in terms of GC content of 16s rRNA.

An assessment that includes relatedness (i.e. whether communities comprise groups of related species) may be more useful in studies which seek to characterise unique assemblages of microflora in individuals and assess themes in these assemblages in people with particular disorders (i.e. the extent of 'dysbiosis') (Begon *et al*., 1990). Similarly assessment of relatedness may be useful in gauging the extent of change i.e. whether loss of a particular band as indicated by other methods induces secondary effects at other trophic levels (Begon *et al*., 1990).

7.9.4 Differences between component peaks

Multivariate methods distinguish specific variations in the pattern of bands on a DGGE gel by condensing variation in the volume and position into a few axes of variation to enable meaningful comparison of the effects of various treatments on diversity and to relate these to variation in specific bands.

This research used stepwise forward 'discriminant analysis' as this rotates the orthogonal axes of variation to maximise differences between the treatments. Hence the method focuses on variations that are able to distinguish between

treatments. The initial and final log converted DGGE band volume profiles for each treatment were compared to quantitatively determine the extent of similarity prior to the commencement and following completion of three months gluten avoidance and to compare them with similarly spaced samples from control subjects. This enabled the simultaneous detection of any common themes of differences in enteral microbial population dynamics over time and between the two treatment groups.

Chapter Eight: THE FAECAL STUDY- Results of analysis of faecal samples

8.1 Dendrographic analysis

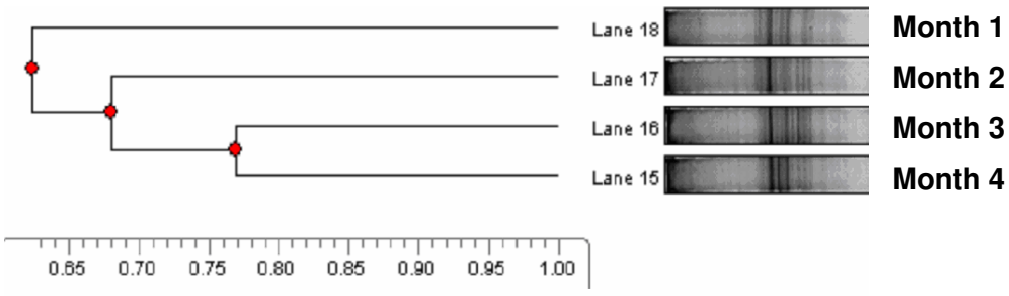
The dendrographic analyses of successive samples within subject for people with CD showed a tendency to ***temporal ordination*** mainly with a closer relationship between early samples but on two occasions with a closer relationship between later samples (Figure 8.1).

Temporal ordination indicates that data is related in time hence the closest resemblance to month 1 is month 2, the closest resemblance to month 2 is month 3 and so on. Thus there is a pattern of branches that cluster, as in subject CSI months 1-4 where the branch distance (distance from the red dot to the photo as measured on the scale beneath) goes strictly in decreasing (or increasing) length from 1 to 4.

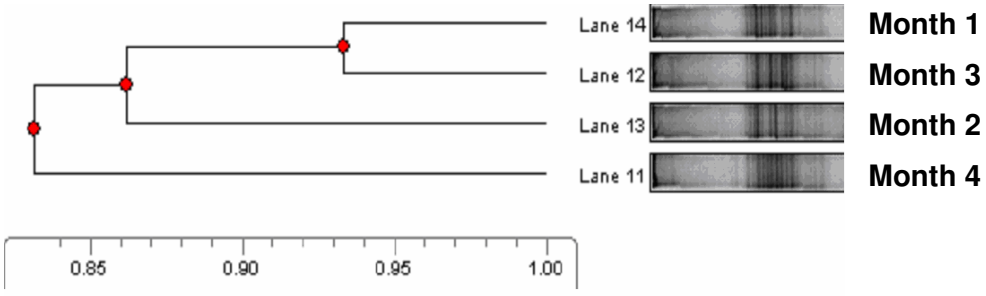
The dendrographic analyses of successive samples within subject for control subjects showed a temporally more random relationship between samples ordination (Figure 8. 2).

Figure 8.1: Dendrograms of DGGE analysis for subjects with CD

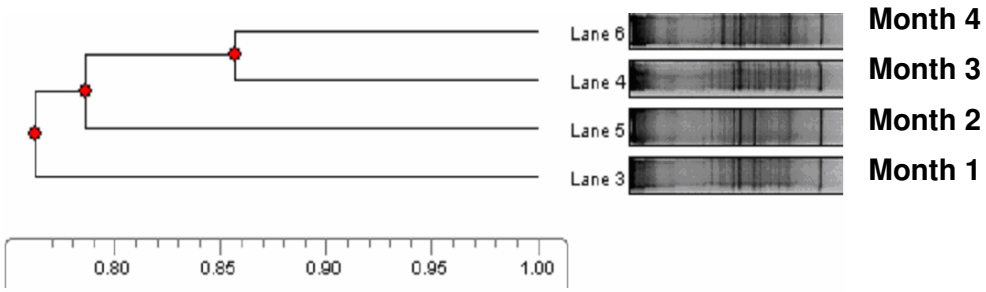
CSI 1-4 (Coeliac subject I)



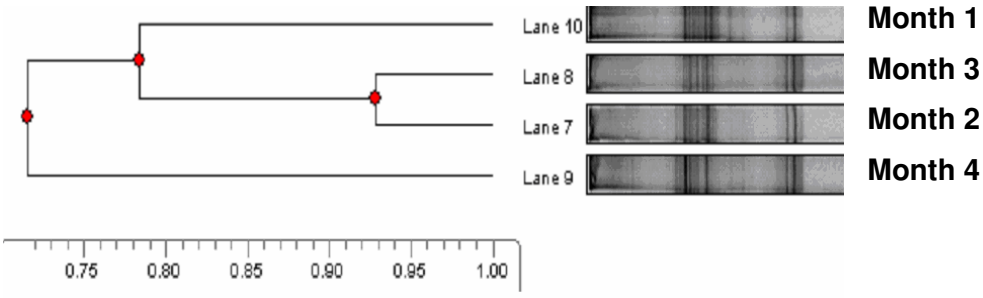
CST 1-4 (Coeliac subject T)



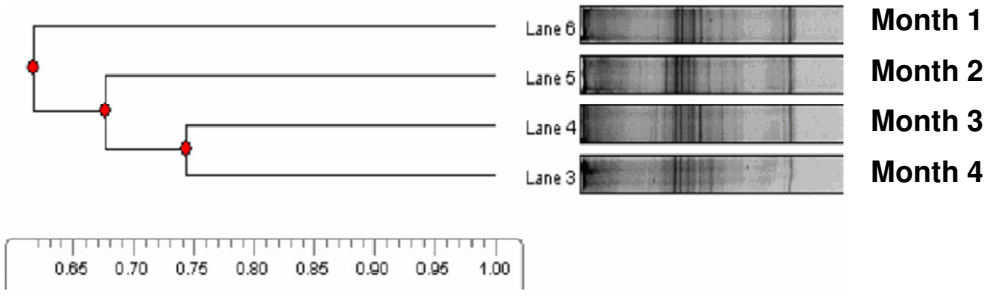
CSU 1-4 (Coeliac subject U)



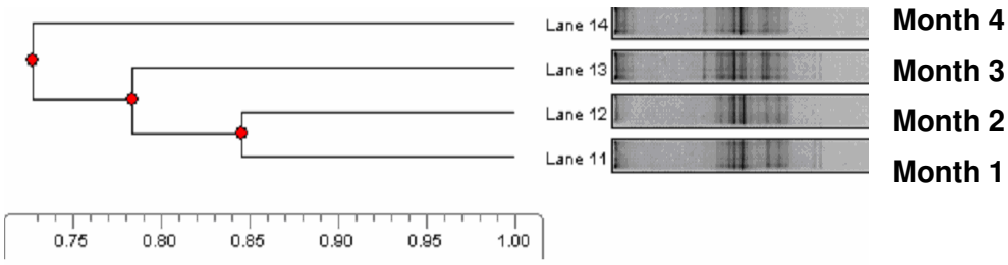
CSW 1-4 (Coeliac subject W)



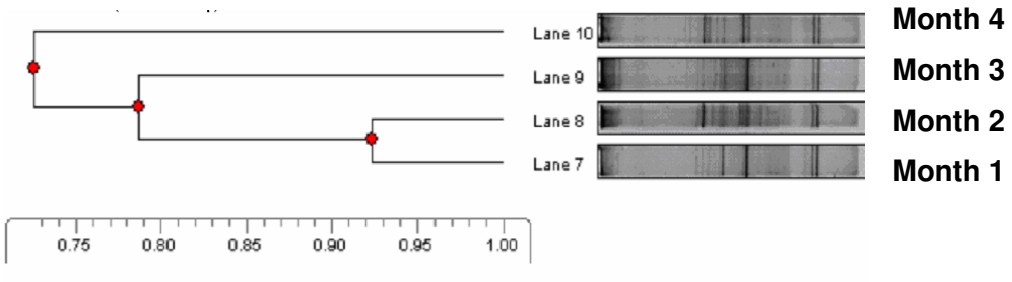
CSZ 1-4 (Coeliac subject Z)



CSAA 1-4 (Coeliac subject AA)



CSAB 1-4 (Coeliac subject AB)



CSAC 1-4 (Coeliac subject AC)

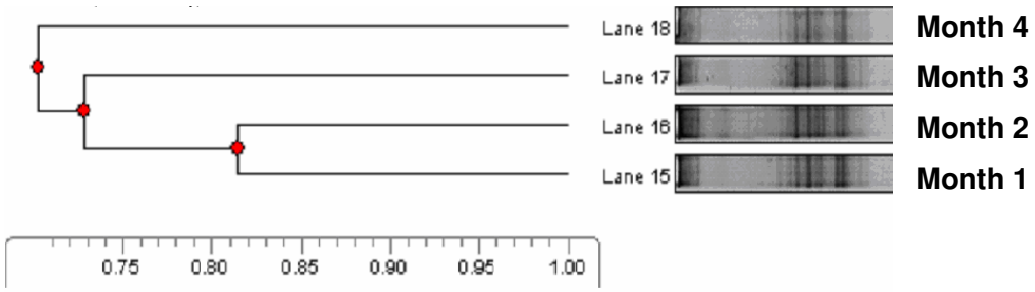
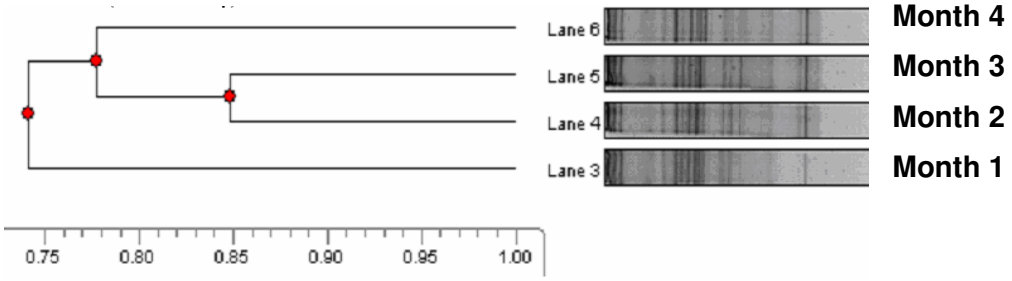
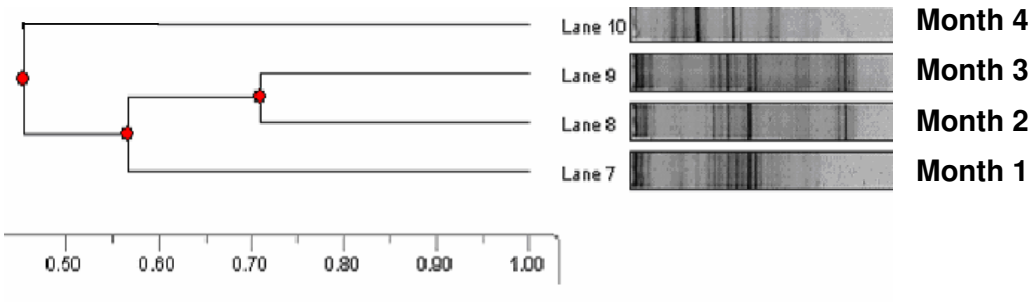


Figure 8.2: Dendrograms of DGGE analysis for subjects <u>without</u> CD

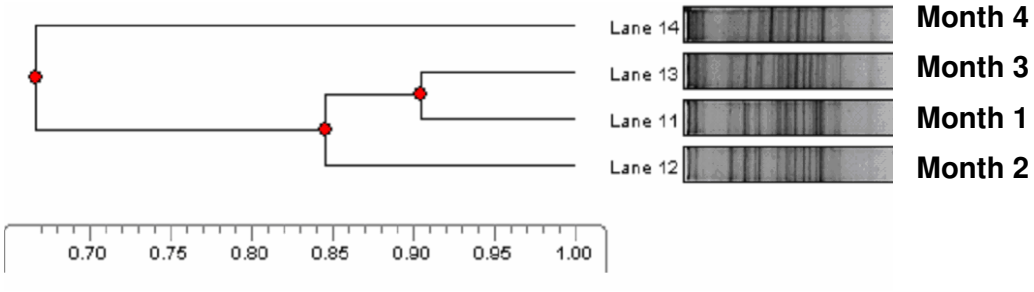
CSCGA 1-4 (control subject A)



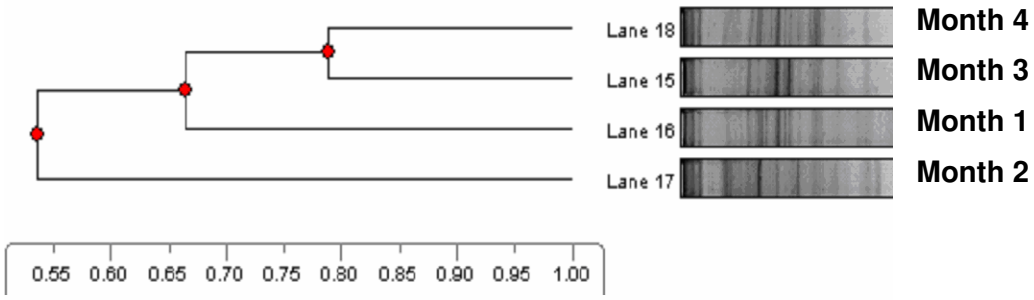
CSCGB 1-4 (control subject B)



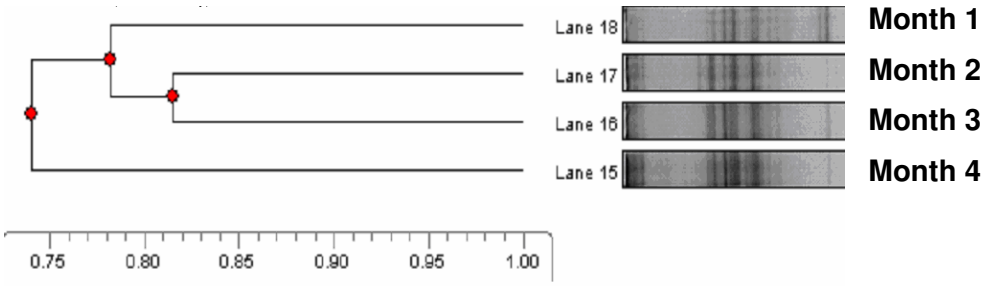
CSCGC 1-4 (control subject C)



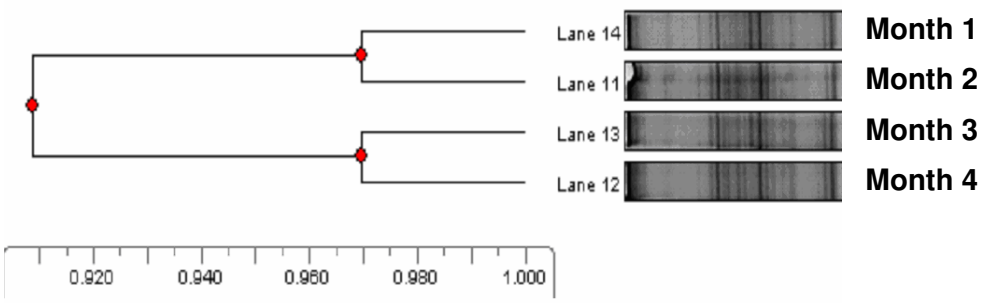
CSCGD 1-4 (control subject D)



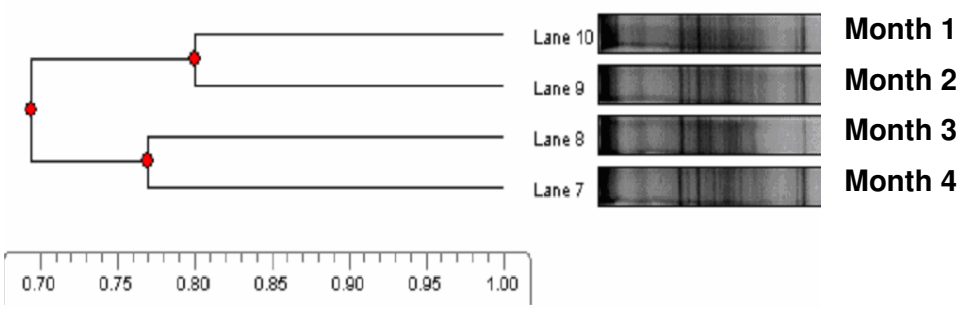
CSCGE 1-4 (control subject E)



CSCGF 1-4 (control subject F)



CSCGG 1-4 (control subject G)



CSCGH 1-4 (control subject H)

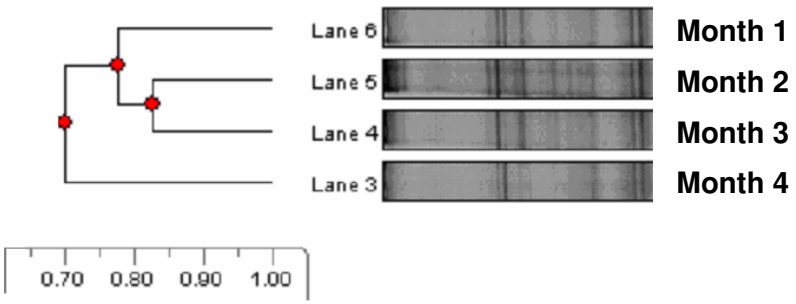


Figure 8.3: Summary of DGGE gel profiles for group of subjects with CD

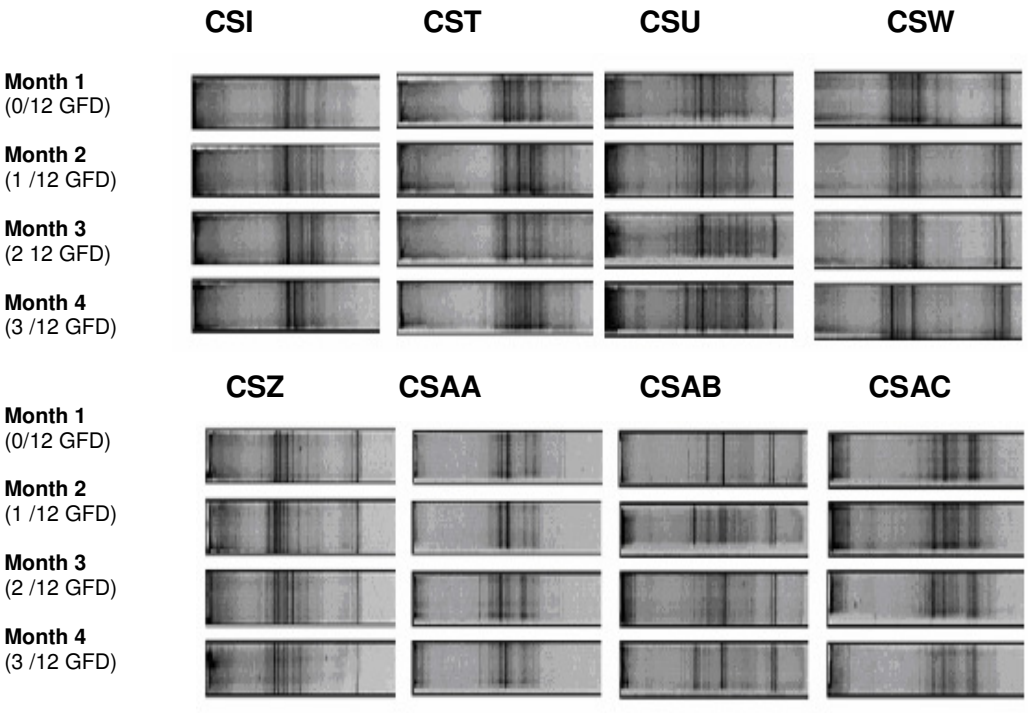


Figure 8.4: Summary of DGGE gel profiles for group of subjects without CD

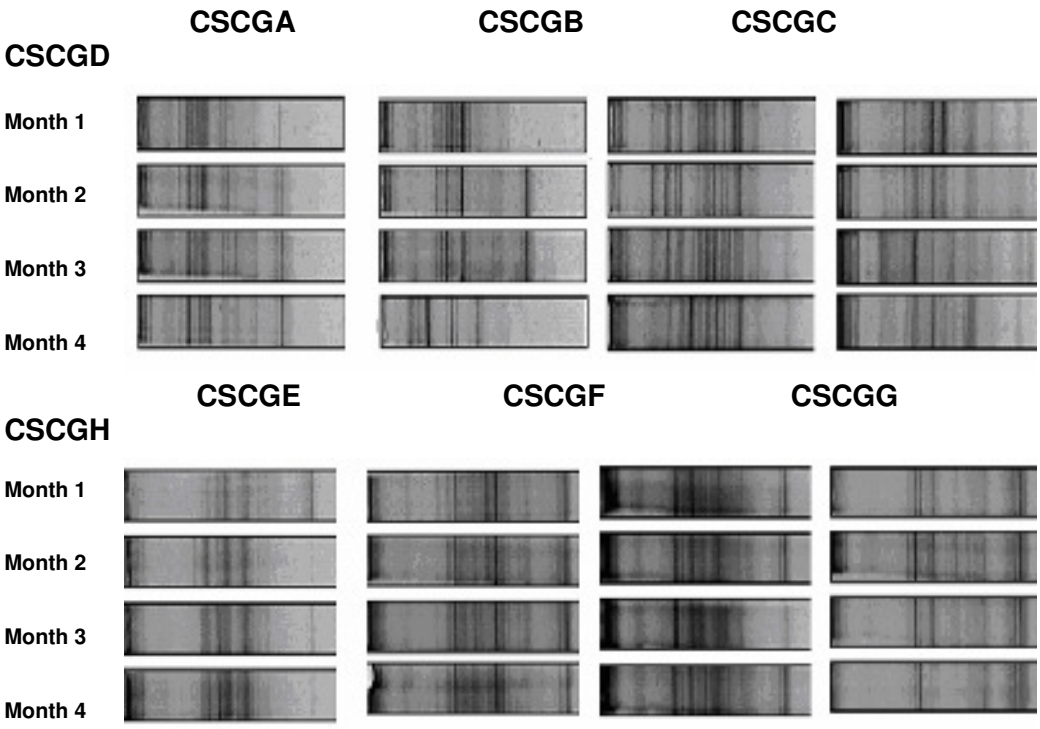


Table 8.1: Similarities of DGGE profiles from subsequent to initial sample on dendrographic analysis

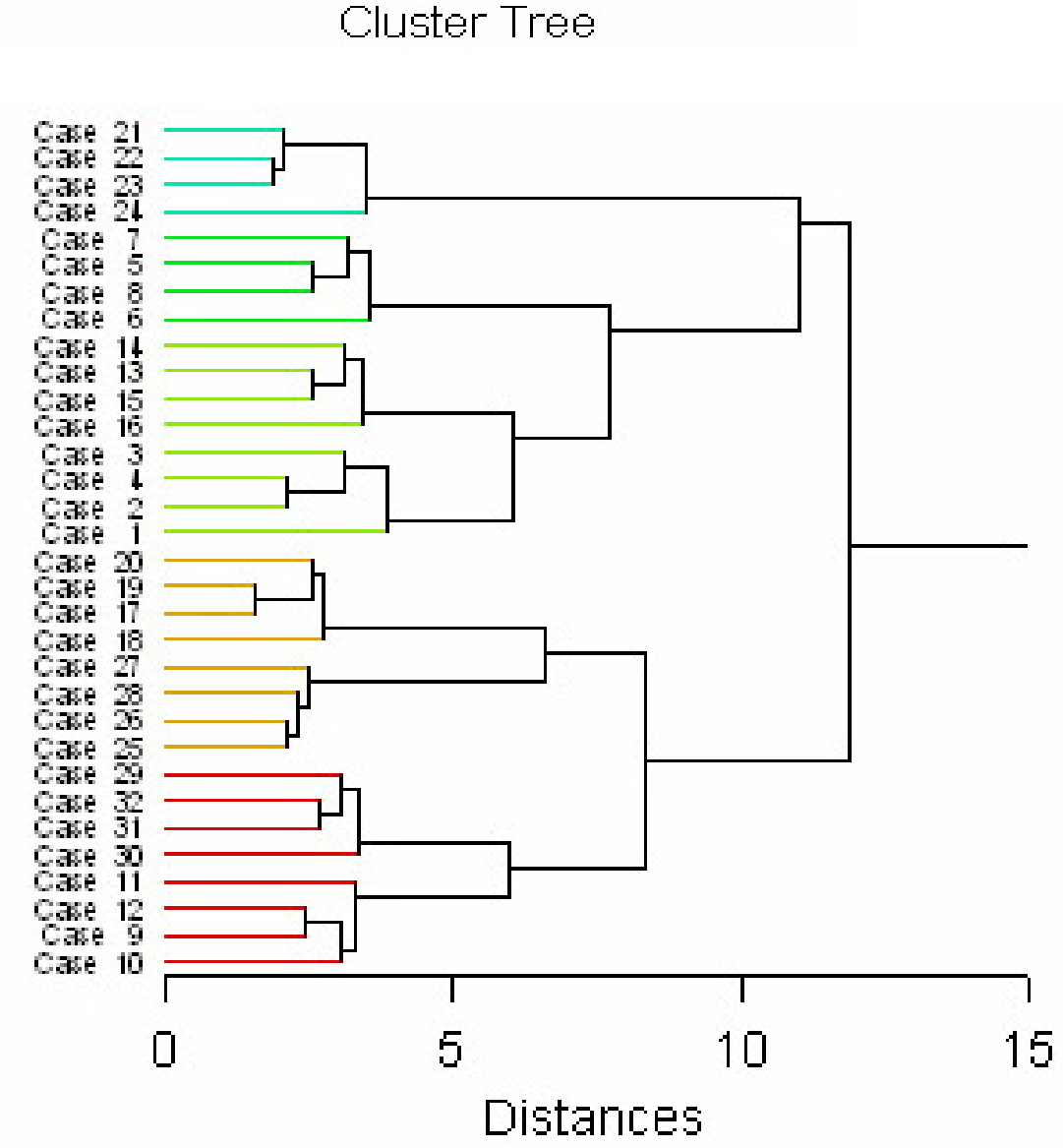
Group	Percentage similarity to initial sample (± se)		
	First month	Second month	Third month
Coeliac	0.79 ± 0.039	0.76 ± 0.036	0.72 ±0.027
Control	0.76 ± 0.037	0.74 ± 0.048	0.71 ± 0.057

Analysis conducted in Phoretics software on a basis of Euclidean distance with group separation based on UPGMA

Figure 8.5: Cluster analyses of DGGE profiles based on faecal microbial 16s RNA from subjects with CD and healthy controls

Cluster analyses of log converted areas under the densiometric curves of DGGE profiles at specific positions on the gel based on Euclidean distance with a Ward joining algorhythm. Case numbers are for eight subjects per treatment in successive sequences of four samples per subject in date order.

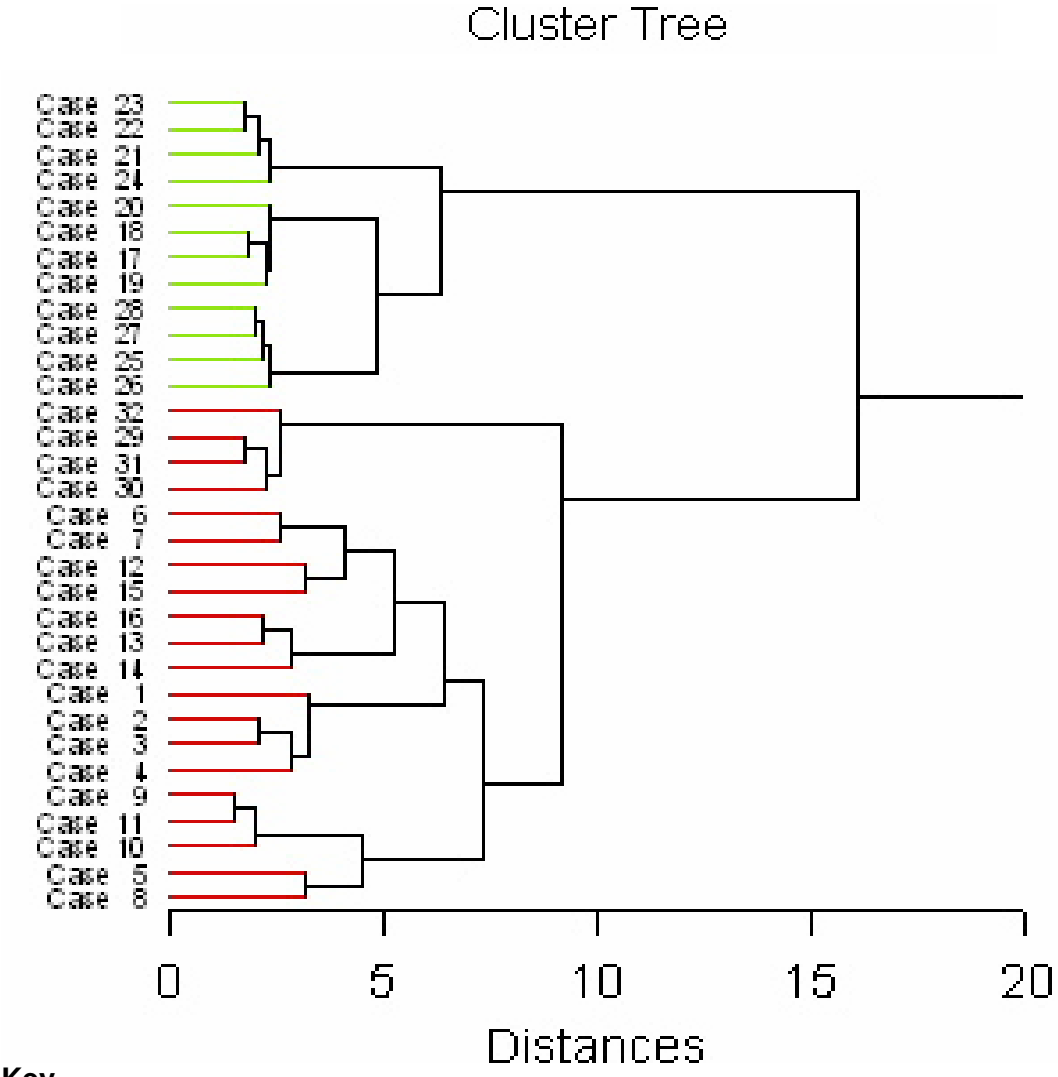
A) ***Samples from subjects with CD taken at diagnosis and monthly for three months following institution of a gluten- free dietary regime***



Key

Case 1, Case 2, Case 3, Case 4	= coeliac subject CSI (months1-4)
Case 5, Case 6, Case 7, Case 8	= coeliac subject CST (months 1-4)
Case 9, Case10, Case11, Case 12	= coeliac subject CSU (months1-4)
Case 13, Case 14, Case 15, Case 16	= coeliac subject CSW (months1-4)
Case 17, Case 18, Case19, Case 20	= coeliac subject CSZ (months1-4)
Case 21, Case 22, Case 23, Case 24	= coeliac subject CSAA (months 1-4)
Case 25, Case 26, Case 27, Case 28	= coeliac subject CSAB (months1-4)
Case 29, Case 30, Case 31, Case 32	= coeliac subject CSAC (months1-4)

B) ***Samples from control subjects at the time of recruitment and monthly for three months thereafter***



Key

Case 1, Case 2, Case 3, Case 4	= control subject CSCGA (months1-4)
Case 5, Case 6, Case 7, Case 8	= control subject CSCGB (months 1-4)
Case 9, Case 10, Case 11, Case 12	= control subject CSCGC (months1-4)
Case 13, Case 14, Case 15, Case 16	= control subject CSCGD (months1-4)
Case 17, Case 18, Case 19, Case 20	= control subject CSCGE (months1-4)
Case 21, Case 22, Case 23, Case 24	= control subject CSCGF (months 1-4)
Case 25, Case 26, Case 27, Case 28	= control subject CSCGG (months1-4)
Case 29, Case 30, Case 31, Case 32	= control subject CSCGH (months1-4)

The pattern of distribution of the cases on the Cluster tree (Figure 8.5 A and B) differs between the coeliac and the non-coeliac control groups. The length of the lines that separate the cases indicate the closeness between them. Short lines indicate that they are similar whereas longer lines indicate they are less similar.

8.2 Diversity

Simpsons Index of Diversity (I-D) was significantly lower on repeated measures ANOVA (df 1,14, f= 8.89, p= 0.01) in samples from people with CD (0.849± 0.011) than the control group (0.908±0.003). Fewer microflora tended to dominate the faecal community in people with CD, hence the probability of randomly selecting two individual bacteria (i.e. microbial diversity) was lower in people with CD. There were no significant changes in the values of Simpsons Index between successive samples on Post-Hoc Bonferroni testing either in people with CD or control subjects.

8.3 Multivariate analysis

The results of the multivariate comparison of distribution patterns of faecal microflora at diagnosis with those after gluten removal showed significant differences in overall distribution patterns of faecal microflora in the two treatment groups.

Hence the 'between groups' F values (Table 8.2) indicated significant discrimination between samples taken from people with CD and those taken from the control group based on the first axis of discrimination (Figure 8.3).

The 'between groups' F values showed it was possible to distinguish between the initial and final samples from people with CD (i.e. gluten avoidance resulted in a significant change in a number of enteral microbial species). However, it was not possible to distinguish between the initial and final samples of the control group.

Hence the jackknifed classification matrix showed successful distinction between people with CD and controls, but a less successful distinction between times of sampling (Table 8.2C).

It is noteworthy that there was successful discrimination of samples taken after three months of gluten avoidance from all other samples (Table 8.2) (Fig 8.3) and that this was based on a lower score on Factor 1 likely resulted from the presence of band B790 (Table 8.2b) which had a high negative loading and F to remove value. Hence people with CD who had undergone three months of gluten avoidance were more likely to have this micro-organism (band 790) in their faeces than people from the other treatment group.

Table 8.2: Results of a multivariate (discriminant*) analysis of the quantitative patterns of distribution of faecal microflora of people with CD and those of control subjects sampled at monthly intervals over three consecutive months

***Stepwise forward on log converted peak volumes and positions**

A) Between groups F-matrix - (df, 27, 59 Approx F= 6.24, p<0.0005)

	Control		Coeliac	
	Recruitment (a)	after 3mths (b)	At diagnosis (c)	after 3mths (d)
Control (a)	0.000			
Control (b)	0.54	0.000		
Coeliac(c)	11.91	7.90	0.000	
Coeliac(d)	27.78	23.47	8.60	0.000

B) Details of Band differences between treatments

Band no	F to remove	Standardised Discriminant function	Presence of band (in eight subjects)			
			Coeliac		Control	
			c	d	a	b
B049	5.19	0.995	1	0	0	0
B074	6.42	-1.254	1	2	0	0
B310	4.40	-0.940	1	3	0	0
B333	4.26	0.415	0	3	7	4
B491	6.67	-0.556	4	2	1	2
B600	2.86	-0.511	5	5	0	1
B612	16.01	-1.665	2	1	0	0
B685	6.95	-0.979	2	2	0	0
B790	30.69	-1.957	1	3	0	0

a = initial control sample; **b** = control sample after 3 months;
c = coeliac sample at diagnosis; **d** = coeliac sample after 3 months (3/12)

C) Jack-knifed classification matrix

actual group	Predicted group				
	a	**b**	**c**	**d**	**% correct**
a	6	2	0	0	75
b	2	6	0	0	75
c	0	2	3	3	38
d	1	0	1	7	88
Total	8	10	4	10	69

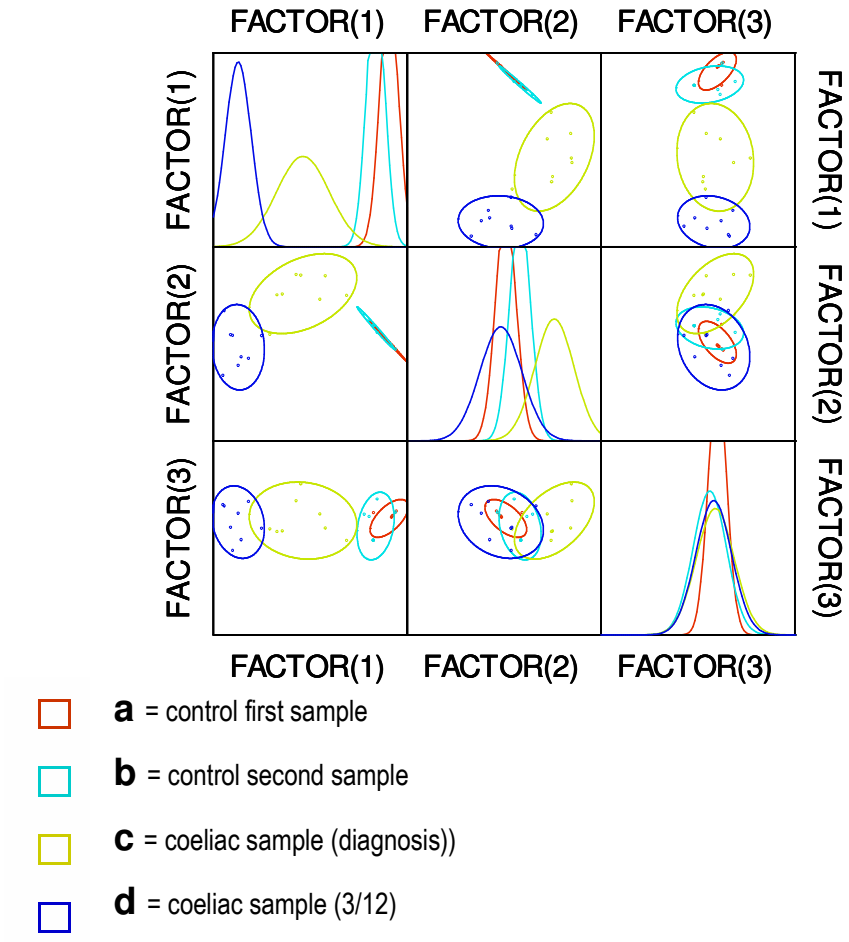
It is also evident that there was a greater level of distinction between the samples taken from people with CD (after gluten avoidance) and those taken from the control group, when compared to samples taken from people with untreated CD (i.e. at diagnosis) and the control group. Thus, while gluten avoidance produced symptomatic improvement, the faecal microflora of people with CD did not become more similar to those of the controls, mainly due to persistence of five species of micro-organisms that were not found in samples from control subjects.

The first axis of discrimination, which was responsible for the bulk of separation between the treatment groups, had high factor loadings for peak 131(B612) and 135 (B790). Seven of the nine DGGE bands that distinguished between the faecal microflora of coeliac and control groups were more likely to occur in people with CD than in control subjects. The remaining band was more intense in the control group than the coeliac group.

Table 8.2 shows the three bands more likely to occur in people with CD (i.e. B612, B685, B790). Three of these distinctive bands were positioned adjacent to the two *Lactobacillus* markers used for standardisation of the DGGE.

Figure 8.6 plots the individual scores on the principal axis of variation from the discriminant analysis of the DGGE profiles for both treatment groups. Factors 1, 2 and 3 are the three orthogonal axes of discrimination. Factor 1 represents the main method of separation used in this analysis.

Figure 8.6: Canonical Scores Plot of individual scores on the principal axis of variation from a discriminant analysis of the DGGE profiles of subjects with coeliac disease and control groups



8.4 Discussion of results

The significant decrease in microbial diversity in people with CD differs from the increase in diversity reported in a study on children (Sanz *et al*., 2007) and of people with other chronic inflammatory conditions of the bowel (Martinez- Medina *et al*., 2006; Bullock *et al*., 2004). However these researchers measured diversity as the number of bands present rather than using a formal index. These results, in showing no change in diversity following a period of gluten avoidance, do not fit with the suggestion that the resolution of mucosal inflammation may induce change in faecal microflora composition (Collado *et al*., 2007; Nadal *et al*., 2007). However, the longstanding decrease in general microbial diversity in people with CD fits with the hypothesis that people with CD express a number of phenotypes different from

those of normal subjects and that people with CD express differing patterns of fucosylation in their enteral mucins (Forsberg *et al*. 2004).

The lack of any significant differences in the distribution patterns of faecal microflora over four months indicates that the institution of a GFD does not change the general distribution pattern of enteral microflora to any greater extent than control subjects. Hence there is no evidence of extensive restructuring of communities. Any change in the distribution pattern of faceal microflora in people with active CD is more likely due to the addition or replacement of a few micro-organisms.

The persistence of distinctive microflora over three months of gluten avoidance when inflammatory change is likely to have subsided runs counter to the hypothesis that differences in the enteral microflora of people with CD (compared to the control group) are a result of the pro-inflammatory status of their mucosa (Kagnoff 2005).

These findings are not contrary to the hypothesis that particular enteral micro-organisms may be involved in the presentation of CD either causally or coincidentally (Collado *et al*., 2007; Nadal *et al*., 2007). Hence a number of microflora may be harboured by people with CD as a result of the presence of unique carbohydrate structures in the glycocalyx and mucous layer that facilitate their adhesion (Forsberg *et al*. 2004).

The similarities in the positions of the bands on the DGGE denaturation gradients that distinguished the coeliac group to those of *Lactobacilli* species on the calibration ladder and hence their similarity in their G/C ratio indicates they may be related to this genus.

Further work is required to sequence and more precisely identify these bands, but it is noteworthy that particular species of *Lactobacillus* have previously been associated with CD in childhood, namely *L. curvatus* (Sanz *et al*. 2007).

Furthermore, particular species of *Lactobacilli* (i.e. *L. fermentum* and *Bifidobacterium lactis*) prevent the appearance of morphological changes in Caco-2 cell clusters and the associated change in the expression of tight junction proteins which are associated with gliadin-induced damage, possibly by their greater ability to detoxify gliadin or gliadin-derived peptides than other species (Lindfors *et al*.,

2008). Particular species of *Lactobacilli* (i.e. *L. casei*) also polarise CD_4 T-helper cell responses towards a Th_1 phenotype without inducing mucosal ulceration characteristic of CD (D'Arienzo *et al*., 2008).

8.5 Potential limitations of the the faecal study

8.5.1 Small size of study

Recruiting was carried out through private gastroenterologists in Wellington and Palmerston North as people presented with symptoms indicative of CD however it difficult to find enough newly diagnosed people in the time allocated for recruitment. The success of this study was therefore reliant on the gastroenterologists approaching potential subjects. Only 8 people were willing to participate in the study over a one year period. A larger study group would have provided a more representative sample of people with CD, however as the study was a 'within subjects' design it was still possible to gain results that could be statistically validated. Because of the small size of the study, a control group of age and gender matched participants was included for comparison to ensure that changes observed over the four months of sampling were not simply due to stochastic drift.

8.5.2 Potential contamination of faecal samples during collection and transit

Subjects were asked to collect faecal samples in a home-based setting and courier these to the researcher. It is only assumed that the subjects followed their instructions and collected the samples as requested but there is no way of checking this. There is also no way of knowing if the environment where the faecal samples were collected was clean and free of potential contaminants or if the samples were delivered directly to the researcher or if there was a delay. Participants were asked to notify the researcher of the day when their samples were to be delivered but not the time. Once the samples arrived at the researcher they were immediately frozen at -18℃ pending transport to Massey University.

Chapter Nine: THE DIETARY STUDY

As prevouisly indicated CD is an immune mediated disorder involving a complex series of interactions, triggered by the consumption of gluten in genetically predisposed people. Without the consumption of gluten, CD would cease to exist so this is clearly a dietary related issue and is associated with a number of nutrient deficiencies.

9.1 Aims and rationale of the dietary study

9.1.1 Aims

The aim of the dietary study was to analyse dietary intake to determine if a lack of nutrients exists at diagnosis (before institution of a GFD) and at monthly intervals over three consecutive months post diagnosis (on a GFD). This with the view to determining whether resolution from inflammation associated with removal of gluten from the diet is similarly associated with an improved nutrient intake and status.

9.1.2 Rationale

It is known that CD is associated with nutrient deficiencies resulting from malabsorption due to intestinal inflammation and damage which can worsen the clinical picture. Research indicates that untreated CD is commonly associated with deficiencies of iron and folate, with less common (but still significant) deficiencies of vitamins A, D, E, K, B_{12}, B_6, and the mineral calcium, also being reported (Halfdanarson et al., 2007; Saibeni *et al*., 2005). People newly diagnosed with CD should be routinely evaluated for nutrient deficiencies (Presutti *et al*., 2007), hence the reason for the inclusion of an anaysis of dietary intake in this research.

9.2 Collection of data for the Dietary Study

Dietary information was collected using quantitative diet records and a qualitative food-frequency questionnaire which both have accepted pros and cons. An estimated diet record assesses the actual or usual intake of food while a food frequency questionnaire (FFQ) identifies food patterns associated with an inadequate intake of specific nutrients (Black 2001). Both methods are easy, quick and inexpensive to administer and have a reasonably low respondent burden so compliance is usually quite high over short time frames, however their accuracy depends on the conscientiousness of the subject, their ability to estimate quantities and it assumes a good level of literacy as they are self-administered (Black 2001).

These two methods were incorporated into a custom-designed questionnaire (see Appendix N).

Participants (the same ones as in the faecal study) recorded all food and drink consumed in the 3-days immediately prior to collection of each faecal sample as three to four days is the optimum time for gaining useful information using an estimated food (diet) record (Black 2001). Each participant weighed, measured or estimated the serving size using given examples (i.e. household scales, or measures-cups, teaspoons or tablespoons, or weights marked on packages, or describing the size of a serve- 1 slice of toast bread).

This method potentially provides good information but can have lower compliance as it takes time and effort to fill the charts in (Black 2001). (For limitations of methods see Table 9.1 in Appendix G). A specifically designed FFQ was included to assess usual eating habits. A list of specific foods was provided and participants indicated if they had consumed any of these food items in the week prior to the sampling period to determine the type of food typically consumed. This information was also used to fill gaps in the diet record where insufficient detail was given (Nelson *et al*., 1997).

9.3 Analysis of Dietary data

Dietary intake was analysed from the 3-day estimated food record carried out at diagnosis then at monthly intervals for three consecutive months post-diagnosis, following commencement of a GFD. The aim of this analysis was two-fold. Firstly, to determine any apparent nutrient deficiencies in the diet of subjects at the time of diagnosis of CD and secondly to gauge significant changes in nutrient intake over the ensuing three months post-diagnosis, with the view to determining whether resolution from inflammation associated with removal of gluten from the diet was similarly associated with a change in nutrient status.

Dietary data was assessed using **"Foodworks Professional" (Xyris software package 2007)** (see Appendices K and L for a summary of dietary data). Hypotheses were formulated as part of dietary analysis from the coeliac group. A similar set of data was collected from the control group however the amount of

gluten consumed was omitted. Nutrient intake (for both groups) was compared to the 1997 National Nutrition Survey (MOH 1999) and the National Nutrition Guidelines for Healthy Adults (MOH 2003). A rating system was devised to assess changes in gluten intake (in the coeliac group) from diagnosis through the post-diagnosis months.

9.3.1 Dietary rating

The exact amount of gluten present in 'gluten containing foods' is difficult to determine because it is not tested. Gluten only requires testing and listing if the food is to be sold as 'low gluten', or 'gluten-free', or if the manufacturer makes a claim relating to the gluten content in the product. It was not feasible to test all foods for their gluten content in this project so a scoring system was devised.

Gluten Rating system

0 = no known gluten
1 = trace amounts of gluten
2 = small amounts of gluten
3 = moderate amounts of gluten
4 = large amounts of gluten
5 = significant amounts of gluten

Each food was assigned one of these numbers based on its gluten content and a cumulative total derived for each day of data collection. So if a subject ate 5 foods containing gluten, the cumulative score for these 5 foods was determined for the day (see Table 9.2 in Appendix G).

9.4 Justification for choice of dietary data analysis

Treatment for CD is strict adherence to a lifelong GFD, which enables people to remain symptom-free and healthy. In most cases, a GFD eliminates symptoms within six to twelve months. However, a GFD may not be nutritionally balanced as it removes a high proportion of grain-based foods and reduces the intake of the essential nutrients supplied by these foods (Hallert *et al.*, 2002). The average daily intake of grain-based foods (in the US) is estimated at 250 grams (g) per person; of this intake, 180 g contains gluten which is removed on a GFD (Koehler 2005). Although other grains are substituted for wheat, rye, barley and oats, these grains are generally poorer sources of fibre, B-group vitamins, iron, calcium, magnesium, zinc and selenium (USDA 2001) (see Table 9.3 in Appendix G).

Untreated CD is commonly associated with deficiencies of iron and folate, and less commonly with deficiencies of vitamins A, D, E, K, B_{12}, B_6, and calcium due to malabsorption (Halfdanarson *et al*., 2007; Saibeni *et al*., 2005). Thus, people newly diagnosed with CD should be routinely evaluated for nutrient deficiencies (Presutti *et al*., 2007).

9.4.1 Iron malabsorption

IDA is the most frequent extra-intestinal symptom in adults with CD (Clark 2008; Halfdanarson *et al*., 2007; Annibale *et al*., 2001b; Bottaro *et al*., 1999). In many cases, IDA is the presenting symptom of CD thus screening for CD should be routinely carried out in adults who present with IDA (Grisolano *et al*., 2004; Mody *et al*., 2003; Kolho *et al*., 1998; Garrido *et al*., 1997; Schmitz *et al*., 1994). Iron malabsorption in untreated CD is mostly due to villous atrophy and crypt hyperplasia in the proximal small intestine which causes a decreased absorptive surface area and reduced absorption (Grisolano *et al*., 2004). People with iron malabsorption characteristically have low serum iron levels, elevated total iron-binding capacity and low ferritin levels (Cook 2005).

Sari *et al*., (2000) examined people newly-diagnosed with asymptomatic CD accompanied by 3-years of unexplained severe IDA and found that a strict GFD led to increased serum iron levels, resolution of anaemia, and restoration of normal mucosal morphology. Recovery from anaemia usually occurs within 6-12 months on a GFD due to normalisation of the intestinal mucosa (Sari *et al*., 2000). Thus the recommended treatment of IDA associated with CD is a GFD and iron supplementation until the iron stores are restored (Halfdanarson *et al*., 2007; Annibale *et al*., 2001b) (For haematologic manifestations in CD see Table 9.4 in Appendix G).

9.4.2 Folate deficiency

Folate deficiency is a frequent finding in untreated and newly diagnosed CD (Haapalahti *et al*., 2005; Gregory *et al*., 2002; Howard *et al*., 2002; Kemppainen *et al*., 1998; Bode *et al*., 1996; Hoffbrand 1974). Two early studies found folate deficiency common in children with CD but it does not usually result in anaemia (Stevens 1979; Pittschieler 1986).

9.4.3 Vitamin B_{12} deficiency

Vitamin B_{12} deficiency was considered rare in CD as it is a disorder of the proximal small intestine and the distal ileum is relatively spared (Dickey *et al*., 2004). This is not the case, as vitamin B_{12} deficiency is common in CD and frequently results in anaemia (Dickey *et al*., 2004). Recent studies suggest that 8-41% of previously untreated subjects with CD were deficient in vitamin B_{12} (Dickey 2002; Dahele *et al.,* 2001). This is consistent with earlier findings reporting 11% incidence of vitamin B_{12} deficiency in 50 consecutively diagnosed patients with CD (Bode *et al*., 1996). Vitamin B_{12} deficiency should be suspected in all people with CD who have haematologic and neurologic abnormalities. Vitamin concentrations mostly normalise on a GFD but in some cases supplementation is required. The cause of vitamin B_{12} deficiency in CD is unknown but may include bacterial overgrowth, autoimmune gastritis, or perhaps dysfunction of the distal small intestine (Dickey *et al*., 2004).

9.4.4 The impact of elevated homocysteine levels in CD

Homocysteine levels are often elevated in untreated and newly diagnosed CD and may serve as a diagnostic clue (Saibeni *et al*., 2005). Hyperhomocysteinemia could, in part, be attributed to deficiencies in vitamin B_{12}, B_6 and folate (Saibeni *et al*., 2005; Wilcox *et al*., 2006). Dickey *et al*., (2008) compared plasma homocysteine levels and biomarker status of metabolically related B-vitamins (folate, vitamin B_{12}, B_6 and B_2) in treated and untreated CD patients and healthy controls. They found no evidence of compromised vitamin B_6 or vitamin B_2 status. However, they noted that homocysteine concentrations were inversely associated with both serum, red cell folate and with serum vitamin B_{12} levels.

Hyperhomocysteinaemia is a risk factor for cardiovascular disease (particularly stroke) and is implicated in recurrent miscarriage and osteoporotic fracture, all of which are recognised manifestations of CD (Dickey *et al*., 2008). Thus, reducing the risk of homocysteine-related disease is another reason for aggressive diagnosis and treatment of CD, as gluten exclusion conclusively normalises folate, vitamin B_{12} and homocysteine levels (Dickey *et al*., 2008; Wilcox *et al*., 2006; Saibeni *et al*., 2005).

9.4.5 Vitamin B_6 deficiency

Reinken *et al*., (1976) compared vitamin B_6 levels in children with active CD to those in remission and a control group with normal duodenal mucosa. Children with untreated CD had significantly decreased pyridoxal phosphate levels in serum and the duodenal mucosa when compared with both children in remission and controls. The activity of pyridoxal-kinase, however, was significantly increased in both the serum and the duodenal mucosa when compared with controls, but not compared with children in remission. These children had the same increase in pyridoxal-kinase activity as children with acute CD suggesting vitamin B_6 deficiency. The children with CD in remission still had an increased activity of pyridoxal-kinase which may be a compensating mechanism caused by vitamin B_6 deficiency prior to the implementation of a GFD.

Adults with CD excrete abnormal amounts of tryptophan metabolites after loading with this amino acid, suggesting vitamin B_6 deficiency (Reinken *et al*., 1976). The excretion of tryptophan metabolites normalises after administration of vitamin B_6 (Reinken *et al*., 1976). Hallert *et al*., (1983) found supplementation with vitamin B_6 in adults with CD improved their mental state and alleviated the psychological symptoms frequently associated with CD indicating a possible causal relationship between adult CD and concomitant depressive symptoms. Clayton (2006) recently examined this association of vitamin B_6 malabsorption with CD and found people with CD have increased requirements for pyridoxine and /or pyridoxal phosphate and that as pyridoxal phosphate is a co-factor for over a hundred enzyme-catalysed reactions in the body, a deficiency of vitamin B_6 has wide ranging physiological implications (Clayton 2006).

9.4.6 Fat-soluble vitamin deficiency

9.4.6.1 Vitamin A

CD is linked with Vitamin A deficiency resulting in visual disorders, most commonly due to malabsorption (Sass *et al*., 2000; Purvin 1999). In untreated CD, damage to the intestinal mucosa causes a reduced absorptive surface area and decreased nutrient absorption. While the small intestine adapts and compensates for the loss of functional surface area, this process takes time and fat malabsorption frequently occurs in the interim. Healing of the intestinal mucosa is inhibited by a deficiency in

Vitamin A. The removal of gluten (the causative agent) and supplementation with Vitamin A frequently repairs the gut and improves clinical outcomes in CD (Sass *et al*., 2000).

9.4.6.2 Vitamin D

Although dietary vitamin D contributes very little to vitamin D status in most people, untreated CD is indirectly linked with vitamin D deficiency (Garrison *et al.,* 1995). Vitamin D absorption depends on normal bile secretion and fat absorption, so decreased absorption in CD causes flushing out of unabsorbed fats, calcium soaps and vitamin D in steatorrhoeic stools (Garrison *et al.,* 1995). While normalisation of the intestinal mucosa after gluten removal controls the steatorrhoea and enables vitamin D levels to normalise, in many cases damage associated with impaired mineralisation is permanent. In children, this may result in the inability to develop optimal bone mass while in adults a loss of bone mass can occur, both of which increase the risk of osteoporosis, osteopenia and osteomalacia (Shane 1996).

9.4.6.3 Vitamin E

Untreated CD is associated with severe vitamin E deficiency which reverses on a GFD and with vitamin E supplementation, again a direct consequence of fat malabsorption (Kleopa *et al*., 2005). It has been suggested that vitamin E deficiency is associated with the neurological complications of CD (Mauro *et al*., 1991).

9.4.6.4 Vitamin K

Vitamin K deficiency and intestinal bleeding are known in CD due to malabsorption (Djuric *et al*., 2007; Avery *et al*., 1998). Hypoprothrombinemia (caused by vitamin K malabsorption) is a well-documented complication of CD however overt vitamin K deficiency bleeding is rare (Djuric *et al*., 2007; Granel *et al*., 2005; Lubel *et al*., 2005; Vaynshtein *et al*., 2004). Disruption of IM and associated changes in vitamin K production may contribute to this deficiency bleeding in CD (Djuric *et al*., 2007). Prolonged prothrombin time was reported in 18.5% of adults and 25.6 % of children with CD (Ertekin *et al*., 2006; Cavallaro *et al*., 2004). This prolonged prothrombin time occurs almost exclusively in people with a typical presentation of CD rather than in people with 'silent' clinical forms of CD (Hussaini *et al*., 1999). Hypoprothrombinemia in CD responds well to a GFD (Djuric *et al*., 2007).

9.4.7 Deficiency of other micronutrients

9.4.7.1 Calcium deficiency

Calcium malabsorption, hypocalcemia and skeletal demineralisation are all recognised features of untreated CD. Calcium deficiency due to reduced absorption is noted even in treated CD. Thus, low bone density is common in both children and adults with untreated and newly diagnosed CD (Pazianas *et al*., 2005; Shane 1996). While there are long-term benefits of gluten withdrawal on calcium metabolism in CD, the establishment of a GFD may not normalise calcium absorption and bone mineral density even with increased intake (Pazianas *et al*., 2005).

Molteni *et al*., (1995) found that at diagnosis, people with CD had intestinal calcium malabsorption (using the strontium test) but after starting a GFD, the normalisation of calcium absorption and the decrease of mid-molecule parathyroid hormone (PTH) suggested a normalisation of mineral metabolism, although a positive effect on bone mineral density was not evident at that time (after 12 months on GFD). It is also noted that all people in this study had significantly abnormal mean haemoglobin, serum potassium, magnesium, plasma calcium, urinary calcium, and phosphorus levels at diagnosis. There is also evidence that people with untreated CD excrete more calcium in faeces than 'normal' people do (Shane 1996).

9.4.7.2 Magnesium deficiency

Osteoporosis and magnesium deficiency often occur in malabsorption syndromes. Rude *et al*., (1996) demonstrated that people with CD have reduced intracellular free magnesium ions (Mg^2+), despite being clinically asymptomatic on a GFD. Bone mass was also reduced. Magnesium therapy resulted in a rise in PTH, suggesting that the intracellular magnesium deficit was impairing PTH secretion in these people. An increase in bone density in response to magnesium therapy indicates that magnesium depletion may be a contributing factor in osteoporosis associated with CD (Rude *et al*., 1996).

9.4.7.3 Selenium and Carnitine

Significantly lower levels of selenium are found in both treated and untreated CD compared to controls (Yuce *et al*., 2004). Selenium deficiency is attributed to malabsorption in untreated CD. Yuce *et al*., (2004) found the serum levels of selenium in children with CD were decreased. Levels were similar in children with

and without diarrhoea as a presenting symptom. Low serum levels of selenium are associated with an increased incidence of GI tumours in people with CD (Rayman 2000; Sher 2000; Cortigiani *et al*., 1989).

For those on a GFD, selenuim deficiency can be caused by the diet itself due to removal of grain-based foods that are valuable sources of selenium. New Zealand soils are generally low in selenium which affects the concentration in food and the availability of selenium in the food supply (Vannoort *et al*., 2000). In the general New Zealand population, selenium levels are improving due to increased use of Austrialian wheat and wheat-based products with higher selenium content (MOH 2003). This source is not available to people on a GFD.

Yuce *et al*., (2004) also found that the serum levels of carnitine in children with CD were reduced due to malabsorption. In another study, serum total carnitine (but not free carnitine) was significantly decreased in a group of adults with active CD (Lerner *et al*., 1993) and more recently both free and total carnitine deficiency was found in an infant with CD (Fitzgerald *et al*., 2003).

9.4.7.4 Zinc and copper

Trace metal deficiency is common in CD, with both zinc and copper deficiency having been observed (Solomons *et al.,* 1976). Copper deficiency has been described in adults and children with CD and may result in anaemia and thrombocytopenia (low blood-platelet level) (Fisgin *et al*., 2004; Goyens *et al*., 1985).

Chapter Ten: DIETARY STUDY- Results of statistical analysis of dietary data

Both sample groups were analysed to determine a normal distribution. Variables were screened for outliers and tested for normality using the Lillefors test and a Post-Hoc Bonferroni sought to determine whether significant differences existed between group means in an analysis of variance setting. This would allow for comparisons of means while controlling the type-1-error rate (Howell 2002). However data was not normally distributed, so parametric analysis were not considered appropriate. Non-parametric analyses were carried out in the form of a Kruskall-Wallis One Way Analysis of Variance (K-W).

Such non-parametric tests are a powerful and robust measure. The Kruskall-Wallis is 95% as powerful as equivalent parametric tests (i.e. ***t***- test and analysis of variance). The relative power of the Kruskall-Wallis test declines to 65% as the size of the sample increases, however, there should be little hesitation using it to replace ***t***- tests in this situation where the sample size is small and the distributions of ***t*** and F may be skewed (Klugh 1986).

Because it is non-parametric, the Kruskal-Wallis test does not assume a normal population, unlike the analogous one-way analysis of variance. However, the test does assume an identically-shaped distribution for each group, except for any difference in medians (Siegel *et al*., 1988).

10.1 Statistical Analysis of the Coeliac Group

(For a summary of results see Appendix I).

10.1.1 Gluten Intake

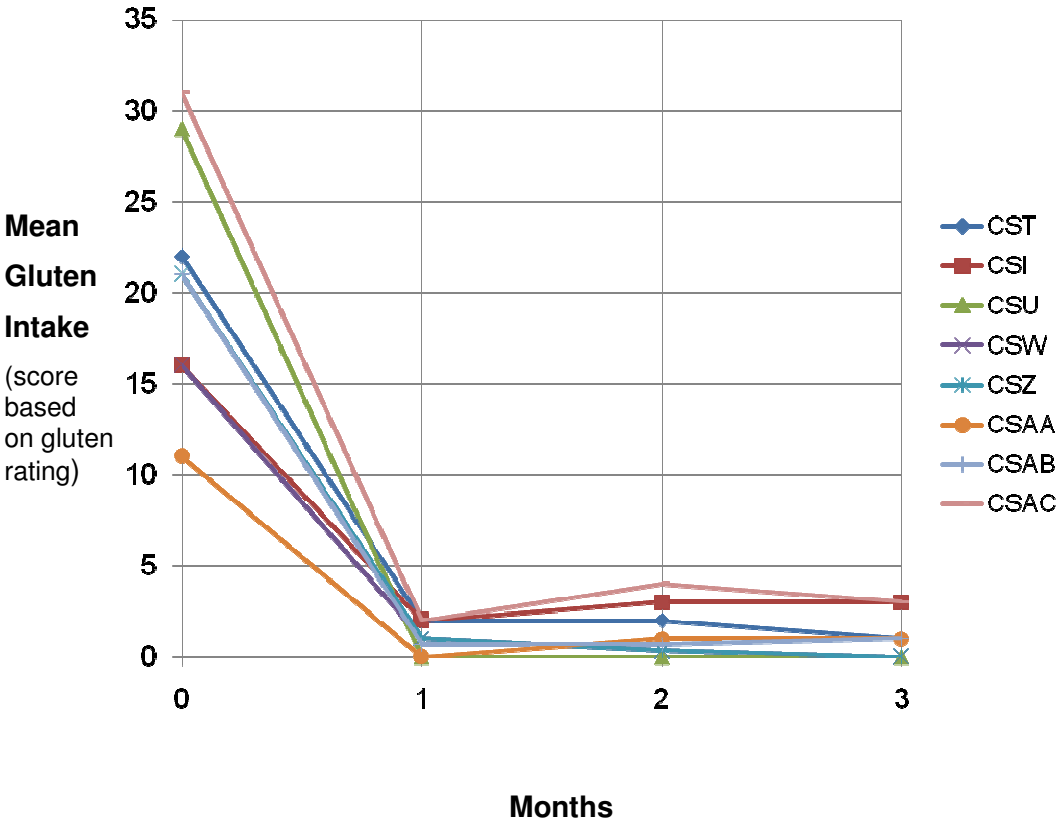
Hypothesis One: It is proposed that gluten intake will decrease immediately after diagnosis, but increase slightly in months 3 and 4 as new foods are included in the diet which could inadvertently contain gluten.

Results and discussion: Highly significant differences were found on Kruskall-Wallis tests of variance between month 1-2 (N= 48; Mann-Whitney U score= 576.000; P < 0.0005), month 1-3 (N=48; Mann-Whitney U score 576.000; P < 0.0005) and month 1-4 (N= 48; Mann-Whitney U score = 576.000; P< 0.0005)

indicating a fall in gluten consumption after month 1 (see Appendix H and I). No significant differences occurred between month 2-3, (N= 48; Mann-Whitney U score= 259.500; P= 0.534) month 2- 4 (N = 48; Mann-Whitney U score = 302.500; P= 0.745), or months 3-4, (N= 48; Mann-Whitney U score = 325.500; P= 0.404)

Some inadvertent consumption of gluten would have been expected. Most participants were not being very adventurous in their food choices and were selecting foods known to be gluten-free (i.e. meat, fruit and vegetables). Thus, the initial part of the hypothesis is upheld whilst the latter part is refuted. Figure 10.1 graphically illustrates the mean gluten intake for all coeliac subjects over the four consecutive months of testing.

Figure 10.1: Mean gluten intake for the coeliac group

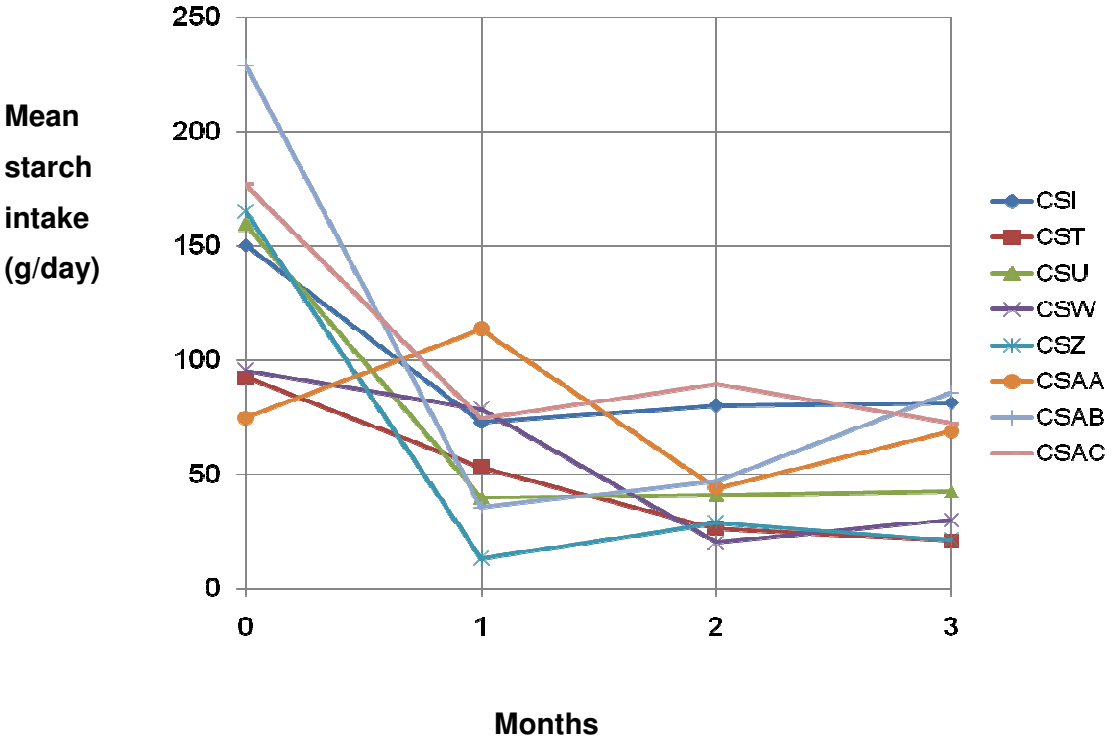


10.1.2 Starch Intake

Hypothesis Two proposes that starch intake will decrease on a GFD because all wheat, barley, rye and oat-based foods that are good sources of starch are removed.

Results and discussion: Highly significant differences were found on Kruskall-Wallis tests of variance between month 1-2 (N= 48; Mann-Whitney U score= 505.000; P < 0.0005), month 1-3 (N=48; Mann-Whitney U score = 544.000; P < 0.0005) and month 4 (N= 48; Mann-Whitney U score = 518.000; P< 0.0005) indicating that starch intake fell after the first month (see Appendix I). These results are as expected, with the exception of subject CSAA who had an increased intake from diagnosis to one month post-diagnosis due to an increased consumption of fries to compensate for the removal of breads and cereals. Thus this hypothesis is confirmed. Figure 10.2 shows the mean starch intake in grams/day over the consecutive months of testing.

Figure 10.2: Mean Starch intake



10.1.3 Total fibre intake

Hypothesis Three: proposes a decrease in the total fibre intake on a GFD because commercially available GF foods, (especially carbohydrate-based foods) are generally low in dietary fibre as fibre-rich grains have been removed. These foods are more likely to be consumed by newly diagnosed indivduals while they adjust to their new dietary regime.

Results and discussion: There were no significant differences between month 1-2, (N= 48; Mann-Whitney U score= 310.000;P= 0.650), month 1-3 (N = 48; Mann-Whitney U score = 344.000;P= 0.284), or month 1-4 (N = 48; Mann-Whitney U score= 365.000;P = 0.112) (see Appendix H and I). This is a little surprising as research shows that non-gluten containing grains have a lower fibre content than their gluten containing counterparts (USDA 2001). However, an increase in fruit and vegetable consumption may compensate for the removal of gluten containing grains. This appears to be the case with this study. Thus this hypothesis is rejected.

10.1.4 Total energy intake

Hypothesis Four: total energy intake will increase on a GFD for two reasons;

(1) The amount of food consumed increases; removing gluten alleviates symptoms and discomfort decreases so the person may begin to eat more food.

(2) The type of food consumed changes; many commercially available GF foods are energy-dense foods.

Results and discussion: No significant differences were found between month 1-2, (N= 48; Mann-Whitney U score= 281.000;P= 0.885), month 1-3 (N = 48; Mann-Whitney U score = 297.000;P= 0.853), or month 1-4 (N = 48; Mann-Whitney U score= 298.000;P = 0.837) (see Appendix H and I). Total energy intake should increase because the amount eaten increases and the type of food changes. Prior to diagnosis, gastric upset, abdominal discomfort and bloating are common. Diagnosis and gluten removal alleviates symptoms so more food is eaten. However, many commercially available GF foods are energy dense. The short time-frame of the study does not allow for the assessment of overall weight gain following the implementation of a GFD.

Alternatively, total energy intake could decrease on a GFD because the newly diagnosed subject is unsure of what foods to safely eat. They may be completely overwhelmed by not being able to eat familiar foods so choose not to eat rather than eating the wrong foods. Also the energy-rich carbohydrate-based foods removed from the diet may not be replaced in the same quantities as previously consumed. Results found no significant differences (either increases or decreases) in intake between consecutive months, thus both these hypotheses are rejected.

10.1.5 Total fat intake

Hypothesis Five: proposes an increase in total fat intake on a GFD because the participant may eat foods higher in fat (e.g. fries or snack foods) until they become accustomed to buying or making GF foods. Their total fat intake should decrease as their knowledge of GF foods expands and their confidence increases.

Results and discussion: No significant differences were found between month 1-2, (N= 48; Mann-Whitney U score= 281.000;P= 0.885), month 1-3 (N = 48; Mann-Whitney U score = 297.000;P= 0.853), or months 1-4 (N = 48; Mann-Whitney U score= 298.000;P = 0.837) (see Appendix H and I). Total fat intake neither increased nor decreased, thus this hypothesis is rejected.

10.1.6 Saturated fat intake

Hypothesis Six: proposes that more saturated fat will be consumed on a GFD because when carbohydrates are removed from the diet, the participant may compensate for the loss of energy by consuming more meat and dairy products higher in saturated fat. Alternatively, they may eat commercially available GF foods high in saturated fat.

Results and discussion: No significant differences were found between month 1-2, (N= 48; Mann-Whitney U score= 305.000;P= 0.537),month 1-3 (N = 48; Mann-Whitney U score = 319.000;P= 0.523), or month 1-4 (N = 48; Mann-Whitney U score= 289.000;P = 0.984) (see Appendix H and I). More saturated fat should be consumed on a GFD when carbohydrates are removed however, results did not confirm this so the hypothesis is rejected.

10.1.7 Carbohydrate intake

Hypothesis Seven: proposes an initial decrease in carbohydrate intake on a GFD, but a subsequent increase in months 3 and 4 because gluten-containing cereal-based foods are removed. As GF foods based on corn, rice or potato, and sugary commercial GF foods are added back in, carbohydrate intake should increase slightly.

Results and discussion: Significant differences were found on Kruskall-Wallis tests of variance between month 1-2 (N= 48; Mann-Whitney U score= 409.000;P=0.013), however no significant difference was found between month 1-3 (N=48; Mann-Whitney U score = 378.000; P=0.063) or month 1-4 (N= 48; Mann-Whitney U score = 325.000;P=0.445) indicating carbohydrate intake fell after the

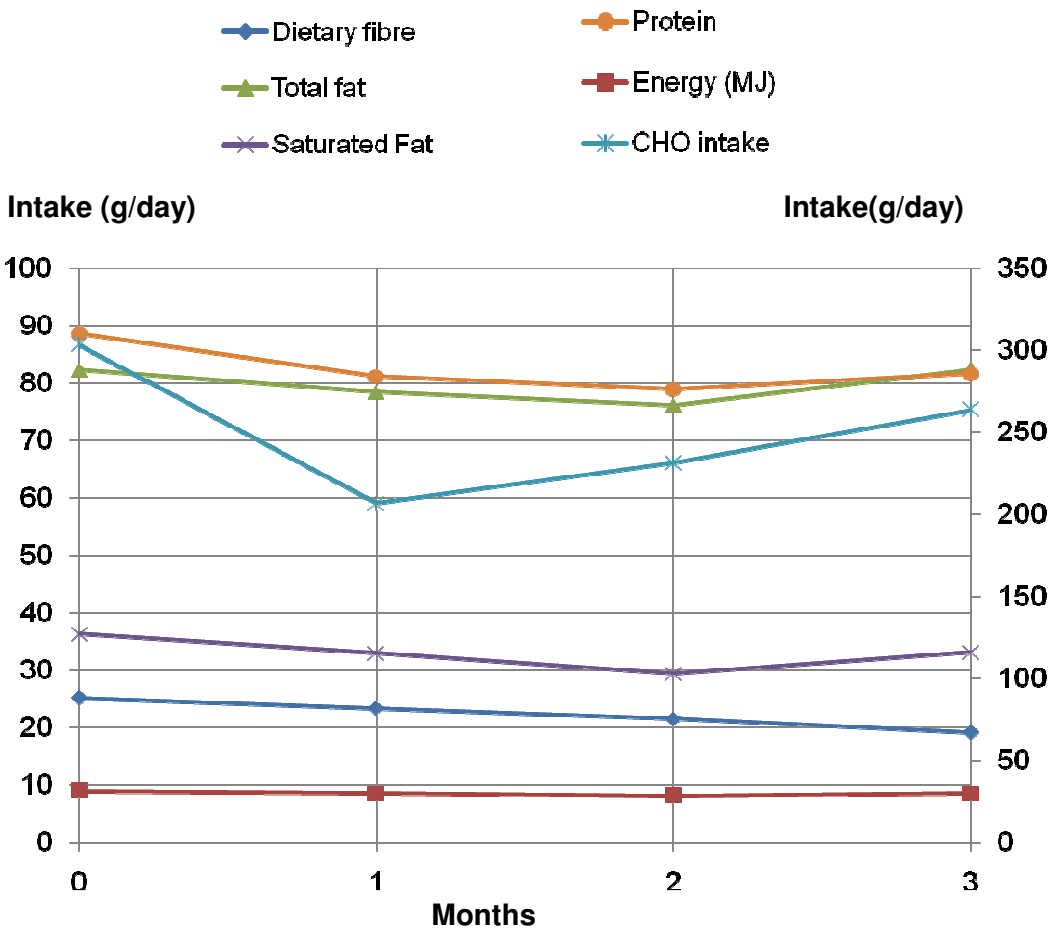
first month but not between consecutive months (see Appendix H and I). Thus, the initial part of the hypothesis is confirmed whilst the latter part is rejected.

10.1.8 Protein intake

Hypothesis Eight: proposes that protein intake will increase on a GFD because the participant will likely consume more protein-rich foods (eggs, fish, chicken, meats, nuts, dairy products) when cereal-based foods are removed.

Results and discussion: No significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N = 48; Mann-Whitney U score =319.000; P=0.523), month 1-3 (N= 48; Mann-Whitney U score =335.000; P= 0.332) or month 1-4 (N= 48; Mann-Whitney U score = 332.000; P=0.364), thus this hypothesis is rejected (see Appendix H and I).

Figure 10.3: Intake of specified nutrients



10.1.9 Vitamin C intake

Hypothesis Nine: proposes that the vitamin C intake will remain the same or increase slightly on a GFD due to the participant consuming more fruit and vegetables (high in vitamin C), when cereal-based foods are removed because they know these foods are GF.

Results and discussion: No significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N = 48; Mann-Whitney U score =295.000; P=0.885), between month 1-3 (N= 48; Mann-Whitney U score =223.000; P= 0.180) or between month 1-4 (N= 48; Mann-Whitney U score = 296.000; P= 0.869) (see Appendix H and I).

Thus the first part of the hypothesis is confirmed but no increase was detected so the latter part is rejected.

10.1.10 Vitamin B_6 intake

Hypothesis Ten: proposes that the vitamin B_6 intake will decrease on a GFD because gluten-containing foods high in vitamin B_6 are eliminated from the diet. These foods are replaced with corn, rice, and potato-based carbohydrates that are poorer sources of vitamin B_6.

Results and discussion: No significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N = 48; Mann-Whitney U score =305.500; P=0.718), month 1-3 (N= 48; Mann-Whitney U score =304.000; P= 0.741) or month 1-4 (N= 48; Mann-Whitney U score = 314.000; P= 0.592), thus this hypothesis is rejected. This is a little surprising and contrasts with established literature (Halfdanarson *et al*., 2007; Saibeni *et al*., 2005; USDA 2001). A decrease between diagnosis and subsequent months would have been expected. Further research would be useful to monitor changes in intake of B-group vitamins in CD over a longer time-frame and with a larger sample group (see Appendix H and I).

10.1.11 Vitamin B_{12} intake

Hypothesis Eleven: proposes that vitamin B_{12} intake will decrease on a GFD because people may be unsure of what to safely eat and avoid eating marinated and coated (battered or crumbed) meat products, or meat from grain-fed animals or they may chose to become vegetarian (vitamin B_{12} is only found in animal products). Fortified ready-to-eat cereals that are a valuable source of B_{12} are also removed on a GFD.

Alternatively, people on a GFD may consume more meat and dairy products to compensate for the removal of cereal-based foods which could result in an increased intake.

Results and discussion: No significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N = 48; Mann-Whitney U score =295.000; P=0.885), month 1-3 (N= 48; Mann-Whitney U score =379.500; P= 0.059) or month 1-4 (N= 48; Mann-Whitney U score = 332.000; P= 0.364). Thus both the initial hypothesis and the alternative hypothesis are rejected. This is surprising as some change in vitamin B_{12} intake would have been expected. This finding could warrant further investigation using a bigger sample group and a longer sampling period (see Appendix H and I).

10.1.12 Calcium intake

Hypothesis Twelve: proposes that calcium intake will not change after the implementation of a GFD because gluten-free sources of calcium exist.

Results and discussion: No significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N= 48; Mann-Whitney U score= 318.000;P= 0.536), month 1-3 (N=48; Mann-Whitney U score = 295.000; P=0.885) or month 1-4 (N= 48; Mann-Whitney U score = 281.000;P=0.885), thus this hypothesis is upheld (see Appendix H and I).

10.1.13 Sodium intake

Hypothesis Thirteen: proposes that the sodium intake will increase after the implementation of a GFD as people beginning a GFD may eat more snack-foods (i.e. potato chips or fries), that are high in sodium as these foods are readily available and cheap. Many commercially available GF foods are relatively high in sodium so this could increase intake.

Results and discussion: Significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N= 48; Mann-Whitney U score= 392.000;P= 0.032), month 1-3 (N=48; Mann-Whitney U score = 394.000; P = 0.029) but not between month 1-4 (N= 48; Mann-Whitney U score = 374.000;P=0.076). This is opposite to what was proposed so the hypothesis is rejected which is surprising. It is possible that intake decreased due to the removal of commercially prepared wheat-based snacks, cakes, biscuits and breads which can be high in sodium.

Further investigation could be warranted using a bigger sample group over a longer timeframe (see Appendix H and I)

10.1.14 Iron intake

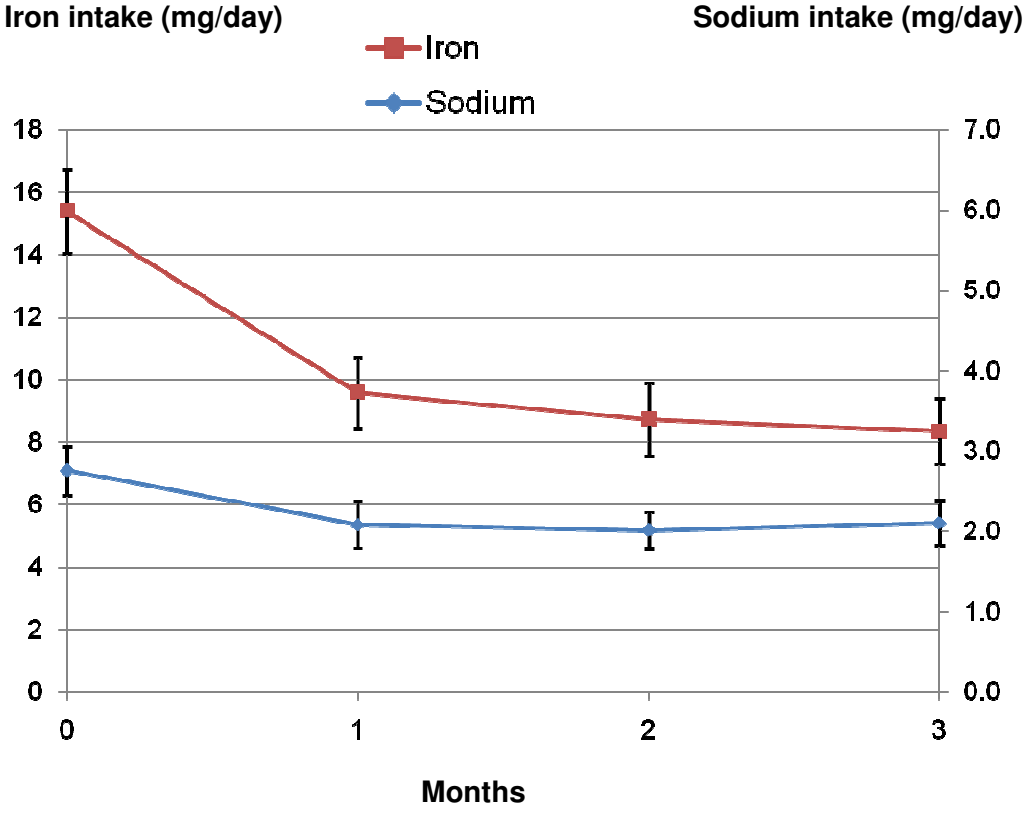
Hypothesis Fourteen: proposes that iron intake will remain unchanged or perhaps increase slightly on a GFD. When gluten-containing cereals are removed from the diet, there should not be a significant drop in iron intake as these foods are easily substituted with other gluten-free sources of iron. However, iron levels in newly diagnosed CD are often low due to malabsorption so higher intakes may be needed to replenish iron stores (Halfdanarson *et al*., 2007; Annibale *et al*., 2001b). There may be an increase as people newly diagnosed with CD increase their intake of meat and vegetables to compensate for the removal of gluten-containing grains.

Results and discussion: Very significant differences were found on Kruskall-Wallis testing of variance between month 1-2 (N= 48; Mann-Whitney U score= 480.000; P= 0.000), month 1-3 (N=48; Mann-Whitney U score = 486.500; P = 0.000) and month 1-4 (N= 48; Mann-Whitney U score = 491.000; P=0.000) (see Table 10.14 in Appendix H and Appendix I).

It is not known why such a significant decrease in iron intake occurred after the implementation of a GFD. It could be a result of the removal of iron-fortified breakfast cereals and other wheat-based products. This is particularly important as the gut inflammatory changes associated with CD affect iron absorption. This may confirm the recommendation that iron supplementation should be considered in the months following diagnosis of CD (Halfdanarson *et al*., 2007; Annibale *et al*., 2001b). Further investigation into the reasons underlying this significant decrease in iron intake would be warranted.

Figure 10.4 shows the mean sodium and iron intakes over the four months of sampling (measured in mg/day).

Figure 10.4: Mean sodium and iron intake



10.2 Statistical and Dietary Analysis of the Control Group

Non-parametric tests were performed on the data sets for all nutrients.

No significant differences were found using the Kruskall-Wallis tests of variance in the control group for any of the thirteen nutrients tested (for a summary of results see Appendix J).

Intakes remained relatively stable over the sampling period. Thus it is clear that distinct differences in the intake of some nutrients exist for people with CD compared with those in the control group. It is assumed that the non-coeliac control group is representative of the general population and the coeliac group representative of those people with CD. Thus significant changes in intake of nutrients do not occur in the general population over time, but significant changes in intake do occur for gluten, starch, carbohydrates, sodium and iron in people with CD. There may be some effect of seasonal variation in the intake of certain nutrients but this is not apparent in the non-coeliac control group. To confirm whether results from both sampling groups are representative of the general population, nutrient intakes were compared against

national intakes as established by the Ministry of Health and with the results of the National Nutrition Survey 1997.

10.3 Comparison of findings with the National Nutrition Survey and the National Nutrition Guidelines

Nutrient intakes for both the coeliac group and the control group was compared with the 1997 National Nutrition Survey (NNS 97) (MOH 1999) and the National Nutrition Guidelines for Healthy Adults (MOH 2003) to assess whether intake is adequate in terms of recommended daily intakes for New Zealanders, whether subjects were consuming a typical New Zealand diet, to compare the two sample groups (i.e. coeliac and control) against national data and to see if there are obvious differences between the two test groups (see Table 10.20).

Table 10.20: Comparison of nutrient intakes with the 1997 NNS and the RDIs or nutrient reference values.

Nutrient	RDI/NRV	NNS 1997	Coeliac group	Control group
Energy (kJ/day)				
Male	9100-13700	11630		
Female	7200- 11300	7700		
Mean	8700	10325	8587	7990
Protein (g/day)				
Male	55	105		
Female	45	71		
Mean	50	88	83	87
Total fat (g/day)				
Male		110		
Female		72		
Mean	70	91	80	83
Saturated fat (g/day)		47		
Male		30		
Female	24	39	33	34
Mean				
Carbohydrate (g/day)		305		
Male		214		
Female	310	260	251	207
Mean				
Sugars (g/day)				
Male		131		
Female		99		
Mean	90	114	118	79
Fibre (g/day)				
Male		23		
Female		18		
Mean	30	20	22	22

Nutrient	RDI/NRV	NNS 1997	Coeliac group	Control group
Water (ml/day)				
Male	3000	NA		
Female	2200			
Mean			1948	1661
Vitamin C (mg/day)				
Male	45	111		
Female	45	95		
Mean	45	102	100	110
Vitamin B_6 (mg/day)	1.6	1.7		
Male	1.6	1.2		
Female	1.6	1.4	1.6	1.1
Mean				
Vitamin B_{12} (ug/day)	2	5		
Male	2	3		
Female	2	4	4.8	4.5
Mean				
Vitamin A (ug/day)				
Mean	800	939	993	982
Vitamin E (mg/day)				
Male		11.2		
Female		8.6		
Mean	10	9.7	8.2	9.9
Sodium (mg/day)				
Range	920- 2300			
Mean		3473	2242	2447
Iron (mg/day)				
Male	8	14.6		
Female	18	9.9		
Mean	12	12	10.5	12
Magnesium (mg/day)		365		
Male		265		
Female	320	309	290	334
Mean				
Calcium (mg/day)				
Male		691		
Female		857		
Range	800-1000			
Mean		766	832	746
Zinc (mg/day)				
Male		14.5		
Female		9.8		
Mean	12	12	10	12

10.4 Discussion of nutrient intake and comparisons

The energy intake for both the coeliac group and the control group was lower than the intake determined by the NNS and was closer to the RDI. This lower intake could be a result of under-reporting. Results for protein intake for both the coeliac

and non-coeliac group were higher than the RDI however they were similar to the NNS intake.

The New Zealand Nutrition Taskforce recommends that the proportions of **total fat** should be 30-33 percent of total energy however the fat intake of New Zealand adults is above the upper limit of the levels recommended (Ministry of Health 2003). Total fat intake for both sample groups was slightly higher than the RDI but lower than the NNS results.

Types of fat differ between various age groups however in the New Zealand population (as determined by the NNS) saturated fat was a major contributor to total daily fat intake compared to lower monounsaturated and polyunsaturated fat. It is recommended that polyunsaturated fatty acids contribute approximately 6-10% of total energy. The 1997 NNS found that the consumption of food containing polyunsaturated fatty acids (at 5% of total energy) was lower than recommended. **Saturated fatty acids** plus trans fatty acids should contribute no more than 12% of total energy. Consumption of food containing saturated fatty acids still dominates total fat intake and is above the recommended level. Both the study groups had intakes higher than the RDI but lower than the NNS results. The recommendation for **mono-unsaturated fatty acid** is between 10-20% of total intake. Consumption of food containing mono-unsaturated fatty acids needs to be increased to meet recommendations (Ministry of Health 2003), or a decreased saturated fat to an increased percentage of monounsaturated fat should prevail. For all groups, the percentage energy from polyunsaturated fat is below the level recommended by the New Zealand Nutrition taskforce (1991) guideline (6-10%) (Ministry of Health 2003).

The New Zealand Nutrition Taskforce (Ministry of Health 1999) recommends that New Zealand adults obtain 50-55% of energy from **carbohydrates**. The NNS, the coeliac and control groups all had intakes lower than this recommended level. The mean intake for the control group was lower than the intake for the coeliac group which is not at all what would have been expected as members of the control group were not eliminating carbohydrate-rich gluten containing foods from their diet.

The intake of sucrose and other free sugars should be no more than 15% of total daily energy intake (Ministry of Health 1999). The sugar intake for the coeliac group was the highest followed by the control group. Only the control group intake was less than the RDI.

The recommended guideline for dietary **fibre** in New Zealand is 25-30 g per day (Ministry of Health 2003). This recommended dietary fibre intake was based on a definition of fibre that included resistant starch and other components in addition to non-starch polysaccharides (NSP). Research (NNS 1997) shows that average dietary fibre intake in New Zealand is typically low. Approximately half the NSP intake occurs as insoluble NSPs with the remainder as soluble NSPs which is consistent with the findings of this study where mean fibre intake for both groups were lower than the RDI.

The NNS97 indicates that the usual daily median intake of **vitamin C** for New Zealanders and for both study groups is considerably higher than the RDI. The usual daily intake of **vitamin B_6** as determined by the NNS 97 is close to the Reference Nutrient Intake and within the RDI range. The RDI was met by the coeliac group but both the results from the NNS and the control group were lower than the RDI. The daily intake for **vitamin B_{12}** inboth study groups were all higher than the RDI.

Both the sample groups and the NNS had intakes of **vitamin A** over the RDI. All intakes are very similar and whilst higher than the RDI they are well within the safe upper limit for vitamin A (3000 µg/day). The intakes for **vitamin E** in both study groups and for the NNS was very close to the RDI The coeliac group intake was lower than the control group but comparable to the intake for the general New Zealand population as determined by the 1997 NNS.

Researchers assessing **sodium** intake for the NNS 97 found that determining intake by dietary assessment methods was difficult to perform because discretionary salt is added to food, and cooked meals may have an unknown salt content. For this reason, dietary sodium intake was not included in the NNS97. Sodium excretion in the urine is known to be the best indicator of sodium intake (Caggiula *et al*., 1985; Shortt *et al*., 1988). A regional study in New Zealand found a mean sodium excretion of 3105 mg/ day which corresponds to a mean sodium intake of 3473 mg/ day (Thomson *et al*., 1998). Analysis of sodium intake based on urinary excretion was not possible for this research but according to analysis of

dietary intake from Foodworks 2007, results show an estimated intake above both the MOH targets (Ministry of Health 2003) and the RDI.

Both the control group and the general New Zealand had **iron** intakes that met the RDI whilst the coeliac group had a lower intake. This is consistent with research (Sari *et al*., 2000) indicating that iron levels are often low in people with CD and that supplementation may be required in the post diagnosis months as dietary intake may not be adequate and malabsorption may still be present.

Magnesium intake in the coeliac group and the general population were lower than the RDI but the control group was higher. **Calcium** intakes for the control group and the NNS were below the RDI. However, the intake for people with CD was just met the RDI.
The general New Zealand population and the control group had a zinc intake which met the RDI but the coeliac group had a lower intake.

10.5 Potential limitations of the dietary study

10.5.1 Potential sources of error in dietary sampling and analysis

As with all methods used to measure food intake, the methods chosen have inherent limitations. This was indeed the case in this study where in places only very broad estimates of quantities were given thus inaccuracies in estimating serving sizes occurred. This made it difficult when entering the information into the Foodworks programme as detailed serving sizes were needed. The Foodworks programme in itself is limited in its accuracy as it does not take into account seasonal variation of foods, strain variation in nutrient content, ripening variation, or shelf life variation. It does, however, give a representation based on an average product. There are often differences between the composition of diets analysed using a food composition database and chemical analysis, especially in fat, protein and vitamin values. The inability to accurately measure nutrients present in very small quantities is one reason for this discrepancy. Thus the only totally accurate means of assessing dietary would have been to monitor and collect all food and beverages consumed over the 3 day sampling period and perform a chemical analysis to determine all its constituent nutrients or to carry out a saliva test test to

confirm the accuracy of the dietary record. This was simply not possible for this study as it was carried out under free-living conditions rather than in an institutional setting.

10.5.2 Limitations in the use of the Foodworks food composition database

10.5.2.1 Variability in the composition of food as it is actually eaten (i.e. the amount of fat trimmed off meat during preparation).

10.5.2.2 Limited coverage of food items- food composition data is not often available for unusual foods or new food products. This was the case with gluten-free food. The Foodworks programme did not have a database for gluten-free foods programmed into it so these foods had to be entered individually from the nutritional information provided on the packaging of the product or from the recipe used to make it. Some vitamins and minerals were not listed on the packaging or some ingredients were not recognised by the Foodworks programme thus underestimating the content of some nutrients.

10.5.2.3 Nutrient availability- Nutrient values derived from food composition databases represent the maximum amount of that nutrient available to the body rather than the amount actually absorbed and utilised. This could be especially relevant in people with CD as they can have problems with malabsorption of nutrients.

10.5.3 Under-reporting of food intake

Another potential source of error in the collection of dietary data is that of under-reporting of food intake. Under-reporting, under eating and consequently under estimating nutrient intake is widespread in self-administered dietary studies and the problems are not unique to 3-day dietary recall methodology (NNS 1997). This limitation should be recognised when interpreting nutrient data. This could have been minimised using the Goldberg cutoff method which identifies obviously implausible intake values by evaluating energy intake against estimated energy requirements. However this method only identifies extreme under-reporting and it

has poor sensitivity (Coulston *et al*., 2008). There is also huge variation in the type and amount of food consumed from day-to-day over the 3-day sampling periods and the foods eaten on these 3 days may not be representative of the typical diet for the person. The only way to overcome this would have been to sample over a longer time frame but this could have reduced reliability as it would have increased respondent burden.

Chapter Eleven: CONCLUSIONS AND FUTURE DIRECTIONS FOR THE STUDIES

11.1 Conclusions

This thesis presents the results from a small clinical study investigating changes in faecal microflora in people with CD and a dietary survey to assess possible changes in nutrient intake over several months on a GFD.

11.1.1 The Faecal Study

Specifically, the faecal study sought to further our understanding of the pathology of CD in regard to its secondary effects on the diversity of enteral microflora via changes in immune tolerance and to determine whether the pro-inflammatory conditions associated with CD abrogate tolerance to enteral microflora. It proposed that enteral mucosal pro-inflammatory change in CD (i.e. at diagnosis) is probably associated with a decrease in enteral microbial diversity and that the resolution of pro-inflammatory conditions after implementation of a GFD would be accompanied by increased diversity of enteral microflora that may help restore tolerance.

However, no increase in the diversity of enteral microflora was found from diagnosis through the months on a GFD which argues against the main hypothesis. The fact that no change was found in the diversity of enteral microflora following gluten withdrawal is contrary to the proposal that resolution of mucosal inflammation triggers a change in the composition of intestinal microflora. There was no evidence of restructuring of the microbial community occurring during resolution from inflammation. Overall, there were no significant differences in the distribution of faecal microflora during testing within subjects indicating that a GFD does not alter the general pattern of enteral microflora in people with CD any more than in the control subjects in this time-frame.

It was interesting to find that the diversity of faecal microflora as measured by the Simpson Index was significantly lower in people with CD than in control subjects. Changes in a few individual organisms and persistence of distinctive bands of microflora were identified from diagnosis and over the months on a GFD. People with CD had several different species of microflora that were not present in the faeces of control subjects. The similarities of the bands that distinguish the coeliac

group from the control group and their proximity to *Lactobacilli* markers on the gel ladders indicate that these species are likely to be *Lactobacilli*. The presence of these 'unique' species is probably related to people with CD expressing different patterns of fucosylation in their enteral mucins and confirms established research that specific species of microflora are involved in the presentation of CD either casually or coincidentally (Forsberg *et al*., 2004). This finding that distinctive bands of microflora were present in the faeces of the coeliac group but not in the control group lends support to the hypothesis that associated genetic changes in the pattern of fucosylation of mucous may result in lifelong differences in faecal microflora, however, it remains to be determined whether these microflora once acquired exacerbate gluten sensitivity.

11.1.2 The Dietary Study

In terms of nutrient changes after the implementation of a GFD, some interesting findings were noted. Contemporary research identifies that nutrient deficiencies are common in newly diagnosed CD and can continue in the months immediately following diagnosis due to malabsorption and probably alteration in diet. As expected there was a significant decrease in gluten intake from diagnosis to one month post-GFD because gluten was excluded from the diet, but there was no increase in gluten intake between later months which would have been expected as participants with CD began experimenting with a wider range of foods. It is prudent to note that these subjects were not being adventurous in their eating habits and were sticking to very basic meals. This may have changed if the study had been continued over a longer time-frame.

As expected, significant decreases in starch and carbohydrate intake were found in the coeliac group following the implementation of a GFD, because large quantities of these macronutrients are removed on a GFD. Although many of these foods are replaced with GF alternatives, it takes time to adjust to new foods and they are often poorer sources of starch and carbohydrates so more must be consumed to maintain intake levels. By comparison no changes were found in the control group over the sampling period.

This study found no significant changes in the intake for any B-group vitamins, vitamin C or any of the fat soluble vitamins in either group. This was surprising for the coeliac group and is contrary to established research. A decrease in vitamin B_6 intake was expected as significant sources are removed when gluten-containing grains are eliminated. Although these foods are replaced with corn, rice, and potato-based carbohydrates, these are poorer sources of vitamin B_6.

Whilst iron deficiency is frequently reported in association with untreated and treated CD, the significant decrease in iron intake over the study period was not expected. The reasons why such a significant decrease in iron intake occurred after the implementation of a GFD can only be surmised and could warrant further investigation. There does not appear to be a huge change in the type of food being eaten, except that many cereal-based foods have been replaced with alternative grain sources. While these gluten-free alternatives are poorer sources of absorbable iron, red meat and leafy green vegetables are still being consumed in similar quantities post-diagnosis as pre-diagnosis. It is unlikely that this alone would account for the significant decrease in iron that was observed. Iron intake may have decreased as subjects omitted iron-fortified wheat-based breakfast cereals. This is particularly important as the inflammatory gut changes associated with CD affect iron absorption and may indicate the need to fortify gluten-free grain-based foods with iron.

An increase in sodium intake was predicted after the implementation of a GFD as people newly diagnosed with CD replace breads and cereal-based foods with GF alternatives that are higher in salt, or eat salty snack foods while familiarising themselves with GF alternatives. The opposite was found. Sodium intake initially decreased as salted wheat-based snacks were omitted but intakes went back to baseline as subjects became accustomed to a GFD.

Nutrient intake was compared with the RDIs established by the MOH Health for Healthy Adults and with the average intake of New Zealanders as determined by the 1997 National Nutrition Survey (NNS 97). The pattern of intake for both the coeliac and control groups in this study was consistent with the intake patterns of the general New Zealand population. There were no glaring examples of excess or inadequate intakes of macronutrients or micronutrients, although some intakes

were either slightly low or high. Energy, carbohydrate, fibre, vitamin E, magnesium, zinc and iron intake for the coeliac group were slightly lower than recommended, whilst protein, total fat, saturated fat, vitamin C and vitamin A were higher than the RDIs but comparable to the NNS.
Sodium intake was within the acceptable range for both sample groups and was lower than the intake from the NNS. This indicates that the nutrient intake for both the coeliac group and the control group are representative of the larger population.

This has been a successful study in that it has furthered our understanding of the complex aetiology underlying CD. It has used an appropriate method for genetic analysis (i.e. PCR-DGGE) and dietary analysis (Foodworks 2007), and whilst the hypothesis was not upheld, there were nevertheless important findings. This project has created as many questions as it has found answers and opened the door to future investigation in this field.

11.2 Future directions for this research

11.2.1 The Faecal Study

The finding of species unique to people with CD is exciting for future research. The most obvious direction for further research would be identification of the specific species of microflora present in the CD group but not the controls (i.e. band 790, 135, 131, 137 etc). It could be useful to examine the characteristics of these specific micro-organisms to determine why they are found in subjects with CD but not controls (i.e. their niche) and to investigate both their positive and negative interactions within the intestinal environment. It could also be helpful to investigate whether these bands are present in other household members eating the same meals as this would establish if there is an environmental influence on intestinal microflora.

A longitudinal study charting the extent of villous atrophy and inflammation at diagnosis and after implementation of a GFD (via a series of follow up gastroscopies) would allow for a more extensive understanding of the relationship between intestinal microflora and the inflammatory status of the gut mucosa and how these change as the integrity of the intestinal mucosa is restored. Some may question whether this is an ethically acceptable method of data collection, however

follow-up gastroscopies are routinely performed at yearly intervals to chart histological changes. It may also be possible to determine if the presence of species unique to CD are related to the extent of intestinal inflammation, or if they are present in a different group of people newly diagnosed with CD. It could be that the 'cause and effect' of the presence of these unique species can be determined (i.e. are these specific microflora involved in the aetiology of CD, or does the presence of CD predispose people to this unique set of microflora).

Another avenue worth pursuing could be investigating the effect of supplementation with probiotics on microbial diversity and inflammation in people with newly diagnosed CD and over an extended time-frame. It may be possible to determine whether supplementation influences the presence of the species unique to people with CD, or to evaluate the effects of immune suppressant therapies commonly used to reduce acute inflammatory changes, on the rate of restoration of normal enteral microflora and see if probiotics promote repopulation of the gut with beneficial micro-organisms that improves the clinical outcome for people with CD.

11.2.2 The Dietary Study

In terms of the dietary study, it would also be beneficial to repeat this research using a larger sample group over a longer study period, or to perform the study in a controlled environment rather than a free-living situation. The effects of CD usually resolve within 6 -12 months on a GFD so a longer time period would allow changes in nutrient intake and absorption to be charted and to establish what changes occur in the microbial population once inflammation has resolved.

A longitudinal study would allow for weight changes to be charted and nutrient levels to be analysed from serum and actual food intake at diagnosis and over an extended period of time using a much larger sample and control group. This would allow a comparison between intake and absorption of nutrients and could show whether malabsorption is present at diagnosis and whether it continues to be an issue. If nutrient deficiencies are found to be present, the effect of nutrient supplementation could be monitored.

Every new piece of information discovered about CD improves our understanding of this disease but it also highlights how much remains unknown about this diverse and complex disorder.

Chapter Twelve: REFERENCES

Abouzied, M., Carroll, M., Mozola, M. (2004). A Sandwich ELISA test for detection and quantitation of gluten in food products and ingredients. *Online @ http://www.aoac.org/.*

Abreu, M., Fukata, M., Arditi, M. (2005). TLR Signaling in the Gut in Health and Disease. *J Immunol, 174*, 4453-4460.

Ackerman, Z., Eliakima, R., Stalnikowicz, R.*, et al.* (1996). Role of small bowel biopsy in the endoscopic evaluation of adults with iron deficiency anemia. *Am J Gastroenterol, 91* (10), 2099-2102.

Addesa, J., Proctor, D. (2004). Chapter 39 - Small Intestine and Colonic Diseases. In M. Legato, Bilezikian, J (Ed.), *Principles of Gender-specific Medicine* (Vol. 1, pp. 415 - 416). San Diego, USA: Academic Press.

Addesa, J., Proctor, D. (2004). Chapter 39 -Small Intestine and Colonic Diseases. In M. Legato, Bilezikian, J (Ed.), *Principles of Gender-Specific Medicine* (Vol. volume 1, pp. 415-418). San Diego, USA: Academic Press.

Afkarian, M., Sedy, J., Yang, J.*, et al.* (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4 T cells. *Nat Immunol, 3* (6), 549-557.

Akira, S., Takeda, K., Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol, 2* (8), 675-680.

Akiyama, H., Isuzugawa, K., Harikai, N.*, et al.* (2004). Inter-laboratory evaluation studies for development of notified ELISA methods for allergic substances (wheat). *Shokuhin Eiseigaku Zasshi, 45* (3), 128-134.

Alaedini, A., Green, P. (2005). Narrative Review: Celiac Disease: Understanding a Complex Autoimmune Disorder. *Ann Int Med, 1442* (4), 289-299.

Aleanzi, M., Demonte, A., Esper, C.*, et al.* (2001). Celiac disease: Antibody recognition against native and selectively deamidated gliadin peptides. *Clin Chem, 47*, 2023–2028.

Allez , M., Tieng , V., Nakazawa, A.*, et al.* (2007). CD4(+)NKG2D(+) T Cells in Crohn's Disease Mediate Inflammatory and Cytotoxic Responses Through MICA Interactions. *Gastroenterology, 132* (7), 2346-2358.

Alonso, L., Cuesta, E., Gilliland, S. (2003). Production of free conjugated linoleic acid by Lactobacillus acidophilus and Lactobacillus casei of human intestinal origin. *J. Dairy Sci, 86*, 1941-1946.

Alpan, O., Rudomen, G., Matzinger, P. (2001). The Role of Dendritic Cells, B Cells, and M Cells in Gut-Oriented Immune Responses. *J Immunol, 166*, 4843-4852.

Altuntas, B., Kansu, A., Ensari, A.*, et al.* (1998). Celiac disease in Turkish short-statured children and the value of antigliadin antibody in diagnosis. *Acta Paediatr, 40* (5), 457-460.

Anderson, R. (2000). In vivo challenge in coeliac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nature Med, 6*, 337-342.

Anderson, R. (2005). The way to the future. *Coeliac Solution, 2* (1), 1-4.

Anderson, R. (2006). Seminar on coeliac disease presented to the New Zealand Coeliac Society. Wellington: unpublished.

Annibale, B., Capurso, G., Chistolini, A.*, et al.* (2001). Gastrointestinal causes of refractory iron deficiency anemia in patients without gastrointestinal symptoms. *Am J Med, 111* (6), 439-445.

Annibale, B., Lahner, E., Chistolini, A.*, et al.* (2003). Endoscopic evaluation of the upper gastrointestinal tract is worthwhile in pre-menopausal women with iron-deficiency anaemia irrespective of menstrual flow. *Scand J Gastroenterol, 38* (3), 239-245.

Annibale, B., Severi, C., Chistolini, A.*, et al.* (2001). Efficacy of gluten-free diet alone on recovery from iron deficiency anemia in adult celiac patients. *Am J Gastroenterol, 96* (1), 132-137.

Arato, A., Korner, A., Veres, G.*, et al.* (2002). Frequency of coeliac disease in Hungarian children with type 1 diabetes mellitus. *Eur J Pediatr, 162* (1), 1-5.

Arentz-Hansen, H., Fleckenstein B, Molberg, O et al. (2004). The molecular basis for oat intolerance in patients with celiac disease. *Plos Med*.

Arentz-Hansen, H., Korner, R., Molberg, O.*, et al.* (2000). The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J. Exp Med, 191*, 603-612.

Arentz-Hansen, H., McAdam, S., Molberg, O.*, et al.* (2002). Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterol, 123*, 803-809.

Arenz, S., Ruckerl, R., Koletzko, B.*, et al.* (2004). Breast-feeding and childhood obesity: a systemic review. *Int J Obes Relat Metab Disord, 28*, 1247-1256.

Arienzo, R., Maurano, F., Luongo, D.*, et al.* (2008). Adjuvant effect of Lactobacillus casei in a mouse model of gluten sensitivity. *Immunol Lett, 119*, 78-83.

Artan, R. (1998). Antigliadin antibody measurement as a screening test for childhood coeliac disease. *Int Med J, 5* (3), 209-212.

Ascher, H., Hahn-Zoric, M., Hanson, L.*, et al.* (1996). Value of serologic markers for clinical diagnosis and population studies of coeliac disease. *Scandinavian Journal of Gastroenterology, 31* (1), 61-67.

Ashabani, A., Abushofa, U., Abusrewill, S.*, et al.* (2003). The prevalence of coeliac disease in Libyan children with type-1 diabetes mellitus. *Diabetes Metab Res Rev, 19*, 69-75.

ASSEATTA. (2003). HLA nomenclature. In *Australasian and South East Asian Tissue Typing Association*: online @ www.aseatta.org.au/nomencla.htm.

Auricchio, S., Greco, L., Troncone, R. (1988). Gluten-sensitive enteropathy in childhood. *Pediatr Clin North Am, 35*, 157-187.

Auricchio, S., Troncone, R. (1996). History of Coeliac disease. *Eur J Pediatr*(155), 427-428.

Australian Government, New Zealand Ministry of Health, NHMRC. (2005). *Nutrient Reference Values for Australia and New Zealand*. Sydney.

Avaniss-Aghajani, E., Jones, K., Holtzmann, A.*, et al.* (1996). Molecular technique for rapid identification of Mycobacteria. *J Clin Microbiol, 34*, 98 102.

Avery, R., Duncan, W., Alving, B. (1998). Severe vitamin K deficiency induced by occult celiac disease. *Am J Hematol, 53* (1), 55.

Axelsson, L., Mahida, Y. (2002). Flora; role in colonisation resistance andother effects; production of antimicrobial peptides. *Microbiol Ecol Health Dis, 2*, 216-222.

Bäckhed, F., Ley, R., Sonnenburg, J.*, et al.* (2005). Host-bacterial mutualism in the human intestine. *Science, 307*, 1915-1920.

Baeuerle, P., Henkel, T. (1994). Function and Activation of NF-kappaB in the Immune System. *Ann Rev Immunol, 12*, 141-179.

Bahia, M., Rabello, A., Brasileiro, F.*, et al.* (2001). Serum antigliadin antibody levels as a screening criterion before jejunal biopsy indication for celiac disease in a developing country. *Brazilian Journal of Medical and Biological Research, 34* (11), 1415-1420.

Bai, J., Zeballos, E., Fried, M.*, et al.* (2005). WGO-OMGE Practice Guideline CELIAC DISEASE. *World Gastroent News, 10*(2), S1-8.

Balas, A., Vicario, J., Zambrano, A.*, et al.* (1997). Absolute linkage of celiac disease and dermatitis herpetiformis to HLA-DQ. *Tissue Antigens, 50* (1), 52-56.

Bamias, G., Cominelli, F. (2006). Novel strategies to attenuate immune activation in Crohn's disease. *Curr Opin Pharm, 6*, 401-407.

Barbeau, W., Bassaganya-Riera, J., Hontecillas, R. (2007). Putting the pieces of the puzzle together – a series of hypotheses on the etiology and pathogenesis of type 1 diabetes . *Medical Hypotheses, 68* (3), 607 - 619.

Bardella, M., Trovato, C., Cesana, B.*, et al.* (2001). Serological markers for coeliac disease: is it time to change? *Dig Liver Dis, 33* (5), 426-431.

Barera, G., Bonfanti, R., Viscardi, M.*, et al.* (2002). Occurrence of celiac disease after onset of type 1 diabetes:6 year prospective longitudinal study. *Pediatrics, 109*, 833-838.

Bargou, R., Emmerich, F., Krappmann, D.*, et al.* (1997). Constitutive Nuclear Factor- B-RelA Activation Is Required for Proliferation and Survival of Hodgkin's Disease Tumor Cells. *J. Clin. Invest, 100*(12), 2961-2969.

Barnes, P., Karin, M. (1997). Nuclear factor kappaB- A pivotal transcription factor in chronic inflammatory diseases. *New Eng J Med, 336*, 1066-1071.

Bateman, E., Ferry, B., Hall, A.*, et al.* (2004). IgA antibodies of coeliac disease patients recognise a dominant T cell epitope of A-gliadin. *Gut, 53*, 1274-1278.

Beattie, R. (2006). The changing face of coeliac disease. *Arch Dis Child, 91*, 955-956.

Beaugerie, L., Petit, J. (2004). Microbial-gut interactions in health and disease: Antibiotic-associated diarrhoea. *Best Practice & Research Clinical Gastroenterology, 18* (2), 337-352.

Begon, M., Harper, J., Townsend, C. (1990). *Ecology: Individuals, populations and communities*. Boston: Blackwell Scientific Publications.

Belury, M. (2002). Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. *Annu. Rev. Nutr, 22*, 505-531.

Benjamini, E., Coico, R., Sunshine, G. (2000). Chapter 10; Activation and function of T and B cells. In *Immunology: A short course* (4 ed). Canada: Wiley-Liss Inc.

Berger, R., Schmidt, G. (1996). Evaluation of six anti-gliadin antibody assays. *J Immunol Methods, 191*(1), 77-86.

Bergseng, E., Xia, J., Kim, C.*, et al.* (2005). Main Chain Hydrogen Bond Interactions in the Binding of Proline-rich Gluten Peptides to the Celiac Disease-associated HLA-DQ2 Molecule. *J Biol Chem, 280* (23), 21791- 21796.

Berne, R., Levy, M. (2000). *Principles of Physiology* (3 ed). Missouri ,: Mosby.

Berti, I., Horvath, K., Green, P.*, et al.* (1999). Prevalence of celiac disease among first and second degree relatives in the USA. *Gastroenterol, 116*, A861.

Berti, I., Horvath, K., Green, P.*, et al.* (2000). Prevalence of celiac disease among risk groups and the general population in USA. *Invest Med, 48* (206), 220A.

Berti, I., Horvath K, Green P *et al*. (2000). Differences of celiac disease's clinical presentation among pediatric and adults relatives of CD patients in U.S.A. *J Invest Med, 48*, 215A.

Bettelheim, K., Breadon, A., Faiers, M.*, et al.* (1974). The origin of O serotypes of Escherichia coli in babies after normal delivery. *Journal of Hygiene, 72* (1), 67-70.

Biagi, F., Pezzimenti, D., Campanella, J.*, et al.* (2001). Endomysial and tissue transglutaminase antibodies in coeliac sera: a comparison not influenced by previous serological testing. *Scand JGastroenterol, 36* (9), 955-958.

Bibiloni, R., Mangold, M., Madsen, K.*, et al.* (2006). The bacteriology of biopsies differ between newly diagnosed, untreated Crohn's disease and ulcerative colitis patients. *J Med Microbiol, 55*, 1141-1149.

Bibiloni, R., Walter, J., Tannock, G. (2004). Chapter 6: The Gut Microflora. In M. Nakano, Zuber, P (Ed.), *Strict and Facultative Anaerobes: Medical and Environmental Aspects* (pp. 125-144). Beaverton, USA: Horizon Bioscience (CRC Press).

Bingley, P., Williams, A., Norcorss, A.*, et al.* (2004). Undiagnosed coeliac disease at age seven: population based prospective birth cohort study. *BMJ, 328*, 322-323.

Biology-Online (2006). Lysozyme. *http:// www.Biology-onlineorg/dictionary/Lysozyme*.

Bird, A., Brown, I., Topping, D. (2000). Starches, resistant starches, the gut microflora and human health. *Curr Issues Intest Microbiol, 1* (1), 25-37.

Bischoff, S., Sellge, G. (2003). Chapter 2: Immune Mechanisms in Food-Induced Disease. In D. Metcalfe, Sampson H, Simon R (Ed.), *Food Allergy: Adverse Reactions to Foods and Food Additives* (3 ed). Massachusetts, USA: Blackwell Publishing Inc.

Bizzaro, N., Villaltab, D., Tonuttic, E. (2003). Association of celiac disease with connective tissue diseases and autoimmune diseases of the digestive tract. *Autoimmunity Reviews, 2* (6), 358-363.

Björkstén, B., Sepp, E., Julge, K.*, et al.* (2001). Allergy development and the intestinal microflora during the first year of life. *Journal of Allergy and Clinical Immunology, 108* (4), 516-520.

Black, A. (2001). Dietary Assessment for Sports Dietetics. *British Nutrition Foundation Bulletin, 26*, 29-42.

Bode, S., Gudmand-Hoyer, E. (1994). Evaluation of the gliadin antibody test for diagnosing coeliac disease. *Scand J Gastroenterol, 29* (2), 148-152.

Bode, S., Gudmand-Hoyer, E. (1996). Symptoms and haematologic features in consecutive adult coeliac patients. *Scand J Gastroenterol, 31*, 54–60.

Bodger, K., Bromelow, K., Wyatt, J.*, et al.* (2001). Interleukin 10 in Helicobacter pylori associated gastritis: immunohistochemical localisation and in vitro effects on cytokine secretion. *J Clin Pathol, 54*, 285-292.

Bollinger, R., Everett, M., Palestrant, D.*, et al.* (2003). Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology, 109* (4), 580-587.

Bonamico, M., Ferri M, Nenna R, et al. (2004). Tissue transglutaminase autoantibody detection in human saliva: a powerful method for celiac disease screening. *J Pediatr, 144*, 632-636.

Bonamico, M., Tiberti, C, Picarelli, A *et al*. (2001). Radioimmunoassay to detect antitransglutaminase autoantibodies is the most sensitive and specific screening method for celiac disease. *Am J Gastroenterol, 96* (5), 1536-1540.

Booijink, C., Zoetendal, E., Smidt, H.*, et al.* (2006). Functional analysis of the GI-tract microbiota. *Reprod Nutr Dev, 46*, S3-S35.

Book, L., Hart, A., Black, J.*, et al.* (2001). Prevalence and clinical characteristics of celiac disease in Downs syndrome in a U.S. study. *Am J Med Genetics, 98* (1), 70-74.

Borghesi, C., Taussig, M., Nicoletti, C. (1999). Rapid appearance of M cells after microbial challenge is restricted at the periphery of follicle-associated epithelium of Peyer's patch. *Lab Invest, 79*, 1393-1401.

Borriello, S. (2002). Chapter 1: The normal flora of the gastrointestinal tract. In A. Hart, Stagg, A, Graffner, H *et al* (Ed.), *Gut Ecology*. London: Martin Dunitz Ltd.

Bottaro, G., Cataldo, F., Rotolo, N.*, et al.* (1999). The clinical pattern of subclinical/silent celiac disease: an analysis on 1026 consecutive cases. *Am J Gastroenterol, 94*, 691–696.

Bottaro, G., Volta, U., Spina, M.*, et al.* (1997). Antibody pattern in childhood celiac disease. *J Pediatr Gastro Nutr, 24* (5), 559-562.

Bouma, G., Strober, W. (2003). The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol, 3*, 521-533.

Brandtzaeg, P. (2002). Current understanding of gastrointestinal immunoregulation and its relation to food allergy. *Ann NY Acad Sci, 964*, 13-45.

Brewer, V., Thomas, F., Garber, A.*, et al.* (2004). The detection of wheat using commercial ELISA-based assays. *Online @ http://www.aoac.org/.*

Brissoni, B. (2005). *Characterization of tollip in interleukin-1 receptor/toll like receptor signalling pathways.*, University of Medicine; online at http://www2unilch/cyberdocuments/pratique/acces/biologie_medecine/These_Brissonipdf, Lausanne.

Brocke, S., Gijbels, K., Allegretta, M.*, et al.* (1996). Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature, 379*, 343-346.

Brostoff, J., Gamlin, L. (2000). *Food Allergies and Food Intolerances; The complete guide to their identification and treatment*. Rochester, Vermont: Healing Arts Press.

Bry, L. (1996). Development of E Coli in the human intestine. *Molecular biology online at www.madsci.org*.

Bry, L., Falk, P., Midtvedt, T.*, et al.* (1996). A model of host-microbial interactions in an open mammalian ecosystem. *Science, 273*, 1380-1383.

Bullock, N., Booth, J., Gibson, G. (2004). Comparative composition of bacteria in the human intestinal microflora during remission and active ulcerative colitis. *Curr Issues Intest Microbiol, 5*, 59-64.

Burns, B., Carr-Davies, E. (1996). Nutritional care in diseases of the nervous system. In K. Mahan, Escott-Stump, S (Ed.), *Krause's Food, Nutrition and Diet Therapy* (pp. 863-888). Philadelphia: WB Saunders Co.

Buttriss, J. (2002). *British Nutrition Foundation; Adverse reactions to food*. London: Blackwell Science.

Caggiula, A., Wing, R., Nowalk, M.*, et al.* (1985). The measurement of sodium and potassium intake. *Am J Clin Nutr, 42*, 391-398.

Carlsson, A., Axelsson, I., Borulf, S.*, et al.* (2001). Serological Screening for Celiac Disease in Healthy 2.5-Year-Old Children in Sweden. *Pediatr, 107* (1), 42-45.

Carman, R., Simon, M., Fernández, H.*, et al.* (2004). Ciprofloxacin at low levels disrupts colonization resistance of human fecal microflora growing in chemostats. *Regulatory Toxicology and Pharmacology, 40* (3), 319-326.

Carroccio, A., Lacono, G., D'Amico, D.*, et al.* (2002). Production of anti-endomysial antibodies in cultured duodenal mucosa: usefulness in coeliac disease diagnosis. *Scand J Gastroenterol, 37* (1), 32-38.

Carroccio, A., Lacono, G., Montalto, G.*, et al.* (1993). Immunologic and absorptive tests in celiac disease: can they replace intestinal biopsies? *Scand J Gastro, 28* (8), 673-676.

Carroccio, A., Vitale,G, Di Prima, L et al. (2002). Comparison of anti-transglutaminase ELISAs and an anti-endomysial antibody assay in the diagnosis of celiac disease: a prospective study. *Clin Chem, 48* (9), 1546-1550.

Cataldo, F., Ventura, A., Lazzari, R.*, et al.* (1995). Antiendomysium antibodies and coeliac disease: solved and unsolved questions. An Italian multicentre study. *Acta Paediatr, 84*, 1125-1131.

Catassi, C., Doloretta, M., Ratsch, I.*,et al.* (2001). The distribution of DQ genes in the Saharawi population provides only a partial explanation for the high celiac disease prevalence. *Tissue Antigens, 58* (6), 402-406.

Catassi, C., Fabiani, E, Ratsch, I *et al*. (1996). The coeliac iceberg in Italy. A multicenter antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl, 412*, 29-35.

Catassi, C., Fanciulli, G., D'Appello, A.*, et al.* (2000). Antiendomysium versus antigliadin antibodies in screening the general population for coeliac disease. *Scand J Gastroenterol, 35* (7), 732-736.

Catassi, C., Kryszak, D., Louis-Jacques, O.*, et al.* (2007). Detection of coeliac disease in primary care: a multicenter case-finding study in North America. *Am J Gastroenterol, 102* (7), 1454-1460.

Catassi, C., Ratsch, I., Fabiani, E. (1994). Coeliac disease in the year 2000: exploring the iceberg. *Lancet, 343*, 200-203.

Catassi, C., Ratsch, I., Fabiani, E.*, et al.* (1995). High prevalence of undiagnosed coeliac disease in 5280 Italian students screened by antigliadin antibodies. *Acta Paediatr, 84*, 672-676.

Catassi, C., Ratsch I, Gandolfi L. (1999). Why is coeliac disease endemic in people of the Sahara? *Lancet, 354*, 647-648.

Cavallaro, R., Iovino, P., Castiglione, F.*, et al.* (2004). Prevalence and clinical associations of prolonged prothrombin time in adult untreated celiac disease. *Eur J Gastroenterol Hepat, 16*, 219-223.

Cerutti, F., Bruno, G., Chiarelli, F.*, et al.* (2004). Younger age at onset and sex predict celiac disease in children and adolescents with type 1 diabetes: an Italian multicenter study. *Diabetes Care, 27*, 1294-1298.

Chamaillard, M., Girardin, S., Viala, J.*, et al.* (2003). Nods, Nalps and Naips:intracellular regulators of bacterial-induced inflammation. *Cell Microbio, 5* (9), 581-592.

Chan, A., Butzner, J., McKenna, R.*, et al.* (2001). Tissue transglutaminase enzyme-linked immunosorbent assay as a screening test for celiac disease in pediatric patients. *Pediatrics, 107* (1), E8.

Chartrand, L., Agulnik, J., Vanounou, T.*, et al.* (1997). Effectiveness of antigliadin antibodies as a screening test for celiac disease in children. *Can Med Assn J, 157* (5), 527-533.

Chirdo, F., Rumbo, M., Carabajal, P.*, et al.* (1999). Analysis of anti-gliadin antibodies by immunoblot analysis and enzyme-linked immunosorbent assay using gliadin fractions as antigens. *J Pediatr Gastro Nutr, 29* (2), 171-177.

Ciacci, C., Cirillo, M., Auriemma, G.*, et al.* (1996). Celiac disease and pregnancy outcome. *Am J Gastroenterol, 91*, 718-722.

Ciccocioppo, R., Di Sabatino, A., Ara, C.*, et al.* (2003). Gliadin and tissue transglutaminase complexes in normal and coeliac duodenal mucosa. *Clin Exp Immunol, 134* (3), 516-524.

Clark, M., Hirst, B. (2003). Expression of junction-associated proteins differentiates mouse intestinal M-cells from enterocytes. *Histochem Cell Biol, 118*, 137-147.

Clark, M., Jepson, M. (2003). Intestinal M cells and their role in bacterial infection. *Int J Med Microbiol, 293*, 17-39.

Clark, R., Bishop-Bailey, D., Estrada-Hernandez, T.*, et al.* (2000). The Nuclear Receptor PPAR gamma and Immunoregulation: PPAR-gamma Mediates Inhibition of Helper T cell responses. *J Immunol, 164*, 1364-1371.

Clark, S. (2008). Iron Deficiency Anemia. *Nutr Clin Prac, 23* (2), 128-141.

Clayton, P. (2006). B6-responsive disorders: a model of vitamin dependency. *J Inherit Metab Dis, 29* (3), 317-326.

Clemente, M., De Virgiliis, S., Kang, J.*, et al.* (2003). Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut, 52*, 218-223.

Cleusix, V., Lacroix, C., Vollenweider, S.*, et al.* (2007). Glycerol induces reuterin production and decreases Escherichia coli population in an in vitro model of colonic fermentation with immobilized human feces. *FEMS Microbiology Ecology, 63* (1), 56–64.

Coakley, M., Ross, R., Nordgren, M.*, et al.* (2003). Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. *J. Appl. Microbiol, 94*, 138-145.

Cobrin, G., Abreu, M. (2005). Defects in mucosal immunity leading to Crohn's disease. *Immunol Reviews, 206*, 277-295.

Coenen, J., Koenen, H., van Rijssen, E.*, et al.* (2006). CTLA-4 engagement and regulatory CD4(+) CD25(+) T cells independently control CD8(+)-mediated responses under costimulation blockade. *J Immunol, 176* (9), 5240-5246.

Collado, M., Calabuig, M., Sanz, Y. (2007). Differences between the fecal microbiota of coeliac infants and healthy controls. *Curr Issues Intest Microbiol, 8*, 9-14.

Collado, M., Meriluoto, J, Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Letters Appl Microbiol, 45*, 454- 460.

Collado, M., Sanz , Y. (2007). Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent in situ hybridization coupled with flow cytometry techniques. *Vet Microbiol, 121*(3-4), 299-306.

Collin, P., Kaukinen, K, Valimaki, M *et al.* (2002). Endocrinological Disorders and Celiac Disease. *Endo Rev, 23* (4), 464-483.

Collin, P., Reunala, T., Pukkala, E.*, et al.* (1994). Coeliac disease-associated disorders and survival. *Gut, 35*, 1215-1218.

Collin, P., Reunala T, Rasmussen, M et al. (1997). High incidence of adult coeliac disease: Augmented approach. *Scand J Gastroenterol, 32*, 1129 - 1133.

Collin, P., Thorell, L, Kaukinen, K et al. (2004). The safe threshold for gluten contamination in gluten-free products. Can trace amounts be accepted in the treatment of coeliac disease? *Aliment Pharmacol Ther, 19*, 1277-1283.

Collin, P., Vilska, S., Heinonen, P.*, et al.* (1996). Infertility and celiac disease. *Gut, 39*, 382-384.

Collins, K., O' Mahony, L. (2002). Chapter 4: The 'unculturables'. In A. Hart, Stagg, A, Graffner, H *et al* (Ed.), *Gut Ecology*. London: Martin Dunitz Ltd.

Colonna, M., Pulendran, B., Iwasaki, A. (2006). Dendritic cells at the host-pathogen interface. *Nature Immunol, 7*, 117-120.

Comstock, L., Kasper, D. (2006). Bacterial Glycans: Key Mediators of Diverse Host Immune Responses. *Cell, 126* (5), 847-850.

Cook, D., Prosser, D., Forster, R.*, et al.* (2000). CCR6 mediates dendritic cell localization, lymphocyte homeostasis and immune responses in mucosal tissue. *Immunity, 12,* 495-503.

Cook, J. (2005). Diagnosis and management of iron-deficiency anaemia. *Best Pract Res Clin Haematol, 18*, 319–332.

Coppa, G., Bruni, S., Morelli, L.*, et al.* (2004). The first prebiotics in humans: human milk oligosaccharides. *Journal of Clinical Gastroenterology, 38* (6), S80-S83.

Corazza, G., Frisoni, M., Treggiari, E.*, et al.* (1993). Subclinical celiac sprue. Increasing occurrence and clues to its diagnosis. *J Clin Gastro, 16* (1), 16-21.

Corazza, G., Strocchi, A., Gasbarrini, G. (1987). Fasting breath hydrogen in celiac disease. *Gastroenterology, 93*, 53-58.

Corazza, G., Valentini, R., Andreani, M *et al*. (1995). Subclinical coeliac disease is a frequent cause of iron-deficiency anaemia. *Scand J Gastroenterol, 30* (2), 153-156.

Corfield, A., Carroll, D., Myerscough, N.*, et al.* (2001). Mucins in the gastrointestinal tract in health and disease. *Frontiers in Bioscience, 6*, D1321-1337.

Corrao, G., Corazza, G., Bagnardi, V.*, et al.* (2001). Mortality in patients with coeliac disease and their relatives: a cohort study. *Lancet, 358* (9279), 356-361.

Cortigiani, L., Nutini, P., Caiulo, V.*, et al.* (1989). Selenium in celiac disease. *Minerva Pediatr, 41* (11), 539-542.

Coulston, A., Boushey, C (2008). *Nutrition in the Prevention and Treatment of Disease.* (2 Ed. pp 121-125) Academic Press. San Diego.

Coyne, M., Reinap, B., Lee, M.*, et al.* (2005). Human symbionts use a host-like pathway for surface fucosylation. *Science, 307* (5716), 1778-1781.

Cranney, A., Zarkadas, M., Graham, I.*, et al.* (2007). *The Canadian Celiac Health Survey*

Crosby, W. (1957). Intraluminal biopsy of the small intestine. *Am J Dig Dis, 2*, 236-241.

Cummings, J., Macfarlane, G., Macfarlane, S. (2003). Intestinal bacteria and ulcerative colitis. *Curr Issues Intest Microbiol, 4*, 9-20.

Cummings, J., Pomare, E., Branch, W.*, et al.* (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut, 28*, 1221-1227.

Cummins, A., Thompson, F., Butler, R.*, et al.* (2001). Improvement in intestinal permeability precedes morphometric recovery of the small intestine in coeliac disease. *Clin Sci, 100*, 379-386.

Cunningham-Rundles, S. (1998). Nutrition and the immune system of the gut. *Nutrition, 14* (7), 573-579.

Dahele, A., Ghosh, S. (2001). Vitamin B12 deficiency in untreated celiac disease. *Am J Gasterol, 96* (3), 745-750.

Daims, H., Ramsing, N., Schleifer, K.*, et al.* (2001). Cultivation-Independent, Semiautomatic Determination of Absolute Bacterial Cell Numbers in Environmental Samples by Fluorescence In Situ Hybridization. *Appl Environ Microbiol, 67* (12), 5810-5818.

D'Arienzo, R., Maurano, F., Luongo, D.*, et al.* (2008). Adjuvant effect of *Lactobacillus casei* in a mouse model of gluten sensitivity. *Immunol Lett, 119*, 78-85.

Dave, S. (2006). *Exogenous and endogenous danger signals in inflammatory bowel disease.* University of Pittsburgh, Pittsburgh.

Davies, H., Halablab, M., Clarke, J.*, et al.* (1999). *Infection and Immunity*. London: CRC Press.

De Angelis, M., Coda, R., Silano, M.*, et al.* (2006). Fermentation by selected sourdough lactic acid bacteria to decrease the intolerance to rye and barley flours. *J Cereal Sci, 43*, 301-314.

De Stefano, D., Maiuri, M., Lovine, B.*, et al.* (2006). The role of NF kappa B, IRF-1 and STAT-1 alpha transcription factors in the iNOS gene induction by gliadin and IFN-gamma in RAW 264.7 macrophages. *J Mol Med, 84*, 65-74.

Delves, P., Roitt, I., Martin, S.*, et al.* (2006). *Roitt's Essential Immunology* (6 ed). London: Blackwell Publishing.

Denzer, K., Kleijmeer, M., Heijnen, H.*, et al.* (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci, 113*, 3365-3374.

Department of Agriculture, U. S. (2001). USDA Nutrient Database for Standard Reference, Release 14. Nutrient Data Laboratory. *Agricultural Research Service, http://www.nal.usda.gov/fnic/foodcomp*.

des Rieux, A., Fievez, V., Garinot, M.*, et al.* (2006). Nanoparticles potential oral delivery systems of proteins vaccines: A mechanistic approach. *J Control Rel, 116*, 1-27.

Dethlefsen, L., Eckburg, P., Bik, E.*, et al.* (2006). Assembly of the human intestinal microbiota. *Trends Ecology Evol, 21* (9), 517-523.

Devillard, E., McIntosh, F., Duncan, S.*, et al.* (2007). Metabolism of Linoleic Acid by Human Gut Bacteria: Different Routes for Biosynthesis of Conjugated Linoleic Acid. *J Bacteriol, 189* (6), 2566-2570.

Dewar, D., Pereira, S., Ciclitira, P. (2004). The pathogenesis of coeliac disease. *Int J Biochem Cell Bio, 36*, 17-24.

Di Cagno, R., De Angelis, M., Alfonsi, G.*, et al.* (2005). Pasta made from durum wheat semolina fermented with selected lactobacilli as a tool for a potential decrease of gluten intolerance. *J Agric Food Chem, 53*, 4393-4402.

Dicke, W., Weijers, H., Van de Kamer, J. (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta Paediatr, 42* (1), 34-42.

Dickey, W. (2002). Low serum vitamin B12 is common in coeliac disease and is not due to autoimmune gastritis. *Eur J Gastroenterol Hepatol, 14* (4), 425-427.

Dickey, W., Hughes, D. (2004). Histology of the terminal ileum in coeliac disease. *Scand J Gastroenterol, 39*, 665-667.

Dickey, W., Kenny, B., McMillan, S.*, et al.* (1997). Gastric as well as duodenal biopsies may be useful in the investigation of iron deficiency anaemia. *Scand J Gastroenterol, 32* (5), 469-472.

Dickey, W., Ward, M., Whittle, C.*, et al.* (2008). Homocysteine and related B-vitamin status in coeliac disease: Effects of gluten exclusion and histological recovery. *Scand J Gastroenterol, 43* (6), 682-688.

Dickey, W., Wylie, J., Collins, J.*, et al.* (1994). Lewis phenotype secretor status and celiac disease. *Gut, 35*(6), 769-770.

Didierlaurent, A., Sirard, J., Kraehenbuhl, J.*, et al.* (2002). How the gut senses its content. *Cell Microbio, 4* 2), 61-72.

Dieterich, W., Ehnis, T., Bauer, M.*, et al.* (1997). Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med, 3*, 797 - 801.

Dietrich, W., Ehnis, T., Bauer, M.*, et al.* (1997). Gliadin is a preferred substrate for tissue transglutaminase, the autoantigen in coeliac disease. *Gastroenterology, A359*.

Diosdado, B., Wijmenga, C. (2005). Molecular mechanisms of the adaptive, innate and regulatory immune response in the intestinal mucosa of celiac disease patients. *Exp Rev Mol Diag, 5* (5), 681-700.

Djuric, Z., Sasa, Z., Vuka, K. (2007). Celiac disease with diffuse cutaneous vitamin K deficiency bleeding. *Adv Ther, 24* (6), 1286-1289.

Drago, S., El Asmar, R., Di Pierro, M.*, et al.* (2006). Gliadin, zonulin and gut permeability:
Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J*
Gastroenterol, 41 (4), 408-419.

Duarte, J., Deshpande, P., Guiyedi, V.*, et al.* (2007). Total and functional parasite specific IgE responses in Plasmodium falciparum-infected patients exhibiting different clinical status. *Malaria J, 6* (1), 1-13.

Eckburg, P., Bik, E., Bernstein, C.*, et al.* (2005). Diversity of the human intestinal microbial flora. *Science, 308*, 1635-1638.

Eigenmann, P., Zamora, S., Belli, D. (1999). Food Hypersensitivities. *Annales Nestle, 57*, 57-67.

Eliakima, R., Sherer, D. (2001). Celiac disease: infertility and pregnancy. *Gynae Obstet Invest, 51*, 3-7.

Elliot, D., Urban, J., Argo, C.*, et al.* (2000). Does the failure to acquire helminthic parasites predispose to Crohn's disease? *FASEB J, 14*, 1848-1855.

Ellis, H., Pollock, E., Engel, W.*, et al.* (2003). Investigation of the putative immunodominant T cell epitopes in coeliac disease. *Gut, 52*, 212-217.

Ensari, A., Marsh, M., Moriarty, K.*, et al.* (1998). Studies *in vivo* of omega gliadins in gluten sensitivity (coeliac sprue disease). *Clin Sci, 95*, 419-424.

Erb, K. (2007). Helminths, allergic disorders and IgE mediated immune responses: where do we stand? *Eur J Immunol, 37* (5), 1170-1173.

Ertekin, V., Selimoglu, M. (2006). Prevalence of prolonged prothrombin time in children with coeliac disease. *Eur J Gastroenterol Hepat, 18*, 579-580.

Eun, C., Han, D., Lee, S.*, et al.* (2006). Attenuation of Colonic Inflammation by PPAR gamma in Intestinal Cells: Effect on Toll-like Receptor Pathway. *Digest Dis Sci, 54* (4), 693-697.

Fagarasan, S., Kinoshita, K., Muramatsu, M.*, et al.* (2001). *In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature, 413*, 639-643.

Fanaro, S., Chierici, R., Guerrini, P.*, et al.* (2003). Intestinal microflora in early infancy: composition and development. *Acta Paediatrica, 91*(441), 48-55.

FAO/WHO. (1998). Codex Alimentarius. *Online @ www.nzfsa.govt.nz*.
FAO/WHO, J. (1998). Food Standards Program; Codex Committee on Nutrition and Foods for Special Dietary Uses: Draft revised standard for gluten free foods. *CX/NFSDU 98/4*, 1-4.

Farthing, M., Rees, L., Edwards, C.*, et al.* (1983). Male gonadal function in coeliac disease. 2. Sex hormones. *Gut, 24*, 127-136.

Fasano, A. (1996). Where have all Amercian celiacs gone? *Arch Dis Child, 412*, 20-24.

Fasano, A. (2001). Celiac Disease: the Past, the Present, the Future. *Pediatrics, 107*(4), 768-770.

Fasano, A. (2006). Systemic autoimmune disorders in celiac disease. *Curr Opin Gastroenterol, 22* (6), 674-679.

Fasano, A., Berti, I., Gerarduzzi, T.*, et al.* (2003). Prevalence of Celiac Disease in At-Risk and Not-At-Risk Groups in the United States; A Large Multicenter Study. *Arch Intern Med., 163*, 286-292.

Favier, C., Vaughan, E., De Vos, W.*, et al.* (2002). Molecular monitoring of succession of bacterial communities in human neonates. *Applied and Environmental Microbiology, 68* (1), 219-226.

Feleszko, W., Jaworska, J., Hamelmann, E. (2006). Toll-like receptors- novel targets in allergic airway disease (probiotics, friends and relatives). *Eur J Pharm, 533*, 308-318.

Ferary, S., Thibout, E., Auger, J. (1998). Direct Analysis of Odors Emitted by Freshly Cut Allium Using Combined High-performance Liquid Chromatography and Mass Spectrometry. *Rapid Communications Mass Spectrometry, 10* (11), 1327 - 1332.

Ferguson, R., Holmes, G., Cooke, W. (1992). Celiac disease, fertility and pregnancy. *Scand J Gastroenterol, 17*, 65-68.

Fernandez, F., Kennedy, H., Hill, M.*, et al.* (1985). The effect of diet on the bacterial flora of ilestomy fluid. *Micrbiol Ailments Nutr, 3*, 47.

Fernandez, F., Kennedy, H., Truelove, S.*, et al.* (1983). The effect of changes in the amount of dietary protein on the composition of ilestomy fluid. *Trans Nutr Soc, 42*, 108A.

Ferranti, P., Mamone, G., Picariello, G. (2007). Mass spectrometry analysis of gliadins in celiac disease. *J Mass Spectrom, 42* (12), 1531-1548.

Finegold, S., Attebery, H., Sutter, V. (1974). Effect of diet on human fecal flora: comparison of Japanese and American diets. *Am J Clin Nutr, 27*, 1456-1469.

Finegold, S., Sutter, V., Sugihara, H.*, et al.* (1977). Fecal microflora in Seventh Day Adventist populations and control subjects. *Am J Clin Nutr, 30*, 1781-1792.

Fisgin, T., Yarali, N., Duru, F.*, et al.* (2004). Hematologic manifestation of childhood celiac disease. *Acta Haematol, 111*, 211-214.

Fitzgerald, J., Troncone, R., Roggero, P.*, et al.* (2003). Secondary carnitine deficiency due to celiac disease. *J Pediatr Gastroenterol Nutr, 36*, 636-646.

Fitzsimmons, C., Mc Beath, R., Joseph, S.*, et al.* (2007). Factors affecting IgE and IgG responses to allergen-like Schistosoma mansoni antigens: molecular structure and patterns in vivo exposure. *Int Arch Allergy Immunol, 142* (1), 40-50.

Fleckenstein, B., Molberg, O., Qiao, S.*, et al.* (2002). Gliadin T Cell Epitope Selection by Tissue Transglutaminase in Celiac Disease: Role of enzyme specificity and pH influence on the transamidation versus deamidation reactions. *J Biol Chem, 277* (37), 34109-34116.

Flint, H. (2004). Polysaccharide breakdown by anaerobic microorganisms inhabiting the mammalian gut. *Adv Appl Microbiol, 56*, 89-120.

Forsberg, G., Fahlgren, A., Horstedt, P.*, et al.* (2004). Presence of bacteria and innate immunity of intestinal epithelium in childhood coeliac disease. *Am J Gastroenterol, 99*, 894-904.

Fort, T. (2007). What Is the Nucleotide ATP?
The Energy Currency of Organic Molecule Adenosine Triphosphate. *Online @ http://biochemistry.suite101*.

Foster, G., Germain, C., Jones, M.*, et al.* (2000). Human T cells elicit IFN-alpha secretion from dendritic cells following cell to cell interactions. *Eur J Immunol, 30* (11), 3228-3235.

Franks, A., Harmsen, H., Raangs, G.*, et al.* (1998). Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S r RNA-targeted oligonucleotide probes. *Appl Environ Microbiol, 64*, 3336-3345.

Fraser, J., Engel, W., Ellis, H.*, et al.* (2003). Coeliac disease: In vivo toxicity of the putative immunodominant epitope. *Gut, 52*, 1698-1702.

Freed, D. (1999). Do dietary lectins cause disease? *BMJ, 318*, 1023-1024.

FSANZ. (2002). Regulations relating to gluten intake. *Online @ www.foodstandards.govt.nz*.

Fujihashi, K., Kiyono, H., Aicher, W.*, et al.* (1989). Immunoregulatory function of CD3+, CD4- and CD 8- T cells. gamma/delta T cell receptor-positive T cells from nude mice abrogate oral tolerance. *J Immunol., 143*, 3415-3422.

Fujihashi, K., Taguchi, T., Aicher, W.*, et al.* (1992). Immunoregulatory functions for murine intraepithelial lymphocytes: gamma/delta T cell receptor-positive (TCR+) T cells abrogate oral tolerance while alpha/beta TCR+T cells provide B cell help. *J Exp Med, 175*, 695-707.

Gabrovská, D., Rysová, J., Burkhard, M.*, et al.* (2004). Collaborative study of a newly developed ELISA kit for gluten determination. *J Food Ag Environ, 2* (1), 113-115.

Gandolfi, L., Bocca, A., Pratesi, R. (2000). Screening of celiac disease inchildren attending the outpatient clinic of a university hospital. *J Pediatr Gastroenterol Nutr, 31*, S212.

Gandolfi, L., Pratesi, R., Cordoba, J. (2000). Prevalence of celiac disease among blood donors in Brazil. *Am J Gastroenterol, 95*, 689-692.

Garcia -Tsao, G., Wiest, R. (2004). Gut microflora in the pathogenesis of the complications of cirrhosis. *Best Practice & Research Clinical Gastroenterology, 18*(2), 353-372.

Garrido, C., Gaya, J., Liompart, A.*, et al.* (1997). Prevalence of monosymptomatic celiac disease in patients with iron deficiency anemia. *Gastroenterol Hepatol, 20*, 172–174.

Garrison, R., Somer, E. (1995). Chapter 3: The fat soluble vitamins. In *The Nutrition Desk Reference* (pp. 80-81). Connecticut: McGraw-Hill Professional.

Gasbarrini, A., Sanz Torre, E., Trivellini, C.*, et al.* (2000). Recurrent spontaneous abortion and intrauterine fetal growth retardation as symptoms of coeliac disease. *Lancet, 256*, 399-400.

Gebert, A., Fassbender, S., Werner, K.*, et al.* (1999). The development of M cells in Peyer's patches is restricted to specialized dome associated crypts. *Am J Pathol, 154*, 1573-1582.

Gee, S. (1888). On the coeliac affection. *St Bartholomew's Hospital reports, 24*, 17–20.

Gendel, S., Carlson, D., Dennis, S.*, et al.* (2005). USFDA: Approaches to Establish Thresholds for Major Food Allergens and for Gluten in Food. *Online @ www.cfsan.fda.gov*.

Germain, R., Margulies, D. (1993). The biochemistry and cell biology of antigen processing and presentation. *Ann Rev Immunol, 11*, 403-450.

Gerrard, J., Sutton, K. (2005). Addition of transglutaminase to cereal products may generate the epitope responsible for coelaic disease. *Trends in Food Sci Technol, 16* (11), 510-512.

Gibson, G. (1998). Dietary modulation of the human microflora using prebiotics. *Br J Nutr, 80*, S209-S212.

Gibson, G. (2004). Fibre and effects on probiotics (the prebiotic concept). *Clinical Nutrition Supplements, 1* (2), 25-31.

Gillett, H., Freeman, H. (2000). Comparison of IgA endomysium antibody and IgA tissue transglutaminase antibody in celiac disease. *Can J Gastroenterol, 14* (8), 668-671.

Gilroy, D., Colville-Nash, P., Chivers, W.*, et al.* (1999). Inducible cyclo-oxygenase may have anti-inflammatory properties. *Nat Med, 6*, 698- 671.

Goldin, B. (2003). Microflora of the intestine; Role and Effects. In B. Caballero, Trugo, L, Finglas, P (Ed.), *Encyclopedia of Food Sciences* (2 ed., pp. 3904-3911). San Diego: Academic Press.

Goldstein, D. (2004). Toll-like receptors and other links between innate and acquired alloimmunity. *Curr Opin Immunol, 16* (5), 538-544.

Gonczi, J., Skerritt, J., Mitchell, J. (1991). A reliable screening test for coeliac disease: enzyme-linked immunosorbent assay to detect anti-gliadin antibodies in serum. *Aust NZ J Med, 21* (5), 723-731.

Gorelik, L., Flavell, R. (2000). Abrogation of TGF beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity, 12*, 171-181.

Gould, H., Sutton, B., Beavil, A.*, et al.* (2003). The biology of IgE and the basis of allergic disease. *Ann Rev Immunol, 21*, 579-628.

Goyens, P., Brasseur, D., Cadranel, S. (1985). Copper deficiency in infants with active celiac disease. *J Pediatr Gastroenterol Nutr, 4*, 677-680.

Grakoui, A., Bromley, S., Sumen, C.*, et al.* (1999). The immunological synapse: a molecular machine controlling T cell activation. *Science, 285* (5425), 221-227.

Granel, B., Rossi, P., Frances, Y.*, et al.* (2005). Bilateral massive adrenal haemorrhage revealing coeliac disease. *QJM, 98*, 70-71.

Green, P., Stavropoulos, S., Panagi, S.*, et al.* (2001). Characteristics of adult celiac disease in the USA: results of a national survey. *Am J Gastroenterol, 96*, 126–131.

Gregory, J., Quinlivan, E. (2002). In vivo kinetics of folate metabolism. *Annu Rev Nutr, 22*, 199–220.

Greicius, G., Arulampalam, V., Pettersson, S. (2004). A CLAs act: feeding away inflammation. *Gastroenterology, 127*, 994-996.

Griffin, M., Casadio, R., Bergamini, C. (2002). Transglutaminases: nature's biological glues. *Biochem J, 368*, 377-396.

Grisolano, S., Oxentenko, A., Murray, J.*, et al.* (2004). The usefulness of routine small bowel biopsies in evaluation of iron deficiency anemia. *J Clin Gastroenterol, 38*(9), 756-760.

Grodzinsky, E., Jansson, G., Skogh, T.*, et al.* (1995). Anti-endomysium and anti-gliadin antibodies as serological markers for coeliac disease in childhood: a clinical study to develop a practical routine. *Acta Paediatr, 84*, 294-298.

Groh, V., Bahram, S., Bauer, S.*, et al.* (1998). Recognition of stress-induced MHC molecules by intestinal epithelial gamma delta cells. *Science, 279*, 1737-1740.

Guandalini, S. (2008). Historical Perspective of Celiac Disease. In A. Fasano, R. Troncone & D. Branski (Eds.), *Frontiers in Celiac Disease. Pediatr Adolesc Med* (Vol. 12, pp. 1-11). Basel: Karger.

Guarner, F., Malagelada, J. (2003). Gut flora in health and disease. *The Lancet, 361*(9356), 512-519.

Guarner, F., Malagelada, J. (2003). Role of bacteria in experimental colitis. *Best Practice & Research Clinical Gastroenterology, 17* (5), 793-804.

Gutierrez, O., Pipaon, C., Inohara, N.*, et al.* (2002). Induction of Nod 2 in myelomonocytic and intestinal epithelial cells via nuclear factor- kappa B activation. *J Biol Chem, 277*, 41701- 41704.

Haapalahti, M., Kulmala, P., Karttunen, T.*, et al.* (2005). Nutritional status in adolescents and young adults with screen-detected celiac disease. *J Pediatr Gastroenterol Nutr, 40*, 566–570.

Haas, S. (1924). The value of the banana in the treatment of coeliac disease. *Am J Dis Child, 24*, 421-437.

Hadjivassiliou, M., Grunewald, R., Davies-Jones, G. (2002). Gluten sensitivity as a neurological illness. *J Neurol Neurosurgery Psych, 72*, 560-563.

Hadjivassiliou, M., Williamson, C., Woodroofe, N. (2004). The immunology of gluten sensitivity: beyond the gut. *Trends in Immunology, 25* (11), 578-582.

Halfdanarson, T., Litzow, M., Murray, J. (2007). Hematologic manifestations of celiac disease. *Blood, 109* (2), 412-421.

Haller, D. (2006). Intestinal epithelial cell signaling and host derived negative regulators under chronic inflammation: to be or not to be activated determines the balance towards commensal bacteria. *Gastroent Motil, 18*, 184-199.

Hallert, C., Aström, J., Walan, A. (1983). Reversal of psychopathology in adult coeliac disease with the aid of pyridoxine (vitamin B6). *Scand. J. Gastroenterol, 18* (2), 299–304.

Hallert, C., Grant, C., Grehn, S.*, et al.* (2002). Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Aliment Pharmacol Ther, 16* (7), 1333-1339.

Hallstrom, O. (1989). Comparison of IgA-class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. *Gut, 30* (9), 1225-1232.

Halstensen, T., Farstad, I., Scott, H.*, et al.* (1990). Intraepithelial TcR alpha/beta+ lymphocytes express CD45RO more often than the TcR gamma/delta+ counterparts in coeliac disease. *Immunol, 71*, 460-466.

Halstensen, T., Scott, H., Brandtzaeg, P.*, et al.* (1990). Human CD+8 intraepithelial T lymphocytes are mainly CD45RA-RB+ and show increased co-expression of CD45RO in coeliac disease. *Eur J Immunol, 20*, 1825-1830.

Hamer, R. (2005). Coeliac disease: background and biochemical aspects. *Biotech Adv, 23*, 401-408.

Hansson, T., Dahlbom, I., Hall, J.*, et al.* (2000). Antibody reactivity against human and guinea pig tissue transglutaminase in children with celiac disease. *J Pediatr Gastro Nutr, 30* (4), 379-384.

Harder, T., Bergmann, R., Kallischnigg, G.*, et al.* (2005). Duration of breastfeeding and risk of overweight: a meta-analysis. *Am J Epidemiol, 162*, 397-403.

Harmsen, H., Harmsen, G., Gibson, P.*, et al.* (2000). Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human faecal bacteria. *FEMS Microbiol Lett, 183* . 125–129.

Harmsen, H., Harmsen, A, Wildeboer-Veloo, J., *et al*. (2000). Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Appl Environ Microbiol, 66*, 4523–4527.

Harmsen, H., Wildeboer-Veloo, A., Raangs, G.*, et al.* (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *Journal of Pediatric Gastroenterology and Nutrition, 30* (1), 61-67.

Harrington, L., Hatton, R., Mangan, P.*, et al.* (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol, 6* (11), 1023-1032.

Hartmann, G., Weiner, G., Krieg, A. (1999). CpG DNA: A potent signal for Growth, Activation and Maturation of Human Dendritic Cells. *Proc Nat Acad Sci, 96*, 9305-9310.

Hausch, F., Shan, L., Santiago, N.*, et al.* (2002). Intestinal digestive resistance of immunodominant gliadin peptides. *Am J Physiol Gastrointest Liver Physiol, 283*, 996-1003.

Hawkes, N., Swift, G., Smith, P.*, et al.* (2000). Incidence and presentation of coeliac disease in South Glamorgan. *Eur J Gastroenterol Hepatol, 12* (3), 345-349.

Hawrelak, J., Myers, S. (2004). The causes of intestinal dysbiosis:a review. *Altern Med Rev, 9* (2), 180-197.

Hayashi, H., Sakamoto, M., Benno, Y. (2002). Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. *Micrbiol Immunol, 46*, 819-831.

Hebuterne, X. (2003). Gut changes attributed to aging: effects on intestinal microflora. *Curr Opin Clin Nutr Met Care, 6*, 49-54.

Heneghan, M., McCarthy, C., Moran, A. (2000). Relationship of blood group determinants on *Helicobacter pylori* lipopolysaccharide with host Lewis phenotype and inflammatory response. *Infect Immun, 68* (2), 937-941,.

Hernandez, L., Green, P. (2006). Extraintestinal manifestations of celiac disease. *Curr Gastroenterol Reports, 8* (5), 383-389.

Hernell, O., Forsberg, G., Hammarstrom, S.*, et al.* (2002). Coeliac disease: a model to study oral tolerance. In A. Hart, Stagg, A, Graffner, H et al (Ed.), *Gut Ecology*. London: Martin Dunitz Ltd.

Hershberg, R., Cho, D., Youakim, M.*, et al.* (1998). Highly polarized HLA class II antigen processing and presentation by human intestinal epithelial cells. *J Clin Invest, 102*, 792-803.

Heyman, M. (2001). How dietary antigens access the mucosal immune system. *Proc Nat Acad Sci, 60*, 419-426.

Hill, I., Bhatnagar S, Cameron D et al. (2002). Celiac disease: working group report of the First World Congress of Paediatric Gastroenterology, Hepatology and Nutrition. *J Paediatr Gastroenterol Nutr, 35*, S78-88.

Hill, I., Fasano, A., Schwartz, R.*, et al.* (2000). Prevalence of celiac disease in at risk groups of children in the Unites States. *Pediatric Res, 136*, 86-90.

Hill, I., Horvath, K., Fasano, A. (1995). Epidemiology of celiac disease. *Am J Gastroenterol, 90*, 163-164.

Hill, M., Marsh, P. (1990). *Human Microbial Ecology*: CRC Press.

Hoffbrand, A. (1974). Anaemia in adult coeliac disease. *Clin Gastroenterol Hepatol, 3*, 71–89.

Holtmeier, W., Kabelitz, D. (2005). T cells link innate and adaptive immune responses. *Chem Immunol Allergy, 86*, 151-183.

Hooper, L., Gordon, J. (2001). Commensal host-bacterial relationships in the gut. *Science, 292*, 1115-1118.

Hooper, L., Midtvedt, T, Gordon, J. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Ann Rev Nutr, 22*, 283-307.

Hooper, L., Wong, M., Thelin, A.*, et al.* (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science, 291*, 881-884.

Howard, M., Turnbull, A., Morley, P.*, et al.* (2002). A prospective study of the prevalence of undiagnosed coeliac disease in laboratory defined iron and folate deficiency. *J Clinl Path, 55* (10), 754-757.

Howdle, P. (2003). Celiac (Coeliac) Disease. In *Encyclopedia of Food Sciences and Nutrition* (pp. 987-994): Academic Press. San Diego.

Howell, D. (2002). Multiple comparisons with repeated measures. In *Statistical Methods for Psychology* (5 ed). Belmont, CA: Duxbury Press.

Hudson, M., Marsh, P. (1995). Carbohydrate metabolism in the colon. In G. Gibson, Macfarlane, G (Ed.), *Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology* (pp. 61-73). Boca Raton, FL: CRC Press.

Hue, S., Mention, J., Monteiro, R.*, et al.* (2004). A direct role for NKG2D/MICA interaction in villous atrophy during coeliac disease. *Immunity, 21*, 367-377.

Hugot, J. (2004). Inflammatory bowel disease: a complex group of genetic disorders. *Best Practice & Research Clinical Gastroenterology, 18* (3), 451-462.

Hunter, M. (2006). Real-time PCR. *Microbio Immunol, Online @ http://www.pathmicro.med.sc.edu/pcr/realtime-home.htm*.

Huse, M., Lillemeier, B., Kuhns, M.*, et al.* (2006). T cells use two directionally distinct pathways for cytokine secretion. *Nature Immunol, 7*, 247- 255.

Hussaini, S., Ahmed, S., Heatley, R. (1999). Celiac disease and hypoprothrombinemia. *Nutrition, 15*, 389-391.

Iltanen, S., Holm, K., Partanen, J.*, et al.* (1999). Increased density of jejunal gamma/delta+ T cells in patients having normal mucosa-marker of operative autoimmune mechanisms? *Autoimmunity, 29* (3), 179-187.

Immer, U., Vela, C., Mendez, E.*, et al.* (2003). PWG collaborative trial of gluten in gluten-free food through cocktail ELISA. *Verlag Wissenschaftliche Scripten*(23-33).

Isolauri, E., Sutas, Y., Kankaanpaa, P.*, et al.* (2001). Probiotics: effects on immunity. *Am J Clin Nutr, 73,* 444S-50S.

Ivarsson, A., Hernall, O., Stenlund, M.*, et al.* (2002). Breast-feeding protects against coeliac disease. *Am J Clin Nutr, 75*, 914-921.

Ivarsson, A., Hernall, O, Nystrom, L et al. (2003). Children born in the summer have increased risk for coeliac disease. *J Epidemiol; Community Health, 57*, 36-39.

Iwasaki, A., Kelsall, B. (2000). Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP) -3 alpha, MIP-3 beta and secondary lymphoid organ chemokine. *J Exp Med, 191,* 1381-1393.

Izcue, A., Coombes, J., Powrie, F. (2006). Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Reviews, 212*, 256-271.

Jabri, B., De Serre, N., Cellier, C.*, et al.* (2000). Selective expansion of intraepithelial lymphocytes expressing the HLA-E specific natural killer receptor CD94. *Gastroenterol, 118*, 867-879.

Janatuinen, E., Kemppainen,T., Pikkarainen, P.*, et al.* (2000). Lack of cellular and humoral immunological responses to oats in adults with coeliac disease. *Gut, 46* (3), 327-331.

Janatuinen, E., Kemppainen, T, Julkunen, R., *et al*. (2002). No harm from five year ingestion of oats in coeliac disease. *Gut, 50* (3), 332-335.

Janatuinen, E., Pikkarainen, P., Kemppainen, T.*, et al.* (1995). A Comparison of Diets with and without Oats in Adults with Celiac Disease. *N Engl J Med, 333* (16), 1033-1037.

Janeway, C., Travers, P., Walport, M.*, et al.* (2005). *Immunobiology: The immune system in health and disease* (6 ed). New York: Garland Science.

Jenkins, H., Hawkes, N., Swift, G. (1998). Incidence of coeliac disease. *Arch Dis Child, 79*, 198.

Jewell, A. (2005). Is the liver an important site for the development of immune tolerance to tumours? *Medical Hypotheses, 64* (4), 751-754.

Jiang, H., Chess, L. (2004). An integrated view of suppressor T cell subsets in immunoregulation. *J Clin Invest, 114* (9), 1198-1208.

Jiang, J., Bjorck, L., Fonden, R. (1998). Production of conjugated linoleic acid by dairy starter cultures. *J. Appl. Microbiol, 85*, 95-102.

Johansson-Lindbom, B., Svensson, M., Wurbel, M.*, et al.* (2003). Selective generation of gut tropic T cells in gut-associated lymphoid tissue(GALT): requirement for GALT dendritic cells and adjuvant. *J Exp Med, 198*, 963-968.

Jones, M., Elkus, B., Lyles, J.*, et al.* (1995). Celiac Disease (Gluten Sensitive Enteropathy) Information Guide. *American Celiac Association online @ www.enabling.org/ia/celiac*.

Kagnoff, M. (1992). Celiac disease; A gastrointestinal disease with environmental, genetic and immunological components. *Gastroent Clin Nth Am, 21* (2), 405-424.

Kagnoff, M. (1993). Celiac disease pathogenesis: the plot thickens. *Gastroenterol, 123,* 939-43.

Kagnoff, M. (2005). Overview and pathogenesis of celiac disease. *Gastroenterol, 128*, S10-S18.

Kagnoff, M. (2007). Celiac disease: pathogenesis of a model immunogenetic disease. *J Clin Invest, 117*, 41-49.

Kaminogawa, S. (1996). Food Allergy, Oral tolerance and Immunomodulation-Their molecular and cellular mechanisms. *Biosci Biotech Biochem, 60* (11), 1749-1756.

Kaminogawa, S. (2000). Food allergens and the mucosal immune system. *Biofactors, 12*, 29-32.

Kang, H., Fei, H., Saito, I.*, et al.* (1993). Comparison of HLA class II genes in Caucasoid, Chinese and Japanese patients with primary Sjögren's syndrome. *J Immunol, 150*, 3615- 3623.

Karell, K., Louka, A., Moodie, S.*, et al.* (2003). HLA types in celiac disease patients not carrying the DQA*05-DQB1*02(DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. *Hum Immunol, 64*, 469-477.

Karlsson, H., Larsson, P., Wold, A.*, et al.* (2004). Pattern of cytokine responses to gram-positive and gram-negative commensal bacteria is profoundly changed when monocytes differentiate into dendritic cells. *Infect Immunol, 72* (5), 2671-2678.

Kasarda, D. (2003). Celiac disease and safe grains. *Online @ http://wheat.pw.usda.gov/ggpages/topics/Celiac.vs.grains.html*.

Kaufmann, S. (1996). Review: Gamma /delta and other unconventional T lymphocytes: What do they see and do? *Proc Nat Acad Sci, 93*, 2272-2279.

Kaukinen, K., Collin, P., Holm, K.*, et al.* (1999). Wheat starch containing flour products in treatment of coeliac disease and dermatitis herpetiformis: a long-term follow-up study. *Scand J Gastro, 34*, 163-169.

Kaukinen, K., Collin, P., Mykkanen, A.*, et al.* (1999). Celiac disease and autoimmune endocrinologic disorders. *Dig Dis Sci, 44* (7), 1428-1433.

Kaukinen, K., Turjanmaa, K, Maki, M., *et al*. (2000). Intolerance to cereals is not specific for coeliac disease. *Scand J Gastroenterol, 35* (9), 942-946.

Keeley, J. (2004). Good bacteria trigger proteins to protect the gut. *Howard Hughes Medical Institute, EurekAlert*.

Kelly, D., Campbell, J., King, T.*, et al.* (2004). Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nature Immunol, 5* (1), 103 -112.

Kelly, D., Conway, S. (2005). Bacterial modulation of mucosal innate immunity. *Molecular Immunology, 42*, 895-901.

Kelly, D., Conway, S., Aminov, R. (2005). Commensal gut bacteria: mechanisms of immune modulation. *Trends Immunology, 26* (6), 326-333.

Kelsall, B., Biron, C., Sharma, O.*, et al.* (2002). Dendritic cells at the host-pathogen interface. *Nat Immunol, 3*, 699-702.

Kemp, M., Jeffy, B., Romagnolo, D. (2003). Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: effects on the expression of G1-restriction points in breast and colon cancer cells. *J Nutr, 133*, 3670-3677.

Kemppainen, T., Kosma, V., Janatuinen, E.*, et al.* (1998). Nutritional status of newly diagnosed celiac disease patients before and after the institution of a celiac disease diet: association with the grade of mucosal villous atrophy. *Am J Clin Nutr, 67*, 482–487.

Kenamore, B. (1940). A biospy forceps for the flexible gastorscope. *Am J Dig Dis, 7*, 539.

Kendall, M., Cox, P., Schneider, R.*, et al.* (1972). Gluten subfractions in coeliac disease. *Lancet, 2,* 1065-67.

Kepczyk, T., Kadakia, S. (1995). Prospective evaluation of gastrointestinal tract in patients with iron-deficiency anemia. *Dig Dis Sci, 40* (6), 1283-1289.

Kertulla, T., Holm, K., Partanen, A.*, et al.* (1998). Circulating T-lymphocyte subsets in coeliac disease patients and healthy family members. *Clin Exp Immunol, 111* (3), 536.

Kleopa, K., Kyriacou, K., Zamba-Papanicolaou, E.*, et al.* (2005). Reversible inflammatory and vacuolar myopathy with vitamin E deficiency in celiac disease. *Nerve Muscle, 31* (2), 260-265.

Klugh, H. (1986). *Statistics:The Essentials of Research*. New Jersey: Lawrence Erlbaum Associates.

Knight, D., Girling, K. (2003). Gut flora in health and disease. *The Lancet, 361,* (9371), 1831.

Kobayashi, K., Chamaillard, M., Ogura, Y.*, et al.* (2005). NOD-2 dependent regulation of innate and adaptive immunity in the intestinal tract. *Science, 307*, 731-734.

Koch, C., Platt, J. (2007). T cell recognition and immunity in the fetus and mother. *Cell Immunol, 248*, 12-17.

Koehler, K. (2005). U.S. Food Drug Administration; Mean per capita consumption of selected foods and gluten-forming grain proteins in the U.S., 2000, based upon disappearance data. *Unpublished data, online @ www.cfsan.fda.gov*.

Kolho, K., Farkkila, M., Savilahti, E. (1998). Undiagnosed coeliac disease is common in Finnish adults. *Scand J Gastroenterol, 33*, 1280–1283.

Kolho, K., Savilahti, E. (1997). IgA endomysium antibodies on human umbilical cord: an excellent diagnostic tool for celiac disease in childhood. *J Pediatr Gastro Nutr, 24* (5), 563-567.

Koning, F., Schuppan, D., Cerf-Bensussen, N.*, et al.* (2005). Pathomechanisms in coeliac disease. *Best Pract Res Clin Gastroenterol, 19* (3), 373-387.

Korponay-Szabo, I., Kovacs, J., Czinner, A.*, et al.* (1999). High prevalence of silent celiac disease in preschool children screened with IgA/IgG antiendomysuim antibodies. *J Paediatr Gastroenterol Nutr, 28*, 26-30.

Krahenbuhl, J., Neutra, M. (2000). Epithelial M-Cells: Differentiation and Function. *Annual Review of Cell and Developmental Biology, 16*, 301-332.

Kumar, P. (2006). Coeliac disease and lymphoma. *European Journal of Gastroenterology & Hepatology, 18* (2), 131-132.

Kumar, V., Lerner, A., Valeski, J.*, et al.* (1989). Endomysial antibodies in the diagnosis of celiac disease and the effect of gluten on antibody titers. *Immunol Invest, 18* (1-4), 533-544.

Labeta, M., Vidal, K., Rey-Nores, J.*, et al.* (2000). Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J Exp Med, 191*, 1807-1812.

Ladinser, B., Rossipal, E., Pittschieler, K. (1994). Endomysium antibodies in coeliac disease: an improved method. *Gut, 35* (6), 776-778.

Laitinen, K., Hoppu, U., Hämäläinen, M.*, et al.* (2006). Breast milk fatty acids may link innate and adaptive immune response regulation: analysis of soluble CD14, prostaglandin E2, and fatty acids. *Pediatr Res, 59*, 723-727.

LeBouder, E., Rey-Nores, J., Raby, A.*, et al.* (2006). Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specially and differentially modulated by human milk. *J Immunol, 176*, 3742-3752.

LeBouder, E., Rey-Nores, J., Rushmere, M.*, et al.* (2003). Soluble forms of Toll-like receptor (TLR) 2 capable of modulating TLR2 signaling are present in human plasma and breast-milk. *J Immunol, 171*, 6680-6689.

Lelouard, H., Sahuquet, A., Reggio, H.*, et al.* (2001). Rabbit M cells and dome enterocytes are distinct cell lineages. *J Cell Sci, 114*, 2077-2083.

Lenoir-Wijnkoop, I., Hopkins, M. (2003). *The Intestinal Microflora- Understanding the Symbiosis- Nutrition and Health Collection*. London: John Libbey & Company Ltd.

Lentle, R. (2008). Personal discussion with Dr Lentle relating to the types of molecular methods available to identify and classify intestinal microflora. *Massey University*.

Leonardi, S., Bottaro, G., Patané, R.*, et al.* (1990). Hypertransaminasemia as the first symptom in infant celiac disease. *J Pediatr Gastroenterol Nutr, 11*, 404-406.

Lerner, A., Gruener, N., Lancu, T. (1993). Serum carnitine concentrations in celiac disease. *Gut, 34*, 933-935.

Lerner, A., Kumar, V., Lancu, T. (1994). Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. *Clin Exp Immunol, 95* (1), 78-82.

Letley, D., Rhead, J., Twells, R.*, et al.* (2003). Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *J Biol Chem, 278*, 26734-26741.

Levitt, M., Gibson, G., Christl, S. (1995). Gas metabolism in the large intestine. In G. Gibson, Macfarlane, G (Ed.), *Human Colonic Bacteria: Role in Nutrition, Physiology, and Pathology* (pp. 131-149). Boca Raton: CRC Press.

Lewis, S., Heaton, K. (1999). The metabolic consequences of slow colonic transit. *Am J Gastroenterol, 94*, 2010-2016.

Ley, R., Bäckhed, F., Turnbaugh, P.*, et al.* (2005). Obesity alters gut microbial ecology. *Proc Nat Acad Sci, 102* (31), 11070-11075.

Ley, R., Turnbaugh, P., Klein, S.*, et al.* (2006). Microbial ecology: human gut microbes associated with obesity. *Nature, 444* (7122), 1022-1023.

Li, J., Barreda, D., Zhang, H.*, et al.* (2006). B lymphocytes from early vertebrates have potent phagoctyic and microbicidal abilities. *Nat Immunol, 7*, 1116-1124.

Li, Q., Venna, I. (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol, 2* (10), 725-734.

Li Voon Chong, J., Leong, K., Wallymahmed, M.*, et al.* (2002). Is coeliac disease more prevalent in young adults with coexisting Type 1 diabetes mellitus and autoimmune thyroid disease compared with those with Type 1 diabetes mellitus alone? *Diabet Med, 19* (4), 334-337.

Lin, T., Lin, C., Lee, C. (1999). Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic acid. *Food Chem, 67*, 1-5.

Lindberg, T., Nilsson, L., Borulf, S.*, et al.* (1985). Serum IgA and IgG gliadin antibodies and small intestinal mucosal damage in children. *J Pediatr Gastro Nutr, 4* (6), 917-922.

Linder, M. (1991). Nutrition and metabolism of proteins. In M. Linder (Ed.), *Nutritional Biochemistry and Metabolism* (2 ed., pp. 87-110). Norwalk, CT: Appleton and Lange.

Lindfors, K., Blomqvist, T., Juuti-Uusitalo, S.*, et al.* (2008). Live probiotic *Bifidobacterium lactis* bacteria inhibit the toxic effects induced by wheat gliadin in epithelial cell culture. *Clin Exp Immunol, 152*, 552-558.

Lindquist, B., Rogozinski, T., Moi, H.*, et al.* (1994). Endomysium and gliadin IgA antibodies in children with coeliac disease. *Scand J Gastroenterol, 29*, 452-456.

Linnemeyer, P., Pollack, S. (1993). Prostaglandin E-2 induced changes in the phenotype, morphology and lytic activity of IL-2 activated natural killer cells. *J Immunol, 150*, 3747-3754.

Logan, R. (1992). Problems and pitfalls in epidemiological studies of coeliac disease. *Dyn Nutr Res, 2*, 14-24.

Logan, R., Rifkind, E., Turner, I.*, et al.* (1989). Mortality in celiac disease. *Gastroenterol, 97*(2), 265-271.

Logan, R., Tucker, G., Rifkind, E.*, et al.* (1983). Changes in clinical features of coeliac disease in adults in Edinburgh and the Lothians 1960-79. *BMJ, 286*, 95-97.

Louka, A., Sollid, L. (2003). HLA in coeliac disease: unravelling the complex genetics of a complex disorder. *Tissue Antigens, 61*, 105-117.

Lubel, J., Burrell, L., Levidiotis, V. (2005). An unexpected case of macroscopic haematuria. *Med J Aust, 183*, 321-323.

Ludvig, M., Sollid, M., Gary, M. (2004). A role for bacteria in celiac disease. *Am J Gastroenterol, 99* (5), 905-906.

Lundin, K., Scott, H., Hansen, T.*, et al.* (1993). Gliadin-specific, HLA-DQ (alpha 1*050, beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp Med, 178*, 187-196.

Lundin, K., Sollid, L. (2003). Gliadin peptide specific intestinal T cells in coeliac disease. *Gut, 52*, 162-163.

Macfarlane, G., Gibson, G. (1994). Metabolic activities of the normal colonic flora. In S. Gibson (Ed.), *Human Health: The Contribution of Microorganisms* (pp. 17-53). London: Springer-Verlag.

Macfarlane, S., Furie, E., Kennedy, A.*, et al.* (2005). Mucosal bacteria in ulcerative colitis. *Br J Nutr, 93*, S67-72.

Macfarlane, S., Macfarlane, G. (1995). Proteolysis and amino acid fermentation. In G. Gibson, Macfarlane, G (Ed.), *Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology* (pp. 75-100). Boca Raton, FL: CRC Press.

Mackay, I. (2007). *Real-Time PCR in Microbiology: From diagnosis to characterization*: Horizon Scientific Press.

MacKenzie, D. (2006). The gluten-free diet. *Unpublished data; Information supplied to patients referred to Lifestile Nutrition Consultancy for implementation of a gluten free dietary regime*.

Mackie, R., Sghir, A., Gaskins, H. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *American Journal of Clinical Nutrition, 69* (5), 1035S-1045S.

Mahmud, F., Murray, J., Kudva, Y.*, et al.* (2005). Celiac disease in type 1 diabetes mellitus in a North American community: prevalence, serologic screening, and clinical features. *Mayo Clin Proc, 80*, 1429-1434.

Maiuri, L., Ciacci, C., Ricciardelli, I.*, et al.* (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet, 362*, 30-37.

Majamaa, H., Isolauri, E. (1997). Probiotics: A novel approach in the management of food allergy. *J Allergy Clin Immunol, 99* (2), 179-185.

Maki, M., Holm, K., Lipsanen, V.*, et al.* (1991). Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. *Lancet, 338* (8779), 1350-1353.

Maki, M., Kallonen, K., Lahdeaho, M.*, et al.* (1988). Changing pattern of childhood coeliac disease in Finland. *Acta Paediatr Scand, 77*, 408-412.

Mäki, M., Mustalahti K, Kokkonen, J., *et al*. (2003). Prevalence of Celiac Disease among Children in Finland. *NEJM, 348* (25), 2517-2524.

Malnick, S., Atali, M., Lurie, Y.*, et al.* (1998). Celiac sprue presenting during puerperium. *J Clin Gastro, 26*, 164-166.

Mantis, N., Frey, A., Neutra, M. (2000). Accessiblilty of glycolipid and oligosaccharide epitopes on rabbit villus and follicle associated epithelium. *Am J Physiol Gastrointest Liver Physiol, 278*, G915-923.

Marks, J., Shuster, S., Watson, A. (1966). Small bowel changes in dermatitis herpetiformis. *Lancet, ii*, 1280-1282.

Marsh, M. (1992). Gluten, major histocompatibilty complex and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity(coeliac sprue). *Gastroenterology, 102*, 330-354.

Marsh, M. (1995). The natural history of gluten sensitivity; defining, refining, and re-defining. *Q J Med, 88*, 9-13.

Marsh, T. (1999). Terminal restriction fragment length polymorphism (T-RFLP): an emerging method of characterising diversity among homologous populations of amplification products. *Curr Opin Microbiol, 2*, 323-327.

Martinez-Medina, M., Aldeguer, X., Gonzalez-Huix, F.*, et al.* (2006). Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis, 12* (12), 1136-1145.

Matysiak-Budnik, T., Candahl, C., Cellier, C.*, et al.* (2005). Limited efficiency of prolyl endopeptidase in detoxification of gliadin peptides in celiac disease. *Gastroenterology, 129*, 786-796.

Mauro, A., Orsi, L., Mortara, P.*, et al.* (1991). Cerebellar syndrome in adult celiac disease with vitamin E deficiency. *Acta Neurol Scand, 84* (2), 167-170.

Mayer, L. (1998). Current concepts in mucosal immunity- Antigen presentation in the intestine; new rules and regulations. *Am J Physiol Gastroenterol Liver Physiol, 274* (1), G7-9.

Mayer, L. (2003). Mucosal Immunity. *Pediatrics, 111* (6), 1595-1600.

Mayer, L., Walker, A. (2005). Chapter 1: Development and physiology of mucosal defense: an introduction. In J. Mestecky, Lamm, M, Strober, W et al (Ed.), *Mucosal Immunity* (3 ed., pp. 6): Academic Press.

Mazzetti, D., Giorgetti, G., Gregori, M.*, et al.* (1992). Subclinical coeliac disease. *Ital J Gastroenterol, 24* (6), 352-354.

McClure, R. D. (2005). Chapter 2: Endocrinology of male infertility. In P. Patton, Battaglia, D (Ed.), *Office Andrology*. New Jersey: Humana Press.

McCracken, S., Drury, C., Lee, H.*, et al.* (2003). Pregnancy is associated with suppression of the nuclear factor kappa B/I kappa B activation pathway in peripheral blood mononuclaer cells. *J Reprod Immunol, 58*, 27-47.

McIntyre, A., Long, R. (1993). Prospective survey of investigations in outpatients referred with iron deficiency anaemia. *Gut, 34* (8), 1102-1107.

McMillan, S., Haughton, D., Biggart, J.*, et al.* (1991). Predictive value for coeliac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. *BMJ, 303* (6811), 1163-1165.

McRae, B., Nagai, T., Semnani, R.*, et al.* (2000). Interferon-alpha and -beta inhibit the in vitro differentiation of immunocompetent human dendritic cells from CD14(+) precursors. *Blood, 96*, 210-217.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews; Immunology, 1*, 135-145.

Meini, A., Pillan, N., Villanacci, V.*, et al.* (1996). Prevalence and diagnosis of celiac disease in IgA-deficient children. *Ann Allergy Asthma Immunol, 77* (4), *333-6*.

Mensink, R. (2005). Metabolic and health effects of isomeric fatty acids. *Curr Opin Lipidol, 16*, 27-30.

Meresse, B., Curran, S., Ciszewski, C.*, et al.* (2006). Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med, 203*, 1343-1355.

Messer, M., Baume, P. (1976). Oral papain in gluten intolerance. *Lancet, 2*, 1022.

Metcalfe, D., Sampson, H., Simon, R. (2003). *Food Allergy: Adverse Reactions to Foods and Food Additives* (3 ed). Massachusetts, USA: Blackwell Publishing Inc.

Mills, K., McGuirk, P. (2004). Antigen-specific regualtory T cells- their induction and role in infection. *Semin Immunol, 16*, 107-117.

Mizoguchi, E., Mizoguchi, A., Preffer, F.*, et al.* (2000). Regulatory role of mature B-cells in a murine model of inflammatory bowel disease. *Int Immunol, 12*, 597-605.

Moal Lievin-Le, V., Servin, A. (2006). The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin Microbiol Rev, 19* (2), 351-337.

Mody, R., Brown, P., Wechsler, D. (2003). Refractory iron deficiency anemia as the primary clinical manifestation of celiac disease. *J Pediatr Hematol Oncol, 25*, 169–172.

Ministry of Health (MOH) (2003); Food and Nutrition Guidelines for Healthy Adults; A Background Paper: Wellington, New Zealand

Ministry of Health (MOH) (1999); NZ Food: NZ People; key results of the 1997 National Nutrition Survey. Online at *www.moh.govt.nz*.

Molberg, O., Kett, K., Scott, E.*, et al.* (1997). Gliadin specific, HLADQ2-restricted T cells are commonly found in small intestine biopsies from coeliac disease patients but not from controls. *Scand J Immunol, 46*, 103-108.

Molberg, O., McAdam, S., Sollid, L. (2000). Role of tissue transglutaminase in celiac disease. *J Paediatr Gastroenterol Nutr, 30*, 232-240.

Molberg, O., Uhlen, A., Jensen T., *et al*. (2005). Mapping of gluten T-cell epitopes in bread wheat ancestors: implications for celiac disease. *Gastroenterology, 128*, 393-401.

Molteni, N., Bardella, M., Vezzoli, G.*, et al.* (1995). Intestinal calcium absorption as shown by stable strontium test in celiac disease before and after gluten-free diet. *Am J Gastroenterol, 90* (11), 2025-2028.

Monsuur, A., De Bakker, P., Alizadeh, B.*, et al.* (2005). Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet, 37*, 1341-1344.

Montoya, M., Schiavoni, G., Mattei, F.*, et al.* (2002). Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood, 99* (9), 3263-3271.

Moreau, M. (2000). Intestinal flora, probiotics and effects on the intestinal IgA immune response. *Arch Pediatr, 7* (2), 247S -248S.

Morein, B., Blomqvist, G., Hu, K. (2007). Immune responsiveness in the neonatal period. *J Comp Pathol, 137*, S27-S31.

Morita, C., Mariuzza, R., Brenner, M. (2000). Antigen recognition by human gamma/delta T cells: pattern recognition by the adaptive immune system. *Semin Immunopathol, 22*, 191-217.

Morrow, A., Ruiz-Palacios, G., Jiang, X.*, et al.* (2005). Human-milk glycans that inhibit pathogen binding protect breast feeding infants against infectious diarrhea. *J Nutr, 135*, 1304-1307.

Mowat, A. (2003). Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews Immunology, 3*, 331-341.

Mowat, A. (2003). Coeliac disease- a meeting point for genetics, immunology, and protein chemistry. *Lancet, 361*, 1290-1292.

Mowat, A. (2005). Dendritic cells and immune responses to orally administered antigens. *Vaccine, 23* (15), 1797-1799.

Mowat, A., Donachie, A., Parker, L.*, et al.* (2003). The role of dendritic cells in regulating mucosal immunity and tolerance. *Novartis Foundation Symposium, 252*, 291-305.

Mowat, A., Viney, J. (1997). The anatomical basis of intestinal immunity. *Immunol Rev, 156*, 145-166.

Mueller, S., Saunier, K., Hanisch, C.*, et al.* (2006). Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol, 72* (2), 1027-1033.

Murray, J., Van Dyke, C., Plevak, M.*, et al.* (2003). Trends in the identification and clinical features of celiac disease in a North American community, 1950-2001. *Clin Gastroenterol Hepatol, 1* (1), 19-27.

Murray, J., Watson, T., Clearman, B.*, et al.* (2004). Effect of a gluten-free diet on gastrointestinal symptoms in celiac disease. *Am J Clin Nutr, 79* (4), 669-673.

Murray, P. (2005). NOD proteins: an intracellular pathogen-recognition system or signal transduction modifiers? *Curr Opin Immunol . 17* (4), 352-358.

Mustalahti, K., Reunanen, A., Heuer, M.*, et al.* (2004). Prevalence of coeliac disease in four European countries. *The 11th International Symposium: Coeliac Disease, Belfast, Northern Ireland*, P60.

Muyzer, G., Smalla, K. (1998). Application of denaturing gradient gel electrophoresis(DGGE) and tempertaure gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek, 73*, 127-141.

Nadal, I., Donant, E., Ribes-Koninckx, C.*, et al.* (2007). Imbalance in the composition of the duodenal microbiota of children with coeliac disease. *J Med Microbiol, 56* (12), 1669-1674.

Nagler-Anderson, C. (2001). Man the barrier! Strategic defences in the intestinal mucosa. *Nature Reviews; Immunology, 1* (1), 159-167.

Nailis, H., Coenye, T., Van Nieuwerburgh, F.*, et al.* (2006). Development and evaluation of different normalization strategies for gene expression studies in Candida albicans biofilms by real-time PCR. *BMC Mol Biol, 7*, 25.

Nanthakumar, N., Dai, D., Newburg, D. (2002). The role of indigenous microflora in the development of murine intestinal fucosyl- and sialyl-transferases. *FASEB, Online express article 10.1096/fj.02-0031fje.*

Neish, A., Gewirtz, A., Zeng, H.*, et al.* (2000). Prokaryotic regulation of epithelail response by inhibition of I kappa B-kappa ubiquitination. *Science, 289*, 1560-1563.

Nelson, M., Bingham, S. (1997). Assessment of food consumption and nutrient intake. In B. Margetts & M. Nelson (Eds.), *Design Concepts in Nutritional Epidemiology* (pp. 123-169). Oxford: Oxford University Press.

Neutra, M., Mantis, N., Frey, A.*, et al.* (1999). The composition and function of M cell apical membranes: implications for microbial pathogenesis. *Semin Immunol, 11*, 171-181.

Neutra, M., Mantis, N., Kraehenbuhl, J. (2001). Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol, 2*, 1004-1009.

Newburg, D., Ruiz-Palacios, G., Morrow, A. (2005). Human milk glycans protect infants against enteric pathogens. *Ann Rev Nutr, 25*, 37-58.

Nolan, T., Hands, R., Bustin, S. (2006). Quantification of mRNA using real-time RT-PCR. *Nat Protoc, 1*, 1559-1582.

Nordgård, L., Traavik, T., Nielsen, K. (2005). Nucleic acid isolation from ecological samples-vertebrate gut flora. *Methods in Enzymology, 395*, 38-48.

Norgard, B., Fonager, K., Sorensen, H.*, et al.* (1999). Birth outcomes of women with celiac disease: A nationwide historical cohort study. *Am J Gastroenterol, 94* (9), 2435-2440.

Norris, J., Barriga, K., Hoffenberg, E.*, et al.* (2005). Risk of celiac disease autoimmunity and timing of gluten introduction in the diet of infants at increased risk of disease. *JAMA, 293*, 2343-2351.

Not, T., Horvath, K., Hill, I.*, et al.* (1998). Celiac disease risk in the USA: high prevalence of antiendomysium antibodies in healthy blood donors. *Scand J Immunol, 33*, 494-498.

Noverr, M., Huffnagle, G. (2004). Does the microbiota regulate immune responses outside the gut? *Trends Microbiol, 12* (12), 562-568.

Noverr, M., Huffnagle, G. (2005). The 'microflora hypothesis' of allergic diseases. *Clin Exp Allergy, 35*, 1511-1520.

Nussenzweig, M. (1997). Immune responses; tails to teach a B cell. *Curr Bio*. *7*:R355

O'Mahony, S., Arranz, E., Barton, J.*, et al.* (1991). Dissociation between systemic and mucosal humoral immune responses in coeliac disease. *Gut, 32*, 29-35.

Osborn, A., Moore, E., Timmis, K. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ Microbiol, 2* (1), 39-50.

Osman, A., Günnel, T., Dietl, A.*, et al.* (2000). B cell epitopes of gliadin. *Clin Exp Immunol, 121* (2), 248-254.

Owen, C., Martin, R., Whincup, P.*, et al.* (2005). The effect of infant feeding on the risk of obesity across the life course: a quantitative review of published evidence. *Pediatrics, 115*, 1367-1377.

Oxentenko, A., Grisolano, S., Murray, J.*, et al.* (2002). The insensitivity of endoscopic
markers in celiac disease. *Am J Gastroenterol, 97* (4), 933-938.

Panga, X., Dingb, D., Weia, G.*, et al.* (2005). Molecular profiling of Bacteroides spp. in human feces by PCR-temperature gradient gel electrophoresis. *J Microbiol Method, 61*(3), 413-417.

Papadakis, K., Landers, C., Prehn, J.*, et al.* (2003). CC chemokine receptors 9 expression defines a subset of peripheral blood lymphocytes with mucosal T cell phenotype and Th1 or T-regulatory 1 cytokine profile. *J Immunol, 171,* 159 - 62.

Papadakis, K., Prehn, J., Moreno, S.*, et al.* (2001). CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. *Gastroenterol, 121*, 246-250.

Papadopoulos, G., Wijmenga, C., Koning, F. (2001). Interplay between genetics and the environment in the development of celiac disease: perspectives for a healthy life. *J Clin Invest, 108* (9), 1261-1266.

Pare, P., Douville, P., Caron, D.*, et al.* (1988). Adult coeliac sprue: changes in the pattern of clinical recognition. *Gastroenterology, 10*, 395.

Parham, P. (2005). *The Immune System*. New York: Garland Science Publishing.

Pariza, M. (2004). Perspective on the safety and effectiveness of conjugated linoleic acid. *Am J Clin Nutr, 79*, 1132S-1136S.

Pauleau, A., Murray, P. (2003). Role of NOD 2 in the response of macrophages to toll-like receptor agonists. *Mol Cell Bio, 23*, 7531-7539.

Paulley, J., Fairweather, F., Leemin, A. (1957). Postgastrectomy steatorrhoea and patchy jejunal atrophy. *Lancet, 1*, 406-407.

Paveley, W. (1988). From Aretaeus to Crosby: a history of coeliac disease. *BMJ, 297*, 1646-1649.

Pazianas, M., Butcher, G., Subhani, J.*, et al.* (2005). Calcium absorption and bone mineral density in celiacs after long term treatment with gluten-free diet and adequate calcium intake. *Osteoporos Int, 16* (1), 56-63.

Peach, S., Tabaqchali, M. (1982). Mucosa-associated flora of the human gastrointestinal tract in health and disease. *Eur J Chemother Antibiot, 2*, 41.

Peltonen, R., Ling, W., Hanninen, O. (1992). An uncooked vegan diet shifts the profile of human fecal microflora: computerized analysis of direct stool sample gas-liquid chromatography profiles of bacterial cellular fatty acids. *Appl Environ Microbiol, 58*, 3660-3666.

Peraaho, M., Collin, P., Kaukinen, K.*, et al.* (2004). Oats can diversify a gluten-free diet in celiac disease and dermatitis herpetiformis. *J Am Diet Assoc, 104* (7), 1148-1150.

Perdue, M. (1999). Mucosal immunity and inflammation III. The mucosal antigen barrier: crosstalk with mucosal cytokines. *Am J Physiol Gastrointest Liver Physiol, 277* (1), G1-G5.

Petrilli, V., Papin, S., Tschopp, J. (2005). The inflammasome. *Curr Bio, 15* (15), R581.

Picarelli, A., Sabbatella, L., Di Tola, M.*, et al.* (2000). Celiac disease diagnosis in misdiagnosed children. *Pediatric Research, 48* (5), 590-592.

Pittman, F., Holub, D. (1965). Sjögren's syndrome and adult celiac disease. *Gastroenterology, 48*, 869- 876.

Pittschieler, K. (1986). Folic acid concentration in the serum and erythrocytes of patients with celiac disease. *Padiatr Padol, 21*, 363–366.

Pittschieler, K., Ladinser, B. (1996). Coeliac disease: screened by a new strategy. *Acta Paediatr, 412*(Suppl), 42-45.

Podolsky, D. (2002). Inflammatory bowel disease. *N Engl J Med, 347*, 417-429.

Pollock, D. (1977). The liver in coeliac disease. *Histopathology, 1*, 421-430.

Presutti, J., Cangemi, J., Cassidy, H.*, et al.* (2007). Celiac Disease. *Am Fam Physician, 76*, 1795 -1802, 1809 -1710.

Przemioslo, R., Lundin, K., Sollid, L.*, et al.* (1995). Histological changes in small bowel mucosa induced by gliaden sensitive T lymphocytes can be blocked by anti-interferon antibody. *Gut, 36*, 874-879.

Pulendran, B., Kumar, P., Cutler, C.*, et al.* (2001). Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J Immunol, 167*, 5067-5076.

Purvin, V. (1999). Through a Shade Darkly. *Survey of Ophthalmology, 43* (4), 335-340.

Quinn, C., Cotter, P., Stevens, F.*, et al.* (2007). Coeliac disease in the older patient. *Rev Clin Geront*, 1-10.

Raimondi, F., Paludetto, R., Fasano, A. (2004). Intestinal permeability and child health; a dangerous liaison. *Ital J Pediatr, 30*, 142-146.

Rampertab, S., Pooran, N., Brar, P.*, et al.* (2006). Trends in the Presentation of Celiac Disease. *Am J Medicine, 119* (4), 355. e359-314.

Rang, H. (2003). *Pharmacology*. Edinburgh: Churchill Livingstone.

Ransford, R., Hayes, M., Palmer, M.*, et al.* (2002). A controlled, prospective screening study of celiac disease presenting as iron deficiency anemia. *J Clin Gastroenterol, 35* (3), 228-233.

Rashid, M., Cranney, A., Graham, I.*, et al.* (2003). Canadian Celiac Health Survey: pediatric data. *J Pediatr Gastroenterol Nutr, 37*, 369.

Ravikumara, M., Tuthill, D., Jenkins, H. (2006). The changing clinical presentation of coeliac disease. *Arch Dis Child, 91*, 969-971.

Rayman, M. (2000). The importance of selenium to human health. *Lancet, 356* (9225), 233-241.

Reif, S., Lerner, A. (2004). Tissue transglutaminase-the key player in celiac disease: a review. *Autoimmunity Reviews, 3* (1), 40-45.

Reinecker, H., Podolsky, D. (1995). Human intestinal epithelial cells express functional cytokine receptors sharing the common gamma C chain of the interleukin 2 receptors. *Proc Natl Acad Sci, 92*, 8353-8357.

Reinken, L., Zieglauer, H., Berger, H. (1976). Vitamin B6 nutriture of children with acute celiac disease, celiac disease in remission, and of children with normal duodenal mucosa. *Am J Clin Nutr, 29* (7), 750-753.

Reis e Sousa, C., Sher, A., Kaye, P. (1999). The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Curr Opin Immunol . 4*, 392-399.

Rescigno, M., Urbano, M., Valzasina, B.*, et al.* (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol, 2*, 361-367.

Rewers, M., Liu, E., Simmons, J.*, et al.* (2004). Celiac disease associated with type 1 diabetes mellitus. *Endocrinol Metab Clin Nth Am, 33*(1), 197-214.

Rich, E., Christie, D. (1990). Anti-gliadin antibody panel and xylose absorption test in screening for celiac disease. *J Pediatr Gastro Nutr, 10*(2), 174-178.

Riestra, S., Fernandez, E., Rodrigo, L.*, et al.* (2000). Prevalence of Coeliac disease in the general population of northern Spain. Strategies of serologic screening. *Scand J Gastroenterol, 35*(4), 398-402.

Rigottier-Gois, L., Le Bourhis, A., Gramet, G.*, et al.* (2003). Fluorescent hybridization combined with flow cytometry and hybridization of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. *FEMS Microbiol Ecol, 43*, 237–245.

Rigottier-Gois, L., Rochet, V., Garrec, N.*, et al.* (2003). Enumeration of Bacteroides species in human faeces by fluorescent in situ hybridisation combined with flow cytometry using 16S rRNA probes. *Syst Appl Microbiol, 26* (1), 110–118.

Riordan, S., McIver, C., Wakefield, D.*, et al.* (2001). Small intestinal mucosal immunity and morphometry in luminal overgrowth of indigenous gut flora. *Am J Gastroenterol, 96* (2), 494-500.

Rizzello, C., de Angelis, M., Di Cagno, R.*, et al.* (2007). Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. *Appl Environ Microbiol, 73* (14), 4499 - 4507.

Roediger, W., Moore, J., Babidge, W. (1997). Colonic sulfide in the pathogenesis and treatment of ulcerative colitis. *Dig Dis Sci, 42*, 1571-1579.

Roos, S., Jonsson, H. (2002). A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus components. *Microbiol, 148*, 433-442.

Rosberg-Cody, E., Ross, R., Hussey, S.*, et al.* (2004). Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid-producing bifidobacteria. *Appl Environ Microbiol, 70*, 4635-4641.

Ross, M., Pawlina, W. (2003). *Histology: A Text and Atlas* (4 ed). Philadelphia: Lippincott Williams & Wilkins.

Rossi, T., Slbini, C., Kumar, V. (1993). Incidence of celiac disease identified by the presence of serum endomysial antibodies in children with chronic diarrhea, short stature, or insulin dependent diabetes mellitus. *J Paediatr, 123*, 262-264.

Rostom, A., Dubé, C., Cranney, A.*, et al.* (2005). The diagnostic accuracy of serologic tests for celiac disease: A systematic review. *Gastroenterol, 128* (4), S38-S46.

Rostom, A., Murray, J., Kagnoff., M., American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. Gastroenterology 131(6): 2007.

Roux, M., Slobdanik, N., Gauffin Cano, P.*, et al.* (2003). Chapter 7; Mucosal Immune System and Malnutrition. In R. Fuller, Perdigon, G (Ed.), *Gut Flora, Nutrition, Immunity and Health*. Oxford: Blackwell Publishing Ltd.

Rubin, C., Brandborg, L., Flick, A.*, et al.* (1962). Studies of celiac sprue. III. The effect of repeated wheat instillation into the proximal ileum of patients on a gluten free diet. *Gastroenterology, 43*, 621-641.

Rude, R., Olerich, M. (1996). Magnesium deficiency: possible role in osteoporosis associated with gluten-sensitive enteropathy. *Osteoporos Int, 6* (6), 453-461.

Ruoff, M. (1996). Chapter 5- Small Bowel. In A. Gelb (Ed.), *Clinical Gastroenterology in the Elderly* (pp. 78-80). New York: Marcel Dekker.

Russell, D., Parnell, W., Wilson, N., *et al*. (1999). NZ Food: NZ People- Key results of the 1997 National Nutrition Survey, *Minstry of Health*, Wellington, New Zealand.

Russell, M., Nagler-Anderson, C., Anderson, P.*, et al.* (1993). Cytotoxic potential of intraepithelial lymphocytes (IELs): Presence of TIA-1, the cytolytic granule-associated protein, in human IELs in normal and diseased intestine. *Am J Pathol, 143* (2), 350-354.

Russo, P., Chartrand, L., Seidman, E. (1999). Comparative analysis of serologic screening tests for the initial diagnosis of celiac disease. *Pediatrics, 104*, 75-78.

Sacchetti, L., Calcagno, G., Ferrajolo, A.*, et al.* (1998). Discrimination between celiac and other gastrointestinal disorders in childhood by rapid human lymphocyte antigen typing. *Clin Chem, 44* (8 Pt 1), 1755-1757.

Saibeni, S., Lecchi, A., Meucci, G.*, et al.* (2005). Prevalence of hyperhomocysteinemia in adult gluten-sensitive enteropathy at diagnosis: role of B12, folate, and genetics. *Clin Gastroenterol Hepatol, 3* (6), 574-580.

Sallusto, F., Lanzavecchia, A. (1999). Mobilizing Dendritic Cells for Tolerance, Priming, and Chronic Inflammation. *J Exp Med, 189* (4), 611-614.

Salmaso, C., Ocmant, A., Pesce, G.*, et al.* (2001). Comparison of ELISA for tissue transglutaminase autoantibodies with antiendomysium antibodies in pediatric and adult patients with celiac disease. *Allergy, 56* (6), 544-547.

Salminen, S., Isolauri, E., Onnela, T. (1995). Gut flora in normal and disordered states. *Chemotherapy, 41*, 5-15.

Sanders, D., Hurlstone, D., Stokes, R.*, et al.* (2002). Changing face of adult coeliac disease: experience of a single university hospital in South Yorkshire. *Postgrad Med J, 78* (915), 31-33.

Sanz, Y., Sanchez, E., Marzotto, M.*, et al.* (2007). Differences in faecal bacterial communities in coeliac and healthy children as detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunol Med Microbiol, 51*, 562-568.

Sari, R., Yildirim, B., Sevinc, A.*, et al.* (2000). Gluten-free diet improves iron-deficiency anaemia in patients with coeliac disease. *J Health Popul Nutr, 18* (1), 54-56.

Sartor, R. (2004). Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics and prebiotics. *Gastroenterol, 126*, 1620-1633.

Sass, J., Heinz-Erian, P. (2000). Cause of Vitamin A deficiency in celiac disease. *Br J Ophthalmol, 84*, 1075.

Sategna-Guidetti, C., Grosso, S. (1994). Changing pattern in adult coeliac disease: A 24-year survey. *Eur J Gastroenterol Hepatol, 6* (1), 15-19.

Sategna-Guidetti, C., Grosso, S., Bruno, M.*, et al.* (1995). Comparison of serum anti-gliadin, anti-endomysium, and anti-jejunum antibodies in adult celiac sprue. *J Clin Gastro, 20* (1), 17-21.

Sblattero, D., Berti, I., Trevisiol, C. (2000). Human tissue transglutaminase ELISA: a powerful mass screening diagnostic assay for celiac disease. *Am J Gasterol, 98*, 1253-1257.

Scanlan, P., Shanahan, F., O' Mahony, C*., et al.* (2006). Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. *J Clin Microbiol, 44*, 3980-3988.

Schein, J. (1947). Syndrome of non-tropical sprue with hitherto undescribed lesions of the intestine. *Gastroenterol, 8*, 438-460.

Schell, M., Karmirantzou, M., Snel, B*., et al.* (2002). The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Nat Acad Sci, 99* (22), 14422-14427.

Schmitz, U., Ko, Y., Seewald, S*., et al.* (1994). Iron-deficiency anemia as the sole manifestation of celiac disease. *Clin Investig, 72* (7), 519-521.

Schuster, S., Marks, J. (1970). Dermatogenic enteropathy. *Gut, 11*, 292-298.

Schwarzenberg, S., Brunzell, C. (2002). Type 1 Diabetes and Celiac Disease: Overview and Medical Nutrition Therapy. *Diabetes Spectrum, 15*, 197-201.

Schwiertz, A., Gruhl, B., Lobnitz, M*., et al.* (2003). Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatric Research, 54* (3), 393-399.

Scott, K., Manunta, M., Germain, C. (2005). Qualitatively distinct patterns of cytokines are released by human dendritic cells in response to different pathogens. *Immunology, 116* (2), 245-254.

Sears, C. (2005). A dynamic partnership: Celebrating our gut flora. *Anaerobe, 11*(5), 247-251.

Shahbazkhani, B., Maghari, M., Nasseri-Moghaddam, S*., et al.* (2000). Prevalence of celiac disease among Iranian patients with chronic diarrhea. *J Paediatr Gastroenterol Nutr, 31*, S3.

Shan, L., Matthews, I., Khosla, C. (2005). Structural and mechanistic analysis of two prolyl endopeptidases: role of interdomain dynamics in catalysis and specificity. *Proc Nat Acad Sci, 102*, 3599-3604.

Shan, L., Molberg, O., Parrot, I*., et al.* (2002). Structural basis for gluten intolerance in celiac sprue. *Science, 297*, 2275-2279.

Shanahan, F. (2000). Nutrient Tasting and signaling mechanisms in the gut v. mechanisms of immunologic sensation of intestinal contents. *Am J Physiol Gastrointest Liver Physiol, 278*, G191-196.

Shanahan, F. (2002). The host-microbe interface within the gut. *Best Practice & Research Clinical Gastroenterol, 16* (6), 915-931.

Shane, E. (1996). Osteoporosis and Osteomalacia in Patients with Celiac Disease. In M. Jones, B. Elkus & J. Lyles (Eds.), *Metabolic Bone Disease Program*. NY: Columbia-Presbyterian Medical Center.

Shaw, S., Hermanowski-Vosatka, A., Shibahara, T.*, et al.* (1998). Migration of intestinal intraepithelial lymphocytes into a polarized epithelial monolayer. *Am J Physiol Gastroenterol Liver Physiol, 38*, G584-G591.

Shehata, M. (2005). Rel/Nuclear factor-Kappa B apoptosis pathways in human cervical cancer cells. *Cancer Cell International, 5* (10), 1-13 online at http://www.cancerci.com/content/15/11/10.

Sher, K., Mayberry, J. (1994). Female fertility, obsteric and gynaecological history in celiac disease: a case control study. *Digestion, 55*, 243-246.

Sher, L. (2000). Selenium and human health. *Lancet, 356*, 233-241.

Shewry, P., Tatham, A., Barro, F.*, et al.* (1995). Biotechnology of breadmaking: unravelling and manipulating the multi-protein gluten complex. *Biotechnology, 13*, 1185-1190.

Shina, A., Mon, S., Dawson, S.*, et al.* (2001). Use of temporal temperature gradient gel electrophoresis to identify flaA and fim3 sequence types in Bordetella bronchiseptica. *App Microbiol, 32*(6), 384–387.

Shiner, M. (1956). Duodenal biospy. *Lancet, (i)*, 17-19.

Shortman, K., Caux, C. (1997). Dendritic Cell Development: Multiple pathways to nature's adjuvants. *Stem Cells, 15* (6), 409-419.

Shortt, C., Madden, A., Fylnn, A.*, et al.* (1988). Influence of dietary sodium intake on urinary calcium excretion in selected Irsih individuals. *Eur J Clin Nutr, 42*, 595-603.

Siegel, S., Castellan, N. (1988). *Nonparametric Statistics for the Behavioural Sciences* (2 ed). New York: McGraw Hill.

Sjostrom, H., Lundin, K., Molberg, O.*, et al.* (1998). Identification of a gliadin T-cell epitope in celiac disease: general importance of gliadin deamidation for intestinal T cell recognition. *Scand J Immunol, 48* (2), 111-115.

Skerritt, J., Hill, A. (1991). Enzyme immunoassay for determination of gluten in foods: collaborative study. *J Assoc Off Anal Chem, 74* (2), 257-264.

Skerritt, J., Tatham, A. (1992). Chapter 12: Cereal seed storage proteins. In M. Van Regenmortel (Ed.), *Structure of Antigens* (pp. 350-377): CRC Press.

Sloan-Lancaster, J., Allen, P. (1996). Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Ann Rev Immunol, 14*, 1-27.

Smith, E., Macfarlane, G. (1997). Dissimilatory amino acid metabolism in human colonic metabolism. *Anaerobe, 3*, 327-337.

Smits, H., van Beelen, A., Hessle, C.*, et al.* (2004). Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. *Eur J Immunol, 34* (5), 1371-1380.

Snoeck, V., Goddeeris, B., Cox, E. (2005). The role of enterocytes in the intestinal barrier function and antigen uptake. *Micro Infect, 7*, 995-1004.

Sokal, R., Rohlf, F. (1995). *Biometry* (3 ed). New York: Freeman.

Sollid, L., Gray, G. (2004). A role for bacteria in celiac disease. *Am J Gastroenterol, 99* (5), 905-906.

Sollid, L., Jabri, B. (2005). Is celiac disease an autoimmune disorder? *Curr Opin Immunol, 17*, 595-600.

Sollid, L., Lie B. (2005). Celiac disease genetics:current concepts and practical applications. *Clin Gastroenterol Hepatol, 3*, 843-851.

Sollid, L., Markussen, G., Ek, J.*, et al.* (1989). Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med, 169*, 345-350.

Sollid, L., Thorsby, E. (1993). HLA susceptibility genes in coeliac disease: genetic mapping and role in pathogenesis. *Gastroenterol, 105*, 910-922.

Solomons, N., Rosenberg, I., Sandstead, H. (1976). Zinc nutrition in celiac sprue. *Am J Clin Nutr, 29* (4), 371-375.

Spaenij-Dekking, E., Kooy-Winkelaar, E., Nieuwenhuizen, W.*, et al.* (2004). A novel and sensitive method for the detection of T cell stimulatory epitopes of alpha/beta and gamma gliadin. *Gut, 53*, 1267-1273.

Spaenij-Dekking, E., Kooy-Winkelaar, Y., van Veelan, P.*, et al.* (2005). Natural variation in toxicity of wheat: potential for selection of non toxic varieties for celiac disease patients. *Gastroenterology, 129*, 797-806.

Spiekermann, G., Walker, W. (2001). Oral Tolerance and Its Role in Clinical Disease. *J Ped Gastro Nutr, 32* (3), 237-255.

Srinivasan, U., Jones, E., Carolan, J.*, et al.* (2006). Immunohistochemical analysis of coeliac mucosa following ingestion of oats. *Clin Exp Immunol, 144*, 197-203.

Stagg, A. (2002). Chapter 6: Modification of host cell function by normal flora- a molecular perspective. In A. Hart, Stagg, A, Graffner, H et al (Ed.), *Gut Ecology*. London: Martin Dunitz Ltd.

Stagg, A., Hart, A., Knight, S.*, et al.* (2004). Interactions between dendritic cells and bacteria in the regulation of intestinal immunity. *Best Prac Research Clin Gastroenterol, 18* (2), 255-270.

Stagg, A., Kamm, M., Knight, S. (2002). Intestinal dendritic cells increase T cell expression of alpha-4 beta-7 integrin. *J Exp Med, 332*, 1445.

Stanford. (2002). Using spectrophotometer to quantitate DNA and RNA: http://www.stanford.edu/~teruel1/Protocols/pdf/Using%20spectrophotometer%20to%20quantitate%20DNA%20and%20RNA.pdf.

Starr, C. (2005). Unit VII; Principles of Ecology; Chapter 40- Community Structure and Biodiversity: Section 40.3 "Competitive Interactions". In *Biology: Concepts and Applications*: Thomson Brooks/Cole.

Stebbings, S., Munro, K., Tannock, G.*, et al.* (2002). Comparison of the faecal microflora of patients with ankylosing spondylitis and controls using molecular methods of analysis. *Rheumatol, 41*, 1395-1401.

Steinhoff, U. (2005). Who controls the crowd? New findings and old questions about the intestinal microflora. *Immunol Lett, 99*(1), 12-16.

Stenhammar, C., Fallstrom, S., Jansson, G.*, et al.* (1986). Celiac disease in children of short stature without gastrointestinal symptoms. *Eur J Pediatr, 145*, 185.

Stepniak, D., Spaenij-Dekking, L., Mitea, C.*, et al.* (2006). Highly efficient gluten degradation with newly identified prolyl endopeptidase: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol, 291*, G621-629.

Stevens, D. (1979). Nutritional anaemia in childhood coeliac disease [abstract]. *Proc Nutr Soc, 38*, 102A.

Still, G. (1918). The Lumleian lectures on coeliac disease. *Lancet, ii*, 163-166, 193-167, 227-169.

Storsrud, S., Hulthen, L., Lenner, R. (2003). Beneficial effects of oats in the gluten-free diet of adults with special reference to nutrient status, symptoms and subjective experiences. *Br J Nutr, 90* (1), 101-107.

Strachan, D. (2000). Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax, 55*(Suppl 1), S2 -10.

Strobel, S., Mowat, A. (1998). Immune responses to dietary antigens:oral tolerance. *Immunol Today, 19*, 173-181.

Strober, W., Fuss, I., Blumberg, R. (2002). The immunology of mucosal models of inflammation. *Ann Rev Immunol, 20*, 495-549.

Suenaert, P., Bulteel, V., Lemmens, L.*, et al.* (2002). Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *The American Journal of Gastroenterology, 97*(8), 2000-2004.

Sulkanen, S., Halttunen, T., Laurila, K.*, et al.* (1998). Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterol, 115* (6), 1322-1328.

Sumner-Smith, M., Rafalski, J., Sugiyama, T.*, et al.* (1985). Conservation and variability
of wheat alpha/beta gliadin genes. *Nucleic Acids Res, 13*(4), 3905-3916.

Sumnik, Z., Cinek, O., Bratanic, N.*, et al.* (2006). Risk of celiac disease in children with type 1 diabetes is modified by positivity for HLA-DQB1*02-DQA1*05 and TNF -308A. *Diabetes Care, 29*, 858-863.

Suskovic, J., Kos, B., Goreta, J.*, et al.* (2001). Role of Lactic Acid Bacteria and Bifidobacteria in Synbiotic Effect. *Food Tech Biotech, 39* (3), 227-235.

Swartz-Basile, D., Wang, L., Tang, Y.*, et al.* (2003). Vitamin A deficiency inhibits intestinal adaptation by modulating apoptosis, proliferation, and enterocyte migration. *Am J Physiol Gastrointest Liver Physiol 285: G424-G432*.

Swidsinski, A., Ladhoff, A., Pernthaler, A.*, et al.* (2002). Mucosal flora in inflammatory bowel disease. *Gastroenterol, 122*, 44-54.

Swinson, C., Levi, A. (1980). Is coeliac disease underdiagnosed? *BMJ, 281*, 1258-1260.

Szatmari, I., Gogolak, P., Im, J.*, et al.* (2004). Activation of PPAR gamma specifies a dendritic cell subtype capable of enhanced induction of iNKT cell expansion. *Immunity, 21* (1), 95-106.

Tagkalidis, P., Gibson, P., Bhathal, P. (2007). Microscopic colitis demonstartes a TH1 mucosal cytokine profile. *J Clin Pathol, 60*(4), 382-387.

Talley, N., Valdovinos, M., Petterson, T.*, et al.* (1994). Epidemiology of celiac sprue: a community-based study. *Am J Gastroenterol, 89*, 843-846.

Tanaka, Y., Imai, T., Baba, M.*, et al.* (1999). Selective expression of liver and activation-regulated chemokine (LARC) in intestinal epithelium in mice and humans. *Eur J Immunol, 29* (2), 633-642.

Tannock, G. (2003). Chapter 1: The Intestinal Microflora. In P. Fuller R, G (Ed.), *Gut Flora, Nutrition, Immunity and Health* (pp. 1-15). Oxford: Blackwell Publishing.

Tannock, G. (2007). What immunologists should know about bacterial communities of the human bowel. *Sem Immunol, 19*(94-105).

Tannock, G., Munro, H., Harmsen, G.*, et al.* (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. *Appl Environ Microbiol, 66*, 2578-2588.

Tanure, M., Silva, I., Bahia, M.*, et al.* (2006). Prevalence of celiac disease in Brazilian children with type 1 diabetes mellitus. *J Pediatr Gastroenterol Nutr, 42*, 155-159.

Taylor, K., Truelove, S., Thompson, D.*, et al.* (1961). An immunological study of coeliac disease and idiopathic steatorrhoea; serological reactions to gluten and milk proteins. *BMJ, 2*, 1727-1731.

Tesei, N., Sugai, E., Vazquez, H.*, et al.* (2003). Antibodies to human recombinant tissue transglutaminase may detect coeliac disease patients undiagnosed by endomysial antibodies. *Aliment Pharm Ther, 17* (11), 1415-1423.

Thompson, C., Colls, A. (1998). *Twenty four hour urinary sodium excretion in 700 residents of Otago and Waikato. Report prepared for the Ministry of Health*. Dunedin: Dept Human Nutrition, University of Otago.

Thompson, T. (2003). Oats and the gluten-free diet. *J Am Diet Assoc, 103*, 376-379.

Thompson-Chagoyan, O., Maldonado, J., Gil, A. (2005). Aetiology of inflammatory bowel disease(IBD): role of intestinal microbiota and gut-associated lymphoid tissue immune response. *Clin Nutr, 24* (3), 339-352.

Tjellstrom, B., Stenhammar, L., Hogberg, L.*, et al.* (2005). Gut microflora associated characterisitics in children with celiac disease. *Am J Gastroenterol, 100*, 2784-2788.

Tlaskalova-Hogenova, H., Tuckova, L., Lodinova-Zadnikova, R.*, et al.* (2002). Mucosal immunity: its role in defense and allergy. *Int Arch Allergy Immunol, 128* (2), 77-89.

Toivanen, P., Vaahtovuo, J., Eerola, E. (2001). Influence of Major Histocompatibility Complex on Bacterial Composition of Fecal Flora. *Infect Immun, 69* (4), 2372-2377.

Trevisiol, C., Not, T., Berti, I.*, et al.* (1999). Screening for coeliac disease in healthy blood donors at two immuno-transfusion centres in north-east Italy. *Ital J Gastro Hepatol, 31* (7), 584-586.

Troncone, R., Ferguson, A. (1991). Anti-gliadin antibodies. *J Pediatr Gastroenterol Nutr, 12*, 150-158.

Troncone, R., Gianfrani, G., Mazzarella, L.*, et al.* (1998). The majority of gliadin specific T cell clones from the celiac small intestinal mucosa produce both gamma interferon and IL-4. *Dig. Dis Sci, 43*, 156-162.

Tschopp, J., Martinon, F., Burns, K. (2003). Nalps: A novel protein family involved in inflammation. *Nat Rev Mol Cell Bio, 4*, 95-104.

Tuohy, K., Gibson, G. (2006). Chapter 6; Functions of the Human Intestinal Flora: The use of Probiotics and Prebiotics. In A. Crozier, Clifford, M, Ashihara, H (Ed.), *Plant Secondary Metabolites: occurence, structure and role in the human diet* (pp. 174): Blackwell Publishing.

Turnbaugh, P., Ley, R., Mahowald, M.*, et al.* (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature, 444* (7122), 1027-1031.

Tursi, A., Brandimarte, G., Giorgetti, G. (2003). High prevalence of small intestinal bacterial overgrowth in celiac patients with persistence of gastrointestinal symptoms after gluten withdrawal. *Am J Gastroenterol, 98* (4), 839-843.

Tuthill, D., Hawkes, N., Jenkins, H. (1999). The rise of coeliac disease following serological testing. *Arch Dis Child, 80*, 22.

Tye Din, J. (2005). Report from Digestive Disease Week. *Coeliac Solution, 2* (1).

Uhlig, H., McKenzie, B., Hue, S.*, et al.* (2006). Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity, 25* (2), 309-318.

Uhlig, H., Powrie, F. (2003). Dendritic cells and the intestinal bacterial flora: a role for localized mucosal immune responses. *J Clin Invest, 112*.

Unknown. (2003). Codex Alimentarius Commission; Report of the 25th session of the Codex Committee on nutrition and foods for special dietary uses. *Alinorm, Online @ www.codexalimentarius.net*.

Unknown. (2005). The normal gut flora. *Glasgow University; Available online through web archive*.

Unsworth, D., Lock, R., Harvey, R. (2000). Improving the diagnosis of coeliac disease in anaemic women. *Br J Haematol, 111*(3), 898-901.

Vaahtovuo, M., Korkeamäki, E., Munukka, M.*, et al.* (2005). Quantification of bacteria in human feces using 16S rRNA-hybridization. DNA-staining and flow cytometry. *J Microbiol Method, 63*, 276–286.

Vader, W., de Ru, A., van der Wal, Y.*, et al.* (2002). Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J Exp Med, 195* (5), 643-649.

Vader, W., Kooy, Y., Van Veelen, P.*, et al.* (2002). The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterol, 122*, 1729-1737.

Vader, W., Stepniak, D., Kooy, Y.*, et al.* (2003). The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. *Proc Natl Acad Sci, 100* (21), 12390-12395.

Valdimarsson, T., Franzen, L., Grodzinsky, E.*, et al.* (1996). Is small bowel biopsy necessary in adults with suspected celiac disease and IgA anti-endomysium antibodies? *Dig Dis Sci, 41*, 83-87.

Valdimarsson, T., Lofman, O, Toss, G et al. (1996). Reversal of osteopenia with diet in adult coeliac disease. *Gut, 38* (3), 322-327.

Valentini, R., Andreani, M., Corazza, G.*, et al.* (1994). IgA endomysium antibody: a valuable tool in the screening of coeliac disease but not its follow-up. *Ital J Gastroenterol, 26* (6), 279-282.

van Bodegraven, A., Curley, C., Hunt, K., *et al*. (2006). Genetic variation in myosin IXB is associated with ulcerative colitis. *Gastroenterol, 131* (6), 1768-74.

Van de Wal, Y., Kooy, Y., Van Veelen, P.*, et al.* (1998). Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc Natl Acad Sci, 95* (17), 10050-10054.

van Heel, D., Franke, L, Hunt, K., *et al*. (2007). A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet, 39*, 827-829.

van Heel, D., Hunt, K., Greco, L.*, et al.* (2005). Genetics in coeliac disease. *Best Pract Res Clin Gastroenterol, 19*, 323-339.

van Heel, D., West J. (2006). Recent advances in coeliac disease. *Gut, 55*, 1037-1046.

Van Mook, W., Bourass-Bremer, I., Bos, L.*, et al.* (2001). The outcome of esophago-gastroduodenoscopy (EGD) in asymptomatic outpatients with iron deficiency anemia after a negative colonoscopy. *Eur J Intern Med, 12* (2),122-6.).

Van Niel, G., Heyman, M. (2002). The epithelial cell cytoskeleton and intracellular trafficking II. Intesinal epithelial cell exosomes perspectives on their structure and function. *Am J Gastrointest Liver Physiol, 283* (2), G251-255.

Van Niel, G., Raposo, G., Hershberg, R.*, et al.* (2001). Intestinal epithelial cells secrete exosome like vesicles. *Gastroenterol, 121*, 337-349.

Vanhoutte, T., Huys, G., De Brandt, E.*, et al.* (2004). Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group specific 16S rRNA gene primers. *FEMS Microbiol Ecol, 48*, 437-446.

Varona, R., Villares, R., Carramolino, L.*, et al.* (2001). CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J Clin Invest, 107*, R37-R45.

Vaughan, E., Schut, F., Heilig, G. (2000). A molecular view of the intestinal ecosystem. *Curr Issues Intest Microbiol, 1*, 1-12.

Vaynshtein, G., Rosenbaum, H., Groisman, G.*, et al.* (2004). Celiac sprue presenting as severe hemorrhagic diathesis due to vitamin K deficiency. *J Isr Med Assn, 6*, 781-783.

Vazquez-Torres, A., Jones-Carson, J., Baumler, A.*, et al.* (1999). Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. *Nature, 401* (6755), 804-808.

Vedantam, G., Hecht, D. (2003). Antibiotics and anaerobes of gut origin. *Current Opinion in Microbiology, 6*(5), 457-461.

Veltkamp, C., Tonkonogy, S., De Jong, Y.*, et al.* (2001). Continuous stimulation by normal luminal bacteria is essential for the development and perpetuation of colitis in Tg (epsilon26) mice. *Gastroenterol, 120* (4), 900-913.

Ventura, A., Maguzzi, G., Greco, L.*, et al.* (1999). Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. *Gastroenterol, 117*, 297-303.

Vickerstaff Joneja, J. (2003). *Dealing with Food Allergies; A practical guide to detecting culprit foods and eating a healthy, enjoyable diet*. Boulder, CO: Bull Publishing Co.

Villadangos, J., Schnorrer, P., Wilson, N. (2005). Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol Review, 207*, 191-205.

Vitoria, J., Arrieta, A., Ortiz, L.*, et al.* (2001). Antibodies to human tissue transglutaminase for the diagnosis of celiac disease. *J Pediatr Gastro Nutr, 33* (3), 349-350.

Vladutiu, A. (2000). Immunoglobulin D: Properties, measurement, and clinical relevance. *Clin Diagnos Lab Immunol, 7* (2), 131-140.

Vogelsang, H., Genser, D., Wyatt, J.*, et al.* (1995). Screening for celiac disease: a prospective study on the value of noninvasive tests. *Am J Gastroenterol, 90* (3), *394-8.*

Volta, U., Bellentani, S, Bianchi F., *et al*. (2001). High prevalence of celiac disease in Italian general population. *Dig Dis Sci, 46* (7), 1500-1505.

Volta, U., Molinaro, N., De Franceschi, L.*, et al.* (1995). IgA anti-endomysial antibodies on human umbilical cord tissue for celiac disease screening. Save both money and monkeys. *Dig Dis Sci, 40* (9), 1902-1905.

Vora, P., Youdin, A., Thomas, L.*, et al.* (2004). Beta-defensin expression is regulated by TLR signalling in intestinal epithelial cells. *J Immunol, 173*, 5398-5405.

Wain, H., Bruford, E., Lovering, R.*, et al.* (2002). Guidelines for human gene nomenclature. *Genetics, 79* (4), 464-470.

Walls, R. (1997). *Allergies and their management*. Sydney, Australia: MacLennan & Petty Pty Ltd.

Walter, J., Tannock, G., Tilsala-Timisjarvi, A.*, et al.* (2000). Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species specific PCR primers. *Appl Environ Microbiol, 66*, 297-303.

Wang, J., Johnson, L. (1992). Role of transglutaminase and protein cross-linking in the repair of mucosal stress erosions. *Am J Physiol, 262,* 19-25.

Wang, W., Uzzau, S., Goldblum, S.*, et al.* (2000). Human Zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci, 113*, 4435-4440.

Washington, H. (1984). Diversity, biotic and similarity indices: a review with special relevance to aquatic ecosystems. *Water Research, 18*, 653-694.

Wehkamp, J., Salzman, N., Porter, E.*, et al.* (2005). Reduced Paneth cell alpha defensins in ileal Crohn's disease. *Proc Nat Acad Sci, 102* (50), 18129-18134.

Weijers, H., van de Kamer, J. (1965). Some considerations of celiac disease. *Am J Clin Nutr, 17* (1), 51-54.

Wein, M., Sterbinsky, S., Bickel, C.*, et al.* (1995). Comparison of human eosinophil and neutrophil ligands for P-selectin: ligands for P-selectin differ from those for E-selectin. *Am J Respir Cell Mol Biol, 12* (3), 315-319.

Westberg, L., Baghaei, F., Rosmond, R.*, et al.* (2001). Polymorphisms of the androgen receptor gene and the estrogen receptor ß gene are associated with androgen levels in women. *J Clin Endocrinol Metab, 86* (6), 2562-2568.

Wexler, H. (2007). *Bacteroides*: the Good, the Bad and the Nitty-Gritty. *Clin Microbiol Rev, 20* (4), 593-621.

Whelan, A., Willoughby, R., Weir, D. (1996). Human umbilical vein endothelial cells: a new easily available source of endomysial antigens. *Eur J Gastro Hepat, 8* (10), 961-966.

Wiedermann, U. (2003). Mucosal immunity - mucosal tolerance. A strategy for treatment of allergic diseases. *Chem Immunol Allergy, 82*, 11-24.

Wiese, U., Wulfert, M., Prusiner, S.*, et al.* (1995). Scanning for mutations in the human prion protein open reading frame by temporal temperature gradient gel electrophoresis. *Electrophoresis, 16* (10), 1851-1860.

Wilcox, G., Mattia, A. (2006). Celiac sprue, hyperhomocysteinemia, and MTHFR gene
variants. *J Clin Gastroenterol, 40* (7), 596-601.

Wilkinson, L. (1990). *Systat: the system for statistics*. Evanston, IL: Systat Inc.

Williams, C. (1997). Celiac disease: Past, present and future. *Can J Gastroenterol, 11* (8), 647-649.

Wilson, M. (2002). *Bacterial Adhesion to Host Tissues; Mechanisms and Consequences* (Vol. 1). London: University College Press.

Wilson, M. (2005). Chapter 1*;* An introduction to the human-microbe symbiosis. In *Microbial Inhabitants of Humans* Cambridge: Cambridge University Press.

Wilson, M. (2005). Chapter 7; The indigenous microbiota of the GI tract. In *Microbial Inhabitants of Humans.* Cambridge: Cambridge University Press.

Woese, C. (1987). Bacterial evolution. *Microbiol Rev, 51* (2), 221-271.

Wolters, V., Vooijs-Moulaert, A., Burger, H.*, et al.* (2002). Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both the guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease. *Eur J Pediatr, 161* (5), 284-287.

Wood, I., Doig, R., Motteram, R.*, et al.* (1949). Gastric biospy. *Lancet, (i)*, 18-21.

Wright, T. (2001). *Food Allergies; enjoying life with a severe food allergy*. London: Class Publishing.

Wynne, A., McCartney, A., Brostoff, J.*, et al.* (2004). An *in vitro* assessment of the effects of broad-spectrum antibiotics on the human gut microflora and concomitant isolation of a Lactobacillus plantarum with anti-Candida activities. *Anaerobe, 10* (3), 165-169.

Xian, C., Mardell, C., Read , L. (1999). Specificity of the localization of transforming growth factor-alpha immunoreactivity in colon mucosa. *J Histochem Cytochem, 47*, 949-958.

Xu, J., Bjursell, M., Himrod, J.*, et al.* (2003). A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science, 299*, 2074-2076.

Yachha, S., Mohindra, S., Srivastava, A.*, et al.* (2000). Effects of a gluten-free diet on growth and small bowel histology in children with celiac disease in India. *J Paediatr Gastroenterol Nutr, 31*, S23.

Yamaguchi, Y., Kato, K., Shindo, M.*, et al.* (1998). Dynamics of the carbohydrate chains attached to the FC portion of immunoglobulin G as studied by NMR spectroscopy assisted by selective 13C labeling of the glycans. *J Biomolecular NMR, 12* (3), 385-394.

Yoshino, K., Nishigaki, K., Husimi, Y. (1991). Temperature sweep gel electrophoresis: a simple method to detect point mutations. *Nucleic Acids Res, 19* (11), 3153.

Yuan, Q., Walker, W. (2004). Innate immunity of the gut: mucosal defense in health and disease. *J Pediatr Gastroenterol Nutr, 38*, 463-473.

Yüce, A., Demir, H., Temizel, I.*, et al.* (2004). Serum carnitine and selenium levels in children with celiac disease. *Indian J Gastroenterol, 23* (3), 87-88.

Zarkadas, M., Case, S. (2005). Celiac disease and the gluten-free diet; an overview. *Top Clin Nutr, 20* (2), 127-138.

Zenclussen, A., Schmacher, A., Zenclussen, M.*, et al.* (2007). Immunology of pregnancy: cellular mechansims allowing fetal survival within the maternal uterus. *Exp Rev Mol Med, 9* (10), 1-14.

Zhou, L., Nazarian, A., Smale, S. (2004). Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. *Mol Cell Bio, 24* (6), 2385-2396.

Zimmer, K., Naim, H., Weber, P.*, et al.* (1998). Targeting of gliadin peptides, CD8, alpha/beta-TCR and gamma/delta-TCR to Golgi complexes and vacuoles within celiac disease enterocytes. *Fed Am Soc Exp Bio, 12*, 1349-1357.

Zoetendal, E., Akkermans, W., De Vos, M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host specific communities of active bacteria. *Appl Environ Microbiol, 64*, 3854-3859.

Zoetendal, E., Akkermans, A, Akkermans van-Vliet, W., *et al*. (2001). The host genotype affects the bacterial community in the human gastrointestinal tract. *Micro Ecol Health Dis, 13*, 129-134.

Zoetendal, E., Cheng, B, Koike, S., *et al*. (2004). Molecular ecological analysis of the gastrointestinal microbiota. *J Nutr, 134*, 465-472.

Chapter Thirteen: APPENDICES

Appendix A; Glossary of terms

Absorptive enterocytes

Represent the majority (80%) of IECs. They facilitate absorption, perform a passive barrier function and are actively involved in immune processes (Abreu *et al*., 2005).

Adenosine triphosphate (ATP)

Adenosine triphosphate is the major unit of energy used in all living cells. It is not energy itself, but rather it temporarily "stores" energy in its phosphor-diester bonds (the bonds between the phosphate groups of ATP). When these bonds are broken, they release energy that fuels chemical reactions, and creates ADP (which has one less phosphate group). ATP is produced in the cell's organelles called mitochondria (Fort 2007).

Adverse reactions

These include any untoward effects from ingestion of foods which may arise by whatever mechanism. They include food intolerances and food allergy (Walls 1997).

Anaphylaxis

This is the clinical manifestation of an IgE-mediated response to allergen, expressed as a generalized reaction affecting primarily respiratory and cardiovascular systems which if untreated results in shock and respiratory failure (Walls 1997).

Allergen

This is the term used to describe anything that triggers the immune system to respond with an allergic reaction (Vickerstaff-Joneja 2003).

Allochthonous

Species that live in the gut lumen. They are transient species that are foreign to the environment in which they are found and were formed in a place other than where they were reside. They may be ingested micro-organisms, or they may

be translocated from another area. Allochthonous species do not usually adhere to the gut wall so they are more easily flushed from the digestive tract. They are however, better able to withstand the gastric acidity of the bile shower (Bibiloni *et al*., 2004).

Autochthonous

Species that establish and colonise the gut by adhering to the gut wall. They reproduce *in situ*, and are mostly found where they form. When attached and replicating on the intestinal surface, microbes are able to persist in a flowing habitat, whereas non-adherent microbes are more likely to be flushed away by the flow of intestinal secretions (Biblioni *et al*., 2004).

Anergy

This describes an unresponsive state of immune cells to antigen stimulation. It is induced when a T- cell's antigen receptor is stimulated, which inhibits T-cell responses pending a "second signal" from the antigen-presenting cell (APC). The delivery of the second signal by the APC rescues the activated T cell from anergy, allowing it to produce the cytokines necessary for the growth of additional T-cells (Janeway *et al*., 2005).

Antibody

This is a protein molecule made by the body to help fight disease causing bacteria and viruses. The antibody binds to a specific target called an antigen. Their presence rouses immune cells that attack the pathogen. What goes wrong in allergies is that the body makes the antibody IgE in response to harmless antigen. IgE is usually found on the surface of mast cells. If the IgE molecules on the surface of mast cells bind to their specific antigen, they stimulate the mast cell to release chemical messengers. Such messengers normally organise an effective immune response but they can also cause the damaging symptoms of allergy (Brostoff *et al*., 2000).

Antigen

Any substance that provokes the body to produce antibodies against it. The major groups of antigen are proteins (Brostoff *et al*., 2000).

***Antigen-presenting cells (APCs*)**

These are cells which digest antigen and display antigen fragments on their own surface (Linnemeyer *et al*., 1993).

Atopy

The term used to define clinical manifestations of allergy (Walls 1997).

Avidity

Is a term used to describe the combined strength of multiple bond interactions in proteins. Avidity is distinct from affinity, which is a term used to describe the strength of a single bond. As such, avidity is the combined syngeristic strength of bond affinities rather than the sum of bonds. It is commonly applied to antibody interaction, where multiple, weak, non-covalent bonds form between antigen and antibody. Individually, each bond is quite readily broken, however when many are present at the same time the overall effect results in synergistic, strong binding of antigen to antibody (e.g. IgM is said to have low affinity but high avidity because it has 10 weak binding sites as opposed to the 2 strong binding sites of IgG, IgE, and IgD) (Delves *et al*., 2006).

Basophils

These are white blood cells originating from stem cells in bone marrow. Their granules release preformed mediators and produce the lipid mediators called leukotrienes, prostaglandins and platelet-activating factor. They produce cytokines and influence mast cell and eosinophil function. Basophils are present between the epithelial cells and in the mucous overlying epithelial surfaces and are the first cells that the antigen come in contact with (Vickerstaff-Joneja 2003).

B-cells

These are lymphocytes that mature into plasma cells and produce antibodies (Benjamini *et al*., 2000).

Cellular Adhesion molecules (CAMs)

These are proteins located on the cell surface that bind with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. These proteins are transmembrane receptors made up of three domains; an intracellular domain that interacts with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts either with other CAMs of the same kind (homophilic binding) or with other CAMs or the extracellular matrix (heterophilic binding) CAMs *include* **IgCAMs, Integrins, Cadherins and Selectins** (Wein *et al*., 1995).

Immunoglobulin CAMs **(IgCAMs)** are either homophilic or heterophilic and bind integrins or different IgCAMs. This group of proteins includes neural cell adhesion molecules (NCAMs), intercellular cell adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), platelet-endothelial cell adhesion molecule (PECAM-1), L1CAM, CHL1, myelin-associated glycoprotein (MAG), plus nectins and nectin like molecules (Wein *et al*., 1995).

Integrins are cell surface receptors that interact with the ECM and mediate various intracellular signals. They define cellular shape, mobility, and regulate the cell lifecycle. These intregral membrane proteins are attached to the cellular plasma membrane through a single transmembrane helix. Integrin is involved in the attachment of cells to other cells, and also plays a role in the attachment of a cell to the extracellular matrix. Besides the attachment role, integrin also has a role in signal transduction, a process by which a cell transforms one kind of signal or stimulus into another. The integrins are unusual membrane proteins because the signals they convert travel in both outside-in: transducing information from the ECM to the cell, and inside-out: "revealing" the status of the cell to the extracellular world. This allows cells to make rapid and flexible responses. It is more common for cells to make new receptors on their surfaces, or remove them if they need to alter their ability to respond to the environment. There are many types of integrin, and many cells have multiple types on their surface.

Integrins are vitally important in humans and have been extensively studied (Wein *et al*., 1995).

Cadherins are a class of type-1 transmembrane proteins. They play important roles in cellular adhesion, ensuring that cells within tissues are bound together. They are dependent on calcuim ions (Ca^{2+}) to function, hence their name. The most important members of this family are E-cadherins (epithelial), P-cadherins (placental) and N-cadherins (neural).The cadherin superfamily includes cadherins, protocadherins, desmogleins, and desmocollins, and more. In structure, they share *cadherin repeats*, which are the extracellular Ca^{2+}-binding domains. There are multiple classes of cadherin molecule, each designated with a one-letter prefix (generally noting the type of tissue with which it is associated). Cadherins within one class will bind only to themselves. For example, an N-cadherin will bind only to another N-cadherin molecule. Because of this specificity, groups of cells that express the same type of cadherin molecule tend to cluster together during develpoment, whereas cells expressing different types of cadherin molecules tend to separate (Wein *et al*., 1995).

The ***selectins*** are a family of heterophilic CAMs that bind fucosylated carbohydrates, e.g. mucins. They are calcium-dependent. The three family members are E-selectin (endothelial), L-selectin (leukocyte) and P-selectin (platelet). The best-characterised ligand for the three selectins is P-selectin glycoprotein ligand-1 (PSGL-1), which is a mucin-type glycoprotein expressed on all white blood cells. Neutrophils and eosinophils bind to E-selectin. One of the reported ligands for E-selectin is the sialylated Lewis X Ag (sLe(x)). Eosinophils, like neutrophils, use sialylated, protease-resistant structures to bind to E-selectin, although the eosinophil expresses much lower levels of these structures on its surface. Ligands for P-selectin on eosinophils and neutrophils are similar sialylated, protease-sensitive, endo-beta-galactosidase-resistant structures, clearly different than those reported for E-selectin, and suggest disparate roles for P-selectin and E-selectin during recruitment during inflammatory responses (Wein *et al*., 1995)

Colonocytes

These are intestinal epithelial cells of the colon (Kemp *et al*., 2003).

Commensal microflora

These are symbiotic organisms resident in the gut, which help maintain mucosal integrity, by participating in a network of lympho-epithelial and bacterial signaling and by providing a conditioning effect. These intestinal bacteria form a protective layer against pathogens, referred to as the 'mucosal barrier effect', as well as inducing and maintaining oral tolerance (Isolauri *et al*., 2001).

Cytokines

Are chemical messengers which are specific to the type of T-cell. They are dependent on the nature of the antigen and the way it is presented by the antigen presenting cells (APC) (Wright 2001).

Defensins

Are antimicrobial peptides (AMPs) that actively destroy bacteria, fungi, and viruses by either inducing lethal membrane damage or binding to cytoplasmic targets of bacteria. This effectively inhibits enzymatic activity, and reduces the synthesis of the bacterial cell wall, nucleic acid and protein. α-defensins are produced inside neutrophils and paneth cells whereas β-defensins are common in epithelial cells. These two types of defensins differ in size and cysteine motifs. Some are able to attract monocytes and T-cells by chemotaxis. The expression of defensins also appears to be regulated by nucleotide oligomerisation domain (NOD2) proteins (Cobrin *et al*., 2005).

Dendrites

These are long branched fingerlike projections on dendritic cells that can insinuate between the tight junctions of enterocytes to project in to the lumen of the gut and sample gut contents (Roux *et al*., 2003).

***Dendritic cells (DCs*)**

Are immune cells (APCs). Their main function is to process antigenic material and present it to other cells of the immune system. DCs are present in small quantities in tissues that are in contact with the external environment such as the skin, nose, lungs, stomach and intestines. They can also be found in an immature state in the blood. Once activated, they migrate to the lymphoid tissues where they interact with T-cells and B-cells to initiate and shape an adaptive immune response (Snoeck *et al*., 2005).

Dietary lectins

Are carbohydrate-binding proteins found in seeds and tubers, like cereals, potatoes and beans. Lectins have toxic and inflammatory properties, they are resistant to cooking and digestive enzymes and are in most food (Freed 1999).

Dysbiotic

Is the term used to describe a deleterious alteration in the balance of microflora within the intestine (Hawrelak *et al*., 2004).

Endocytosis

Is a process where cells absorb material (molecules such as proteins) from the outside by engulfing it within their cell membrane. It is used by all cells of the body as most cells require large polar molecules that cannot pass through the hydrophobic plasma or cell membrane.

There are three types of endocytosis: namely, macro-pinocytosis, caveolar endocytosis, and clathrin-mediated endocytosis. The absorption of material from the outside environment of the cell is commonly occurs in two phases: phagocytosis and pinocytosis (Snoeck *et al*., 2005).

Enterocytes

(Also called intestinal absorptive cells) are the predominant cells in the small intestinal mucosa but can also be found in the large intestine. They are tall simple columnar epithelial cells that are responsible for the final digestion and absorption of nutrients, electrolytes and water. The luminal surface is covered by a glycocalyx which contains digestive enzymes. Microvilli on the apical

surface increase the surface area for digestion and transport of molecules from the intestinal lumen. These cells also have a secretory role (Ross *et al*., 2003).

Enteroendocrines

Are the endocrine producing cells which export peptide hormones Snoeck *et al*., 2005).

Eosinophils

Come from bone marrow precursors and their production and activation is dependent on cytokines produced by T-cells and mast cells. They are attracted to sites of allergic inflammation, where their release of highly potent enzymes leads to denudation of mucosal cells from the basement membrane and inflammation (Vickerstaff-Joneja 2003).

Epitope

A structure on the surface of an antigen that an antibody recognises and attaches it self to (Brostoff *et al*., 2000).

Exosomes

Are small membrane vesicles produced and released by epithelial cells, believed to play a role in the induction of unresponsiveness (Didierlaurent *et al*., 2002).

FC portion

This forms part of the structure of immunoglobulin G. Immunoglobulin G consists of two distinct regions The Fab portion and the FC portion. The Fab portion carries the recognition site for antigenic determinants, whereas the FC portion promotes effector functions through interactions with the complement system or cellular receptors. The FC portion which is composed of two domains possesses one conserved glycosylation site in each of these domains where complex oligosaccharides are expressed (Yamaguchi *et al*., 1998).

Follicle-associated epithelium

Is the epithelium that overlies mucosal lymphoid tissues, such as the Peyer's patches and the isolated lymphoid follicles in the intestine. Lymphoid tissues

induce the differentiation of normal intestinal epithelium into FAE, which is specialised in antigen capture and transport (Clark *et al*., 2003).

Food allergy

This is an abnormal reaction resulting from heightened immunologic responses to glycoprotein components within foods (called allergens). Two types exist: IgE-mediated immediate reactions and delayed reactions to foods (Metcalfe *et al*., 2003).

Food intolerance

Is a reproducible adverse reaction to a specific component of food which is not psychologically mediated (Buttriss 2002).

Glycoprotein

a protein linked to a sugar (Vickerstaff-Joneja 2003).

Glycosylation

Is the enzymatic process that links saccharides to produce glycans, either free or attached to proteins and lipids (Forsberg *et al*., 2004).

Goblet cells

Are a type of secretory cell found in the epithelium of the intestinal tract which produce mucins (Snoeck *et al*., 2005).

Gut-associated lymphoid tissue (GALT)

Is immune system of the digestive tract. It consists of lymphoid tissue associated with the gut such as Peyer's patches, mesenteric lymph nodes and the appendix (Johansson-Lindbom *et al*., 2003).

Haptens

Are small sized food molecules that piggy back on a protein or polypeptide (Vickerstaff-Joneja 2003).

Heteroduplex

This is a double-stranded molecule of nucleic acid that originates from the genetic recombination of single complementary strands derived from different sources, (i.e. from different homologous chromosomes or even from different organisms). An example is the heteroduplex DNA strand formed in hybridisation processes, used for biochemical-based phylogenetic analyses (Westburg 2001).

Hypersensitivity

Is an increased immune response leading to clinical effects usually as a result of tissue damage. Various immunological mechanisms are involved in these reactions. Four types of processes are involved; immediate hypersensitivity (IgE); antibody-mediated (IgA and IgM); immune complex disease (antigen-antibody complement complex); delayed hypersensitivity cell mediated (T-cells and accessory cells) (Buttriss 2002).

Intestinal epithelial cells (IECs)

Are the cells of the intestinal epithelium. They provide a protective barrier against the entry of harmful substances in the form of secretory products (mucins and defensins). IECs include absorptive enterocytes, dendritic cells, M-cells, goblet cells, trefoil peptides enteroendocrines and paneth cells (Abreu *et al*., 2005).

In vitro

Refers to studies using cell cultures or extracts rather than whole organisms. It means “in glass" (McRae *et al*., 2000).

In vivo

Refers to studies performed using whole organisms i.e. "in the body" (Pulendran *et al*., 2001).

Immunological synapse

Is the specialised junction between a T-cell and an antigen-presenting cell (APC). The immunological synapse consists of a central cluster of T-cell

receptors surrounded by a ring of adhesion molecules. Immunological synapse formation is an active and dynamic mechanism that allows T-cells to distinguish potential antigenic ligands (Grakoui *et al*., 1999)

Lymphocytes

Are the intelligent cells of an immune response. They recognise foreign antigen and direct and modulate the resulting immune response. There are two types B and T-cells (Neutra *et al*., 2001).

Lysozyme

Is a glycosidase that works in concert with mucin. It catalyses the hydrolysis of complex polysaccharides that form bacterial cell walls, effectively destroying their structural integrity and killing the bacteria. Lysozyme aids in the elimination of foreign microbes and is particularly effective in antibacterial defense against gram-positive bacteria (Biology Online 2006).

Mast cells

Play a crucial role in allergic responses by producing a variety of potent biological substances that affect tissues and direct immune responses. They are one source of mediators for allergic reactions (Neutra *et al*., 2001).

M-cells

Are resident in the follicle-associated epithelium (FAE) that overlays Peyer's patches where antigen presenting cells (APCs) process and present antigen to naïve T-cells (Snoeck *et al*., 2005).

MICA and ***MICB***

Are stress proteins expressed on epithelial cells. IL-15 induces the expression of MICA (Meresse *et al*., 2006).

Mucins

Are glycoproteins with unique molecular structures, characteristic protein domains and tissue specific glycosylation. Mucins may be secreted or membrane-bound. In the colon, many mucin oligosaccharide chains are sulphated and are involved in binding trefoil factors that play a protective role

Mucins provide saccharides as a source of energy to intestinal microflora that adhere to mucous. Advances in the study of mucin-gene expression and mature mucin synthesis have increased our understanding of the role these proteins play in mucosal disease at sites throughout the GI tract. Recent studies have implicated membrane-bound mucins in cellular signaling, suggesting that they may be important as sensor mechanisms in response to the presence of pathogens or injury (Moal *et al*., 2006).

Nalps

Are a large subfamily of cytoplasmic proteins that have a vital role in human defense processes by activating inflammatory caspases. They contain an amino-terminal pyrin domain (PYD) and differ from TLRs in that they have no toll/interleukin receptor (TIR) (Tschopp *et al*., 2003).

Neutrophils

Come from bone marrow precursors and are attracted to sites of allergic inflammation. They add to the inflammatory process by release of cytolytic enzymes and cytokines (Neutra *et al*., 2001).

NKG2D

Is a C-type lectin-like activating immuno-receptor that binds with MICA which is a stress-induced major histocompatibility complex-related molecule expressed on normal intestinal epithelial cells (IECs) and recognised by the NKG2D-activating receptor on CD8(+) T-cells, gamma/delta T-cells, and natural killer cells) (Allez *et al*., 2007).

Nucleotide-binding oligomerisation domain protein 2 (NOD2)

(also known as Caspase recruitment domain protein 15 or CARD15). This protein has an important role in the immune system. It is an intracellular pattern recognition receptor that recognizes molecules containing the specific structure called muramyl dipeptide (MDP) that is found in certain bacteria. **NOD1** encodes a protein with two caspase recruitment domains (CARD) and six leucine-rich repeats (LRRs). NOD1 is involved in an immune response to intracellular bacterial lipopolysaccahrides (LPS) by recognizing their MDP and activating the NFκB protein. The **NOD2** gene is specifically linked to

inflammatory diseases such as inflammatory bowel disease and Crohn's disease (Dave 2006).

Paneth cells

Are the specialised epithelial cells found in the base of intestinal crypts of the small intestine that express toll-like receptors (TLRs) and are characterised by granules containing defensins, antimicrobial cryptdins, digestive enzymes and growth factors(Cobrin *et al*., 2005).

Peyer's patches

Are lymphoid organs of immune surveillance that reside in the intestinal epithelium and facilitate immune responses (Snoeck *et al*., 2005).

Phagocytosis

Is the cellular process of engulfing solid particles by the cell membrane to form an internal phagosopme (which is a food vacuole). The phagosome is usually delivered to the lysosome, (an organelle involved in the breakdown of cellular components), which fuses with the phagosome. The contents are subsequently degraded and either released extracellularly via exocytosis, or released intracellularly to undergo further processing. Phagocytosis is used in acquiring nutrients for some cells, as well as in the immune system where it is a major mechanism used to remove pathogens and cellular debris. It is a specific form of endocytosis involving the internalisation of solid matter, (such as bacteria) and is therefore distinct from other forms of endocytosis such as pinocytosis, which is the vesicular internalisation of liquids (Parnham 2005).

Polyclonal antibodies

(or antisera) are antibodies derived from different B-cell lines. They are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognising a different epitope (Parnham 2005).

Rel A

Is a subunit of the activated form of NF-κB. RelA contains trans-activation domains in its C termini (Li *et al*., 2002).

STAT6

Stands for 'signal transducer and activator of transcription'-6) (Afkarian *et al*., 2002).

Secretory immunoglobulin A

Is a subclass of IgA that is found primarily in secretions. sIgA may contribute to biofilm formation in the gut. This form of IgA is protected from proteolytic degradation by the presence of a secretory component (Bollinger *et al*., 2003).

Sensitisation

Is the process brought about when processed antigens are presented to T-cells by macrophages and DCs in association with the MHC class II antigens. The next time the subject is exposed to the allergen, an altered immune response occurs, resulting in the release of mediators and cytokines which produce an allergic inflammatory response (Walls 1997).

T-cells

These are lymphocytes. Two types of T-cells exist, one is cytotoxic to target cells and suppress an immune response. These have the $CD+_8$ surface marker. The other is known as helper cells and carry the $CD+_4$ surface marker. Two subgroups of CD_4+ cells exist, the Th_1 and Th_2 cells. They can be distinguished on the basis of the cytokines they produce which also determine their function. Th_1 cells induce cell mediated immune responses, Th_2 cells facilitate antibody production, especially IgE antibodies (Wright 2001).

T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4 T-cells (Feleszko *et al*., 2006).

Tight junctions **(**or **zonula occludens)**

Are a type of junctional complex between two adjoining cells. All IECs are connected by tight junctions. Tight junctions seal the apical poles of enterocytes and prevent the lateral diffusion of glycolipids and proteins between apical and basolateral domains of plasma membranes. These highly regulated gates prevent intestinal permeability and maintain the barrier function of the intestinal mucosa (Snoeck *et al*., 2005).

Tolerance

Immune tolerance is the ability of the immune system to suppress responses to prevent over-reactivity to innocuous antigen. The induction (and maintenance) of tolerance is crucial to immune function (Wiedermann 2003).

Transcytosis

Is the process by which macromolecules are transported across the interior of a cell. Vesicles are employed to engulf the macromolecules on one side of the cell, draw them through the cell and eject them on the other side. Transcytosis is most commonly observed in cells of the epithelium (Heyman 2001).

Trefoil peptides

Are three small proteins secreted by goblet cells which are needed for epithelial protection, growth and repair Snoeck *et al*., 2005).

Appendix B- Tables from Chapter 2; Epidemiology

Table 2.1; Sensitivity, specificity, positive and negative predictive value from studies using the IgA antibody assay (IgA AGA) and the IgG (IgG AGA) antibody assay in children with CD (adapted from Rostom *et al.*, 2007)

Study details (Author, year; country)	IgA-AGA				IgG-AGA			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Altuntas *et al.*, 1998;Turkey	23	90	75	48	100		55	
Artan *et al.*, 1998;Turkey	58	51	42.4	66.7	83	59	55.6	85.2
Ascher *et al.*,1996;Sweden	100	94.4	95.7	100	100	66.7	75.6	100
Bahia *et al.*, 2001;Brazil	95.5	95.6	91.3	97.9	90.9	97.8	95.2	95.7
Berger *et al.*, 1996;Switzerland	76	67	74	59	69	59	68	53
Bode, *et al.*, 1993;Denmark	64	99	90	97	71	99	100	98
Carroccio *et al.*, 1993;Italy	68	91.7	86.1	79.7	88.9	46.7	55.6	84.8
Chartrand *et al.*, 1997;Canada	80	92	67	96	83	79	45	96
Chirdo *et al.*, 1999;Argentina	75	87.1	84	80	85.7	80.6	80	86
Gaetano *et al.*, 1997; Italy	92	68	85.2	80.9	100	36	75.7	100
Gonczi *et al.*, 1991; Australia	95	92.4	76	98.6	100	92.4	76.9	100
Hansson *et al.*, 2000;Sweden	95.5	73.9	77.8	94.4	81.8	82.6	81.8	82.6
Lerner *et al.*, 1994; USA, Israel	52	94	87	74	88	92	88	92
Lindberg *et al.*, 1985;Sweden	88	88			93	89	93.1	88.6
Lindquist *et al.*, 1993;Sweden	86.5	92.7	93.7	85				
Meini *et al.*, 1996;Italy	0	100	0	91.7	100	80	31.2	100
Picarelli *et al.*, 2000;Italy	22.2	66.7	50	36.3	33.3	58.3	54.5	36.8
Poddar *et al.*, 2002;India	94	91.5	92	93.5				
Rich *et al.*, 1990;USA	53	93	72.7	85.7	100	58	44	100
Russo *et al.*, 1999;Canada	83.3	84.5	64.5	93.8	93.8	83.3	85.9	66.7
Wolters *et al.*, 2002; Netherlands	83	86	81	81	81	83	80	86

Table 2.2: Sensitivity, specificity, positive and negative predictive value using the IgA antibody assay and the IgG antibody assay in studies of adults with CD (adapted from Rostom *et al*., 2007).

Study details (Author, year; country)	IgA-AGA				IgG-AGA			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Bardella *et al*., 2001;Italy	95	89	76	98				
Bode *et al*., 1994; Denmark	46	98	75	92	62	97	73	94
Dahele *et al*., 2001;Scotland	61	86	88.5	42.7				
Gonczi *et al*., 1991; Australia	92	88.2	85.2	93.8	100	69.7	69.4	100
Kaukinen *et al*., 2000; Finland	83	45	75	92	17	86	14	93.5
Maki *et al*., 1991; Finland	30.8	87.2	22.2	91.3	46.2	89	33.3	93.3
McMillan *et al*., 1991; Ireland	100	100	100	100	57	85	64	81
Sategana-Guidetti 1995; Italy	55	100	100	55.9	78	80.7	87.6	67.6
Valdimarsson 1996; Sweden	79	70	28	96				
Vogelsang *et al*., 1995; Austria	81.6	83	81.6	83	73.5	73.6	72	75

Table 2.3: Sensitivity, specificity, positive and negative predictive value using IgA antibody assay (IgA AGA) and IgG antibody assay (IgG AGA) in studies including both children and adults (adapted from Rostom *et al*., 2007).

Study details (Author, year; country)	IgA-AGA				IgG-AGA			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Ascher *et al*., 1996 ; Sweden					96.4	69.2	72.6	95.7
Carroccio *et al*., 2002 ; Italy					76	75	73.4	77.3
Cataldo *et al*., 2000; Italy	0	100	0	33.3	100	100	100	100
Sulkanen *et al*., 1998; Finland	84.5	81.6	75.2	89	69	73.4	63	78.3
Tesei *et al*., 2003 ; Argentina					84	86	89	79

Table 2.4: Studies using IgA-EMA(ME) or IgA-EMA (HU) in adults (adapted from Rostom *et al.*, 2007).

	IgA-EMA(ME)						**IgA-EMA (HU**				
Author, year; country	**Sen**	**Spec**	**PPV**	**INPV**	**Prev (%)**	**Author, year; country**	**Sen**	**Spec**	**PPV**	**NPV**	**Prev (%)**
Bardella *et al.*, 2001;Italy	100	97.2	93	100	28.7	Dahele *et al.*, 2001; Scotland	87	100	100	81.3	55.3
Biagi *et al.*, 2001;Italy	94.6	100	100	94.5	49.1	Gillbert, 2000; Canada	100	100	100	100	33.3
Carroccio *et al.*, 2002;Italy	100	100	100	100	11.6	Kaukinen *et al.*, 2000; Finland	88.9	100	100	98.9	7.6
Hallstrom *et al.*, 1989; Finland	90.6	100	100	88.9	51.8	Ladinser *et al.*, 1994; Italy	90	100	100	98	18.9
Ladinser *et al.*, 1994;Italy	100	100	100	100	21.1	Salmaso *et al.*, 2001; Italy	87	100	100	95.1	24.7
McMillan, 1991; Ireland	89.2	100	100	95.3	28.1	Volta *et al.*, 1995; Italy	95	100	100	97.1	35.6
Sategana-Guidetti 1995;Italy	100	100	100	100	63.7						
Valdimarsson 1996;Sweden	74	100	100	96	9.7						
Valentini *et al.*, 1994;Italy	99	100	100	96.7	76.2						
Vogelsang *et al.*, 1995; Austria	100	100	100	100	48						
Volta *et al.*, 1995;Italy	95	100	100	97.1	35.6						

Table 2.5: Studies using IgA-EMA(ME) and IgA-EMA(HU) in children

(adapted from Rostom *et al.*, 2007).

	IgA-EMA(ME)					**IgA-EMA(HU)**			
Author, year; country	**Sens**	**Spec**	**PPV**	**NPV**	**Author, year; country**	**Sens**	**Spec**	**PPV**	**NPV**
Ascher *et al.*, 1996; Sweden	95.4	100	100	94.7	Gaetano *et al.*, 1997; Italy	94	100	100	89.2
Bonamico *et al.*, 2001; Italy	95.1	98.2	90	44.3	Iltanen *et al.*, 1999 Finland	100	77.1	60.1	100
Carroccio *et al.*, 1993; Italy	100	96.7	95.7	100	Kolho *et al.*, 1997; Finland	100	100	100	100
Chan *et al.*, 2001; Canada	89	97	80	98	Russo *et al.*, 1999; Canada	45.8	95.8	78.6	84
Chirdo *et al.*, 2000; Argentina	92.4	100	100	85.2	Salmaso *et al.*, 2001; Italy	100	100	100	100
Di Leo *et al.*, 2003; Italy	100	96.5	93.5	100					
Gaetano *et al.*, 1997; Italy	96	96	97.9	92.3					
Hallstrom *et al.*, 1989; Finland	100	100	100	100					
Hansson *et al.*, 2000; Sweden	95.5	100	100	95.8					
Kolho *et al.*, 1997; Finland	100	95	100	100					
Kumar *et al.*, 1989; USA, Israel	96	96	89	87					
Lerner *et al.*, 1994; USA, Israel	97	97	98	97					
Lindquist *et al.*, 1993; Sweden	98.1	98.1	92.7	94.4					
Russo *et al.*, 1999; Canada	75	75	88.7	69.2					
Vitoria *et al.*, 2001; Italy	100	100	100	100					
Whelan *et al.*, 1996; Ireland	100	100	100	100					
Wolters *et al.*, 2002; Netherlands	92	92	90	90.5					

Table 2.6: Studies using IgA-EMA(ME) and IgA-EMA(HU) in both children and adults (adapted from Rostom *et al*., 2007).

	IgA-EMA (ME)					IgA-EMA-(HU)			
Author, year; country	**Sens**	**Spec**	**PPV**	**NPV**	**Author, year; country**	**Sens**	**Spec**	**PPV**	**NPV**
Cataldo, 2000; Italy	0	100	0	33.3	Sblaterro, 2000; Italy	93	100	100	80
Dickey, 2001; Northern Ireland	75.3	98.3	98.2	76	Sulkanen,1998; Finland	92.6	99.5	99.2	94.9
Ascher, 1996; Sweden	98.2	100	100	98.5					
Carroccio 2002; Italy	88	99	98.7	90					
Tesei, 2003; Argentina	86	100	100	83					

Table 2.7: Studies for IgA-tTG-GP and IgA-tTG-HR in adults (adapted from Rostom *et al*., 2007).

	IgA-tTG-GP					IgA-tTG-HR			
Author, year; country	**Sens**	**Spec**	**PPV**	**NPV**	**Author, year; country**	**Sens**	**Spec**	**PPV**	**NPV**
Bardella, 2001; Italy	100	98.2	83.3	100	Carroccio, 2002; Italy	100	97	80	100
Biagi, 2001; Italy	87.5	98.1	98	87.1	Gillbert, 2000; Italy	95.2	100	95.2	100
Carroccio, 2002; Italy	100	92	60	100	Kaukinen, 2000; Finland	100	100	100	100
Dahele, 2001; Scotland	81	97	97.9	74.1					
Salmaso, 2001; Italy	87	97	90.9	94.9					

Table 2.8: Studies for IgA-tTG-GP and IgA-tTG-HR in children (adapted from Rostom *et al*., 2007).

	IgA-tTG-GP					IgA-tTG-HR			
Author, year; country	**Sens**	**Spec**	**PPV**	**NPV**	**Author, year; country**	**Sens**	**Spec**	**PPV**	**NPV**
Bonamico, 2001; Italy	90.3	100	100	30.3	Hansson, 2000; Sweden	95.5	95.7	95.5	95.7
Chan, 2001; Canada	89	94	67	98	Vitoria, 2001; Italy	95	100	100	93
Hansson, 2000; Sweden	90.9	95.7	95.2	91.7	Wolters, 2002; Netherlands	96	100	100	96
Salmaso, 2001; Italy	95	100	100	94.1					
Wolters, 2002; Netherlands	96	92	92.6	95.7					

Table 2.9: HLA studies with biopsied cases of CD and controls (adapted from Rostom *et al*., 2007)

Author, year; country	DQ2 in CD	DQ2 in controls	Sens	Spec	PPV	NPV
Iltanen, 1999; Finland	90.48	29.85	90.5	70	49	96
Sacchetti, 1998; Italy	86.89	18.75	87	81	95	62
Catassi 2001; Nth Africa	91	38.9				

Table 2.10: Chance of developing another autoimmune disorder along with CD

(Ventura *et al*., 1999)

Age at diagnosis of CD (in years)	% chance of developing another autoimmune disorder
2-4	10.5%
4 -12	16.7%
12 – 20	27%
20 +	34%

Table 2.11: Studies showing prevalence of CD in Type-I diabetes

(adapted from Rostom *et al.*, 2007)

Author, year; country	Total patients	Age group	Screening test(s)	Prevalence by serology	Prevalence by biopsy
Acerini, 1998; UK	167	Children	EMA or AGA	0.0659	0.0479
Agardh, 2001; Sweden	162	Children	AGA, EMA, or tTG IgG or IgA	0.0494	0.037
Aktay, 2001; USA	218	Mixed; mostly children	EMA	0.078	0.0459
Arato, 2003; Hungary	205	Children	EMA	0.1171	0.0829
Bao, 1999; USA	847	Mixed	tTG	0.1157	0.0177
Barera, 2002; Italy	273	Children	EMA, second EMA	0.0549	0.033
Barera,1991; Italy	498	Children	AGA IgA then if neg IgG AGA	0.0602	0.0321
Carelo, 1996; Spain	141	Children	IgA AGA if positive twice	0.0851	0.0284
Cronin, 1997; Ireland	101	Mixed; mostly adults	EMA	0.0792	0.0495
De Block, 2001; Belgium	399	Mixed	EMA	0.0226	0.0075
De Vitis, 1996; Italy	1114	Mixed	IgA, IgG then IgA EMA	0.1086	0.0566
Frazer-Reynolds, 1998; Canada	263	Children	EMA	0.0646	0.0456
Gillett, 2001; Canada	233	Children	EMA or AGA	0.0815	0.0601
Hansen, 2001; Denmark	104	Children	EMA or tTG	0.0962	0.0865
Jager, 2001	197	Mixed	tTG	0.0964	
Juan, 1998; Spain	93	Children	EMA	0.0753	0.0645
Kaukinen, 1999; Finland	62	Adults	EMA	0	0.1129
Kordonouri, 2000; Germany	520	Mixed; mostly children	tTG	0.0442	0.0173
Lampasona, 1999; Italy	287	Mixed; mostly children	tTG IgA or IgG	0.0836	n/a

Author, year; country	Total patients	Age group	Screening test(s)	Prevalence by serology	Prevalence by biopsy
Li Voon Chong, 2002; UK	509	Adults	EMA	0.0138	n/a
Lorini, 1996; Italy	133	Mixed;mostly children	AGA IgA or IgG	0.0376	n/a
Not, 2001; Italy	491	Mixed	EMA	0.057	0.057
Page, 1994; Mixed	1785	N/a	AGA	0.0409	0.0073
Rensch, 1996; USA	47	Adults	EMA	0.0638	0.0638
Roldan, 1998; Spain	177	Children	IgA, IgG AGA, and with EMA)	0.1073	0.0395
Rossi, 1993 Italy	211	Children, some adults	EMA	0.0474	0.0142
Sategna-Guidetti, 1994; Italy	383	Adults	EMA	0.0313	0.0261
Saukkonen, 1996; Finland	776	Children	AGA or ARA	0.0979	0.0245
Schober, 2000; Austria	403	Mixed; mostly children	EMA	0.0298	0.0149
Sigurs, 1993; Sweden	459	Children	AGA	0.0414	0.0458
Sjoberg, 1998; Germany	848	Adults	AGA - IgG or IgA; EMA	0.0259	0.0083
Spiekerkoetter, 2002; Germany	205	Children	tTG IgA or IgG	0.0634	0.0293
Talal, 1997; USA	185	Adults	EMA	0.0486	0.0216
Valerio, 2002; Italy	383	Children	EMA or IgG AGA	n/r	0.0836

Appendix C- Tables from Chapter 4; Microbiology of the gut

Table 4.1: Genera of microflora most frequently present in the GI tract (adapted from Wilson 2005b)

Organism	Characteristics	Optimum conditions for growth	Organism	Characteristics	Optimum conditions for growth
Bacteroides	Non motile; non sporing; anaerobic gram-negative pleomorphic rods	temperature 25-45°C pH range is 5.5-7.5	**Lactobacillus**	Gram-positive facultative anaerobic or microaerophilic; non-spore-forming bacterium	temperature 45 °C pH 4-5 or lower
Bifidobacterium	Non sporing, non motile, pleomorphic gram- positive rods; mostly obligate anaerobes	temperature 37-41°C pH 4.0-8.5 optimal growth at pH 6.5-7.0 Acid tolerant, but not acidophilic	**Methanogenic bacteria including methanobrevibacter and methanosphaera**	Methanobrevibacter are non-motile, anaerobic gram-positive short rods Methanosphaera are anaerobic, gram-positive cocci	temperature 37-39°C pH 7.0 temperature 37°C pH 6.5-6.9
Clostridium	Obligately anaerobic, spore forming gram- positive rods; most species are motile	temperature 15 - 45 °C pH 6.0	**Peptococcus**	anaerobic, gram-positive, coccoid bacteria, non sporing; non motile	temperature 37°C
Desulphovibrio	Motile, anaerobic, gram-negative curved rods	temperature 30-38°C	**Peptostreptococcus**	obligately anaerobic, gram-positive, coccoid bacteria, non sporing, non motile	temperature 37°C
Enterococcus	Facultatively anaerobic, non-sporing; catalase negative gram-positive cocci;	temperature 35-37°C	**Propionibacterium**	gram positive, anaerobic, rod shaped (bacillus)	

Organism	Characteristics	Optimum conditions for growth	Organism	Characteristics	Optimum conditions for growth
Enterobacteriaceae including Escherichia, Citrobacter, Enterobacter, and Proteus	30 genera; 150 species; non sporing, facultatively anaerobic gram- negative; most species are motile	temperature 25-37°C	**Ruminococcus**	Non sporing, non-motile, anaerobic gram-positive cocci	temperature 20–40 °C (optimum 37 °C) pH 5·5–8·0 (optimum 6·5)
Eubacterium	anaerobic gram-positive rods; 2 types exist- saccharolytic and asaccharolytic	temperature 37°C	**Streptococcus**	Spherical gram-postive bacteria facultatively anaerobic; non sporing, non-motile,	temperature 25-45°C
Fusobacterium	anaerobic, gram-negative non-spore forming bacterium		**Serratia**	gram-negative, facultative anaerobes, motile, rod shaped	temperatures 20-37 C pH 9
Helicobacter pylori	Microphilic; gram-negative, motile, non sporing curved rod	Temperature 37°C pH 7.0;range 6.0-8.0	**Veillonella**	Anaerobic, non-motile gram-negative cocci	

Table 4.2: Predominant genera of microflora in human faeces (Wilson 2005b)

	Abundance in faeces (log_{10} per g dry weight of faeces)			**Abundance in faeces (log_{10} per g dry weight of faeces)**	
Organism	**Mean**	**Range**	**Organism**	**Mean**	**Range**
Bacteroides spp.	11.3	9.2-13.5	*Propionibacterium* spp.	9.4	4.3-12.0
Eubacterium spp.	10.7	5.0-13.3	*Actinomyces* spp.	9.2	5.7-11.1
Bifidobacterium spp.	10.2	4.9-13.4	*Streptococcus* spp.	8.9	3.9-12.9
Ruminococcus spp.	10.2	4.6-12.8	*Methanobrevibacter* spp.	8.8	7.0-10.5
Peptostreptococcus spp.	10.1	3.8-12.6	*Escherichia* spp.	8.6	3.9-12.3
Peptococcus spp.	10.0	5.1-12.9	*Desulphovibrio* spp	8.4	5.2-10.9
Clostridium spp	9.8	3.3-13.1	*Fusobacterium* spp	8.4	5.1-11.0
Lactobacillus spp.	9.6	3.6-12.5			

Table 4.3: Most frequently isolated species in human faeces (Wilson 2005b)

Genus/group	**Most frequently isolated species**
Bacteroides	*B. thetaiotaomicron, B. vulgatus, B. distasonis, B. eggerthii, B. fragilis, B. ovatus*
Eubacterium and *Eubacterium* like organisms	*Col. aerofaciens, Eg. lenta, Eub. contortum, Eub. cylindroids, Eub. rectale, Eub. biforme, Eub. ventriosum*
Bifidobacterium	*Bif. adolescentis, Bif. infantis, Bif. catenulatum, Bif. pseudocatenulatum, Bif. breve, Bif. longum*
Clostridium	*Cl. ramosum, Cl. bifermentans, Cl. butyricum, Cl. perfringens, Cl. difficile, Cl. indolis, Cl. septicum, Cl. sporogenes*
Enterococcus	*Ent. faecalis, Ent. faecium*
Fusobacterium	*F. prausnitzii, F. mortiferum, F. necrophorum, F. nucleatum, F. varium, F. russii*
Gram positive anaerobic cocci	*Pep. productus, Micromonas micros (Pep.micros), Anaerococcus prevotii (Pep prevotii), Schleiferella asaccharolytica (Pep asaccharolytica), Finegoldia magna (Pep magnus)*
Ruminococcus	*Rum. albus, Rum. obeum, Rum. torques, Rum. flavfaciens, Rum. gnavus Rum. bromii*
Enterobacteria	*E. coli, Enter. aerogenes, Pr. mirabilis*
Lactobacillus	*L. acidophilus, L. brevis, L. casei, L. salivarius, L. plantarum, L. gasseri, L. johnsonii, L. delbrueckii*

Table 4.4: pH of different regions of the GI tract (Wilson 2005a)

Site	pH	Predominant microflora colonising the site
Stomach	1-2	*Helicobacter* spp., acidophilic streptococci
Duodenum	5.7-6.4	acidophilic streptococci and lactobacilli
Ileum	7.3-7.7	Streptococci, coliforms, anaerobes (*Veillonella* spp., *Clostridium* spp., *Bacteroides* spp)
Ascending colon	5.6	Anaerobes (*Bacteroides* spp., *Bifidobacterium* spp.,*Clostridium* spp., *Eubacterium* spp)
Transverse colon	5.7	Anaerobes (*Bacteroides* spp., *Bifidobacterium* spp.,*Clostridium* spp., *Eubacterium* spp)
Caecum	5.7	Anaerobes (*Bacteroides* spp., *Clostridium* spp.) Facultative anaerobes *(E coli, lactobacilli, enterococci*)
Descending colon	6.6	Anaerobes (*Bacteroides* spp., *Bifidobacterium* spp.,*Clostridium* spp., *Eubacterium* spp)

Table 4.5: Positive interactions occurring between members of the intestinal microflora (Wilson 2005b)

Process	Benefit to other organisms
Quorum sensing	Production of bacteriocins and virulence factors
Oxygen utilisation by anaerobes/facultative anaerobes	Creates an environment suitable for the growth of microaerophiles and anaerobes
Degradation of polysaccharides	Provides monosaccharides
Degradation of proteins	Provides amino acids
Degradation of mucins and other glycoproteins	Provides sugars, amino acids and sulphate
Excretion of metabolic end products (e.g. lactate, ethanol, hydrogen, ammonia)	Serve as nutrient sources for other organisms

Table 4.6: The dominant and minor genera of the climax community (Lenoir- Wijnkoop *et al.*, 2003)

Species	**The climax microflora (dominant genera)** (expressed in Log^{10} bacteria/g faeces)	**Species**	**The climax microflora (minor genera)** (expressed in Log^{10} bacteria/g faeces)
Bacteroides	10-11	Veillonella	5-7
Bifidobacteria	10-11	Enterococci	5-7
Fusobacteria	7-10	Bacilli	ND
Eubacteria	7-10	Micrococci/Staphylococci	ND
Lactobaclli	7-9	Methanogens	5
Streptococci	6-8	Sulfate-reducing bacteria	5
Clostridia	6-10	Anaerobic cocci	
Enterobacteria	6-8		

Table 4.7: Comparison of counts per gram of faeces for selected bacteria of faecal microflora (Goldin 2003)

Bacterial genera	Log counts- Adults	Log counts - Elderly
Lactobacillus	5.8	7.5
Enterobacter	7.8	8.2
Streptococcus	7.9	7.4
Clostridium perfringens	4.4	6.6
Bifidobacterium	10.0	9.4

Table 4.8: Factors contributing to alterations in the indigenous microflora in the elderly (Wilson 2005a)

Factor	Consequences
Immuno-senescence	Possibly influences balance of microflora
Malnutrition	Impaired immune response; affects composition of secretions thereby influencing microflora
Decreased gastric-acid production	Increased colonisation of stomach including species normally destroyed
Increased bacterial adhesion	Intestinal dysbiosis
Decreased intestinal motility, alterations in mucous composition	Enables colonisation of the small intestine with potentially pathogenic species

Table 4.9: The effects of various diets on intestinal microflora (Hawrelak *et al*., 2004)

Micro-organisms	American / mixed Western diet	American Seventh day Adventist Vegetarian diet	English/ Mixed Western diet	Japanese/ Japanese diet	Japanese/ Mixed Western diet	Ugandan/ Vegetarian diet	Indian Vegetarian diet
Total anaerobes	10.2 a	-	10.1a	9.9a 11.4 b	11.5a	9.3a	9.7a
Total aerobes	7.5a	-	8.0a	9.4a 9.6b	9.6b	8.2a	8.2a
Bacteroides spp	9.8a	11.7 b	9.8a 9.7b	9.4a 10.1b	11.1b	8.2a 8.2a ψ	9.2a
Enterococci	5.5a	6.5a	5.8a 5.7a ψ	8.1a 8.4b	8.4b	7.0a 7.0b ψ	7.3a
Bifidobacteria	10.0a	8.1b	9.8a 9.9a ψ	9.7a 8.2b	9.5b	9.3a 9.3a ψ	9.6a
Lactobacilli	7.3a	10.0b	6.5a 6.0a ψ	7.4a 5.7b	4.0b	7.2a 7.2a ψ	7.6a
Clostridia	-	-	5.0a 4.4a	5.1a 9.7b	9.5b	4.6a 4.0a	5.0a

a –log ^{10} mean count/g wet weight of faeces; - no data; ψ a significant difference between groups b- log 10 mean count/g weight of faeces

Table 4.10: Short chain fatty acids and other organic substrates in the human intestine (Cummings *et al.*, 1987)

	Small intestine			Large intestine			
	Jejunum	Ileum	Caecum	Ascending	Tranverse	Descending	Sigmoid
Acetate	0.6	7.9	69.1	63.4	57.9	43.5	50.1
Propionate	-	1.5	25.3	26.7	23.1	14.2	19.5
Iso-butyrate	-	0.3	2.1	1.8	2.6	2.3	1.9
Butyrate	-	2.3	26.1	24.5	24.4	14.7	17.9
Iso-valerate	-	0.1	2.7	2.7	3.4	3.5	3.7
Valerate	-	0.2	4.5	3.6	4.2	2.8	4.3
Iso-caproate	-	0.1	.06	0.2	-	-	0.9
Caproate	-	0.3	1.4	1.7	1.7	0.3	1.5
Lactate	2	13.5	4.5	3.1	3.5	3.1	1.5
Succinate	3.7	8.3	0.9	3.1	1.7	1.9	2.1

Table 4.11: Influence of diet on the composition of microflora in the large intestine (Wilson 2005b)

Type of diet	**Effect on microflora**
High meat diet	Increased ratio of obligate: facultative anaerobes
Fibre supplements	Increase in total viable count of *Clostridium* spp
Addition of gum arabic	Increase in proportion of organisms able to degrade this polymer
Vegetarian diet	Decreased prevalence of anaerobic gram-positive cocci and *Fusobacterium* spp; Increased prevalence of *Actinomyces* spp
Inulin supplementation	Increase in *Bifobacterium* spp

Appendix D - Tables from chapter 5; Pathogenesis of CD

Table 5.1: Identified gluten epitopes in key studies.

Peptide sequence	Origin	Notes
LQLQPFPQP**Q**LPY	α- gliadin	Original overlapping
PQP**Q**LPYPQPQLPY	α- gliadin	Immunodominant epitopes
QPQQSFP**Q**QQ	γ-gliadin	Minor epitope
VQG**Q**GIIQPP**Q**QPAQL	γ-gliadin	Deamidation not required
QQPF**QQ**Q**Q**QPLPQ	glutenin	No response in adults
QQQQPPFS**Q**QQ**Q**SPFSQQQQ	glutenin	No response in adults
QPQPFPQQSE**Q**S**Q**QPFQPQPF	unknown	
Q**Q**XSQP**Q**XPQ**QQQ**XP**Q**QPQQF	unknown	Deamidation not required
PFRP**Q**QPYPQPQPQ	α-gliadin	Epitope unknown; deamidation not essential

Q designates glutamine residues that are deamidated by tTG. The epitopes tend to cluster in proline and glutamine-rich regions of the gluten genome. There is considerable sequence homology between epitopes. Many of the epitopes contain numbers of **Q** × **P** sequences, which act as optimal substrates for tTG. The minimum requirement for T-cell reactivity is an oligopeptide of nine residues, although optimal size is 10–15 residues (Dewar *et al*., 2004).

Appendix E- Tables from Chapter 6; Treatment of CD

Table 6.2: Foods recommended on a GFD (MacKenzie 2006)

Serving size	Foods to eat	Tips
Breads, cereals, rice, and pasta; (6-8 servings daily) **One serving;** • 1 slice bread • 1 C breakfast cereal • ½ C cooked cereal, or pasta • ½ bun or bagel or pita bread	• Breads or bread products made from corn, rice, soy, arrowroot, corn or potato starch, pea, potato or whole bean flour, tapioca, sago, rice bran, cornmeal, buckwheat, millet, flax, sorghum, amaranth and quinoa • Pappadums, taco shells, plain unflavoured corn chips • Hot cereals made from soy, hominy, hominy grits, brown and white rice, buckwheat groats, millet, cornmeal, and quinoa flakes. • Puffed corn, rice or millet and other rice and corn products made with allowed ingredients • Rice, rice noodles and GF pasta • Rice crackers (plain only with NO MSG), rice cakes, popped corn cakes, GF cakes	• Use corn, rice, soy, arrowroot, tapioca and potato flours or a mixture of these available as GF mixes, • Commercial products are available; look for • Bakels GF Baking mix • Horley's bread mix • Healtheries Simple Baking mix and bread mix • Healtheries Simple pasta range • Orgran self-raising and plain flour, Organ pasta range • 'Spoilt for choice' muffin, pancake and baking mixes • Freedom Foods products • San Remo pasta range See Manufactured Food database
Vegetables; (at least 5 servings daily) **One serving;** • 1 C raw leafy • ½ C cooked or chopped • ¾ C juice	• All plain, fresh, frozen, or canned vegetables made with no gluten containing additives (Wash thoroughly to avoid cross contamination with gluten containing foods that may have occurred during storage and transport).	• Buy plain frozen or canned vegetables and season with GF herbs and spices Fresh herbs are excellent, as are Massels stock cubes and gravy mixes. (Check all spices for thickeners, stabilisers, bulking agents that may contain gluten)
Fruit; (2-4 servings daily) **One serving** • 1 medium sized piece • ½ C canned ¾ C juice	• All fresh fruits, frozen or canned fruit without gluten containing additives listed in the ingredient list • All pure fruit juices • Berry fruits and fruit smoothies/icies are recommended	• Check canned and frozen goods carefully as some have hidden sources of gluten in thickeners, fillers, preservatives, stabilisers, colourings and flavourings.

Milk and dairy products; (2 servings daily) **One serving** • 1 C milk or yoghurt • 25 g cheese	• All natural unflavoured milk and milk products, including UHT products • Real cream • Plain yoghurt and flavoured products free of gluten containing additives • Butter • Aged cheese (edam gouda, colby) • Soft cheeses like Camembert, Brie, Double cream, (check ingredients as some contain gluten) Cream cheese and cottage cheese (check ingredients some light versions may contain gluten)	Refer to the Manufactured Food Database for a list of certified gluten-free products at www.mfd.co.nz • This is updated regularly, but still read ingredient lists carefully
Meats, poultry, fish, legumes (dry beans), eggs, nuts; (2 servings daily) **One serving** 75g cooked food (lean, fat removed) 1 egg ½ C cooked beans 2 T peanut butter ¼ C nuts = 25g meat	• All fresh unprocessed, unseasoned, unmarinated, uncrumbed meat, poultry, fish and shellfish • GF sausages, or those without fillers • GF cold meats with no stuffings or marinades (especially soy sauce) • Eggs • Legumes (dried peas and beans) • Salted unflavoured nuts	• When dining out select meat fish or poultry without seasonings, coatings, sauces or gravies
Fats, snacks sweets, condiments and beverages Limit to special occasions	• Butter margarine, homemade salad dressings, soups and desserts made with allowed ingredients (see MFD). • Sugar honey jelly, jam, hard candy, plain chocolate, coconut, molasses, marshmallows, meringues, but check ingredients some contain gluten containing additives • Use Tatua Farm Hollandaise and Cheese sauce, or Watties ' Bit on the Side' sauces or Watties Tomato sauce	• Store gluten free foods in the fridge or freezer as they do not contain preservatives. • Store biscuits or dried products in air tight containers. • Remember to avoid sauces, gravies canned fish and other products with HVP made from wheat. • If in doubt always refer to the database

Table 6.3: Foods to avoid on a GFD (MacKenzie 2006)

	Foods to avoid
Breads, cereals, rice, and pasta	• All breads and baked products made with or containing wheat, triticale, rye, oats, barley, wheat germ or bran, all wheat derived flours, including wholemeal and stoneground, wheat starch, wheaten cornflour, oat bran, bulgar, farina, wheat-based semolina, spelt, kamut • Cereals made from wheat, oats, rye, barley, triticale, spelt, and kamut; cereals with malt extract added, or malt and caramel flavourings or colourings • Pasta made from any of the above flours • All crackers, except those in permitted list.
Vegetables	• Any creamed or breaded vegetables unless specified GF • All seasoned vegetables including French fries (Check these carefully, as many have gluten added)
Fruit	• Commercial fruit pie fillings and dried fruit unless specified GF • No powdered fruit drinks or syrups containing additives
Milk and dairy products;	• Flavoured milks especially malted ones • Frozen yoghurt • Thickened creams, lite creams • Canned creams, spray creams • Cream cheeses and cottage cheese containing flavour enhancers, or stabilisers that could potentially contain gluten
Meats, poultry, fish, legumes (dry beans), eggs, nuts;	Any meats prepared with wheat, oats, rye barley, or gluten containing stabilisers or fillers, including some frankfurters, cold cuts, sandwich ham, meat spreads, sausages, self-basting turkey or chicken, some egg substitutes
Fats, snacks sweets, condiments and beverages	• Commercial salad dressings, prepared soups, condiments, sauces, seasonings prepared with gluten containing ingredients (see MFD for permitted foods) • Avoid all sauces containing malt vinegar, look for white vinegar or acetic acid. • All dried commercial soup, sauces, and stocks contain gluten unless specified GF • Hot cocoa mixes, non-dairy cream, cream substitutes, flavoured instant coffee, some herbal teas or alcohol distilled from cereals, especially gin, vodka, whiskey, beer • Malted beverages • Licorice

Appendix F- Tables from Chapter 7; The Faecal Study

Table 7.1: A summary of possible methods for analysing microbial communities

(Dethlefsen *et al.*, 2006)

Method	Main use	Advantages	Limitations
Traditional methods			
Cultivation	Identify and quantify taxa	Provides isolates for further characterisation; can focus on recovering strains with desired traits	Incomplete and biased community representation; many types of media needed to maximize species recovery
Microscopy (general stains)	Estimate abundance of all microbes or recognisable types	Quantification without cultivation or broad-range PCR bias	Cannot distinguish between many taxa with different traits and ecological roles; low throughput
16S r RNA methods			
Oligonucleotide hybridization including FISH, flow cytometry and microarrays	Detect and quantify known phylogenetic groups	Can be high throughput; can reveal spatial relationships; phylogenetic identification of visible cells	Detects only taxa that hybridise to chosen probes, typically 6-18 genus-or family-level groups detected
5' exonuclease PCR includes DGGE and similar techniques as well as tRFLP	Detect and quantify known phylogenetic groups	Rapid; high throughput	Detects only taxa that hybridise to chosen probes, typically 6-18 genus-or family-level groups detected
Community profiling	Comparing	Rapid; inexpensive	Broad range PCR bias;

(also called quantitative PCR, RT-PCR or TaqMan assay) This is the biochemical amplification of particular DNA sequences	communities	assessment of abundant 16SrRNA sequence variants	additional work needed to identify groups represented in profiles; hard to compare analyses done at different times
16SrRNA sequencing	Phylogenetic identification of microbes; generates data for other 16S rRNA-based methods	Identificaiton to strain level; can detect taxa; analysis possible at multiphylogenetic levels	Broad range PCR bias; Expensive; labourious

Appendix G- Tables from Chapter 9; The Dietary Study

Table 9.1: Uses and limitations of methods commonly used to assess the food composition of individuals (adapted from Black 2001)

Methods	Procedures	Uses	Advantages	Limitations
24 hour recall	Subject or caretaker recalls food intake of previous 24 hours. Quantities are estimated in household measures using food models as memory aids and or to assist in quantifying portion sizes. May be written or verbal. Nutrient intakes calculated using food composition databases	Useful for assessing average usual intakes of a large population, provided that the sample is truly representative and that the days of the week are adequately represented. Used for international comparisons of relationship of nutrient intakes to health and susceptibility to chronic disease	Inexpensive, easy, quick, with low respondent burden so that compliance is high Large coverage possible; Can be used with illiterate people, Element of surprise so people are less likely to modify their eating pattern	Relies on memory and hence unsatisfactory for the elderly or young children Single 24 hour recalls are likely to omit foods consumed infrequently Multiple replicate 24 hour recalls required to estimate usual intakes of individuals
Estimated food record	Record of all food and beverages consumed(including snacks) over periods from 1 to 7 days Quantities are estimated in household measures. May be written or verbal. Nutrient intakes calculated using food composition databases	Used to assess actual or usual intakes of individuals depending on the number of measurement days Data on usual intakes used for diet counselling and statistical analysis involving correlation and regression	Inexpensive, easy quick, with reasonably low respondent burden so that compliance is usually quite high over shorter time frames.	Accuracy depends on conscientiousness of the subject and their ability to estimate quantities. Longer time frames result in a higher respondent burden and lower co-operation. Subjects must be literate
Weighed food record	All food consumed over a defined period is weighed by the subject, caretaker or assistant (depending on the setting). Food samples may be saved individually or as a composite for direct chemical analysis. Alternatively nutrient intakes can	Used to assess actual or usual intakes of individuals depending on the number of days measurement.	Accurate, but time consuming Heavy respondent burden.	The person's physical condition must allow them to weigh all food prior to eating Subjects may change their usual eating patterns to simplify weighing or to impress the investigator. Expensive for respondent and to analyse Requires literate,

	be calculated using food composition databases			motivated and willing participants
Dietary history	Interview method consisting of 24 hour recall of actual intake, plus information on overall usual eating pattern	Used to describe usual food and /or nutrient intakes over a relatively long time period which can be used to estimate prevalence of inadequate intakes. Such information is used for national food policy development, food fortification planning and to identify food patterns associated with inadequate intakes	Uses trained administrator so quite accurate Able to be used over a longer time frame to gather large amounts of information.	Labour intensive, tir consuming Results dependent on skill of observer Can be expensive to set up and administer
Food frequency Questionnaire	Uses a comprehensive list or list of specific food items to record intakes over a given period of time (day, week month, year). Record is obtained by interview or by self administered questionnaire Questionnaire can be semi-quantitative when subjects are asked to quantify usual portion sizes of food items, with or without the use of food models	Designed to obtain qualitative, descriptive data on usual intakes of food or classes of foods over a long period of time Useful in epidemiological studies for ranking subjects into broad categories of low, medium and high intakes of specific foods, food components, or nutrients for comparison with the prevalence and/or mortality statistics of a specific disease.	Can identify food patterns associated with inadequate intakes of specific nutrients. Inexpensive to prepare and administer Can be used on large populations Method is rapid with low respondent burden and high response rate	Accuracy is lower than other methods Requires a literate population Enables respondent to modify answers to please investigator. Low response rate

Table 9.2: Summary chart of gluten intake (based on devised rating system)

	Gluten intake													
	Month One				Month Two			Month Three			Month Four			
Sub Code	Day 1	Day 2	Day 3	Ave	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Ave
CSI	14	16	18	16	2	2	2	3	3	3	3	3	3	3
CST	24	18	23	22	2	2	2	2	2	2	0	0	0	1
CSU	28	30	29	29	0	0	0	0	0	0	1	1	0	0
CSW	7	23	17	16	0	0	3	0	1	0	0	0	0	0
CSZ	23	20	20	21	1	1	1	0	1	0	0	0	0	0
CSAA	12	12	10	11	0	0	0	1	0	2	0	1	3	1
CSAB	24	22	16	21	0	2	0	0	2	0	0	3	0	1
CSAC	32	37	23	31	3	3	0	3	6	3	3	0	4	3

Rating system

1 = trace amounts of gluten present in the food
2 = small amounts of gluten present in the food
3 = moderate amounts of gluten present in the food
4 = large amounts of gluten present in the food
5 = very significant amounts of gluten present in the food

Table 9.3: Nutrient content of selected cereal grains (Gendel *et al*., 2005:Karsada 2002)

Nutrient /100g	Wheat	Oats	Brown Rice	Barley	Maize	Rye	Sorghum	Millet
Fibre (g)	13	11	3.5	16	NA	15	NA	9
Thiamin (mg)	0.4	0.8	0.4	0.2	0.4	0.3	0.2	0.4
Riboflavin (mg)	0.1	0.1	0.1	0.1	0.2	0.3	0.1	0.3
Niacin (mg)	6.7	1.0	4.7	4.6	3.6	4.3	2.9	4.7
Pantothenic acid (mg)	0.9	1.4	1.5	0.3	0.4	1.5	NA	0.9
Pyridoxine (mg)	0.4	0.1	0.5	0.3	0.6	0.3	NA	0.4
Folate (µg)	43	56	20	23	19	60	NA	85
Vitamin E (mg)	1.4	0.7	0.7	0.1	0.8	1.9	NA	0.2
Calcium (mg)	34	54	28	29	7	33	28	8
Iron (mg)	5.4	4.7	1.6	2.5	2.7	2.7	4.4	3
Magnesium(mg)	144	177	143	79	127	121	NA	114
Phosphorus(mg)	508	523	299	221	210	374	287	285
Potassium (mg)	435	429	246	280	287	264	350	195
Sodium(mg)	2	2	6	9	35	6	6	5
Zinc(mg)	4.2	4	2	2.1	2.2	3.7	NA	1.6
Selenuim (µg)	89	NA	23	38	16	35	NA	2.7

Table 9.4. **Haematological manifestations of CD** (Halfdanarson *et al*., 2007)

Problem	**Frequency**	**Comments**
Anaemia: iron deficiency, folate deficiency, vitamin B_{12} deficiency, and other nutritional deficiencies	Common	The anaemia is most commonly secondary to iron deficiency but may be multifactorial in etiology. Low serum levels of folate and vitamin B_{12} without anemia are frequently seen. Anaemia due to other deficiencies appears to be rare
Thrombocytopenia	Rare	May be associated with other autoimmune phenomena
Thrombocytosis	Common	May be secondary to iron deficiency or hyposplenism
Thromboembolism	Uncommon	Aetiology is unknown but may be related to elevated levels of homocysteine or other procoagulants
Leukopenia/neutropenia	Uncommon	Can be autoimmune or secondary to deficiencies of folate, vitamin B_{12}, or copper
Coagulopathy	Uncommon	Malabsorption of vitamin K
Hyposplenism	Common	Rarely associated with infections
IgA deficiency	Common	May be related to anaphylactic transfusion reactions
Lymphoma	Uncommon	The risk is highest for intestinal T-cell lymphomas

Appendix H- Raw data for statistical analysis of dietary intake

Table 10.5: Starch intake in grams (g)

	Starch intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	109.1	218.1	123.2	57.7	82.8	77.9	72.5	87.5	80.02	85.6	85.6	72.4
CST	93.0	91.6	92.8	57.7	40.3	61.0	26.6	26.6	25.69	11.7	42.2	9.1
CSU	195.7	141.3	141.3	12.9	52.0	54.7	27.3	47.9	47.93	47.1	41.5	38.9
CSW	37.9	149.8	98.2	110.9	65.4	59.4	26.2	30.7	3.53	46.9	33.4	9.6
CSZ	182.5	78.4	233.7	10.5	24.5	4.7	17.8	41.8	26.09	57.2	1.9	4.4
CSAA	88.4	82.6	53.3	74.4	101.9	164.1	61.6	37.0	33.11	35.9	45.3	125.3
CSAB	274.4	273.8	139.3	30.1	41.1	35.5	37.4	53.9	49.17	150.9	68.3	38.3
CSAC	181.4	188.1	160.4	117.3	82	23.6	81.5	105	81.22	44.9	96.0	76.3

Table 10.6: Dietary fibre intake in grams (g)

	Dietary fibre intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	17.97	25.65	25.5	44.81	19.75	18.93	33.06	20.4	21.76	17.81	17.81	23.47
CST	15.7	29.22	13.92	23.84	13.96	16.73	21.52	17.54	16.94	22.25	18.69	18.47
CSU	44.16	35.39	42.53	37.46	44.6	40.97	43.7	47.98	46.51	33.61	29.37	29.08
CSW	6.36	14.09	14.42	21.23	10.59	17.84	18.26	14.92	14.07	10.18	17.71	12.78
CSZ	30.79	21.1	27.13	9.25	7.54	3.08	16.3	26.02	5.89	17.09	16.23	13.19
CSAA	17.2	15.24	15.81	19.12	32.2	42.41	15.51	5.49	12.06	19.55	19.87	13.42
CSAB	37.81	44.93	14.3	17.05	19.39	15.69	12.93	21.88	15.1	21.2	18.32	8.51
CSAC	31.22	27.8	37.95	37.99	24.05	24.25	22.83	23.48	23.79	20.28	22.91	19.35

Table 10.7: Energy intake in kilojoules (kJ) x10^4

	Energy intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	1.13	1.21	0.87	1.45	1.16	1.17	1.04	0.82	1.30	1.35	1.38	0.89
CST	0.57	0.77	0.48	0.54	0.44	0.75	0.69	0.60	0.62	0.56	0.54	0.68
CSU	1.36	0.82	0.89	0.92	0.98	1.02	1.06	1.53	1.45	1.06	0.95	1.13
CSW	0.58	0.75	0.51	0.66	1.48	0.75	0.64	0.46	0.49	0.52	0.71	0.60
CSZ	1.25	0.37	1.41	0.46	0.24	0.27	0.70	0.53	0.23	0.74	0.71	0.58
CSAA	0.56	0.47	0.73	0.90	0.72	1.28	0.95	0.50	0.86	1.05	1.01	0.64
CSAB	1.75	1.27	0.81	0.87	1.08	1.15	0.76	0.77	1.02	1.26	1.07	0.66
CSAC	1.09	1.22	0.85	0.73	0.97	0.78	0.94	0.94	0.82	0.92	1.10	0.65

Table 10.8: Total fat intake in grams (g)

	Total fat intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	137.0	89.2	55.5	167.3	91.0	97.3	94.7	79.5	116.3	143.3	143.3	92.1
CST	50.5	92.1	56.4	26.9	32.1	60.8	28.1	37.5	42.0	50.7	48.1	60.7
CSU	12.4	55.1	59.5	68.9	69.6	73.9	69.0	139.6	127.9	89.7	55.6	91.0
CSW	84.5	65.4	49.5	59.3	149.5	60.0	47.0	42.8	51.9	52.9	83.3	81.8
CSZ	156.9	27.7	181.2	65.9	18.1	26.9	86.7	68.8	25.1	80.4	67.6	80.3
CSAA	67.6	36.5	70.2	93.3	78.1	141.6	114.6	56.2	81.1	128.4	101.2	50.8
CSAB	196.3	106.5	61.4	83.4	131.9	114.2	71.7	91.2	115.2	73.4	103.7	54.4
CSAC	89.0	101.0	74.3	44.0	69.4	58.2	80.6	86.5	73.1	54.8	132.2	57.2

Table 10.9: Saturated Fat intake in grams (g)

	Saturated fat intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	57.99	39.32	22.83	60.36	31.46	38.01	30.94	29.73	34.49	47.17	47.17	32.78
CST	18.88	42.28	17.98	9.31	11.27	22.7	13.62	14.87	18.29	19.4	22.97	32.96
CSU	47.75	19.93	21.15	19.27	19.14	27.72	23.37	51.92	44.59	42.63	26.96	44.69
CSW	38.72	26.35	24.29	24.43	93.63	32.8	23.75	17.56	23.16	19.29	37.87	31.85
CSZ	59.76	6.96	84.52	18.96	6.49	13.39	27.16	15.03	8.18	15.05	15.09	24.17
CSAA	28.5	15.86	16.57	32.31	31.66	59.45	51.02	26.3	32.83	57.04	46.36	26.29
CSAB	87.13	45.81	22.62	38.51	66.7	59.38	35.11	23.33	56.41	28.23	39.26	28.02
CSAC	48.04	40.97	37.55	18.75	31.61	24.8	35.77	34.53	34.86	25.35	59.82	26.85

Table 10.10: Carbohydrate intake in grams (g)

	Carbohydrate intake											
	Month One			Month Two			Month Three			Month Four		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	848.0	405.2	403.4	272.1	412.5	283.0	299.6	241.8	403.1	406.5	406.5	245.4
CST	229.0	141.4	254.1	135.1	170.8	114.1	318.4	226.1	225.5	158.6	162.6	196.3
CSU	389.1	284.8	294.8	299.0	321.8	384.7	345.7	465.7	447.6	290.0	332.7	324.7
CSW	92.5	240.3	146.4	196.8	137.3	140.7	96.1	203.0	122.4	188.7	458.9	232.5
CSZ	265.0	121.6	317.1	62.6	70.2	91.8	118.4	133.0	47.0	161.9	189.4	107.3
CSAA	199.2	172.2	364.3	137.0	147.0	158.6	225.5	110.9	237.2	276.7	288.1	198.7
CSAB	505.5	436.6	252.3	233.6	218.9	308.4	208.2	183.9	251.9	457.1	324.5	224.1
CSAC	317.4	361.8	239.3	213.0	243.7	211.6	267.5	201.7	170.1	280.6	213.1	203.4

Table 10.11: Protein intake in grams (g)

	Protein intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	101.4	117.5	101.3	109.0	97.4	77.2	112.2	73.6	127.6	96.3	96.3	82.9
CST	98.2	89.1	48.2	35	49.9	63.8	27.8	50.5	53.9	62.4	51.8	72.5
CSU	160.5	85.1	110.6	95.2	106.9	97.0	129.8	133.5	130.9	140.9	114.2	146.0
CSW	65.2	63.5	50.9	20.6	85.4	82.2	79.5	44.5	39.2	40.3	37.5	54.4
CSZ	133.4	38.9	127.2	30.9	38.9	14.3	91.9	34.1	34.2	98.3	79.2	55.2
CSAA	48.0	55.1	119.4	132.9	82.4	82.0	91.8	65.1	94.8	61.3	83.6	70.6
CSAB	106.2	89.1	96.0	95.8	130.8	122.0	88.1	72.2	99.1	138.0	82.3	48.6
CSAC	71.3	76.1	71.9	59.3	144.6	95.3	97.3	54.7	67.7	64.0	139.1	43.6

Table 10.12: Vitamin C intake in milligrams (mg)

	Vitamin C intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	184.7	232.1	306.1	195.6	194.3	129.8	55.6	84.3	40.5	26.6	26.6	126.1
CST	31.5	7.0	11.1	68.2	13.5	78.3	111.1	160.1	160.1	34.6	40.5	39.0
CSU	94.0	73.1	169.7	144.7	161.3	126.8	234.1	185.3	243.7	146.8	338.5	153.5
CSW	163.6	175.6	24.5	14.0	121.1	141.9	170.7	67.0	34.3	8.8	58.3	23.5
CSZ	76.2	134.1	17.0	8.9	55.5	3.9	232.0	137.3	52.4	29.8	129.5	115.2
CSAA	61.0	60.7	33.9	62.6	5.8	20.0	82.0	12.3	35.4	43.7	37.0	62.6
CSAB	14.4	31.4	45.1	32.4	58.3	38.5	84.1	216.2	94.9	119.5	115.8	10.5
CSAC	89.3	123.4	102.8	411.2	52.5	268.4	86.4	86.1	116.2	187.8	142.5	129.9

Table 10.13: Vitamin B_6 intake (mg)

	Vitamin B_6 intake											
	Month One			Month Two			Month Three			Month Four		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	1.86	2.15	2.12	2.33	2.4	1.57	2.81	2.06	2.74	2.29	2.29	1.82
CST	1.2	1.05	0.44	1.26	0.77	1.05	0.99	1.84	1.87	1.5	0.83	0.66
CSU	2.4	1.91	2.73	2	2.3	2.19	1.92	1.88	3.59	2.07	1.88	1.76
CSW	0.71	0.88	1.16	0.59	0.85	1.53	1.47	0.48	0.79	0.97	1.04	1.22
CSZ	3.87	0.76	1.46	0.56	0.74	0.51	0.9	0.64	0.35	1.54	0.79	0.52
CSAA	1.25	1.21	1.4	3.06	1.09	1.5	1.86	1.52	2.49	1.76	1.08	1.64
CSAB	1.99	0.99	1.58	1.42	0.81	1.38	0.96	1.42	1.56	3.02	0.93	0.26
CSAC	2.2	2.88	1.4	4.71	1.5	4.67	1.69	1.58	1.83	3.51	4.12	1.43

Table 10.14: Vitamin B_{12} intake (µg)

	Vitamin B_{12} intake											
	Month One			**Month Two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	2.85	5.94	2.33	4.5	2.89	3.77	5.48	2.64	3.31	3.53	3.53	3.38
CST	3.96	4.06	2.48	1.77	6.02	3.31	1.01	5.58	6.14	7.06	5.36	5.49
CSU	3.99	2.59	4.92	14.36	14.53	4.44	2.24	1.92	3.94	2.34	2.39	2.92
CSW	4.94	2.76	4.92	0.12	2.04	1.46	3.7	1.92	1.22	4.43	1.62	2.34
CSZ	6.5	0.6	12.42	0.44	1.76	0.71	3.16	0.86	0.74	3.12	3.89	2.13
CSAA	2.84	8.9	8.16	9.48	4.32	3.02	5.84	3.45	2.47	4.08	3.13	1.34
CSAB	4.02	1.52	2.9	2.15	10.43	5.43	3.99	1.85	4.71	7.75	2.68	1.52
CSAC	2.13	3.56	3.84	2.89	5.22	7.32	3.8	1.93	1.08	2.94	10.43	2.06

Table 10.15: Calcium intake in milligrams (mg) (x10^3)

	Calcium intake											
	Month One			**Month two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	.37	1.09	1.13	1.47	1.33	1.02	1.03	0.79	1.41	1.23	1.23	1.39
CST	.56	0.56	0.16	0.27	0.36	0.67	0.27	0.32	0.51	0.40	0.37	1.23
CSU	.24	1.06	1.31	0.94	1.06	1.09	1.66	1.57	1.43	1.71	1.36	1.68
CSW	.55	0.41	0.54	0.14	1.16	0.55	0.52	0.22	0.65	0.23	0.14	0.50
CSZ	.79	0.60	1.24	0.30	0.22	0.23	0.62	0.44	0.21	0.46	0.60	0.88
CSAA	.32	0.50	0.34	0.45	0.39	0.66	1.30	1.04	0.71	0.97	1.18	0.46
CSAB	.12	0.98	1.28	1.20	2.44	2.18	1.34	0.36	1.41	1.24	0.59	1.05
CSAC	.64	0.90	0.76	0.58	0.58	0.47	0.57	0.54	0.50	0.67	0.75	0.45

Table 10.16: Sodium intake in micrograms (µg) (x10^3)

	Sodium intake											
	Month One			**Month two**			**Month Three**			**Month Four**		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	3.34	3.35	2.02	3.91	2.79	1.34	1.24	3.53	2.57	3.64	3.65	1.89
CST	0.97	3.82	1.57	0.61	1.64	1.28	0.54	1.48	1.37	1.12	1.48	2.56
CSU	5.16	3.65	3.47	1.80	2.45	1.59	1.76	2.13	2.68	3.66	2.45	1.77
CSW	1.83	2.78	1.51	0.59	2.49	1.70	1.60	0.84	2.24	1.21	0.92	0.78
CSZ	2.80	1.27	5.82	2.07	1.42	1.54	1.36	1.80	0.27	1.05	0.68	1.50
CSAA	1.48	0.95	1.80	1.13	0.56	2.03	1.96	0.91	4.84	1.86	5.76	1.04
CSAB	3.47	3.23	2.41	2.42	2.37	4.91	1.86	2.63	2.48	2.09	3.64	1.19
CSAC	3.06	2.89	3.53	1.65	6.67	1.09	4.43	2.45	1.30	0.98	2.45	3.24

Table 10.17: Iron Intake in milligrams (mg)

Iron	Month One			Month two			Month Three			Month Four		
Sub code	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
CSI	23.1	20.6	11.6	12.5	12.8	13.3	19.4	11.8	14.5	12.7	12.7	12.6
CST	12.2	19.8	13.3	7.81	7.0	7.28	3.51	6.95	7.74	5.47	4.95	7.62
CSU	26.7	17.4	19.8	9.2	10.7	10.7	11.3	13.4	12.0	13.4	10.6	11.3
CSW	11.3	8.88	4.95	4.91	6.84	7.42	13.8	6.62	3.97	2.26	4.78	5.28
CSZ	21.6	11.5	16.7	8.0	4.87	2.82	10.0	5.46	2.3	11.1	9.52	6.09
CSAA	15.4	11.7	12.1	19.8	8.22	11.5	9.76	4.37	5.98	6.25	5.16	5.43
CSAB	17.5	14.7	13.4	7.1	6.9	11.1	6.1	9.8	7.4	15.2	9.0	1.7
CSAC	18.3	14.4	12.4	12.6	17.5	9.12	12.41	5.04	6.0	5.21	16.0	5.9

Appendix I: Results of Kruskall-Wallis statistical analysis for coeliac group

Table 10.18: Summary of statistical analysis for coeliac group

Nutrient	Change	Mann-Whitney U score	Probability
Gluten	**Significant ↓**		
	Month 1-2	576.000	< 0.0005
	Month 1-3	576.000	< 0.0005
	Month 1-4	576.000	< 0.0005
	Not significant		
	Month 2-3	259.500	0.534
	Month 2-4	302.500	0.745
Starch	**Significant ↓**		
	Month 1-2	505.000	< 0.0005
	Month 1-3	544.000	< 0.0005
	Month 1-4	518.000	< 0.0005
Fibre	**Not significant**		
	Month 1-2	310.000	0.650
	Month 1-3	344.000	0.284
	Month 1-4	365.000	0.112
Energy	**Not significant**		
	Month 1-2	281.000	0.885
	Month 1-3	297.000	0.853
	Month 1-4	298.000	0.837
Total fat	**Not significant**		
	Month 1-2	289.000	0.984
	Month 1-3	291.000	0.951
	Month 1-4	271.500	0.734
Saturated fat	**Not significant**		
	Month 1-2	305.000	0.537
	Month 1-3	319.000	0.523
	Month 1-4	289.000	0.984
Carbohydrate	**Significant ↓**		
	Month 1-2	409.000	0.013

	Not significant	378.000	0.063
	Month 1-3	325.000	0.445
	Month 1-4		
Protein	**Not significant**		
	Month 1-2	319.000	0.523
	Month 1-3	335.000	0.332
	Month 1-4	332.000	0.364
Vitamin C	**Not significant**		
	Month 1-2	295.000	0.885
	Month 1-3	223.000	0.180
	Month 1-4	296.000	0.869
Vitamin B_6	**Not significant**		
	Month 1-2	305.500	0.718
	Month 1-3	304.000	0.741
	Month 1-4	314.000	0.592
Vitamin B_{12}	**Not significant**		
	Month 1-2	295.000	0.885
	Month 1-3	379.500	0.059
	Month 1-4	332.000	0.364
Calcium	**Not significant**		
	Month 1-2	318.000	0.536
	Month 1-3	295.000	0.885
	Month 1-4	281.000	0.885
Sodium	**Significant ↓**		
	Month 1-2	392.000	0.032
	Month 1-3	394.000	0.029
	Not significant		
	Month 1-4	374.000	0.076
Iron	**Very significant ↓**		
	Month 1-2	480.000	0.000
	Month 1-3	486.500	0.000
	Month 1-4	491.000	0.000

K-W Calculations for Macronutrients

(A) GLUTEN

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	2004.000
b	**24**	862.000
c	**24**	941.000
d	**24**	753.000

Kruskal-Wallis Test Statistic = 57.101
Probability is 0.000 assuming Chi-square Distribution with 3 df

Kruskal-Wallis One-way Analysis of Variance for 48 Cases
Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

The categorical values encountered during processing are
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	876.000
b	**24**	300.000

Mann-Whitney U test statistic = 576.000
Probability is 0.000
Chi-square approximation = 35.847 with 1 df significant

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	876.000
c	**24**	300.000

Mann-Whitney U test statistic = 576.000
Probability is 0.000
Chi-square approximation = 35.699 with 1 df significant

Kruskal-Wallis One-way Analysis of Variance for 48 Cases
Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d
The categorical values encountered during processing are
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	876.000
d	**24**	300.000

Mann-Whitney U test statistic = 576.000
Probability is 0.000
Chi-square approximation = 36.267 with 1 df significant

Kruskal-Wallis One-way Analysis of Variance for 48 Cases
Data for the following results were selected according to:
(TYPE$ = 'b') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
b, c
The categorical values encountered during processing are
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
b	**24**	559.500
c	**24**	616.500

Mann-Whitney U test statistic = 259.500
Probability is 0.534

Chi-square approximation = 0.386 with 1 df

Kruskal-Wallis One-way Analysis of Variance for 48 Cases
Data for the following results were selected according to:
(TYPE$ = 'c') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
c,d

The categorical values encountered during processing are
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
c	**24**	625.500
d	**24**	550.500

Mann-Whitney U test statistic = 325.500
Probability is 0.404
Chi-square approximation = 0.695 with 1 df

(B) STARCH

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1867.000
b	**24**	1041.000
c	**24**	827.000
d	**24**	921.000

Kruskal-Wallis Test Statistic = 36.618
Probability is 0.000 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	805.000
b	**24**	371.000

Mann-Whitney U test statistic = 505.000
Probability is 0.000
Chi-square approximation = 20.022 with 1 df very significant dec

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	844.000
c	**24**	332.000

Mann-Whitney U test statistic = 544.000
Probability is 0.000
Chi-square approximation = 27.867 with 1 df very significant dec

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	818.000
d	**24**	358.000

Mann-Whitney U test statistic = 518.000
Probability is 0.000
Chi-square approximation = 22.494 with 1 df significant

(C) ENERGY

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1160.000
b	**24**	1218.000
c	**24**	1091.000
d	**24**	1187.000

Kruskal-Wallis Test Statistic = 0.472
Probability is 0.925 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	581.000
b	**24**	595.000

Mann-Whitney U test statistic = 281.000
Probability is
Chi-square approximation = 0.021 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	597.000
c	**24**	579.000

Mann-Whitney U test statistic = 297.000
Probability is 0.853
Chi-square approximation = 0.034 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	598.000
d	**24**	578.000

Mann-Whitney U test statistic = 298.000
Probability is 0.837
Chi-square approximation = 0.043 with 1 df

(D) PROTEIN

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1263.000
b	**24**	1137.000
c	**24**	1067.000
d	**24**	1093.000

Kruskal-Wallis Test Statistic = 1.091
Probability is 0.779 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases

Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	619.000
b	**24**	557.000

Mann-Whitney U test statistic = 319.000
Probability is 0.523 Not significant
Chi-square approximation = 0.409 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	635.000
c	**24**	541.000

Mann-Whitney U test statistic = 335.000
Probability is 0.332 not significant
Chi-square approximation = 0.939 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	632.000
d	**24**	544.000

Mann-Whitney U test statistic = 332.000
Probability is 0.364 not significant
Chi-square approximation = 0.823 with 1 df

(E) TOTAL FAT

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1151.500
b	**24**	1145.000
c	**24**	1130.000
d	**24**	1229.500

Kruskal-Wallis Test Statistic = 0.320
Probability is 0.956 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases

Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	589.000
b	**24**	587.000

Mann-Whitney U test statistic = 289.000
Probability is 0.984 not s**i**gnificant
Chi-square approximation = 0.000 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	591.000
c	**24**	585.000

Mann-Whitney U test statistic = 291.000
Probability is 0.951 not significant
Chi-square approximation = 0.004with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases

Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	571.500
d	**24**	604.500

Mann-Whitney U test statistic = 271.500
Probability is 0.734 not significant
Chi-square approximation = 0.116 with 1 df

(F) SATURATED FAT

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1243.000
b	**24**	1099.000
c	**24**	1061.000
d	**24**	1253.000

Kruskal-Wallis Test Statistic = 1.557
Probability is 0.669 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases

Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	605.000
b	**24**	523.000

Mann-Whitney U test statistic = 305.000
Probability is 0.537 not **s**ignificant
Chi-square approximation = 0.381with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	619.000
c	**24**	557.000

Mann-Whitney U test statistic = 319.000
Probability is 0.523 not significant
Chi-square approximation = 0.409 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	589.000
d	**24**	587.000

Mann-Whitney U test statistic = 289.000
Probability is 0.984 not significant
Chi-square approximation = 0.000 with 1 df

(G) CARBOHYDRATE

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1412.000
b	**24**	914.500
c	**24**	1058.000
d	**24**	1271.500

Kruskal-Wallis Test Statistic = 7.869
Probability is 0.049 assuming Chi-square Distribution with 3 df
Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	709.000
b	**24**	467.000

Mann-Whitney U test statistic = 409.000
Probability is 0.013 significant
Chi-square approximation = 6.225 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	678.000
c	**24**	498.000

Mann-Whitney U test statistic = 378.000
Probability is 0.063 Just about significant
Chi-square approximation = 3.444 with 1 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	625.000
d	**24**	551.000

Mann-Whitney U test statistic = 325.000

Probability is 0.445 not significant
Chi-square approximation = 0.582 with 1 df

(H) FIBRE

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1319.000
b	**24**	1218.000
c	**24**	1096.000
d	**24**	1023.000

Kruskal-Wallis Test Statistic = 2.762
Probability is 0.430 assuming Chi-square distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-way Analysis of Variance for 48 Cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	610.000
b	**24**	566.000

Mann-Whitney U test statistic = 310.000
Probability is 0.650
Chi-square approximation = 0.206 with 1 df not significant
Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	644.000
c	**24**	532.000

Mann-Whitney U test statistic = 344.000
Probability is 0.284
Chi-square approximation = 1.333 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	665.000
d	**24**	511.000

Mann-Whitney U test statistic = 365.000
Probability is 0.112
Chi-square approximation = 2.521 with 1 df not significant

Micronutrients

(A) VITAMIN C

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1114.000
b	**24**	1105.000
c	**24**	1367.000
d	**24**	1070.000

Kruskal-Wallis Test Statistic = 3.008
Probability is 0.390 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
b	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885
Chi-square approximation = 0.021with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	523.000
c	**24**	653.000

Mann-Whitney U test statistic = 223.000
Probability is 0.180
Chi-square approximation = 1.796with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	596.000
d	**24**	580.000

Mann-Whitney U test statistic = 296.000
Probability is 0.869
Chi-square approximation = 0.027with 1 df not significant

(B) VITAMIN B6

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
The categorical values encountered during processing are
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1223.500
b	**24**	1124.500
c	**24**	1185.000
d	**24**	1123.000

Kruskal-Wallis Test Statistic = 0.388
Probability is 0.943 assuming Chi-square Distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	605.500
b	**24**	570.500

Mann-Whitney U test statistic = 305.500
Probability is 0.718

Chi-square approximation = 0.130 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	604.000
c	**24**	572.000

Mann-Whitney U test statistic = 304.000
Probability is 0.741
Chi-square approximation = 0.109 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	614.000
d	**24**	562.000

Mann-Whitney U test statistic = 314.000
Probability is 0.592
Chi-square approximation = 0.287 with 1 df not significant

(C) VITAMIN B12

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
The categorical values encountered during processing are
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1306.500
b	**24**	1225.000
c	**24**	975.000
d	**24**	1149.500

Kruskal-Wallis Test Statistic = 3.220
Probability is 0.359 assuming Chi-square distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
b	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885

Chi-square approximation = 0.021with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	679.500
c	**24**	496.500

Mann-Whitney U test statistic = 379.500
Probability is 0.059
Chi-square approximation = 3.560with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	632.000
d	**24**	544.000

Mann-Whitney U test statistic = 332.000
Probability is 0.364
Chi-square approximation = 0.823 with 1 df not significant

(D) CALCIUM

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
The categorical values encountered during processing are
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1192.500
b	**24**	1079.000
c	**24**	1154.000
d	**24**	1230.500

Kruskal-Wallis Test Statistic = 0.675
Probability is 0.879 assuming Chi-square distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	618.000
b	**24**	558.000

Mann-Whitney U test statistic = 318.000
Probability is 0.536
Chi-square approximation = 0.383 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
c	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885
Chi-square approximation = 0.021 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1

Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	581.000
d	**24**	595.000

Mann-Whitney U test statistic = 281.000
Probability is 0.885
Chi-square approximation = 0.021 with 1 df NOT significant

(E) SODIUM

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
The categorical values encountered during processing are
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1460.000
b	**24**	1049.000
c	**24**	1073.000
d	**24**	1074.000

Kruskal-Wallis Test Statistic = 6.294
Probability is 0.098 assuming Chi-square distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	692.000
b	**24**	484.000

Mann-Whitney U test statistic = 392.000
Probability is 0.032

Chi-square approximation = 4.599 with 1 df significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	694.000
c	**24**	482.000

Mann-Whitney U test statistic = 394.000
Probability is 0.029
Chi-square approximation = 4.777 with 1 df significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	674.000
d	**24**	502.000

Mann-Whitney U test statistic = 374.000
Probability is 0.076 not significant
Chi-square approximation = 3.145 with 1 df

(F) IRON

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
The categorical values encountered during processing are
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1745.000
b	**24**	1034.000
c	**24**	915.500
d	**24**	961.500

Kruskal-Wallis Test Statistic = 24.555
Probability is 0.000 assuming Chi-square distribution with 3 df

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'b')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, b

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	780.000
b	**24**	396.000

Mann-Whitney U test statistic = 480.000
Probability is 0.000
Chi-square approximation = 15.673 with 1 df very significant decrease

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	786.500
c	**24**	389.500

Mann-Whitney U test statistic = 486.500
Probability is 0.000
Chi-square approximation = 16.754 with 1 df very significant decrease

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	791.500
d	**24**	384.500

Mann-Whitney U test statistic = 491.500
Probability is 0.000 very significant decrease
Chi-square approximation = 17.609 with 1 df

Appendix J: Results of Kruskall- Wallis analysis for the non-coeliac control group

Table 10.19: Summary of statistical analysis for control group

Nutrient	Change	Mann-Whitney U score	Probability
Starch	**Not Significant**		
	Month 1-2	311.000	0.635
	Month 1-3	281.000	0.885
	Month 1-4	298.000	0.837
Fibre	**Not significant**		
	Month 1-2	307.500	0.503
	Month 1-3	269.000	0.695
	Month 1-4	293.000	0.918
Energy	**Not significant**		
	Month 1-2	298.000	0.837
	Month 1-3	295.000	0.885
	Month 1-4	328.000	0.409
Total fat	**Not significant**		
	Month 1-2	342.000	0.266
	Month 1-3	302.000	0.773
	Month 1-4	328.000	0.409
Saturated fat	**Not significant**		
	Month 1-2	310.000	0.650
	Month 1-3	317.000	0.550
	Month 1-4	333.500	0.348
Carbohydrate	**Not significant**		
	Month 1-2	294.000	0.902
	Month 1-3	313.000	0.606
	Month 1-4	312.000	0.621
Protein	**Not significant**		
	Month 1-2	246.000	0.386
	Month 1-3	314.000	0.592
	Month 1-4	290.000	0.967

Vitamin C	**Not significant**		
	Month 1-2	337.500	0.307
	Month 1-3	363.000	0.122
	Month 1-4	309.000	0.665
Vitamin B_6	**Not significant**		
	Month 1-2	262.500	0.599
	Month 1-3	248.000	0.409
	Month 1-4	249.000	0.421
Vitamin B_{12}	**Not significant**		
	Month 1-2	289.500	0.975
	Month 1-3	313.500	0.599
	Month 1-4	270.500	0.718
Sodium	**Not significant**		
	Month 1-2	336.000	0.322
	Month 1-3	323.000	0.470
	Month 1-4	351.000	0.194
Calcium	**Not significant**		
	Month 1-2	298.000	0.837
	Month 1-3	295.000	0.885
	Month 1-4	328.000	0.409
Iron	**Not significant**		
	Month 1-2	248.000	0.409
	Month 1-3	306.500	0.703
	Month 1-4	295.000	0.885

K-W Calculations for Macronutrients

(A) ENERGY

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1247.000
b	**24**	1208.000
c	**24**	1143.000
d	**24**	1058.000

Kruskal-Wallis Test Statistic = 1.101
Probability is 0.777 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	598.000
b	**24**	578.000

Mann-Whitney U test statistic = 298.000
Probability is 0.837
Chi-square approximation = 0.043 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
c	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885
Chi-square approximation = 0.021 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	628.000
d	**24**	548.000

Mann-Whitney U test statistic = 328.000
Probability is 0.409
Chi-square approximation = 0.680 with 1 df not significant

(B) PROTEIN

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	24	1150.000
b	24	1315.000
c	24	1059.000
d	24	1132.000

Kruskal-Wallis Test Statistic = 1.882
Probability is 0.597 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	546.000
b	**24**	630.000

Mann-Whitney U test statistic = 246.000
Probability is 0.386
Chi-square approximation = 0.750with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	614.000
c	**24**	562.000

Mann-Whitney U test statistic = 314.000
Probability is 0.592
Chi-square approximation = 0.287with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	590.000
d	**24**	586.000

Mann-Whitney U test statistic = 290.000
Probability is 0.967
Chi-square approximation = 0.002 with 1 df

(C) TOTAL FAT

Kruskal-Wallis One-Way Analysis of Variance for 96 cases

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1272.000
b	**24**	1060.000
c	**24**	1229.000
d	**24**	1095.000

Kruskal-Wallis Test Statistic = 1.690
Probability is 0.639 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	642.000
b	**24**	534.000

Mann-Whitney U test statistic = 342.000
Probability is 0.266
Chi-square approximation = 1.240 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	602.000
c	**24**	574.000

Mann-Whitney U test statistic = 302.000
Probability is 0.773
Chi-square approximation = 0.083 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	628.000
d	**24**	548.000

Mann-Whitney U test statistic = 328.000
Probability is 0.409
Chi-square approximation = 0.680with 1 df

(D) SATURATED FAT

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1260.500
b	**24**	1182.000
c	**24**	1141.000
d	**24**	1072.500

Kruskal-Wallis Test Statistic = 0.995
Probability is 0.802 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	610.000
b	**24**	566.000

Mann-Whitney U test statistic = 310.000
Probability is 0.650
Chi-square approximation = 0.206 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:

TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	617.000
c	**24**	559.000

Mann-Whitney U test statistic = 317.000
Probability is 0.550
Chi-square approximation = 0.358 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	633.500
d	**24**	542.500

Mann-Whitney U test statistic = 333.500
Probability is 0.348
Chi-square approximation = 0.880 with 1 df

(E) CARBOHYDRATES

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1,219.000
b	**24**	1,195.000
c	**24**	1,099.000
d	**24**	1,143.000

Kruskal-Wallis Test Statistic = 0.465
Probability is 0.927 assuming Chi-square Distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	594.000
b	**24**	582.000

Mann-Whitney U test statistic = 294.000
Probability is 0.902
Chi-square approximation = 0.015 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	613.000
c	**24**	563.000

Mann-Whitney U test statistic = 313.000
Probability is 0.606
Chi-square approximation = 0.266 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	612.000
d	**24**	564.000

Mann-Whitney U test statistic = 312.000
Probability is 0.621
Chi-square approximation = 0.245 with 1 df

(F) STARCH

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1190.000
b	**24**	1088.000
c	**24**	1212.000
d	**24**	1166.000

Kruskal-Wallis Test Statistic = 0.470
Probability is 0.925 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	611.000
b	**24**	565.000

Mann-Whitney U test statistic = 311.000
Probability is 0.635
Chi-square approximation = 0.225 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	581.000
c	**24**	595.000

Mann-Whitney U test statistic = 281.000
Probability is 0.885
Chi-square approximation = 0.021 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	598.000
d	**24**	578.000

Mann-Whitney U test statistic = 298.000
Probability is 0.837
Chi-square approximation = 0.043 with 1 df

(G) FIBRE

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1170.500
b	**24**	1082.000
c	**24**	1228.000
d	**24**	1175.500

Kruskal-Wallis Test Statistic = 0.590
Probability is 0.899 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	607.500
b	**24**	520.500

Mann-Whitney U test statistic = 307.500
Probability is 0.503
Chi-square approximation = 0.449 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	569.000
c	**24**	607.000

Mann-Whitney U test statistic = 269.000
Probability is 0.695
Chi-square approximation = 0.153 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	593.000
d	**24**	583.000

Mann-Whitney U test statistic = 293.000
Probability is 0.918
Chi-square approximation = 0.011 with 1 df

Micronutrients

(A) VITAMIN C

Kruskal-Wallis One-way Analysis of Variance for 96 Cases

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1309.500
b	**24**	1139.500
c	**24**	963.000
d	**24**	1244.000

Kruskal-Wallis Test Statistic = 3.682
Probability is 0.298 assuming Chi-square Distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	637.500
b	**24**	538.500

Mann-Whitney U test statistic = 337.500
Probability is 0.307
Chi-square approximation = 1.042 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	663.000
c	**24**	513.000

Mann-Whitney U test statistic = 363.000
Probability is 0.122
Chi-square approximation = 2.392 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	609.000
d	**24**	567.000

Mann-Whitney U test statistic = 309.000
Probability is 0.665
Chi-square approximation = 0.188 with 1 df

(B) VITAMIN B6

Kruskal-Wallis One-way Analysis of Variance for 96 Cases
Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1059.500
b	**24**	1130.000
c	**24**	1229.000
d	**24**	1237.500

Kruskal-Wallis Test Statistic = 1.165
Probability is 0.761 assuming Chi-square Distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	562.500
b	**24**	613.500

Mann-Whitney U test statistic = 262.500
Probability is 0.599
Chi-square approximation = 0.277 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	548.000
c	**24**	628.000

Mann-Whitney U test statistic = 248.000
Probability is 0.409
Chi-square approximation = 0.680 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	549.000
d	**24**	627.000

Mann-Whitney U test statistic = 249.000
Probability is 0.421
Chi-square approximation = 0.647 with 1 df

(C) VITAMIN B12

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Kruskal-Wallis One-Way Analysis of Variance for 96 cases

Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1172.500
b	**24**	1154.000
c	**24**	1082.000
d	**24**	1151.500

Kruskal-Wallis Test Statistic = 0.421
Probability is 0.936 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	589.500
b	**24**	586.500

Mann-Whitney U test statistic = 289.500
Probability is 0.975
Chi-square approximation = 0.001with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	613.500
c	**24**	562.500

Mann-Whitney U test statistic = 313.500
Probability is 0.599
Chi-square approximation = 0.276 with 1 df significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	570.500
d	**24**	605.500

Mann-Whitney U test statistic = 270.500
Probability is 0.718
Chi-square approximation = 0.130 with 1 df

(D) CALCIUM

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1221.000
b	**24**	1195.000
c	**24**	1185.000
d	**24**	1055.000

Kruskal-Wallis Test Statistic = 0.888
Probability is 0.828 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	598.000
b	**24**	578.000

Mann-Whitney U test statistic = 298.000
Probability is 0.837
Chi-square approximation = 0.043with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
c	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885
Chi-square approximation = 0.021with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	628.000
d	**24**	548.000

Mann-Whitney U test statistic = 328.000
Probability is 0.409
Chi-square approximation = 0.680 with 1 df

(E) SODIUM

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1310.000
b	**24**	1101.000
c	**24**	1183.000
d	**24**	1062.000

Kruskal-Wallis Test Statistic = 1.936
Probability is 0.586 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	636.000
b	**24**	540.000

Mann-Whitney U test statistic = 336.000
Probability is 0.322
Chi-square approximation = 0.980 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	623.000
c	**24**	553.000

Mann-Whitney U test statistic = 323.000
Probability is 0.470
Chi-square approximation = 0.521with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	651.000
d	**24**	525.000

Mann-Whitney U test statistic = 351.000
Probability is 0.194
Chi-square approximation = 1.688 with 1 df

(F) IRON

Categorical values encountered during processing are:
MONTH$ (4 levels)
a, b, c, d

Kruskal-Wallis One-Way Analysis of Variance for 96 cases
Dependent variable is A1
Grouping variable is MONTH$

Group	Count	Rank Sum
a	**24**	1149.500
b	**24**	1312.000
c	**24**	1072.000
d	**24**	1122.500

Kruskal-Wallis Test Statistic = 1.734
Probability is 0.629 assuming Chi-square distribution with 3 df

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	548.000
b	**24**	628.000

Mann-Whitney U test statistic = 248.000
Probability is 0.409
Chi-square approximation = 0.680 with 1 df not significant

Data for the following results were selected according to:
(TYPE$ = 'a') OR (TYPE$ = 'c')

Categorical values encountered during processing are:
TYPE$ (2 levels)
a, c

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	606.500
c	**24**	569.500

Mann-Whitney U test statistic = 306.500
Probability is 0.703
Chi-square approximation = 0.146 with 1 df not significant

Data for the following results were selected according to:

(TYPE$ = 'a') OR (TYPE$ = 'd')
Categorical values encountered during processing are:
TYPE$ (2 levels)
a, d

Kruskal-Wallis One-Way Analysis of Variance for 48 cases
Dependent variable is A1
Grouping variable is TYPE$

Group	Count	Rank Sum
a	**24**	595.000
d	**24**	581.000

Mann-Whitney U test statistic = 295.000
Probability is 0.885
Chi-square approximation = 0.021with 1 df not significant

Appendix K: Dietary data for coeliac study sample group with coeliac disease

Table 1: Macronutrient intake at monthly intervals for each participant

Coeliac study sample group with coeliac disease									
subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSI 01									
Day 1	11352.7	101.4	137.03	57.99	272.11	162.52	17.97	109.12	1916.45
Day 2	12128	117.53	89.23	39.32	412.52	193.86	25.65	218.18	1939.81
Day 3	8795.04	101.35	55.52	22.83	283.06	159.23	25.5	123.29	1813.29
daily av	10758.62	106.75	93.92	40.04	322.56	171.87	23.03	120.38	1889.84
CSI 02									
Day 1	14548.3	109.02	167.33	60.36	848.01	209.27	44.81	57.77	1607
Day 2	11676.3	97.46	91.06	31.46	405.28	257.76	19.75	82.8	2386.81
Day 3	11715.1	77.23	97.39	38.01	403.61	245.68	18.93	77.96	1510.77
daily av	12646.64	94.56	118.59	43.27	552.3	237.56	27.83	72.84	1834.98
CSI 03									
Day 1	10418.9	112.2	94.79	30.94	299.61	189.72	33.06	72.56	1879.18
Day 2	8236.97	73.6	79.5	29.73	241.89	133.86	20.4	87.53	1773.22
Day 3	13025.8	127.64	116.35	34.49	403.18	250.26	21.76	80.02	2257.45
daily av	10560.6	104.48	96.88	31.71	314.89	191.28	25.07	80.03	1969.95
CSI 04									
Day 1	13580.8	96.37	143.31	47.17	406.58	248.07	17.81	85.63	1794.99
Day 2	13580.8	96.37	143.31	47.17	406.58	248.07	17.81	85.63	1794.99
Day 3	8928.06	82.92	92.13	32.78	245.4	163.03	23.47	72.46	1462.81
daily av	12029.92	91.88	126.25	42.37	352.85	219.72	19.69	81.24	1684.26

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CST 01									
Day 1	5783.46	98.21	50.52	18.88	135.16	42.26	15.7	93.03	1310
Day 2	7787.46	89.19	92.13	42.28	170.82	79.18	29.22	91.67	725
Day 3	4821.86	48.26	56.43	17.98	114.18	21.33	13.92	92.88	562.39
daily av	6130.92	78.55	66.36	26.38	140.05	47.59	19.61	92.52	866.02
CST 02									
Day 1	5430.67	35	26.91	9.31	229.06	129.71	23.84	57.79	1347.55
Day 2	4405.64	49.94	32.16	11.27	141.49	20.61	13.96	40.33	763.64
Day 3	7560.23	63.88	60.8	22.7	254.1	123.62	16.73	61.03	1323.56
daily av	5798.84	49.6	39.95	14.42	208.21	91.31	18.18	3.05	1144.91
CST 03									
Day 1	6917.42	27.88	28.14	13.62	318.44	149.61	21.52	26.61	1805.32
Day 2	6038.47	50.5	37.53	14.87	226.18	114.69	17.54	26.65	1207.74
Day 3	6255.35	53.98	42.02	18.29	225.59	115.07	16.94	25.69	942.94
daily av	6403.74	44.12	35.89	15.59	256.73	126.45	18.66	26.67	1318.67
CST 04									
Day 1	5694.88	62.4	50.73	19.4	158.65	49.27	22.25	11.78	1159.95
Day 2	5476.91	51.87	48.1	22.97	162.61	38.07	18.69	42.24	1171.81
Day 3	6872.93	72.59	60.71	32.96	196.38	102.9	18.47	9.13	1321.9
daily av	6014.9	62.28	53.17	25.11	172.54	63.41	19.8	21.05	1217.88
CSU 01									
Day 1	13647.6	160.56	12.49	47.75	389.15	187.81	44.16	195.71	2887.53
Day 2	8211.13	85.11	55.11	19.93	284.85	139.98	35.39	141.35	2390.95
Day 3	8971.94	110.62	59.56	21.15	294.85	147.3	42.53	142.85	2895.24
daily av	10276.9	118.76	78.38	29.61	322.95	158.36	40.69	159.96	2724.57

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSU 02									
Day 1	9276.89	95.23	68.93	19.27	299.04	144.47	37.46	12.93	2491.95
Day 2	9853.03	106.9	69.66	19.14	321.86	153.92	44.6	52.09	2716.8
Day 3	10274.6	97.08	73.9	27.72	384.73	217.37	40.97	54.7	2727.32
daily av	9801.52	99.73	70.82	22.04	323.21	171.92	41.01	39.9	2645.36
CSU 03									
Day 1	10616.2	129.83	69.07	23.37	345.75	218.5	43.7	27.32	3035.79
Day 2	15331.4	133.51	139.64	51.92	465.77	275.05	47.98	47.93	3040.23
Day 3	14548	130.95	127.95	44.59	447.6	272.63	46.51	29.36	2945.11
daily av	13498.58	131.42	112.22	39.95	419.7	255.39	46.06	34.87	3007.04
CSU 04									
Day 1	10607.1	140.91	89.76	42.63	290.09	151.11	33.61	47.11	3084.57
Day 2	9572.35	114.2	55.67	26.96	332.72	196.54	29.37	41.52	3348.4
Day 3	11334.2	146.08	91.04	44.69	324.72	140.54	29.08	38.91	2911.53
daily av	10504.58	133.73	78.82	38.09	315.84	162.72	30.68	42.51	3114.83
CSW 01									
Day 1	5802.4	65.25	84.5	38.72	92.52	53.63	6.36	37.96	1229.42
Day 2	7514.37	63.51	65.49	26.35	240.31	89.73	14.09	149.82	959.51
Day 3	5140.45	50.96	49.59	24.29	146.48	48.22	14.42	98.2	1897.66
daily av	6152.4	59.91	66.52	29.78	159.77	63.85	11.62	95.32	1362.19
CSW 02									
Day 1	6686.74	20.62	59.32	24.43	188.73	55.98	21.23	110.92	752.17
Day 2	14836.5	85.42	149.51	93.63	458.93	173.95	10.59	65.4	1297.8
Day 3	7529.41	82.21	60.08	32.8	232.96	132.03	17.84	59.42	1595.59
daily av	9684.21	62.75	89.63	50.28	293.53	120.65	16.55	78.57	1215.18

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSW 03									
Day 1	6400.67	79.54	47.02	23.75	196.83	115.09	18.26	26.29	2222.39
Day 2	4660.21	44.54	42.84	17.56	137.37	43.7	14.92	30.72	1646.56
Day 3	4990.2	39.23	51.99	23.16	140.78	60.91	14.07	3.53	1910.07
daily av	6400.66	54.43	47.28	21.49	158.32	73.23	15.75	20.18	1926.34
CSW 04									
Day 1	5243	40.31	52.94	19.29	96.1	36.17	10.18	46.97	1347.09
Day 2	7159.33	37.54	83.37	37.87	203.05	100.17	17.71	33.45	1835.01
Day 3	6068.82	54.4	81.83	31.85	122.45	73.99	12.78	9.63	1703.54
daily av	6157.16	44.08	72.71	29.67	140.53	70.11	13.55	30.01	1628.54
CSZ 01									
Day 1	12512.5	133.48	156.95	59.76	265.01	82.44	30.79	182.58	1460.69
Day 2	3702.86	38.94	27.78	6.96	121.62	43.16	21.1	78.4	3562
Day 3	14196.6	127.27	181.29	84.52	317.1	83.2	27.13	233.72	1365.36
daily av	10137.37	99.89	122	50.41	234.57	69.6	26.34	164.9	2129.67
CSZ 02									
Day 1	4698.04	30.95	65.95	18.96	62.6	24.23	9.25	10.56	1243.56
Day 2	2497.16	38.97	18.12	6.49	70.24	33.75	7.54	24.58	790.5
Day 3	2799.33	14.37	26.99	13.39	91.85	50.2	3.08	4.7	1315.99
daily av	3331.51	28.09	37.02	12.94	74.89	36.06	6.62	13.28	1116.68
CSZ 03									
Day 1	7097.58	91.93	86.73	27.16	118.43	81.93	16.3	17.84	2855.69
Day 2	5384.02	34.15	68.87	15.03	133.08	62.07	26.02	41.82	2781.41
Day 3	2311.5	34.21	25.18	8.18	47.07	16.13	5.89	26.09	406.05
daily av	4931.03	53.42	60.25	16.79	99.52	53.37	16.07	28.58	2014.38

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSZ 04									
Day 1	7430.49	98.36	80.43	15.05	161.98	62	17.09	57.27	2370.24
Day 2	7118.6	79.24	67.64	15.09	189.42	78.85	16.23	1.98	2582.49
Day 3	5810.95	55.24	80.35	24.17	107.3	42.69	13.19	4.42	2702.32
daily av	6786.68	77.8	76.14	18.1	152.9	61.18	15.5	21.22	2551.68
CSAA 01									
Day 1	5651.41	48.09	67.61	28.5	137.07	42.73	17.2	88.47	815.06
Day 2	4736.09	55.12	36.58	15.86	147.09	64.4	15.24	82.69	942.18
Day 3	7308.19	119.47	70.27	16.57	158.65	104.7	15.81	53.32	569.93
daily av	5898.56	74.22	58.15	20.64	147.6	70.61	16.08	74.82	775.72
CSAA 02									
Day 1	9022.32	132.94	93.32	32.31	199.23	124.6	19.12	74.43	1328.13
Day 2	7268.96	82.48	78.18	31.66	172.29	33.87	32.2	101.93	528.89
Day 3	12859.7	82.06	141.63	59.45	364.32	120.73	42.41	164.13	647.04
daily av	9717	99.16	104.37	41.14	245.28	93.06	31.24	113.49	834.68
CSAA 03									
Day 1	9597.46	91.82	114.66	51.02	225.57	158.2	15.51	61.62	852.55
Day 2	5047.18	65.12	56.26	26.3	110.98	74.05	5.49	37.08	496.16
Day 3	8676.4	94.87	81.13	32.83	237.28	61.11	12.06	33.11	794.55
daily av	7773.68	83.93	84.01	36.71	191.27	97.78	11.01	43.93	714.42
CSAA 04									
Day 1	10523.7	61.34	128.49	57.04	276.78	172.39	19.55	35.93	854.01
Day 2	10169.3	83.64	101.29	46.36	288.13	44.96	19.87	45.32	782.36
Day 3	6436.02	70.66	50.85	26.29	198.73	73.05	13.42	125.39	938.17
daily av	9043.04	71.88	93.54	43.23	254.54	96.79	17.61	68.88	858.18

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSAB 01									
Day 1	17529.9	106.27	196.32	87.13	505.59	231.19	37.81	274.49	4127.28
Day 2	12716.8	89.13	106.52	45.81	436.6	162.8	44.93	273.82	2826.73
Day 3	8107.07	96.06	61.45	22.62	252.35	113.04	14.3	139.34	2235.53
daily av	12784.61	97.15	121.43	51.85	398.18	169	32.34	229.21	3063.17
CSAB 02									
Day 1	8721.88	95.84	83.44	38.51	233.67	110.54	17.05	30.13	3060.58
Day 2	10861.9	130.81	131.99	66.7	218.9	102.73	19.39	41.19	2819.59
Day 3	11562	122.08	114.29	59.38	308.44	173.37	15.69	35.53	3378.74
daily av	10381.96	116.24	109.91	54.86	253.67	128.87	17.37	35.61	3086.3
CSAB 03									
Day 1	7695.53	88.17	71.75	35.11	208.24	109.76	12.93	37.48	2821.42
Day 2	7732.35	72.26	91.21	23.33	183.97	68.96	21.88	53.96	2534.23
Day 3	10277.3	99.13	115.27	56.41	251.97	104.98	15.1	49.17	2079.69
daily av	8568.42	86.52	92.74	38.28	214.72	94.57	16.63	46.87	2478.44
CSAB 04									
Day 1	12698.6	138.08	73.46	28.23	457.16	199.24	21.2	150.99	4651.4
Day 2	10710.9	82.35	103.75	39.26	324.57	163.44	18.31	68.35	3820.48
Day 3	6608.72	48.63	54.44	28.02	224.12	119.56	8.51	38.3	2431.36
daily av	10006.1	89.68	77.21	31.83	335.28	160.74	16.01	85.88	3634.41
CSAC 01									
Day 1	1094.9	71.37	89.07	48.04	317.49	135.99	31.22	181.49	2474.32
Day 2	12233.7	76.19	101.06	40.97	361.82	173.78	27.8	188.11	3419.87
Day 3	8537.75	71.9	74.36	37.55	239.38	78.76	37.95	160.45	2715.13
daily av	10571.12	73.15	88.16	42.18	306.22	129.51	32.32	176.68	2869.77

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSAC 02									
Day 1	7310.15	59.31	44.08	18.75	280.65	163.05	37.99	117.32	2147.54
Day 2	9793.95	144.63	69.49	31.61	213.16	59.71	24.05	82	2404.17
Day 3	7886.72	95.36	58.24	24.8	203.45	131.73	24.25	23.63	1428.1
daily av	8330.27	97.76	57.27	25.05	232.41	118.16	28.76	74.31	1993.27
CSAC 03									
Day 1	9411.09	97.38	80.6	35.77	213.02	60.97	22.83	81.51	2239.68
Day 2	9426.98	54.73	86.53	34.53	243.76	68.22	23.48	105	2106.83
Day 3	8298.22	67.77	73.13	34.86	211.65	59.94	23.79	81.22	2125.15
daily av	9045.43	73.29	80.08	35.05	222.8	63.04	23.36	89.24	2157.22
CSAC 04									
Day 1	9288.15	64.03	54.89	25.35	267.57	172.79	20.28	44.99	3240.56
Day 2	11086	139.12	132.28	59.82	201.76	59.35	22.91	96.06	1867.02
Day 3	6559.99	43.6	57.2	26.85	170.17	66.41	19.35	76.39	2372.13
daily av	8978.07	82.24	81.45	37.33	213.16	99.51	20.84	72.48	2493.23

Table 2: Micronutrient intake at monthly intervals for each participant

Coeliac study sample group with coeliac disease												
Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSI 01												
Day 1	184.71	1.86	2.85	426.17	2.7	6.6	3336.15	4371.68	23.15	475.92	1376.58	10.96
Day 2	232.13	2.15	5.94	538.4	1.69	6.94	3392.84	4471.62	20.67	345.56	1097.66	18.55
Day 3	306.14	2.12	2.33	1021.23	0.69	14.27	2019.52	4576.02	11.66	437.26	1131.09	9.02
daily av	240.99	2.04	3.71	661.94	1.69	9.27	2916.16	4473.1	18.49	419.58	1201.77	12.84
CSI 02												
Day 1	195.66	2.33	4.5	401.93	0.14	9.34	3909	4256.51	12.54	480.99	1476.49	9.85
Day 2	194.33	2.4	2.89	561.77	0.12	8.17	2795.06	5167.48	12.82	530.79	1337.63	9.86
Day 3	129.83	1.57	3.77	1116.31	0.03	7.7	1339.77	4072.52	13.35	416.21	1023.73	7.96
daily av	173.27	2.1	3.72	693.33	0.96	8.4	2681.55	4498.83	12.9	475.99	1279.28	9.22
CSI 03												
Day 1	55.61	2.81	5.49	1927.14	0.08	12.67	1243.7	5680	19.4	487.13	1030.29	14.02
Day 2	84.33	2.06	2.64	528.05	0.31	5.42	3531.12	3831.6	11.89	309.82	794.36	8.35
Day 3	40.51	2.74	3.32	679.94	0.12	11.89	2568.73	6349.3	14.52	660.77	1419.54	11.34
daily av	60.14	2.54	3.81	1045.04	0.16	9.99	2447.84	5286.96	15.27	485.9	1081.46	11.32
CSI 04												
Day 1	26.67	2.29	3.52	525.06	0.16	8.54	3647.31	4408.21	12.71	415.05	1233.16	10.63
Day 2	26.67	2.29	3.52	525.04	0.16	8.54	3647.31	4408.21	12.71	415.05	1233.16	10.63
Day 3	126.14	1.82	3.39	1484.05	0.42	12.62	1898.88	4205.14	12.68	504.24	1394.09	9.76
daily av	59.83	2.13	3.47	844.72	0.24	9.9	3064.49	4340.51	12.69	444.78	1286.8	10.34

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CST 01												
Day 1	31.55	1.2	3.97	316.26	0.27	5.59	972.76	1925.47	12.26	239.8	561.15	11.36
Day 2	7.02	1.05	4.07	520.02	1.92	5.67	3816.65	2375.83	19.82	331.19	561.25	9.6
Day 3	11.11	0.44	2.48	616.46	1.82	4.81	1565.91	1432.3	13.31	148.01	169.41	5.93
daily av	16.59	0.89	3.5	484.28	1.33	5.35	2118.44	1911.19	15.13	239.66	430.6	8.98
CST 02												
Day 1	68.27	1.26	1.78	256.11	0.01	5.96	615.47	2552.2	7.81	160.14	270.08	3.33
Day 2	13.53	0.77	6.02	1123.83	4.86	4.56	1641.99	1143.82	7	106.92	361.94	3.09
Day 3	78.32	1.05	3.31	404.18	0.27	7.13	1284.66	2732.89	7.28	152.14	670.74	5.59
daily av	53.37	1.03	3.71	594.7	1.71	5.87	1180.7	2142.96	7.36	139.73	434.25	4
CST 03												
Day 1	111.13	0.99	1.02	235.37	0.13	1.63	539.48	1370.92	3.51	115.36	270.38	1.58
Day 2	160.15	1.84	5.59	317.57	5.41	5.09	1487.27	2258.21	6.95	117.21	321.67	3.16
Day 3	160.15	1.87	6.15	372.07	4.83	5.16	1368.83	2061.52	7.74	169.32	515.88	3.78
daily av	143.81	1.56	4.25	308.33	3.45	3.95	1131.86	1896.88	6.06	153.96	369.3	2.84
CST 04												
Day 1	34.65	1.5	7.06	431.23	6.6	4.86	1127.75	1966.76	5.47	134	407.15	4.01
Day 2	40.57	0.83	5.37	390.56	5.29	4.06	1478.98	2188.93	4.95	127.83	377.25	3.29
Day 3	39.07	0.66	5.49	781.92	0.88	2.97	2559.81	3257.3	7.62	178.43	1233.58	8.86
daily av	38.09	0.99	5.97	534.56	4.25	3.96	1722.18	2470.99	6.01	146.75	672.65	5.38
CSU 01												
Day 1	94.05	2.4	3.99	1011.15	3.6	11.18	5163.75	6620.32	26.72	633.13	1244.26	21.49
Day 2	73.15	1.91	2.6	519.38	1.38	9.43	3645.56	4834.4	17.43	421.46	1062.42	10.45
Day 3	169.74	2.73	2.92	934.45	1.44	10.81	3466.35	6019.22	19.83	481.8	1318.23	12.92
daily av	112.31	2.34	3.17	821.65	2.14	10.47	4091.88	5824.64	21.17	512.24	1208.3	14.95

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSU 02												
Day 1	144.75	2	14.37	1751.98	7.04	12.66	1801.1	5115.15	9.2	322.17	942.15	9.37
Day 2	161.32	2.3	14.53	1160.11	7.04	12.23	2456.64	6028.1	10.75	385.7	1064.96	10.92
Day 3	126.82	2.19	4.44	637.47	1.56	7.35	1590.23	5719.65	10.78	360.54	1097.36	8.75
daily av	144.29	2.16	11.11	1183.18	5.21	10.74	1949.32	5620.97	10.24	356.13	1034.82	9.67
CSU 03												
Day 1	234.15	1.92	2.24	2719.94	3.68	10.48	1768.32	7718.2	11.39	412.3	1669.16	9.83
Day 2	185.33	1.88	1.92	2987.67	3.93	15.28	2132.6	7727.83	13.45	719.34	1571.16	12.79
Day 3	243.78	3.59	3.94	3806.16	5.14	17.27	2681.37	8526.86	12.07	781.31	1432.46	12.54
daily av	221.08	2.46	2.7	3171.25	4.25	14.34	2194.09	7990.78	12.3	637.64	1557.59	11.52
CSU 04												
Day 1	146.86	2.07	2.34	3616.97	3.44	8.61	3664.27	5426.45	13.42	421.44	1712.22	22.25
Day 2	338.52	1.88	2.4	1131.32	0.56	10.33	2426.51	5900.78	10.6	368.57	1362.29	11.51
Day 3	153.53	1.76	2.93	3180.43	1.9	10.75	1770.67	5464.38	11.38	378.84	1683.71	17.83
daily av	212.97	1.9	2.55	2642.9	1.96	9.89	2620.48	5597.2	11.8	389.61	1586.07	17.19
CSW 01												
Day 1	163.7	0.71	4.95	884.76	4.08	7.03	1824.63	1752.63	11.31	328.9	557.53	9.29
Day 2	175.63	0.88	2.76	156.97	0.85	3.19	2779.99	1657.39	8.88	203.27	416.52	10.92
Day 3	24.52	1.16	4.93	1237.57	1	4.71	1510.18	2963.93	4.98	203.89	541.98	4.28
daily av	121.28	0.91	4.21	759.76	1.97	4.97	2038.27	2124.64	8.38	245.35	505.34	8.16
CSW 02												
Day 1	14.04	0.59	0.12	158.74	0.22	2.81	586.03	2702.15	4.91	177.41	145.99	3.41
Day 2	121.11	0.85	2.04	1497.89	5.85	5.92	2498.28	3619.42	6.84	253.27	1168.47	8.62
Day 3	141.91	1.53	1.46	1545.54	1.1	10.43	1704.99	3579.47	7.42	249.05	554.25	6.67
daily av	92.35	0.98	1.2	1067.38	2.39	6.39	1596.43	3300.34	6.4	226.57	622.9	6.23

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSW 03												
Day 1	170.72	1.47	3.7	1935.96	1.17	5.71	1608.56	3227.65	13.88	184.39	520.9	10.79
Day 2	67.1	0.48	1.92	3040.73	1.18	3.38	839.03	1976.82	6.62	113.59	224.52	5.22
Day 3	34.34	0.79	1.23	499.36	2.51	6.63	2247.29	1948.81	3.97	140.7	652.51	4.22
daily av	90.71	0.91	2.28	1825.35	1.61	5.24	1608.55	2384.43	8.15	146.22	465.97	6.74
CSW 04												
Day 1	8.8	0.97	4.43	250.37	1.71	3.56	1212.99	1622.21	2.26	102.74	233.72	1.92
Day 2	58.34	1.04	1.62	1357.36	0.28	8.68	916.28	2750.76	4.78	154.76	145.11	5.22
Day 3	23.51	1.22	2.34	563.84	3.68	2.91	785.91	2175.34	5.28	281.19	500.13	8.09
daily av	30.21	1.07	2.79	723.86	1.88	5.05	971.72	2182.76	4.01	179.56	292.98	5.07
CSZ 01												
Day 1	76.27	3.87	6.52	706.64	1.98	15.39	2804.3	3186.96	21.64	437.03	792.72	20.68
Day 2	134.16	0.76	0.61	287.26	0.23	4.78	1269.6	1992.82	11.55	171.61	608.7	4.18
Day 3	17.07	1.46	21.42	1985.88	7.34	12.79	5822.16	3550.89	16.72	438.79	1249.31	18.5
daily av	75.83	2.02	9.51	993.25	3.18	10.98	3298.68	2910.22	16.64	349.14	883.57	14.45
CSZ 02												
Day 1	8.91	0.56	0.44	108.68	1.1	8.86	2070.4	1328.36	8	114.11	307.3	3.28
Day 2	55.6	0.74	1.76	205.26	0.55	4.28	1421.7	2156.3	4.87	109.33	225.16	4.87
Day 3	3.9	0.51	0.71	307.32	0.73	4.25	1544.26	1059.76	2.82	74.68	231.83	1.94
daily av	22.8	0.6	0.97	207.08	0.79	5.88	1678.78	1514.8	5.22	99.37	254.76	3.36
CSZ 03												
Day 1	232.07	0.9	3.17	457.45	2.14	7.81	1361.21	3441.15	10.03	216.69	627.2	11.08
Day 2	137.33	0.64	0.86	319.11	0.29	14.37	1804.87	2112.54	5.46	163.11	442.06	3.99
Day 3	52.44	0.35	0.74	303.9	0.03	2.98	270.21	861.93	2.3	65.08	217.78	3.49
daily av	140.61	0.63	1.59	360.15	0.82	8.38	1145.42	2138.53	5.93	148.29	429.01	6.18

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSZ 04												
Day 1	29.89	1.54	81.8	845.74	0	9.48	1057.82	2583.39	11.1	203.67	462.01	17.87
Day 2	129.56	0.79	3.9	147.3	0.01	10.46	683.87	2633.24	9.52	199.49	604.88	8.43
Day 3	115.23	0.52	2.14	360.53	1.17	10.21	1508.74	1875.46	6.09	189.17	886.33	5.83
daily av	91.56	0.94	29.27	451.18	0.39	10.04	1083.47	2364.02	8.9	197.44	651.14	10.7
CSAA 01												
Day 1	61.07	1.25	2.84	489.15	0.08	5.83	1478.6	2813.75	15.42	192.32	320.88	8.58
Day 2	60.7	1.21	8.91	627.71	0.95	6.89	950.21	2565.2	11.74	206.99	508	9.95
Day 3	33.99	1.4	8.16	442.53	0.01	10.08	1803.14	2827.97	12.15	326.68	340.38	11.69
daily av	51.91	1.28	6.63	519.79	0.34	7.59	1410.65	2735.64	13.1	241.99	389.75	10.07
CSAA 02												
Day 1	62.68	3.06	9.49	707.63	2.33	4.79	1133.45	5873.67	19.89	521.55	450.71	25.03
Day 2	5.82	1.09	4.32	255.47	1.28	5.01	562.71	4489.38	8.22	253.23	390.84	12.61
Day 3	20.08	1.5	3.02	407.75	2.5	4.56	2034.65	4450.2	11.53	322.65	662.67	8.28
daily av	29.52	1.88	5.61	456.94	2.03	4.78	1243.6	4937.74	13.21	365.81	501.4	15.3
CSAA 03												
Day 1	82.03	1.86	5.84	511.14	0.16	10.32	1967.49	3982.65	9.76	261.39	1302.56	16.88
Day 2	12.32	1.52	3.46	368.55	0.68	4.65	913.49	1670.61	4.37	117.88	1048.67	10.93
Day 3	35.49	2.49	2.47	480.27	0.75	7.93	4840.86	2718.63	5.98	215.88	717.26	7.85
daily av	43.28	1.95	3.92	453.32	0.53	7.63	2573.94	2790.63	6.7	218.38	1022.83	11.89
CSAA 04												
Day 1	43.72	1.76	4.09	1057.6	10.31	13.64	1864.35	2704.3	6.25	275.7	974.39	6.81
Day 2	37.08	1.08	3.13	1338.96	1	7.86	5759.28	1857.41	5.16	157.62	1185.73	9.5
Day 3	62.68	1.64	1.35	240.53	0	5.26	1045.4	2602.42	5.43	210.85	465.8	5.02
daily av	47.82	1.49	2.85	879.03	3.77	8.92	2889.67	2388.04	5.61	214.72	875.31	7.11

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSAB 01												
Day 1	14.45	1.99	4.03	224.47	0.15	15.89	3466.07	4539.04	17.57	468.82	1123.12	10.24
Day 2	31.48	0.99	1.52	514.46	1.35	10.98	3225.54	2974.92	14.71	441.73	982.73	12.35
Day 3	45.14	1.58	2.9	866.4	1.83	6.73	2401.9	2718.09	13.45	232.24	1281.68	10.92
daily av	30.36	1.51	2.81	535.11	1.1	11.2	3031.16	3410.68	15.24	380.93	1129.17	11.16
CSAB 02												
Day 1	32.48	1.42	2.16	1182.94	0.95	6.26	2418.57	2492.58	7.16	213.77	1203.48	9.72
Day 2	58.33	0.81	10.43	775.67	0.66	7.85	2370.96	2259.45	6.95	277.13	2449.18	18.82
Day 3	38.57	1.38	5.43	1142.14	4.13	7.2	4911.48	3204.77	11.15	297.71	2186.8	15.68
daily av	43.12	1.2	6	1033.56	1.94	7.1	3233.67	2652.26	8.42	262.87	1946.48	14.74
CSAB 03												
Day 1	84.13	0.96	3.99	706.62	1.76	10.21	1867.46	2708.44	6.16	177.59	1349.42	13.01
Day 2	216.22	1.42	1.85	1003.98	2.18	7.89	2633.58	2960.07	9.8	332.09	365.88	9.84
Day 3	94.98	1.56	4.72	799.54	2.66	8.99	2480.28	3040.59	7.42	232.08	1417.57	14.64
daily av	131.77	1.31	3.52	836.71	2.19	9.02	2327.1	2903.04	7.79	247.25	1044.29	12.49
CSAB 04												
Day 1	119.55	3.02	7.76	1196.58	1.52	8.17	2097.05	3676.07	15.2	301.61	1241.26	28.89
Day 2	115.86	0.93	2.68	523.01	1.67	5.58	3637.96	2283.81	9.04	205.82	599.25	8.81
Day 3	10.52	0.26	1.52	409.17	0.64	2.98	1196.32	716.16	1.7	84.34	1056.01	6.12
daily av	81.97	1.4	3.98	709.58	1.27	5.57	2310.44	2225.34	8.64	197.25	965.5	14.6
CSAC 01												
Day 1	89.31	2.2	2.13	2186.67	1.25	6.65	3056.46	3946.41	18.39	377.07	641.2	8.21
Day 2	123.48	2.88	3.57	2331.83	3.63	16.74	2891.76	5498.62	14.48	413.74	901.13	8.07
Day 3	102.89	1.4	3.84	1516.41	0.37	7.69	3529.97	4091.83	12.43	263.27	766.8	10.44
daily av	105.22	2.15	3.18	2011.64	1.75	10.36	3159.36	4512.28	15.1	351.35	769.7	8.9

Subject code	Vitamin C (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSAC 02												
Day 1	411.29	4.71	2.89	2207.61	0.04	17.42	1648.9	6457.88	12.62	429.59	585.52	10.1
Day 2	52.52	1.5	5.23	1626.97	37.21	9.32	6669.01	4516.51	17.58	310.24	583.81	6.55
Day 3	268.43	4.67	7.32	1308.34	2.73	9.16	1092.41	3589.57	9.12	269.82	477.51	11.67
daily av	244.07	3.62	5.14	1714.3	13.32	11.96	3136.77	4854.65	13.1	336.55	584.94	9.44
CSAC 03												
Day 1	86.47	1.69	3.81	1507.23	18.11	10.44	4437.22	3841.91	12.41	262.74	577.66	6.22
Day 2	86.17	1.58	1.94	1607.46	0.16	12.1	2448.94	3126.25	5.04	211.56	549.48	4.71
Day 3	116.27	1.83	1.08	1402.82	0.16	9.93	1300.14	3641.4	6	211.46	506.85	6.74
daily av	96.3	1.7	2.27	1505.83	6.14	10.82	2728.76	3536.52	7.81	228.58	544.66	5.88
CSAC 04												
Day 1	187.84	3.51	2.94	1911.85	0.71	6.2	987.46	4044.63	5.21	332.92	673.46	6.95
Day 2	142.59	4.12	10.44	1888.62	3.75	14.94	2451.89	4609.75	16.03	269.51	759.37	21.54
Day 3	129.92	1.43	2.07	1464.75	0.15	4.45	3241.65	2919.21	5.94	204.54	454.44	5.21
daily av	153.45	3.01	5.14	1755.07	1.53	8.52	2226.99	3857.86	9.06	268.99	629.09	11.23

Appendix L: Dietary data for coeliac study sample group <u>without</u> coeliac disease (control group)

Table 1: Macronutrient intake at monthly intervals for each participant

Coeliac study sample group without coeliac disease (Control group)									
subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGA 01									
Day 1	18541.98	83.14	294.4	105.03	366.86	180.18	23.1	186.69	1934.03
Day 2	10115.7	59.2	101.44	37.98	319.73	158.06	21.54	161.61	1873.08
Day 3	8320.87	73.37	87.77	43.36	229.85	140.04	10.64	89.77	1883.78
daily av	12326.18	71.9	161.2	62.12	305.47	159.42	18.42	146.02	1896.96
CSCGA 02									
Day 1	9460.14	106.78	108.66	41.33	216.48	122.43	16.08	85.86	1995.92
Day 2	12971.4	138.99	125.66	49.78	346.92	164.15	27.47	182.78	2118.63
Day 3	8573.51	70.63	67.08	22.92	292.2	97.85	18.13	194.36	1692.44
daily av	10335.01	105.46	100.46	38	285.19	128.14	20.56	154.33	1935.66
CSCGA 03									
Day 1	13151.6	93.65	179.9	63.99	290.84	73.95	35.93	216.94	1384.16
Day 2	9636.66	85.68	116.02	52.44	232.4	76.39	17.9	155.95	406.23
Day 3	9668.74	60.92	112.22	39.41	267.97	24.58	38.59	243.32	560.99
daily av	10819.01	80.08	136.04	51.94	263.74	58.3	30.8	205.4	783.79
CSCGA 04									
Day 1	8590.38	61.59	78.02	36.45	280.17	134.43	22.25	144.33	2209.89
Day 2	13404.6	95.75	185.28	66.56	292.98	92.38	50.07	200.14	1762.27
Day 3	1196.5	120.36	129.97	56.13	303.37	132.14	31.94	171.24	1715.93
daily av	11303.87	92.56	131.08	53.04	292.17	119.64	34.75	171.9	1896.02
CSCGB 01									
Day 1	11952.9	155.67	129.67	60.79	271.21	96.53	31.34	174.62	2828.91
Day 2	9928.93	109.09	108.91	52.94	242.07	111.17	26.92	130.83	2137.52
Day 3	7250.53	59.6	73.98	25.76	209.09	39.03	17.19	170.05	2267.88
daily av	9710.81	108.12	104.18	46.49	240.79	82.24	25.15	158.5	2411.43

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGB 02									
Day 1	12235.6	131.48	124.41	43.82	324.17	69.73	35.82	254.08	2311.54
Day 2	15474	124	188.55	85.16	380.79	54.42	36.68	326.29	2360.96
Day 3	5761.26	58.21	37.71	19.1	203.48	43.08	24.37	160.39	2315.62
daily av	11156.96	104.56	116.88	49.35	302.81	55.74	32.28	246.92	2329.37
CSCGB 03									
Day 1	7473.66	70.73	79.77	26.66	199.16	62.62	23.12	136.44	2513.99
Day 2	10722.7	115.98	107.08	43.46	287.02	53.51	27.23	233.49	2442.26
Day 3	7615.49	64.34	83.86	20.42	203.24	59.92	29.19	143.26	2467.74
daily av	8603.95	83.68	90.23	30.18	229.8	58.68	26.51	171.06	2474.66
CSCGB 04									
Day 1	6781.95	63.57	50.58	13.26	230.91	102.23	22.24	128.65	2032.03
Day 2	4097.27	53.83	35.97	17.11	109.67	28.04	13.36	81.53	2040.12
Day 3	8412.04	102.11	68.99	27.07	248.56	78.05	23.26	170.38	2093.38
daily av	6430.41	73.17	51.84	19.14	196.37	69.43	19.61	126.85	2055.17
CSCGC 01									
Day 1	5803.32	62.73	61.29	20.95	126.21	8.91	7.43	117.25	1100.49
Day 2	7429.64	81.17	91.96	39.8	156.63	22.4	24.17	134.02	1940.31
Day 3	10177.2	79.95	117.23	45.6	267.55	126.27	22.82	135.68	1927.77
daily av	7803.4	74.61	90.15	35.45	183.46	52.52	18.14	128.98	1656.18
CSCGC 02									
Day 1	5817.86	63.25	59.26	28.53	153.06	85.24	22.26	67.8	1858.55
Day 2	9475.44	92.52	143	61.57	153.98	47.57	20.23	106.54	2011.52
Day 3	3718.53	29.35	45.69	21.56	91.16	22.33	9.32	68.79	1398.25
daily av	6337.27	61.7	82.64	37.22	132.73	51.71	17.26	81.04	1756.1

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGC 03									
Day 1	6345.15	71.43	80.47	43.26	128.43	80.46	9.13	48.12	1551.07
Day 2	5286.31	56.63	43.46	17.95	144.75	60.62	17.29	84.24	907.4
Day 3	5265.36	56.13	62.64	20.38	96.95	28.86	6.65	67.98	1250.42
daily av	5632.27	61.41	62.18	27.19	123.37	56.64	11.02	66.78	1236.29
CSCGC 04									
Day 1	5694.85	64.99	45.28	13.8	176.02	27.21	10	148.7	1139.32
Day 2	7499.18	98.02	97.39	27.55	113.02	43.21	17.45	68.47	1152.89
Day 3	5414.71	53.98	74.34	17.18	81.28	53.03	6.61	27.24	988.34
daily av	6202.91	72.32	72.33	19.5	123.44	41.48	11.35	81.47	1093.51
CSCGD 01									
Day 1	4492.25	64.69	49.2	16.69	94.17	13.37	14.63	80.77	778.26
Day 2	5536.3	59.68	69.55	22.23	114.46	66.28	11.35	48.29	1001.05
Day 3	5694.17	68.27	68.73	39.47	116.43	47.48	20.04	68.97	849.64
daily av	5240.9	64.21	62.52	26.12	108.35	42.38	15.34	66.01	876.31
CSCGD 02									
Day 1	7530.19	86.89	77.61	38.7	190.82	87.55	13.15	103.31	1018.96
Day 2	6053.75	128.15	65.11	20.74	69.14	4.6	3.39	64.51	568.13
Day 3	4150.63	73.02	41.66	16.19	82.37	19.9	15.93	62.43	545.09
daily av	5911.52	96.01	61.46	25.21	114.11	37.35	10.82	76.75	710.72
CSCGD 03									
Day 1	4190.5	82.85	42.92	11.68	70.5	25.29	16	45.16	589.01
Day 2	521.67	83.33	60.88	28.79	96.55	18.57	11.6	77.66	548.83
Day 3	4760.62	41.08	66.59	25.25	92.81	21.15	11.98	71.15	553.72
daily av	4744.26	69.08	56.79	21.9	86.62	21.76	13.19	64.65	548.83

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGD 04									
Day 1	8800.92	76.51	117.01	54.28	188.55	43.73	21.46	144.61	544.46
Day 2	3933.95	72.26	32.53	7.99	89.21	31.59	6.55	57.62	452.37
Day 3	6124.94	95.2	53.16	19.28	2	28.36	12.91	123.32	716.22
daily av	6286.6	81.32	67.56	27.18	143.14	34.56	13.63	108.51	571.01
CSCGE 01									
Day 1	12620.88	108.51	119.85	64.44	380.39	214.08	26.44	166.18	1992.06
Day 2	8344.03	71.62	52.91	23.46	311.69	133.26	22.15	178.43	1686.88
Day 3	11316.43	199.07	117.95	55.89	214.23	113.17	17.46	101.09	2021.21
daily av	10760.45	126.4	96.9	47.93	302.1	153.5	22.01	148.56	1900.05
subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGE 02									
Day 1	10495.7	106.27	133.37	64.05	222.13	121.49	16.57	100.31	1456.99
Day 2	10794.8	107.53	119.56	64.73	269.85	147.51	18.96	122.04	1951.25
Day 3	8217.51	89.68	23.4	9.32	353.41	218.4	14.31	134.67	2466.96
daily av	9836.05	101.16	92.11	46.03	281.79	162.46	16.61	119	1958.39
CSCGE 03									
Day 1	4651.82	47.69	36.62	14.31	149.01	32.82	11.24	116.25	1640.56
Day 2	9973.03	70.98	100.87	46.12	301.65	93.19	30.28	208.55	1726.76
Day 3	15540.4	118.63	187.44	103	372.01	205.37	24.81	165.95	2511.41
daily av	10055.09	79.1	108.31	54.47	274.22	110.45	22.2	163.58	1959.57
CSCGE 04									
Day 1	11588.9	105.85	116.76	62.81	326.74	166.52	21.94	159.64	2358.35
Day 2	10333.7	75.92	116.44	64.2	281.24	152.54	17.07	128.5	1969.92
Day 3	5630.99	78.21	31.96	11.91	188.69	82.62	19.01	105.96	1744.78
daily av	9184.56	86.66	88.38	46.3	265.55	133.89	19.34	131.36	2024.34

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGF 01									
Day 1	6717.61	77.72	52.5	16.29	196.64	79.83	22.55	116.91	2589.1
Day 2	7538.34	99.02	50.81	19.88	239.85	77.88	21.51	142.03	2668.95
Day 3	10512.8	116.01	77.27	29.41	342.05	95.07	26.68	247	2754.98
daily av	8256.26	97.58	60.19	21.86	259.51	90.92	23.57	168.64	2671
CSCGF 02									
Day 1	10929.7	135.67	60.36	26	223.56	89.49	21.09	134.1	2895.93
Day 2	8450.94	125.79	56.48	23.48	255.35	85.09	20.69	169.93	2491.86
Day 3	6847.46	107.84	47.85	21.42	196.4	89.92	18.83	106.35	1871.13
daily av	8742.7	123.1	54.89	23.63	225.1	88.16	20.2	136.79	2419.63
CSCGF 03									
Day 1	13895.56	154.35	152.02	63.72	338	94.33	38.37	243.74	2208.87
Day 2	9210.38	111.65	101.96	29.61	212.34	101.63	26.88	110.84	2093.33
Day 3	8128.42	91.28	87.3	22.78	200.9	93.69	26.28	107.17	2190.06
daily av	10411.46	119.09	113.76	38.7	250.41	96.54	30.5	153.91	2164.08
CSCGF 04									
Day 1	7481.89	95.49	58.69	24.04	222.58	81.85	22.27	140.98	2008.21
Day 2	8646.4	131.81	74.89	35.71	219.3	81.59	30.36	137.52	2027.86
Day 3	6292.16	75.36	48.35	17.05	194.41	91.53	18.71	102.91	2678.89
daily av	7473.31	100.88	60.64	25.6	212.09	84.85	23.78	127.13	2238.32
CSCGG 01									
Day 1	8403.75	116.43	90.2	19.28	185.45	75.14	40.92	110.29	1390.52
Day 2	6083.18	75.19	47.34	16.45	184.29	62.63	20.84	121.54	936.17
Day 3	8278.96	86.79	72.06	33.35	248.64	67.48	25.68	181.12	1205.6
daily av	7588.62	92.8	69.86	23.05	206.12	68.41	29.14	137.65	1177.43

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGG 02									
Day 1	5985.55	78.64	39.91	10.82	192.07	66.14	36.79	125.89	1691.88
Day 2	6267.89	62.77	60.37	27.06	178.16	71.35	35.09	106.77	1654.71
Day 3	7165.63	71.73	65.64	29.47	211.32	74.03	23.22	137.09	1277.76
daily av	6473.02	71.04	55.3	22.48	193.85	70.5	31.7	123.25	1541.45
CSCGG 03									
Day 1	5613.16	70.03	40.29	10.54	177.22	58.48	22.36	118.73	1343.93
Day 2	6408.71	121.64	50.05	17.25	150.76	54.6	24.97	96.13	1336.13
Day 3	5716.91	58.94	43.53	16.08	187.13	67.19	22.04	119.85	1460.22
daily av	5912.92	83.53	44.62	14.62	171.7	60.09	23.12	111.56	1380.09
CSCGG 04									
Day 1	8094.16	103.07	72.33	30.89	220.92	70.92	39.99	149.96	1943.88
Day 2	6642.63	70.64	61.5	26.22	190.38	66.15	22.86	124.11	1041.22
Day 3	10202.1	110.02	102.8	44.98	271.64	107.42	26.02	164.18	1192.59
daily av	8312.96	94.57	78.87	34.02	227.64	81.5	29.62	146.08	1392.56
CSCGH 01									
Day 1	5237.1	63.06	52.38	25.85	133.95	63.41	27.66	70.25	1888.35
Day 2	6214.01	85.56	64.71	30.61	142.3	61.72	21.55	80.66	2225.18
Day 3	7165.97	95.11	93.04	42.51	125.36	42.96	16.92	82.43	1660.3
daily av	6205.69	81.24	70.04	32.98	133.87	56.02	22.04	77.78	1924.61
CSCGH 02									
Day 1	6030.49	91.97	48.96	18.13	155.14	72.3	20.98	82.91	1766.8
Day 2	5903.19	69.09	60.65	30.14	149.46	97.98	17.06	51.6	1911.2
Day 3	7875.01	80.79	82.46	39.71	207.2	65.97	25.67	141.18	1155
daily av	6602.89	80.61	64.02	29.32	170.6	78.74	21.23	91.89	1611

subject code	Energy (kj)	Protein (g)	Total fat (g)	Saturated fat (g)	Carbohydrate (g)	Sugars (g)	Fibre (g)	Starch (g)	Water (ml)
CSCGH 03									
Day 1	11797.7	139.46	84.97	29.6	379.32	73.55	37.2	305.76	1971.22
Day 2	7801.83	96.2	85.79	40.87	179.38	58.63	20.69	120.71	1142.7
Day 3	7316.48	68.89	87.2	40.24	174.97	41.85	14.56	133.08	1043.23
daily av	8972.01	101.51	85.98	36.9	244.55	58.01	24.15	186.51	1385.71
CSCGH 04									
Day 1	9644.7	98.34	114.19	46.65	224.56	117.28	24.12	107.51	2193.08
Day 2	6273.06	51.9	75.37	21.64	152.27	47.1	14.97	105.35	1292.24
Day 3	5678.04	81.78	44.14	14.93	159.53	89.25	11.97	70.41	1056.01
daily av	7198.59	77.34	77.89	27.74	178.78	84.54	17.02	94.42	1513.77

Table 2: Micronutrient intake at monthly intervals for each participant

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGA 01												
Day 1	11.35	1.2	2.37	1439.61	3.03	35.29	2952.08	2528.08	15.04	344.91	351.99	12.3
Day 2	113.61	1.55	2.18	615.22	1.93	13.78	2461.12	2461.12	9.71	270.86	830.91	6.96
Day 3	19.26	0.96	1.78	423.4	0.55	4.29	3436.11	3436.11	8.38	199.48	1020.39	9.62
daily av	48.07	1.24	2.11	826.08	1.84	17.79	2949.76	2949.77	11.04	271.75	734.43	9.63
CSCGA 02												
Day 1	141.35	1.35	2.97	1852.35	3.05	17.38	1948.4	3084.31	11.81	316.84	1017.76	14.93
Day 2	122.69	1.65	3.65	1630.70	1.00	13.77	2539.66	3560.44	13.83	455.05	1022.99	16.65
Day 3	86.02	1.71	2.24	222.20	0.09	7.10	3776.02	2693.62	9.67	281.96	590.43	9.70
daily av	116.68	1.57	2.95	1235.08	1.38	12.75	2754.69	3112.78	11.76	351.28	877.05	13.76
CSCGA 03												
Day 1	288.46	2.04	2.57	398.51	0.48	30.19	3483.43	5931.10	16.57	549.28	882.82	10.99
Day 2	21.41	1.23	5.37	581.25	0.48	12.33	2950.71	1736.72	12.42	239.91	884.74	15.25
Day 3	57.44	2.25	0.39	201.01	0.08	18.33	3285.74	5070.39	11.15	425.67	636.75	8.96
daily av	122.43	1.84	2.77	393.59	0.34	20.28	3239.96	4246.07	13.37	404.95	801.43	11.73
CSCGA 04												
Day 1	1177.36	10.99	11.23	646.31	2.17	4.82	3393.76	2219.50	13.70	224.53	560.69	8.70
Day 2	1030.45	13.14	10.87	258.94	0.49	17.59	2580.11	4338.85	18.89	501.17	827.49	12.03
Day 3	99.78	3.49	6.55	1172.98	1.52	19.24	2237.48	4466.06	19.93	461.33	1073.64	21.07
daily av	769.19	9.20	9.54	692.74	1.39	13.88	2737.12	3674.80	17.50	395.68	820.60	13.93
CSCGB 01												
Day 1	163.07	2.30	10.18	884.28	0.97	11.36	2642.98	5655.72	22.03	586.47	708.60	30.23
Day 2	67.06	1.85	1.62	1867.42	1.88	9.88	2663.53	3598.17	12.95	365.97	1229.00	12.10
Day 3	49.24	0.60	0.53	444.18	1.96	12.42	2069.47	1937.13	6.43	213.29	404.12	7.43
daily av	93.12	1.58	4.10	1065.29	1.60	11.22	2458.66	3730.34	13.80	388.57	780.57	16.58

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGB 02												
Day 1	164.44	2.31	5.37	1155.57	2.97	24.97	1910.01	4268.85	15.66	425.64	972.32	17.66
Day 2	11.72	1.89	5.03	800.88	3.73	10.11	1101.06	4976.93	19.71	456.00	769.93	11.66
Day 3	52.36	0.65	0.86	1710.46	1.25	4.02	2064.31	2408.25	9.52	298.09	713.71	8.58
daily av	76.17	1.62	3.75	1222.30	2.64	13.03	1691.79	3884.68	14.96	393.24	818.65	12.63
CSCGB 03												
Day 1	127.87	1.80	3.03	1180.73	0.48	16.50	1747.04	4179.59	12.76	357.39	805.73	9.95
Day 2	49.43	2.40	1.81	1962.23	1.93	15.30	1489.01	4003.84	13.07	501.12	730.83	12.99
Day 3	91.07	2.12	1.37	960.10	1.79	17.02	1180.47	3831.28	10.87	362.29	591.78	8.13
daily av	89.45	2.10	2.07	1367.68	1.40	16.27	1472.17	4004.90	12.23	406.93	709.45	10.35
CSCGB 04												
Day 1	80.98	2.14	0.58	668.71	1.62	12.69	1799.24	2743.21	8.62	291.88	378.84	7.09
Day 2	85.38	0.68	1.08	980.24	1.21	6.75	3720.61	1763.87	5.60	232.30	448.31	6.73
Day 3	83.43	2.23	8.45	768.72	1.40	10.54	1189.63	3158.88	13.25	335.24	616.40	11.72
daily av	83.26	1.68	3.36	805.89	1.41	9.99	2236.49	2555.32	9.15	286.47	481.18	8.51
CSCGC 01												
Day 1	44.67	2.73	5.01	98.86	2.91	2.24	2560.24	635.86	5.56	98.40	82.60	6.99
Day 2	264.11	1.21	2.97	693.97	2.31	4.85	2560.03	3338.13	9.98	214.70	1003.33	9.90
Day 3	118.89	1.06	3.30	491.49	1.07	24.81	2521.34	4467.33	11.02	455.40	1108.77	9.92
daily av	142.55	1.67	3.76	428.10	2.09	10.63	2547.2	2813.77	8.85	256.16	731.56	8.93
CSCGC 02												
Day 1	338.66	1.29	3.87	826.99	4.92	13.05	1388.34	3213.19	6.92	389.89	765.96	11.20
Day 2	97.84	2.47	9.31	2141.50	5.00	17.70	1856.94	4986.42	21.82	804.84	2715.60	31.38
Day 3	7.51	0.24	0.72	423.39	0.10	1.12	1685.07	895.99	3.53	98.80	171.77	4.30
daily av	148.00	1.33	4.63	1130.63	3.34	10.62	1643.45	3031.87	10.76	431.17	1217.78	15.62

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGC 03												
Day 1	81.96	1.35	3.44	669.64	5.58	6.30	2327.97	3315.18	5.08	407.60	1060.39	8.64
Day 2	35.48	1.37	1.67	329.71	4.90	3.94	1241.8	3211.95	5.60	331.78	277.19	4.87
Day 3	50.06	2.68	6.80	364.47	3.03	12.15	3152.16	1805.80	5.50	254.68	418.65	5.50
daily av	55.83	1.80	3.97	454.60	4.50	7.47	2240.64	2777.64	5.39	331.35	585.40	6.34
CSCGC 04												
Day 1	15.64	1.28	2.43	155.92	1.37	5.80	1747.04	1335.02	12.94	228.31	290.64	15.34
Day 2	24.29	1.35	3.75	121.17	0.38	18.66	1489.01	2562.07	14.78	412.83	488.26	14.60
Day 3	1.04	2.56	3.95	51.14	2.75	17.11	1180.47	1048.59	4.89	265.55	230.55	14.60
daily av	13.65	1.73	3.38	109.41	1.50	13.86	1472.17	1648.56	10.87	302.23	336.49	11.98
CSCGD 01												
Day 1	77.66	1.03	1.84	1191.81	3.23	3.08	1799.24	1761.05	6.57	129.43	205.07	6.86
Day 2	129.55	1.16	4.32	537.07	3.55	12.26	3720.61	2029.29	10.35	165.99	317.48	7.67
Day 3	176.49	0.85	1.17	882.92	0.21	7.71	1189.63	2001.26	6.10	220.67	629.20	8.79
daily av	127.90	1.01	2.44	870.60	2.33	7.68	2236.49	1930.54	7.67	172.03	383.92	7.77
CSCGD 02												
Day 1	116.10	1.97	8.95	337.44	12.48	7.11	2560.24	3066.66	8.33	260.36	492.56	5.18
Day 2	0.85	2.10	12.99	414.84	8.87	7.62	2560.03	1622.11	9.62	166.23	328.62	10.75
Day 3	37.16	0.89	6.40	2532.19	4.87	5.14	2521.34	1816.78	10.90	154.70	450.29	7.20
daily av	51.37	1.65	9.45	1094.82	8.74	6.63	2547.2	2168.52	9.62	193.76	423.82	7.71
CSCGD 03												
Day 1	122.44	1.19	1.52	1572.55	7.28	6.59	1388.34	2188.20	6.57	163.86	219.57	4.61
Day 2	38.08	2.28	16.30	872.36	16.01	11.28	1856.94	2110.64	9.13	280.99	523.39	7.45
Day 3	55.55	0.48	1.28	721.66	3.61	8.05	1685.07	1619.05	7.63	150.08	240.62	5.81
daily av	72.02	1.32	6.37	1055.52	8.97	8.64	1643.45	1972.63	7.78	198.31	327.86	5.96

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGD 04												
Day 1	54.32	0.83	3.72	1082.72	0.62	10.65	2327.97	1958.49	9.51	199.81	604.42	14.59
Day 2	55.52	0.69	2.38	355.97	8.17	5.03	1241.8	1715.39	5.98	142.62	247.56	4.44
Day 3	63.09	1.74	9.71	1553.98	8.13	7.83	3152.16	3035.13	10.79	206.85	444.21	8.88
daily av	57.64	1.08	5.27	997.55	5.64	7.83	2240.64	2236.34	8.76	183.09	432.06	9.30
CSCGE 01												
Day 1	190.93	2.08	3.11	2299.48	3.56	10.44	4126.05	4228.25	12.37	361.85	1396.88	13.49
Day 2	145.40	1.84	1.32	361.73	3.23	7.41	3685.04	3465.00	9.10	299.11	893.19	8.56
Day 3	24.56	2.39	27.47	569.10	3.13	6.24	3318.69	2939.80	25.72	299.02	794.85	51.32
daily av	120.30	2.10	10.64	1076.77	3.31	8.03	3709.92	3544.35	15.73	319.99	1028.31	24.49
CSCGE 02												
Day 1	12.85	1.61	6.97	1268.43	1.24	10.05	2369.48	2360.34	16.12	228.55	518.99	24.85
Day 2	41.19	1.07	2.69	1584.67	2.71	8.50	3598.28	3612.64	12.07	295.50	1053.79	13.97
Day 3	78.62	1.02	1.21	135.14	0.07	2.17	2298.33	3553.62	9.60	288.52	1112.32	8.39
daily av	44.22	1.23	3.62	996.01	1.34	6.90	2755.36	3175.53	12.60	270.85	895.03	15.73
CSCGE 03												
Day 1	69.22	0.29	0.92	357.23	1.09	4.22	2049.03	1610.02	4.97	181.31	868.52	6.06
Day 2	49.85	1.11	2.51	657.32	0.85	8.05	2182.63	4186.90	11.54	310.09	694.12	7.44
Day 3	213.83	2.70	8.29	1838.96	2.27	14.53	2621.57	4925.90	17.97	363.60	970.63	25.56
daily av	110.97	1.36	3.90	951.17	1.40	8.93	2284.4	3574.27	11.50	285.00	844.42	13.02
CSCGE 04												
Day 1	98.81	1.97	2.26	1693.19	2.68	10.48	3130.47	4538.14	10.53	350.08	945.65	10.30
Day 2	81.20	1.41	3.15	1197.79	3.45	7.60	3303.09	3049.90	8.80	286.11	1239.35	9.57
Day 3	143.36	1.56	1.12	514.58	0.71	5.67	1609.89	3462.41	6.32	285.26	691.42	6.76
daily av	107.80	1.65	2.18	1135.18	2.28	7.92	2681.51	3683.48	8.55	307.15	958.80	8.78

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGF 01												
Day 1	133.08	1.50	7.38	682.45	2.48	7.62	2148.81	4081.37	15.59	298.45	801.41	12.03
Day 2	103.12	2.05	1.67	394.19	2.29	6.20	1868.98	3447.04	16.66	360.06	654.67	12.42
Day 3	105.07	2.16	4.63	331.57	2.28	5.68	3886.04	3842.47	19.92	343.61	775.47	19.25
daily av	113.76	1.90	4.56	496.40	2.35	6.50	2634.6	3790.30	17.39	334.04	743.85	14.56
CSCGF 02												
Day 1	144.95	2.10	7.99	357.61	3.29	5.00	3090.54	4142.51	21.16	366.12	1235.04	20.48
Day 2	122.93	2.41	6.24	465.66	2.05	6.05	1886.91	4698.30	20.50	364.57	914.87	16.70
Day 3	103.17	2.09	7.42	652.62	2.05	6.69	1635.21	3583.68	17.26	290.08	888.32	17.96
daily av	123.68	2.20	7.22	491.96	2.32	5.92	2204.21	4141.50	19.64	340.26	1012.74	18.38
CSCGF 03												
Day 1	83.56	2.97	11.72	4154.58	11.27	16.98	4686.21	4303.52	19.69	506.96	1095.19	16.09
Day 2	109.82	2.00	7.19	530.70	1.73	11.80	2862.82	3613.25	13.53	434.90	1211.61	13.60
Day 3	111.01	2.39	7.60	1152.63	1.95	13.03	3207.7	4107.38	14.53	455.11	992.47	13.78
daily av	101.46	2.45	8.84	1945.97	4.99	13.94	3585.57	4008.05	15.92	465.65	1099.75	14.49
CSCGF 04												
Day 1	83.57	1.79	7.05	331.94	1.69	5.45	2846.07	3335.33	13.46	300.30	1031.42	12.84
Day 2	157.94	2.48	8.56	1689.36	1.91	6.94	2203.65	4558.38	20.48	360.56	834.72	19.18
Day 3	170.49	1.87	7.68	366.46	1.95	5.83	2697.28	3610.58	12.27	318.55	941.69	12.04
daily av	137.34	2.04	7.76	795.92	1.85	6.07	2582.33	3834.76	15.40	326.47	935.95	14.68
CSCGG 01												
Day 1	171.67	3.13	7.35	1522.71	0.64	18.32	1565.13	4165.95	18.50	472.82	554.71	12.38
Day 2	13.75	2.57	6.11	238.72	0.82	7.36	1419.83	1970.08	17.36	261.51	393.03	13.84
Day 3	29.31	1.89	7.44	484.25	0.99	7.56	2452.32	2006.22	15.10	275.33	775.72	10.97
daily av	71.57	2.53	6.97	748.56	0.81	11.08	1812.42	2714.09	16.99	336.55	574.49	12.40

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGG 02												
Day 1	110.17	2.30	2.00	2027.96	0.64	7.94	1470.89	2621.85	18.02	297.20	509.97	13.66
Day 2	135.40	2.43	1.34	2167.11	0.65	9.64	1054.13	2798.40	15.35	359.88	500.13	8.57
Day 3	28.95	2.32	1.49	451.33	0.95	7.90	1548.48	1997.74	13.42	306.36	572.69	7.78
daily av	91.51	2.35	1.61	1548.80	0.75	8.50	1357.83	2472.66	15.60	321.15	527.60	10.00
CSCGG 03												
Day 1	40.01	2.42	1.28	172.43	0.68	8.01	1513.35	3111.22	13.51	504.56	345.34	6.70
Day 2	50.50	2.84	3.74	798.13	0.65	6.11	1310.66	2962.03	16.54	323.87	362.90	7.86
Day 3	27.90	2.16	1.42	355.01	0.87	6.94	1554.09	1932.10	13.10	261.06	497.76	6.99
daily av	39.47	2.47	2.15	441.85	0.73	7.02	1459.36	2668.45	14.39	363.17	402.00	7.18
CSCGG 04												
Day 1	138.00	2.59	1.93	2167.11	0.65	10.34	1410.95	3133.94	18.33	390.70	531.51	16.12
Day 2	28.04	2.31	1.48	391.67	0.87	8.11	1473.47	1988.70	13.05	300.05	576.24	7.72
Day 3	44.87	3.08	4.66	473.39	1.15	10.83	2243.06	3213.48	19.81	394.48	622.18	11.21
daily av	70.30	2.66	2.69	1010.72	0.8.9	9.76	1709.15	2778.70	17.06	361.74	576.65	11.69
CSCGH 01												
Day 1	146.59	1.20	4.00	4560.74	0.61	8.69	1468.96	3883.40	5.99	347.29	1221.75	7.93
Day 2	156.98	1.40	13.95	2987.27	0.68	7.02	2550.68	4037.18	10.13	374.97	1003.40	12.39
Day 3	67.54	1.27	4.30	1633.60	0.63	5.21	2370.79	2848.85	6.81	351.87	1241.34	10.51
daily av	123.70	1.29	7.42	3060.54	0.64	6.98	2130.14	3589.81	7.64	358.04	1155.50	10.28
CSCGH 02												
Day 1	75.95	1.63	1.38	1061.28	0.10	6.85	1731.25	3706.73	10.25	520.54	913.70	10.55
Day 2	56.69	2.08	1.13	974.75	0.03	5.80	787.7	3425.28	11.74	733.42	640.37	7.51
Day 3	87.62	1.15	2.28	1162.32	1.51	8.40	3409.76	2880.54	12.01	468.47	623.66	11.90
daily av	73.42	1.62	1.59	1066.12	0.55	7.01	1976.23	3337.52	11.33	574. 15	725.91	9.98

subject code	Vitamin C (mg)	Vitamin B_6 (ug)	Vitamin B_{12}(ug)	Vitamin A (ug)	Vitamin D (ug)	Vitamin E (mg)	Sodium (ug)	Potassium (mg)	Iron (mg)	Mag (mg)	Calcium (mg)	Zinc (mg)
CSCGH 03												
Day 1	68.11	1.14	3.66	581.16	0.19	9.52	2903.95	3552.85	12.61	659.43	1134.21	14.52
Day 2	14.01	1.44	3.04	1138.03	1.06	8.19	3141.65	2544.37	10.95	569.12	1477.88	12.51
Day 3	4.82	0.58	2.67	613.62	2.10	7.96	3535.18	1998.83	7.59	461.66	1257.30	10.37
daily av	28.98	1.05	3.12	777.60	1.12	8.56	3193.59	2698.68	10.38	563.40	1289.80	12.46
CSCGH 04												
Day 1	116.96	1.07	2.90	2516.72	0.71	12.21	2909.44	3824.47	9.34	367.34	1291.75	10.36
Day 2	117.67	0.62	1.32	988.13	0.27	9.32	2859.88	1852.81	6.94	180.97	584.85	6.11
Day 3	137.88	1.35	42.60	683.35	0.10	5.30	1352.36	2747.02	9.65	208.12	1072.39	13.49
daily av	124.17	1.01	15.60	1396.07	0.36	8.94	2373.89	2808.10	8.64	252.14	983.00	9.98

Appendix M - Human Nutritional Studies Laboratory Procedure

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Collection of Faecal Samples

Read these instructions through carefully before you begin.
This kit contains the materials necessary for the collection of your next faecal sample.

Prior to the day you chose to take your first sample.

- Remove the unfrozen ice pack from the kit, place it in your freezer. It will not freeze solid.
- Contact Delwyn MacKenzie on 4789781 to confirm the day you are sending the sample so that she can be ready to receive it. If no one is available to take your call, please leave your name and phone number on the answer phone.
- Contact Courier Post on 08002687437 and tell them you have a same day regional pick up to be collected from your place and delivered to 12 Dr Taylor Tce, Johnsonville, Wellington. This is to check that they can pick up and transport the sample on the day you choose.
- Everyone has different times that their bowels move so there are several options for you to follow.

    - If you collect the sample early in the morning you need to ring the courier before 9.00 am that day and they will collect it.
    - If you take the sample before 10.30 am, ring the courier and they will collect it.
    - If you take the sample after lunch or in the evening, you will need to freeze it to prevent deterioration. (Wrap the sample as described in step 6 below). Place in the small snap-lock sealable bag, close the top and place in your freezer. The next morning, place your frozen sample and completed questionnaire in the courier bag and ring the courier for collection.

On the day you chose to take your sample

Obtain the sample as described below

Equipment list

- A black polystyrene tray(food tray from Packaging house)
- A plastic screw-top faecal container with sampling scoop
- A pair of disposable gloves
- 1 small snaplock sealable plastic bag with a label on it
- 1 large snaplock sealable plastic bag with no label
- a brown paper bag
- a rubberband
- 1 ice pack (to be frozen)
- Paper towels
- Bubble wrap
- 1 pre-addressed courier trackpack

Instructions

1. Open the pack and label the faeces 'sterile container' with your name, the time and the date.
2. Take the pack into the toilet with you. Urinate first, then place the black tray into the middle of toilet bowl in a position where it will collect your faeces. Go to the toilet as normal. After you have finished, put on the gloves, lift the tray out and place it on the paper towel that you have placed on the floor in front of you, or on a convenient surface.
3. Now use toilet paper to clean your backside as normal, put the used toilet paper in the toilet.
4. Then unscrew the plastic screw-top 'sterile container' and use the plastic scoop inside this container to half fill it (about one scoop full) with faeces. Put the scoop back in the container and screw the top on tightly to seal the container. Wipe the outside of the container with one of the paper towels to remove any faeces from the outside of the container. Then place the container into the small labeled plastic bag provided and seal it.
5. Tip any remaining faeces from the black collecting tray into the toilet bowl and flush them away along with the toilet paper. Place the used black tray and the paper towels into the brown paper bag.
6. Take your sample out of the small labeled plastic bag. Wrap your pre-frozen icepack around the sample, wrap in the bubble wrap and secure with the rubberband. Place your sample back in the small plastic bag, and seal.
7. Remove the gloves and place them in the paper bag. Close the paper bag, wrap it in newspaper and place it into your outdoor household rubbish bin. Wash your hands.
8. Put your sample in the courier pack. Place your completed questionnaire and consent form in the large unlabeled plastic bag and close the top. Put in the courier bag along with your sample. Store in a cool place, out of direct sunlight. (Preferably place the sealed sample in your freezer)
9. Ring the courier to collect the sample by 9.00 am or 11 am.
10. If you can only take your sample in the afternoon or evening, you will need to place the wrapped sample your deep freeze and ring the courier in the morning.
11. If you make a mistake (such as forgetting to put the sample in the freezer overnight) please ring Delwyn and we will provide you with another sample kit.

Thank you.

The coeliac research team

Appendix N- Food, Symptom and Medication Questionnaire

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Changes in Beneficial gut microflora in people with coeliac disease study

Please answer the following questions in as much detail as possible

1. What times do you usually eat your meals:

   Breakfast _____

   Mid-day meal_____

   Evening meal_____

2. Are there any foods you avoid?

   If yes, please give details (reasons for avoiding)

Food	Reason

(continue on the next page if required)

3. Do you usually prepare your own meals? Y / N

If no, who prepares your meals?

…….

4. Have you eaten any of these foods in the last week?
Please give as much detail as possible.

Food type	**Amount** *E.g. 3 slices, 1 croissant*	**Brand names and other details** E.g. Signature brand or Freya's granary
Breads		
Breakfast cereals		
Pasta		
Cookies		
Crackers		
Gravies		
Sauces		
Yoghurts		

4. Medications:

- What medications (prescription or non-prescription) have you taken in the last week?

.....

.

- In the last fortnight have you taken -

(a) Antibiotics Y / N

If yes, give details;

Name of antibiotic ……

Date taken……..

Dose given ……

(b) Pentasa Y / N

If yes, give details;

Date taken……..

Dose given ……

(c) Azathiaprine Y / N

If yes, give details;

Date taken……..

Dose given ……

5. Symptoms

- Do you usually suffer from any of the following symptoms?

		Comments
○ Flatulence	Y / N	……
○ Constipation	Y / N	……
○ Diarrhoea	Y / N	……
○ Bloating	Y / N	……
○ Abdominal pain	Y / N	……
○ Itchy skin	Y / N	……
○ Headaches	Y / N	……
○ Muscle or bone pains	Y / N	……
○ Fatigue and tiredness	Y / N	……
○ Other?		……

6. Now some questions about your bowel movements (poos)

- How many times do your bowels normally open each day?

Please circle the best answer.

1 2 3 4 5 other

- Is the stool usually formed or loose?
  Please circle the best answer.

  Very loose 1 2 3 4 5 very firm

- Is there any mucous in the stool? Y / N If yes describe the mucous (is it thick or thin, is it jelly like, is it throughout the stool or in clumps, is it just on the outside, is it yellow or clear)

- Is there any blood in the stool? Y / N If yes describe how much (is it the occasional small fleck, lots of small flecks, a few large flecks, lot of large flecks, lots of blood throughout the stool)

8. Instructions for a 3-day dietary history:

- Record all the food and drink that you have eaten in the three days before you take your faecal sample.
- Remember to include all meals, snacks, drinks and alcohol
- Remember to include anything that you may have added to foods such as sauces, gravies, spreads, dressings, etc.

<u>Describing the food and drink</u>

- provide as much detail as possible about the type of food eaten.
  For example; Cheese- Edam cheese,
  Milk- Lite blue top milk
  Bread - Vogels wholegrain bread
  Breakfast cereal – Sanitarium Natural muesli
  Pasta- white spaghetti noodles
  Yoghurt- Fresh 'n' Fruity- Apricot

- Give details of all cooking methods used.

For example; fried, baked, grilled, casseroled, stir-fried, roasted, poached, boiled, steamed. etc.

Try to estimate the amounts you ate. This can be done by

(a) using household measures; For example. cups(C), teaspoons (t), tablespoons (T)
(b) using weights marked on packages; for example 32g muesli bar, 300ml can of Pepsi
(c) using comparisons; for example; butter the size of a walnut, tennis ball size scoop of ice cream
(d) For bread, describe the size of the slice (sandwich, toast)

Also include any medications that you take, and any vitamins or other supplements eg Berocca, Omega 3 fish oil capsules, garlic capsules, a probiotic etc.

If it goes in your mouth, you need to record it.
Remember to record this information for 3 days before you take your faecal sample.
Example:

Date…23 Feb……. Day One	Food and drink consumed	Serving size	Cooking method
Breakfast	*Sanitarium toasted muesli* *Half fat milk* *White bread, Freyas* *Jam, strawberry*	*1 cup* *1 cup* *3 slices* *1 tbsp*	*Toasted*

Date…… Day One	Food and drink consumed (include description)	Serving size	Cooking method
Breakfast			
Snacks			
Lunch			
Snacks			
Evening meal			
Snacks			
Anything else?			

Date…… Day Two	Food and drink consumed (include description)	Serving size	Cooking method
Breakfast			
Snacks			
Lunch			
Snacks			
Evening meal			
Snacks			
Anything else?			

Date…… Day Three	Food and drink consumed (include description)	Serving size	Cooking method
Breakfast			
Snacks			
Lunch			
Snacks			
Evening meal			
Snacks			
Anything else?			

Thank you for completing this questionnaire.

Please return it in the envelope provided.

Delwyn MacKenzie
Institute of Food Nutrition & Human Health
Massey University
Palmerston North

Contact phone: 06 350 5962

This project has been reviewed and approved by the Massey University Human Ethics Committee, PN Protocol No. 06/08.

If you have any concerns about the conduct of this research, please contact Professor Sylvia V Rumball, Chair, Massey University Campus Human Ethics Committee: Palmerston North, telephone 06 350 5249, email humanethicspn@massey.ac.nz

Appendix O: PARTICIPANT CONSENT FORM

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Changes in Beneficial gut microflora in people with coeliac disease

This consent form will be held for a period of five (5) years

- I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction.
- I understand that I may ask further questions at any time.
- I agree to allowing my faecal samples to be analysed for the purposes of coeliac research as outlined in the accompanying information
- I have read the outline of the study and I agree to participate in this research under the conditions outlined.

Signature:		**Date:**	
Full Name – printed please			
Postal address			
Phone number			
Age			
Gender			

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 06/08. If you have any concerns about the conduct of this research, please contact Dr John O'Neill, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 8635, email humanethicsoutha@massey.ac.nz."

Appendix P: Information Sheet

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Changes in Beneficial gut microflora in people with coeliac disease

My name is Delwyn MacKenzie. I am undertaking this research project as part of a Masters of Science in Human Nutrition through the Institute of Food, Nutrition and Human Health (IFNHH) at Massey University.

You have been given this letter and information pack because the specialist you have just seen, considers it probable that you have coeliac disease, a disorder in which your body reacts abnormally to a harmless dietary protein called gluten.

The purpose of this study is to investigate whether having coeliac disease influences the normal population of bacteria that inhabit your intestine. We know the human intestine harbours a large number of bacteria that contribute to your health. For example, helping to prevent bacteria that cause infectious disease from invading your bowel. It seems that your body recognises the normal bacteria as 'friends' rather than foes and 'tolerates' their continuing presence but keeps them restricted to the walls of the intestine. It is also known that when the bowel is inflamed that it becomes more permeable to all bacteria allowing them to get through the walls of the intestine. This provokes the immune system to inactivate and kill these 'invading' bacteria. It is not known whether the normal population of 'friendly' bacteria invade and are treated in this way. If they are, then people with coeliac disease may be missing out on the benefits of having friendly bacteria owing to the fact that the bowel wall is inflamed by gluten in the food they eat.

If after reading this information sheet, you are prepared to participate this study, or you have further questions, you should ring Mrs Delwyn MacKenzie on 04 4789781.

If you choose to participate in the study we will require about 5 hours of your time spread over the next 4 months.

We will ask you to answer a questionnaire about your eating habits and collect a sample of faeces on 4 separate occasions. **The first sample needs to be taken shortly after you get this information pack** in order to get a stool sample before you start a gluten free diet. The other 3 stool samples will be collected at monthly intervals to monitor the response of your friendly bacteria to a diet that avoids gluten.

We realise that it is an imposition to ask you to provide us with a faecal sample, but this is the only way we can investigate changes induced by inflammation. The information gained from this study will benefit those with coeliac disease and may even be relevant in other inflammatory conditions of the intestine.

NOTE: As you do not currently have the results of laboratory testing to confirm the diagnosis of coeliac disease, we will need you to inform us when you receive this confirmation from your specialist. Similarly, we will need to be informed if your diagnosis changes and it turns out that you do not have coeliac disease. If you have already sent the first stool sample to us, but coeliac disease is not confirmed, we will withdraw you from the study and dispose of your initial sample as you will no longer be eligible to participate. When you receive your results, please fill in the coeliac confirmation slip and return it to us in the pre-addressed envelope provided.

Non-participation in the study will in no way affect your ongoing clinical care.

The initial kit contains;

- This information sheet
- A consent form
- Instructions for collection of faecal samples
- A sample collection pack.
- Instructions on how to return your sample to the principal researcher
- Coeliac confirmation slip
- A pre-addressed stamped envelope

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- *decline to answer any particular question;*
- *withdraw from the* ***study at any time***
- *ask any questions about the study at any time during participation;*
- *provide information on the understanding that your name will not be used unless you give permission to the researcher;*
- *be given access to a summary of the project findings when it is concluded*

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 06/08. If you have any concerns about the conduct of this research, please contact Dr John O'Neill, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 ex 8635, email humanethicsoutha@massey.ac.nz

Thank you

Delwyn MacKenzie (Mrs)
MSc Student
Institute of Food, Nutrition and Human Health
Massey University