THE USE OF PROBIOTICS IN INTESTINAL PROTECTION

By

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INTRODUCTION

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ABSTRACT

While studies have suggested that gastrointestinal pathogenicity associated with conditions such as inflammatory bowel disease and chemotherapy induced mucositis can be improved by the use of probiotics, the mechanisms of probiotic protection are not clearly understood. Such knowledge is necessary to achieve maximum benefit from such forms of treatment. These investigations were undertaken to initially establish a non-invasive measure of intestinal barrier function and then to examine the protective effects of different yogurts on conditions which cause loss of barrier function: chemotherapy induced mucositis; inflammatory bowel disease (IBD) patients; and athletes in training.

Different yogurts acted on different regions of the intestine in an animal model of methotrexate-induced small bowel damage. Sheep milk yogurt decreased the severity of proximal small intestinal damage and increased sucrase activity. Probiotic LA1 (Lactobacillus johnsonii strain LA1) yogurt improved intestinal function by maintaining small intestinal permeability with the development of methotrexate-induced damage. These yogurts may enhance mucosal recovery after damage.

IBD patients' intestinal permeability was found to mirror their disease activity and may assist in monitoring disease activity and direct interventions. Intestinal permeability in moderately trained recreational athletes was elevated to a level of severity seen in disease affected intestines. The number of athletes with elevated intestinal permeability increased with the length of training. Colonic microflora metabolism, measured by faecal short chain fatty acids (SCFA), was significantly increased in IBD patients and athletes compared to healthy controls. Probiotic LA1 yogurt significantly decreased faecal SCFA in athletes and IBD patients. SCFA also increased the adhesion of probiotic bacteria, which may affect their colonisation. Probiotic induced intestinal functional changes may be related to SCFA levels.

The function of the small intestine can be compromised to different degrees after chemotherapy drugs, under physiological stress and in disease. Probiotics may improve compromised intestines through enhancing recovery of the intestinal mucosa and the barrier function.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any institution. To the best of my knowledge and belief, this thesis contains no material that had been previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis being available for photocopying and loan.

Emma Southcott

Date: [month, day, year]  Signature:
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ABBREVIATIONS

CD – Crohn’s disease

$^{51}$Cr EDTA - $^{51}$Chromium ethylene diamine tetra acetic acid

CRP – C - reactive protein

ESR – Erythrocyte sedimentation rate

GALT – Gastrointestinal lymphoid tissue

HB – Harvey-Bradshaw

HR – Heart rate

$HR_t$ - Heart rate threshold

HRP – Horse radish peroxidase

IBD – Inflammatory bowel disease

IgA – Immunoglobulin A

IL - Interleukin

L - Lactulose

LAB – Lactic acid bacteria

LGG – *Lactobacillus rhamnosus* strain GG

LA1 – *Lactobacillus johnsonii* strain LA1

LPS - Lipopolysaccharide

L Shirota – *Lactobacillus paracasei* subspecies *paracasei* strain Shirota

M - Mannitol

MTX - Methotrexate

NSAIDs – Non steroidal anti-inflammatory drugs

PCDAI – Paediatric Crohn’s disease activity index

PEG – Polyethylene glycol

PP – Peyer’s patches

Rh - Rhamnose

RI – Refractive index

SCFA – Short chain fatty acids

TNF – Tumor necrosis factor

UC – Ulcerative colitis

YC-180 – mix of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

ZO – Zonula occludins
AIMS OF THESIS

This thesis seeks to better understand small intestinal permeability and its role in barrier function testing in chemotherapy induced mucositis, IBD patients and athletes. It further aims to elucidate the interaction of probiotic organisms in both healthy and compromised intestines. It is hoped this work will increase the understanding of the physiological function of intestinal permeability in relation to the luminal milieu in order to design therapeutic approaches to diseases and conditions with altered permeability.

HYPOTHESES

The hypotheses of this thesis are that:

1. Measurements of small intestinal permeability can detect abnormalities in barrier function induced by chemical damage, stress, and disease.

2. Probiotics will improve small intestinal permeability in animal models of intestinal damage and in patients with intestinal disease.

3. Probiotics will favourably modify the metabolic activity of the intestinal flora.
RESEARCH PLAN

The first study involved developing a methodology and protocol for intestinal permeability testing in a methotrexate-induced damage rat model in order to investigate the integrity of the barrier function. Chapter Two investigated the effect of fasting versus feeding state in rats on performance of the permeability test. The influence of probiotics on intestinal barrier function was evaluated. Chapter Three examined the small bowel damage induced by methotrexate in rats and the effect of probiotic intervention of either *Lactobacillus johnsonii* strain LA1 or *Lactobacillus bulgaricus* (YC-180) on intestinal function.

The second study determined the usefulness of small intestinal permeability in the assessment of inflammatory bowel disease. Chapter Four aimed to assess intestinal permeability as a non-invasive marker of disease activity and disease extent in paediatric inflammatory bowel disease patients. Intestinal permeability was evaluated as a means of monitoring the response to therapy and predicting disease relapse. Chapter Five investigated changes in intestinal function after a probiotic *Lactobacillus johnsonii* strain LA1 supplemented diet in IBD patients and healthy subjects.

The third study (Chapter Six) investigated the effect of training and probiotics on the intestinal barrier function of athletes. Runners had their exercise performance, intestinal permeability and faecal SCFA tested prior to and after a training program. The influence of a probiotic yoghurt (LA1) and placebo yoghurt on exercise performance and barrier function over a four week exercise program was tested.

The fourth study (Chapter Seven) observed the adhesion to intestinal mucus by two probiotic strains; *Lactobacillus johnsonii* strain LA1 and *Lactobacillus bulgaricus* (YC-180). The adhesion of these strains was compared to two of the most researched probiotic strains: LGG and L Shirota. The adhesion was measured with increasing levels of tributyrin, the triglyceride of butyrate, to determine the effect of butyrate on the mucus adhesion of these strains.
Chapter 1 INTRODUCTION

The health of the intestine cannot be easily determined without surgery or other invasive methods. Endoscopy and colonoscopy can provide information about the proximal and distal areas of the gastrointestinal tract, however access to the majority of the small intestine is limited. The intestinal epithelium is an important surface for digestion and absorption, and it also plays a role in intestinal barrier function. The intestinal epithelium restricts the entry of molecules into the body, selectively absorbing needed nutrients with a high degree of efficiency, and preventing the absorption or uptake of macromolecules, antigens and bacteria from the lumen of the gut. Intestinal permeability is a dynamic property of the epithelium and refers to the passage of molecules via non-mediated diffusion (Travis and Menzies 1992). Intestinal permeability tests, which are non-invasive, have become increasingly important tools in screening and assessing the integrity of the epithelium in gastrointestinal diseases (Pearson et al. 1982; Wyatt et al. 1993; Catassi et al. 1997). Permeability tests have enabled researchers to detect altered intestinal permeability in several gastrointestinal disorders (Pounder et al. 1983; Ford et al. 1985; Barau and Dupont 1990). These alterations suggest that the barrier to the absorption of macromolecules, bacteria and antigens may be disrupted and thus no longer prevent their penetration into the mucosa. Increased access of molecules and antigens would increase antigenic stimulation and may result in further increases in permeability (Hollander 1988; Olaison 1990). This thesis investigates three scenarios where intestinal permeability might be altered: chemotherapy induced mucositis; inflammatory bowel disease; and exercise induced stress in athletes.

THE GASTROINTESTINAL BARRIER

The intestine provides a barrier to inhibit ingested bacterial pathogens, antigens, macromolecules and noxious agents from entering the body’s internal environment. The non-immunological components of the gastrointestinal barrier include the epithelial cell layer, gastric acid, motor propulsive activity, intestinal mucus and intestinal bacterial flora (Figure 1.1). In the intestine the epithelial cell layer constitutes the principal barrier to
permeation. The primary function of this barrier is to regulate the flow across transcellular and paracellular pathways. The intracellular tight junctions restrict access to the paracellular pathway (Travis and Menzies 1992; Eckmann et al. 1995; Hecht 1995). Changes in the paracellular pathway, probably mediated through tight junctions, appear to be a major factor determining the altered permeability to larger molecules (Madara et al. 1990; Sadowski and Meddings 1993). Cytokines, inflammatory cells, drugs or physical factors such as osmotic stress, may influence tight junctions in the intercellular space and lead to increased antigen access (Krugliak et al. 1990; Madara et al. 1990; Fasano and Uzzau 1997; Groot et al. 2000). Excessive exposure of antigens to the immune system is thought to lead to gastrointestinal or systemic disease (Olaison 1990; Heyman and Desjeux 2000).

Figure 1.1 The intestinal barrier. The intestinal barrier has several features that protect the body against noxious agents from entering through the intestine. Intestinal motility influences luminal contents’ contact time with the mucosa affecting both absorption and bacterial species colonisation. The luminal contents are broken-down by proteolysis, gastric acid, bile acid and by colonic bacterial population. The principal barrier to absorption is the epithelial cell layer, and molecules can either permeate the cell layer actively or passively through paracellular and transcellular pathways. Other non-immunological components involved in the intestinal barrier are: unstirred layer, thickness and viscosity of the mucus layer, goblet cells, and microvillus membrane.
Figure 1.2 The intestinal barrier: Mucosal immune system. The mucosal immune system provides the immunological components: secretory IgA system; gastrointestinal lymph tissues (GALT); M cells-directed antigen response; Lamina propria lymphoid cells; and intraepithelial lymph cells. If pathogens or antigens cross the epithelial cell layer the mucosal immune system responds. The intestinal contents are sampled by M cells in the Peyer’s Patch. The lamina propria contains several immune cells that are able to respond directly to an antigen and do not respond to food antigens (tolerance). This diagram is adapted from Kagnoff 1989.

**Bacterial population**

The gastrointestinal tract is a complex ecosystem. It is a habitat for more than 400 species of microorganisms. These micro-organisms colonise the gut in the first few days of life, and once established, the proportion of species present in the colon changes with age (Fuller 1989). Germ-free animals have provided the best evidence that the gastrointestinal flora protects against disease. Germ-free animals have no intestinal flora and have been found to be more susceptible to disease than conventional animals. A germ-free mouse can be killed with 10 cells of Salmonella enteritidis, whereas it requires $10^6$ cells to kill a conventional mouse (Collins and Carter 1978; Fuller 1989).
Lactic acid bacteria (LAB), including *Bifidobacteria, Lactobacillus, Streptococcus* and *Enterococcus* form part of the indigenous microflora (Lidbeck and Nord 1993). In 1904 Elie Metchnikoff hypothesised the beneficial effect of LAB on human health (cited in Bibel 1988). He proposed that the replacement of toxin-producing bacteria in the intestine by LAB present in yoghurt could lead to a long and healthy lifestyle (Metchnikoff 1908).

The intestinal microflora established in the gut is very stable, but can be influenced or altered by diet, environmental factors, antibiotic therapy and stress (Tannock and Savage 1974; Goldin and Gorbach 1984b; Cummings and Englyst 1987; Cummins et al. 1988; Parker 1990; Guo et al. 1995; Krause et al. 1995). The microflora is important for obtaining energy from insoluble dietary fractions, removal of toxins, retention of water and electrolytes (Cummings and Englyst 1987; Fuller 1989). During stressful conditions there is a trend for the lactobacilli to decrease and for the coliforms to increase biomass (Tannock and Savage 1974; Fuller 1989). Bacterial populations are also distinctly different between those patients with intestinal diseases and healthy individuals (Gaiffer et al. 1991; Salminen and Deighton 1992; Aguirre and Collins 1993; Lidbeck and Nord 1993).

**Short chain fatty acid production**

The microflora of the human colon ferments dietary components that escape digestion in the small intestine. The major fermentation products are gases (H₂, CH₄, CO₂), increased biomass and short chain fatty acids (SCFA) (Cummings and Englyst 1987). Different isolated species of bacteria make different contributions to the production of SCFA (Sakata 1994). These fatty acids can be modified by changes in bacterial flora (Hove et al. 1994) or in the type of carbohydrate predominating in the diet (Mortensen et al. 1991). The major SCFA produced from fermentation are acetate, propionate, and butyrate which are readily taken up by colonocytes (Roediger 1981; Sakata 1994). Lactate isomers, valerate, iso-butyrate and iso-valerate are produced at lower concentrations (Cummings and Macfarlane 1997). Butyrate is the preferred oxidative fuel of distal colonic epithelial cells, above glucose and glutamine (Roediger 1980b; Roediger 1982). Acetate and propionate are absorbed, enter the portal vein, and appear to make a significant contribution to energy through hepatic metabolism. SCFA have benefits for colonic epithelial health *in vitro* and
in vivo. SCFA, particularly butyrate, may be essential for maintaining mucosal integrity, as colitis that develops from the surgical diversion of the faecal stream from the affected segment of bowel can be reversed by SCFA enemas (Harig et al. 1989; Scheppach et al. 1992; Sakata 1994; Scheppach 1994). Butyrate-promoted cell migration in wounded colonic epithelium and in vitro studies have shown that butyrate inhibited proliferation but improved maturation of colonic epithelial cell lines (Sakata 1987; Sakata 1994; Wilson and Gibson 1997; Inan et al. 2000; Mariadason et al. 2001).

Mucus production

The epithelial surface of the intestine is covered by a mucus gel coating the microvilli of the enterocytes (Figure 1.1 and 1.2). Mucus has a protective role and is secreted along the intestine. In the small and large intestine, the mucus is synthesized by specialised epithelial cells called goblet cells which are scattered between the enterocytes. Mucins (mucus glycoproteins) are the major organic components of mucus and are responsible for the viscoelastic properties of the mucus gel. The mucins are composed of glycoprotein monomers that vary in size and in the composition of their oligosaccharide chains (Allen 1984; Neutra and Forstner 1987). The protein content of mucin is about 20% by weight, while carbohydrate comprises 70 to 80%. The mucus gel is predominately water (up to 95%); mucin content makes up to 5% with the remainder being lipids, free proteins, nucleic acids and salts. Mucus provides a habitat and nutrients for the bacteria belonging to the normal microflora (Tuomola 1999).

Disease states, particularly ulcerative colitis and cancer, can result in changes in mucus properties and decrease in its thickness (Gibson and Pavli 1992). A reduction of the thickness of this layer is achieved by efficient stirring of the mucus, possibly from the contraction of the villi and microvilli (Laboisse et al. 1996). Altered glycoproteins affecting the viscosity of the mucus layer can contribute to the disturbed intestinal barrier (Rhodes 1989; Travis and Menzies 1992; Tytgat et al. 1996; Strugala et al. 1998; Bodger et al. 2002).
Intestinal mucosal integrity

The epithelial cell lining provides a physical barrier to antigens, bacteria and noxious substances (Kagnoff 1989). Behind the epithelial cell lining is the lamina propria, which extends between the crypts and on to the cone of each villus (Figure 1.2). Lamina propria is rich with lymphatic tissue and blood supply and is separated from luminal contact by epithelial cells. The healthy mucosa is normally impermeable or poorly permeable to foreign particles including markers of intestinal permeability. Damage to the integrity of the epithelial cell layer will allow the permeation of foreign particles into the lamina propria and hence the blood stream (Hollander 1988; Hecht 1995). The transport of permeability markers across damaged intestines is also enhanced leading to an increased concentration of these markers in the blood and ultimately the urine. Blood and urine assayed for these markers give a measure of intestinal integrity (Travis and Menzies 1992).

Mucosal immune system

The mucosal immune system supports barrier function by responding to antigen challenge and regulating the immune response (Kagnoff 1989; Sanderson and Walker 1993; Eckmann et al. 1995). The mucosal immune system is capable of removing the majority of antigens and rarely does it respond excessively to an antigen. The mucosal immune system is constantly sampling luminal contents and the antigens are transported across the intestine in a controlled manner (Kagnoff 1993). Sanderson (1997) suggested that the intestinal epithelium responds to luminal factors and signals their presence to the mucosal immune system. The Peyer’s patches are involved with the initiation and expression of the mucosal immune system when antigens and microbial pathogens cross the physical barrier.

Gastrointestinal lymphoid tissue

When food or bacterial antigens cross the intestinal barrier the gastrointestinal lymphoid tissue (GALT) responds as it contains as much lymphoid tissue as the spleen (Kagnoff 1989). The three major populations of lymphoid cells in the GALT are Peyer’s patches
(PP), lamina propria lymphoid cells, and intraepithelial lymph cells. Peyer’s patches are separated from gut luminal contents by specialised cells called M cells (membranous) and are important in the initiation and expression of the mucosal immune response. Mucus-producing goblet cells with M cells are part of the follicle-associated epithelium that covers the lymphocytes in the PP. Figure 1.2 displays the cells present in the lamina propria. Intraepithelial lymphocytes are present between epithelial cells and are separated from direct exposure to gut antigens by the tight junctions of enterocytes (Kagnoff 1989; Kagnoff 1993). Macromolecules, food and bacterial antigens increase number of lymphoid cells in the intestinal tissue (Kagnoff 1989). Macromolecules uptake can be advantageous for the immune system as it is needed in infancy for the passive transfer of maternal antibodies (Thompson et al. 1998). M cells are a part of the trans-epithelial mechanism that facilitates the controlled uptake of growth factors and immunoglobulins from the lumen into PP (Sanderson and Walker 1993).

IgA

The mucosal secretory IgA response is a major protective factor against antigen challenge. The properties of secretory IgA include neutralising viruses and toxins and enhancing the non-specific defence mechanisms of lactoperoxidase and lactoferrin. IgA system creates a regionally initiated immunity to antigens and pathogens that has a long memory (Hobbs 1992; Holmgren and Svennerholm 1992; Pentilla et al. 1995; Mestecky et al. 1999). The memory cells assist in limiting the immune response to the antigen present in the mucosal area (Hobbs 1992; Kagnoff 1993). A raised serum level of IgA can occur arising from a common non-specific response to disease of the small gut such as Crohn’s disease (Hobbs 1969).

**Neuroendocrine system**

Substantial evidence exists illustrating the involvement of the nervous system and neurotransmitters/neuromodulators in regulating epithelial physiology (McKay and Perdue 1993a). The intestinal neuroendocrine system consists of an extensive network of nerves
and specialized epithelial endocrine cells (Garabella 1979; Kimura et al. 1994). In the lamina propria a diffuse collection of lymphocytes, plasma cells and monocytes are within one cell distance of nerve fibres (McKay and Perdue 1993b). The positioning of the neuroendocrine system and the mucosal immune system points to the existence of a communication network between the two systems. The fact that both systems express neuropeptide and cytokine receptors supports the existence of a neuroendocrine–mucosal immune interaction (Kagnoff and Kiyono 1996). Abnormalities in these interactions can promote pathophysiological reactions in the intestine, such as these seen in inflammatory bowel disease (IBD) and food allergy reactions (Bienenstock 1995; Nagura et al. 1996).

**Intestinal Permeability**

Permeability is a dynamic property of the intestinal epithelium which allows molecules to pass by diffusion. Permeability is regarded as the property of semi-permeable membranes, and this is different to permeation which is the act of non-mediated diffusion (Travis and Menzies 1992; Pape et al. 1994; Sun et al. 1998). Differences in mucosal surface area, the thickness of the unstirred water layer, or intestinal transit, have been found to alter the permeation of a marker independently of changes on epithelial permeability (Travis and Menzies 1992).

The intestinal epithelium is considered a heteroporous barrier. The mechanisms of intestinal permeability are still theories. Travis and Menzies (1992) re-examined the pore theory to explain differential diffusion across the intestinal membrane. Pappenheimer et al. (1951) suggested that the epithelial membrane is perforated with a large population of small pores (radius 0.4-0.7 nm) and a small population of large pores (radius 6.5 nm). This theory was derived from observations of a 20-30 fold increase in the permeation of small molecules with radii <0.4 nm, compared to large molecules with radii 0.4–0.8 nm (Pappenheimer et al. 1951 cited in Travis and Menzies 1992). As yet no pores have been seen with a magnitude of 0.5 to 1.5 nm in the intestinal epithelium.

An alternative theory presented by Hollander (1992) suggested that water-soluble permeability probes transverse the epithelial layer predominately through the paracellular
tight junctions, and not through the cell membrane. This hypothesis is based on differences in paracellular structure, and the fact that the permeability of probes in the two regions is governed by molecular size. Hollander (1992) also suggested that the permeability of probes differs along the villus-crypt axis. Anatomic and electrophysiological data indicated that the epithelium has major differences in the size and strand density of the tight junctions between the crypt and villus. The crypt has more permeable tight junctions while the villus epithelium has smaller and more resistant junctions (Marcial et al. 1984; Harris et al. 1988 cited in Hollander 1992). Intestinal absorption takes place primarily in the villus tips. Luminal antigens, peptides, bacterial particles, or water soluble particles could penetrate the mucosa depending on their size and approach (Johansen et al. 1989; Travis and Menzies 1992; Sadowski and Meddings 1993). Large molecules (around 1 nm) are able to penetrate the crypt tight junctions but have limited access from the lumen (Hollander 1988; Fasano and Uzzau 1997). The intestinal permeability for small and large molecules is not uniform along the length of the intestine and colon (Krugliak et al. 1994; Sinko and Hu 1996; Fagerholm et al. 1997). Both pore and tight junction theories highlighted the fact that differential permeability occurs in the healthy intestine, and that the size and radius of molecules affects their absorption (Hollander 1988).

Paracellular permeability

The paracellular pathway is the route of permeation for molecules larger than monosaccharides (molecular mass >180). When these molecules are injected intravenously they remain in the extra-cellular space (Marcial et al. 1984; Elia et al. 1987). Inter-cellular junctions represent a natural interruption in the membrane and a potential pathway for these large molecules. It has been suggested that lactulose, cellobiose, EDTA, raffinose and dextran all use paracellular pathways (Marcial et al. 1984; Elia et al. 1987; Travis and Menzies 1992). The paracellular pathway involves the passage of molecules via tight junctions to the underlying intracellular space, or through gaps in the epithelium caused by exfoliation.
The paracellular pathway corresponds to the rate-limiting step of the permeability barrier. Tight junctions are the site where two cells fuse and provide a discrete barrier (Figure 1.3). The linking of tight junctions to the cytoskeleton has led to the suggestion that tight junctions represent a gating mechanism in the paracellular pathway. Madara (1987) hypothesised that a series of intracellular events might stimulate the peri-junctional ring to contract, with the tension exerted on the lateral membrane resulting in the opening of tight junctions (Madara 1987). Tight junctions are discussed in more detail below.

Once through the tight junctions, molecules then diffuse through the intercellular space, though tortuous and narrow. The molecules transport is dependent on the rate of fluid transport. It has been suggested that a small osmotic gradient or sub-mucosal tissue (hydrostatic) pressure gradients influence paracellular water and solute absorption (Madara and Pappenheimer 1987; Pappenheimer and Reiss 1987). It is thus difficult to estimate the contribution of the intercellular space to the passage of molecules (Naftalin and Tripathi 1985; Weinstein 1987).

The second part of the paracellular pathway involves large molecules permeating through gaps left by dead cells, ulceration or extrusion zones. Normal exfoliation appears to occur without disrupting the barrier because as the cell is extruded the adjacent cells ‘zip-up’ from base to apex (Moore et al. 1989; Travis and Menzies 1992). Proliferation and differentiation of surface mucosal cells re-establish the continuity of the epithelial surface (Moore et al. 1989).

**Tight junctions**

The tight junctions provide a barrier to passive ion flow and an adhesive force that maintains epithelial confluence. Tight junctions circumferentially belt intestinal epithelial cells and allow linking of lateral membranes of adjacent cells through ‘kisses’ or fusion sites (Figure 1.3). Electron microscopy shows that tight junctions between neighbouring epithelial cells are 0.1 to 0.6 µm in length (Madara and Hecht 1989). Freeze fracture images have shown the fusion sites to consist of a net-like web of strands and grooves (Madara and Hecht 1989). The majority of enterocyte tight junctions strands are uniformly
Figure 1.3 Enterocyte cells use tight junction to link the lateral membranes of adjacent cells through 'kisses' or fusion sites. At the tight junctions the linear fusion sites are visualised as anastomosing strands or grooves in fracture planes. Below the tight junctions lie the zonula adherens and intermediate junctions. At the tight junctions, a peri-junctional ring of actin-myosin filaments inserts into the lateral membrane of many epithelial cells. Occludin, a transmembrane protein strand connects the cell cytoskeleton with the junctional complex. Proteins zonula occludins (ZO) are involved in the complex. Other proteins involved in tight junction regulation are found near the complex (7H6, cingulin, rab13, rho and ras). Below the tight junction complex and adherens is the tortuously narrow intercellular space. This figure is a combination of diagrams in the following references (Madara and Hecht 1989; Madara et al. 1990; Fasano 2000).
composed of 4-6 cross-linked strands (Claude 1978; Marcial et al. 1984; Madara and Pappenheimer 1987). The tight junctions in villus goblet cells are highly heterogeneous in structure (Ernst 1980). Goblet cell tight junctions are more permeable compared with epithelial cell tight junctions. Tight junctions represent the physical barriers to passive trans-junctional flow.

The estimated numbers of tight junction strands are greater in the villi (6.03) than in the crypt (4.45) (Hollander 1992). Claude (1978) suggested junctional resistance increase logarithmically as strand count increases arithmetically. In the intestine this would mean many strands would provide more resistance thus providing a better barrier than a few strands. This theory is influenced by cell dimensions and junctional structure and varied from tissue to tissue (Claude 1978 cited in Madara and Hecht 1989).

The uptake of nutrients may be controlled by reversible changes in tight junctions (Madara and Hecht 1989). After a meal luminal substrates concentration exceed the transport maximum for the co-transporters. Sodium co-transporters and substrates create a transjunctional osmotic gradient favouring passive water absorption but also trigger a reversible opening of tight junctions that can no longer sieve out substrates sized solutes carriers by solvent drag. The process is thought vital for glucose and amino acid absorption (Madara and Hecht 1989). The tight junctions may undergo permanent changes with disease or low grade inflammation (Ramage et al. 1988; Madara et al. 1990). The perturbation of tight junction in disease state may results from maturation of intestinal cells in the presence of inflammation (Madara and Hecht 1989; Madara et al. 1990).

It has been suggested that abnormal tight junctions are an aetiological factor in Crohn's disease (Hollander 1988). Marin et al. (1983) and Dvorak et al. (1979) found uninflamed tissue from Crohn's disease (CD) patients differed in number and separation of tight junctions compared to controls. Schmitz et al. (1999) found tight junctions contributed to impaired barrier function in ulcerative colitis. Freeze-fracture electron microscopy identified a decrease in the strand number in villus cells of UC patients, accompanied by a reduction in the depth of the tight junction network. Strand discontinuations are frequently observed in UC patients. This may enable macromolecules such as bacteria and food antigens to cross the intestinal barrier (Fasano and Uzzau 1997; Schmitz et al. 1999). It is
possible that structure and function of tight junctions are active factors in both Crohn’s disease and ulcerative colitis (Schmitz et al. 1999; Soderholm et al. 2002a).

**Transcellular permeability**

For molecules to travel through the cell (i.e. transcellularly) they must be able to cross several barriers: the unstirred water layer, mucin layer and then the apical membrane. Monosaccharides such as L-rhamnose and mannitol are thought to diffuse through enterocyte cell membranes as they have been found to enter erythrocytes. The epithelial cell membranes have pores which small, non-lipid solutes may permeate. Membrane pores could represent water-filled channels, but it is possible that proteins are inserted into the membrane, analogous to a water channel (Naftalin and Tripathi 1985; Travis and Menzies 1992).

Apart from pores, there are at least three other mechanisms by which molecules can cross cell mechanisms. Firstly, molecules with a degree of lipid solubility may diffuse directly through the membrane. Secondly, membrane wounds caused by mechanical forces within the gastrointestinal tract, or abrasion by food and faecal particles, may allow water-soluble macromolecules to enter the epithelial cell, but not to penetrate the basolateral membrane (McNeil and Ito 1989 cited in Travis and Menzies 1992). Thirdly, transcellular transport of large molecules, including protein antigens, appears to occur by endocytosis into vesicles and subsequent exocytosis (Schaerer et al. 1991). M cells in the intestinal epithelium are far more permeable than the epithelial cells which surround them. M cells transport macromolecules and particulate material from the lumen of the gut into Peyers’ patches (Sanderson and Walker 1993; Sanderson 1997).

**Common pathways**

The passage of molecules through the basement membrane, extra-cellular matrix and across the capillary or lymphatic endothelium is common to both paracellular and transcellular pathways. Together these appear to present little impediment to diffusion of
molecules for the size of proteins. Direct study of the intestinal basement has not been
possible, but it appears to contain oval gaps 0.5–5.0 μm in diameter. This is not a problem
for molecules the size of $^{51}$Cr EDTA (359 MW) or smaller, which rapidly equilibrate
within the intestinal space (Granger and Perry 1983 cited in Travis and Menzies 1992).

**Measurement of Intestinal Permeability**

Non-invasive assessment of intestinal barrier function can be achieved by measuring the
permeability of the intestinal epithelial cell layer to a variety of orally administered test
substances of various sizes. Permeability tests have become increasingly useful tools in
screening and assessment of gastrointestinal diseases. This has reduced the need to use
more invasive investigations of the small intestine (Bjarnason et al. 1995).

**Carbohydrates**

Oligosaccharides are used as test substances for non-invasive assessment of the intestinal
permeability (Bjarnason et al. 1995). These sugars are non-metabolisable by mammalian
cells, hydrophilic and lipophobic, with negligible affinity for the monosaccharide transport
system, and are absorbed passively by non-mediated means. Small intestinal permeability
is measured using two non-metabolised sugars that travel along different routes. Lactulose
is a man-made disaccharide with a molecular mass of 342 Da and a diameter of 9.5 – 10.4
Å and transverses epithelia via a paracellular route (Bjarnason et al. 1995). Rhamnose is a
sugar alcohol with a molecular mass of 164 Da (hydrated L-rhamnose Rh.H$_2$O = 182 Da)
and a diameter of 8.3 Å. Mannitol is also a sugar alcohol with a molecular weight of 182
Da, a radius of 4 Å and a molecular volume 206 x 10$^3$ nm$^3$ (Krugliak et al. 1994;
Bjarnason et al. 1995). Endogenous mannitol production in rats and humans is negligible.
Both rhamnose and mannitol transverse intestinal epithelial cells via small pore in the
apical side, and exit the cell through pores in the basolateral membrane. Little difference
has been found between the permeability of mannitol and rhamnose (Maxton et al. 1986;
Hamilton et al. 1987; Andre et al. 1988).
The urinary excretion of these monosaccharides 24 hours after intravenous administration in humans is incomplete with about 74% and 67-100% in L-rhamnose and mannitol respectively (Laker et al. 1982; Cobden et al. 1985; Maxton et al. 1986 cited in Bjarnason et al. 1995). In rats, Meddings and Gibbons (1998) found the recovery of mannitol to be 70 ± 10% and lactulose 72.1 ± 3.7%. They also showed that ¾ of both probes were digested after overnight incubation with rat stool. It is well known that sugars measure only small intestinal permeability and not large intestinal as they are degraded by bacteria in the large intestine (Bjarnason et al. 1995; Meddings and Gibbons 1998). The urinary excretion ratio (L/Rh or L/M) of these sugars gives a measure of the leakiness or integrity of the intestinal barrier (Bjarnason et al. 1995). By using a ratio of two sugars to measure intestinal permeability, factors altering urinary excretion, including gastric emptying, intestinal transit time and renal clearance will affect both sugars equally and thus cancel out these factors.

**Chromium-51- labelled ethylene diamine tetra acetic acid**

Chromium-51- labelled ethylene diamine tetra acetic acid (\(^{51}\)Cr EDTA) is a water soluble substance with a molecular weight of 359. Its properties as a biological marker have been investigated in animal (Løkken 1970; Bjarnason et al. 1985b) and human studies (Elia et al. 1987; Aabakken 1989). Its positive features include high stability, inertness and low radiotoxicity. No affinity is seen for any particular organ, and an intravenously administered dose is excreted un-disassociated via the kidneys in a few hours. Løkken (1970) orally administered the probe in rats and less than 1% was excreted into the gastrointestinal tract of which no absorption was seen in the stomach (Bjarnason et al. 1985b). \(^{51}\)Cr EDTA is also suitable for measuring colonic permeability as it is readily absorbed and bacterial flora is unable to degrade it (Elia et al. 1987; Travis and Menzies 1992). Its stability and distribution compare favourably with phenol red and polyethylene glycol (PEG) 4000 (Maxton et al. 1986 cited in Bjarnason et al. 1995).

Bjarnason and Peters (1984) used \(^{51}\)Cr EDTA as a screening test for untreated coeliac disease. Since then the test has been used to demonstrate increased gastrointestinal permeability in inflammatory bowel disease (Teahon et al. 1991; Teahon et al. 1992),
atopic eczema (Bjarnason et al. 1985a), dermatitis (Griffiths et al. 1988), cystic fibrosis (Leclercq-Foucart et al. 1986; Leclercq-Foucart et al. 1987), and in patients taking non-steroid anti-inflammatory drugs (NSAIDs) (Bjarnason et al. 1985b; Aabakken 1989). The degree of absorption of $^{51}\text{Cr EDTA}$ from the stomach is uncertain, although indirect evidence suggests that this part of the intestine plays a minor role (Bjarnason and Peters 1984; Elia et al. 1987).

Animal studies

Several research studies have investigated the intestinal transport mechanisms in the rat to optimize drug absorption (Madsen et al. 1997; Duizer et al. 1998; Lindmark et al. 1998). In vitro methods using Ussing chambers, isolated perfused loops, and monolayer cell cultures have been useful in expanding our knowledge of passive permeability (Krugliak et al. 1989; Bijlsma et al. 1995; Collett et al. 1996; Sinko and Hu 1996). A disadvantage of these methodologies is that a permeability test cannot be repeated in the same animal over time. A summary of in vivo intestinal permeability tests in healthy animals and models of disease or induced intestinal damage in a number of studies is shown in Table 1.1.

Currently there are several protocols available for conducting non-invasive permeability tests. These differ in the probe type and concentration, urinary collection time, food and water availability. Carbohydrates have been used to test intestinal permeability in fasted, mildly fasted or fed rats over 4-48 hours (Bijlsma et al. 1995; Meddings and Gibbons 1998; Pernet et al. 1998). Bijlsma et al. (1995) kept animals in a mildly fasted state to compare with human studies. The lactulose recovery in rats was considerably greater than that in humans. Pernet et al. (1998) suggested a four-hour collection time for assessing urinary lactulose and mannitol in the rat, as this was when they found a difference in the lactulose recovery after a burn injury. Burn injuries have been associated with increased passive permeability (Levoye et al. 1992). In healthy fed rats the probe excretion over 4-24 hours was 72% for lactulose and 50% for mannitol of total probe recovery in 24 hours (Pernet et al. 1998). Meddings and Gibbons (1998) showed that 50% of the probes were still present in the small intestine of fasted rats three hours after the gavage. At nine hours only the non-metabolisable probe sucralose was detected, and this was in the colon.
Table 1.1  Summary of *in vivo* passive intestinal permeability studies in animal models.

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Time Period</th>
<th>Method</th>
<th>Marker</th>
<th>Results</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L/M</td>
<td>$^{51}$Cr EDTA</td>
</tr>
<tr>
<td>Bjarnason <em>et al.</em> 1985b</td>
<td>Rat</td>
<td></td>
<td>24 hr</td>
<td>RIA</td>
<td></td>
<td>2.06 ± 0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$^{51}$Cr EDTA</td>
<td></td>
<td>4.19 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>Control (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.20 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>Alcohol (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.97 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>Certimide (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>MTX (6)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ramage <em>et al.</em> 1988</td>
<td>Rat</td>
<td></td>
<td>24 hr</td>
<td>RIA</td>
<td></td>
<td>2.25 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Control Nematode</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.5 ± 2.0</td>
</tr>
<tr>
<td>Bijlsma <em>et al.</em> 1995</td>
<td>12 hr</td>
<td>HPLC</td>
<td>Lactulose mannitol</td>
<td></td>
<td>0.030 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Cats (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rats (4)</td>
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<tr>
<td>Rabbits (5)</td>
<td></td>
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<tr>
<td>Guinea pigs (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meddings and Gibbons 1998</td>
<td>Rat</td>
<td>HPLC</td>
<td>Lactulose Mannitol</td>
<td></td>
<td>0.30</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Control Aspirin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indomethacin TNBS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pernet <em>et al.</em> 1998</td>
<td>Control (18)</td>
<td>24 hr</td>
<td>GC</td>
<td>Lactulose</td>
<td>0.198 ± 0.022</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Burn (18)</td>
<td></td>
<td></td>
<td>Mannitol</td>
<td>0.470 ± 0.021</td>
<td></td>
</tr>
</tbody>
</table>

HPLC – High pressure liquid chromatography; GC – Gas chromatography; RIA – Radioimmuno assay
A 24 hr $^{51}$Cr EDTA test has been used to investigate the permeability in animal models of intestinal damage induced by ethanol, non-steroidal anti-inflammatory drugs or nematode infection (Bjarnason et al. 1985b; Ramage et al. 1988; Davies et al. 1994). Løkken (1970) found absorption of $^{51}$Cr EDTA in ligated parts of the rat intestine was limited in the stomach. A greater amount of $^{51}$Cr EDTA was absorbed in duodenal mucosa and absorption declined progressively along the intestine, however Elia et al. (1987) showed that $^{51}$Cr EDTA is readily absorbed in the colon.

Bjarnason et al. (1985) showed $^{51}$Cr EDTA is stable and does not bind to rat intestinal contents or tissue. They found no difference in results between a dose of 2 µL and 20 µL. They assessed several time points and observed that a five hour urine collection gave an average of 50% (range 36 - 63%) of that detected after the 24 hour collection. No activity was measured in the faeces after five hours, but it was measured eight or nine hours after the permeability test. At the five hour point a larger proportion of $^{51}$Cr EDTA was present in the caecum (76.8%) than in any other region in the intestine. The middle and distal intestine had the second largest proportion. Bjarnason et al. (1985) showed that normal mucosa is maximally resistant to the absorption of $^{51}$Cr EDTA and that when rats are treated with ethanol, MTX or a detergent, an increase in $^{51}$Cr EDTA is induced. Ramage et al. (1988) observed the barrier function became impaired from the damage and inflammation. $^{51}$Cr EDTA results in this model were similar to ovalbumin results, leading to the hypothesis that $^{51}$Cr EDTA permeability response reflected the permeability of macromolecule antigens.

MODELS OF COMPROMISED INTESTINAL INTEGRITY

**Methotrexate animal model of intestinal damage**

Methotrexate (MTX) is most widely used as an anti-metabolite, as it induces cell death in cancer cells. It is a folate antagonist and folates are essential for the synthesis of purine nucleotides and thymidylate (needed in DNA and cell division) (Jolivet et al. 1983; Egan
and Sandborn 1996). MTX will slow the replication of rapidly dividing cells, especially cancer cells. MTX in low doses is used as an immuno-modulating agent, and is considered useful in the treatment of IBD when traditional medical therapy has failed. MTX has low lipid solubility and thus does not cross the blood brain barrier easily. The unwanted effects of MTX are that it inhibits DNA synthesis causing myelosuppression and damage to the epithelium of the gastrointestinal tract (Jolivet et al. 1983; Egan and Sandborn 1996). MTX administration leads to a deficiency in folate and to the reduced ability of cells to double their nuclear DNA complement. This reduces cell turnover which causes significant mucosal injury (Rang and Dale 1996). The gastrointestinal tract is affected by MTX because crypt cells rapidly divide. The cells migrate from the crypt to the villus tip of the epithelium in about two days in rats. At the top of the villus the cells slough off so the epithelial lining is replaced in three to five days.

MTX causes acute injury to the intestinal epithelium in the rat and is characterised by reduced mitoses in crypt cells and shortened villi (Taminiau et al. 1980). MTX damage is most severe in the proximal small bowel, with pronounced crypt and villus ablation, and depletion in goblet cells (Taminiau et al. 1980; Howarth et al. 1996; Howarth et al. 1998; Xian et al. 1999). Following MTX-induced injury the subsequent intestinal repair involves crypt cell hyper-proliferation and mucosal regeneration (Xian et al. 2000). Verberg et al. (2000) found that regeneration through hyper-proliferation coincided with villus atrophy and a reduction of enterocyte maturation. They also found that goblet cells were spared at the villus tips and remained functional.

Taminiau et al. (1980) showed that 96 hours after MTX the activity of disaccharidase and alkaline phosphatase was decreased, although MTX was not detectable in the serum. Villus cells had increased expression of thymidine kinase, which is normally only seen in crypt cells. This suggested that cell differentiation did not proceed to completion during rapid cell division and migration. During hyper-proliferation crypts depth was increased as was villus height. Taminiau et al. (1980) suggested that the MTX rat model to have histopathological similarities with radiation enteritis, infective enteritis, and had the mucosal atrophy of coeliac disease.

Vanderhoof et al. (1990) further developed the MTX animal model, by injecting 2.5 mg/kg body weight subcutaneously on three consecutive days. This MTX dosage produced a
consistent mucosal injury within 24 hours of the last injection. The mucosal injury resolved over a two week period. Severe inflammatory changes were apparent after three days, with both polymorphonuclear and lymphocytic infiltrates in the lamina propria and crypt abscess formation. The villi were severely blunted and the surface epithelium was severely damaged. MTX damage was found to occur primarily in the proximal small intestine, and to a lesser degree in the distal small intestine.

Howarth et al. (1996) used Vanderhoof et al. (1992) MTX animal model to test the efficacy of a whey growth factor extract purified from cheese whey. The growth factor intervention improved mucosal architecture, increased sucrase activity and resulted in a lower damage score in the distal small bowel. Xian et al. (2000) found the expression of hepatocyte growth factor and its receptor c-met to be up-regulated at the same time as crypt hyper-proliferation and mucosal recovery. Vanderhoof et al. (1990) demonstrated that dietary deficiency impairs recovery from mucosal injury. These studies showed that elements of food were capable of improving intestinal function after injury.

*MTX and IBD animal models and intestinal permeability*

The methodology of a permeability test can influence the interpretation of the barrier function’s state. Taminiau et al. (1980) investigated MTX depressed active transport (D-glucose) as well as passive mannitol transport in rats after four days of oral MTX administration. Twenty four hours after MTX, the crypt and villus cells had a suppression of active intestinal transport measured by glucose-stimulated transport. *In vitro* studies using Ussing chambers found excessive permeability. Taminiau et al. (1980) suggested the permeability was a result of transcellular changes in the crypt and intercellular changes in the villus. This was not seen 96 hours after MTX as the glucose Na⁺ carrier was synthesized during the rapid phase of proliferation.

Mao et al. (1996) demonstrated that MTX increased $^{51}$Cr EDTA clearance at least five fold in perfusion studies of the proximal small bowel, distal small bowel and colon. Bjarnason et al. (1985) also found the five hour $^{51}$Cr EDTA permeability test was increased in rats 72 hours after a single dose of MTX (25 mg/kg). Erdman et al. (1991) reported significant difference in the passive permeability of mannitol and PEG 900 between MTX and control
rare. The in vivo active uptake with 3-methy-D-glucose was shown to reflect the disruption and recovery of mucosal function in an animal MTX model. In the MTX group 40-50% of the probes were recovered in the lumen after the test, compared to 5% of probes in the controls (Erdman et al. 1991). The results were calculated as serum concentrations over 150 min rather than as percentage dose recovered.

Several animal models of inflamed intestines have shown increased permeability and a drop in fluid and electrolyte absorption from the decreased absorptive surface area following enteric cell damage (Krugliak et al. 1990; Cui et al. 1996; Madsen et al. 1999b). Cui et al. (1996) used an acetic acid model of pancolitis in rats to determine the ileal permeability with Ussing chambers. The decrease in ileal fluid and electrolyte absorption during pancolitis coincided with a rise in the paracellular and transcellular permeability of mannitol and inulin. Interestingly, the increase in permeability was not accompanied by structural changes in the tight junctions. Madsen et al. (1999) found increased permeability in the colon and ileum of the IL-10 mice. Colonic injury developed in the fourth week but no histological damage was detected in the ileum of these mice. Madsen and colleagues have suggested that the physical environment of the mice may influence disease progression. They concluded that it might be the increase in small intestinal permeability through increased antigen uptake that contributes to colonic inflammation (Madsen et al. 1999a; Madsen et al. 2000).

**Inflammatory Bowel Disease**

IBD is a term used to describe the diseases, ulcerative colitis (UC) and Crohn's disease (CD) (Donaldson 1989). In Australia it is estimated that at least 23,000 people have IBD. Incidence rates for UC vary from 0.7 to 15.8 per 100,000 and from 0.08 to 9.7 per 100,000 for CD. The difference is that these rates are assumed to reflect differences in the genetic background and environmental exposure between studies (Dignass and Goebell 1995; Thjodleifsson et al. 1998; Montgomery et al. 2001). Despite many years of research, the pathogenesis of IBD remains unclear. Environmental triggers seem to play a substantial role in the incidence of IBD (Sartor 1997; Breslin et al. 2001; Ahmad et al. 2002), which is increasing in the Western world. There is compelling evidence that genetically defined
factors predispose to the development of both UC and CD (Gasche et al. 2000; Ahmad et al. 2002). Genes associated with IBD susceptibility were thought to encode for products that either play a role in the regulation of mucosal immune responses or contribute to the integrity of the mucosal barrier (Choi and Tarrgo 1994; Grimm et al. 1995; Kam et al. 1995; MacDermott 1996; Yao et al. 1996; Adam et al. 2002; Esters et al. 2002a). The importance of environmental factors as causative agents on IBD is generally accepted, although no definite agents have been identified so far (Sartor 1997; Shanahan 2000). Both pathogenic and normal intestinal flora can induce chronic intestinal inflammation and may participate in disease causation (Duchmann et al. 1995; Linskens et al. 2001; Oliva-Hemker and Fiocchi 2002).

CD is also called chronic regional enteritis where the chronic inflammation occurs along the alimentary tract. CD causes inflammation of the full thickness of the bowel wall. The inflammation process occurs at discrete sites with normal tissue between affected areas (Donaldson 1989; Pavil and Gibson 1992). It typically occurs in the ileocaecal region, in the colon, and less commonly in the small intestine (Pavil and Gibson 1992; Louis et al. 2001). The symptoms for CD are chronic ill health, diarrhoea, abdominal pain, anaemia, and weight loss (Vernia et al. 1988; Hyams et al. 1993). Its aetiology remains obscure. It is a chronic remitting disease and may lead to systemic manifestations such as arthritis, skin lesions and liver disease (Donaldson 1989). In the active state of CD there is a tendency for an increase in the permeability of the small intestine (D’Inca et al. 1992; Meddings 2000). Increased permeability would increase the antigenic stimulation, and result in an inappropriate immune response that may lead to further increase the permeability (Hollander 1988).

UC causes inflammation of the inner lining of the large bowel and may extend to the caecum, but it is uncommon (Cello and Schneiderman 1989; Gibson and Pavli 1992). A non-specific inflammation of the mucosa and submucosa is often followed by extensive superficial ulceration. The muscular and serosal inflammation leads to a rigid short colon. Usually the total colon is involved, beginning in the rectum and proceeding in a retrograde fashion (Cello and Schneiderman 1989). The aetiology is ill-defined and it is believed to be triggered by bacteria but no microbial pathogens have been identified (Sartor 1997; Linskens et al. 2001). Ulcerative colitis is a common cause of chronic diarrhoea in temperate climates and the blood and mucus in the stool are thought to be from
inflammatory reactions in the colonic mucosa. Patients undergo cycles of disease relapses and remittance (Gibson and Pavli 1992; Hamilton 1996). The relapses have become less frequent with the present drug regime of sulphasalazine and in the acute phase, corticosteroids (Hawkey and Hawthorne 1988; Peppercorn 1990; Cunliffe and Scott 2002).

Intestinal permeability in IBD patients

Numerous studies have assessed the mucosal barrier of IBD patients by intestinal permeability with inert probes and have been reviewed by Bjarnason et al. (1995) and Meddings (1997). Several studies have shown that the intestinal permeability of sugar probes is increased in patients with active CD (Pearson et al. 1982; Sanderson et al. 1987; Andre et al. 1988; Hollander 1988; Katz et al. 1989; Olaison 1990; Bjarnason et al. 1995). A selection of these studies of intestinal permeability in a paediatric and adult CD population is summarised in Table 1.2.

One of the difficulties associated with the management of CD is the frequent relapses (Brignola et al. 1986; Sutherland 2000b). Several researchers have suggested the use of intestinal permeability to predict relapses (Wyatt et al. 1993; Fleming et al. 1996; Meddings 1997). Wyatt et al. (1993) followed the incidence of relapse in 72 patients, who had been in remission for at least 6 months. They measured the patients' L/M permeability ratio for a year. They found that 70% of patients with elevated intestinal permeability relapsed within the year. Further application of the intestinal permeability test in assessing disease activity, disease extent, response to therapy, and an early detection of relapse in paediatric patients with IBD does however require delineation (Bjarnason et al. 1995; Sanderson et al. 1987; Murphy et al. 1989).

Previous studies have queried whether or not small intestinal permeability is increased with UC (Howden et al. 1991; D'Inca et al. 1992; Peeters et al. 1995). Wyatt et al. (1993) however predicted the relapse of patients who were judged to have colonic disease. This would suggest that the sugar permeability test may help detect the small intestinal damage these patients. Further work appears to be needed to better establish the effectiveness of the intestinal permeability test in identifying both CD and UC patients.
Table 1.2 Summary of *in vivo* intestinal permeability test in humans studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Solution</th>
<th>Patients (n)</th>
<th>Results – Ratio or Recovery (%)</th>
<th>L/Rh ratio</th>
<th>L/M ratio</th>
<th>^51CrEDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ukabam et al.</td>
<td>10 g L</td>
<td>Normals (16)</td>
<td>N/A</td>
<td>0.008 *</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>1983</td>
<td>0.5 g M</td>
<td>UC (6)</td>
<td></td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mL</td>
<td>CD ileum</td>
<td></td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD colon</td>
<td></td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanderson et al.1987</td>
<td>5.25 g L</td>
<td>Controls (6)</td>
<td>0.04 ± 0.01</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75 g L-rh</td>
<td>CD (14)</td>
<td>0.257 ± 0.037</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Andre et al.</td>
<td>5 g M</td>
<td>Control (100)</td>
<td>N/A</td>
<td>0.021± 0.001</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>1988</td>
<td>5 g L</td>
<td>CD (47)</td>
<td></td>
<td>0.085 ± 0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Katz et al.</td>
<td>7.5 g L</td>
<td>Controls (29)</td>
<td>0.035 **</td>
<td>0.012**</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>1989</td>
<td>1 g L-rh</td>
<td>CD (25)</td>
<td>0.069</td>
<td>0.022</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 g M</td>
<td>Relatives (41)</td>
<td>0.037</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murphy et al.</td>
<td>5 g M</td>
<td>Control (13)</td>
<td>N/A</td>
<td>0.02 *</td>
<td>N/A</td>
<td></td>
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<td>1989</td>
<td>5 g L</td>
<td>CD (17)</td>
<td></td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>65 mL</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Howden et al.</td>
<td>1 g L-rh</td>
<td>Controls (17)</td>
<td>0.029± 0.012</td>
<td>0.014± 0.007</td>
<td>0.35 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>1991</td>
<td>1 g M</td>
<td>UC (14)</td>
<td>0.027± 0.013</td>
<td>0.012± 0.006</td>
<td>0.39 ± 0.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 g L</td>
<td>CD (28)</td>
<td>0.061± 0.115</td>
<td>0.02 ± 0.023</td>
<td>1.67 ± 0.13</td>
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</tr>
<tr>
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<td>^51CrEDTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D’Inca et al.</td>
<td>L; M</td>
<td>Healthy (30)</td>
<td>N/A</td>
<td>0.011 ± 0.006</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td></td>
<td>UC (21)</td>
<td></td>
<td>0.040 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD (104)</td>
<td></td>
<td>0.034 ± 0.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andre et al.</td>
<td>5 g M; 5 g L;</td>
<td>Control (10)</td>
<td>N/A</td>
<td>0.031</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>1990</td>
<td>^51Cr EDTA</td>
<td>UC (9)</td>
<td></td>
<td>0.048</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD (19)</td>
<td></td>
<td>0.045</td>
<td>3.3</td>
<td></td>
</tr>
</tbody>
</table>

N/A= results not available; L=Lactulose; M=Mannitol; L-rh=L-rhamnose. * Ukabam et al. (1983) and Murphy et al. (1989) L/M ratio values are the medium. Howden et al. (1991) values are Mean ± SD. Andre et al. (1990) values are Medians. All other values are Mean ± SEM. Colonic involvement increases permeability variability. All tests are five hours unless stated. ** Katz et al. (1989) sugar ratios are estimates from their figures in the paper.
Bacterial involvement in IBD pathogenesis

The complexity of the colons micro-ecology increases the possibility that subtle alterations in bacterial function may have profound implications for mucosal barrier function and immune response. Several bacterial species have been implicated in CD (Blaser 1997; Sartor 1997; Colombel et al. 2001; Roediger and Macfarlane 2002). These include *Mycobacterium paratuberculosis*, the measles virus and *Listeria monocytogenes*. There is no consistent evidence that IBD is caused by these persistent pathogens, and not all bacteria can be detected with cultures and screening (Sartor 1997; Linskens et al. 2001). It is difficult to determine whether colonic bacterial populations are a predisposing factor for IBD or if the flora had been adjusted in response to the disease.

Gaiffer et al. (1991) found that the bacterial populations in faecal flora in IBD patients (42 CD and 37 UC) were different to that seen in healthy individuals (n=21). They used a semi-quantitative bacteriological method and the scores for *E. coli* and aerobes were similar in all groups. Patients with clinically active CD had significantly higher scores for total aerobes than patients with quiescent CD or UC, or healthy individuals. *Lactobacillus* and *Bifidobacteria* scores were significantly reduced in patients with CD. However, Favier et al. (1997) found no decrease in the *Lactobacillus* populations in CD patients, and showed that bacterial populations and enzyme activity were significantly altered. The decreased in faecal glycosidase activity, especially beta-D-Galactosidase, was more pronounced during flare-ups in CD patients. The enumeration of faecal samples found no difference between controls and patients for *Bacteroides* and *Lactobacillus*, but *Bifidobacteria* was significantly reduced.

Roediger et al. (1984) postulated that UC developed from abnormal luminal bacteria that in turn impaired cell metabolism. In Roediger’s earlier work he had observed that colonocytes needed butyrate, a short chain fatty acid as a source of energy (Roediger 1980a). His theory suggested that luminal bacteria that have a defective short chain fatty acid metabolism could result in the starvation of colonocytes and detrimentally affect mucosal permeability and healing.

Several animal models develop IBD from systemic immuno-regulatory defects have not developed colonic inflammation when they were maintained in a germ-free environment.
The IBD animal models have targeted deletions of IL-2, IL-10, IL-12, T cell receptor (α or β), or have expression of human HLA-B27/β2 microglobulin (Hammer et al. 1990; Mombaerts et al. 1993; Sadlack et al. 1993; Neurath et al. 1995; Stenson 1995; Madsen et al. 1999b). The models showed that the disease was dependent on the presence of microflora and not, in the traditional sense, on tissue invasion or toxin production (Schultz et al. 1998; Madsen et al. 1999b). Germ-free animals exhibited an underdeveloped immune system and their lymphoid tissues were hypocellular. In germ-free rats the mucosal immune system alters when bacteria begin to colonise. Woolverton et al. (1992) observed that the number of lamina propria cells increased and matured when bacteria were introduced. Initially, the lamina propria cells are transiently hyper-reactive to bacterial antigens but these are sufficient to induce immune competency. In the case of the animal models of IBD, immune competency was not reached as their systemic immune system was defective (Woolverton et al. 1992).

**Inflammatory bowel disease immunology**

The immune response of IBD is characterised by T cell and macrophage activation (Jacquot et al. 1996; MacDermott 1996). Human T cell activation requires a cross-linking mechanism with T cell antigen receptor complex and interleukin 1, which are presented by macrophages. IL-1 is present in the inflamed mucosa of a majority of IBD patients to a greater extent than in normal subjects (Grottrup-Wolfers et al. 1996; Mackenzie et al. 1996). The increased local release of cytokines and chemokines mediate the mucosal influx of inflammatory cells, which infiltrate into the muscle layer (Breese et al. 1994; Kam et al. 1995; MacDermott 1996). In active IBD there is an increased migration of monocytes, macrophages and polymorphonuclear neutrophils (PMN’s) from the blood stream into the inflamed mucosa and submucosa (MacDermott 1996; Yao et al. 1996). The macrophage population in the IBD intestine has been described as a heterologous population and the intestinal milieu and the integrity of the epithelial barrier determine its phenotype (Mahida et al. 1989). In IBD the macrophage population expresses receptors rarely seen in normal tissue (Mahida et al. 1989; Grimm et al. 1995). Grimm et al. (1995) reported that CD and UC patients have a population of macrophages that strongly
expresses CD14, a receptor for LPS. The macrophage population in IBD may be part of the amplification of the immune reaction and inflammatory cascade (Schreiber et al. 1995).

Clinical observations suggest that IBD is similar to other chronic inflammatory processes with its two-stage pathogenesis. The first stage is the initiating event, which triggers the inflammatory response the second stage is the amplification of the inflammatory response, which involves a number of inflammatory cells including lymphocytes, mast cells, macrophages and neutrophils. Cytokines have been established as a critical mediator in the cascade of inflammatory events associated with IBD. The inhibition of the inflammatory process is part of a dynamic equilibrium of a cytokine immune cell network that responds to stimuli (Choi and Tarrgo 1994; Kam et al. 1995; Holtmann et al. 2001).

**Sports athletes**

Gastrointestinal disturbances during and after exercise have been reported in 83% of runners and varies from 20-50% in studies of other athletes (Sullivan 1981; Sharman 1982; Brouns et al. 1987; Halvorsen et al. 1990; Green 1992; Halvorsen and Ritland 1992; Peters et al. 1999b). After intense exercise and long distance running a high incidence of gastrointestinal dysfunction including abdominal pain, diarrhoea and flatulence has been observed (Økstendal et al. 1992; Ryan et al. 1996). There is no consensus in the literature that gut problems are related to the intensity and duration of exercise. The gastrointestinal problems experienced by athletes are similar to those experienced by patients with IBD (Table 1.3).

During strenuous exercise, failure of the gastrointestinal barrier may contribute to the development of gut disorders by allowing toxic and antigenic agents to enter the intestinal mucosa and/or systemic circulation (Ryan et al. 1996). Baska et al. (1990) ascertained the gastrointestinal problems in ultra marathoners and found that a high incidence of mucosal erosion with bleeding occurred after a marathon run (Ryan et al. 1996). More recently in endoscopic studies of competitive endurance runners after strenuous exercise, lesions such as hemorrhagic colitis and ischaemic colitis, have been observed (Gaudin et al. 1990; Schwartz et al. 1990; Irving et al. 1991). Ischaemic colitis, use of NSAIDS and alcohol,
are all thought to play a role in the resulting gastrointestinal disturbance (Ryan et al. 1996). Where these factors act within the gastrointestinal tract with respect to intestinal integrity is also an important factor in the type and severity of symptoms experienced. Intestinal permeability has also been used as a diagnostic tool to identify alterations in barrier function of the intestine following strenuous exercise (Øktedalen et al. 1992).

Table 1.3 Intestinal problems occurring in athletes and inflammatory bowel disease patients.

<table>
<thead>
<tr>
<th>Gastrointestinal symptoms</th>
<th>Athletes*</th>
<th>Inflammatory bowel disease†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal cramps</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Bloating</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>✓</td>
<td>✓ blood and mucus</td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>4 during and after exercise</td>
<td>✓</td>
</tr>
<tr>
<td>Gastrointestinal reflux</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Heartburn, chest pain</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Increased frequency of stools</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Mouth ulcers</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Nausea</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Pain in the abdomen</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Tiredness</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Vomiting</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Weight loss</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Intestinal Permeability in Sports Athletes

The intestinal permeability in elite sports athletes was first investigated by Øktedalen et al. (1992). They used a 24 hour $^{51}\text{Cr}$ EDTA urinary collection to give information on the integrity of the whole intestine. They reported a significant increase in the probe excretion following completion of half and full marathons. Pal et al. (1997) found that the intensity of running affected small intestinal permeability. They tested small intestinal permeability and found that L/Rh excretion ratio for six subjects exercising for an hour at their 80% VO$_{2\text{peak}}$ was significantly greater than 40%, 60% VO$_{2\text{peak}}$ and at rest.

The studies conducted by Pals et al. (1997) and Ryan et al. (1996) found high permeability but no gastrointestinal symptoms. Øktedalen et al. (1992) found that of the seventeen runners, one experienced some nausea and another runner experienced dyspepsia during the run. They speculated that the enhanced intestinal permeability may contribute to the pathogenesis of diarrhoea and abdominal cramps. The elevations in intestinal permeability for such sports athletes are either unrelated to or may actually precede the development of gastrointestinal symptoms.

Probiotics

Definition

The term 'probiotic' was first used in its present meaning by Fuller (1989), who defined it as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance'. The definition broadened to 'a viable mono- or mixed culture of live micro-organism which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora'. This definition expands the use of probiotics also to areas other than the intestine and includes the use of either mono- or mixed cultures (Havenaar et al. 1992). Recently, the European probiotic group modified the definition to 'a live microbial food ingredient that is beneficial to health'
The benefits gained from using of probiotics are listed in Table 1.4 and have been tested in \textit{in vivo} and \textit{in vitro} settings and are beyond their inherent general nutrition. When using probiotics it is necessary to consider whether probiotics are safe for the host, and whether they will survive the intestine (Conway \textit{et al.} 1987; Goldin \textit{et al.} 1992; Donohue and Salminen 1996; Goldin 1998). Several \textit{in vitro} tests are required to confirm safety and survival before testing \textit{in vivo} (Crittenden 1998; Hendriksson 1998).

\textbf{Probiotic safety}

Many societies have traditional foods that contain probiotics. Centuries of probiotic consumption by the general population has deemed it as safe. The safety of probiotics was questioned from observations of rare cases of bacteraemia seen in association with lactic acid bacteria, inherent antibiotic resistance, and plasmid-associated resistance to vancomycin (Donohue and Salminen 1996; Donohue 1998). Observations of protein malnutrition found the ecology of the microflora to be disrupted, sometimes leading to bacterial translocation. If bacteria translocate across the luminal wall they can move into systemic organs (Allori \textit{et al.} 2000). Suggested strategies to test the safety of probiotic strains have included studies on the intrinsic properties of the strain, host organism interaction, and pharmacokinetics studies. The development of novel probiotic products requires them to be characterised as safe, to have demonstrated a benefit, and to have attributes for industrial production (Brashears and Gilliland 1995; Donohue 1998).
Table 1.4 The beneficial effects of probiotic species.

<table>
<thead>
<tr>
<th>Beneficial effects</th>
<th>Bacterial species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shorned the duration and severity of diarrhoea</td>
<td><strong>L. rhamnosus</strong> (GG)</td>
<td>Isolauri et al. 1991; Perdigon et al. 1991; Kaila et al. 1995; Majamaa et al. 1995; Rautanen et al. 1998; Oberhelman et al. 1999; Guandalini et al. 2000</td>
</tr>
<tr>
<td></td>
<td><strong>L. reutri</strong></td>
<td>Shormikova et al. 1997</td>
</tr>
<tr>
<td></td>
<td><strong>L. casei</strong> (Shirota)</td>
<td>Sugita and Togawa 1994; Morotomi 1996</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td><strong>B. bifidum, S. thermophilus</strong></td>
<td>Saavedra et al. 1994</td>
</tr>
<tr>
<td>Antibiotic associated diarrhoea</td>
<td><strong>L. rhamnosus</strong> (GG)</td>
<td>Gorbach et al. 1987; Biller et al. 1995</td>
</tr>
<tr>
<td></td>
<td><strong>L. rhamnosus</strong> (GG)</td>
<td>Siitonen et al. 1990; Arvola et al. 1999; Vanderhoof et al. 1999</td>
</tr>
<tr>
<td></td>
<td><strong>L. acidophilus, L. bulgaricus</strong></td>
<td>Gotz et al. 1979</td>
</tr>
<tr>
<td></td>
<td><strong>S. boulardii</strong></td>
<td>Adam et al. 1977; Surawicz et al. 1989</td>
</tr>
<tr>
<td>Travellers diarrhoea</td>
<td><strong>B. Longum, L. acidophilus</strong></td>
<td>Orrage et al. 1994</td>
</tr>
<tr>
<td><strong>Altered enzyme activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose intolerance</td>
<td><strong>L. acidophilus</strong></td>
<td>Marteau et al. 1990; Hove et al. 1994; Montes et al. 1995</td>
</tr>
<tr>
<td>Faecal enzyme activity</td>
<td><strong>L. casei</strong></td>
<td>Djouzi et al. 1997; Spanhaak et al. 1998</td>
</tr>
<tr>
<td></td>
<td><strong>L. acidophilus</strong></td>
<td>Gilliand et al. 1978; Goldin et al. 1980; Goldin and Gorbach 1984a; Goldin and Gorbach 1984b;</td>
</tr>
<tr>
<td>Prevented pathogen adherence</td>
<td><strong>L. rhamnosus</strong> (GG),</td>
<td>Silva et al. 1987; Chauviere et al. 1992; Bernet et al. 1994; Bernet et al. 1997;</td>
</tr>
<tr>
<td></td>
<td><strong>L. casei</strong> (Shirota),</td>
<td>Lehto and Salminen 1997; Tuomola and Salminen 1998; Michetti et al. 1999</td>
</tr>
<tr>
<td></td>
<td><strong>L. acidophilus</strong> (LA1),</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>L. acidophilus</strong> (LB)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>L. fermentum</strong></td>
<td></td>
</tr>
</tbody>
</table>

31
<table>
<thead>
<tr>
<th>Beneficial effects</th>
<th>Bacterial species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immune enhancement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase phagocytic activity</td>
<td><em>L. acidophilus</em> (LA1)</td>
<td>Schiffrin et al. 1995</td>
</tr>
<tr>
<td>Stimulate IgA</td>
<td><em>L. acidophilus</em> (LA1)</td>
<td>Link-Amster et al. 1994; Marteau et al. 1997</td>
</tr>
<tr>
<td></td>
<td><em>L. bulgaricus,</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. thermophilus</em></td>
<td>Perdigon et al. 1994</td>
</tr>
<tr>
<td></td>
<td><em>L. rhamnosus</em> (GG)</td>
<td>Perdigon et al. 1991; Perdigon et al. 1995; Malin et al. 1996b</td>
</tr>
<tr>
<td></td>
<td><em>B. longum</em></td>
<td>Takahashi et al. 1998</td>
</tr>
<tr>
<td></td>
<td><em>S. bouardii</em></td>
<td>Buts et al. 1990</td>
</tr>
<tr>
<td>Induced cytokine production</td>
<td><em>L. rhamnosus</em> (GG)</td>
<td>Miettinen et al. 1996</td>
</tr>
<tr>
<td>Increased B cells in Peyer’s patches</td>
<td><em>L. bulgaricus</em></td>
<td>De Simone et al. 1987; De Simone et al. 1988</td>
</tr>
<tr>
<td></td>
<td><em>S. thermophilus</em></td>
<td></td>
</tr>
<tr>
<td>Macrophage activation</td>
<td><em>L. rhamnosus</em> (GG),</td>
<td>Perdigon et al. 1986b;</td>
</tr>
<tr>
<td></td>
<td><em>L. bulgaricus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. casei, L. acidophilus</em></td>
<td>Perdigon et al. 1986a</td>
</tr>
<tr>
<td>Milk Allergy</td>
<td><em>L. rhamnosus</em> (GG)</td>
<td>Pelto 1998; Pelto 2000</td>
</tr>
<tr>
<td>Bacterial overgrowth</td>
<td><em>L. fermentum</em></td>
<td>Stotzer et al. 1996</td>
</tr>
<tr>
<td>Proliferation/growth</td>
<td>Various</td>
<td>Hargrove and Alford 1978; Wong et al. 1983; Parker 1990; Thoreux et al. 1996</td>
</tr>
</tbody>
</table>

This is a selection of papers displaying the beneficial effects of probiotics.
**Probiotics survival of the intestine**

Survival of bacteria differs among bacterial species and depends on the combination of lactic acid bacteria (Goldin *et al.* 1992; Djouzi *et al.* 1997). The ability of a probiotic or any bacteria to survive and colonise in the intestine is influenced by many normal gastrointestinal functions. These include hormones, diet, gastric acid, enzymatic secretions, peristalsis and colonic fermentation. Host interactions also influence bacterial survival and commensal bacterial flora through non-specific anti-bacterial actions such as lysozyme or the lactoperoxidase system. Physiological factors, the gastrointestinal system, environmental elements, diet and drugs shape the balance of bacterial flora.

Probiotics need to withstand low pH to survive stomach acid, bile acid, diet and by-products of fermentation (Goldin *et al.* 1992). In the intestine of breast-fed infants the pH is approximately 5.0 and this was found to inhibit the growth of predominantly proteolytic organisms such as *Clostridium* (Gothefors 1989). Conway *et al.* (1987) tested the survival of *L. acidophilus* strains, *L. bulgaricus* and *Streptococcus thermophilus*, in human gastric juice *in vivo* and *in vitro*. All strains tested were found to have their survival enhanced by skim milk. The number of bacterial cells adhered to human ileal cells was shown to be three fold higher when the strains were grown in the presence of skim milk. Skim milk (250 mL) can raise stomach pH by 4 to 5 units suggesting that fermented milk products may also increase the survival and adhesion of probiotics (Conway *et al.* 1987).

For bacteria to survive peristalsis they must either adhere or replicate at a rate not to be affected by peristalsis. The mucus layer is the first point of contact between the ingested bacteria and the host mucosa and an important site of adhesion and colonisation (Kiravainen *et al.* 1998; Mikelsaar *et al.* 1998). The bacteria inhabiting the mucus layer can establish in large populations if the replication exceeds the turnover and the erosion of the mucus layer (Mantel 1996).

Bacterial species found in the alimentary tract have specificity to the host environment (Fuller 1991). Probiotic strains are species specific as Tannock *et al.* (1982) found that Lactobacilli isolated from chicken stomachs did not adhere to a rodent stomach and conversely lactobacilli isolated from rodent intestines did not adhere to the stomach tissue from a chicken (Tannock *et al.* 1982; Tannock *et al.* 1984). For probiotics to be tested in
humans, strains must be selected that are known to have survived the intestine and can colonise. Several of the commercial probiotics have been developed from isolates of human faeces (LA1 and GG).

The survival of bacteria was influenced by the presence of the other micro-organisms and can lead to a positive symbiosis. For example, Veillonella is an organism that utilized lactate, and it is commonly found in association with lactate producing organisms such as Streptococcus, Lactobacillus or Bifidobacteria (Gothefors 1989).

Measurement of colonisation involves in vitro studies, which analyse colonic washout and faecal samples (Goldin et al. 1992; Saxelin et al. 1995; Ruseler-Van Embden et al. 1998). Intestinal colonisation has been successfully observed using a colonoscope (Sarem-Damerdj et al. 1995). However this is an expensive method compared to the use of faecal samples. Growing bacteria from faecal samples is restricted to date as there are many live species present that cannot be selected. Strains that survive the intestine are thought to have a better chance of colonising the intestine (Goldin et al. 1992; Conway 1996). Probiotic adhesion to mucus and enterocytes are discussed later.

*Viable versus non-viable probiotic bacteria*

Research into the effects and health benefits of live or viable probiotics has strengthened the view that probiotics have to be viable in order to be efficacious. The need for probiotics to be viable does however have its disadvantages. These include: limited shelf life, strict requirements of transportation conditions, storage and handling. These limitations are notably less for a non viable probiotic. Often non-viable probiotics are used as placbos and controls, though there is mounting evidence of non-viable probiotics eliciting an efficacious response (Ouwehand 1998).

Marteau et al. (1990) showed that both live and heat-killed lactobacilli were able to reduce the symptoms and maldigestion of lactose. Non-viable Lactobacillus acidophilus strain LB was found to inhibit the action of diarrhoea inducing bacteria (Coconnier et al. 1993; Ouwehand 1998). Kalia et al. (1995) found that subjects receiving Lactobacillus casei strain GG (LGG) had a shorter duration of diarrhoea. The only difference was that viable
LGG had higher number of IgA secreting cells compared with rotavirus (Kaila et al. 1995). Perdigon et al. (1986) administered L. casei CRL 431 and L. bulgaricus CRL 423 to mice orally or intraperitoneally. Both viable and non-viable treatments results in increased phagocytic activity, but feeding viable probiotics to mice induced greater macrophage enzymatic activities than those fed non-viable probiotics (Perdigon et al. 1986b). Perdigon et al. (1995) tested both viable and non-viable treatments and found an increased number of cells expressing immunoglobulins. The Peyer patch cells from the viable yoghurt treated group had an increased proliferative response to LPS (Perdigon et al. 1995). This was not seen in the other groups, leading Perdigon et al. (1995) to suggest that heating yoghurt sufficiently reduced its immunomodulating properties.

The cytoplasm or cell walls of heat-killed lactobacilli may still cause an increased antibody production whether viable or not (Lin and Chang 2000). Takahashi et al. (1998) reported a significantly greater humoral response of IgG and IgA to B. longun cytoplasm and L. acidophilus cell wall than in control mice. Lin and Chang (2000) showed that both the intact cells and cell-free extracts of B. longum and L. acidophilus inhibited lipid peroxidation which was used as a measure of anti-oxidative activity. Lin and Chang (2000) suggest the anti-oxidative potential of intestinal lactic acid bacteria would occur whether the strains survived the intestine or not.

**Probiotic mucus adhesion**

For many bacterial species colonisation involves adherence to intestinal mucosa. Adhesion is regarded as important for both pathogenic and probiotic bacteria in order to persist in the intestine. Adhesion of bacteria to the mucus may be a part of the colonisation process. By adhering to the mucus the bacteria must contend with the continual degradation of the mucus layer as well as constant secretion of new mucin glycoproteins (the major components of the mucus layer) being constantly secreted (Kiravainen et al. 1998).

Adherence to cultured intestinal cells *in vitro* has been used as one of the selection criteria for probiotic strains (Conway 1996). Conway et al. (1987) and Sarem-Damerdji et al. (1995) have shown that the ability of probiotics to adhere to intestinal cells differs with
each strain. A summary of the results screening tests for the strains used in this thesis and major strains is shown in Table 1.5. Bernet et al. (1994) measured four strains of *Lactobacillus acidophilus* for adherence to intestinal cell lines. They found the LA1 strain able to adhere to the cells and to mucus secreted from a human goblet cell line HT-29-MTX. This strain was able to interfere with cell association and cell entry of enteropathogenic bacteria such as *E. coli* and *Salmonella typhimurium*. They found its action was preventative, as it was more effective when incubated before or together with the enterovirulent *E. coli* than after infection with *E. coli* (Bernet et al. 1994).

Table 1.5 Probiotic result from adhesion experiments *in vitro* probiotic adhesion

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Cellular adhesion</th>
<th>Mucus Adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> strain LGG</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>L. johnsonii</em> strain LA1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>L. paracasei sub. Paracasei</em> strain Shirota</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>+</td>
<td>??</td>
</tr>
<tr>
<td><em>S. thermophilus</em></td>
<td>-</td>
<td>?</td>
</tr>
</tbody>
</table>

This table displays information collected from the following references (Conway et al. 1987; Sarem-Damerdi et al. 1995; Kiravainen et al. 1998; Tuomola and Salminen 1998; Ouwehand et al. 1999; Tuomola 1999).

Tuomola et al. (1998) found the choice of selection criteria important for determining the use of a probiotic strain. Among the strains studied, the adherence to Caco-2 cell line was more common than the adherence to ileostomy glycoproteins, suggesting the Caco-2 cell line would give more strains positive results. Tuomola et al. (1998) tested the usefulness of the Caco-2 screening and showed that many probiotic bacteria adhere both to intestinal cells and intestinal mucus. They further tested the human intestinal glycoproteins isolated from faeces as an *in vitro* model of intestinal mucus to measure adhesion of *E. coli* and Salmonella strains and the effect of probiotics on this adhesion (Tuomola 1999).

Tuomola et al. (1999) studied *L. rhamnosus* strain GG (LGG) and *L. johnsonii* strain LA1 and showed that they exhibited high adhesion whereas *L. casei* Shirota strain adhered
poorly. The *S-fimbriae E. coli* mediated adhesion was slightly reduced by pre-treatment of the mucus with LGG. Adhesion of *S. typhimurium* was significantly inhibited by LA1 and *L. Shirota*. This inhibition was not related to the adhesion potential of the *Lactobacillus* strains and only 10-23% of adhesion was inhibited. *L. rhamnosus* strain GG did not co-aggregate with *S. typhimurium* to reduce adhesion (Tuomola 1999). Mucus adhesion has been a more selective manner of adhesion but probiotic strains still need to be tested in cell models to determine their successful inhibition of enteropathogens.

**Probiotics and gastrointestinal health**

Probiotics are useful in the prevention and treatment of gastrointestinal disorders in humans. Numerous *in vivo* and *in vitro* studies have shown that the normal intestinal flora is an extremely effective barrier against pathogenic and opportunistic micro-organisms (Fuller 1991). Lactic acid bacteria have been demonstrated to inhibit the *in vitro* growth of many enteric pathogens including *Salmonella typhmurium*, *Staphylococcus aureus*, *Escherichia coli*, and *Clostridium perfringens* (Silva *et al.* 1987).

**Intestinal barrier function**

Few studies have observed the effects of probiotics on the intestinal barrier function. Isolauri *et al.* (1993) measured the transport of horse radish peroxidase (HRP) in jejunal segments in rat pups. Rat pups absorb immunoglobulins and macromolecules up to day 21 when gut closure occurs. When the pups were exposed to cow's milk the HRP uptake was increased ten-fold compared to controls. The addition of *Lactobacillus rhamnosus* strain GG (LGG) to the cow's milk pups reduced the HRP uptake to a level comparable with controls. There was little difference between intestinal segments that contained Peyer's patches and those that were patch-free. The cow's milk did not cause direct damage or change total ionic conductance, an index of paracellular mechanisms. There was an increase in permeability to intact macromolecules. Isolauri *et al.* (1993) observed a decline in intact HRP after LGG was introduced which occurred simultaneously with an increased immune response. LGG significantly increased the frequency of cells secreting β-
lactoglobulin. There was no antigen-specific immune response to cow’s milk with the enhanced permeability. They suggested a link between the intensity of the antigen specific immune response and stabilisation of the mucosal barrier (Isolauri et al. 1993).

Sheep’s milk yoghurt can influence small intestinal permeability in a study by Southcott et al. (1997). Fourteen teenagers had their permeability measured before and after two days of their diet supplemented with sheep’s milk yoghurt. Subjects were required to eat 500g of yoghurt each day. The sheep’s milk was fermented with a probiotic mix YC-180 of L. bulgaricus and Strep. thermophilus to produce the yoghurt. The subjects’ permeability significantly decreased after yoghurt consumption. The action of probiotics in altering permeability may involve individual cells (transcellular movement), tight junctions (paracellular) or epithelium causing cellular adaptation. Probiotics may have also stimulated the immune system, resulting in improvement of the mucosal barrier.

Salminen et al. (1996) suggested that probiotics were a promising treatment of clinical conditions involving altered gut mucosal barrier function. Salminen’s theory involved a pathogen, virus, antigen, or radiation affecting normal intestinal function leading to disturbed microflora and a permeability disorder. He also theorised that the addition of probiotics would restore normal intestinal function. However, the relationship between bacterial flora and the intestinal barrier is yet to be determined. In CD this relationship is considered a contributing factor in the development of the disease (Blaser 1997; Sartor 1997). Probiotic studies in IBD patients are discussed later, however none of these studies investigated permeability.

**Intestinal enzyme activity**

Goldin et al. (1980) found that the metabolic activity of the microflora was influenced by diet and could be altered by lactobacillus supplements. The probiotic LGG has been shown to suppress bacterial enzyme activity. These enzymes include β-glucoronidase, β-glucosidase, urease, faecal glycocholic hydrolase, nitroreductase and tryptic activity. Several of the enzymes play a role in activation of procarcinogens in the large intestine (Goldin and Gorbach 1984a). Goldin et al. (1992) showed that the β-glucoronidase activity did not change in four subjects receiving L. bulgaricus and Strep. thermophilus.
Various types of probiotics do not always cause similar changes in enzyme activity (Gilliland 1990; Gorbach 1990).

Probiotics given to patients diagnosed with disaccharidase deficiency have provided positive results. *Saccharomyces cerevisiae* has shown improvement in the gastrointestinal symptoms in sucrase-isomaltase deficient patients (Harms *et al.* 1997). *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species have increased lactose digestibility of milk products in lactose intolerant patients (Kim and Gilliland 1983; Gilliland and Kim 1984 cited in Rolf 2000). Gallagher *et al.* (1974) reported that lactose intolerant subjects were able to digest lactose in yoghurt better than lactose in milk. These studies seemed to show that probiotics were providing the enzymes for sugar metabolism (Gilliland 1990; Montes *et al.* 1995).

**Immunological effects**

Oral administration of LAB to animals stimulates both non-specific and specific immune responses. This effect is mediated by activation of different cell populations, typically macrophages or dendritic cells, or by regulation of a number of T cell subsets or immunoglobulin-secreting cells, especially in the intestinal mucosa (Meydani and Ha 2000). Macrophages represent one of the first lines of non-specific defence against bacterial invasion and tumours. Macrophages kill bacteria and tumours using similar mechanisms as nitric oxide and reactive oxygen species. Several studies have suggested the increased anti-tumor effect of LAB was due to enhancement of macrophage activity (Perdigon *et al.* 1986b; Goulet *et al.* 1989; Meydani and Ha 2000.) In an allergy model, the action of LGG was suggested to stabilise the intestine. Isolauri *et al.* (1993) hypothesised a link between the intensity of the antigen-specific immune response and the stabilisation of the mucosal barrier. They also showed an enhancement of the circulating antibody secreting cell responses in rotavirus induced diarrhoea using LGG (Kaila *et al.* 1992; Kaila *et al.* 1995).

Probiotic *L. johnsonii* strain LA1 action has been shown to stimulate the mucosal immune system and treat *Helicobacter pylori* (Michetti *et al.* 1999; Felley *et al.* 2001). Schiffrin *et al.* (1997) found anti-infective mechanisms were enhanced after *L. johnsonii* ingestion.
Ingestion of *L. johnsonii* was observed to enhance the immune response to *E. coli* (Schiffrin *et al.* 1995; Schiffrin *et al.* 1997). *L. johnsonii* reduced the inflammation associated with *H. pylori*, though the mechanisms have yet to be demonstrated (Massen *et al.* 1998; Felley *et al.* 2001). The reasons for the anti-bacterial activity exhibited by *L. johnsonii* are not known but may have been due to an anti-microbial substance which is still to be identified (Bernet *et al.* 1997; Michetti *et al.* 1999).

**Probiotics treatment in intestinal damage**

The complex nature of inflammatory bowel disease pathogenesis appears to involve interactions among three crucial elements: host genetic susceptibility, intestinal bacteria, and gut mucosal immune response. Current drug therapy is limited, firstly because it suppresses the host immune response and ignores the effect of enteric bacteria in pathogenesis and secondly because of the lack of organ specificity of immunomodulating drugs leading to side effects. By contrast probiotics confer a health benefit by altering indigenous microflora. Gut microflora species (*Lactobacilli, Bifidobacteria* and others) that have no apparent capacity to induce mucosal inflammation are selected as probiotics. Not all bacterial species are probiotics. Some bacterial products stimulate inflammatory responses and animal models have suggested the role of bacteria in the pathogenesis of IBD. Probiotic therapy has been effective for treating mice deficient in IL-10 and other animal models of IBD.

**Studies in animal models**

Mao *et al.* (1996) evaluated the protective effects of *L. plantarum* (DSM9843) and *L. reuteri* (R2LC) with and without fermentation of an oat base in an MTX-induced enterocolitis. The multiple effects of lactobacilli species show that they improve colonic health on several fronts (Mao *et al.* 1996). Probiotic treatment increased lactobacillus levels in the ileum and colon. Lactobacilli administration improved the intestinal mucosal histology, prevented protein loss, and lowered plasma endotoxin levels. All lactobacilli groups had significantly reduced myeloperoxidase levels compared to the MTX group.
The lactobacilli-fermented oats were more successful at reducing damage than unfermented lactobacilli in the small and large intestine. Interestingly the *L. plantarum* exerted better effects in re-establishing intestinal micro-ecology than administration of *L. reuteri*. Mao *et al.* (1996) believed the administration of lactobacilli significantly improved the bowel nutritional status and the barrier function, by decreasing the number of enteric pathogenic bacteria.

Videla *et al.* (1997) demonstrated that bacteria aggravated bowel inflammation when the mucosa was injured. Early antibiotic treatment, using imipenem and vancomycin, reduced the mucosal lesion scores in colitic mice, but antibiotic treatment was not effective when colitis was established. Sonicated bacterial suspensions inhibited both the growth of bacteria and the effects of colonic inflammation, suggesting that viable bacteria rather than inert antigens were responsible for the inflammation (Videla *et al.* 1997).

The IL-10 knockout mouse model develops a patchy chronic colitis, akin to Crohn’s disease, when reared in a conventional or special pathogen-free environment. Colitis does not develop when animals are reared in a germ-free environment. In the IL-10 mouse model, disease is not present at birth or weaning, but bacterial agents or bi-products of the microflora initiate a chronic inflammation (Madsen *et al.* 1999a; Madsen *et al.* 2000). At two weeks of age the IL-10 mice exhibited an increased number of adherent and translocated aerobic bacteria compared to control mice. A drop in the *lactobacillus* species levels between weeks two and eight occurred when colonic injury developed. This is a critical period during which luminal bacterial may play a pivotal role in the initiation of the mucosal injury.

The antibiotic, lactulose, normal breast milk, and probiotic treatments all result in bacterial repopulation which improves the balance of intestinal microflora (Madsen *et al.* 1998; Madsen *et al.* 1999a; Madsen *et al.* 2000). Exogenous *Lactobacillus reuteri* was used to repopulate the intestine of the IL-10 knockout mice. Probiotic treatments restored the number of total lactobacilli to control levels, normalising colonic adherent and translocated bacteria, and lead to an attenuated development of the disease (Madsen *et al.* 1999a). Madsen *et al.* (1999) concluded that it was not a specific species of lactobacilli that reduced the inflammation, but rather that the total *Lactobacillus* species levels were important.
The animal models of intestinal damage, chemically induced (Mao et al. 1996; Videla et al. 1997) or genetically engineered (Madsen et al. 1999a; Madsen et al. 2000), show that bacteria in the intestine and the balance of the bacterial species may be part of the pathogenesis of the intestinal disease. These studies show that the addition of probiotics is successful in reducing the level of damage. The true mechanisms leading to damage and the prevention by probiotics remain uncertain.

Studies in inflammatory bowel disease patients

Studies of probiotics in IBD patients have had encouraging preliminary results. Probiotic action in IBD is both functional and immunological. The probiotics alter the bacterial population, eradicating pathogens. As a result the bi-products of fermentation are changed. Probiotics can also increase specific IgA secreting cell levels and modify antigen structure (Malin et al. 1996). These studies included clinical trials with several different strains and are discussed in more detail below.

Malin et al. (1996) measured the IgA response in patients with CD after oral therapy with L. casei strain GG. Despite the short duration of 10 days and a small number of patients (14 CD patients vs. seven controls), their experiment showed that increased numbers of IgA secreting cells reacted to β-lactoglobulin and casein. Malin et al. (1996) concluded that the probiotic LGG may have the potential to maintain the gut immunological barrier.

Gionchetti et al. (1998) investigated a new probiotic in a randomised placebo-controlled, double-blind trial as a maintenance treatment for chronic pouchitis. Pouchitis often develops as a complication of ileal reservoir surgery in 10-20% of ulcerative colitis patients. Pouchitis is hypothesised to develop from mucus degradation of the layer over the epithelial cells by bacteria, leading to inflammation (Madden et al. 1990). Patients have symptoms including bloody diarrhoea, lower abdominal pain and fever. Specific probiotic bacterial species have been found not to metabolise mucus and may protect the epithelial cell layer from bacteria (Madden et al. 1990; Ruseler-Van Embden et al. 1995; Rolf 2000). Gionchetti et al. (1998) trialled a probiotic called VSL#3 that contained B. logum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbruekii sub species bulgaricus, L. plantarum and S. salivarius sub species thermophilus. Patients in remission (n=40)
were randomised to take either VSL#3 or a placebo for nine months. Three patients in the probiotic group relapsed, but all twenty patients in the placebo group relapsed. After fifteen days of the VSL#3 treatment the faecal concentrations of lactobacilli, bifidobacteria and \textit{S. thermophilus} significantly increased from basal levels. This mixed probiotic was administered as a powder and no side effects were registered.

Ulcerative colitis patients volunteered to test VSL#3 as an alternative for patients who are allergic or had side effects to 5-ASA (Venturi \textit{et al.} 1999). Twenty UC patients (12M:8F, age range 30-65) in remission received 3g (5x10^{11} \text{ cell/g}) twice daily for twelve months. Patients collected faecal samples (day 10, 20, 40, 60, 75, 90, 365 and 370), attended regular clinical assessment every two months, and underwent endoscopies at six and twelve months or at relapse. At the 20\textsuperscript{th} day of treatment all patients showed a significant increase in the faecal populations of \textit{S. thermophilus}, lactobacilli and bifidobacteria. At twelve months fifteen patients remained in remission and five patients relapsed. The probiotic treatment resulted in no change in bacteroides, coliforms, total aerobes and anaerobes, and no side effects. The mixed probiotic preparation VSL#3 was found to transiently alter the intestinal flora in UC patients. Gionchetti’s and Venturi’s studies indicated the usefulness of a mixed probiotic in its ability to altering bacterial populations and their metabolic activities in the treatment of intestinal inflammation diseases (Gionchetti \textit{et al.} 1998; Venturi \textit{et al.} 1999).

Ulcerative colitis patients have been reported to have high numbers of pathogenic adhesive and enterohaemorrhagic \textit{Escherichia coli} (Burke and Axon 1987; Von Wulffen \textit{et al.} 1989). The presence of \textit{E. coli} strains have been found to be inversely related to the number of non-pathogenic \textit{E. coli} bacteria (Kruis \textit{et al.} 1997). Kruis \textit{et al.} (1997) hypothesised that therapeutic colonisation with non-pathogenic \textit{E. coli} would be beneficial for UC patients. This hypothesis was based on earlier work of Nissle (1930), who observed alteration in the patterns of aerobic intestinal flora and a significant decrease in non-pathogenic \textit{E. coli} in patients with non-infectious bowel disorder. Nissle found a significant improvement in the patients’ symptoms when administered the non-pathogenic \textit{E. coli} strain Nissle 1917 (Nissel 1932). \textit{E. coli} strain Nissle 1917 was found to colonise the intestine and act adversely towards enteropathogenic bacteria (Lodinova-Zadnikova \textit{et al.} 1992).
The double-blind study of Kruis et al. (1997) compared mesalazine (500 mg) to an oral viable preparation of E. coli strain to prevent relapses in UC patients, and found little difference after twelve weeks of treatment. Tolerance of the treatments was good and the frequency of minor side-effects was similar in both groups. The preliminary results showed a non-pathogenic E. coli to have similar efficacy to mesalazine in maintaining remission of UC patients.

Rembacken et al. (1999) compared non-pathogenic E. coli strain Nissle 1917 with mesalazine (2.4 g/day) in active UC patients to determine if a non pathogenic E. coli strain induced remission or prevented relapse over a year. In this study all patients received a weeks’ course of gentamicin at entry to suppress their native E. coli. The patients were a heterogeneous group but were randomised between the treatments. Once patients were in remission their dosing of mesalzine and E. coli was halved. Depending on the severity of their disease patients received additional treatment of hydrocortisone and prednisolone. There was no difference in the remission and relapse rates after active proctitis, moderate colitis or severe UC. Rembacken et al. (1999) suggested that the manipulation of colonic bacteria is linked to relapsing events of UC patients.

Positive manipulation of the flora to prevent relapse using treatment antibiotics or probiotics may develop more effective options for managing IBD. Probiotics have a bonus over antibiotics because they are capable of delivering a nutritional benefit.
SUMMARY

The gastrointestinal barrier has a variety of non-immunological mechanisms that inhibit the growth of bacterial pathogens and prevent entry across the mucosa. In the intestine it is the epithelial cell layer that provide the principal barrier to permeation. Its barrier function can be measured non-invasively to detect abnormalities or a breech in the barrier due to environment or disease. A compromised intestinal barrier may occur in inflammatory bowel disease patients, mucositis patients and athletes. As a result the patients and subjects may thus be placed at greater risk of bacterial/pathogen infection and increased antigenic stimuli.

The integrity of the epithelial cell layer can be determined by markers of small intestinal permeability and may be used as a predictor and index of disease activity and disease severity. Permeability may also be a means of monitoring the response to therapy and predicting disease relapse. Changes in both tight junction integrity and intestinal surface area have been shown to lead to altered permeability, but detailed understanding of the precise mechanism is lacking.

Methotrexate is a chemotherapeutic drug that affects the rapid rate of cell turnover of the gastrointestinal epithelium, in some cases leading to inflammation and ulceration in the intestinal mucosa (Howarth et al. 1996). MTX induces mucosal damage in the rat and provides a model for the study or whether functional changes in the gut occur with the development of disease. Both passive and active transport mechanisms in the epithelium are affected by MTX. Abnormal permeability potentially allows increased numbers of micro-organisms to breech the intestine, and this may further exacerbate intestinal inflammation. Mao et al. (1996) found that the addition of lactobacilli reduced intestinal damage and intestinal permeability. Lactobacilli-fermented oats elicited a greater response than lactobacilli alone. Whether all lactobacilli species can respond in this manner is not known.

Inflammatory bowel disease is a catch-all term for two inflammatory conditions of the intestine: Crohn’s disease and ulcerative colitis. Crohn’s disease is described as discrete sites of inflammation of the bowel wall, producing a cobble-stone pattern and often
causing thickening of the bowel wall. It may affect any site along the intestine, but the small bowel is the primary site in children (Donaldson 1989; Pavli and Gibson 1992; Oliva-Hemker and Fiocchi 2002). Ulcerative colitis is described as a non-specific inflammation of the mucosa and submucosa that is followed by extensive superficial ulceration then muscular and serosal involvement leading to a rigid short colon. Ulcerative colitis, is the common cause of chronic diarrhoea in temperate climates (Gibson and Pavli 1992). Both diseases have a tendency to remit and relapse that becomes less frequent with time and with current drug regimes. The aetiology of UC is ill-defined, but it is believed to be triggered by bacteria, although no microbial pathogens have as yet been identified (Blaser 1997; Sartor 1997; Oliva-Hemker and Fiocchi 2002).

Abnormalities in the structural strands (tight junctions) between enterocytes have been identified in both Crohn’s disease and ulcerative colitis (Dvorak and Dickerson 1979; Madara et al. 1990; Schmitz et al. 1999; Schmitz et al. 2000). In the active state of Crohn’s disease there is a tendency for an increase in the permeability of the small intestine which would increase the antigenic stimulation, and which may result in an inappropriate immune response (Hollander 1988; Olaison 1990). The debate as to whether the alterations in the enterocyte tight junctions lead to the permeability or are a secondary phenomenon is a contentious issue.

Gastrointestinal disturbances during and after exercise have been reported (Øktedalen et al. 1992). After intense exercise and long distance running a high incidence of gastrointestinal dysfunction including abdominal pain, diarrhoea and flatulence has been observed (Øktedalen et al. 1992; Ryan et al. 1996). The gastrointestinal problems experienced by athletes are similar to the problems experienced by IBD patients. The stress of exercise could affect the gastrointestinal tract and lead to altered permeability. During strenuous exercise, failure of the gastrointestinal barrier may contribute to the development of gut disorders by allowing toxic and antigenic agents to enter the intestinal mucosa and/or systemic circulation (Ryan et al. 1996).

Probiotics are defined as a mono or mixed microbial preparation that beneficially assists the host, either animal or human, by stimulating the immune system. The beneficial effects of probiotics have been found to be greater than their inherent nutrition. Probiotics that survive and colonise the intestine are able to alter the indigenous microflora
populations and their metabolic activity. The enteric flora is thought to have a profound effect on gastrointestinal health. Recent theories posit that an unbalanced bacterial flora may play a causative role in IBD (Blaser 1997; Sartor 1997). Restoration of the bacterial balance in individuals may provide a rational approach to prevention and treatment of IBD. In animal models of IBD, the intestinal microflora is needed for this disease to occur (Videla et al. 1997, Madsen et al. 1999; Madsen et al. 2000).

Probiotic bacteria may improve the balance of the bacterial ecology in the colon. There may be an application for probiotics in diseases such as Crohn’s disease. Patients with diarrhoea have a compromised intestinal barrier, and probiotics have shown some success in reducing the severity of diarrhoea particularly that induced by rotavirus injection in childhood. The evidence of probiotics reducing colonic injury in IBD animal models is promising (Mao et al. 1996, Madsen et al. 1999, Madsen et al. 2000; Schultz et al. 1998; Schultz et al. 2002). There are few human studies investigating probiotics and IBD, but they have had encouraging preliminary results. Single and mixed probiotic treatments have manipulated intestinal flora and their metabolic activity to reduce the frequency of relapse in UC patients (Gionchetti et al. 1998; Venturi et al. 1999). Malin et al. (1996) concluded that LGG may have the potential to promote the gut immunological barrier. The true mechanisms leading to damage and the prevention of damage by probiotics remain uncertain.

Salminen et al. (1996) theorised that radiation enteropathy resulted from damage to the intestinal mucosa, changes in the intestinal microflora, and impaired immune response. They further suggested that probiotic bacteria may stabilise the intestinal flora and gut mucosal barrier to strengthen the intestinal defence mechanisms. Figure 1.4 displays the hypothesis of this thesis based on Salminen’s theory. A trigger (drugs, disease and stress) will elevate passive intestinal permeability resulting in a compromised intestinal barrier. The barrier will be unable to prevent the access of antigens, toxins, pathogens and macromolecules to the lamina propria, causing an immune response. The immune response will trigger an inflammatory cascade that results in intestinal damage. This thesis will determine whether passive intestinal permeability is elevated prior to the detection of damage, and whether probiotics will prevent this elevation.
Figure 1.4 The role of intestinal permeability in disease development.
Chapter 2  

**INTRODUCTION**

The dual sugar permeability test was developed to measure small intestinal integrity and the function of the whole small intestine *in vivo* (Fleming *et al.* 1990; Catassi *et al.* 1991; Travis and Menzies 1992). The test is simple, safe, and non-invasive and has been used in humans for the assessment of gut integrity in intestinal diseases such as inflammatory bowel disease and coeliac disease (Pearson *et al.* 1982; Sanderson *et al.* 1987; Howden *et al.* 1991; Van Elburg *et al.* 1992). Animal and human studies have shown that lactulose and mannitol are sensitive indicators of small intestinal damage (Hamilton *et al.* 1987; Catassi *et al.* 1997; Pernet *et al.* 1998). This test involves the oral ingestion of two non-metabolisable synthetic sugars that can travel across the intestinal membrane in small amounts.

High pressure liquid chromatography (HPLC) is the methodology used to assess sugar concentration in the urine and has been established to measure small intestinal permeability in paediatric and adult populations (Fleming *et al.* 1990; Miki *et al.* 1996). This methodology could be modified to allow testing of permeability in rat with methotrexate-induced intestinal damage. Various techniques are currently used to measure intestinal permeability including *in vitro* and *in vivo* (Madara and Hecht 1989; Bijlsma *et al.* 1995; Rubas *et al.* 1995; Sinko and Hu 1996). A major disadvantage of these studies has been that the rats are sacrificed and intestinal permeability determination cannot be repeated in the same animal over time. The advantage of an *in vivo* permeability tests is that it measures the function of the whole intestine and it allows multiple investigations into intestinal permeability in the same animal.

Studies using the sugar permeability test in rats were either fed *ad libitum* or had food intakes restricted prior to the intestinal permeability test, to be similar to human studies (Bijlsma *et al.* 1995; Meddings and Gibbons 1998). In human studies, subjects were fasted overnight to reduce the possibility of contaminating sugars affecting the HPLC analysis (Miki *et al.* 1996). Little is known about the effect of fasting or feeding prior to probe
administration on both sugar probe recovery and urine excretion. The current methodologies for in vivo permeability tests vary in probe type, concentrations, delivery, and urine collection (Davies et al. 1994; Bijlsma et al. 1995; Pernet et al. 1998). In this study multiple probes were used requiring 24 hour urine collection to maximise urine volume and probe collection.

Probiotic bacteria have been suggested as a potential therapy for improving intestinal health (Fuller 1989; Salminen et al. 1998). Research conducted in the laboratory has shown that small intestinal permeability decreased by 50% in healthy children and adolescents fed yoghurt made from Lactobacillus bulgaricus and Streptococcus thermophilus fermented sheep milk (Southcott et al. 1997). The mechanism for this decrease in permeability is unclear. It is known that reducing elevated intestinal permeability could be a treatment strategy for intestinal damage which occurs in diseases such as inflammatory bowel disease (Salminen et al. 1996; Sanderson 1997; Isolauri 1999). However, before sheep milk yoghurt can be trialled in a model of intestinal damage it is important to first determine its effects in healthy rats.

The aims of this study were to determine the effect of feeding and fasting on the performance of the permeability test, and then to investigate the effect of sheep yoghurt on intestinal permeability in the healthy rats.

**METHODS**

**Rats**

Eight Sprague Dawley rats (134.1 ± 1.2 g) were housed in Tecniplast metabolism cages with free access to water and a casein-based diet (Appendix I). Rats were individually maintained in metabolism cages for two days prior to commencing experiments to allow acclimatization. Animal weight, food and water intake were measured daily throughout
the trial. The project was approved by the Animal Ethics Committees at the Women's and Children's Hospital and The University of Adelaide.

**Study 1: Overnight fasting or feeding prior to a permeability test**

The permeability tests for sugar markers and $^{51}$Cr EDTA were conducted on separate days over four weeks. Rats were orally administered with a permeability test solution in the morning (9 am) and urine was collected over 24 h. Prior to the first test rats were randomly assigned to be either fed or fasted overnight before the test with a minimum of 72 h lapsed between the cross over permeability test. Intestinal permeability was tested in the eight rats four times. The four permeability tests were: after an overnight fast with sugars markers; after overnight feeding with sugar markers; after overnight fast with $^{51}$Cr EDTA; and finally after overnight feeding with $^{51}$Cr EDTA.

**Study 2: Effects of a probiotic diet on intestinal permeability in healthy rats**

The diet was supplemented with 2 mL yoghurt orally gavaged at 9:00 am and 4:00 pm for seven days. Sheep milk yoghurt (Pure Island Kangaroo Island, South Australia) contained *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mix (YC180). The content of sheep milk is shown in Appendix II. After seven days of supplementation, intestinal permeability was tested on consecutive days. Food intake was not restricted overnight before the dual sugar permeability test or the $^{51}$Cr EDTA permeability test. The probiotic diet was maintained up to the day of sacrifice which was performed four days after their last permeability test.

*Intestinal permeability test*

A pretest urine sample was collected one day prior to the permeability test. Rats were either fed or fasted overnight prior to being orally gavaged with the test sugar solution at 9
The test sugar solution contained 13.7 mg lactulose (lactulose syrup is known as Duphalac\textsuperscript{R}, Solvay-Duphar, B.V., Holland), 7.2 mg L-rhamnose (Sigma Chemical Co., St. Louis, MO, USA) and 7.2 mg mannitol (Sigma Chemical Co., St. Louis, MO, USA) in 2 mL of water (20 mmol/L concentration). Water was permitted throughout the test to increase urine flow/volume. Food was reintroduced to the fasted rats at 2 pm on the day of the permeability study. Urine was collected for a total of 24 h in a container containing 0.1 mL 10 g/L Thimerosal (BDH Chemical Ltd., Poole, UK) as a preservative. Urine was also collected at 9 am on the following morning. The total urine volume was recorded before storage at -20°C until analysis.

Sample preparation for analysis involved the dilution of thawed urine specimen with Milli-Q water at several dilutions (Table 2.1). The urine was desalted with washed mixed ion-exchange resin (Duolite MB 5113; BDH Chemical, Poole, UK). The mixture was vortex-mixed for 30 s, rested for at least 10 min and centrifuged for 10 min at 3000 x g. The resultant supernatant was filtered twice through a 0.2 µm (pore-size) disposable syringe filters (Acrodisc\textsuperscript{R}; Gelman Sciences, Ann Arbor, MI, USA).

**HPLC analysis**

The HPLC analysis followed a modified protocol of Miki et al. (1996). Briefly, an aliquot of filtered supernate was injected into the manual injector with a 10 µL loop (Model 7125; Rheodyne, Cotati, CA, USA). The HPLC was equipped with an isocratic pump (Model SP 8810; Spectra Physics, San Jose, CA, USA), a polymeric guard column (Direct-Connect\textsuperscript{TM} Cartridge Guard Column; Alltech, Deerfield, IL, USA) and an amine-modified silica column (Kromasil\textsuperscript{TM} NH\textsubscript{2} Column, 5 µm particle size, 250 x 4.6 mm; Alltech, Deerfield, IL, USA) used at ambient temperature. The mobile phase was degassed 70% HPLC-grade acetonitrile (ACN) (E. Merck, Darmstadt, Germany) in distilled-deionized water at a flow rate of 1 mL/min. Detection was by a refractive index (R.I.) detector (LC 1240 R.I. Detector; GBC Scientific Equipment, VIC, Australia) and was recorded on a linear chart recorder (Linear 1200; Alltech Associates, Deerfield, IL, USA). Standard curves were prepared by analyzing appropriate concentrations of each compound in distilled water and plotting the peak-height obtained at 1.0 x 10\textsuperscript{-5} R.I. units/FS sensitivity of the detector. Standard solutions of the sugar probe (5, 2, 1, 0.5, 0.2, 0.1 mmol/L) were made from a 20
mmol/L stock which contained L-rhamnose and mannitol (Sigma Chemical Co., St. Louis, MO, USA) and Lactulose (E. Merck, Darmstadt, Germany). Urine sugar probes concentration was calculated from the standard curves using peak-height analysis. In each sample the total urinary excretion was calculated for each sugar, and results were expressed as percentage of ingested dose present in urine and as excretion ratios, lactulose/L-rhamnose and lactulose/mannitol.

Chromium-51-labelled EDTA permeability

\(^{51}\)Cr EDTA permeability was measured using a modified protocol of Davies et al. (1994). Rats were orally gavaged with 0.5 mL of a solution containing 10 µCi/mL of \(^{51}\)Cr complexed with EDTA (Amrad Pharmacia Biotech and NEN Life Science Products Inc, Boston, MA, USA). 100 µL thiomersal was placed in the urine collection container and urine samples were collected over 24 h following probe administration. \(^{51}\)Cr EDTA was counted by a gamma counter LKB Wallac Multigamma 1261 (Wallac, OY 20101 Turku 10 Finland) for 1 minute in a counting window of 0-2 Mev. At least two 100 µL standards of the administered probe were counted with the urine samples. Relative permeability was determined by calculating the activity present in each urine sample as a percent of the administered dose after correcting for background radiation.

Statistics

Permeability test results were analysed in triplicate and expressed as an average for each rat. Student’s T-test was used to compare fed and fasted rats and fed rats with probiotics rats. Data were expressed as Mean ± SEM. Differences were considered significant at p<0.05.
RESULTS

Permeability analysis

Samples were tested at several dilutions to optimise detection of all three sugars. The different preparation of rat urine and HPLC techniques used to measure intestinal permeability are listed in Table 2.1. The rat urine was yellow in colour, which was due to the presence of proteins and bilirubin in the urine. These proteins affected the RI detector and as a result the baseline was elevated during the elution of mannitol. When samples were analysed again after being re-frozen the colour in the sample was greatly reduced. Human samples arrive at the lab frozen and undergo two freeze/thaw cycles before analysis. These rat samples had only one freeze/thaw cycle, suggesting the proteins causing the yellow colour are denatured or broken down by two freeze/thaw cycles.

Rhamnose elution was a further complication encountered. It seemed likely that sugars eluting at a similar time to rhamnose made rhamnose difficult to measure. The rhamnose levels were very high for both groups and believed to co-elute with dietary sugars (Figure 2.1C). Pretest urine samples were analysed and a spike was found to elute closely to rhamnose (Figure 2.1B), thus rhamnose results were not accurate when rats were fed the casein-based diet.

The optimal preparation of rat urine involved two freeze-thaw cycles prior to dilution. Duolite resin (0.4 g) was added to 2 mL of urine and 1mL of water, vortexed for 30 s then centrifuged. The urine had little colouration and was filtered 0.2 μm before analysis of the HPLC. Separation of the sugars was optimised using an 80% acetonitrile (ACN) and 20% Milli Q water mobile phase (Figure 2.1D).
Table 2.1 Dilutions, resin and permeability conditions used to measure L-rhamnose, mannitol and lactulose levels in rat urine.

<table>
<thead>
<tr>
<th>Urine: H$_2$O Ratio</th>
<th>Resin (g)</th>
<th>ACN:H$_2$O %</th>
<th>L-rhamnose mmol/L</th>
<th>Mannitol mmol/L</th>
<th>Lactulose mmol/L</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>0.6 g</td>
<td>70:30</td>
<td>34.34</td>
<td>masked</td>
<td>ND</td>
<td>L-rhamnose levels high</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>double peak at Mannitol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactulose ND</td>
</tr>
<tr>
<td>1:3</td>
<td>1.0 g</td>
<td>70:30</td>
<td>20+</td>
<td>masked</td>
<td>0.093</td>
<td>L-rhamnose levels high</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactulose low</td>
</tr>
<tr>
<td>1:2</td>
<td>0.6 g</td>
<td>80:20</td>
<td>20+</td>
<td>1.83</td>
<td>0.124</td>
<td>Colouration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L-rhamnose levels high/off scale</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactulose low</td>
</tr>
<tr>
<td>1:1</td>
<td>1.0 g</td>
<td>80:20</td>
<td>20+</td>
<td>2.59</td>
<td>0.331</td>
<td>Colouration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L-rhamnose off scale</td>
</tr>
<tr>
<td>2:1 + 2F/T</td>
<td>0.4 g</td>
<td>80:20</td>
<td>8.17</td>
<td>1.41</td>
<td>0.208</td>
<td>L-rhamnose levels high</td>
</tr>
<tr>
<td>1:0</td>
<td>0.6 g</td>
<td>70:30</td>
<td>ND</td>
<td>ND</td>
<td>0.420</td>
<td>Colouration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L-rhamnose off scale</td>
</tr>
</tbody>
</table>

Optimum

| 2:1                 | 0.4 g     | 80:20         |                   |                 |                 | L-rhamnose levels high   |
| + 2F/T              |           |               |                   |                 |                 | Mannitol peak separates |
|                     |           |               |                   |                 |                 | Lactulose detected       |

Sugar concentrations are the results of one sample. ACN = acetonitrile ND = not detected. F/T = freeze/thaw cycle.
Figure 2.1 Chromatograms of a sugar standard and a 24 hour rat permeability test.

Chromatograms show the elution of three sugars rhamnose (1), mannitol (2) and lactulose (3) from a standard (2 mmol/L in A) and from rat permeability test. All Chromatograms shown were measured at 2x sensitivity for comparison. Rat urine samples were: pretest rat urine (B); undiluted test sample (C); and diluted sample after two freeze/thaws (D).
Study 1: Overnight fasting or feeding prior to a permeability test

Average body weight, food and water intake was collected and compiled from both intestinal permeability tests. When rats were fasted overnight prior to the permeability test they lost 10.7 ± 1.1% of their body weight (151.0 ± 10.5 g to 140.2 ± 10.0 g) compared to when fed ad libitum (145.9 ± 5.8 g to 155.3 ± 5.3 g). In the fasted rats there was a significant decrease in cumulative food intake compared to fed rats until the fifth day (p=0.025). There was no significant difference in water intake.

\(^{51}\)Cr EDTA and dual sugar permeability test results for rats fed ad libitum and fasted overnight are shown in Figure 2.2 and Table 2.2. Urinary recovery of \(^{51}\)Cr EDTA in fed rats was 2.48 ± 0.21% and this was significantly greater compared to fasted rats, 1.62 ± 0.24% (p<0.05). During the \(^{51}\)Cr EDTA permeability test, seven out of the eight fasted rats had reduced urine volume compared to those fed ad libitum. A larger urine volume assists analysis of the probes by flushing through the probe and enabling repeat analysis of the test sample. There was no significant difference between fed and fasted rats for either the lactulose/mannitol permeability ratio or the urinary recovery of mannitol and lactulose.

Study 2: Effects of a probiotic diet on intestinal permeability in healthy rats

Lactulose recovery after a seven day diet supplemented with probiotics was 4.85 ± 1.26% whilst mannitol recovery was 24.80 ± 4.63%. There was no difference in the lactulose/mannitol ratio between fed rats and those given probiotic supplementation (0.134 ± 0.062 vs.0.214 ± 0.062). The recovery of \(^{51}\)Cr EDTA however was significantly reduced by over 40% after probiotic supplementation compared to fed rats (p<0.05) (Figure 2.2).
Table 2.2 Small intestinal permeability results in rats (n=8) fed or fasted prior to test.

<table>
<thead>
<tr>
<th></th>
<th>Fed rats</th>
<th>Fasted rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Volume (mL)</td>
<td>13.6 ± 0.7</td>
<td>13.4 ± 1.3</td>
</tr>
<tr>
<td>Range (mL)</td>
<td>11-16</td>
<td>7-22</td>
</tr>
<tr>
<td>Mannitol (%)</td>
<td>32.7 ± 8.5</td>
<td>53.4 ± 20.8</td>
</tr>
<tr>
<td>Lactulose (%)</td>
<td>4.41 ± 0.90</td>
<td>2.96 ± 0.64</td>
</tr>
<tr>
<td>Lactulose/Mannitol ratio</td>
<td>0.1346 ± 0.0621</td>
<td>0.1233 ± 0.0530</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM.

Figure 2.2 $^{51}$Cr EDTA recovery in fed, fasted and probiotic fed rats.

* $^{51}$Cr EDTA recovery in fed rats was significantly greater than fasted or probiotic fed rats (p<0.05). Values are expressed as Mean ± SEM.
DISCUSSION

The methodology for quantifying probe sugars in urine of rats was successful and provided a easy method and that was serially repeated. Optimal separation of the sugars on the HPLC required the samples to be run with an 80/20 acetonitrile/water mobile phase. This mobile phase slowed the elution, allowing lactulose and mannitol to be resolved from the background dietary sugars. Furthermore, the analysis of urine requires the removal of protein and colouration as they altered the detectors sensitivity (Miki et al. 1996). The removal of the colouration by dilution, freeze/thaw, and resin improved the measurement of the probes.

Several difficulties were encountered when measuring the test probes in rat urine. The HPLC methodology involved the measurement of probe sugars with a column that separates carbohydrates. The casein-based diet is a sugar rich diet that results in some sugars eluting close to the probes. Rhamnose recovery in this experiment was considerably higher than expected and higher when compared to animal other studies (Sorensen et al. 1993). This suggests an alternative diet with less sugar is needed before rhamnose can be used as a probe in measuring intestinal permeability. Rhamnose elutes early in the HPLC analysis (Figure 2.1). For this reason most animal studies have used mannitol and lactulose as the test markers (Bijlsma et al. 1995; Meddings and Gibbons 1998; Pernet et al. 1998). Moreover, fasting prior to the permeability test, did not improve the detection of rhamnose.

Urinary recovery of $^{51}$Cr EDTA was greater in fed rats, than in fasted rats, suggesting that the reduction of $^{51}$Cr EDTA recovery could have been a physiological response to fasting. Rats consume the majority of their food and water during the night and eat little during the day. The fasting of rats overnight prior to and throughout the permeability test could result in rats being without food for up to 36 h. Prolonged starvation or a reduced diet may impose stress and possibly affect intestinal barrier function (Taminiau et al. 1980). This could be exacerbated in animal models of intestinal disease leading to effects of food availability rather than changes in intestinal function. Butler et al. (1992) found that enterocyte proliferation decreased with fasting and increased with re-feeding. The authors concluded that fasting resulted in a more marked suppression of proliferation in the large
bowel of rats compared with the small bowel. This may have contributed to a significant reduction in $^{51}$Cr EDTA recovery whilst the sugar probe recovery remained unaltered.

Lactulose and $^{51}$Cr EDTA cross the intestine via paracellular pathways, controlled by the tight junctions between enterocytes (Travis and Menzies 1992). The structure of the tight junctions differs along the villus-crypt axis, where the crypt is more permeable than the villus (Hollander 1992). The structure of the mucosa in the colon differs from the small intestine with a higher proportion of immature cells. The ratio of immature to mature cells is however decreased in the fasted state (Butler et al. 1992) and this may be reflected by a less permeable large bowel. Pappenheimer and Volpp (1992) have suggested that luminal nutrients are a potent stimulus for altering tight junction permeability (Sadowski and Meddings 1993). The current permeability results suggest the absence of luminal nutrients may also affect colonic paracellular pathways and the intestinal barrier.

Administration of a probiotic for one week showed a significant reduction in $^{51}$Cr EDTA permeability. On the other hand, there was no difference in small intestinal permeability using the dual sugar permeability test. The difference in intestinal permeability between lactulose and $^{51}$Cr EDTA may be due to the site of uptake. $^{51}$Cr EDTA and lactulose could compete for transportation through a common site. Lactulose is only taken up in the small intestine as it is fermented in the large intestine but $^{51}$Cr EDTA is not fermented and can be absorbed throughout the whole intestine (Løkken 1970; Aabakken 1989; Bjarnason et al. 1995). Simultaneous administration of lactulose and $^{51}$Cr EDTA can enable permeability changes in the colon to be distinguished from those involved in the small intestine (Jenkins et al. 1992).

In this study L. bulgaricus may have modified colonic permeability as Garcia-Lafuente et al. (2001) found colonisation of L. brevis reduced colonic permeability to mannitol in a normal rat. They did not measure $^{51}$Cr EDTA with L. brevis, though they confirmed E. coli colonisation altered colonic barrier function as $^{51}$Cr EDTA and mannitol clearance was decreased (Garcia-Lafuente et al. 2001). Studies have shown bacteria may impair colonic barrier function and commensal bacteria may improve the tightness of the barrier (Moore et al. 1995; Garcia-Lafuente et al. 2001; Mangell et al. 2002).
Fatty acids present in milk could also have influenced intestinal permeability (Lindmark et al. 1998). Medium chain fatty acids, such as capric acid (C10) and lauric acid (C12), have the ability to alter tight junctions, although their mechanisms in altering paracellular permeability are not clearly understood (Lindmark et al. 1998). Intestinal permeability may have thus been influenced by *L. bulgaricus*, the higher percentage of short and medium chain fatty acids in sheep milk, or a combination of both of these factors.

$^{51}$Cr EDTA recovery has been used as a surrogate marker of changes in permeability of macromolecular antigens (Ramage et al. 1988). Macromolecular transport involves the molecules crossing the epithelial barrier through extrusion zones at the villus tip and via M cell transportation in Peyer’s patches (Sanderson and Walker 1993). Ramage et al. (1988) suggested that $^{51}$Cr EDTA and ovalbumin had a common route of uptake, even though there is a difference in their molecular weights, 359 mW versus 45,000 mW. Thus the decreased $^{51}$Cr EDTA recovery observed after sheep milk yoghurt may be reduced following macromolecule uptake as LGG was shown to improve intestinal integrity by counteracting the uptake of macromolecules (Isolauri et al. 1993). Sheep milk yoghurt could affect the mechanisms of passive permeability and uptake of these two markers in the intestine by: i) changes in the site of uptake; ii) changes in the mechanism of uptake through the tight junctions; and iii) alterations in the mucosal immunological system.

In summary, small intestinal permeability in rats can be measured using lactulose and mannitol sugar probes by high performance liquid chromatography. Rhamnose is not an effective probe for measuring permeability when rats are fed a casein-based diet. Fasting prior to permeability testing may selectively influence colonic permeability in the rat, as shown by a decrease in $^{51}$Cr EDTA recovery. Small intestinal permeability was found to be unaffected by the absence of food prior to and during a sugar permeability test suggesting that fasting had caused no appreciable small intestinal alteration. The yoghurt used in this study resulted in a reduction of $^{51}$Cr EDTA recovery. Whether probiotics act on the uptake mechanisms of the probes or enhanced the permeability at different sites in the small intestine requires further investigation. The protocol used to test intestinal permeability via HPLC techniques combined with the radioisotope assay will allow us to investigate further the intestinal permeability of methotrexate-induced intestinal damage in rats.
Chapter 3  PROBIOTICS AND METHOTREXATE-INDUCED SMALL BOWEL DAMAGE IN THE RAT.

INTRODUCTION

Probiotic bacteria reduce the duration and severity of acute rotavirus (Isolauri et al. 1991; Kaila et al. 1995; Goldin 1998) and have potential as a novel therapy in inflammatory bowel disease (Sartor 1997; Shanahan 2000). However, there are concerns in administering live bacteria to patients with impaired intestinal barrier function and a compromised immune system. Animal models can determine the actions of probiotic containing yoghurts on intestinal function (Mao et al. 1996; Madsen et al. 1999a). Methotrexate is a chemotherapy drug that induces mucosal damage and transient abnormalities in intestinal permeability (Keefe et al. 1997). Methotrexate produces intestinal damage in the animal model akin to acute viral enteritis and chemotherapy treatment in humans (Taminiau et al. 1980). A methotrexate rat model of intestinal damage was chosen as it provides a suitable experimental setting to test the efficacy of probiotic bacteria.

The methotrexate-induced intestinal damage rat model has been used to investigate treatments that lead to a reduction in tissue damage and improve the regeneration process (Vanderhoof et al. 1990; Howarth et al. 1996). Methotrexate administration has caused alterations in active and passive transporters (Taminiau et al. 1980; Erdman et al. 1991; Mao et al. 1996). Previous studies have measured intestinal permeability at discrete sites of the intestine and thus the function of the whole intestine was not measured (Taminiau et al. 1980; Mao et al. 1996). Moreover, these studies do not determine the changes in the mucosal barrier as the methotrexate-induced damage develops. Using the intestinal permeability protocol from Chapter 2 allows measurement of both small and whole intestinal permeability and it may detect alterations in the intestinal barrier with the development of damage.

In the present study, commercially available yoghurts were used rather than laboratory strains. Sheep yoghurt was chosen as it was previously demonstrated to alter of small intestinal permeability in adolescents (Southcott et al. 1997). L. johnsonii yoghurt was
chosen for to its ability to improve the colonic bacterial environment (Bernet et al. 1994; Marteau et al. 1997; Schiffrin et al. 1997). These yoghurts were tested in a model of methotrexate-induced damage established by Howarth et al. (1996). The aims of this study were to determine: i) the changes in intestinal permeability in the rat induced by methotrexate; and ii) the effect of probiotics on intestinal function in this animal model.

METHODS

Maintenance of rats

Thirty two adult Sprague Dawley rats (body weight 90.8 ± 1.3 g) were individually housed in Tecniplast metabolism cages and allowed three days to acclimatise. This study was approved by the Animal Ethics Committee, Women’s and Children’s Hospital and The University of Adelaide. The rats were housed in the animal care facility at the Women’s and Children’s Hospital, Adelaide, SA and maintained at an environmental temperature of 25°C with a 12 hour light: dark cycle for the duration of the study. Throughout the experimental period rats had continual access to water and casein-based diet (Howarth et al. 1996). Body weight (g), food intake (g), water intake (mL), urine (mL) and faeces (g) excreted were measured daily. Rats were randomly assigned to either: Control, MTX, MTX and cow’s milk yoghurt (MTX + CY) or MTX and sheep milk yoghurt (MTX + SY).

Administration of probiotics

Yoghurt treated rat groups consumed the casein-based diet supplemented with yoghurt for seven days prior to, and six days after, the first methotrexate injection (Day -7 to Day +7) (Figure 3.1). The MTX + CY group consumed commercial brand yoghurt that was produced from cow’s milk fermented by Lactobacillus johnsonii strain LA1, at a concentration of 10^7 organisms/L. MTX + SY was administered sheep milk yoghurt, produced from sheep milk fermented by a mix of bacteria brand YC-180 (Lactobacillus...
bulgaricus and Streptococcus thermophilus) from Chr. Hansen Pty. Ltd. (Windsor Gardens, SA, Australia). Both yoghurts were natural with no flavouring added. The rats were gavaged with yoghurt twice a day; 2 mL of yoghurt in the morning (9 am) and afternoon (4 pm). MTX rats were gavaged with 2 mL H2O in the morning (9 am) and afternoon (4 pm). Rats were not gavaged prior to or during a permeability test.

Methotrexate administration

Methotrexate (Lederle Laboratories, Baulkham Hills, NSW, Australia) was administered once daily to 24 rats by subcutaneous injection (2.5 mg/kg, given as an injection of 0.5 g methotrexate/L sterile isotonic (9 g/L) saline) in the suprascapular region on Day +1, +2 and +3 (Howarth et al. 1996). Treated groups were maintained on their respective diets until Day +7, 96 h after the final methotrexate injection, when they were sacrificed (see Figure 3.1). Control rats (weight 211.1 ± 4.7 g; n=8) were weight-matched to the methotrexate-treated rats from heaviest to lightest, and fed the same amount of casein-based diet ingested by their methotrexate-treated counterpart on each day of the seven day protocol.

Permeability test

Intestinal permeability tests were conducted on four different days in order to assess changes in the barrier function (Figure 3.1). On Day -8 after acclimatization; on Day -1, (after seven days of yoghurt administration for MTX + CY and MTX + SY); and on Day +3 and Day +6. All tests were conducted at the same time of the day and urine was collected 24 h after gavage. The methodology of the permeability test is described in Chapter 2.
**Kill procedure and tissue collection**

Rats were sacrificed by cervical dislocation under CO₂ anaesthesia. The abdomen was opened via a midline incision and the liver and gut were excised and weighed. Gut contents were flushed thoroughly with phosphate buffered saline, pH 6.0. The small intestine was placed on an ice-cold slab and divided into three sections: the duodenum, the gastro-duodenal junction to the ligament of Treitz; the remaining intestine from the ligament of Treitz to the caecum was divided in half to provide jejunum and ileum of equal length. A map of the samples collected is shown in Figure 3.2.

In the duodenum, jejunum and ileum four cm sections were excised and frozen in liquid nitrogen, before being stored at −70°C until determinations of intestinal disaccharidase activity could be made. In the large intestine and along the small intestine one cm of each sections were excised and fixed in methacarn fixative for two h, transferred to 70% ethanol for 48 h, and embedded in paraffin wax for histological analysis (Howarth et al. 1996). A jejunal sample, 15 cm from the ligament of Treitz, was collected for mucosal mast cell counts and fixed in Carnoy’s fixative for 24 h. These histological samples were placed in 100% ethanol before embedding in paraffin.

**Assessment of intestinal severity score**

For histological analysis, methacarn-fixed one cm tissue samples were embedded in paraffin wax, sectioned transversely at two µm, stained with haematoxylin and eosin to expose the structure of the intestinal mucosa, and examined by an impartial observer under a light microscope (Howarth et al. 1996). A semi-quantitative histological assessment of intestinal damage was utilized to obtain an overall score ranging from zero (normal) to three (maximal damage) for 11 histological features (Table 3.1), such that the total maximum severity scores (33) indicated the most severe damage (Howarth et al. 1996). Histological severity scores were assigned for the duodenum, jejunum and ileum and presented as median and range.
Figure 3.1 The experimental time frame used to investigate intestinal permeability and small intestinal damage induced by methotrexate in rats.

![Diagram showing MTX injections and timeline for permeability test and diet]

**Permeability test**
- Baseline
- Day -1
- Day 3
- Day 6

**Diet**
- **MTX**
  - Casein based diet + 2 mL water
- **MTX + CY**
  - Casein based diet + 2 mL cow’s yoghurt
- **MTX + SY**
  - Casein based diet + 2 mL sheep yoghurt

Figure 3.2. Schematic representation of tissue collection protocol.

![Diagram showing tissue collection protocol]

**Samples collected**
- **H** Histology
- **O** Frozen OCT
- **Green** Mast cells
- **Pink** Enzyme

**Scale**
- = 1 cm
Table 3.1 Histological parameters used to derive severity scores.

<table>
<thead>
<tr>
<th>Intestinal wall component</th>
<th>Histological parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosa</td>
<td>Villus fusion and stunting</td>
</tr>
<tr>
<td></td>
<td>Enterocyte disruption</td>
</tr>
<tr>
<td></td>
<td>Reduction in mitotic figures</td>
</tr>
<tr>
<td></td>
<td>Crypt disruption</td>
</tr>
<tr>
<td></td>
<td>Crypt cell disruption</td>
</tr>
<tr>
<td></td>
<td>Crypt cell abscess formation</td>
</tr>
<tr>
<td></td>
<td>V/C ratio</td>
</tr>
<tr>
<td></td>
<td>Lymphocytic and PMN infiltration</td>
</tr>
<tr>
<td></td>
<td>Capillary and lymphatic dilation</td>
</tr>
<tr>
<td>Submucosa</td>
<td>Thickening /oedema</td>
</tr>
<tr>
<td>Muscularis externa</td>
<td>Thickening</td>
</tr>
</tbody>
</table>

*Morphological assessment of the small intestine*

Methacarn-fixed one cm tissue samples used for histological analysis were also used to measure villus height and crypt depth in the duodenum, jejunum and ileum. Generally 10 to 20 well-orientated, full length villi and crypts were measured. Measurements were taken using a linear microscope graticule and villus and crypt lengths were expressed in μm.

*Mucosal immune activity*

Mucosal mast cell counts were used as indicators of mucosal immune activity. Mucosal mast cell's of the gastrointestinal tract require different staining techniques as they are a separate cell line within the mast cell system of the rat (Blaies and Williams 1981). Staining with Astra Blue or Alcian Blue (a basic copper phthalocyanine) followed by counter staining with Safranin O, causes intestinal connective tissue mast cells to stain
violet, distinct from mucosal mast cells which stain blue (Bloom and Kelly 1960; Enerback 1966; Mayrhofer 1980; Wal et al. 1985; Gibson et al. 1987).

Mucosal mast cells were counted after staining with Alcian Blue and Safranin O following the methodology of Cummins et al. (1988). Briefly, a one cm jejunal sample, 15 cm from the ligament of Treitz, was collected. The biopsy was placed in Carnoy’s fixative for 24 h, and then stored in 100% ethanol for 24 h. Tissues were embedded in paraffin wax and histological sections were cut at four μm. After rehydration in distilled water the slides were stained with fresh 0.1% Alcian Blue in 0.7 N HCl (pH 0.3) for 30 min and rinsed in 0.7 N HCl, then counter stained with freshly made Safranin O in 0.125N HCl (pH 1.0) for 30 seconds. Slides were immediately dehydrated by washing with tert-Butyl Alcohol (100% 2 Methly -2-propanol). Mucosal mast cells s were counted in well orientated sections, using fixed field techniques with a linear microscopic graticule (length of field = 500 μm, x 20 objective lens) aligned along the muscularis mucosae of each sample. Thirty fields per specimen were counted, for a coefficient of variance of 25% and the results expressed as cells per mm of muscularis mucosae (Cummins et al. 1988; Cummins et al. 1989a; Thompson et al. 1996).

Assay of intestinal enzyme activity

Duodenum, jejnum and ileum sections (4 cm) were prepared for the assay of sucrase, lactase and alkaline phosphatase (ALP) activity according to the first two steps in Shirazi and Beechley (1991). Briefly, the brush border membrane was isolated via hypo-osmolar shock followed by centrifugation. Three aliquots of brush boarder membrane samples (approx. 250 μL) were stored from each section from each rat. Aliquots were stored in liquid nitrogen prior to being thawed and assayed for sucrase and lactase activity by the method of Dahlqvist (1968) and ALP activity by the method of Forstner et al. (1968). Enzyme activity was expressed as activity per mg of protein. The protein concentration of the enzyme preparation was determined by the method of Bradford (1976). A standard curve of increasing bovine serum albumin concentrations was used to determine the protein content of the sample (Bradford 1976). Sucrase and lactase activity was expressed
as μmol of substrate hydrolysed at 37°C at pH 6.0/ mg protein/hour. ALP activity was expressed as μmol of substrate hydrolysed at 37°C at pH 9.2/ mg protein/hour.

Statistics

The semi-quantitative histological scoring of intestinal damage, involved statistical comparison of data form each region using a non-parametric Mann-Whitney U test. The intestinal permeability and enzyme data were analysed using a linear mixed effects model methodology in the S-PLUS statistical software. The data were log transformed (base e) to produce more symmetric distributions and error variances that were more constant. The individual rats were included as random effects. This allowed some rat to rat variation in recovery rates and the amount of enzyme activity, which then enabled a better examination of the treatment and time differences. Tests of statistical significance were conducted using model-based t-tests or contrasts for individual effects using F-tests within the ANOVA for overall differences due to treatment and times.
RESULTS

Methotrexate response

Semi-quantitative histological assessment showed methotrexate-induced intestinal damage in the small intestine of MTX, MTX + CY and MTX + SY compared to Control (p<0.05) (Table 3.2). MTX + SY group had significantly reduced duodenal severity scores compared to MTX (p<0.05) and the MTX + SY group duodenal scores were significantly less compared to jejunum and ileum scores (p<0.05).

Body weight, food and water intake and urine and faeces excretion were measured in MTX treated rats throughout the trial (Table 3.3). There was no significant difference between treatments at any time point. MTX rats lost 21.7 ± 4.4% of their body weight 96 h after the last injection of MTX compared with 13.5 ± 9.3% for MTX + CY rats and 28.6 ± 6.8% for MTX + SY rats.

Table 3.2 Semi-quantitative histological assessment of methotrexate-induced intestinal damage at different regions of the small intestine.

<table>
<thead>
<tr>
<th></th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>2 (0-6)</td>
<td>1.5 (0-6) †</td>
<td>1.5 (1-7) †</td>
</tr>
<tr>
<td><strong>MTX</strong></td>
<td>8 (2-15) *</td>
<td>11 (1-26)</td>
<td>10.5 (2-21)</td>
</tr>
<tr>
<td><strong>MTX + CY</strong></td>
<td>5 (2-22)</td>
<td>12 (6-25)</td>
<td>11 (2-15)</td>
</tr>
<tr>
<td><strong>MTX + SY</strong></td>
<td>5 (2-9) α</td>
<td>14.5 (5-20) δ</td>
<td>12.5 (5-16) δ</td>
</tr>
</tbody>
</table>

Values are the sum of the scores for 11 independent histological criteria as detailed in Table 3.1. The severity of each histological parameter was scored from 0 to 3. Values are presented as medians with the ranges in parentheses. * Severity scores in the duodenum were significantly higher for MTX rats than Control p<0.05 or MTX + SY scores p=0.029. † Control rats had a significantly lower scores than other groups p=0.02; δ MTX + SY jejunum and ileum scores were significantly higher than the duodenal scores p<0.001.
Table 3.3 Body weight, food and water intake and urine excretion in rats prior to probiotic supplemented diet (baseline) and during the development of methotrexate-induced intestinal damage.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day -1</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>117.6 ± 1.4</td>
<td>164.7 ± 1.9</td>
<td>187.6 ± 2.9</td>
<td>187.6 ± 3.6</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>115.8 ± 1.7</td>
<td>159.9 ± 2.4</td>
<td>184.0 ± 3.6</td>
<td>188.1 ± 6.5</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>113.5 ± 1.8</td>
<td>158.2 ± 2.4</td>
<td>178.8 ± 3.0</td>
<td>181.6 ± 4.5</td>
</tr>
<tr>
<td><strong>Food intake (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>12.3 ± 0.5</td>
<td>16.8 ± 0.6</td>
<td>13.1 ± 1.1</td>
<td>8.0 ± 1.3</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>10.4 ± 0.8</td>
<td>15.0 ± 0.4</td>
<td>13.5 ± 1.2</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>12.0 ± 1.1</td>
<td>15.5 ± 0.6</td>
<td>13.7 ± 0.9</td>
<td>7.7 ± 1.35</td>
</tr>
<tr>
<td><strong>Water intake (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>21.0 ± 1.4</td>
<td>24.7 ± 1.5</td>
<td>25.1 ± 1.4</td>
<td>17.5 ± 1.6</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>18.0 ± 1.16</td>
<td>21.3 ± 0.8</td>
<td>23.0 ± 1.5</td>
<td>15.8 ± 2.3</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>18.4 ± 1.3</td>
<td>20.7 ± 0.8</td>
<td>24.5 ± 1.5</td>
<td>19.2 ± 2.2</td>
</tr>
<tr>
<td><strong>Urine excretion (mL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>12.3 ± 1.0</td>
<td>13.9 ± 1.1</td>
<td>13.0 ± 1.1</td>
<td>10.3 ± 0.4</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>12.1 ± 1.1</td>
<td>11.9 ± 0.6</td>
<td>12.6 ± 0.6</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>11.2 ± 1.0</td>
<td>12.4 ± 0.7</td>
<td>13.3 ± 0.9</td>
<td>14.8 ± 2.0</td>
</tr>
</tbody>
</table>

Values are the average of the three previous days and expressed as Mean ± SEM.

Table 3.4 illustrates the intestinal length and weight on Day +7 after the first methotrexate injection. The intestinal length in MTX + CY rats increased, but when adjusted for body weight, was only significant for the colon (p<0.05). There was no significant difference in the weight of the intestine, spleen, liver or the body weights between the groups (Table 3.4 and 3.4a).
Table 3.4 Intestinal length and weight after methotrexate–induced intestinal damage.

<table>
<thead>
<tr>
<th>Length (cm)</th>
<th>Duodenum</th>
<th>Small Intestine</th>
<th>Colon</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>8.1 ± 0.2</td>
<td>76.6 ± 2.2</td>
<td>14.5 ± 0.3</td>
<td>99.2 ± 2.5</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>8.9 ± 0.1</td>
<td>85.0 ± 3.1</td>
<td>16.9 ± 0.8 *</td>
<td>110.8 ± 3.4</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>8.1 ± 0.3</td>
<td>76.5 ± 2.5</td>
<td>14.9 ± 0.5</td>
<td>99.6 ± 2.9</td>
</tr>
</tbody>
</table>

Weight (g)

<table>
<thead>
<tr>
<th></th>
<th>Spleen</th>
<th>Liver</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>3.10 ± 0.27</td>
<td>6.87 ± 0.34</td>
<td>178.30 ± 4.48</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>3.25 ± 0.27</td>
<td>7.35 ± 0.87</td>
<td>185.21 ± 10.28</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>3.11 ± 0.25</td>
<td>6.64 ± 0.75</td>
<td>173.62 ± 7.36</td>
</tr>
</tbody>
</table>

* MTX + CY colon was significantly longer than other groups (p<0.05). Values are expressed as Mean ± SEM.

Table 3.4a Body weight and weight of internal organs.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Spleen</th>
<th>Liver</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>3.10 ± 0.27</td>
<td>6.87 ± 0.34</td>
<td>178.30 ± 4.48</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>3.25 ± 0.27</td>
<td>7.35 ± 0.87</td>
<td>185.21 ± 10.28</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>3.11 ± 0.25</td>
<td>6.64 ± 0.75</td>
<td>173.62 ± 7.36</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM.

**Intestinal Permeability**

Methotrexate-treated rats showed a significant increase in the L/M excretion ratio on Day +6 from Day +3 (p<0.05) (Figure 3.3). The L/M excretion ratio increased across the time periods in MTX rats (p<0.05). MTX + CY and MTX + SY L/M excretion ratio was
significantly less than MTX (p<0.025). MTX + CY L/M excretion ratio remained significantly less than MTX on Day +6 (p<0.05). Figure 3.4 shows the 24 h urinary excretion of $^{51}$Cr EDTA during and after methotrexate administration in rats. On Day +6, the rats showed a significant increase in $^{51}$Cr EDTA recovery (p<0.05). Lactulose recovery was significantly lower at baseline than at other time points (p<0.025) (Table 3.5). Mannitol recovery at Day +3 was significantly greater than Day +6 and baseline (p<0.05). Mannitol recovery had a positive relationship with body weight, food and water intake and urine and faeces excreted (Figure 3.5). No relationship was observed between L/M excretion ratio, lactulose recovery, and $^{51}$Cr EDTA recovery and body weight, food and water intake and urine and faeces excreted. L/M excretion ratio, lactulose recovery and mannitol recovery did not show a relationship with $^{51}$Cr EDTA recovery (data not shown).

Figure 3.3 Lactulose/mannitol excretion ratio during the development of methotrexate-induced intestinal damage in rats.

* MTX + CY and MTX + SY was significantly less than MTX on Day +3 (p<0.025). † Day +6 was significantly greater than Day +3 in all groups (p<0.05). δ Day +6 results in MTX rats were significantly greater than baseline (p<0.05). § MTX + CY rats were significantly less than MTX rats on Day +6 (p<0.025). Values are expressed as Mean ± SEM.
Figure 3.4 ⁵¹Cr EDTA 24 hour recovery (%) during the development of methotrexate-induced intestinal damage in rats.

* All groups had significantly greater recovery on Day +6 results than Day -1 (p<0.001). Values are expressed as Mean ± SEM.

Table 3.5 Lactulose and mannitol recovery (%) and prior to probiotic supplemented diet (baseline) and during the development of methotrexate-induced intestinal damage in rats.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day -1</th>
<th>Day +3</th>
<th>Day +6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactulose (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>1.78 ± 0.38 *</td>
<td>3.38 ± 0.50</td>
<td>5.05 ± 0.98</td>
<td>3.65 ± 0.68</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>1.99 ± 0.36 *</td>
<td>2.91 ± 0.57</td>
<td>3.49 ± 0.64</td>
<td>2.95 ± 0.52</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>1.57 ± 0.32 *</td>
<td>3.26 ± 0.78</td>
<td>2.94 ± 0.48</td>
<td>3.49 ± 0.55</td>
</tr>
<tr>
<td><strong>Mannitol (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>51.3 ± 12.8</td>
<td>56.3 ± 11.1</td>
<td>61.1 ± 11.1 †</td>
<td>29.7 ± 6.1</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>40.8 ± 15.0</td>
<td>69.9 ± 14.4</td>
<td>85.9 ± 25.6 †</td>
<td>48.9 ± 10.6</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>37.2 ± 14.6</td>
<td>52.7 ± 9.7</td>
<td>80.7 ± 7.6 †</td>
<td>49.0 ± 7.1</td>
</tr>
</tbody>
</table>

* Baseline was significantly lower than at other time points (p<0.025). † Day +3 was significantly greater than Day +6 (p<0.05). Values are expressed as Mean ± SEM.
Figure 3.5 Mannitol and lactulose recovery on Day +6 compared to total bodyweight, food intake, water intake, urine volume and faeces weight, in methotrexate-induced damage in rats.

Day +6 permeability results were plotted against five daily measurements totalled for the entire period. A smoothing spline displays the relationship between the mannitol recovery and each daily measurement. There was a lot of variation about each line and the mannitol relationships were significantly positive and relatively weak, whereas there was no significant relationship with lactulose recovery. Mannitol recovery increased notably with all of the daily measurements.

Morphological assessment of the small intestine

Duodenal crypts were significantly deeper than those measured in the jejunum and ileum (Table 3.6) (p<0.05). Methotrexate-treated rats groups had significantly greater crypt depths than Control rats (p<0.05). Duodenal villus height was significantly greater than jejunum and ileum villus height (p<0.05). Jejunal villus height was significantly reduced by MTX treatment (p<0.05). In the ileum, the villus height in MTX + CY and MTX + SY was significantly greater than Control rats (p<0.05).
Table 3.6  Small intestinal crypt depth (μm) and villus height (μm) in rats with methotrexate-induced intestinal damage.

<table>
<thead>
<tr>
<th>Region</th>
<th>Crypt depth</th>
<th>Villus height</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>188 ± 5 γ δ</td>
<td>647 ± 50 δ</td>
<td>6</td>
</tr>
<tr>
<td>MTX</td>
<td>236 ± 10* γ δ</td>
<td>561 ± 29 γ δ</td>
<td>8</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>236 ± 18* γ δ</td>
<td>621 ± 50 γ δ</td>
<td>7</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>233 ± 21* γ δ</td>
<td>562 ± 60 γ δ</td>
<td>8</td>
</tr>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>124 ± 4</td>
<td>550 ± 46</td>
<td>8</td>
</tr>
<tr>
<td>MTX</td>
<td>151 ± 10*</td>
<td>309 ± 20†</td>
<td>8</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>154 ± 10*</td>
<td>358 ± 27†</td>
<td>7</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>151 ± 8*</td>
<td>354 ± 22†</td>
<td>8</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>112 ± 7</td>
<td>233 ± 10</td>
<td>8</td>
</tr>
<tr>
<td>MTX</td>
<td>143 ± 7*</td>
<td>305 ± 13</td>
<td>8</td>
</tr>
<tr>
<td>MTX + CY</td>
<td>149 ± 5*</td>
<td>311 ± 10*</td>
<td>7</td>
</tr>
<tr>
<td>MTX + SY</td>
<td>160 ± 8*</td>
<td>327 ± 21*</td>
<td>8</td>
</tr>
</tbody>
</table>

Crypt depth and villus height were measured in 10-20 well orientated sections in the duodenum, jejunum and ileum region from each animal. * Significantly greater than Control (p<0.05); † Significantly less than Control (p<0.002); γ Significantly greater than jejunum (p<0.05); δ Significantly greater than ileum (p<0.05). Values are expressed as Mean ± SEM.

Measure of mucosal immune activity

Mucosal mast cells were measured in the jejunum1/jejunum2 region and there was no significant difference between MTX + CY (57 ± 1 cells/mm), MTX + SY (54 ± 1 cells/mm) and MTX (50 ± 1 cells/mm).
Intestinal brush border enzyme activity

Rat sucrase activity in Control rats was similar in the duodenum and jejunum and significantly lower in the ileum (p<0.05) (Figure 3.6). On Day +7 sucrase activity was significantly diminished in all three sections of the intestine (p<0.005). MTX + SY significantly improved sucrase activity in the jejunum when compared to MTX rats (p<0.03). Figure 3.7 shows methotrexate treatment significantly decreased ALP activity in the intestine (p<0.005). Expression of ALP was found to decrease from the duodenum to the ileum (p<0.05). Lactase activity was significantly increased in the small intestine of all MTX treated groups compared to Control (p<0.05) (Figure 3.8).

**Figure 3.6** Small intestinal sucrase activity of control and methotrexate-treated rats.

Enzyme activity is expressed as Mean ± SEM μmol substrate hydrolysed at 37°C at pH 6.0/ mg protein/hour. * Sucrease activity was significantly less than Control rats (p<0.005); † MTX + SY sucrase activity was significantly greater than MTX rats (p<0.03)
**Figure 3.7** Small intestinal ALP activity in control and methotrexate-treated rats.

Enzyme activity is expressed as Mean ± SEM μmol substrate hydrolysed at 37°C at pH 9.2/ mg protein/ hour. * ALP activity was significantly less than Control rats (p<0.05); § Jejunal and ileal enzyme activity was significantly less than duodenum (p<0.05).

**Figure 3.8** Small intestinal lactase activity in control rats and methotrexate-treated rats.

Enzyme activity is expressed as Mean ± SEM μmol substrate hydrolysed at 37°C at pH 6.0/ mg protein/ hour. * Control rats were significantly less than other groups (p<0.05).
DISCUSSION

This study investigated small intestinal changes after administration of probiotics during MTX induced injury. The two yoghurts tested improved intestinal function after injury through different mechanisms. Sheep milk yoghurt (MTX + SY) supplementation resulted in a lower severity score in the duodenum. In the current study methotrexate induced damage in the small intestine, as measured by the histological severity scores and brush border enzymes, was consistent with previous studies (Taminiau et al. 1980; Vanderhoof et al. 1990; Erdman et al. 1991; Howarth et al. 1996).

The L/M excretion ratio showed that methotrexate administration damaged the small intestinal barrier function. Serial in vivo tests revealed that permeability in the MTX-treated rats was elevated on Day +3 and increased further on Day +6. This elevation was not seen with cow’s milk LA1 yoghurt group (MTX + CY) as the L/M excretion ratio was maintained throughout the trial. Treating MTX rats with sheep milk yoghurt (MTX + SY) resulted in a significantly lower L/M excretion ratio on Day +3 but not on Day +6. Probiotic LA1 yoghurt and sheep yoghurt improved permeability in the small intestine during methotrexate treatment but the $^{51}$Cr EDTA levels did not reflect this improvement.

$^{51}$Cr EDTA recovery was increased on Day +6 which suggested a reduction in the effectiveness of the intestinal barrier as $^{51}$Cr EDTA measures whole intestinal permeability. These results are in agreement with perfusion studies in MTX rats (Bjarnason et al. 1985; Mao et al. 1996). Though the L/M excretion ratio peaked on Day +6 in parallel with $^{51}$Cr EDTA recovery in the MTX group, L/M excretion ratio did not coincide with a peak in mannitol or lactulose.

There was a significant positive relationship between mannitol recovery and the daily measurements of body size, food and water intake, urine and faeces excreted. This implies the larger the rat, the more food and water ingested and urine and faeces excreted. Owl and Young (1992) showed body size was related to intestinal length and radius. Mannitol recovery was suggested to be related to surface area (Maxton et al. 1986; Andre et al. 1988; Meddings 1997). The relationship of mannitol with daily measurements may have reflected intestinal size and surface area. L/M excretion ratio, lactulose recovery and $^{51}$Cr
EDTA recovery did not show a relationship with any body measurement, further supporting their validity as good measures of intestinal barrier function.

L/M excretion ratio and lactulose recovery showed no relationship with $^{51}$Cr EDTA recovery. Lactulose and $^{51}$Cr EDTA recovery was found to differ in fed and fasted rats (Chapter 2) and there are two possibilities for the difference. Firstly, the site and rate of uptake may have been different for each probe. If the transport of $^{51}$Cr EDTA was similar to a macromolecular antigen then it would be affected by water fluxes. Bjarnason et al. (1985) suggested water and $^{51}$Cr EDTA share, at least partially, a common absorption pathway. It is possible that enhanced serosal to mucosal water flux could have accounted for the decreased uptake of $^{51}$Cr EDTA when given with other probes. The effect of water flux on these probes in a setting of intestinal damage is unclear although the permeability was exacerbated. Damage to the mucosal barrier would result in increased macromolecule uptake and increased access of bacterial and antigen.

Secondly, the difference in $^{51}$Cr EDTA recovery could have been due to changes in large intestinal permeability. Previous studies have observed that methotrexate-induced intestinal damage was concentrated in rapidly dividing cells of the jejunum (Taminiau et al. 1980; Vanderhoof et al. 1992; Howarth et al. 1996), whereas few studies have observed histological damage in the colon (Mao et al. 1996). Mao et al. (1996) found colonic permeability was elevated in probiotic fermented group compared to control rats but their results were less than half that of the methotrexate-treated group. A change in the $^{51}$Cr EDTA recovery was not detected in the yoghurt rats fed compared to MTX.

In the current study methotrexate reduced sucrase and ALP enzyme activity in the small intestine. ALP activity decreased longitudinally in the small intestine and this was consistent with Young et al. (1981) who found that both sucrase and ALP expression decreased longitudinally from the duodenum to the ileum. Decreased sucrase activity has been used as a reflection of damage in the small intestinal epithelium (Davidson et al. 1977; Langman and Rowland 1990; Schulman et al. 1991; Jourdan et al. 1998). The decrease in enzyme activity was not related to the changes in villus height. Taminiau et al. (1980) suggested methotrexate induced incomplete maturation of enterocytes and this was the reason for the reduction in villus height and sucrase activity on Day +6. The morphology and biochemical processes of villus cells differed regionally along the axis.
(Weiser 1973; Hoffman and Kuksis 1979). Sucrase-isomaltase enzymic activity is predominately found in mid and lower villus cells and is low in crypt cells (Hartmann et al. 1982; Traber 1990). ALP activity, unlike sucrase activity, decreased down the villus-crypt axis (Young et al. 1981; Benjawatanapon et al. 1982; Hartmann et al. 1982). Methotrexate’s effect on sucrase and alkaline phosphatase activity along the villus crypt axis is yet to be defined.

Sheep milk yoghurt treatment increased jejunal sucrase activity in methotrexate-treated rats but this increase in sucrase activity was slight compared control rats activity. Duodenal ALP activity was not enhanced by after sheep milk yoghurt. There was no difference in the brush border enzyme activity between the yoghurt treatments, though intestinal bacteria have been suggested to influence ALP activity (Kozakova et al. 2001). Vievin-Le Moal et al. (2002) found a diarrhoeagenic E. coli induced rearrangement of brush border associated structural and functional proteins and decreased sucrase and ALP expression by greater than 50%. L. acidophilus strain LB antagonised the E. coli associated cellular damage and prevented loss of structure and function (Lievin-Le Moal et al. 2002). The actions of the L. acidophilus maintained intestinal function in the presence pathogen, whether structure and function can be maintained in the presence of chemo therapeutic drugs is yet to be determined.

ALP expression after intestinal damage and the role that it plays in the recovery of the intestinal mucosa is unclear. ALP activity did not coincide with less damage or an improvement in intestinal function (Naruhashi et al. 2000; Nieto et al. 2002). ALP activity may not have correlated to intestinal damage as there are two isoenzymes of rat intestinal ALP (AP-I and AP-II) produced from two distinct genes (Engle and Alpers 1992, Xie, 2000 #482; Wada et al. 2001). The expression of AP-II on surfactant-like particles plays a role in fat absorption in the small intestine (Zhang et al. 1996). Xie and Alpers (2000) suggested intestinal AP-II was regulated at the transcription level. MTX affects DNA replication and may affect the transcription of brush border membrane enzymes. The brush border enzymes have been found to be replaced several times in the life of an enterocyte (James et al. 1971; Alpers and Tedesco 1975). MTX may thus influence the ability of the rat intestines to replace or the synthesis of these complex glycoproteins.
In this study lactase activity was increased after methotrexate treatment. Adult rats have low lactase activity and the enzymes expression decreased in the intestine was correlated with weaning (Cummins et al. 1989b; Freund et al. 1990; Duluc et al. 1991; Thompson et al. 1996; Lee et al. 1997; Jang et al. 2000). It is unusual to see lactase expression increase with intestinal damage as lactase activity in adult humans is decreased in mild and severe duodenal lesions (Langman and Rowland 1990). Alternatively, lactase activity was suggested to be related to dietary intake or fasting (Holt and Yeh 1992; Samulitis-Dos Santos et al. 1992). Methotrexate-induced starvation is a possible cause for increased lactase activity, though unlikely to be the sole reason as the control rats were pair fed. Lactase activity was also stimulated in the yoghurt groups, but interestingly, not at all sites in the small intestine. Lactase activity was higher in rats fed sheep yoghurt in the proximal small intestine and LA1 rats had elevated lactase activity in the jejunum.

There are several other possibilities for the increase in lactase activity. Firstly, the lactose levels in milk are less likely to cause an increase in lactase activity. Lactose levels are low in yoghurts as lactobacilli ferment lactose. Yoghurt was unlikely to remain in the intestine as it was flushed with a saline solution before sections were taken for enzyme activity. Secondly, a diet high in medium chain fats (MCT) has been found to affect gene expression and post-translational events of the lactose-phlorizin hydrolase (Goda et al. 1995). Sheep milk yoghurt had a larger proportion of MCT than cow’s milk, and may have contributed to the increase in lactase activity (Appendix I). Goda et al. (1995) investigated enzyme activity in healthy rats and did not measure the enzyme activity in damage or repair. Thirdly, the lactase activity of adhered bacteria could have partly contributed. The activity of the lactobacilli could not have been large as methotrexate-treated rats not fed yoghurt had elevated lactase activity. The measurement of sucrase, lactase and ALP mRNA and protein may have determined whether MTX affects brush border membrane enzyme expression post MTX injury, and the involvement of these enzymes in intestinal recovery.

In the current study morphometric results showed the villus height in the ileum of all MTX treated rats was greater than controls. Increases in crypt depth have been measured in several MTX studies as a sign of rapid proliferation on Day 6 (Taminiau et al. 1980; Bjarnason et al. 1985b). In this study the villus height did not coincide with improvements in enzyme activity, histological severity score or permeability tests. In the ileum both
yoghurts increased villus height greater compared to controls and MTX rats. The histological severity score did not change with the increase in villus height in the ileum. These two yoghurts may have acted by a compensation mechanism trying to maximise function after the proximal MTX damage. Topping (1996) suggested the presence of probiotics in the intestine increased short chain fatty acid (SCFA) levels. SCFA are known stimulators of proliferation and as the bacterial population in the ileum is greater than the duodenum, it is possible the probiotics indirectly stimulated cell proliferation. Proliferation is a part of recovery after MTX and would explain the probiotic groups increase in crypt depth and villus height.

Probiotics may have affected intestinal function in another manner. Previous studies in rats and humans have found *S. boulardii* exerts trophic effects on the intestine (Buts et al. 1986). These consisted of increases in sucrase and lactase brush border membrane enzyme activities and endoluminal secretion of IgA (Buts et al. 1990; Buts et al. 1999). *S. boulardii* that secrete polyamines, have been found to modulate mucosal healing post surgery (Buts et al. 1999). Polyamines derived from microflora have also been suggested to enhance the recovery of enzyme expression after injury (Buts et al. 1999).

In summary, probiotic yoghurt LA1 improved intestinal function primarily by maintaining small intestinal permeability and increasing the brush border enzymes after MTX injury. Sheep milk yoghurt improved proximal small intestinal function and reduced the severity of damage in the duodenum. This effect could have been due to the sheep milk or the bacteria but since sheep milk or bacterial controls were not used it is only speculation. The combination of sheep milk fermented by these bacteria was more likely to result in intestinal changes than the individual elements. These two yoghurts both improved the intestinal function after injury. The specific actions of yoghurts in recovery from intestinal injury vary from species to species suggesting yoghurt can induce functional changes at the cellular level in the small intestine.
Chapter 4 SMALL INTESTINAL PERMEABILITY IN PAEDIATRIC INFLAMMATORY BOWEL DISEASE PATIENTS

INTRODUCTION

About 25% of cases of IBD specifically Crohn’s disease (CD) and ulcerative colitis (UC), are diagnosed in childhood and adolescence (Oliva-Hemker and Fiocchi 2002). CD is a chronic inflammatory process that has clinical and pathological manifestations in any part of the alimentary tract from the mouth to the anus, but inflammation is mainly found in the ileum and colon (Donaldson 1989; Gasche et al. 2000; Ahmad et al. 2002). UC causes inflammation of the inner lining of the large bowel and may extend to the caecum (Cello and Schneiderman 1989; Gibson and Pavli 1992). The aetiology and pathogenesis for both is yet unknown; however it is generally agreed that an inappropriate immune response or breakdown in normal immune regulation contributes significantly to the pathogenesis of the disease (Gibson and Pavli 1992; Pavli and Gibson 1992; Choi and Tarrgo 1994; Grimm et al. 1995; Oliva-Hemker and Fiocchi 2002).

The clinical assessment of disease activity and response to therapy may be difficult and inaccurate. A number of activity indices have been derived and validated, usually in adult populations (Truelove and Witts 1955; Best et al. 1976; Harvey and Bradshaw 1980; Lichtiger and Present 1990). Their application to childhood disease may however be limited (Lloyd-Still and Green 1979; Hyams et al. 1991; Escher et al. 2002). Conventional laboratory markers for inflammation such as erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) may reflect disease activity, but do not assess intestinal function. A simple, non-invasive, and reproducible laboratory test providing an objective measure of intestinal function would be valuable in children with IBD to assess of disease activity, and their response to drug therapy.

Intestinal permeability measures an important aspect of mucosal barrier function in the intestine (Hollander 1988; Travis 1992; Bjarnason 1995). The dual sugar test is a simple, non-invasive, and reproducible index of intestinal permeability in children (Van Elburg et
Intestinal permeability is elevated in patients with active CD (Pearson et al. 1982; Sanderson et al. 1987; Andre et al. 1988; Murphy et al. 1989; Meddings 1997). Adult CD urinary excretion ratio was found to be higher than controls and differ with disease activity (Pounder et al. 1983; Andre et al. 1988). Murphy et al. (1989) found permeability tests useful in screening for patients with non-specific symptoms and assessment of the extent of disease activity in Crohn’s disease patients. Lactulose recovery and lactulose/mannitol excretion ratio correlated with the activity index (Murphy et al. 1989). Both the Paediatric Crohn’s Disease Activity Index (PCDAI) and Crohn’s Disease Activity Index are useful tools for clinicians and are widely used in the reporting of CD activity (Escher et al. 2002; Su et al. 2002). The application of the intestinal permeability test to the assessment of disease activity, disease extent, response to therapy, and early detection of relapse in paediatric patients with CD would be an advantage in treatment (Sanderson et al. 1987; Murphy et al. 1989).

There have been several other studies of sugar permeability in UC, but results have been variable and inconsistent (Ukabam et al. 1983; Andre et al. 1990; Howden et al. 1991; D’Inca et al. 1992; Peeters et al. 1995). These studies all had different permeability protocols, probe concentrations, subject numbers, variable disease activity level and site of involvement making comparisons difficult.

The aim was to assess the utility of intestinal permeability as a non-invasive marker of disease activity and disease extent in paediatric IBD. This was a means of monitoring the response to therapy and predicting disease relapse.

**METHODS**

**Patients and Methods**

Patients with both new and established diagnoses of IBD (17 CD, 13 male and four female patients mean age 14.2 years (range 9.9 to 18.9 years); 19 UC, seven male and 12 female patients, mean age 11.9 years (range 4.5 to 16.5 years)) were recruited from the Women’s
and Children’s Hospital (WCH) Adelaide, SA. The diagnosis of IBD was based on clinical, radiologic, endoscopic, and histological criteria (Schachter and Kirsner 1975). Principal sites of disease in the patients with CD consisted of orofacial granulomatosis (two), oesophageal disease (two), gastroduodenal (three), ileal (two), ileocolonic (three) and colon (five). Patients with UC were either diagnosed with extensive colitis (n=12), (macroscopic or histological activity extending proximal to the splenic flexure) and left-sided colitis, distal to the splenic flexure (seven). No patients had undergone surgical intervention before or during the study protocol.

The patients with CD were divided into active (at diagnosis or in relapse) and inactive disease (in remission) according to the PCDAI and the Harvey-Bradshaw (HB) (Harvey and Bradshaw 1980; Hyams et al. 1991). Patients with PCDAI ≤ ten were considered to have inactive disease, 11-30 mild disease, and a score of 31 or greater for moderate to severe disease. Patients with UC were divided into inactive (zero), mild (one), moderate (two) or severe (three) colitis according to clinical symptoms and signs, laboratory investigations, and radiological findings referring to Truelove and Witt criteria for the severity of UC (Truelove and Witts 1955). Patients were further subdivided into extensive UC and left-sided (distal) colitis depending on colonoscopic examination and whether the disease was extended proximally or distally to splenic flexure. UC remission was defined as the absence of diarrhoea, bleeding, and abdominal pain with no systemic symptoms and no laboratory abnormalities.

Intestinal permeability was performed in patients at diagnosis, after treatment, in relapse or remission. Other laboratory investigations including complete blood counts, CRP, ESR, and serum albumin were examined at the time of the permeability test. This study was approved by the Research Ethics Committee at the WCH. Informed written consent was obtained from the patients parents or caregivers or patients themselves if aged ten years old.

*Intestinal permeability test*

After an overnight fast, a pre-test urine sample was collected. The subjects then drank the test sugar solution, containing 5 g of lactulose, 1 g of L-rhamnose, and 1 g of mannitol in
100 mL of water (isotonic solution molarity = 330 mOsm/L). After a further half an hour, a liberal intake of water was permitted to increase urine flow. Food intake was allowed after the first three hours. Urine was collected for a total of five hours and stored in a container containing 0.1 mL of 10 g/L Thiomersal as preservative. If the subjects had not voided within the last half an hour of the 5 hours, they were instructed to collect one more void. The total volume was recorded and a 20 mL aliquot was stored at -20°C until analysis.

Sample preparation

De-salting of urine required 0.5 g of washed, mixed ion-exchange resin (Duolite MB 5113; BDH Chemical, Poole, UK) to be added to 2 mL of the thawed urine specimen. The mixture was vortex-mixed for 10 s, and centrifuged for 10 min at 3000 x g. The resultant supernatant was filtered through 0.2 μm (pore-size) disposable syringe filters (Acrodisc™; Gelman Sciences, Ann Arbor, MI, USA).

HPLC analysis

An aliquot of the filtered supernate was injected into the manual injector (Model 7125; Rheodyne, Cotati, CA, USA) with 10-μL loop. The HPLC was equipped with an isocratic pump (Model SP 8810; Spectra Physics, San Jose, CA, USA), a polymeric guard column (Direct-Connect™ Cartridge Guard Column; Alltech, Deerfield, IL, USA) and an amine-modified silica column (Kromasil™ NH2 Column, 5 μm particle size, 250 x 4.6 mm; Alltech, Deerfield, IL, USA) used at ambient temperature. The mobile phase was degassed, acetonitrile (ACN) into distilled-deionized water (70/30 vol), prior to running through the column at a flow rate of 1 mL/min. Detection was by a refractive index (RI) detector (LC 1240 R.I. Detector; GBC Scientific Equipment, VIC, Australia) and was recorded on a linear chart recorder (Linear 1200; Alltech Associates, Deerfield, IL, USA). Standard curves were prepared by analysing appropriate concentrations of each compound in distilled water and plotting the peak-height obtained at 1.0 x 10⁻³ R.I. units/FS sensitivity of the detector. Urinary concentrations of sugar probes were calculated from the standard curves using peak-height analysis. For each subject, total urinary excretion was calculated for each sugar, and results were expressed as the percentage of the ingested dose present in urine and as the excretion ratios, lactulose/L-rhamnose and lactulose/mannitol.
Reference range for normal permeability responses

The reference range for normal permeability results was determined previously (Miki et al. 1998) from studying 36 healthy children and adolescents (18 male, 18 female), ranging in age from 5 to 17 years (median ± SD, 14.39 ± 3.61 years), with no significant medical histories or gastrointestinal symptoms for ≥ 2 weeks. The reference range expressed as Mean ± 2 SD value of the subjects’ sugar recoveries and sugar ratios. These were L-rhamnose 2.7 - 10.9%, mannitol 4.9 - 20.2%, lactulose 0.06 - 0.67%, L/rh excretion ratio 0.0138 - 0.0738 and for L/M excretion ratio 0.065 - 0.0362.

Statistics

The frequency of elevated intestinal permeability ratio between active disease and remission in CD, extensive UC and left sided UC was compared with a \( \chi^2 \) test. The analysis of Spearman’s correlation coefficient by rank was used to compare the permeability ratio with activity index and other laboratory investigations where \( p < 0.05 \) and the correlation coefficient=0.7 were considered significant (Miki et al. 1998). One way ANOVA was used to compare the intestinal permeability results and blood test results between control subjects and active and remission in CD and extensive UC patients. When significance was identified a Tukey test was used for multiple comparisons and \( p < 0.05 \) was significant.

RESULTS

Disease activity index in patients

Patients were separated into subgroups determined by their disease activity. The disease activity in the patients was determined by a gastroenterologist and the time of the permeability test and displayed in Table 4.1. Several subjects were diagnosed before the trial, but have had several permeability tests over time of this study. Remission varied
from three to seven months after diagnosis. In the relapse group, patients are only included if the gastroenterologist indicated a relapse had occurred and the patient met the PCDAI criteria for disease activity. CD patients at diagnosis had high scores for the PCDAI and HB. These scores reduced after treatment and when patients reached remission. When patients were clinically assessed as relapsed their PCDAI scores were higher than the after treatment and when in remission. This was not the case for HB as the scores for relapse and after treatment was similar. The disease severity scores (Truelove and Witts 1955) were used to measure activity in UC patients. Extensive colitis had a higher average score at diagnosis. No distal colitis patients were scored at diagnosis and their relapse scores were higher than at other observed times.

Table 4.1 Disease activity in paediatric inflammatory bowel disease patients.

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis</th>
<th>After treatment</th>
<th>Remission</th>
<th>Relapse</th>
<th>Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crohn’s disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of patients</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>PCDAI *</td>
<td>43 ± 3.8</td>
<td>12.7 ± 2.9</td>
<td>4.7 ± 1.6</td>
<td>22.8 ± 5.2</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>range</td>
<td>(32.5–55)</td>
<td>(5–32.5)</td>
<td>(0–10)</td>
<td>(10–52.5)</td>
<td>(0–5)</td>
</tr>
<tr>
<td>HB modified *</td>
<td>6.2 ± 0.5</td>
<td>3.1 ± 0.6</td>
<td>1 ± 0.3</td>
<td>3.2 ± 1.1</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>range</td>
<td>(5–8)</td>
<td>(1–6)</td>
<td>(0–2)</td>
<td>(0–10)</td>
<td>(0–5)</td>
</tr>
<tr>
<td><strong>Extensive colitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of patients</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Disease severity</td>
<td>1.6</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>range</td>
<td>(1–2)</td>
<td>(0–1)</td>
<td>(0–1)</td>
<td>(0–1)</td>
<td>(0)</td>
</tr>
<tr>
<td><strong>Distal colitis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of patients</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Disease severity</td>
<td>0.3</td>
<td>0.6</td>
<td>1.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>(0–1)</td>
<td>(0–1)</td>
<td>(1–1.5)</td>
<td>(0–1)</td>
<td></td>
</tr>
</tbody>
</table>

*PCDAI (range 0–60) and the modified *Harvey-Bradshaw (HB) (range 0–25) were used to measure the cyclical disease progression over time in Crohn’s disease patients (Harvey and Bradshaw 1980; Hyams et al. 1991).  

The disease severity score was used for ulcerative colitis patients, with activity values remission=0, mild activity=1, Moderate activity=2 and severe activity=3 (Truelove and Witts 1955). Not all patients are presented in all groups.
Intestinal permeability in patients with active disease and remission

The L/rh excretion ratio was significantly increased in eleven of twelve patients with active CD irrespective of the site of their disease (p<0.001) (Figure 4.1). The patient whose L/rh excretion ratio was not increased had no small intestinal involvement and only left-sided colon lesions, predominantly sigmoid colon. A significantly greater number of CD patients had elevated permeability in the active state than in remission (p<0.001). Two CD patients in remission had elevated L/rh excretion ratios of 0.1221 and 0.126, the former had reduced L/rh excretion ratio from diagnosis by 17%. The latter although in remission at the time of the permeability testing, relapsed soon after.

For individuals with extensive UC, the L/rh excretion ratio was increased in eight of nine patients with active disease, whereas L/rh excretion ratio was not increased in ten UC patients in remission. A significant greater number of extensive UC patients were found to have elevated permeability in the active state than in remission (p<0.001). One patient with a low L/rh excretion ratio (0.0245) was measured at the time of diagnosis and had evidence of pancolitis and large intestinal involvement. Two UC patients in remission had elevated L/rh excretion ratios of 0.1258 and 0.1066, had reduced L/rh excretion ratio by 50% from diagnosis and relapse respectively. Only two of nine patients with active left sided UC showed an increased L/rh excretion ratio. These patients with an elevated L/rh excretion ratio were diagnosed with sigmoiditis and with microscopic inflammation of the duodenum present at the time of diagnosis (0.105) or distal relapse (0.161). One left sided UC patient in remission had an elevated L/rh excretion ratio (0.1091), this test was after liver transplant surgery.

The L/rh excretion ratio did not correlate with the PCDAI, ESR, CRP, hematocrit or serum albumin in CD or extensive UC patients with active disease or in remission.
Figure 4.1 Small intestinal permeability in IBD patients with active disease and remission.

* Significantly greater number of CD patients with elevated permeability in the active state than in remission (p<0.001). † Significantly greater number of extensive UC patients with elevated permeability in the active state than in remission (p<0.001). The active disease groups had patients permeability results from tests conducted at diagnosis and whilst in relapse (CD n=10, extensive UC n=9, left sided (distal) UC n=7). Remission groups represents patients results from when their disease state was considered quiescent or mild in activity by clinicians where PCDAI was <10 and disease severity score was 0-1 (CD n=13, extensive UC n=9, left sided UC n=5). The line displays the upper limit of the normal permeability range (0.0138 - 0.0738). Values displayed are patients’ individual results at different time points.
Chronological intestinal permeability results in IBD patients

The L/rh excretion ratio was elevated in CD patients at time of diagnosis (n=4) and when in relapse (n=9). The CD patients L/rh excretion ratio’s improved after initial treatment (Figure 4.2A). The L/M excretion ratio was also elevated at diagnosis and in relapse and within the normal range after treatment and in remission (Figure 4.2B). Seven ulcerative colitis patients were measured at diagnosis with elevated permeability, only four patients permeability was measured after prednisolone treatment. Three of the four patients with extensive UC showed normalisation of L/rh excretion ratio after their initial course of prednisolone. One patient was classed in remission and had an elevated L/rh excretion ratio (Figure 4.2A). This patient had a disease relapse within six months after cessation of prednisolone. The L/M permeability ratio in extensive UC was also elevated at diagnosis and in relapse and within the normal range after treatment and in remission (Figure 4.2B).

Permeability test and blood test results in patients

The L/Rh and L/M excretion ratios in CD and extensive UC active patients were significantly higher than controls (p<0.002) (Table 4.2). Urinary lactulose recovery was greater in active CD patients than controls (p<0.001). Active CD patients’ lactulose recovery, L/Rh and L/M excretion ratio’s were significantly greater than when CD patients where in remission (p<0.05). Extensive UC remission patients permeability ratios were significantly less than active extensive UC patients (p<0.05) and were not significantly different from controls. CD patients had a significantly higher ESR in the active state compared to remission CD patients (p<0.05). Active CD patients had significantly higher CRP concentration than UC patients (p<0.05). Blood tests were not conducted at all permeability time points as a result the number of patients in the active state differed for CD and UC groups.
Figure 4.2 Intestinal Permeability in IBD patients.

A. L/Rh excretion ratio in IBD patients

B. L/M excretion ratio in IBD patients

Values displayed are patients' individual results.
Table 4.2 Permeability and blood test results in paediatric inflammatory bowel disease patients

<table>
<thead>
<tr>
<th>Permeability results</th>
<th>Control</th>
<th>CD Active</th>
<th>CD Remission</th>
<th>Extensive UC Active</th>
<th>Extensive UC Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>n=36</td>
<td>n=11</td>
<td>n=14</td>
<td>n=10</td>
<td>n=11</td>
</tr>
<tr>
<td>L-rh recovery (%)</td>
<td>6.99 ± 0.35</td>
<td>6.35 ± 0.84</td>
<td>7.04 ± 0.85</td>
<td>5.05 ± 0.79</td>
<td>5.09 ± 0.80</td>
</tr>
<tr>
<td>M recovery (%)</td>
<td>15.91 ± 0.82</td>
<td>18.31 ± 0.90</td>
<td>18.56 ± 1.27</td>
<td>12.25 ± 0.86</td>
<td>14.45 ± 1.13</td>
</tr>
<tr>
<td>L recovery (%)</td>
<td>0.27 ± 0.02</td>
<td>1.027 ± 0.279 δ</td>
<td>0.500 ± 0.082 §</td>
<td>0.652 ± 0.196 δ</td>
<td>0.255 ± 0.053</td>
</tr>
<tr>
<td>L/rh ratio</td>
<td>0.0396 ± 0.0028</td>
<td>0.149 ± 0.025 δ</td>
<td>0.0744 ± 0.0090 §</td>
<td>0.1343 ± 0.0246 δ</td>
<td>0.0487 ± 0.0095 §</td>
</tr>
<tr>
<td>L/M ratio</td>
<td>0.0175 ± 0.0013</td>
<td>0.0581 ± 0.0142 δ</td>
<td>0.0299 ± 0.0031 §</td>
<td>0.0518 ± 0.0140 δ</td>
<td>0.0175 ± 0.0031 §</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood concentrations</th>
<th>Normal range</th>
<th>n=11</th>
<th>n=13</th>
<th>n=9</th>
<th>n=9</th>
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</thead>
<tbody>
<tr>
<td>Haemoglobin (g/L)</td>
<td>12-17 g/L</td>
<td>12.75 ± 0.31</td>
<td>13.66 ± 0.26</td>
<td>12.23 ± 0.55</td>
<td>12.31 ± 0.47</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>&lt;20 mm/hr*</td>
<td>20.63 ± 3.80</td>
<td>9.01 ± 2.34 §</td>
<td>17.77 ± 3.65</td>
<td>5.83 ± 1.72</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt;5 mg/L</td>
<td>42.75 ± 11.23 γ</td>
<td>17.27 ± 7.61</td>
<td>6.20 ± 4.75</td>
<td>0.45 ± 0.45</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>30-50 g/L †</td>
<td>37.18 ± 2.10</td>
<td>41.32 ± 1.70</td>
<td>38.11 ± 1.72</td>
<td>44.92 ± 0.69</td>
</tr>
<tr>
<td>Hematocrit (g/L)</td>
<td>0.37-0.44</td>
<td>0.379 ± 0.012</td>
<td>0.391 ± 0.011</td>
<td>0.360 ± 0.014</td>
<td>0.372 ± 0.014</td>
</tr>
</tbody>
</table>

Patients' permeability results were compared to control permeability results used for laboratory reference. There are no blood results for the control subjects so Normal range for inflammatory activity measures as used by Chemical Pathology Department, WCH and references *Pirioni et al. (1990) and †Teahon et al. (1991). § Remission significantly lower than active where p<0.05. γ CD was significantly greater than UC in the active state where p<0.05. δ CD and extensive UC were significantly greater than control where p<0.05. Data are expressed as Mean ± SEM.
DISCUSSION

The dual sugar permeability test detected elevated intestinal permeability in children and adolescents with active CD. The majority of patients had elevated intestinal permeability which result may reflect the diffuse disease process in patients with active CD (Meddings 1997; Ahmad et al. 2002). Intestinal permeability varied with disease activity, where it was higher at diagnosis and relapse than in remission. Treatments regimes reduced intestinal permeability levels, but the permeability results in remission are still elevated compared to healthy controls. This possibly reflects the fact that although patients with clinical remission are probably not in histological remission. Alternatively, disease at other sites of the gastrointestinal tract may evoke subtle changes in an otherwise normal small intestine. This non-invasive intestinal permeability test cannot distinguish between a few sites of intense inflammation versus a large area of diffuse inflammation as it is a measurement of the small intestinal barrier function as a whole.

The non-invasive measurement of intestinal permeability has been suggested for a diagnostic screening test and as a marker of disease activity in children and adolescents with CD (Pearson et al. 1982; Sanderson et al. 1987; Murphy et al. 1989; Meddings 1997). To date there are over five different sugar combinations that have been used to measure small intestinal permeability in CD patients (Table 1.2). All these studies have shown CD patients in the active disease state with elevated lactulose recovery and L/Rh or L/M excretion ratio (Ukab et al. 1983; Peeters et al. 1985; Sanderson et al. 1987; Andre et al. 1988; Murphy et al. 1989; Andre et al. 1990; Howden et al. 1991; D’Inca et al. 1992). The recovery of large permeability markers (macromolecules and protein markers) are also elevated in CD patients (Katz et al. 1989; Malin et al. 1996a; Soderholm et al. 1999). These studies suggest that the intestinal permeability is increased for both small and large molecules and this may be an etiologic factor in patients with CD (Hollander 1988; Olaison 1990).

Conflict exists as to whether active UC have elevated intestinal permeability. Some studies have not shown increased permeability in UC (Andre et al. 1990; Howden et al. 1991; Issenman et al. 1993; Peeters et al. 1995). In this study intestinal permeability was increased in active extensive UC but not when the patients were in remission. Not all
extensive ulcerative colitis patients in this study had elevated lactulose recovery and this was also similar to CD patients’ results. This study showed the site of disease and disease activity affects UC patients intestinal integrity and this could explain the conflict in permeability results from previous studies (Ukabam et al. 1983; Andre et al. 1990; Howden et al. 1991). Increased intestinal permeability observed in active extensive UC patients may be due to the leakiness of the small intestine or morphological changes in the intestinal mucosa similar to that seen in patients with active CD (Travis and Menzies 1992; Bjarnason et al. 1995).

Hollander (1988) hypothesised that CD may be a disorder of the tight junctions between enterocytes. Morphological investigations using electron microscopy have shown abnormalities in mucosal junctions (Dvorak and Dickerson 1979; Marin et al. 1983; Dvorak et al. 1989). Increased separation between cells and abnormalities in the structural strands of the tight junctions were observed in non-inflamed tissue from CD patients (Dvorak and Dickerson 1979; Marin et al. 1983; Dvorak et al. 1989). Tight junctions in inflamed colonic tissue from UC patients were structurally altered in the surface and crypt regions (Schmitz et al. 1999). The specimens from controls had two more horizontal strands in the tight junctions on average than UC patients. The structural changes in the UC patients’ tissue were related to functional changes (Schmitz et al. 1999; Schmitz et al. 2000). Functional changes observed in the mucosa of CD patients were found to be more reactive to luminal stimuli than controls (Soderholm et al. 2002a). This tight junction vulnerability could be a secondary response to inflammation (Soderholm et al. 2002a).

Hollander et al. (1988) suggested that intestinal permeability seen in patients with CD were not secondary to inflammation and could explain the familial aggregation of the disease.

Whether there is a familial abnormality with tight junctions or abnormal barrier function in IBD relatives has been of interest to several groups. To date results in the relatives of IBD patients have shown permeability differential depending on the test probe used (Hollander et al. 1986; Katz et al. 1989; Teahon et al. 1992; Hollander 1993; May et al. 1993; Peeters et al. 1995; Thjodleifsson et al. 1998; Breslin et al. 2001; Katz et al. 2001; Secondulfo et al. 2001). Clinically healthy relatives when compared to non-related controls were found to have increased intestinal permeability to PEG 400 (Hollander et al. 1986), L/M permeability (Peeters et al. 1995) but there was no specific genetic pattern. Thjodleifsson et al. (1998) measured permeability and inflammation and found that 12% of first degree
relatives of Icelandic CD patients had increased intestinal permeability. Calprotectin was used as a quantitative index of intestinal inflammation and 41% of relatives had evidence of sub-clinical intestinal inflammation. Thjodleifsson et al. (1998) concluded the pattern of inflammation to be compatible with an autosomal dominant mode of inheritance. The incidence of subclinical inflammation was greater in relatives of CD patients than the general population (Katz et al. 2001). The relationship between incidence of CD and increased intestinal permeability or inflammation is yet to be determined.

Increased intestinal permeability in CD patients and relatives may result from environmental factors or an inherited mucosal immune defect, as IBD patients share both genetic and environmental factors with their affected and unaffected siblings (Peeters et al. 1995; Thjodleifsson et al. 1998; Montgomery et al. 2001; Ahmad et al. 2002). Studies with multiple IBD affected families have up to 80% concordance between siblings (Ahmad et al. 2002). Genetic studies have shown that mutations in the NOD2 gene increased IBD susceptibility (Adam et al. 2002; Esters et al. 2002a; Esters et al. 2002b; Silverberg et al. 2002; Sugimura et al. 2002; Van Heel et al. 2002; Vermeire et al. 2002). The NOD2 gene encodes a protein that recognises intracellular bacterial components (Van Heel et al. 2002). A defect in this gene may contribute to an altered immune response to a bacterial and antigen challenge. The integrity of the intestinal barrier is important as this protects the GALT and immune cells from direct contact with luminal contents. An alteration in the integrity or intestinal permeability may increase the probability for a toxin or antigen to cross the intestinal barrier and promote an inflammatory cascade. That cascade would allow the intestine to be directly affected by pro-inflammatory mediators such as cytokines, reactive oxygen metabolites, nitric oxide, eicosanoids and culminated in endotoxin hyperreactivity. Genetic data may give insight into the heterogeneity of IBD and the interaction of intestines integrity and mucosal immune defects.

The results suggest that increased intestinal permeability ratio reflects active disease in both CD and extensive UC and disease extent in patients with active UC. The L/rh excretion ratio did not correlate with the PCDAI, ESR, CRP, hematocrit, or serum albumin in active CD or active extensive UC. Intestinal permeability probably reflects an index of mucosal leakiness or microscopic damage and not severity or extent of inflammatory changes. In some patients with CD and extensive UC, after their acute phase treatments were complete, serial permeability tests showed a decrease in permeability ratios.
However, in two patients with CD, two patients with extensive UC, and one with left sided colitis increased permeability ratios were still present despite apparent quiescent disease. The ability of intestinal permeability test to monitor disease must be confirmed by increasing the number of patients in each group and longitudinal follow-up with serial permeability tests, although the value of intestinal permeability ratios as a useful prognostic tool in CD has been suggested by Wyatt et al. (1993). The measurements of intestinal permeability in paediatric IBD, a marker of disease activity in CD and extensive UC, may be useful for monitoring the disease and predicting disease relapse in CD and extensive UC.
Chapter 5  

INFLAMMATORY BOWEL DISEASE: EFFECTS OF SHORT-TERM PROBIOTIC ADMINISTRATION.

INTRODUCTION

The pathogenesis of inflammatory bowel (IBD) disease remains poorly understood. Current theories have suggested that inflammatory bowel disease occurs as a consequence of an interaction between environmental factors and genetically determined factors (Gibson and Pavli 1992; Peeters et al. 1995; Thjodleifsson et al. 1998; Montgomery et al. 2001; Ahmad et al. 2002; Oliva-Hemker and Fiocchi 2002). There is a body of evidence supporting a necessary rate for environmental factors in both Crohn’s disease (CD) and ulcerative colitis (UC) (Blaser 1997; Sartor, 1997; Shanahan 2000). No specific microbe has yet been implicated in the pathogenesis of IBD. The components of the luminal stream, particularly the microflora, are involved in the aetiology and progression of IBD, as patients often show partial response to antibiotic agents (Sartor 1997; Sutherland 2000a; Colombel et al. 2001; Goodgame et al. 2001; Holtmann et al. 2001; Linskens et al. 2001). Susceptibility of IBD patients to luminal bacteria-induced injury could result from deficiencies in mucosal barrier function, epithelial restitution, immune responsiveness, or as a result of a genetic predisposition (Duchmann et al. 1995; Blaser 1997; Sartor 1997; Oliva-Hemker and Fiocchi 2002).

In Chapter 4, IBD patients’ intestinal permeability was shown to be dependant on disease activity. Even in remission, the intestinal permeability of CD patients was significantly higher than controls. An improvement in abnormal mucosal barrier function may be advantageous for these patients. A diet supplemented with yoghurt decreased small intestinal permeability in healthy subjects (Southcott et al. 1997). It is not know whether probiotics could affect passive permeability in IBD patients but Malin et al. (1996) showed improvement in intestinal barrier function with Lactobacillus rhamnosus strain GG (LGG).

Recent clinical studies have trialled the use of antibiotics and probiotics as treatments to improve the health of IBD patients (Kruis et al. 1997; Gionchetti et al. 1998; Rembacken et al. 1999; Venturi et al. 1999; Linskens et al. 2001). These studies found probiotic
treatment altered resident microflora, however they did not measure intestinal permeability.

Colonic bacteria in IBD patients differ from healthy controls and also differ with disease activity (Gaiffer et al. 1991). IBD treatments that aim to modify the intestinal flora balance will transform the activity and profile of metabolic products. The administration of probiotics may alter the faecal SCFA concentrations in IBD patients toward a profile characteristic of healthy individuals. This raises the question as to whether the administration of a probiotic would alter the bacterial balance and metabolic activity, and improve the integrity of the small bowel as measured by intestinal permeability.

This study aimed to assess changes in faecal SCFA and small intestinal permeability after administration of probiotic yoghurt for a week in paediatric patients with inflammatory bowel disease and in healthy children.

**METHODS**

**Subjects**

The study recruited twenty healthy children (16.33 ± 0.25 years; seven male, 13 female), with no history of gastrointestinal problems. Subjects were enrolled only after informed consent was obtained by a parent or legal guardian. IBD patients were recruited from the Centre for Paediatric and Adolescent Gastroenterology (CPAG), Women's and Children's Hospital. These patients were in remission and aged between five and eighteen. The patient's diagnosis of IBD was based upon clinical, radiological, histological, endoscopic and other markers of disease activity (Schachter and Kirsner 1975). Sixteen patients (13.16 ± 0.91 years; nine male, seven female) with parental written consent were enrolled. All patients maintained their prescribed medication dosage, start and stop date were also recorded. A summary of medication and site of disease at diagnosis is shown in Table 5.1.
The research protocol was approved by the Human Research Ethics committee of the Women’s and Children’s Hospital (WCH), Adelaide, South Australia.

**Experimental Procedure**

Subjects were required to collect faecal and urine samples before and after a short-term yoghurt supplemented diet. Baseline faecal samples were collected within two days before the first permeability test. Samples were collected without being contaminated with water, placed in biohazard bags and frozen immediately. The day before each permeability test, a low residue diet was recommended from 8 am to 12 am and subjects fasted from midnight. At the beginning of the procedure subjects were required to empty their bladder and collect a pre-test urine sample in a 30 mL sterile tube with Thiomersal added as preservative. The pre-test sample was used to check if any materials may interfere with the peaks of the probe sugars in the HPLC assay. The time was noted (t=0) when subjects drank the 100 mL test solution containing 6.68 g lactulose and 1 g of L-rhamnose in water. Subjects refrained from drinking water for the first 30 min after consuming the sugar solution. Following this initial 30 min, subjects were encouraged to drink water as required for the next 2 ½ h. After three hours normal eating and drinking were allowed. All urine was collected over five hours post sugar challenge in a large container with 0.1 mL of Thiomersal. All urine samples were kept frozen until analysis.

Subjects were then provided with 14 x 150 g containers of flavoured commercial yoghurt containing the bacteria *Lactobacillus johnsonii* (LA1) and were required to eat two containers a day for a week. Probiotic yoghurt ingestion started on the afternoon of the first test session. Subjects were required to follow the low residue diet and have two tubs of yoghurt on the 7th Day. On the second test day (8th day) subjects completed another permeability test and collected faecal samples on that day or the following day.

**Intestinal Permeability**

See Chapter 4 for details of the analysis of the intestinal permeability test.
**Faecal SCFA analysis**

Faecal samples were assayed for the SCFA acetate, butyrate, propionate and iso-valerate using gas chromatography. Each sample (1-1.6 g) was homogenized in 3 mL of water and 20 μL of 10mM methyl valeric acid was used as an internal standard. A 0.2 mL aliquot was centrifuged and the supernatant collected, 0.1 μL of the supernatant was injected onto a BP21 column. The column was programmed from 100°C to 160°C with increased temperature of 12 °C/min. The eluted peaks were identified against pure standards. The concentrations of individual SCFA were determined by comparing their peak area with those of the standard solution, taking into account the peak area of the internal standard.

**Statistics**

Means and standards errors of the mean (SEM) were calculated for permeability and SCFA results. Values were expressed as Mean ± SEM. Paired tests were used to compare between baseline and after probiotics and unpaired T tests were used to compare between subjects and patients where significance was p<0.05.

**RESULTS**

Two of the sixteen IBD patients withdrew due to time constraints prior to the commencement of the trial. Eleven (85%) of the IBD patients completed the sample collection for the trial according to the protocol. One IBD patient had an adverse event by becoming ill during the trial and was unable to continue. Two patients failed to collect their faecal samples but complied with all other aspects of the trial. Fourteen (70%) of the healthy controls completed all sample collections for the trial.
### Table 5.1 A summary of the inflammatory bowel disease patients' site of disease at diagnosis.

*Patient's have extensive ulcerative colitis. Patients' length of disease and medication was correct for the time of the study. Intestinal disease activity of inflammatory bowel disease patients at diagnosis was collected using a standardised form by the diagnosing gastroenterologist (Schachter and Kirsner 1975). Site and levels of the disease activity was collected from a patient registration sheet filled out at diagnosis. The coding: blank = no involvement; 1=macroscopically normal, microscopic inflammation; 1a=loss of vascular pattern ± friability, ± erythema, No microscopic inflammation; 2=loss of vascular pattern, ± friability, ± erythema, ± aphthous ulcers +microscopic inflammation; 3=Granular mucosa, increased friability ± exudate ± pus; 4=discrete areas of ulcerative evident/cobblestoning.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Age at diagnosis</th>
<th>Length of disease</th>
<th>Daily dose</th>
<th>Medication</th>
<th>Oral facial</th>
<th>Oesophagus</th>
<th>Stomach</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>ileum</th>
<th>Terminal ileum</th>
<th>Colon</th>
<th>Ascending Colon</th>
<th>Transverse Colon</th>
<th>Descending colon</th>
<th>Sigmoid colon</th>
<th>Rectum</th>
<th>Pancolitis</th>
<th>Fistula Perianal</th>
<th>Tag Perianal</th>
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<tbody>
<tr>
<td>CD</td>
<td>10.48 y</td>
<td>1.59 y</td>
<td>0.5 g</td>
<td>Mesalazine</td>
<td>1</td>
<td>1</td>
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<tr>
<td>CD</td>
<td>11.48 y</td>
<td>1.5 y</td>
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<td>none</td>
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<td>1</td>
<td>2</td>
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<td>CD</td>
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<tr>
<td>UC*</td>
<td>4.0 y</td>
<td>2.43 y</td>
<td>1 g</td>
<td>Dipentum</td>
<td>3</td>
<td>3</td>
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<tr>
<td>UC*</td>
<td>5.3 y</td>
<td>12.68 y</td>
<td>N/A</td>
<td>Salazopyrin</td>
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<td>3</td>
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<tr>
<td>UC*</td>
<td>11.0 y</td>
<td>1.59 y</td>
<td>0.5 g</td>
<td>Salazopyrin</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<tr>
<td>UC*</td>
<td>13.65 y</td>
<td>2.43 y</td>
<td>N/A</td>
<td>Mesalazine</td>
<td>3</td>
<td>3</td>
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<tr>
<td>UC</td>
<td>3.18 y</td>
<td>12.43 y</td>
<td>0.25 g</td>
<td>Salazopyrin</td>
<td>3</td>
<td>4</td>
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<tr>
<td>UC</td>
<td>6.0 y</td>
<td>1.85 y</td>
<td></td>
<td>N/A</td>
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<tr>
<td>UC</td>
<td>7.99 y</td>
<td>6.51 y</td>
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<td>N/A</td>
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<tr>
<td>UC</td>
<td>8.7 y</td>
<td>0.86 y</td>
<td>1.5 g</td>
<td>Salazopyrin</td>
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<tr>
<td>UC</td>
<td>10.47 y</td>
<td>0.76 y</td>
<td>N/A</td>
<td>Salazopyrin</td>
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<tr>
<td>UC</td>
<td>12.78 y</td>
<td>1.08 y</td>
<td>1.5 g</td>
<td>Dipentum</td>
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</table>
**Faecal SCFA**

The faecal SCFA levels before and after a short-term diet supplemented with LA1 are shown in Table 5.2. Inflammatory bowel disease patients propionate levels were significantly higher than controls (p<0.05). Probiotics in the diet resulted in a significant reduction in butyrate levels in faecal SCFA (p<0.02) in IBD patients as shown in Table 5.2. No changes in SCFA concentration were seen in healthy controls after probiotic administration.

**Table 5.2** Faecal short chain fatty acid concentration in inflammatory bowel disease patients and control subjects at baseline and after a diet of yoghurt supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>39.6 ± 5.4</td>
<td>11.3 ± 1.4 *</td>
<td>10.0 ± 1.9</td>
</tr>
<tr>
<td>Control</td>
<td>35.3 ± 5.0</td>
<td>7.6 ± 1.2</td>
<td>8.2 ± 1.2</td>
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</table>

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</thead>
<tbody>
<tr>
<td><strong>Probiotics</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IBD</td>
<td>32.1 ± 4.5</td>
<td>9.1 ± 1.6</td>
<td>6.8 ± 1.2 **</td>
</tr>
<tr>
<td>Control</td>
<td>36.4 ± 3.7</td>
<td>7.7 ± 1.0</td>
<td>8.5 ± 1.4</td>
</tr>
</tbody>
</table>

* Significantly higher in IBD patients than control subjects where p=0.016. ** Significant decrease in butyrate concentration in IBD patients after probiotics p=0.048. Data was presented as Mean ± SEM mM/L.

**Intestinal Permeability**

Small intestinal permeability values before and after probiotic supplementation are shown in Table 5.3. At baseline the lactulose/L-rhamnose ratio was significantly higher in IBD patients compared with controls (p<0.05). Crohn’s disease lactulose recovery exceeded the upper limit of the normal range mentioned in Chapter 4. Both control and UC patients had intestinal permeability values within the normal range. A probiotic yoghurt diet did not significantly change intestinal permeability.
Several patients had their intestinal permeability measured over time and their results are displayed chronologically in Figure 5.2. One of the CD patients had a history of high permeability which probiotics did not alter (Figure 5.2A). Two extensive colitis patients had an intestinal permeability result higher than at diagnosis (Figure 5.2B, 5.2C). At the time of the probiotic trial these patients with high intestinal permeability may have been at risk of relapse.

Table 5.3 Small intestinal permeability results from inflammatory bowel disease patients and control subjects before and after a probiotic (LA1) supplemented diet.

<table>
<thead>
<tr>
<th></th>
<th>Rhamnose (%)</th>
<th>Lactulose (%)</th>
<th>L/rh ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s Disease</td>
<td>6.80 ± 1.43</td>
<td>1.656 ± 0.719</td>
<td>0.1688 ± 0.0712</td>
<td>3</td>
</tr>
<tr>
<td>Extensive colitis</td>
<td>5.95 ± 0.7</td>
<td>0.699 ± 0.150</td>
<td>0.1210 ± 0.0310</td>
<td>4</td>
</tr>
<tr>
<td>Distal colitis</td>
<td>7.07 ± 0.99</td>
<td>0.430 ± 0.071</td>
<td>0.0553 ± 0.0037</td>
<td>6</td>
</tr>
<tr>
<td>Total IBD group</td>
<td>6.66 ± 0.57</td>
<td>0.717 ± 0.192</td>
<td>0.1068 ± 0.0216</td>
<td>13</td>
</tr>
<tr>
<td>Controls</td>
<td>7.99 ± 0.78</td>
<td>0.347 ± 0.072</td>
<td>0.0431 ± 0.0079</td>
<td>14</td>
</tr>
</tbody>
</table>

|                      |              |               |                 |    |
| Probiotic            |              |               |                 |    |
| Crohn’s disease      | 4.58 ± 1.40  | 1.163 ± 0.662 | 0.1463 ± 0.0872 | 3  |
| Extensive colitis    | 7.27 ± 1.25  | 0.819 ± 0.173 | 0.1155 ± 0.0195 | 4  |
| Distal colitis       | 7.00 ± 0.59  | 0.429 ± 0.066 | 0.0539 ± 0.0076 | 6  |
| Total IBD group      | 6.47 ± 0.62  | 0.646 ± 0.153 | 0.0989 ± 0.0214 | 13 |
| Controls             | 8.15 ± 1.33  | 0.426 ± 0.092 | 0.0513 ± 0.0095 | 14 |

* Significantly higher than control subjects (p<0.05). Values were expressed as Mean ± SEM.
Figure 5.1 Permeability test results before and after a probiotic (LA1) in inflammatory bowel disease patients and control subjects.

* Significantly greater than controls as baseline (p<0.05). Values displayed are patients and subjects individual results.
Figure 5.2 Chronological permeability results compared with the probiotic trial results in CD and extensive UC patients. A, Individual results from two Crohn’s disease patients. B, Individual results of three extensive colitis patients. C, Two years small intestinal permeability results for an extensive colitis patient.
DISCUSSION

This study showed that probiotic yoghurt LA1 administration modified faecal SCFA levels in patients with IBD. Faecal SCFA changed after LA1 shifted towards levels seen in healthy controls. In contrast, no changes were seen in healthy controls further supporting the notion that the metabolic activity of the colonic flora is an important factor in the aetiology of IBD. There was a significant difference in the L/rh excretion ratio seen between CD, extensive UC and distal UC compared to controls. This was due to the elevated permeability of the first two disease categories.

The production and fermentation pattern of SCFA are determined by the substrates available for fermentation and the individuals intestinal flora (Goldin et al. 1980; Goldin and Gorbach 1984b; Flourie et al. 1986). Healthy colonic epithelial cells avidly absorb and oxidise SCFA (Roediger 1982). SCFA measured in the faeces are remnants from fermentation, absorption, and oxidation. Early research on the levels of SCFA in the faecal samples of IBD patients has conveyed conflicting results (Roediger 1980b; Roediger et al. 1982; Vernia et al. 1988). It appears disease activity, environmental factors and small patient groups have contributed to the variances. This chapter has shown SCFA results with less variability than the previous studies (Treem et al. 1994).

IBD patients had greater SCFA levels than controls but this was only significant for propionate. The CD patients involved in this study had disease present in the terminal ileum and the extensive UC patients had been diagnosed with pancolitis. A study by Treem and co-workers had selected IBD patients with disease activity at similar sites. Treem et al. (1994) found that CD and UC patients had significantly lower concentrations of acetate and higher concentrations of butyrate compared to controls. Patients with moderate or severe UC had acetate levels significantly reduced but this was not seen in CD patients. All patients in this study were in remission, so unlike Treem et al. (1994), no change in SCFA was seen with severity of disease.

After probiotics the three CD patients and six of the eight UC patients decreased their SCFA levels and shown a 20% drop in both acetate and butyrate, and a 4% drop in propionate. Two UC patients whose SCFA levels increased after probiotics have been
diagnosed with ulcerative colitis for over six and twelve years earlier. Disease duration may also affect the evolution of ulcerative colitis as CD pattern and behaviour dramatically changes with time (Louis et al. 2001).

The intestinal permeability at baseline showed IBD patients had an elevated lactulose/L-rhamnose ratio. The high permeability of patients was comparable with Chapter 4 and other permeability trials in inactive IBD paediatric patients (Pounder et al. 1983; Sanderson et al. 1987). Crohn's disease patients had low rhamnose and high levels of lactulose recovered but variability was high due to the small sample size. The IBD patients were a heterogenous group and as a result the permeability test showed greater variation in IBD patients than controls. Ukabam et al. (1983) observed a difference in permeability between UC and CD patients and the sites of disease in UC were related to the intestine permeability response. Similarly the UC patients and control subjects in this study did not have abnormal small intestinal permeability results and also had no history of small intestinal damage. The L/rh excretion ratio was elevated in CD patients with small intestinal disease involvement.

A week of probiotic yoghurt ingestion resulted in no significant difference in the intestinal permeability. Two of three CD patients permeability reduced to within the normal range. This probiotic had little effect on small intestinal permeability in UC patients. Different yoghurts may take different lengths of time to affect intestinal permeability as sheep milk yoghurt decreased in small intestinal permeability in healthy subjects in two days (Southcott et al. 1997). The optimal time frame in a healthy subject may also differ to an IBD patient. Chapter 4 observed the effectiveness of drug treatment and other studies have used several weeks for testing new treatment strategies for IBD patients (Sanderson et al. 1987; Teahon et al. 1991; Lorenz-Meyer et al. 1996; Griffiths 2000; Heuschkel et al. 2000). Sanderson et al. (1987) gave children with active CD an elemental diet for six weeks for a positive change in their permeability. Their intestinal permeability corresponded with improvements in other indices of disease activity, in particular ESR, body weight and disease activity. Teahon et al. (1991) found an elemental diet for four weeks in CD patients decreased their $^{51}$Cr EDTA permeability by half.

One week's administration is short-term compared to other probiotic studies. Most probiotic-IBD studies have been performed in ulcerative colitis patients. Venturi et al.
used a mix of eight strains of viable bacteria (VSL#3) administered to UC patients intolerant to 5-ASA for 12 months. Gionchetti et al. (1998) found that VSL#3 administered for nine months to be effective in the maintenance of pouchitis in UC patients. The development of treatments for IBD patients need to identify the short-term and long term effects. Probiotic LA1 did not change small intestinal permeability but needs to be tested over a longer term to compare with other IBD treatments.

This study showed that SCFA concentrations were elevated in the faeces of IBD patients compared to healthy individuals. This difference between healthy subjects and IBD patients may be evidence of either a differences SCFA production rate or a different rate of SCFA absorption by colonocytes and bacteria. Colonic bacterial population influences SCFA production and IBD patients colonic bacterial populations and luminal enzyme activity have been found to differ from healthy individuals (Gaiffer et al. 1991; Favier et al. 1997). This current study has shown that probiotics lowered the faecal SCFA levels in IBD patients. Antibiotics are also known to reduce faecal SCFA concentrations and cause significant change in the pattern of SCFA (Goldin and Gorbach 1984b; Midvedt et al. 1986). Previous studies have shown probiotics altered healthy humans' and animals' colonic flora and products of fermentation (Gilliland et al. 1978; Lidbeck and Nord 1993; Sakata et al. 1999). This study shows that probiotics has the ability to change the by-products of the bacterial fermentation in IBD patients and possibly the intestinal flora itself.

The lowered faecal butyrate levels in IBD patients after probiotics may represent enhanced absorption and oxidation of carbohydrates to SCFA. Roediger et al. (1982) theorised the absorption and oxidation of SCFA was impaired in UC patients. If SCFA are not absorbed or oxidised they remain in the lumen and pass with the faeces. A possible mechanism for the probiotics affecting SCFA levels involves the adjustment of the bacterial population. Modifying the colonic flora will also modify SCFA production, which is associated with pH. Probiotic species ability to lower pH has been observed in vitro and in vivo settings (Perez-Chaia et al. 1995; Danone 1996). Moreover the absorption of SCFA though trans-epithelial transport into colonocytes was regulated by pH (Chu et al. 1998). Thus probiotics' ability to change the pH of the colon may change the uptake of SCFA for colonocytes oxidation. Probiotics lowering the pH in the colon has been shown to inhibit pathogen growth (Fernandez et al. 1987; Klaenhammer 1988; Conway 1996). The growth
of some lactobacilli species are affected by the pH and SCFA levels (Perez-Chaia et al. 1995; Topping 1996). Thus the probiotics could modify the bacterial population directly, though growth or indirectly by changing the colonic environment.

The addition of probiotics significantly changed the butyrate levels. This was interesting as butyrate has been identified to affect several levels of enterocyte function. Butyrate has been proposed to induce proliferation, cell migration and regulate the transcription of genes (D'argenio et al. 1996; Wilson and Gibson 1997; Fusunyan et al. 1999; Koyama et al. 2000; Mariadason et al. 2001). If LA1 does stimulate butyrate absorption it could increase the metabolites for colonocytes and affect cellular transcription. Several probiotics species can also induce proliferation but this has not been observed in probiotic LA1 (Parker 1990; Jacquot et al. 1996; Puri et al. 1996; Thoreux et al. 1996; Ichikawa et al. 1999; Ren et al. 2001). Conversely, little is known of the effect butyrate has on probiotic LA1 as it may stimulate bacterial growth.

The administration of probiotics caused marked changes in IBD patient's fermentation and intestinal function. The significance of these functional changes in IBD patients and lack of changes in healthy controls remains to be determined. Future research is needed to understand the effect that probiotic yoghurt LA1 have on the mucosal barrier function.
Chapter 6  SMALL INTESTINAL PERMEABILITY AND THE EFFECT OF PROBIOTICS DURING EXERCISE.

INTRODUCTION

A high incidence of gastrointestinal dysfunction has been reported in athletes after intense exercise, including abdominal pain, diarrhoea and flatulence (Table 1.3) (Brouns et al. 1987; Green 1992; Halvorsen and Ritland 1992; Peters et al. 1999a; Peters et al. 1999b). Surveys of athletes have indicated that more gastrointestinal symptoms occur after severe exertion or during an increase in training (Sharman 1982). These symptoms are similar to those experienced by patients with inflammatory bowel disease (Hyams et al. 1991). There is no consensus in the literature that intestinal problems are related to the intensity and/or duration of exercise.

Intestinal permeability tests have identified altered intestinal integrity following strenuous exercise (Øktedalen et al. 1992; Pals et al. 1997). Strenuous exercise may contribute to alterations in the gastrointestinal barrier thus allowing toxic and antigenic agents to enter the intestinal mucosa and/or systemic circulation (Ryan et al. 1996). Little is known about the effect of repetitive intensive exercise, on gastrointestinal function and health.

Probiotic bacteria have been shown to be beneficial in diarrhoeal disease (Perdigon et al. 1990; Isolauri et al. 1993). The addition of probiotic yoghurt could be of assistance to runners that experience gastrointestinal disorders and may positively influence small intestinal permeability (Southcott et al. 1997). To date there have been no reports on the effect of yoghurt supplementation in runners or athletes.

This chapter contains two studies that aim to assess the effect of duration of exercise on small intestinal permeability. The first study investigates small intestinal permeability in healthy adults after a single session of moderate exercise and in recreational runners after an eight week training program. The second study determines the effect of L. johnsonii (LA1) and L. bulgaricus yoghurts on small intestinal permeability and exercise performance in training athletes over four week program using a randomised placebo controlled study.
METHODS

Ethics

Two studies were collaborations between the Centre for Paediatric and Adolescent Gastroenterology, Women’s and Children’s Hospital and Centre for Research in Education and Sports Science, The University of South Australia. All experimental procedures were approved by the Research Ethics Committee at the Women’s and Children’s Hospital, North Adelaide, SA; Human Research Ethics Committee of University of South Australia; and Human Research Ethics Committee at The University of Adelaide.

Study 1: Intestinal permeability, moderate exercise and endurance training

Subjects

Six healthy adults (HA) (4 M and 2 F, range 19-25 years) gave informed consent to participate in this study. All subjects exercised regularly and could run 10 km. Subjects had no gastrointestinal disorders, no previous bowel surgery, and no antibiotic or aspirin usage in the preceding six weeks. Each subject was required to attend the Gastroenterology laboratory for testing.

Twenty male recreational runners (RR) (range 20-31 years) who had been participating in regular physical activity were recruited. Subjects were screened prior to enrolment in the study. The criteria required subjects to have exercised for a minimum three month period, to overcome the problem of neural adaptation which contributes to large variations in performance improvements. Runners were well trained and were able to move into a higher intensity training program with little risk of injury. Runners were selected with no known cardiovascular disease, respiratory disease, gastrointestinal disease, on medication/antibiotics, illness or injured. All subjects were medically screened using modified pre-exercise screening questionnaire prior to undertaking exercise (Olds and Norton 1999). The protocol and potential risks and benefits were fully explained to each subject before they provided written informed consent.
Experimental Procedure

Healthy adults attended two consecutive sessions approximately one week apart (Table 6.1). Subjects fasted overnight prior to the test day and ate a standard breakfast at 7 am, containing no permeability probe sugars, and arrived at the Gastroenterology laboratory at 9 am. Subjects collected a pretest urine sample prior to each session and the control session was performed before the exercise treatment. On the control day, subjects were requested to abstain from exercise and conduct normal activities. On the exercise day, subjects ran a simulation fun run over 10 km. Following the run or normal activities subjects were rested for half an hour. Once recovered, subjects ingested the sugar permeability test and urine was collected over five hours.

The exercise training program was conducted over eight weeks and small intestinal permeability was measured before (Day 1) and after the training program (Day 60) (Table 6.1). The exercise training program followed the guidelines of the American College of Sports Medicine. This program recommended the quantity and quality of exercise, for developing and maintaining cardiorespiratory, muscular fitness, and flexibility in healthy adults (Etchison and Curd 1998). To maintain the training intensity for eight weeks each subject attended the University of SA laboratory on three separate occasions: weeks zero, four and eight. Tests were conducted at the same time of day to avoid circadian effects. At each testing session, body mass and stature were measured for body mass index. Each subject then performed an incremental treadmill running test to volitional exhaustion. Expired air was collected for determination of gas exchange parameters and fingertip blood samples were taken for determination of blood lactate concentration. The methodology involved is described in the forthcoming section on the measurement for the endurance program. The following day subjects commenced a three day per week running training program. Subjects did not use any additional nutritional supplements during the study period and a diary was kept of their daily food intakes.
Training program

Runners were provided with a heart rate (HR) monitor (Polar Beat, Polar Electro Finland) which they used throughout their training program. Each subject ran for 45 min, three times per week, and carried out a weights program three times a week. The running program involved a five minute warm up of jogging and stretching, then continuous running for 45 min at a level of intensity that had their heart rate corresponding to their lactate threshold (HRt).

After training, runners jogged for five minutes and then stretched. During the first four weeks of the study they ran at the HR, determined at week zero. During the second four weeks of the study they ran at the HR, determined at week four. This adjustment at Day 31 allowed the runners to train at corrected threshold and resistance level for the last four weeks. The weight program involved using upper body, abdominal and lower back muscles workout using 35% of maximum or own body weight for resistance.

Table 6.1 Subjects, treatments, exercise regimes and testing for Study 1.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Adults</th>
<th>Recreational Runners</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject numbers</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Treatment</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Exercise/training</td>
<td>10 km run</td>
<td>8 weeks (Days 3-58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 x week - weights</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 x week - 45 min run at HRt</td>
</tr>
<tr>
<td>Permeability test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Week 1</td>
<td>Day 1</td>
</tr>
<tr>
<td>Exercise/training</td>
<td>Week 2</td>
<td>Day 60</td>
</tr>
<tr>
<td>Exercise Testing</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Post training</td>
<td></td>
<td>Day 59</td>
</tr>
</tbody>
</table>
Measurement for endurance program

Body mass and stature

Body mass was measured using electronic digital scales (AND Mercury, FV-150, Tokyo, Japan). Stature was measured using a stadiometer (SECA, Hamburg, Germany) with subjects in the free standing position (Norton and Olds 1996).

Treadmill running tests

Baseline physiological data were collected whilst subjects stood quietly on the treadmill (Quinton Instruments, Model 1860, Washington USA) for three minutes prior to commencing each running test. Subjects then commenced running at a speed of 10 km/hr and 0% grade. The treadmill speed remained constant throughout the test and the work load was incremented every three minutes by increasing the slope of the treadmill by 1% grade until the subjects reached volitional exhaustion.

Technical errors of measurement for the distance covered and the effective peak speed were determined from two tests, separated by one week, carried out on five subjects who did not participate in the study protocol. As was the case for subjects who participated in the study protocol, the first test was conducted two weeks after a familiarisation trial. The relevant technical errors of measurement were, 2.6% for the distance covered, and 1% for effective peak speed.

Cardiorespiratory variables

Measurement of VO₂ and carbon dioxide production (VCO₂) were recorded as 30 s averages throughout each treadmill run and the values averaged over the final 30 s of each work load were recorded as the measured values. Subjects breathed through a low resistance respiratory valve (Hans Ruldolph 2700 series, Kansas City, USA) with a pre-calibrated large flow turbine transducer (P.K. Morgan Mark 2, Seaford, Australia) attached to the inspiratory port to measure ventilatory volumes. Expired air was directed at a 2.6 L mixing chamber (Sportech, Canberra, Australia) from which dried gas was sampled continuously (~ 500 mL/min) and passed to an O₂ analyser (Ametek S-3A/I, Pittsburgh, USA) and a CO₂ analyser (Ametek CD-3A, Pittsburgh, USA), both of which were
calibrated prior to each exercise test with commercially-produced gas mixtures of known O₂ and CO₂ percentages (BOC Gases, Adelaide, Australia). The electrical outputs from the ventilation meter and gas analysers were integrated using a personal computer which calculated the necessary ventilatory variables. Heart rate was recorded as five second averages during a three minute rest period prior to each treadmill run and during each run a Sport Tester heart rat microcomputer and chest transmitter (Polar Accurex Plus, Polar Electro, Oulu, Finland). The five second average at the end of the three minutes rest period prior to each treadmill run, at the end of each three minute work load, and at the end of exercise were taken to be measured values for HR.

Blood collection and analysis

Blood lactate concentration was determined from fingertip blood samples (50 μL) taken at the end of the three minutes period prior to each treadmill run, at the end of each three minute work load during the runs, and immediately upon the cessation of exercise. 25 μL of each sample was immediately passed through an automated lactate analyser (Yellow Springs International, Model 1500 Sport, Yellow Springs, USA). The remainder of each sample was discarded.

Lactate threshold

A log-log transformation (Bever et al. 1985) of the VO₂ and the blood lactate concentration during the treadmill runs were used to determine the VO₂ corresponding to lactate threshold. Linear region analysis of the HR vs VO₂ response during exercise was then used to determine the HR corresponding to the VO₂ at the lactate threshold. This HR was used as the training for the running training programs.

Permeability test

Subjects were required to fast overnight and refrain from taking products containing alcohol or aspirin for 48 hours prior to testing. The permeability protocol and analysis followed the methodology of Miki et al. (1996) and is described in Chapter 4.
**Study 2: Probiotics treatment during endurance training**

**Subjects**

Male subjects (n=77) aged between 16 and 34 years old were enrolled in this study. All had been participating in regular physical activity for at least three months prior to study commencement. Subjects’ age, height, and weight for the three groups are shown in Table 6.5. Recreational runners were selected with no known cardiovascular disease, respiratory disease, and gastrointestinal disease, not on medication/antibiotics or suffering from any illness or injury. Subjects were excluded if they were lactose intolerant or cows milk protein intolerant (CMPI). All subjects were medically screened using a modified pre-exercise screening questionnaire prior to undertaking exercise (Norton and Olds 1996; Olds and Norton 1999). The protocol and potential risks and benefits were fully explained to each subject before they provided written informed consent. At the time of enrolment subjects were allocated randomised numbers, according to a randomisation schedule.

**Table 6.2** Subjects, treatments, exercise regimes and testing for Study 2.

<table>
<thead>
<tr>
<th>Days</th>
<th>15</th>
<th>16</th>
<th>17-33</th>
<th>34</th>
<th>35</th>
<th>36-41</th>
<th>42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Maltodextrin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Placebo yoghurt</td>
<td><em>L. bulgaricus</em></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Probiotic yoghurt</td>
<td><em>L. johnsonii</em></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Training at HR</td>
<td>3x week -45 min</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>2x week -5x 1 km</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Exercise test</td>
<td>Treadmill</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Sampling</td>
<td>SIP</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>fSCFA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Collection</td>
<td>Blood</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

SIP = small intestinal permeability test, fSCFA = faecal short chain fatty acids
Experimental protocol

The exercise training program was conducted over four weeks and each subject attended the laboratory on two separate occasions; test 1 on Day seven and test 2 on Day 35. A time line of the study design including testing and sample collection is shown in Table 6.2. Small intestinal permeability and faecal short chain fatty acids were measured at specific time points during a four week training program to determine the effect of the supplements on these measures of intestinal function. Athletes daily diary recorded any gastrointestinal or health problems during the trial, and to thus determine the effect of probiotics and yoghurt on the level of adverse events. Tests were conducted at the same time of day to avoid circadian effects. At each testing session body mass and stature were measured for body mass index. Each subject then performed an incremental treadmill running test to volitional exhaustion whilst expired air was collected for determination of gas exchange parameters, and fingertip blood samples were taken for determination of blood lactate concentration. Procedures for measurements of body mass, stature, treadmill running, cardiorespiratory variables and lactate threshold were described in Study 1. The following day subjects commenced a five day per week running training program. Subjects did not use any additional nutritional supplements during the study period and food intakes were recorded daily for subsequent dietary analysis.

Training program

Subjects were provided with a HR monitor (Polar Beat, Polar Electro, Finland) and ran for 45 minutes, three times per week at a heart rate that corresponds to lactate threshold (HRt), during the four week period from Day seven to Day 35. In addition to the three weekly runs at HRt, subjects ran 5 x 1000 m at maximal pace twice per week. The rest period between each 1000 m repetition was equivalent to twice the time taken to run the previous repetition (i.e. 1:2 work: rest duty cycle).
Study Products

All test products were safe for human consumption and are over the counter commercial products. The products were delivered weekly from the factory to the test centre at CRESS for distribution to subjects. The products for Probiotic and Placebo yoghurt groups were refrigerated at temperatures not exceeding 8°C during transportation and storage. Probiotic yoghurt study product was produced by Nestle Chilled Dairy Business Group, Mulgrave Victoria 3170. Each batch of the study product was documented with the standard factory release certificate, including counts of living bacteria: *Streptococcus thermophilus* and *Lactobacillus johnsonii*. Placebo yoghurt study product was a commercial 'Traditional Natural Set Yoghurt' produced by Nestle Chilled Dairy Business Group, Mulgrave Victoria 3170. Each batch of the study product was also documented with the standard factory release certificate, including counts of living bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Control study product treatment contained a soluble fibre. Maltodextrin DE 15 powder supplements were used and each pack contained 30 two gram sachets. Two packs were marked with the individual subject’s unique identification number, according to the randomisation schedule. Each subject was issued with two packs of study product and instructions for mixing were issued in the subject diary.

Permeability test

Subjects were required to fast overnight and refrain from taking products containing alcohol or aspirin for 48 hours prior to testing. The permeability protocol and analysis followed the methodology of Miki *et al.* (1996) and described in Chapter 4.

Stool collection for faecal short chain fatty acids

Stool samples were collected on study Days 6, 16 and 35. Stools were placed in standard stool specimen containers labelled with the subject’s name, study identification number and the date of the stool collection. Each stool sample was snap frozen and stored in the freezer (-20°C) within the Gastroenterology Unit and analysed for faecal SCFAs at a later
date. A gas chromatograph was used for analysis and the results were expressed as a percentage of fatty acids present (Tagerman and Nagengast 1996).

Gas chromographic analysis of faecal short chain fatty acids

The measurement of SCFA followed the methodology of Tagerman and Nagengast (1996). Briefly, a homogenate was prepared from 1 g of faecal material with distilled water. Depending on the faecal consistency the sample was diluted with distilled water from 1:1 to 1:6. One mL of the faecal suspension was transferred into an eppendorf and spun at 10,000 g for one minute. The supernatant was removed and an internal standard was added to give a final concentration of 0.1 M prior to centrifugation at 10,000 g for 1 minute. The sample was further purified by repeated spinning of the supernatant two or three time until sample was clear. Though clear of debris, the sample had colouration and 0.1 μL was injected onto the BP20 Capillary column (Alltech, Deerfield, IL, USA). The internal standard used were either 2-ethylbutyric acid or 4-methyl n-valeric acid (Sigma Chemical Co., St. Louis, MO, USA) for comparison with the standard SCFA solution. For standards a volatile acid standard mix (Sigma Chemical Co., St. Louis, MO, USA) was used. This standard contained 10 mM of formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid and heptanoic acid. Results were expressed as percent of fatty acids present compared to the standard.

Subject diaries

Individual subject diaries were prepared for use in the subject’s home. The amount of study product taken, bowel actions passed and their type and adverse events were recorded. Any treatment and concomitant medications were also entered. Reminders were added to ensure exercise routines were adhered to and times for urine and stool collection were not missed. Following the subject’s completion of all study requirements, baseline complaints, study product doses, adverse events and concomitant medications were entered in the individual’s case report file and completed as appropriate.
Compliance monitoring

Compliance was monitored by ensuring all used and unused trial product containers, assigned to each subject, were collected. Instructions were given to each subject regarding the collection and storage of faecal and urine samples.

Statistics

Paired students t-test was used in Study to compare before and after exercise or training and unpaired Mann-Whitney-U-tests was used to compare between healthy adults and recreational runners, where significance was p<0.05. Spearman’s rank correlation was used for comparing percentage change in permeability and exercise results in runners post training. This correlation is a non-parametric procedure that replaces observation with their rank in the calculation of the correlation coefficient. Study 2 used one way analysis of variance (ANOVA) tests to assess changes within a group or changes between groups at a specific time point. Tukey’s test was used as a post hoc test for significance, where significance was p<0.05. A $\chi^2$ test was used to compare the frequency of elevated permeability results between treatment groups where significance was p<0.05.

RESULTS

Study 1: Intestinal permeability, moderate exercise and endurance training

There was no difference in the L/Rh excretion ratio between baseline and after a period of moderate exercise in healthy adults (Figure 6.1). However, the L/Rh excretion ratio in athletes was significantly higher prior to the exercise training program compared to the
healthy adults (0.0911 ± 0.017 vs. 0.033 ± 0.005 p<0.02). There was no difference in the L/Rh excretion ratio between baseline and after training in recreational runners.

Recreational runners’ exercise parameters had significantly improved at the completion of the program. The time taken to exhaustion was significantly greater (28.17 ± 1.91 min vs. 30.77 ± 1.82 min, p<0.005) and thus the subjects ran significantly further (4.19 ± 0.32 km vs. 4.63 ± 0.30 km, p<0.005). Subjects expended significantly greater amount of energy on the treadmill after the program (154.6 ± 20.2 kj) compared to the initial test (132.6 ± 19.7 kj) (p<0.009). There was no difference in the \( \hat{\text{VO}_2\text{peak}} \) (51.15 ± 1.61 mL/kg/min vs. 51.70 ± 1.34 mL/kg/min) or VO\(_2\) at lactate threshold (38.41 ± 1.45 L/min vs. 37.67 ± 1.18 L/min) after training.

**Figure 6.1** Small intestinal permeability in healthy adults and recreational runners.

![Bar chart with error bars showing permeability ratios](image)

The upper limit of the normal range for used permeability results (0.0138 - 0.0738) shown by a dashed line. * The small intestinal permeability of the recreational runners (RR) (n=20) at baseline and after exercise was significantly greater than the healthy adults (HA) (n=6) where p<0.02. Values are expressed as Mean ± SEM.
Recreational runners baseline L/Rh excretion ratio was 0.0911 ± 0.017 and after training it increased to 0.141 ± 0.026. Using the normal range (0.0138-0.0738) (Miki et al. 1996; Miki et al. 1998) 35% of these athletes were above the upper limit, before the training program. At the completion of the eight-week training program 55% had abnormal permeability.

The percentage change in L/Rh excretion ratio after an eight week training program was analysed using Spearman’s rank coefficient. There was a significant difference from when the correlation was greater than 0.447 or less then -0.447 where p=0.025. A large change in the L/Rh excretion ratio was correlated with a small improvement in the exercise parameters. The specific test battery results which correlate are VO₂peak, time, distance and energy expended on the treadmill (p<0.025).

Three RR reported gastrointestinal problems ranging from vomiting to gastrointestinal bleeding (Table 6.3). Eight subjects reported to have experienced the flu or a virus and four reported sore throats or colds (Table 6.4). There was no relationship between adverse events and intestinal permeability results.

**Table 6.3** Gastrointestinal adverse events reported by 20 recreational runners during an eight week training program.

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Severity</th>
<th>Number of cases</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal cramps</td>
<td>Mild</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>Mild</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stomach bug</td>
<td>Moderate</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Stomache ache</td>
<td>Mild</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vomiting</td>
<td>Severe</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 6.4  Non-gastrointestinal adverse events experience by 20 recreational runners during an eight week training program.

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Severity</th>
<th>Number of cases</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatigue/ lethargic/tiredness</td>
<td>Mild</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Flu</td>
<td>Mild</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>6</td>
<td>3, 5, 5, 7, 12, 19</td>
</tr>
<tr>
<td>Headache</td>
<td>Moderate</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Neck pain</td>
<td>Mild</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sore throat/cold</td>
<td>Mild</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>2</td>
<td>4, 5</td>
</tr>
<tr>
<td>Bruised/sore foot</td>
<td>Moderate</td>
<td>2</td>
<td>1, 10</td>
</tr>
<tr>
<td>Ankle injury</td>
<td>Moderate</td>
<td>2</td>
<td>4, 13</td>
</tr>
<tr>
<td>Hamstring strain</td>
<td>Moderate</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Shin pain</td>
<td>Mild</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sore knee</td>
<td>Mild</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Sore muscles</td>
<td>Moderate</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
Study 2: Probiotics treatment during endurance training

The study was satisfactory completed by 64 subjects, while 13 subjects withdrew. The majority of subjects adhered to the study protocol. Twelve subjects missed one or more doses of the study product. The number of subjects who took all doses for Control, Placebo yoghurt and Probiotic yoghurt were eleven, ten and fourteen respectively. The subject demographics for the three groups are displayed in Table 6.5. There was no difference in height and body mass between any of the groups at test 1 on Day 7 or test 2 on Day 35.

After four weeks of training, the distance covered during the test 2 treadmill run had increased in all groups (p<0.0001), but the magnitude of the increase was not significantly different between the groups (Table 6.6). Nor was there a difference in the distance covered between the groups. The mechanical work done during the treadmill run had significantly increased in all groups by test 2 (p<0.0001), but there was no difference between the groups. \(\dot{V}O_{2\text{peak}}\) was significantly higher in Probiotic yoghurt compared to Placebo yoghurt during the treadmill run at test 1 (p=0.0002) (Table 6.6). \(\dot{V}O_{2\text{peak}}\) had significantly increased by the second test after Placebo yoghurt (p=0.001) but no change was observed in Probiotic yoghurt or Control. The increase in \(\dot{V}O_{2\text{peak}}\) of Placebo yoghurt at Day 35 brought their values into line with those of the other groups, such that there was no difference between any of the groups by Day 35.

Table 6.5 Subjects demographics prior to and after 4 weeks of training.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Placebo yoghurt</th>
<th>Probiotic yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>27.13 ± 6.0</td>
<td>28.00 ± 4.1</td>
<td>28.07 ± 4.26</td>
</tr>
<tr>
<td>(range)</td>
<td>(18-34)</td>
<td>(19-32)</td>
<td>(20-33)</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td>177.24 ± 6.88</td>
<td>180.7 ± 5.64</td>
<td>181.23 ± 6.58</td>
</tr>
<tr>
<td>(range)</td>
<td>(163-188)</td>
<td>(172-195)</td>
<td>(170-191)</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>76.34 ± 9.77</td>
<td>82.34 ± 12.37</td>
<td>82.26 ± 7.20</td>
</tr>
<tr>
<td>test 2</td>
<td>76.46 ± 9.67</td>
<td>81.88 ± 12.22</td>
<td>82.84 ± 7.20</td>
</tr>
</tbody>
</table>

Subjects demographics are expressed as Mean ± SD
Table 6.6 Exercise performance results from an incremental treadmill run to exhaustion prior to and after 4 weeks of training.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Placebo yoghurt</th>
<th>Probiotic yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Distance (km)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>5.03 ± 0.31</td>
<td>4.50 ± 0.25</td>
<td>5.27 ± 0.26</td>
</tr>
<tr>
<td>test 2</td>
<td>5.35 ± 0.31 †</td>
<td>4.93 ± 0.25 †</td>
<td>5.65 ± 0.27 †</td>
</tr>
<tr>
<td><strong>Work done (kJ)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>177.0 ± 20.2</td>
<td>149.1 ± 16.3</td>
<td>208.1 ± 21.1</td>
</tr>
<tr>
<td>test 2</td>
<td>217.2 ± 21.7 †</td>
<td>180.6 ± 18.8 †</td>
<td>240.6 ± 22.3 †</td>
</tr>
<tr>
<td><strong>Time to exhaustion (min)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>33.18 ± 1.89</td>
<td>29.91 ± 1.49</td>
<td>34.63 ± 1.62</td>
</tr>
<tr>
<td>test 2</td>
<td>36.44 ± 1.85 †</td>
<td>32.34 ± 1.51 †</td>
<td>36.9 ± 1.66 †</td>
</tr>
<tr>
<td>$$\dot{V}O_{2peak} (mL·kg^{-1}·min^{-1})$$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>54.4 ± 1.8</td>
<td>51.8 ± 1.6 *</td>
<td>55.1 ± 1.5</td>
</tr>
<tr>
<td>test 2</td>
<td>55.3 ± 1.6</td>
<td>54.5 ± 1.6 †</td>
<td>55.1 ± 1.5</td>
</tr>
<tr>
<td>$$\dot{V}O_{2peak} at lactate threshold (%)$$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test 1</td>
<td>73.9 ± 1.9</td>
<td>71.4 ± 2.5</td>
<td>71.5 ± 2.9</td>
</tr>
<tr>
<td>test 2</td>
<td>73.4 ± 1.9</td>
<td>72.3 ± 2.2</td>
<td>71.3 ± 2.1</td>
</tr>
</tbody>
</table>

* Significantly different from Probiotic yoghurt (p=0.0002) and Control (p=0.003).
† Significantly different from test 1 (p=0.001). Values are expressed as Mean ± SEM.

**Intestinal Permeability**

Lactulose recovery was significantly lower in Probiotic yoghurt runners on Day 16 compared to Day 6 (p<0.05) (Figure 6.2). The Probiotic yoghurt group had significantly less abnormal results than the other treatment groups (p<0.05). No significant differences were seen in small intestinal permeability throughout the study period using both L/rh and L/M excretion ratios as markers.
**Figure 6.2** Lactulose urinary recovery in athletes on Days 6, 16, 34 and 42.

![Graph showing lactulose urinary recovery](image)

* Probiotic yoghurt urinary lactulose recovery on Day 16 was significantly lower than Day 6 (p<0.05). † The number of subjects with elevated permeability in the Probiotic yoghurt group was significantly greater than Placebo yoghurt or Control (p<0.01). Values are expressed as Mean ± SEM.

**Faecal Short Chain Fatty Acids**

Acetic acid levels were significantly greater in Control on Day 34 compared to Day 6 (p=0.036) (Figure 6.3A). On Day 34 propionic acid levels were significantly lower for Probiotic yoghurt compared to Control (p=0.039) (Figure 6.3B). Butyric acid levels in Controls on Day 34 were significantly greater than Day 16 (p=0.049) (Figure 6.3C). Isobutyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid and heptanoic acid showed no changes with training or between treatment groups. Similarly, the molar ratios were unchanged suggesting no marked alteration in the balance of these fermentation products.
Figure 6.3 Short chain fatty acid concentration of acetic acid (A), propionic acid (B), and butyric acid (C) from stool samples collected on Days 6, 16 and 34.

* Control acetic acid levels were significantly higher on Day 34 than Day 6 (p<0.05). ** Control propionic acid levels on Day 34 were significantly greater than Probiotic yoghurt (p<0.05). † Control butyric acid levels on Day 34 were significantly greater than Day 16 (p<0.05). Values expressed are Mean ± SEM.

Adverse Events

No serious adverse events were reported by any subject throughout the duration of the study (Table 6.7 and Table 6.8). Ten subjects reported no adverse events and these were: three in Probiotics yoghurt, five in Placebo yoghurt and two in Control group. Loose stools
or diarrhoea were reported by ten subjects. Blood in stools (one) and anal bleeding (two) were also reported. There was no difference in the incidence of illness between the groups during the study period or duration of illness between groups.

Table 6.7 Gastrointestinal adverse events reported by runners during the four weeks of training and probiotics.

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Control</th>
<th>Placebo yoghurt</th>
<th>Probiotic yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal cramps</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Abdominal pain/sore</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Stomach cramps</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anal bleeding</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Bloated</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Blood in stool</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Frequent stools</td>
<td>3</td>
<td>1, 1, 2</td>
<td>2</td>
</tr>
<tr>
<td>Loose bowel action</td>
<td>8</td>
<td>1-2</td>
<td>1</td>
</tr>
<tr>
<td>Vomiting (food poisoning)</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>No Gastrointestinal events</strong></td>
<td>7</td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

Adverse events are expressed as the number of subjects who reported the adverse event and the number of days they experienced this event. The severity of the complaint was stated as mild in subject diaries.
Table 6.8 Non-gastrointestinal adverse events reported by runners during four weeks of training and probiotics.

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Control</th>
<th>Placebo yoghurt</th>
<th>Probiotic yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Days</td>
<td>No.</td>
</tr>
<tr>
<td>Bruised/ sore foot</td>
<td>2</td>
<td>1, 10</td>
<td>1</td>
</tr>
<tr>
<td>Cough</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Hamstring strain</td>
<td>1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>9</td>
<td>1-2</td>
<td>6</td>
</tr>
<tr>
<td>Influenza</td>
<td>2</td>
<td>2, 3</td>
<td></td>
</tr>
<tr>
<td>Shin pain</td>
<td>2</td>
<td>10, 24</td>
<td></td>
</tr>
<tr>
<td>Sore knee</td>
<td>1</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Sore muscles</td>
<td>4</td>
<td>1, 1, 2, 2</td>
<td>2</td>
</tr>
<tr>
<td>Sore throat</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>None</strong></td>
<td><strong>2</strong></td>
<td><strong>5</strong></td>
<td><strong>3</strong></td>
</tr>
</tbody>
</table>

Adverse events are expressed as the number of subjects who reported the adverse event and the number of days they experienced this event.
DISCUSSION

Small intestinal permeability in recreational runners was significantly higher than in healthy adults and recreational runners exercise performance was also significantly improved after eight weeks of training. A subset of recreational runners (n=7) had elevated baseline L/Rh excretion ratio permeability prior to training which was significantly higher than the healthy adults. At the completion of the eight week training program 55% of athletes showed permeability that was above the upper limit of the normal range. This data suggests that normal training programs of an endurance athlete, defined here as a recreational runner, reduces barrier function and increases the likelihood of intestinal damage. The severity the disturbance to barrier function in recreational runners was similar to that seen in patients with IBD in remission (Figure 6.4).

Figure 6.4 Recreational runners (RR) intestinal permeability compared with Crohn’s disease (CD) patients.

Crohn’s disease patients’ permeability results were displayed in Chapter 4. The highest value of the normal permeability range is displayed by a dashed line.
The individual SCFA concentrations which are the by-products of colonic fermentation over the trial period were remarkably similar between groups. However, opposite effects were seen between probiotics and controls at the completion of the trial. The Control group SCFA levels were increased to similar levels to those seen in IBD patients in remission (Chapter 5), while Probiotic yoghurt LA1 administration resulted in significantly lower faecal SCFA levels on Day 34 maintaining levels similar to normal profiles. The increase seen in faecal SCFA after intense training may be related to a physiological inflammation which enables more substrate to reach the colon and generate a higher production rate of SCFA. Alternatively the SCFA may not be utilized by the colonic mucosa.

After four weeks of training all groups improved their VO2peak, distance ran, time to exhaustion and energy expended on the treadmill. This improvement in exercise parameters was not enhanced by any of the dietary supplementations. There was an elevation in maximal oxygen uptake seen in athletes taking the placebo yoghurt but not the probiotic yoghurt (LA1) or control (maltodextrin).

There was no clear evidence that permeability was altered after four weeks of exercise alone or with probiotics/yoghurt in runners. These results may however reflect the design and duration of the study. An eight week intensive training program did not significantly change intestinal permeability, however the change in the L/Rh excretion ratio was inversely correlated with the change in time, distance and energy expended on the treadmill. Recreational runners with an increase in permeability had small improvements in their exercise test. Conversely subjects who had a small change in permeability had larger improvements in time, distance and energy expended on the treadmill. This was independent of subjects whose initial permeability was elevated. The change in intestinal permeability may have developed in response to a combination of the level of intensity on training and length of the training program.

At four weeks (study 2) no correlations were found between exercise performance and intestinal permeability, though probiotic yoghurt administration significantly decreased lactulose recovery. Probiotic yoghurt may have had early effects during the training
program as a steady state was reached for the duration. The small intestinal permeability was not measured in RR after 4 weeks (study 1), and this may have assisted in predicting the influence of yoghurt on an athlete's susceptibility to developing increased small intestinal permeability which appears to occur between 35 and 60 days.

Previous studies have concentrated on acute events surrounding intense exercise whereas this study assessed the effect of accumulative training on intestinal permeability (Moses et al. 1991; Øktedalen et al. 1992; Pals et al. 1997). Pals et al. (1997) found that small intestinal permeability was significantly altered when subjects exercised at 80% VO\textsubscript{2peak} compared to rest and exercising at 40% and 60% VO\textsubscript{2peak}. In the present study, permeability was elevated in recreational runners at rest, after exercising for eight weeks at 70% of their VO\textsubscript{2peak}. This suggests repetitive exercise at a high intensity can affect intestinal integrity.

The current studies did not measure whole intestine or colonic permeability, however Øktedalen et al. (1992) found that whole intestinal permeability using $^{51}$Cr EDTA was affected by a single intense exercise event. The authors reported a significant increase in $^{51}$Cr EDTA excretion from control values of 3.10 ± 0.43% to 8.26 ± 0.92% following completion of half or full marathons (p=0.001). The control values and marathon results, when compared with other $^{51}$Cr EDTA studies, show that permeability was similar to IBD patients (Bjarnason et al. 1983; Maxton et al. 1986; Andre et al. 1990). Marathon runners have a high training load and their permeability results were elevated at rest. This implies that these marathon runners had elevated intestinal permeability prior to the event, probably due to their training and this was further exacerbated by the marathon competition.

The intensity and duration of exercise in training or competing in an event may predispose athletes to gastrointestinal problems. Gastrointestinal complaints have been associated with endurance sports required aerobic capacity comparable to marathon running such as long distance running, cross-country skiing, cycling and triathlons (Brouns et al. 1987; Halvorsen et al. 1990; Green 1992; Halvorsen and Ritland 1992; Peters et al. 1999a; Peters et al. 1999b). Three recreational runners had gastrointestinal problems and there were four runners who experienced nausea or fatigue over the eight weeks (study 1) (Table 6.3). In study 2, one third of the runners (n=17) had gastrointestinal problems and five subjects
experiences lethargy or fatigue during the four week training program (Figure 6.7). These adverse events were not related to the treatment, though loose bowel actions were more common in the control group than other treatments. No gastrointestinal symptoms were recorded after an acute exercise testing in healthy adults (study 1) which was in agreement in with other studies using non endurance athletes (Ryan et al. 1996; Pals et al. 1997). It is thus possible that prolonged elevated permeability could lead to significant gut damage in high performance athletes.

The type of exercise is known to influences the gastrointestinal problems experienced by the athlete (Green 1992; Halvorsen and Ritland 1992; Peters et al. 1999b). Long distance running was associated with lower gastrointestinal symptoms whereas cyclists experience both upper and lower gastrointestinal symptoms (Peters et al. 1999a; Peters et al. 1999b). Several studies have shown runners to experience more gastrointestinal disturbances than other sports (Green 1992; Halvorsen and Ritland 1992; Peters et al. 1999a). The type of training required for endurance athletes may thus play a role in the development of either upper or lower gastrointestinal disturbances.

Athletes are at risk of intestinal injury, firstly because during maximal exercise blood flow to the gut is reduced by 80% (Sharman 1982). Ischaemia has been suggested as a stress that might lead to gastrointestinal damage (Fogoros 1980; Kolkman et al. 1999) and a compromised intestinal barrier (Willoughby et al. 1996; Pals et al. 1997). Intestinal mucosal injury that results from local ischaemia can be detected early by an increased intestinal permeability, followed by morphological, histological and biochemical abnormalities (Haglund 1994; Szabo et al. 1997).

Secondly, athletes must exercise for 1-2 hours to receive an endurance base for many sports. The exercise time required would increase an athlete’s risk of intestinal injury as the extent and duration of ischaemia determines the depth of injury (Haglund 1994). In prolonged ischaemia epithelial cells along the villus are lifted until the villus core disintegrates, which returns to a normal appearance by eighteen hours (Park and Haglund 1992; Haglund 1994). As most endurance athletes would be exercising again within 18 hours, the intestine has little time to recover.
Thirdly, at the completion of exercise when blood flow returns to normal, the reperfusion component is believed to increase the generation of oxygen derived free radicals. Gastrointestinal mucosal ischaemia is thought to be the common denominator for the process that causes a reduced mucosal barrier. Dehydration and loss of body weight coupled with increased body temperature may also be major factors in gastrointestinal disturbances resulting from the stress of exercise (Fogoros 1980; Reher et al. 1989; Irving et al. 1991; Gebruers and Hall 1992; Green 1992; Van Nieuwenhoven et al. 2000). In previous studies a high permeability result was detected when subjects were immediately after exercising rather than at rest (Ryan et al. 1996; Pals et al. 1997). In the present study an elevation in permeability was measured in athletes after regular training rather than immediately after exercise. It is not known if dehydration (Reher et al. 1989; Gebruers and Hall 1992), loss of body weight (Green 1992), increased body temperature (Fogoros 1980; Pals et al. 1997), or ischaemia (Haglund 1994; Willoughby et al. 1996; Pals et al. 1997; Kolkman et al. 1999) predisposes athletes to increased intestinal permeability. However the duration, frequency, recovery time and intensity of training appears to influence the degree of intestinal permeability.

Probiotic yoghurt LA1 may initially improve small intestinal integrity but does not maintain this reduced permeability when the exercise program is intensified. Four weeks of training and concomitant probiotic yoghurt administration can reduce elevated SCFA levels. Whether probiotics can maintain permeability at a healthy level will need to be determined over long term training. As probiotics may balance SCFA production during exercise, intestinal function may be improved, and gastrointestinal disturbances minimised perhaps by reducing some of the symptoms related to colonic fermentation.

The present study showed that the integrity of the small intestine is compromised (i.e. more permeable) in athletes in training, similar to that seen in a diseased small intestine. It is possible that the abnormalities occurring in healthy runners are either due to physiological adaptations to physical training or are normal responses to extreme exertion. While performance was not compromised in the present study, it may be useful to monitor permeability of athletes in training to tailor their work load to avoid overtraining and prolonged gut damage. Similarly, susceptibility of individual athletes to illness may also be related to an elevated intestinal permeability and ways to suppress these changes may be useful in helping to maintain training regimens. Further studies are warranted to correlate
intestinal permeability with both performance parameters and coincidental illness in endurance athletes.
Chapter 7  

**BUTYRATE ALTERS THE ADHESION PROPERTIES OF LACTOBACILLUS STRAINS TO MUCUS.**

**INTRODUCTION**

*L. johnsonii* yoghurt did not change the intestinal permeability in either healthy controls or inflammatory bowel disease patients (Chapter 5). However, sheep milk decreased intestinal permeability in healthy adolescents (Southcott *et al.* 1997). Why was there a difference in permeability between the *L. bulgaricus* sheep milk yoghurt and *L. johnsonii* yoghurt?

One possible reason could be their ability to survive the intestine. Studies have found *L. bulgaricus* and *S. thermophilus* to be poor at adhering to intestinal cells compared to *L. rhamnosus* strain GG (Conway *et al.* 1987). *L. bulgaricus* was thought not to affect the intestine as it did not adhere, however *L. johnsonii* had a high percentage of adhesion (Tuomola and Salminen 1998). Tuomola *et al.* (1999) compared to Caco-2 adhesion model with a mucus adhesion model and found more strains tested positive for adherence to Caco-2 cells than the glycoproteins thus Caco-2 would select more strains. They recommended that the glycoprotein model would be a better method of screening for bacteria that would positively affect the intestinal ecology.

An *in vitro* model of intestinal mucus has been developed from human intestinal glycoproteins extracted from faeces. Probiotic bacteria have been shown to adhere to mucus *in vivo* when isolated from faecal samples (Kiravainen *et al.* 1998). This chapter uses this methodology to test the ability of *L. bulgaricus* and *L. johnsonii* to adhere to mucus as this was considered closer to the *in vivo* environment.

The different quantities and composition of the fats present in milk may influence the bacterial strains during yoghurt production. Sheep milk has a higher percentage of short and medium chain fats compared to milk from cows and humans (Appendix II). The short chain fatty acid butyrate was investigated for two reasons. Firstly the level of butyrate in sheep milk is almost double that of cow’s milk (Appendix II). Secondly, butyrate has been demonstrated to assist in repair and treatment of colonic inflammation (Wilson and Gibson
1997) and to induce proliferation (Mariadason et al. 2001). Fats in milk are found as triglycerides, so tributyrin was used as the source of butyrate.

This study aims to determine the effect that tributyrin has on probiotic and yoghurt strains adherence to intestinal mucus. The presence of the triglyceride of butyrate may alter the bacteria or their ability to adhere to the intestinal mucus. The two strains *L. bulgaricus* and *L. johnsonii* were investigated and compared to a high adhesion control *L. rhamnosus* strain GG and a low adhesion control *L. paracasei* subspecies *paracasei* strain Shirota.

**MATERIALS AND METHODS**

*Micro-organisms*

*Lactobacillus rhamnosus* strain GG ATCC 53103, *Lactobacillus delbrueckii* ssp. bulgaricus and *Streptococcus thermophilus* YC-180, *Lactobacillus johnsonii* LA1, and *Lactobacillus paracasei* strain Shirota YIT 9018 were grown overnight, anaerobically in MRS broth at 37°C to the late logarithmic phase.

Bacteria were cultured overnight in de Man, Rogosa and Sharpe broth (MRS; Merek Germany) containing 10 µL ml⁻¹ of tritiated thymidine (5⁻³H, 117 Ci mmol⁻¹, Amershon international, UK) for radiolabelling and Tributyrin (C4:0 Sigma Chemical Co., St. Louis, MO, USA) 0, 5,10, 20 and 40 µL in 1.5 mL of bacterial suspension. After cultivation, bacteria were harvested by centrifugation (2000 X g for 10 min), washed three times and resuspended in Hank’s balanced salt solution HEPES (N – (2-hydroxyethyl)piperazine-N°-2-(ethane sulfonic acid)) (HH; 10 mM HEPES; pH 7.4). The concentration of each bacterial suspension was adjusted to correspond to an absorbance of 0.25 ± 0.01 at 600 nm. (Virta et al. 1998)
Mucus adhesion assay

Intestinal mucus was isolated from faecal samples from 12 healthy individuals by extraction and dual ethanol precipitations. The adhesion of radiolabelled bacteria (metabolic labelling with [3H]-thymidine) was studied according to the methodology of Kirjavinen et al. (1999). Briefly, the mucus suspension was diluted (0.5 mg mL⁻¹) in HEPES-Hanks buffer and 100 μL of the suspension immobilized in polystyrene microtitre plate wells by incubating the plates overnight at 4°C. The wells were then washed twice with HEPES-Hanks buffer, 100 μL bacteria (absorbance at 600 nm adjusted to 0.25 ± 0.01; 1-2 x 10⁸ CFU ml⁻¹) were added and incubated at 37°C for 1 h. Non-adherent bacteria were removed by washing twice with HEPES-Hanks buffer, and the radioactivity of lysed (250 μl of 1% SDS – 0.1 M NaOH; incubated at 60 °C for 1h), adhered bacteria was measured by liquid scintillation. The adhesion ratio (%) was calculated by comparing radioactivity of lysed bacterial suspensions (in quadruplicate) to the radioactivity of bacteria added to the wells (four parallel samples). The experiments were repeated three times.

Statistical analysis

The results are presented as averages from three independent analyses, which were performed in quadruplicate. Two-factor analysis of variance was used to elevate the statistical significance of the differences among the tested strains and different tributyrin concentrations.
RESULTS

The effect of tributyrin concentrations on adhesion to human intestinal mucus by probiotic and dairy strains as illustrated in Figure 7.1. *L. rhamnosus* mucus adhesion was 17.1% and significantly greater than the other strains tested, in the absence of tributyrin (p<0.0001). YC-180 adherence to mucus significantly increased at all tributyrin concentrations tested (p<0.05). *L. rhamnosus* significantly increased adhesion at low concentration (26 nM) of tributyrin but decreased significantly (54%) at the high concentration of 216 nM when compared to control (p<0.05). Low tributyrin concentrations significantly increased *L. johnsonii* adhesion (p<0.05). Mucus adhesion was decreased in *L. casei* strain Shirota at 107 nM (p<0.05). Tributyrin had little effect on *L. johnsonii* and *L. casei* strain Shirota at the other concentrations tested when compared to control.

**Figure 7.1** The effect of tributyrin concentrations on mucus adhesion by *Lactobacillus* strains.

The equivalent mass (µg) of butyrate for each concentration was 23.6, 48.1, 97.1 and 195.6. † Significantly greater than other groups (p<0.0001). * Significantly greater than control (0) (p<0.05). § Significantly less than control (0) (p<0.01).
Luminal contents and intestinal conditions determine bacterial growth and may alter mucus composition and production. The in vivo adhesion of bacterial to the mucus could also be altered by the luminal contents. Previous in vitro adhesion studies which observed probiotic bacteria adhering to human intestinal cells showed skim milk was a good vehicle for increasing the bacterial survival in the low pH of gastric acid. (Conway et al. 1987)

The presence of tributyrin, a triglyceride of butyrate, caused a strain specific influence on mucus adhesion. Thus confirming previous research that high adhesion control, L. rhamnosus did bind more avidly to human intestinal mucus than L. johnsonii and L. paracasei strain Shirota (Kiravainen et al. 1998; Tuomola 1999). Low concentrations of tributyrin enhanced the number of bacteria adhering in all strains tested except L. paracasei strain Shirota. The presence of tributyrin at all concentrations tested significantly increased mucus adhesion by YC-180 (p<0.05). Interestingly, higher levels of tributyrin reduced the percentage of adhesion in L. rhamnosus and L. paracasei strain Shirota.

The mucus adhesion model found that the L. bulgaricus and L. johnsonii the level of adherence to mucus can be altered by factors in the milk. The butyrate in sheep milk could have increased the adhesion of a lactobacilli strain to the intestine. It is possible that the differing fat composition of ovine and bovine milk may alter other properties of the yoghurt strains besides adhesion. For instance a higher growth rate combined with increased mucus adhesion might further enhance colonisation.

A high fat diet has been found to increase the diversity of probiotic bacteria adherence in the intestine (Krause et al. 1995). Fats have also increased the adherence of lactic acid bacteria to mouse stomachs (Brockett and Tannock 1981; Brockett and Tannock 1982). Krause et al. (1995) found in pigs that a diet high in fats slowed transit of the chyme and suggested that this increase in the transit time would lead to an associated increase probiotic adherence. Short chain fatty acid enhanced Lactobacillus bacterial adhesion to mucus, independent of delayed transit. It is possible that fatty acids could increase probiotic adherence throughout the intestine.
A milk triglyceride can increase probiotic adhesion to healthy human intestinal mucus *in vitro* in specific strains. This study highlights the influence of factors in milk being a determinant in enhancing the colonisation and efficacy of individual probiotic organisms. Improved colonisation may be a feature in probiotic strains improving the intestinal barrier function. The change in intestinal permeability after sheep milk yoghurt and not cow’s milk yoghurt is from a combination of the milk, bacterial species and fermentation. Once the combination and interaction of factors on probiotic bacteria adherence has been established, further *in vitro* studies should be conducted to determine how probiotic bacteria and fat composition affect the barrier function *in vivo* in healthy and diseased intestines.
This thesis has investigated factors that can induce alterations in barrier function in the small intestine. These functional changes have been examined using animal models of damage, healthy humans, humans undergoing physiological stress and humans with intestinal disease. This has allowed analysis of the integrity of the small intestine at several different levels. Maintenance of the integrity of the intestine and minimising increased permeability are important factors in gut homeostasis. The absorptive function of the small intestine can be compromised to different degrees under physiological stress and in disease. This thesis has investigated permeability arising from different kinds of perturbation and found that barrier function was compromised after MTX administration in rats, in IBD patients and athletes in training. In all of these settings the influence of probiotics in maintaining or improving the intestinal barrier was assessed. The effect of probiotic administration on alterations in faecal SCFA was also examined in healthy humans, recreational runners in training and in inflammatory bowel disease patients.

The physiological effects of fasting and feeding on intestinal permeability have not previously been examined. Fasting reduced whole intestinal permeability as determined by $^{51}$Cr EDTA, and this change was due to altered colonic permeability as no change in small intestinal permeability was observed using the dual sugar test (Chapter 2). This alteration in colonic permeability may be related to the ratio of mature to immature cells. Butler et al. (1992) showed that fasting diminished colonic proliferative activity up to three fold in comparison, cell turnover in the small intestine was reduced by only 15% reduction. Hollander (1992) postulated that the immature crypt cells are more permeable than villus cells (Figure 8.1) which may explain the apparent relationship between proliferation and permeability in the colon.
Figure 8.1 Hollander's proposed model of tight junction differences between villus and crypt. This diagram is modified from Hollander (1992). There is a difference in the size and strand density between villus and crypt cells. Small molecules penetrate small resistant tight junctions at the villus tip. Larger (10Å) and smaller compounds can cross crypt tight junctions. The majority of intestinal absorption takes place at the villus tips and access to the crypt cells is limited (Claude 1978; Harris et al. 1988; Hollander 1992). Tight junctions hold the key for mucosal integrity and paracellular permeability. Cytokines, inflammatory cells, drugs or physical factors, such as osmotic stress, may influence tight junctions in the intercellular space and lead to increased antigen access (Madara et al. 1990; Sadowski and Meddings 1993; Blaser 1997; Sartor 1997; Shanahan 2000).
In chapter 3, methotrexate increased small intestinal permeability in association with mucosal damage and this was in agreement with previous studies (Taminiau et al. 1980; Erdman et al. 1991; Naruhashi et al. 2000). According to the Hollander's model, the recovery phase after methotrexate-induced damage was related to a more permeable epithelial layer because rapid cell proliferation in the small intestine increases the proportion of immature to mature cells.

No apparent colonic damage was observed in this model after methotrexate administration, therefore the increased whole intestinal permeability during recovery may be related to the fact that the methotrexate caused rats to stop eating and therefore they were fasted. As previously mentioned Butler et al. (1992) showed a six fold change in proliferation in the colon in healthy rats upon re-feeding after fasting. Therefore the increase in whole intestinal permeability observed reflects changes in barrier function of the colon resulting from fasting/re-feeding rather than from methotrexate-induced damage to the colonic epithelium.

The changes in small intestinal permeability are more easily explained as a consequence of the repair process, with measurement of intestinal permeability reflecting the extent and severity of mucosal damage. Probiotic LA1 yoghurt improved intestinal function after methotrexate-induced damage by maintaining a normal small intestinal permeability (Chapter 3). Probiotic LA1 action may have improved small intestinal mucosal repair using similar mechanisms to probiotics in diarrhoeal disease. Probiotics have been shown to reduce the incidence, duration and severity of symptoms in diarrhoeal disease (Isolauri et al. 1991; Kaila et al. 1995; Isolauri et al. 2002). Therefore, the probiotic bacterial action may be a two fold process in diarrhoeal disease: firstly preventing the loss of barrier function; and secondly enhancing intestinal mucosal recovery.

Sheep milk yoghurt acted on different sites in the intestine compared with probiotic LA1 yoghurt, and that it affects small and whole intestinal permeability (Chapters 2 and 3). Sheep milk yoghurt decreased the severity of damage to the proximal small intestine and increased sucrase activity, which is a marker of intestinal recovery (Davidson et al. 1977; Taminiau et al. 1980). These findings suggest that the sheep milk yoghurt improve cell maturation, thus potentially decreasing the number of immature cells and reducing intestinal permeability. The specific actions of probiotics vary from bacterial species to
species (Conway et al. 1987; Conway 1996). Future studies could examine the mechanisms whereby probiotics lead to repair of the intestine and their possible application in prevention or treatment of gut infections and allergy (Isolauri et al. 2002).

Small intestinal permeability in children and adolescents with active CD was elevated irrespective of the predominant site of involvement (Chapter 4). Soderholm et al. (2000) suggested that elevated small intestinal permeability was related to alterations in the tight junction structure. The tight junction structure in CD and UC patients contains fewer fusion sites and more incomplete strands than healthy subjects (Schmitz et al. 1999; Schmitz et al. 2000; Soderholm et al. 2002a). Defective tight junctions in CD and UC may be a genetically determined predisposing factor for IBD, allowing an increased antigenic load to permeate the gut barrier and stimulate the mucosal immune system. This may in part explain the elevated permeability seen in relatives of IBD patients as the tight junctions control the paracellular permeability (Katz et al. 1989; Hollander 1993; Issenman et al. 1993; May et al. 1993; Thjodleifsson et al. 1998; Breslin et al. 2001; Katz et al. 2001; Montgomery et al. 2001; Secondulfo et al. 2001). Several populations of IBD patients have been studied to determine the genetic predisposition of IBD (Dignass and Goebell 1995; Ahmad et al. 2002; Esters et al. 2002b). Recent genetic studies in IBD patients and families have found that a common mutation in NOD2 gene. NOD2 gene encodes a monocyte protein that identifies intracellular bacterial components and stimulated cytokine production to respond to the bacterium (Adam et al. 2002; Esters et al. 2002a; Esters et al. 2002b; Silverberg et al. 2002). NOD2's involvement in the regulation of the mucosal immune response to bacteria may be important in relation to an increased antigenic load via abnormal tight junctions. Disease expression, permeability results and genetic studies indicate that IBD is a complex disease with several possible aetiologies. The mechanisms of intestinal permeability and how these are altered in IBD patients is still to be determined.

Chronic changes in intestinal permeability occurred in a proportion of moderately well-trained recreational athletes (Chapter 6). Pals et al. (1997) demonstrated that acute
changes in intestinal permeability were influenced by duration and intensity of exercise. The effect of accumulative training resulted in elevated small intestinal permeability in runners similar to that seen in a disease-affected small intestine. Øktedalen et al. (1992) found that whole intestinal permeability in elite runners was elevated leading up to an intense exercise event (marathon) and that it was further exacerbated by the event. Permeability changes in runners before and after the marathon were similar to IBD patients' results (Bjarnason et al. 1983; Maxton et al. 1986; Andre et al. 1990). A proportion of recreational runners had elevated small intestinal permeability at rest which is in agreement with Øktedalen et al. (1992). This increase intestinal permeability may have developed in response to a combination of the level of intensity of training and length of the training program. Whether these changes occur solely in the small intestine or the large intestine, or whether both are involved is yet to be determined.

Small intestinal permeability in the recreational athletes showed high individual variability. Some runners were more susceptible to developing elevated permeability that may contribute to the pathogenesis of diarrhoea and abdominal cramps, common symptoms in elite runners. The stress induced by exercise in terms of loss of barrier function and intestinal permeability has received relatively little attention. The concept of a physiological inflammation occurring in response to stress has only recently been shown to occur in an animal model (Semin et al. 2001; Soderholm et al. 2001; Soderholm et al. 2002b). Saunders et al. (1994) has shown that stress increases colonic permeability and other studies have shown that dietary and surgical stress affect intestinal function (Spitz et al. 1996; Groot et al. 2000; Rocha et al. 2001).

An alternative reason for marathon runners experiencing diarrhoea during and after training may involve an alteration in colonic flora. These athletes had elevated faecal SCFA levels similar to IBD patients, though these athletes are not known to have impaired SCFA absorption or oxidation (Chapters 5 and 6). The metabolic activity of the colonic microflora of IBD patients is known to differ from that of healthy subjects (Treem et al. 1994). This difference in faecal SCFA may be evidence of either an impaired absorption of SCFA (Roediger 1982) or differences in the production rate from the bacterial populations (Goldin and Gorbach 1984b; Floch 1990). Leading to the second hypothesis that the changes in SCFA metabolism observed were due to an exercise-induced
inflammatory response in athletes. Interestingly, the dietary supplementation of LA1 yoghurt maintained faecal SCFA concentrations with training.

The relationship between intestinal bacteria and SCFA is notable and the type of SCFA may influence aspects of probiotic action. Tributyrin, a triglyceride of butyrate, enhanced probiotic bacterial adhesion to intestinal mucus (Chapter 7) and that this may favour the persistence of probiotic bacteria in the intestine. In turn the promotion of probiotic colonisation may alter the colonic environment to complement the adhered bacteria and encourage survival of endogenous lactobacilli and bifidobacteria species. The by-product of bacterial fermentation, such as butyrate, produces an acid environment that stimulates lactobacilli bacterial growth and may further enhance the persistence of probiotic bacteria.

Probiotics are capable of altering the metabolic activity of colonic microflora (Chapters 5 and 6) and the addition of probiotics to the diet of IBD patients and athletes could improve intestinal function. Probiotic LA1 has been shown to alter faecal SCFA (Chapters 5 and 6). The placebo yoghurt did not affect SCFA levels, supporting the idea that not all lactobacilli species are able to favourably alter the metabolic activity of the large intestinal flora (Conway et al. 1987; Ouwehand 1998). The patterns of SCFA alterations were different between athletes and patients with IBD. Disease, stress and surgery are all known to affect the bacterial flora and its metabolites (Tamnock and Savage 1974; Guo et al. 1995; Rocha et al. 2001; Isolauri et al. 2002). In both IBD patients and athletes, probiotic yoghurt however either maintained or restored the normal SCFA concentrations.

It is possible that the functional changes which occur in the intestine of healthy runners are either physiological adaptation to physical training or normal responses to extreme exertion. Athletic performance was compromised (Chapter 6) and it may thus be useful to monitor intestinal permeability of training athletes to tailor their workload to achieve peak performance. Similarly, the susceptibility of individual athletes to illness may also be related to an elevated intestinal permeability and changes in the colonic flora but further work is needed to clarify this (Ramsbottom et al. 1989; Eathorne 1994; Newsholme 1994; Hooper and Mackinnon 1996; Morton 1997). The changes in faecal SCFA and possibly bacterial populations may modulate the immune response. Differences in neonatal gut
microflora were found to precede the development of atopic disease (Kalliomaki et al. 2001; Isolauri et al. 2002). Isolauri et al. (2002) speculated that the balance of intestinal bacteria may play a crucial role in the maturation of human immune functions. Interestingly, probiotics have been shown to significantly improve atopic eczema, reducing symptoms compared to placebo, and decreasing both the systemic markers and intestinal allergic inflammation (Majamaa and Isolauri 1997; Isolauri et al. 2000; Isolauri et al. 2002).

Tight junctions may undergo permanent changes reflecting disease severity and region of the disease (Madara and Hecht 1989; Madara et al. 1990). The tight junction protein structure in CD and UC patients was found to be altered compared to healthy subjects (Schmitz et al. 1999; Soderholm et al. 2002a). Changes to the tight junction structure in the presence of both IL-2 and IL-15 pro-inflammatory cytokines have also been reported (Nishiyama et al. 2001). The presence of cytokines may perpetuate permanent alterations in tight junction assembly in inflammatory diseases. The perturbations of tight junctions after both physiological stress and in disease states may in part result from an increased proportion of immature intestinal cells associated with inflammation.

Hollander’s theory of differences in permeability along the villus crypt axis (Figure 8.1) could be applied to explain the difference in permeability and proliferation in a fasting/re-feeding animal model and the post-methotrexate reparation. The theory can also be applied to IBD patients and explain the increased intestinal permeability in IBD patients. Probiotic administration may influence intestinal permeability through tight junction mechanisms and/or via SCFA. SCFA enhanced intestinal epithelial cell proliferation in rats (Ichikawa et al. 1999) and stimulated cell differentiation in the colon. The permeability along the crypt-villus axis is important in health and disease.

Salminen et al. (1996) suggested that probiotic bacteria may stabilise the intestinal flora and gut mucosal barrier to strengthen the intestinal defence mechanisms. Figure 8.2 expands Salminen’s theory by indicating the how probiotics may prevent a compromised intestinal barrier developing. This thesis has found probiotics prevented the elevation in passive intestinal permeability and enhanced intestinal recovery after injury.
Probiotics prevent
- adhesion of pathogens to enterocytes or mucus
- Drug-induced inc. intestinal permeability
- Alterations in intestinal flora from stress

Probiotics prevent further
Increases in permeability

Figure 8.2 Theoretical interaction of probiotics with intestinal function

Future studies

Future studies will focus on permeability alterations in relation to enterocyte proliferation and properties of immature cells using the fasting/re-feeding rat model and the methotrexate small bowel damage model. The addition of freeze-fracture analysis would provide information on tight junction structure and expand our knowledge of intestinal
physiology. These techniques would also be useful in diarrheal disease and inflammatory bowel disease.

The differential permeability changes in the animal fasting model emphasised the importance of observing colonic permeability. Colonic permeability has been investigated in coeliac patients using a sucralose test (Smecout et al. 2002). Application of such a test to athletes and IBD patients could provide valuable information on large intestinal health.

The findings in this thesis have showed the necessity of trialling probiotics over short and long term periods to determine their mechanism of action, as different yoghurts have different time courses to induce changes in intestinal function. Obviously, the optimal time frame for probiotic administration in a healthy subject will differ compared to patients with intestinal damage or disease. Probiotic studies in IBD patients have measured disease severity, bacterial population, and mucosal immune system, but not intestinal function. While LA1 did not improve intestinal permeability in IBD patients, it does not preclude other strains from improving intestinal permeability. IBD models have demonstrated evidence that individual probiotic species and collections of species can alter intestinal function and reduce disease severity (Schultz et al. 1998; Madsen et al. 1999a; Madsen et al. 1999b; Schultz and Sartor 2000; Madsen et al. 2001; Schultz et al. 2002). A collection of probiotic bacteria that work together would be more efficacious in IBD for two reasons. Firstly, IBD is a heterogeneous disease and the site of disease is not uniform in CD or UC (Chapter 5 and Sachar et al. 1992; Gasche et al. 2000; Ahmad et al. 2002). Secondly, not all probiotics act on the same site (Chapter 3).

The field of probiotics is rapidly expanding, with new strains being isolated from human faeces. Investigations of these new strains may identify individual strains that complement treatment of specific diseases. Steidler et al. (2000) genetically engineered Lactococcus lactis, a non-pathogenic, non-invasive bacterium to administer IL-10 locally, as opposed to systemically, providing a new therapeutic approach to intestinal disease (Shanahan 2000; Steidler et al. 2000). Bacillus subtilis spores are being sold as probiotics for humans and animals and, as they are dormant life forms, they have been suggested as an alternative to conventional probiotics and antibiotics (Casula and Cutting 2002).
There is however little use in developing new probiotic bacterial strains and combinations of probiotics unless their properties are fully investigated by conducting double blind placebo controlled trials in healthy individuals and in disease states. The interaction of probiotic bacteria and the intestinal environment of the host, in health and disease, still needs to be determined.
Chapter 9 References


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Marteau, P, Flourie, B, Pochart, P, Chastang, C, Desjeux, J F and Rambaud, J C (1990). Effect of the microbial lactase (EC 3.2.1.23) activity in yoghurt on the intestinal absorption...


to Chromium 51-labeled ethylenediamine-tetraacetic acid and ovalbumin during acute inflammation and anaphylaxis. *Gastroenterology* 94: 1368-75.


APPENDIX I

The composition of the laboratory made 18% casein-based diet described fully by Tomas et al. (1984) and Howarth et al. (1996).

<table>
<thead>
<tr>
<th>Composition of purified</th>
<th>minerals (mg/kg)</th>
<th>vitamins (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diet (g/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cornflour Starch: 514</td>
<td>KH₂PO₄: 17, 155</td>
<td>Thiamine HCl: 70</td>
</tr>
<tr>
<td>Casein: 180</td>
<td>CaCO₃: 14, 645</td>
<td>Riboflavin: 30</td>
</tr>
<tr>
<td>Sucrose: 152</td>
<td>NaCl: 12, 530</td>
<td>Niacin Nicotinic acid: 50</td>
</tr>
<tr>
<td>Wheat bran: 50</td>
<td>MgSO₄. 7H₂O: 499</td>
<td>Panthecnic acid: 150</td>
</tr>
<tr>
<td>Peanut Oil: 50</td>
<td>Fe₃O₄H₂O₇. 6H₂O: 312</td>
<td>Pyridoxal HCl: 15</td>
</tr>
<tr>
<td>DL-Methione: 2.5</td>
<td>CaPO₄: 170</td>
<td>Hydroxocobalamin: 0.02</td>
</tr>
<tr>
<td>Choline Chloride: 1</td>
<td>MnSO₄. H₂O: 61</td>
<td>Inositol: 400</td>
</tr>
<tr>
<td>Cod Liver Oil: 4.4</td>
<td>CuSO₄: 78</td>
<td>p-aminobenzoic acid: 50</td>
</tr>
<tr>
<td></td>
<td>KI: 0.25</td>
<td>Folic acid: 10</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₆Mo₇O₂₄.4H₂O: 1.25</td>
<td>Biotin: 0.4</td>
</tr>
<tr>
<td></td>
<td>Na₂SeO₃: 0.5</td>
<td>Glucose: 225</td>
</tr>
</tbody>
</table>

10mg Zn/kg or 100mg Zn/kg diets were made by adding appropriate concentrations of Zn in the form of ZnCl₂ (Sigma Chemical Co., USA). Cornflour starch and casein were purchased from Impak Foods, SA, Australia. Sucrose and wheat bran were obtained from local Franklins supermarket. Peanut oil was purchased from Nuttelex Food Products Pty. Ltd. Vic, Australia and Cod liver oil was obtained from Faulding Phamaceuticals, SA Australia. All other chemicals were obtained from Sigma Chemical Co., USA.
### APPENDIX II

Average gross composition, minerals and vitamins in milk (100 g)

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<thead>
<tr>
<th></th>
<th>Sheep</th>
<th>Goats</th>
<th>Cows</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solids, total,%</td>
<td>19.30</td>
<td>12.97</td>
<td>12.01</td>
<td>12.50</td>
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<tr>
<td>Energy, kcal</td>
<td>108</td>
<td>69</td>
<td>61</td>
<td>70</td>
</tr>
<tr>
<td>kJ</td>
<td>451</td>
<td>288</td>
<td>257</td>
<td>291</td>
</tr>
<tr>
<td>Protein, total,%</td>
<td>5.98</td>
<td>3.56</td>
<td>3.29</td>
<td>1.03</td>
</tr>
<tr>
<td>Lipids, total,%</td>
<td>7.00</td>
<td>4.14</td>
<td>3.34</td>
<td>4.38</td>
</tr>
<tr>
<td>Carbohydrates,%</td>
<td>5.36</td>
<td>4.45</td>
<td>4.66</td>
<td>6.89</td>
</tr>
<tr>
<td>Ash,%</td>
<td>0.96</td>
<td>0.82</td>
<td>0.72</td>
<td>0.20</td>
</tr>
<tr>
<td>Ca, mg</td>
<td>193</td>
<td>134</td>
<td>119</td>
<td>32</td>
</tr>
<tr>
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<td>0.05</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Mg, mg</td>
<td>18</td>
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<td>P, mg</td>
<td>158</td>
<td>111</td>
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<td>14</td>
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<tr>
<td>K, mg</td>
<td>136</td>
<td>204</td>
<td>152</td>
<td>51</td>
</tr>
<tr>
<td>Na, mg</td>
<td>44</td>
<td>50</td>
<td>49</td>
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<tr>
<td>Zn, mg</td>
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<tr>
<td>Ascorbic acid, mg</td>
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<td>1.29</td>
<td>0.94</td>
<td>5.00</td>
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<tr>
<td>Thiamin, mcg</td>
<td>80</td>
<td>40</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Riboflavin, mg</td>
<td>0.355</td>
<td>0.138</td>
<td>0.162</td>
<td>0.036</td>
</tr>
<tr>
<td>Niacin, mg</td>
<td>0.417</td>
<td>0.277</td>
<td>0.084</td>
<td>0.177</td>
</tr>
<tr>
<td>Pantothenic acid, mg</td>
<td>0.407</td>
<td>0.310</td>
<td>0.314</td>
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</tr>
<tr>
<td>Vitamin B6, mcg</td>
<td>80</td>
<td>60</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Folacin, mcg</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin B12, mcg</td>
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<td>0.065</td>
<td>0.357</td>
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<tr>
<td>Vitamin A, RE, mcg</td>
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<td>44</td>
<td>52</td>
<td>58</td>
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<tr>
<td>Vitamin D, mcg</td>
<td>0.18</td>
<td>0.11</td>
<td>0.03</td>
<td>0.04</td>
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<tr>
<td>Vitamin E, mg</td>
<td>0.11</td>
<td>0.03</td>
<td>0.09</td>
<td>0.34</td>
</tr>
<tr>
<td>Vitamin C, mg</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>Goats</td>
<td>Cows</td>
<td>Human</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td><strong>Saturated FA, g</strong></td>
<td></td>
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</tr>
<tr>
<td>C4:0, g</td>
<td>4.60</td>
<td>2.67</td>
<td>2.08</td>
<td>2.01</td>
</tr>
<tr>
<td>C6:0, g</td>
<td>0.20</td>
<td>0.13</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>C8:0, g</td>
<td>0.14</td>
<td>0.09</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>C10:0, g</td>
<td>0.14</td>
<td>0.10</td>
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<td>0.01</td>
</tr>
<tr>
<td>C12:0, g</td>
<td>0.40</td>
<td>0.26</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>C14:0, g</td>
<td>0.24</td>
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<td>0.25</td>
</tr>
<tr>
<td>MCT total</td>
<td></td>
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<tr>
<td>(C6-C14), g</td>
<td>1.58</td>
<td>0.89</td>
<td>0.61</td>
<td>0.64</td>
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<tr>
<td>C16:0, g</td>
<td>1.62</td>
<td>0.91</td>
<td>0.88</td>
<td>0.92</td>
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<tr>
<td>C18:0, g</td>
<td>0.90</td>
<td>0.44</td>
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<td>0.29</td>
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<tr>
<td><strong>Monounsat. FA, g</strong></td>
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<tr>
<td>C16:1, g</td>
<td>1.72</td>
<td>1.11</td>
<td>0.96</td>
<td>1.66</td>
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<tr>
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<td>0.08</td>
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<tr>
<td>C20:1, g</td>
<td>1.56</td>
<td>0.98</td>
<td>0.84</td>
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</tr>
<tr>
<td>C22:1, g</td>
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<td>-</td>
<td>trace</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Polyunsat. FA, g</strong></td>
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<tr>
<td>C18:2, g</td>
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<td>C20:5, g</td>
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<td>trace</td>
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<td>trace</td>
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<td>C22:6, g</td>
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<tr>
<td><strong>Cholesterol, mg</strong></td>
<td>11</td>
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<td>13</td>
<td>20</td>
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</tbody>
</table>

The tables were collated from the following references (Kon and Cowie 1961; Posati and Orr 1976; Renner 1982; Alichanidis and Polychroniadou 1995; Haenlien 1996; IDF 1996).