Rationale, efficacy and safety of probiotics in the treatment of Inflammatory Bowel Disease

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Abstract

Inflammatory Bowel Disease (IBD) affects approximately one in five hundred inhabitants, in the Western world. Since conventional treatment is harsh and not curative, growing attention has been given to probiotics as a safe alternative. Probiotics are live microorganisms with a beneficial effect on the host by improving the intestinal microbial balance. We have performed a literature search of clinical trials involving probiotics treatment of IBD with focus on Crohn’s Disease (CD) and Ulcerative Colitis (UC). We found that several probiotics (E. coli Nissle 1917, BIFICO, BFM, VSL#3, Trichuris suis and LGG) showed efficacy in maintaining remission in UC, but not in CD.

We also evaluated the literature on probiotics and their mechanisms of action. We describe how animal models show probiotics to 1) inhibit microbial pathogens growth 2) increase epithelial barrier function and 3) modulate the immune response of intestinal epithelia and mucosal immune cells. This seem to support the “hygiene hypothesis” claiming that modern lifestyle is too sterile, whereby the immune system does not develop properly thus causing IBD in predisposed individuals.
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Abbreviations

AP-1: Activator protein 1
CAMPs: Cationic antimicrobial peptides
CARD: Caspase activating recruitment domain
CD: Crohn’s disease
CD40: Cluster of difference 40
COX-2: Cyclooxygenase-2
DCs: Dendritis cells
FAE: Follicle-associated epithelium
GALT: Gut-associated lymphoid tissue
GI: Gastrointestinal tract
HLA: Human leukocyte antigen
IBD: Inflammatory Bowel Disease
ICAM-1: Intercellular adhesion molecule
IgA: Immunoglobulin A
IκB: Inhibitor of kB
IKK: Inhibitor of kB kinase
IL: Interleukin
ILF: Isolated lymphoid follicle
INF-α: Interferon-alpha
iNOS: Inducible NO synthase
 IRAK4: IL-1 receptor-associated kinase-4
IRF: Interferon regulatory factor
JNK: c-Jun N-terminal kinase
LPS: Lipopolysaccharide
LRR: Leucine-rich repeat
M cells: Microfold cells
Mal: Myd88-adaptor-like
MALT: Mucosa-associated lymphoid tissue
MAPK: Mitogen-activated protein kinase
MEKK: Mitogen-activated protein kinase kinase
MHC: Major histocompatibility complex
MyD88: Myeloid differentiation primary-response protein 88
NF-κB: Nuclear factor κB
NOD: Nucleotide oligomerization domain
OCTN: Organic cation/carnitine transporters
PAMP: Pathogen-associated molecular patterns
PPAR-γ: Peroxisome proliferative activated receptor-γ
PRR: Pattern recognition receptors
RelA/RelB/c-Rel
RIP1: Receptor-interacting protein 1
RIP2 or RICK: Receptor-interacting protein 2
SAA: Serum amyloid A
SCID: Severe Combined Immunodeficiency
SED: Subepithelial dome
SIGIRR: Single immunoglobulin IL-1R-related protein
TAK1: Transforming growth factor-β-activated kinase-1
TCR: T-cell receptor
TGF-β: Transforming growth factor-β
T H1: T helper 1
T H2: T helper 2
TICAM2: TIR-containing adaptor molecule-2
TIR: Toll-IL-1 receptor
TLR: Toll-like receptor
TNF: Tumor necrosis factor
TOLLIP: Toll-interacting protein
TRAF6: Tumor necrosis factor receptor associated factor-6
TRAM: TRIF-related adaptor molecule
TRIF: TIR-containing adaptor inducing IFN-β
Ub-TRAF: Ubiquitinated TRAF6
UC: Ulcerative colitis
VCAM-1: Vascular cell adhesion molecule
1. Introduction

Inflammatory Bowel Disease (IBD) is a group of chronic diseases confined to the gastrointestinal system, characterised by periods of active disease interrupted by periods of remission without symptoms. Crohn’s disease (CD) and ulcerative colitis (UC) are the two major forms. The two diseases have different etiology and location in the gut; UC is confined to the colon whereas CD can be located in both the small and large intestine. In Denmark there are approximately 8,000 patients with UC (~450 new cases per year) and there are approximately 6,000 patients with CD (~350 new cases per year). This frequency of roughly 1/500 persons is found in most of the Western world with third world countries catching up as they adopt a Western lifestyle. The pathogenesis of IBD is not fully understood; numerous theories exists that blame factors such as modern day hygiene and various dietary factors that supposedly play a modulating role in the disease process. It is believed that IBD is caused by an overly aggressive acquired active immune (T cell) response, in genetically disposed individuals as a response to certain bacteria among the gut flora as well as environmental factors.

![Figure 1](image)

**Figure 1** Factors believed to be contributing to the development of IBD. A subset of commensal bacteria induces an immune response in genetically susceptible individuals. Environmental factors are also thought to contribute to the disease development (figure adapted from Sartor, 2006).

At the beginning of the 20th century the Russian Nobel prize winner Elie Metchnikoff hypothesized that consummation of fermenting bacteria in dairy products by Bulgarian peasants were responsible for their longevity. In 1965 D. Lily and R. Stillwell introduced the
term probiotics as an antagonist to the word antibiotics. And since the 1970s, probiotics have been applied as an alternative treatment.

Today probiotics appear as a promising dietary factor that can be used as a complementary therapy in the treatment of IBD. Probiotics are defined as a live microbial feed supplement that beneficially affects the host by improving the intestinal microbial balance. The idea of replacing or supporting human intestinal gut flora is likely to have been inspired by the beneficial digestive effects attributed to the ingestion of cultured foods such as dairy products. Several different microorganisms including bacteria, fungi and whipworms have been applied in trials and proposed for treatments of IBD, but most commonly therapies are based on lactic acid producing bacteria such as *Lactobacillus*, *streptococcus*, *Lactococcus*, *Bifidobacterium*, *Escherichia coli* Nissle 1917 and the yeast *Saccharomyces boulardii*.

A greater understanding of the mechanisms behind probiotic action on the gastrointestinal microflora is required in order to determine, which probiotic are the most beneficial. The beneficial mechanisms of probiotics are currently thought to be one or more of the following: production of various antimicrobial metabolites, competitive exclusion of enteric pathogens, neutralization of dietary carcinogens, and modulation of mucosal immune responses.(Rachmilewitz *et al.*, 2004). The beneficial effect of probiotics has been demonstrated mainly in the prevention and treatment of pouchitis and in maintaining remission of mild to moderate ulcerative colitis. Probiotic treatment seems to have less effect in patients with Crohn's disease.
2. The aim of the project

*Which current evidence supports the use of probiotics for the clinical treatment of Inflammatory Bowel Disease?*

Treatment of inflammatory bowel disease (IBD) usually involves the use of immunosuppressive drugs, which are associated with adverse side effects, and in severe cases even surgery. Novel treatments of IBD with probiotics as a substitute or complement to conventional drugs are receiving growing attention.

Through a review of current literature and controlled clinical studies of patients with IBD, we will describe the effects of probiotics on gastrointestinal physiology and patophysiology and present the theoretical basis of using probiotics in treatment of IBD. Finally we will present suggestions for future clinical trials and discuss whether probiotics should be viewed as an alternative treatment or a supplement.
3. Pathology of Inflammatory Bowel Diseases

Inflammatory Bowel Diseases (IBD) consists primarily of the two diseases, Crohn’s Diseases (CD) and ulcerative colitis (UC). The diseases are caused by inflammation in the gastrointestinal tract, but the exact etiology is unknown. Even CD and UC both have common features as inflammation in the bowel, genetic factors, dysregulated immune system, and almost similar symptoms such as abdominal pain and diarrhoea, but still they have their own distinguishing (Neuman, 2007).

Patients with IBD are characterized by a defective immune response leading to inflammation and tissue damage. The immune system opposes the bacteria that are normally found in intestines, leading to an immunological imbalance. Both CD and UC are characterized by a TH1 profile (see section 6.12). Medical treatment is not curative but aims at ameliorating the symptoms, obtaining and preserving remission (Viscido et al., 2005; Sartor, 2006).

3.1 Crohn’s disease

Annually there are 10 new incidences of CD per 100,000 inhabitants, in Denmark. There are several different types of the disease; depending on the affected area of the gut. In approximately fifty percent of cases the Ileocolic Crohn's Disease is located in the ileum and in the large intestine. Crohn's ileitis affecting the ileum only is seen in approximately thirty percent all of patients and in the remaining cases Crohn's colitis, which affects the large intestine alone. However combinations of the three diagnoses are seen, where the entire gut is affected. CD affect the submucosal layer to a greater extent than the mucosal layer; a characteristic feature of CD is the demarcated granulomatous lesions that are surrounded by normal-appearing mucosal tissue that leads to an inflamed bowel surface as cobblestone appearance (Fig. 2A)

The primary symptoms in CD are intermittent diarrhea, coliclypain, weight loss, fluid and electrolyte disorders, malaise, and low-grade fever. Complications of CD include narrowing of the bowel, which may lead to bowel obstruction, abnormal passageways (fistulae) between the bowel and other structures such as the skin, which may lead to surgery.
To diagnose CD, sigmoidoscopy is used to visualize the affected areas and to obtain biopsies, and CT-scan to detect an inflammatory mass or abscess. Medical treatment methods to promoting the healing are also used (see chapter 4 for details on treatment) (Porth, 2007).

3.2 Ulcerative Colitis

Annually there are 17 incidences of UC per 100,000 inhabitants, in Denmark. UC affects the large intestine with ulcers or open sores. The inflammation can format to large areas and give very big ulceration (Fig. 2B). There are several different known forms of UC: Proctosigmoiditis colitis affecting the rectum and the sigmoid colon. Left-sided colitis is characterized by continuous inflammation beginning at the rectum and extending as far as the splenic flexure and Pan-ulcerative colitis affecting the entire colon. The primary symptoms in UC are diarrhoea, 30 to 40 bowel movements a day. UC affects the mucosal layer of the bowel, and the stools contain blood and mucus. The severity of the disease varies from mild to fulminating, the disease has been divided into three types: mild chronic, chronic intermittent, and acute fulminating. The common form is the mild chronic, in which the symptoms as diarrhoea and bleeding are in mild form. This form can be cured, when the others gives complication. Cancer of the colon is one of the feared complications.

The diagnosis usually is confirmed by proctosigmoidoscopy. Medical treatments are similar to those used in CD (Porth, 2007).

Figure 2 A: Crohn’s disease. B: Ulcerative colitis. UC can be distinguished from CD by the large unbroken area with inflammation, and the localization of the inflammation to the upper layer of the mucosa. CD shows a more broken pattern of inflammation (pictures from Porth, 2007).
4. Conventional treatment of IBD

The conventional drugs used for treating IBD are: aminosalicylates, corticosteroids, TNF-α antibodies, immunosuppressants, antimicrobials and surgery. They are rather effective but not curative. They are used to obtain remission and maintaining remission. The drugs used for treating UC and CD are broadly the same but different response occurs. The drugs will be described below.

4.1 Aminosalicylates

These drugs are used to treat mild to moderate active IBD, and for achieving and maintaining remission. There is seen a better respond of the treatment with aminosalicylates in patients with UC than in patients with CD.

The active therapeutic component of aminosalicylates is mesalazine (also known as mesalamine; 5-aminosalicylic acid (5-ASA)). The mechanisms of action of mesalazine are still unknown, but it may involve reduced cytokine formation by inhibition of leukocyte chemotaxi, reduced free radical generation and inhibition of the production of inflammatory mediators such as prostaglandin and leukotrienes. It may also function as a peroxisome proliferative activated receptor-γ (PPAR-γ) agonist, decreases the activation of interleukin 1 (IL-1) and tumour necrosis factor (TNF), induces apoptosis and act as a weak inhibitor of nuclear factor κB (NFκB) activity.

Mesalazine is usually well tolerated and has no serious side effect. The side effect is dose dependent and patients may experience headache, nausea, vomiting and diarrhoea (Nielsen and Munck, 2007) (Waller et al., 2005).

4.2 Corticosteroids

Corticosteroids are used to patient with sever active IBD or to patient where aminosalicylates are ineffective. Corticosteroids are only used to induce remission because of the significant side effects such as elevated blood pressure, glaucoma, osteoporosis, mood swings and
anxiety. Once remission is obtained aminosalicylates or immunosuppressants drugs must be used to maintain remission.

The immunomodulatory actions of corticosteroids are probably due to the inhibition of pro-inflammatory transcription factors such as activator protein 1 (AP-1) and NFκB (Waller et al., 2005).

### 4.3 TNF-α antibodies

Infliximab is a monoclonal antibody. It is used to induce remission in both UC and CD and it can obtain remission up to 3 month.

TNF-α is an important cause of inflammation in CD and infliximab inhibits the binding of TNF-α to its receptors. This probably reduces the production of pro-inflammatory cytokines IL-1 and IL-6, leukocyte migration and infiltration and eosinophil and neutrophil activation.

About 15% of patients given infliximab experiences side effects as fever, chills, pruritis or urticaria. Another problem is the formation of antibody with may cause allergic reactions and/or loss of efficacy (Waller et al., 2005).

### 4.4 Immunosuppressants

The most used immunosuppressants in IBD are azathioprine and 6-mercaptopurine. They take 12 to 16 weeks to work because they suppress the bone marrow and as a result the immune response. They are used as second-line drugs once remission has been induced by another medication. They can also help patients to get free of corticosteroids treatment. Side effect such as nausea, vomiting, skin rashes and a hypersensitivity syndrome effects 10 % of the patient during the first 6 weeks. Potential side effect as liver problems or pancreatitis is rare and frequent monitoring is recommended due to this side effects (Waller et al., 2005).
4.5 Antimicrobials

Metronidazole can be moderately effective in treating perineal CD and after ileum resection. They are often used in combination with other medical treatment. The mechanisms of action are unknown. Side effect such as nausea, vomiting, metallic tastes, rashes and intolerance to alcohol can occur (Waller et al., 2005).

4.6 Surgery

More than 15 % of all UC patients and 50 % of all CD patients will receive surgical treatment at some point (Marteau et al., 2006).

Surgery for UC is performed to keep patient symptom free and it is also recommended because of the high risk of cancer in infected tissue. A colectomy or an ileal-anal pouch surgery is performed, which is the removal of the entire colon and rectum. Colectomy is where an end-ileostomy is created on the lower abdomen outside of the patients’ body. The patients have to wear a small bag where the chyme is collected. An ileal-anal pouch surgery is where the small intestine is attached to the anus and the patient can defecate normally afterwards. But there may be up to seven watery bowel movements a day because the colon is not there to absorb water¹.

Surgery for CD is performed for several reasons; the medical therapy does not control the patients’ symptoms or developed complications such as blockage, abscess, perforation or bleeding into the intestine. Resection is the surgery used to CD where the diseased part of the intestine is removed. Symptoms may return after surgery².


5. Genetic factors of IBD

IBD is a multifactorial disease since both genetic factors as well as environmental factors such as smoking habits and the nature of gut flora seems to underlie the pathophysiology of IBD. But a positive familial history of IBD is the most well established risk factor for development of IBD. Children whom have parents that are both suffering from IBD have an estimated 33% risk of developing IBD themselves before the age of 28. Furthermore individuals who have relatives with Crohn’s disease are subjected to not only higher risk of Crohn’s development but also to development of ulcerative colitis. Several susceptibility genes have been indicated and it has been proposed that Crohn’s and UC are both polygenic diseases sharing some of the same susceptibility genes. With the use of genome-wide scans several loci termed IBD1-8 have been associated with IBD. Genome-wide scans mediate the finding of candidate genes. Genetic variation of candidate genes might provide clues to the molecular function or dysfunction of specific proteins (Russell et al., 2004).

IBD1 locus is found in the centromere of chromosome 16 and contains the gene for NOD2/CARD15 (see section 6.10). Three polymorphisms of this gene have been associated with the onset of Crohn’s disease. Two missense mutations Arg702Trp and Gly908Arg and one frameshift mutation Leu1007fsincC. The frameshift mutations introduces a premature stop codon, leading to a truncated protein (Russell et al., 2004). Two of these polymorphisms are located in the LRR domain and the latter upstream of this, but all three of them results in reduced NF-κB activity. NOD2/CARD15 promotes expression of cytokines and thereby inflammation. But studies however have demonstrated that NOD2 is able to negatively regulate TLR9 responses while positively regulating other TLR responses. NOD mRNA is found mainly in Paneth cells, which are mostly concentrated in the ileum. This could be a explanation to the observed linkage of NOD2 mutations with Crohn’s disease (Watanabe et al., 2005).

IBD2 is a locus associated with susceptibility to ulcerative colitis only. No candidate genes have been found yet though (Russell et al., 2004)..
IBD3 is located on chromosome 6 and surrounds the major histocompatibility complex (MHC), which is a gene region that plays a role in the immune system. A subset of genes in MHC is the human leukocyte antigen (HLA) genes, which encodes the MHC class I and II proteins. An HLA allele called HLADRB1*1030 has been associated with ulcerative colitis. Mutations in the promoter region of the gene encoding TNF-α, which is also located in IBD3 locus, have also been linked to susceptibility to IBD (Russell et al., 2004).

IBD4 locus is located in chromosome 14 and has been linked to susceptibility to Crohn’s disease.

IDB5 region is located in chromosome 5 and contains a cluster of genes coding for cytokines, which have been associated with the development of Crohn’s disease (Russell et al., 2004). This region also contains a cluster of genes encoding for organic cation/carnitine transporters called OCTN1 and OCTN2. Gene alleles SCL22A4 and SCL22A5 coding for OCTN1 and OCTN2 respectively are associated with susceptibility to Crohn’s disease. The carnitine transporters OCTN1 and 2 transport acyl-Carnitine across the inner membrane of mitochondria. The polar zwitterion l-Carnitine therefore has an important role in the transport of long-chain fatty acids into mitochondria for subsequent β-oxidation. OCTN1 and 2, which is also found in intestinal therefore has a role in epithelia bioenergetic metabolism. In rat colonic mucosa it has been demonstrated that inhibition of β-oxidation could cause ulceration, destruction of mucus cells and acute colitis. It is therefore suggested that a mutation leading to dysfunction of OCTN1 and 2 could lead to Crohn’s disease. Interestingly it has been demonstrated that a specific mutation Leu503Phe in OCTN1 leads to an 6-9 amino acids antigenic determinant/epitope shared with two bacteria Campylobacter jejuni and Mycobacterium paratuberculosis both known to be implicated in development of IBD. It is therefore thought that infection by one of those pathogens could lead to production of antibodies, which could cross-react with the already dysfunctional OCTN1 causing further harm (Lamhonwah et al., 2005).
6. Intestinal immunity

The gastrointestinal lining presents the largest surface area exposed to the outside environment. Most pathogens enter the human organism through the mucus membrane of this lining. The intestinal immune system is though not only obligated to protect against these pathogens, but also to ensure homeostasis with the more than $10^{14}$ commensal bacteria of the intestine.

The intestinal epithelia cells are joined together by tight junctions forming a physical barrier. The apical surface of the epithelia is covered by mucus produced by goblet cells and a network of polysaccharide called glycocalyx. The main protein component of mucus is a glycosylated macromolecule called mucin functions together with the glycocalyx to trap both pathogens and commensal bacteria. Other components of the mucus are cationic antimicrobial peptides (CAMPs) such as defensins and immunoglobulin A (IgA) both aiding in protection against pathogens. Most commensal bacteria reside on the luminal side of the mucus layer, and one of the main features distinguishing commensal bacteria from pathogens, is the ability to gain access to and penetrate the apical surface of the epithelia. But some commensal bacteria are able to reach the epithelia and modify the immunity of the intestines to benefit of the host as will be discussed later (Magalhaes et al., 2007).

The intestinal epithelia not only function as a physical barrier but also - in conjunction with the mucosa-associated lymphoid tissue (MALT) - as an inducer of innate and adaptive immunity. MALT is the largest lymphoid structure in the human organism and is subdivided in to the gut-associated lymphoid tissue (GALT), which is associated with the gastrointestinal tract. GALT again can be divided into inductive sites where antigens are sensed and immunity induced, and the effector sites where especially lymphocytes differentiate and mediate their response (Magalhaes et al., 2007).
6.1 Inductor sites of GALT

The follicle-associated epithelium (FAE) is mostly found in ileum of small intestine covering the Peyers patches but is also found in the colonic patches (not shown in figure 3) of the colon and in the remaining GI covering isolated lymphoid follicle (ILF). FAE contains microfold cells (M cells), which captures soluble antigens, bacteria and Toll-like receptor ligands (see next section) from the lumen. M cells transport theses substances by transcytosis in a toll-like receptor dependent manner (Tyrer et al., 2006) to the subepithelial dome (SED), which is part of Peyers patch or colonic patches and presents them to dedritic cells (DCs) or macrophages in the SED. Macrophages facilitates destruction of the foreign substances while DCs have toll-like receptors that recognizes toll-like receptor ligands and as a response turns on expression of cytokines.

These cytokines can determine whether naïve Helper T cells of the underlying Peyers patch or colonic patch differentiates into T helper 1 (T_{H1}), T helper 2 (T_{H2}) or T regulatory cells (for more details regarding activation and differentiation of naïve T helper cells see the section “T_{H1} or T_{H2} response”). Peyers patches, colonic patches, mesenteric lymph nodes and ILF all have T cells, which mediate adaptive immunity in response to antigen-presentation mainly by DCs. T cells in return can active B cells also resident in these immune compartments (Magalhaes et al., 2007).

6.2 Effector sites of GALT

The effector compartment of GALT includes the epithelium and underlying lamina propria. The epithelium harbours intraepithelial lymphocytes, which detect damage to the epithelia. The lamina propria contains IgA secreting plasmocytes, T cells, B cells, macrophages, and DCs. DCs of the lamina propria extend their dendrites through the tight junctions of epithelial cells and captures pathogens of the lumen. DCs then migrate to the inductor sites and induces immune response (Magalhaes et al., 2007).
In the following section will be given a detailed explanation on how enterocytes and cells such as DCs, neutrophils and macrophages detect pathogens and subsequently induce immune responses. The particulars may seem excessive, but are a necessity in order to understand the underlying genetics of IDB, the pathogenicity of pathogens and to appreciate the complex and beneficial effect of probiotics.

6.3 Pattern recognition receptors

At all times the GI is exposed to an immense number of microbes, both pathogens and nonpathogens. In order to sense these microbes’ epithelial cells as well as macrophages and neutrophils has specific receptors called pattern recognition receptors (PRR) recognizing different patterns of molecules mostly on the surface of these microbes. The molecules
recognized are different from those of the host, whereby the host’s innate immune system can recognize self from nonself.

6.4 Toll-like receptors

Toll-like receptors (TLR) are all type I transmembrane proteins of the Interleukin-1 receptor (IL-1R) family (West et al., 2006) and is an ancient type of PRR. The first of its kind to be identified was *Drosophila* Toll receptor a protein involved in dorso-ventral polarity of embryo. All multicellular organisms’ posses toll or TLR involved in innate immunity. In plants TLR receptors are involved in defence against fungus, bacteria and virus. The common ancestry of TLR’s suggests that these receptors evolved prior to the divergence of plants and animals more than a billion years ago. This long term conservation indicates that TLR has a very important role in innate immunity (Alberts et al., 2002).

Human’s posses ten types of TLR (TLR1-10). All of them are receptors with a large extracellular domain consisting of leucine-rich repeats known as LRR motif and an intracellular signaling domain termed Toll-IL-1 receptor (TIR). TLR1, TLR2 and TLR4 resides in the membrane apex of epithelia cells facing to GI lumen, whereas TLR5 is located at the basolateral membrane. TLR3, TLR7, TLR8 and TLR9 resides in the membrane of endosomes (Magalhaes et al., 2007).

Toll-like receptors recognizes molecular patterns on pathogens known as pathogen-associated molecular patterns (PAMP) or pathogen-associated immunostimulants and molecular patterns of nonpathogens known as commensal-associated molecular patterns (CAMP). There is a great overlap of PAMP and CAMP since pathogen molecular patterns are also shared with nonpathogens. Different TLR in general recognizes different ligands thereby initiating a signal pathway, which ultimately leads to the production of general inflammatory mediators as well as an appropriate response to the specific type of pathogen. Some TLR though may bind to several different ligands (Kalliomäki and Walker, 2005). And furthermore different TLRs may heterodimerize to increase the diversity of recognized ligands and perhaps also increasing to diversity of signal pathways induced (West et al., 2006) Some of the most significant ligands appears from table 1. Some ligands such a teichoic acids are specific for gram-positive bacteria, others like lipopolysaccharide (LPS) are specific for gram-negative
bacteria, and some are present in both bacteria types such as peptydoglycan, formylated methionine and CpG DNA motif. The N-terminal amino acid in eukaryotes is a methionine whereas it is a formylated methionine in prokaryotes. CpG motif is an unmethylated dinucleotide, which is twenty times less common in vertebrate DNA than in bacterial DNA. Fungi have ligands such as zymosan, glucan and chitin. Many parasites has ligands, among them Plasmodium which has glycosylphosphatidylinositol on its surface (Alberts et al., 2002).

<table>
<thead>
<tr>
<th>Pattern recognition receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Bacterial lipopeptides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Bacterial cell wall lipoteichaic acid, lipoproteins</td>
</tr>
<tr>
<td>TLR3</td>
<td>Bacterial cell wall peptidoglycan, double-stranded RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Bacterial lipopolysaccharide</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacylated and triacylated bacterial lipopeptides</td>
</tr>
<tr>
<td>TLR7</td>
<td>Guanosine-rich and uridine-rich single-stranded viral RNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Guanosine-rich and uridine-rich single-stranded viral RNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Bacterial and viral DNA</td>
</tr>
<tr>
<td>TLR10</td>
<td>Not known</td>
</tr>
<tr>
<td>Nod1</td>
<td>GM-tri-DAP (mesodiaminopimelate-containing N-acetylglucosamin-N-acetyl-ramic acid tripeptide). A peptidoglycan hydrolysis product found in gram-negative bacteria and some gram-positive bacteria</td>
</tr>
<tr>
<td>Nod2</td>
<td>Myramyl dipeptide in cell wall peptidoglycans from all bacteria</td>
</tr>
<tr>
<td>Peptidoglycan recognition protein</td>
<td>Bacterial peptidoglycan</td>
</tr>
</tbody>
</table>

Table 1 Mammalian pattern recognition receptors and their ligands

6.5 NOD receptors

Other pattern recognition receptors are the soluble receptors of the complement, which resides in the blood. And still others are nucleotide oligomerization domain 1 (NOD1) and nucleotide oligomerization domain 2 (NOD2) receptors, which are found in the cytosol of intestinal
epithelia. These receptors have been renamed to caspase activating recruitment domain 4 (CARD4) and caspase activating recruitment domain 15 (CARD15) respectively.

6.6 Signal transduction

PAMPs activate TLRs in two different manners. PAMP binding to TLR causes dimerization of the receptors and induces structural conformation change of cytoplasmic TLR domain, which facilitates binding of adaptor proteins, or preformed dimers binds PAMP thereby leading to conformation change and subsequent recruitment of adaptor proteins (West et al., 2006). The intracellular domain of different toll-like receptors TIR, transduces signals from the extracellular domain to intracellular signal proteins thereby activating different signal pathways, myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway...
and/or MyD88-independent signal pathway. Eventually the transduced signal activates the transcription factors nuclear factor (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor 3 (IRF3). As seen in figure 4, MyD88-dependent signal pathway activates NF-κB and AP-1 thereby initiating transcription of proinflammatory genes, whereas MyD88-independent signal pathway activates NF-κB and IRF-3 thereby initiating transcription of proinflammatory genes and interferon beta (IFN-β) respectively. NOD1 and NOD2 leads to transcription of proinflammatory genes by activating NF-κB directly (Kalliomäki and Walker, 2005).

6.7 TLR signaling (MyD88-dependent signaling)

TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11 signals through the adaptor protein MyD88 (see figure 5). MyD88 has a C-terminal TIR domain that in a homotypic manner interacts with the TIR domain of TLR and an N-terminal death domain that interacts with death domains of other proteins. TLR2 and TLR4 have an additional adaptor called TIR domain-containing adaptor protein (TIRAP) or Myd88-adaptor-like (Mal) that bridges TLR and MyD88. MyD88 interacts with the serine/threonine IL-1 receptor-associated kinase-4 (IRAK-4) and IRAK-4 subsequently phosphorylates IRAK-1, which again autophosphorylates itself and recruits tumour necrosis factor receptor associated factor-6 (TRAF). Ultimately a Myd88/IRAK-4/IRAK-1/TRAF-6 complex is formed. TRAF6 is dimerized and binds to ubiquitin-conjugating enzyme 13 (UBC13) and a UBC-like protein (UEV1A), whereby TRAF6 itself is ubiquitinated. The ubiquitinated TRAF6 (Ub-TRAF6) dissociates from the complex and binds to another complex constituted by transforming growth factor-β-activated kinase-1 (TAK1) and two adaptor proteins, TGF-β binding protein-1 (TAB1) and TAB2 not shown in the figure. TAK1 is a MAPK kinase kinase (MAPKKK), which phosphorylates MAPK kinase kinases (MAPKK) called MEKKs or another protein called NIK. The MEKKs and NIK are not shown in figure 5. MEKKs or NIK then phosphorylates a MAPK called p38 and c-Jun N-terminal kinase (JNK). JNK then activates transcription factor AP-1 (Barton and Medzhitov, 2003).

TAK1 complexed with a MEKK or NIK also phosphorylates and activates the β subunit of the inhibitor of κB kinase (IKK) complex. IKK then phosphorylates inhibitor of κB (IκB),
which leads to degradation of IκB by ubiquitin/proteasome system and thereby release of the NF-κB subunits p65 (Rel-a) and p50. When the p65/p50 heterodimer is released it translocates to the nucleus where it binds to the promoter or enhancer regions of immune genes such as tumour necrosis factor-α (TNF-α) (West et al., 2006).

Myd88/IRAK-4/IRAK-1/TRAF-6 complex also activates the transcription factors interferon regulatory factor 5 (IRF5) and IRF7 leading to synthesis of cytokine and IFN-α.

Figure 5 NOD1/NOD2 and TLR4 signaling pathways. NOD1/NOD2 activates NF-κB through RICK and IKK. TLR4 utilizes both MyD88 and TRIF. MyD88 recruits IRAK-4 and IRAK-1, which then associates with TRAF6 and ECSIT. TRAF6 then activates TAK-1, which in turn activates IKK, JNK and p38 eventually leading to NF-κB and AP-1 activation. TRIF activates both RIP-1 and TBK-1 leading to activation of NF-κB, AP-1 and IRF-3. The activated transcription factors enter the nucleus and initiate the transcription of inflammatory genes (figure adapted from West et al., 2006).
6.8 TLR signaling (MyD88-independent signaling)

TLR3 and TLR4 signals in a MyD88-independent manner (see figure 5). An adaptor called TRIF-related adaptor molecule (TRAM) or TIR-containing adaptor molecule-2 (TICAM-2) acts as a bridge in transducing signal from TLR3 to TRIF. It is not completely understood how TIR-containing adaptor inducing IFN-β (TRIF) activates the transcription factors NF-κB and IRF3. Some studies have suggested that TRIF interact directly with TRAF6 consequently activating NF-κB. Other studies suggests that TRIF interacts with receptor-interacting protein 1 (RIP1) and that RIP1 actives TRAF6 ultimately activating NF-κB. TRIF also activates TRAF-family-member-associated NF-κB activator TANK-binding kinase (TBK-1), which phosphorylates IRF3. IRF3 phosphorylation leads to production of the cytokine interferon-β (IFN-β) (West et al., 2006).

6.9 TLR Signal transduction inhibitors

Excessive inflammation which can be very harmful to the host is regulated by inhibitors of TLR signal transduction. There are several inhibitors of TLR signal transduction pathways in enterocytes, macrophages, dendritic cells (DCs) e.g. some being transmembrane receptors others being cytoplasmic proteins. Among the transmembrane are the single immunoglobulin IL-1R-related protein (SIGIRR), which is thought recruited by TLR4 and inhibiting its signal pathway by sequestering IRAK-1 and TRAF6. ST2L likewise block TLR4 signaling by sequestering the adaptors MyD88 and TIRAP (West et al., 2006). Others are the cytokine IL-10 and transforming growth factor-β (TGF-β) (Kalliomäki and Walker, 2005). IL-10 executes its immunosuppressive effect in multiples ways including suppression of protein translation, destabilization of mRNA and inhibition of gene transcription. For instance IL-10 is known to inhibit activation of NF-κB and the MAPK p38 thereby inhibiting TLR induced signal transduction. Consequently IL-10 inhibits production of proinflammatory cytokines and activation of macrophages and neutrophils (Weaver et al., 2007). More indirectly IL-10 is immunosuppressive through induction of TGF-β secretion in lamina propria T cells. TGF-β mediates its effect by different signal cascades among them MAPK pathways. One effects of TGF-β is to induce degradation of TLR2 (Clavel and Haller, 2007).
The cytoplasmic inhibitors are IRAK-M found in monocytes and macrophages only and thought to interfere with the interaction of IRAK-1 and TRAF6. Supressor of cytokine signaling (SOCS-1) is thought either to block IRAK-1 activation or inhibiting TLR2 and TLR4 signaling by mediating degradation of the adaptor TIRAP. Toll-interacting protein (TOLLIP) inhibits TLR2 and TLR4 signaling by forming a complex with IRAK-1 thereby impeding its autophosphorylation (West et al., 2006). Other cytoplasmic inhibitors are the peroxisome proliferator activated receptor-γ (PPAR-γ) and the zinc finger protein A20 (Kalliomäki and Walker, 2005). PPAR-γ is a nuclear receptor that promotes export of the RelA subunit of NF-κB from the nucleus to the cytosol and thereby attenuate the transcriptional activity of NF-κB.

Figure 6 TLR signal transduction inhibitors. TLR signalling can be dampened by negative regulators. TOLLIP, IRAK-M and SOCS-1 are cytoplasmic molecules that block IRAK-1 and thereby inhibit NF-κB activation. SIGIRR and ST2L are transmembrane receptors that inhibit TLR signalling by either blocking MyD88/IRAK-4/IRAK-1 activation or by sequestering MyD88 (figure adapted from West et al., 2006).
6.10 NOD1/NOD2 signaling

NOD1/CARD4 and NOD2/CARD15 are cytoplasmic receptors that recognize bacterial components through a LRR motif consistently with TLR receptors (see figure 5). At the N-terminal these receptors have caspase activating recruitment domains that interacts with a serine threonine kinase termed receptor interacting protein-2 (Rip2) or RICK. Rip2 interacts with NFκB resulting in transcription of pro-inflammatory cytokines eventually and eventually activating innate immunity (Russell et al., 2004).

6.11 NFκB response

NFκB has overwhelming many target genes and therefore likewise many function in both innate and adaptive immunity. Therefore only a selection of implications will be discussed in this section. NFκB existing in almost all cell types comprises five members in mammals, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. NFκB protein forms different combinations of homo- and heterodimers each having a specific response corresponding to the type of invading pathogen. The complexes of p50/p65, p50/c-rel, p65/p65, and p65/c-rel are all transcriptionally active with p50/p65 being the most inducible, while p50 and p52 homodimers are transcriptionally repressive. NFκB has several target genes expressing proteins such as the cytokines (e.g., IL-1, IL-2, IL-6, IL-12, TNF-α, LTα, LTβ, GM-CSF and INF-γ), adhesion molecules (e.g., intercellular adhesion molecule such as ICAM-1, vascular cell adhesion molecule such as VCAM-1, and endothelial leukocyte adhesion molecule such as E-selectin), chemokines (e.g., IL-8), acute phase proteins (e.g., serum amyloid A (SAA)), and inducible enzymes (e.g., inducible NO synthase (iNOS) and cyclooxygenase-2 ((COX-2)), β-defensins and several TLR. These proteins are components of the innate immunity, some of them being essential for the migration of phagocytic leukocytes such as macrophages and neutrophils to the site of infection others facilitating activation/costimulation of leukocytes. Chemokines acts as chemoattractants on specific leukocytes. The cytokine TNF-α can itself activate NF-κB through some of the intracellular signaling proteins being part of the TLR signaling pathway. Expression of TNF-α therefore initiates a positive feedback loop (Liang et al., 2004).
NF-κB is not only involved in innate immunity but also function in adaptive immunity. NF-κB in thymocytes of the thymus regulates transcription of target genes involved in maturation of T cells. Since NF-κB regulates expression of IL-2 in T cells, it also has a role in proliferation and differentiation of T cell. The RelA member of NF-κB regulates the transcription of major histocompatibility complex class 1 (MHC class1) and CD40 in fibroblasts (Liang et al., 2004). MHC class 1 and 2 are transmembrane proteins that present antigens to T cells thereby activating those. CD40 is a transmembrane costimulatory protein that is also necessary for activation of T cell, but also in the activation of B cells by helper T cells (Alberts et al., 2002). Furthermore NFκB also seems to have a function in development of memory cells.

6.12 TH1 or TH2 response

Activation and differentiation of naïve Helper T cells require three types of signals. Stimulation or signal 1, which is mediated by T-cell receptor (TCR) interacting with antigen-MHC class II complex. Costimulation or signal two is mediated by cluster of differentiation 80 (CD80) or CD86 of DCs interacting with CD28 on naïve Helper T cells. (Corthay, 2006). The nature of the third signaling way called polarization is not fully understood. But seems to depend on both the nature of antigen-MHC class II complex, type of costimulation and type of cytokine produced by DCs (Janeway, 2005). The cytokine IL-12 produced by DCs stimulate the development of TH1 cells. TH1 and TH2 cells secrete different types of cytokines and thereby initiate two different kinds of responses (see figure 7). TH1 cells secrete mostly IFN-γ and TNF-α, which activates macrophages to eliminate the pathogens harboured within its phagosome. These cytokines also activate cytotoxic T cells to kill pathogen infected cells. In this manner TH1 cells mostly protects the host against intracellular pathogens. TH2 cells secrete IL-4, IL-5, IL-10 and IL-13. These cytokines stimulates B cells to secrete most types of antibodies and thereby protects the host against extracellular pathogens (Alberts et al., 2002).

DCs can also induce various regulatory T (Treg) cells. Treg cells can suppress both TH1 and TH2 response, either by direct cellular interaction or by secretion of the anti-inflammatory cytokines IL-10 and TGF-β (Guarner et al., 2006). It is believed that IBD patients lack this anti-inflammatory regulation and that an uncontrolled TH1 response cause’s the chronic
inflammation and tissue damage seen in both CD and UC. The hygiene hypothesis suggests that reduced exposure to relatively harmless microorganisms in modern society can be associated with a defective development of immunoregulatory pathways in IBD (Macpherson and Harris, 2004; Guarner et al., 2006).

![Decision-making in the adaptive immune system](image)

**Figure 7** Decision-making in the adaptive immune system. Antigen-presenting DC cells take up foreign antigens and degrade them to immunogenic peptides that are presented to T-cells. These differentiate into T-helper-1 or T-helper-2 cells with different cytokine secretion. Certain DCs can induce regulatory T-cells, which via cytokines IL-10 and TGF-β can suppress both TH1 and TH2 response. Induction is indicated with green arrows, and suppression with red arrows (figure adapted from Guarner et al., 2006).

### 6.13 Distinguishing commensals from pathogens

One way of distinguishing commensals from pathogens is by compartmentalization of PRRs. TLR 3 and TLR7-9 are resident in endosome, TLR5 is located at the basolateral membrane while expression of TLR2 and TLR4 seems to be attenuated in intestinal epithelial cell. Finally NOD1 and NOD2 are located in the cytoplasm. This physically hinders commensal bacteria from adversely inducing immune response and facilitates response against invading pathogens.

Pathogens differ genetically from commensals in their possession of pathogenecity genes often assembled in pathogenicity islands. Pathogenicity islands can be transferred from one bacterium to another by horizontal genetic transfer (transformation, conjugation and
transduction) and their genes encode virulence factors. Virulence factors are proteins such as adhesion molecules, toxins, enzymes and secretion systems that all aids the pathogen in invasion and colonization of intestinal tissue leading to local or systemic infection. Different adhesion molecules mediate the initial adhesion of the pathogen to the intestinal mucosa. Five secretion systems called type I-V secretion system acts in different manners to transfer virulence factors from the pathogen to the intestinal epithelia. Secretion usually occurs subsequently from adhesion. The secreted virulence factors often target epithelia actin filaments of the cytoskeleton thereby enabling to pathogen to move within or between host cells. Or even create cellular compartments within epithelia cells for pathogenic residence. Invasion of the intestinal epithelia usually occurs through the M cells of FAE. Pathogens generally apply one of two different strategies being either invasive or non-invasive. Invasive bacteria such as Salmonella and Shigella are characterized by the ability to invade epithelia and thrive in the hostile environment of Peyer’s patches and lymph nodes. Non-invasive bacteria inject virulence factors into epithelia cells but stay extracellular themselves. (Magalhaes et al., 2007)
7. Mechanisms of action of probiotics

This chapter will present mechanisms suggested as essential factors in the beneficial effects of probiotics. The mechanisms will be divided into three categories: (1) Inhibiting microbial pathogens growth (2) Increasing epithelial barrier function (3) Modulating immune response of intestinal epithelia and mucosal immune cells.

7.1 Inhibiting microbial pathogens growth.

The simplest mechanism for beneficial probiotic action is by competing with the pathogens for nutrition and space, thereby preventing their colonization of the GI. In the pathogenesis of intestinal infections, preliminary steps include penetration through the mucus layer and the adhesion of the pathogenic bacteria to the epithelial cells in the gut. In theory, preventing this adhesion could constitute a therapeutic strategy.

Pathogens attach to epithelial cells on a limited number of receptors situated on the cell surface. One of the principles of probiotic therapy is the occupation of these receptors by probiotic strains of bacteria preventing invasive pathogens from settling.

- Boudeau et al., 2003 reported in an in vitro study the capacity of adhesion compared between a pathogenic strain LF82 of Escherichia coli (E. coli) and the non-pathogenic subspecies E. coli Nissle 1917. The non-pathogenic strain was shown to impair colonization of the pathogen species by forming a biofilm on the cell surface layer of the epithelial cells, thus occupying all adhesive space. The supposed mechanism explaining the dominance of E. coli Nissle 1917 was believed to be an adhesive factor with higher affinity to receptors on the epithelial cell surface (Boudeau et al., 2003). This may be a protective mechanism shared by other probiotic species.

- Fujiwara et al., 1997 reported the existence of a 100 kDa protein produced by Bifidobacterium longum, that in vitro displayed an inhibitory effect on the binding of an E. coli strain (pb 176) to the glycolipid binding receptor, GA1. In 2000 the protein was tested on an enterocyte-like cell with equivalent results, suggesting that some of
the beneficial effects attributed to probiotics may arise from this mechanism (Fujiwara et al., 2001; Bai and Ouyang, 2006).

- Shiba et al., reported that *B. infantis* 1222 inhibited the growth of the bacteroid *B. vulgatus* in co-culture and in the gut of gnotobiotic mice. Gut colonization with *B. vulgatus* increases the number of Peyer’s patches (PP) cells carrying PNA⁺ (peanut agglutinin)/anti-κ⁺ phenotype, which represent plasma cell-like B cells, which increases the production of antibodies. *B. Infantis* 1222 protects the epithelial layer and PP from being invaded by bacteroids such as *B. vulgatus* by decreasing the number of cells carrying PNA⁺/anti-κ⁺ phenotype in PP, thereby suppressing the antibody stimuli induced by *B. vulgatus* (Shiba et al., 2003).

### 7.2. Increasing epithelial barrier function

The intestinal tract is perpetually exposed to microorganisms of the lumen. The intestinal tissue possesses different levels of physical barriers that prevent from constant infection by these microorganisms. The first line of barrier is constituted by the intestinal epithelial cell that produces mucus and glycocalyx which traps bacteria, which are subsequently eliminated by intestinal epistalsis and water hydrous flow. Microvilli present at the apical surface of epithelial cell create a hinder for attachment of bacteria. And most importantly are the firm joinment of epithelial cells by the transmembrane molecular complexes termed tight junctions (Magalhaes et al., 2007). Tight junctions forms sealing strands encircling epithelia cells and functioning as a selective permeability barrier occluding larger objects such as bacteria (Alberts et al., 2002).

- Fermentating commensal bacteria produce short chain fatty acids such as butyrate (see section 8.6) Bordin et al. demonstrated that different types of cells (HeLa and fibroblasts) treated with sodium butyrate had increased expression of the tight junction components occludin, ZO-1, ZO-2 and cingulin. ZO-1 and ZO-2 acts as linkers between transmembrane components of tight junctions and actin proteins of the cytoskeleton. It was shown that the effect was mediated by sodium butyrate acting as an histone deacetylase inhibitor (Bordin et al., 2004). Acetylation of histone facilitates
binding of transcription factors and promotes transcription (Alberts et al., 2002). Inhibition of a histone deacetylase therefore likewise promotes transcription.

- Bruewer et al. demonstrated that pro-inflammatory cytokines IFN-γ and TNF-α compromise the epithelial barrier function. Elevated levels of cytokines were associated with internalisation of transmembrane proteins such as junction adhesion molecule 1 and occludin, involved in tight junction formation (Bruewer et al., 2003). The exact mechanism of how probiotic strains can lower the levels of IFN-γ and TNF-α is not understood. However Yan and colleagues have in two studies shown that Lactobacillus rhamnosus GG (LGG) secretes proteins that inactivate cytokine-activated pathways, initiated by IFN-γ and TNF-α. In the 2007 paper they reported isolating two proteins (p75 and p40) secreted from LGG (and L. casei 334, L. casei 339), both having an inhibitory effect on TNF-induced apoptosis in intestinal epithelial cells but also enhancing the proliferation and survival of the latter due to the activation of Akt and inhibition of MAPK (p38). L. acidophilus was shown to have a similar effect, but required bacterial-epithelial cell contact for the effect (Yan and Polk, 2002; Yan et al., 2007).

- Madsen et al. demonstrated that IL-10 gene-deficient mice treated with VSL#3 resulted in improvement of the intestinal barrier function and reduction of cytokines IFN-γ and TNF-α. They also demonstrated VLS#3 preventing invasion of Salmonella Dublin in T84 monolayer cells. This was attributed to either improvement in barrier integrity or conversely by binding of probiotic bacteria to surface receptors to block S. dublin invasion (Madsen et al., 2001).

### 7.3 Modulating immune response of intestinal epithelia and mucosal immune cells.

Probiotics can modulate inflammatory response in intestinal epithelia and immune cells by interfering with proteins of signal cascades mainly TLR and NOD causing inflammation. Interference can occur at any level of signaling from receptors such as TLRs og NODs
through intracellular signaling proteins such as IκB to transcription factors such as NF-κB. Attenuation of these pathways can lead inhibited expression of pro-inflammatory molecules as cytokines and chemotaxins. In contrast activation of signal cascades can lead to increased expression of immunosuppressive cytokines. A great number of publications addresses this issue, and only some examples will be given in the following of mechanisms.

- Kelly et al., demonstrated that induction of inflammation in rat intestines by infection with *Salmonella enterica* Seravo enteriditis (*S. enteriditis*) could be attenuated by treatment with *B. thetaiotaomicron*. Rats challenged with *S. enteriditis* only showed extensive histological injury to crypts and villi followed by infection of lamina propria. Rats being administered with both *S. enteriditis* and *B. thetaiotaomicron* showed preserved intestinal structure although some infection was seen. They also demonstrated that the cell line Caco-2 (Human colonic adenocarcinoma cells) cultured with *S. enteriditis* only showed increased nuclear translocation of PPARγ concimitant with RelA (see section 6.9). In contrast Caco-2 co cultured with *S. enteriditis* and *B. thetaiotaomicron* promoted nuclear export of PPARγ and RelA. It is therefore proposed that presence of *B. thetaiotaomicron* in a PPARγ-dependent manner mediates attenuation of inflammation in rat intestine (Kelly *et al.*, 2004).

- Neish et al., reported that different nonvirulent Salmonella species can inhibit ubiquination of IκB hindering its degradation and thereby preventing subsequent nuclear translocation of NF-κB in human intestinal epithelia cells (Neish *et al.*, 2000).

- Rachmilewitz et al., reported that the beneficial effect of VSL#3 and *E. coli* (DH5α) is due to the mediating effect of their DNA, which has immunostimulatory activities depending on TLR9. TLR9 signaling plays an essential role in mediating the anti-inflammatory effect of probiotics and suggests that viable probiotics are as effective as nonviable probiotics (γ-irradiated). This finding could play a role for infants and immunocompromised patients, because nonviable probiotics are safer to use due to the reduced risk of bacteraemia (Rachmilewitz *et al.*, 2004).
• Katakura et al., has shown that TLR9 induced by bacterial DNA lead to MyD88 activation, which again activated downstream DNA-dependent protein kinase (DNA-PK). DNA-PK interacted with transcription factors IRF1 and IRF8 promoting expression of the immunosuppressive type I interferon α and β (IFNα and IFNβ) (Katakura et al., 2005).

• Rakoff-Nahoum et al., reported that TLRs often mediate signaling through MyD88 dependent signal cascades (see section 6.7). The anti-inflammatory effect MyD88 has also been demonstrated by MyD88-deficient mice being more susceptible to DSS induced colitis than wild type mice (Rakoff-Nahoum et al., 2004).

• Sturm et al., reported in a study demonstrated that *E. coli* Nissle 1917 could modulate cytokine expression, cell cycling and proliferation of peripheral blood T cells (PBC). PBCs are resident in the systemic circulation but are recruited to mucosa upon inflammation. Anti-CD3-stimulated PBCs treated with *E. coli* Nissle 1917 medium showed decreased expression of pro-inflammatory TNF-α, IL2, IFN-γ but increased expression of immunosuppressive IL10. Cell cycle was inhibited by decreased expression of cyclin D1 and cyclin B2 and therefore restriction of T cell proliferation. In a parallel study it was shown that PBCs from TLR2 knockout mice treated with *E. coli* Nissle 1917 had normal cell cycle progression, whereas PBCs from wildtype mice had reduced cell cycle progression. It was therefore proposed that the inhibitory effect of *E. coli* Nissle 1917 on PBCs were TLR2-dependent. Since bacterial lipoproteins (BLP) demonstrated a inhibition of PBC mimicking that of *E. coli* Nissle 1917, it was suggested that *E. coli* Nissle 1917 exerted its effect by BLPs (Sturm et al., 2005).

• Yan et al., reported that they isolated two proteins (p75 and p40) secreted from *Lactobacillus rhamnosus* GG (LGG), both having an inhibitory effect on TNF-induced apoptosis in intestinal epithelial cells but also enhancing the proliferation and survival of the latter due to the activation of Akt and inhibition of MAPK (p38). Akt was activated by 10-1000ng/mL of p75 and 1-100ng/mL of p40 in a dose-dependent manner. Also *L casei* 334, *L casei* 339 but not *L acidophilus* supernatants were able to promote Akt activation and prevent TNF-induced apoptosis (Yan et al., 2007).
• McCarthy et al., demonstrated, in a double blind, placebo controlled trial, that *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* 433118 had beneficial effects on colitis in IL-10 knockout mice. The improvement was a result of reduced levels of Th-1 pro-inflammatory cytokine (INF-γ, TNF-α and IL-12). In all groups the level of TGF-β (Th-3) was maintained. *B. infantis* 35625 had a larger effect in reducing pro-inflammatory cytokine production than *L. salivarius* 433188 (McCarthy et al., 2003).

• Delmasso et al., reported that *S. boulardii* treatment inhibits inflammation by decreasing the activation of NF-κB, reducing the level of cytokine expression of TNF-α, IL-1β, IFN-γ and IL-6 in the colon of CD4⁺CD45RB⁺ transferred severe combined immunodeficiency (SCID) mice with developed colitis. *S. boulardii* produces a small water soluble molecule called SAIF (Saccharomyces anti-inflammatory factor), which mediates the inhibition of NF-κB. This inhibition involves prevention of IκBα degradation (see section 6.7) and thereby hindering of nuclear transport of NF-κB (Sougioultzis et al., 2006). The beneficial effect of *S. boulardii* is dose-dependent and the probiotic must be ingested daily for maintaining the anti-inflammatory effect. *S. boulardii* limits the infiltration of TH1 cells into the inflamed colon. This effect is caused by accumulation of IFN-γ producing TH1 cells within the mesenteric lymph nodes. These findings suggest that *S. boulardii* may be used as a therapeutic tool in IBD (Dalmasso et al., 2006).
8. Probiotics species

Probiotics have been defined as live microorganisms, which when administered in adequate amounts, confer a health benefit to the host. In order to assure safety for the patients a reliable probiotic product requires correct identification of the probiotic species applied and acknowledgement of the administered amounts. Since probiotics are not recognized as drugs but merely as nutritional supplements, no documentation regarding efficacy are required by the Food and Drug Administration (FDA) or national health agencies (Del Piano et al., 2006). Probiotics species such as lactobacilli, *E. coli* Nissle 1917 and bifidobacteria are often isolated from healthy gut flora and has a history of lack of toxicity. A safe probiotic should not give rise to allergies and should not be able to colonize outside the GI. Probiotics should be either acid tolerant or be encapsulated to ensure their safe passage through the stomach (Del Piano et al., 2006). Some probiotic species are selected based on their inability to colonize the intestinal tract such as *Saccharomyces boulardii* and *Trichuris suis* (helminths from pigs).

Animal and *in vitro* models are used to determine which probiotics (bacteria and yeast) have a beneficial influence on experimental colitis. The models are also utilized to improve the use and safety of probiotics, to detect side effects and to reveal the underlying mechanism of their beneficial effect. The most commonly used probiotics, which also are the organisms present in our selection of clinical trials, will be described below. All probiotics may posses all three mechanism of action, but mechanism of action will be listed according to our findings in section 7. Furthermore different subspecies of the same group may not posse’s alike mechanisms of action. This is likely due to subspecies differences in surface proteins and secreted metabolic products.

8.1 Lactobacilli species

Lactobacilli species are anaerobe gram-positive rods (1-9 μm). They are acid fermentative and therefore also acid tolerant, which allows their colonization of the entire GI including the duodenum at a low pH approaching 2. Lactobacilli only represent about 0.01% of the intestinal commensal flora, they are rarely pathogenic and mostly found in dairy products.
Commonly used Lactobacilli species as probiotics include: *L. acidophilus*, *L. bulgaricus*, *L. rhamnosus GG*, *L. johnsonii*, *L. lactis*, *L. plantarum*, and *L. reuteri*.

- Mechanism of action contributed to lactobacilli species are 7.2 Increasing epithelia barrier function and 7.3 Modulating immune response of intestinal epithelia and mucousal immune cells.

### 8.2 Bifidobacteria species

Bifidobacteria species are anaerobic gram-positive bacteria and inhabitants of a healthy human colon accounting for app. 10% of the commensal microflora (Shiba *et al.*, 2003). They are bile and acid tolerant, and survive the passage through the GI.

- Mechanism of action contributed to bifidobacteria is 7.1 Inhibiting microbial pathogen growth and 7.3 Modulating immune response of intestinal epithelia and mucosal immune cells.

### 8.3 *Escherichia coli Nissle 1917*

The strain is a non-pathogenic member of the gram-negative bacteria species *E. coli*. It was originally isolated during Word War I from a soldier who withstood a severe outbreak of diarrhoea (Boudeau *et al.*, 2003)

- Mechanism of action contributed to bifidobacteria is 7.1 Inhibiting microbial pathogen growth, 7.2 Increasing epithelia barrier function and 7.3 Modulating immune response of intestinal epithelia and mucosal immune cells.

### 8.4 *Saccharomyces boulardii*

*S. boulardii* is a non-pathogenic, non-systemic yeast, first isolated from lychee and mangosteen fruit in 1923. It is known to be beneficial in preventing and treating a variety of
diarrheal diseases by maintaining and restoring the natural flora in the large and small intestine.

- Mechanism of action contributed to \( S. \ boulardii \) is 7.3 Modulating immune response of intestinal epithelia and mucosal immune cells.

8.5 VSL#3

VSL#3 is a probiotic cocktail containing viable lyophilized gram-positive bacteria of 4 strains of lactobacilli (\( L. \ casei, L. \ plantarum, L. \ acidophilus, \) and \( L. \ delbrueckii \ ssp. \ bulgaricus \)), 3 strains of bifidobacteria (\( B. \ longum, B. \ breve, \) and \( B. \ infantis \)) and one strain of \( Streptococcus \ salivarius \ ssp. \ Thermophilus \).

- Since VSL#3 is a combination of several probiotics species the mechanism of action contributed to VSL#3 is 7.1 Inhibiting microbial pathogen growth, 7.2 Increasing epithelia barrier function and 7.3 Modulating immune response of intestinal epithelia and mucosal immune cells.

8.6 Prebiotics

Prebiotics are substances destined to promote growth and metabolism of the commensal bacteria flora. Most prebiotics are present as nondigested carbohydrates such as fructo-oligosaccharides, inulin, psyllium or germinated barley extracts, which are all substances that support the populations of Lactobacilli and Bifidobacteria. Apart from improving the conditions of survival for these beneficial bacteria, one of the interesting potentials of prebiotic treatment lies in its stimulating effect on the production of short chain fatty acids such as butyrate. The term symbiotic defines the therapeutic assembly of probiotics and prebiotics, combining the beneficial properties of both methods.

- Anti-inflammatory properties are also attributed to butyrate through inhibition of the binding activity of NF-kB to DNA in epithelial cells, thus reducing the production of proinflammatory cytokines such as IL-6 and IL-8 (Kanauchi et al., 1999).
In accordance with these findings, Kanauchi et al., reports on basis of endoscopic observations that bacterial production of butyrate in the colon had a beneficial effect on mucosal damage in Ulcerative Colitis (Kanauchi et al., 2003). To this day however, the experimental data supporting the beneficial use of prebiotics is even less elucidated than that of probiotics (Sartor, 2004).
9. Clinical studies

We want to assess the clinical effectiveness of probiotics in the treatment of IBD (with focus on CD and UC). In order to do that, we searched the PubMed\(^1\) database in April 2007 for reports on clinical studies.

9.1 Search methods for finding studies

To locate relevant articles we used the search term “probiotic*” and in an attempt to find as many articles as possible we also added terms for specific organisms (since not all authors use the term “probiotics”): “trichuris*”, “lactobacillus*”, “saccharomyces*” and “escherichia*”. We then narrowed the search by adding search terms for the individual diseases: “ulcerativ*” or “crohn*”. The search was further refined by adding the search terms; “random*” and “NOT review”. So in an attempt to find articles reporting on randomized clinical trials using probiotics on ulcerative colitis, we would use the search string: “probiotic* OR trichuris* OR lactobacillus* OR saccharomyces* OR escherichia* AND ulcerativ* AND random* NOT review”. A few additional articles were found by manual search (i.e. we found them mentioned in other articles).

9.2 Study selection

In order to assess the relevance and quality of the resulting studies we rated them using the Jadad-scale (Jadad et al., 1996). The Jadad-scale is an attempt to create an instrument to evaluate clinical trials in an easy and unbiased way. Instead of judging the articles on whom the authors are, which institutions they are from or dazzling figures in the articles; the Jadad-scale evaluate simple methodical parameters: whether the trial was properly randomized and blinded, and whether all enrolled patients are described – also the ones that drop out of the trial. We chose to use studies that scored 2 or more points on the scale (see Appendix B for more details about the Jadad-scale).

\(^1\) PubMed is a public database maintained by the National Center for Biotechnology Information (NCBI). It gives free access to the over 12 million abstracts in MEDLINE. The database can be accessed at: http://www.pubmed.gov.
9.3 Description of studies

We found a total of 30 articles reporting on studies of probiotic treatment of IBD (16 on CD and 14 on UC). Not all trials fulfilled our selection criteria of 2 points on the Jadad-scale and some of them were duplicates describing the same studies. Below we list the trials that we chose to include in this analysis, with a short description and mention the excluded trials, with a short explanation of why they were not included.

9.3.1 Crohn’s Disease

Of the 16 reports six were excluded from the analysis. One study evaluated the effect of dietary yeast on CD and was not a probiotic intervention trial (Barclay et al., 1992). Another study tested the effect of VSL#3 on arthralgia in patients with IBD (Karimi et al., 2005). The third study was an un-blinded study containing both CD and UC patients (Zocco et al., 2003). Two were duplicate reports on an open label pilot study, without control group (Gupta et al., 2000; Guandalini, 2002). The last excluded study was a derivative of an included study (Prantera and Scribano, 2002). The included reports are described below. (Best et al., 1976)

Plein and Hotz 1993

In this single centre, randomized, double-blind, placebo-controlled study 20 patients with mildly active CD were treated with Saccharomyces boulardii as a supplement to conventional treatment. All patients received 250 mg Saccharomyces boulardii three times a day for two weeks. Symptoms in the form of frequency of bowel movements and CDAI were compared before and after, and an improvement was registered (Bowel movements went from 5.0 ± 1.4 to 4.1 ± 2.3 evacuations/day and CDAI\(^1\) went from 193 ± 32 to 168 ± 59). The 17 remaining patients were then allocated randomly into a placebo group of 7 patients and a treatment group of 10 patients continuing Saccharomyces boulardii for seven weeks. After 10 weeks the two groups were evaluated: the treatment group had a frequency of bowel movements of 3.3 ± 1.2 evacuations/day and a CDAI of 107 ± 85. The placebo group had 4.6 ± 1.9 evacuations/day and a CDAI of 180 ± 61. The amelioration of symptoms after the initial two weeks treatment with Saccharomyces boulardii was significant, but was largely due to

\(^{1}\) Crohn’s Disease activity index was suggested by Best et al., 1976 as a clinical index for CD. It uses a number of clinical and biochemical values to assess the disease.
improvement in frequency of bowel movements and to a lesser extent in CDAI. In the final comparison between treatment and placebo, the small number of subjects and large variations within the two groups rendered the result insignificant. No adverse effects were reported (Plein and Hotz, 1993).

Malchow 1997
In this single centre, randomized, double-blind, placebo-controlled study 28 patients with active CD (CDAI > 150) were treated with prednisolone (60 mg/day) + probiotics OR prednisolone + placebo. The patients were randomly assigned into two groups; a group of 16 patients who received prednisolone + *Escherichia coli* strain Nissle 1917 (two capsules of 100 mg/day (2.5x10^10 viable bacteria per capsule)) AND a control group of 12 patients who received prednisolone + placebo. Both groups were assessed 11 times during the one year study. The CDAI index was calculated and remission was defined as CDAI < 150. In the *E. coli* group 75% reached remission (12/16) and in the placebo group 91.7% (11/12). Of the patients that reached remission in the *E. coli* group 33.3% experienced relapse (4/12). In the placebo group it was 63.6% (7/11). However, because of the low number of patients these differences were not significant. No adverse effects were reported (Malchow, 1997).

Guslandi 2000
In this single centre, randomized, single-blind (investigator), controlled study 32 patients with CD in remission (CDAI < 150) received maintenance treatment either with mesalazine OR with reduced mesalazine and *Saccharomyces boulardii*. Patients had to be in remission 3 month prior to the beginning of the study and had to be off immunosuppressant or steroid treatment for 3 month before entry. Patients were randomly allocated into two groups; a control group of 16 patients who received full dose of mesalazine (3g/day) AND a group of 16 patients who received reduced mesalazine (2g/day) and *Saccharomyces boulardii* (1g/day). Both groups were assessed 3 times during the six month study; at entry and after 3 and 6 months. CDAI was calculated and relapse was defined as CDAI > 150 with an increase of 100 points over baseline for more than two weeks. In the *Saccharomyces boulardii* group 6.25%

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1 When the patient reacted to prednisolone treatment the dose was tapered to 5 mg/day for one month prior to discontinuation. If symptoms worsened treatment with high concentrations was resumed.

2 Two patients in the *E. coli* group were in remission at the beginning of the trial, but were included in the analysis on an intention-to-treat basis. Without these 85.7% in the *E. coli* group reached remission (12/14).
experienced relapse (1/16) and in the control group it was 37.5% (6/16). Because of the low number of patients this result was not significant. No adverse effects were reported (Guslandi et al., 2000).

Rizzello 2000
In this single centre, randomized, single-blind (investigator), controlled study 40 patients with surgically induced remission were treated with either an antibiotic followed by VSL#3 OR with mesalazine. Patients were randomly allocated into two groups; a treatment group of 20 patients who received rifaximin (1.8 g/day) for 3 month followed by VSL#3 (6 g/day (3x10^{11} viable bacteria/g)) for 9 month AND a control group of 20 patients who received mesalazine (4 g/day) for 12 month. Both groups were assessed by an independent physician after 3 and 12 month in this one year study and relapse was defined endoscopically. After one year 20% (2 after 3 months and 2 after 12 months) in the antibiotic/VSL#3 group and 40% (8 after 3 months) in the mesalazine group experienced relapse. This result was not statistically significant. No side effects were reported in the antibiotic/VSL#3 group, two patients in the mesalazine group experienced mild nausea (Rizzello et al., 2000).

Prantera 2002
In this single centre, randomized, double-blind, placebo-controlled pilot study 45 patients with surgically induced remission were treated with either *Lactobacillus rhamnosus* strain GG (LGG) OR placebo. Patients were excluded if they required antibiotic treatment for more than 10 days after surgery, steroids for more than 30 days or were not able to start oral nutrition within 10 days after surgery. Patients were allocated into two groups; a treatment group of 23 patients who received LGG (2.46g twice a day (1.2x10^{10} colony forming units/day)) AND a control group of 22 patients receiving placebo. Both groups were assessed after 13, 26, 39 and 52 weeks of treatment in this one year study. Patients were assessed clinically and endoscopically. Clinical remission was defined as CDAI < 150 and endoscopic remission as < 1 in the Rutgeert’s scoring system (Rutgeerts et al., 1990). At the end of the study 83.3% of the probiotic group was in clinical remission (15/23) and 89.4% in the placebo group (17/22). Of the patients in clinical remission 40.0% were in endoscopic remission in the probiotics group (6/15) and 64.7% in the placebo group (11/17). There was no significant benefit of the probiotic treatment. No trial related side effect were reported (Prantera et al., 2002).
In this single centre, randomized, double-blind, placebo-controlled study 11 patients with moderate to active CD (CDAI 150 - 300) were treated with antibiotics and corticosteroids to induce remission and then with *Lactobacillus rhamnosus* strain GG (LGG) OR placebo to maintain remission. All patients started on a 12 week tapered corticosteroid treatment, beginning with 60 mg/day. The first two week of the study they also received antibiotic treatment (ciprofloxacin 500mg twice a day and metronidazole 250 mg three times a day). Following the antibiotic treatment the patients were randomly allocated into two groups; a treatment group of 5 patients received LGG (2x10⁹ colony forming units/day) AND a group of 6 patients received placebo. During the six months study patients were assessed after 2, 4, 8, 12, 18 and 26 weeks. CDAI was calculated and relapse was defined as an increase in CDAI of > 100 points. After 6 months 80% of the patients in the LGG group sustained remission (4/5) and 83.3% in the placebo group (5/6). Of the patients that reached remission in the LGG group 50% experienced relapse (2/4); this number was 60% in the placebo group (3/5). There was no significant difference between LGG and placebo treatment. Apart from mild bloating experienced by a few patients in both groups, no side effects were reported (Schultz *et al.*, 2004).

In this multi-centre, randomized, double-blind, placebo-controlled study 75 children (age range 5 - 21 yr) with CD in remission (Paediatric Crohn’s Disease Index (PCDAI) ≤ 10 points) were treated with *Lactobacillus rhamnosus* strain GG (LGG) OR placebo to maintain remission. Patients were allowed treatment with aminosalicylates, 6-mercaptopurine, azathioprine and low-dose alternate day corticosteroids. Patients were randomly allocated into two groups; a treatment group of 39 patients received one capsule of LGG twice per day (10¹⁰ bacteria and 295 mg inulin/capsule) AND a group of 36 patients received placebo. The study was designed as a 2 year study with examinations every 3 month (or at time of relapse). Relapse was defined as PCDAI > 30 points one time or as PCDAI > 15 points on any 2 consecutive examinations more than 1 week apart. The study was stopped before time by the Data and Safety Monitoring Board overseeing the study, because of slow recruitment and lack of efficacy. At this point the median follow-up time for the LGG group was 9.8 months and 11.7 for the placebo group. In the LGG group 39% experienced relapse (12/39) and in the
placebo group this number was 17% (6/36). This was not a significant difference. No adverse effects related to LGG were reported (Bousvaros et al., 2005).

*Marteau 2006*

In this multi-centre, randomized, double-blind, placebo-controlled study 98 patients with CD in remission after surgery received treatment with either *Lactobacillus johnsonii* LA1 (LA1) or placebo. No other treatment was allowed in the study. Patients were randomized into two groups; a treatment group of 48 patients receiving two packets per day of LA1 (2x10⁹ colony forming units per packet) AND a control group of 50 patients receiving placebo. Patients were examined at inclusion and after 3 and 6 months, in this six months study. CDAI was calculated and clinical recurrence was defined as CDAI > 200 points. Ileocolonoscopy was performed after six months or in the case of clinical recurrence, and endoscopic recurrences were scored using the Rutgeert’s scoring system. Endoscopic recurrences were observed in 49% of the patients in the LA1 group (21/43) and in the placebo group it was 64% (30/47). This was not a significant difference. No adverse effects related to the treatment were reported (Marteau et al., 2006).

*Van Gossum 2006*

In this multi-centre, randomized, double-blind, placebo-controlled study 70 patients with CD in remission after surgery were treated with either *Lactobacillus johnsonii* LA1 (LA1) or placebo. No other medication was allowed during the study. After surgery patients were randomly allocated into two groups; 34 patients received LA1 (10¹⁰ colony forming units/day) AND 36 patients received an identical placebo. The patients were examined endoscopically after 12 weeks or on relapse, with relapse defined as CDAI > 150 with an increase of 70 points or higher over base line. En endoscopic recurrences were scored using the Rutgeert’s scoring system. On an intention-to-treat analysis the percentage of patients with severe recurrences were 21% in the LA1 group (6/28) and 15% in the placebo group (4/27). There was no significant benefit of the treatment. No adverse effect were reported (Van Gossum et al., 2006).
Chermesh 2007

In this multi-centre, randomized, double-blind, placebo-controlled study 30 patients with CD in remission after surgery were treated with either Synbiotic 2000\(^1\) or placebo. The patients were randomized in a 2:1 ratio to receive either one daily dose of Synbiotic 2000 \((n = 20)\) OR placebo \((n = 10)\). The study was planned to run over 24 months. Patients were assessed endoscopically (Rutgeert’s scoring system), clinically (bowel movements, abdominal pain and weight) and by laboratory tests (complete blood count, C-reactive protein, haemoglobin and albumin). Only nine patients completed the study; seven from the treatment group and two from the placebo group. From the endoscopic, clinical and laboratory data collected underway the researchers concluded that was no significant benefit from the treatment with Synbiotic 2000 in preventing post surgical recurrence of CD. Reasons for the 21 withdrawal were: self-withdrawal (8), exacerbation (7), arthritis and arthralgia (2), pregnancy (2), fistula (1) and postoperative complications (1). Apparently the withdrawals were not related to the treatment (Chermesh et al., 2007)

9.4.1 Ulcerative colitis

Seven of the fourteen reports were excluded from the analysis for various reasons. One study, which also came up in the CD search was not an intervention trial (Karimi et al., 2005). Three of the trials did not include a control group (Venturi et al., 1999; Guslandi et al., 2003; Bibiloni et al., 2005). Three studies was not blinded (Ishikawa et al., 2003; Tursi et al., 2004; Zocco et al., 2006) and the last excluded study was a duplicate report on an included trial (Macfarlane et al., 2005). The included trials are described below.

Kruis 1997

In this multi-centre, randomized, double-blind, double-dummy controlled study 120 patients with UC in remission (defined as CAI \(\leq 4\)\(^2\) were treated with E. coli Nissle 1917 or with mesalazine. No other treatments were allowed during the 12 week study period. 17 patients

\(^1\)Synbiotic 2000 is a mixture of probiotics and prebiotics. One daily dose in the study contained 4 lactic bacteria: \(10^{10}\) _pedacoccus pentoseceus_, \(10^{10}\) _Lactobacillus raffinolactis_, \(10^{10}\) _Lactobacillus paracasei ssp. Paracasei_ 19 and \(10^{10}\) _Lactobacillus plantarum_ 2362; and the 4 prebiotics 2.5 g \(\beta\)-glucans, 2.5 g inulin, 2.5 g pectin and 2.5 g resistant starch.

\(^2\)Clinical activity index as defined by Rachmilewitz, 1989. The index rates clinical occurrences such as number of stools per week, blood in stool and abdominal pain/cramps.
were excluded before the beginning of the trial, but after randomization, because of CAI \( \geq 4 \). The treatment group \((n=50)\) received 200 mg/day of *E. coli* Nissle 1917 \((5\times10^{10} \text{ cfu/day})\) plus a mesalazine placebo. The control group \((n=53)\) received 500 mg mesalazine three times a day and a placebo indistinguishable from the *E. coli* preparation. CAI was calculated at weeks 0, 2, 4, 8 and 12. Remission was defined as CAI \( \geq 4 \). In the treatment group 16.0\% \((8/50)\) experienced relapse; in the control group it was 11.3\% \((6/53)\). There was no statistically significant difference between the two groups. No adverse effects were reported (Kruis *et al.*, 1997).

**Rembacken 1999**

In this single-centre, randomized, double-blind, double-dummy controlled study 116 patients with active UC were treated with *E. coli* Nissle 1917 or with mesalazine. Remission was achieved by treatment with prednisolone for maximum 12 weeks \((30\) or \(60 \text{ mg/day depending on severity of symptoms})\). At remission 57 patients were randomized to receive 400 mg/day of *E. coli* Nissle 1917 \((5\times10^{10} \text{ cfu/day})\) plus a mesalazine placebo. The 59 patients in the control group received 800 mg mesalazine three times a day and an *E. coli* placebo. Patients were reviewed monthly by their clinical symptoms and in the case of relapse by sigmoidoscopy \((\text{to confirm relapse})\). 39 patients in *E. coli* group reached remission \((68\%)\) and in the mesalazine group it was 44 \((75\%)\). In the *E. coli* group 26 \((67\%)\) patients experienced relapse before the end of the 12 month study. In the mesalazine group it was 32 patients \((73\%)\). These were statically comparable results. No adverse effects were reported (Rembacken *et al.*, 1999).

**Kruis 2004**

In this multi-centre, randomized, double-blind, double-dummy controlled study 327 patients with UC in remission \((\text{defined as CAI} \leq 4)^1\) were treated with *E. coli* Nissle 1917 or with mesalazine. Patients were randomly allocated into two groups: a control group with 165 patients receiving 500 mg mesalazine three times a day plus an *E. coli* placebo AND a group of 162 patients who received 200 mg/day of *E. coli* Nissle 1917 \((5\times10^{10} \text{ cfu/day})\) plus a mesalazine placebo. The trial lasted 12 month and patients were assessed after 1,2,3,6 and 9

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\(^1\) Clinical activity index as defined by Rachmilewitz, 1989. The index rates clinical occurrences such as number of stools per week, blood in stool and abdominal pain/cramps.
months and CAI was calculated. Relapse was defined as CAI ≥ 4 or an increase in CAI of more than 3 points. Patients were also evaluated endoscopically\(^1\) to confirm relapse. 110 patients in the \textit{E. coli} group and 112 in the mesalazine group completed the study. 36.4 patients in the \textit{E. coli} group experienced relapse (40/110) compared with 33.9% in the mesalazine group (38/112). This was significantly equivalent. No adverse effects were reported (Kruis \textit{et al.}, 2004).

\textit{Cui} 2004

In this single-centre, randomized, single-blind, placebo-controlled study, thirty patients with UC in remission received maintenance treatment with BIFICO (Entreococci, Bifidobacteria and Lactobacilli) or with a placebo. Remission was induced by treatment with a combination of sulphasalazine and glucocorticoids. When in remission the patients were randomly allocated into two groups: a treatment group of 15 patients who received 1.26 g/day of BIFICO and a control group of 15 patients who received an identical placebo (starch). The patients were assessed clinically and endoscopically after 2 weeks and at the end of the 8 week study (or at relapse). Biopsy samples were taken and analysed for expression levels of NF-κB and IL-10. Three patients in the BIFICO group experienced relapse (20%) compared with 14 (93.3%) in the placebo group. This was a significant difference. It was also shown that the level of NF-κB mRNA was significantly decreased in the BIFICO group and that the level of IL-10 was elevated compared with the control group. No adverse effects were reported (Cui \textit{et al.}, 2004).

\textit{Kato} 2004

In this multi-centre, randomized, double-blind, placebo-controlled study twenty patients with mild to moderate active UC were treated with bifidobacteria-fermented milk (BFM) or with a similar placebo, both in addition to conventional treatment (3-4 g/day of salazosulphapyridine or 2250-3000 mg/day of mesalazine). Patients were randomly allocated into two groups: a treatment group who received 100 mL/day of BFM and a placebo group who received 100 mL/day of a similar placebo. Patients were assessed at week 4, 8 and 12 in this three month study. The patients were evaluated clinically and endoscopically. At the end of the study the CAI score in the BFM group had decreased from 7.9 ± 0.8 to 3.7 ± 0.4 compared to the

\(^1\) Endoscopic indicators were: granularity, vascular pattern, vulnerability of mucosa and mucosal damage.
placebo group that went from 7.9 ± 0.6 to 5.8 ± 0.8. This was a significant difference. The endoscopically score in the BFM group went from 4.4 ± 0.3 to 3.1 ± 0.3 compared to the placebo group that went from 3.6 ± 0.3 to 3.0 ± 0.4. No adverse effects were reported (Kato et al., 2004).

Furrie 2005
In this multi-centre, randomized, double-blind, placebo-controlled study eighteen patients with active UC were treated with a probiotic (Bifidobacterium longum) and a prebiotic (Synergy 1) OR a placebo, for a period of one month. No other treatment was allowed. Patients were randomly allocated into two groups: a treatment group that received 2x10^{11} viable Bifidobacterium longum in a capsule and a sachet with 6 g of synergy 1 and a placebo group who received similar placebos. The patients were assessed at the beginning of the study and after one month. Patients were scored clinically and endoscopically and biopsy samples were taken for mRNA analysis. The clinical scores were not significantly different between the two groups: the synbiotic group started at 5.6 ± 3.7 and ended at 5.3 ± 3.4. The placebo group started at 4.9 ± 3.2 and ended at 3.1 ± 2.5. The endoscopic score in the synbiotic group went from 4.5 ± 1.4 to 3.1 ± 2.5 compared with the placebo group that went from 2.6 ± 2.1 to 3.2 ± 2.2. This was not significant either, probably because of the low number of patients. However the mRNA levels of defensins 2, 3 and 4 were significantly reduced in the synbiotic group as compared to the placebo group and the mRNA levels of pro-inflammatory cytokines TNF-α and IL-1 were also significantly lower in the synbiotic group. No adverse effects were reported (Furrie et al., 2005).

Summers 2005
In this single-centre, randomized, double-blind, placebo-controlled study 54 patients with active UC (defined by UCAI ≥ 4) were treated with ova from the pig helminth Trichuris suis or with a placebo. Patients were allowed to continue conventional treatment such as mesalazine or prednisolone (< 25 mg/day) if the treatment had been given for more than 8 weeks prior to the start of the study. Patients were randomly allocated into two groups: a

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1 Synergy 1 is a fructo-oligosaccharide/inulin mix designed to stimulate the probiotic bacteria.

2 Ulcerative colitis activity index (UCAI) is a clinical index described by Sutherland et al., 1987. It indexes 4 variables: stool frequency, severity of bleeding, mucosal appearance and the physician’s overall assessment of the disease activity.
treatment group of 30 patients who received 2500 *T. suis* ova every second week for 12 weeks or a placebo group of 24 patients who received a placebo. The patients UCAI were calculated at 0, 6 and 12 weeks. In the ova treatment group 13 of 30 patients (43.3%) experienced a decrease in UCAI compared to 4 of 24 patients in the placebo group (16.7%). This was a significant difference. However the difference in the proportion of patients who achieved remission (UCAI ≤ 2) was not significant. No adverse effects from the treatment were reported (Summers *et al.*, 2005).

### 9.5 Analysis

From the data presented in the selected clinical studies we will address the following issues:

- Are some types or species of probiotics more efficient in the treatment of CD and UC?
- Are combinations of several strains of probiotics more efficient than single strains treatment?
- Are large doses of probiotics more efficient than low doses?

#### 9.5.1 Clinical studies with Crohn’s Disease

The selected clinical studies with CD are listed in table 2. The studies are ordered after probiotic species – with the lowest concentration first.

From the clinical data we conclude that LGG, LA1 and Synbiotic 2000 have no efficacy in treatment of CD with the administered doses. The ten-fold difference in doses in the LGG and LA1 studies did not show any visible difference. VSL#3 that also contains lactobacilli species showed some efficacy (though not significant) but because of the experimental design it was not possible to determine if this was due to the initial antibiotic treatment or the following probiotic treatment. *E. coli* Nissle 1917 was more efficient than placebo in one small study. *S. boulardii* also showed some efficacy in two studies with almost similar doses.

The efficacy for these last three studies were not significant because of the low number of subjects enrolled, this was a general problem in all the studies with CD. None of the probiotic...
species or cocktails showed significant efficacy as compared to placebo/Mesalazine\(^1\) in obtaining remission or maintaining remission. It therefore seems that neither the probiotic strains nor concentrations used affect the mechanisms causing CD. **Conclusion: from these data probiotics cannot be recommended as clinical treatment for CD.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Size (n)</th>
<th>Duration (weeks)</th>
<th>Probiotic</th>
<th>Control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plein and Hotz, 1993</td>
<td>20</td>
<td>10</td>
<td><em>S. boulardii</em> (750 mg/day)</td>
<td>Placebo</td>
<td>+</td>
</tr>
<tr>
<td>Guslandi <em>et al.</em>, 2000</td>
<td>32</td>
<td>26</td>
<td><em>S. boulardii</em> (1000 mg/day)</td>
<td>Mesalazine</td>
<td>+</td>
</tr>
<tr>
<td>Malchow, 1997</td>
<td>28</td>
<td>52</td>
<td><em>E. coli Nissle 1917</em> (5x10(^{10}) cfu/day)</td>
<td>Placebo</td>
<td>+</td>
</tr>
<tr>
<td>Rizzello <em>et al.</em>, 2000</td>
<td>40</td>
<td>52</td>
<td>VSL#3 (3x10(^{11}) cfu/day)</td>
<td>Mesalazine</td>
<td>+</td>
</tr>
<tr>
<td>Schultz <em>et al.</em>, 2004</td>
<td>11</td>
<td>26</td>
<td>LGG (2x10(^{9}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
<tr>
<td>Pranterra <em>et al.</em>, 2002</td>
<td>45</td>
<td>52</td>
<td>LGG (1.2x10(^{10}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
<tr>
<td>Bousvarus <em>et al.</em>, 2005</td>
<td>76</td>
<td>40(^*)</td>
<td>LGG (2x10(^{10}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
<tr>
<td>Marteau <em>et al.</em>, 2006</td>
<td>98</td>
<td>26</td>
<td>LA1 (2x10(^{9}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
<tr>
<td>Van Gossum <em>et al.</em>, 2006</td>
<td>70</td>
<td>12</td>
<td>LA1 (10(^{10}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
<tr>
<td>Chermesh <em>et al.</em>, 2007</td>
<td>30</td>
<td>104</td>
<td>Synbiotic 2000 (4x10(^{10}) cfu/day)</td>
<td>Placebo</td>
<td>‖</td>
</tr>
</tbody>
</table>

*Table 2* Clinical studies with Crohn's disease. Cfu: colony forming units. Explanation to the results: ‖ means same as placebo/mesalazine; + means better than placebo/mesalazine – but not significantly better. \(^*\)Terminated ahead of time 40 weeks was the average treatment time.

\(^1\) Mesalazine has no significant effect on CD (Akobeng and Gardener, 2005).
9.5.2 Clinical studies with Ulcerative colitis.

The selected clinical studies with UC are listed in table 3. The studies are ordered after probiotic species – with the lowest concentration first.

<table>
<thead>
<tr>
<th>Study</th>
<th>Size (n)</th>
<th>Duration (weeks)</th>
<th>Probiotic</th>
<th>Control</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kruiz et al., 1997</td>
<td>120</td>
<td>10</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Mesalazine</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5x10^10 cfu/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kruiz et al., 2004</td>
<td>327</td>
<td>52</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Mesalazine</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5x10^10 cfu/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rembacken et al., 1999</td>
<td>116</td>
<td>52</td>
<td><em>E. coli</em> Nissle 1917</td>
<td>Mesalazine</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10x10^10 cfu/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cui et al., 2004</td>
<td>30</td>
<td>8</td>
<td>BIFICO</td>
<td>Placebo</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.26 g/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kato et al., 2004</td>
<td>20</td>
<td>12</td>
<td>BFM</td>
<td>Placebo</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10^10 cfu/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furrie et al., 2005</td>
<td>18</td>
<td>4</td>
<td><em>B. longum</em></td>
<td>Placebo</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2x10^{11} CFU/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summers et al., 2005</td>
<td>54</td>
<td>40</td>
<td><em>Trichuris suis</em></td>
<td>Placebo</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2500 ova/day)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Clinical studies with ulcerative colitis. Cfu: colony forming units. Explanation to the results: + means better than placebo – but not significantly better; ++ means significantly better than placebo or same as mesalazine.

There were three studies with *E. coli* Nissle 1917 with a total of 563 subjects. The doses were comparable and all three studies the control group received treatment with Mesalazine. Therefore we conclude that *E. coli* Nissle 1917 was comparable to Mesalazine in efficacy. Two minor studies compared BIFICO and BFM to placebo, concluded that they both were significantly better than placebo in maintaining remission (BIFICO) or as a supplement to conventional treatment (BFM). The combination of VSL#3 and low-dose Balsalazide was compared to normal dose of either Balsalazide or Masalazide alone. VSL#3/Balsalazide showed the same efficacy as the single standard treatments. *Trichuris suis* was significantly better than placebo treatment in lowering the UCDAI index in patients with active colitis, but
not in obtaining remission. LGG showed better efficacy in prolonging the remission period for patient with UC than patients receiving Mesalazine alone.

It seems that probiotics is better in maintaining and prolonging remission than to obtain remission in UC patients, but unlike for CD the efficacy in the former was significantly better than placebo and the same as standard treatments such as Mesalazine. **Conclusion: this indicates that probiotics can be used as a valid alternative or supplement to Mesalazine in preventing relapse of UC.**
10. Discussion

The mechanisms of action of probiotics have mainly been elucidated though laboratory experiments and to a lesser extent though clinical studies. In the following we will attempt a synthesis of the theory and the clinical studies. We will take offset in the three main groups of probiotic mechanisms as described in chapter 7.

1. Inhibiting microbial pathogens’ growth.

Even though no specific bacterial species have been associated with CD or UC; it is known that the bacterial flora of IBD patients is altered compared to normal intestinal flora. Adherent and intramucosal bacteria especially *E. coli*, *Bacteroides ssp.* and *Enterobacterium ssp.* were elevated in patients with active IBD. In CD the level of Bifidobacteria were found to be decreased (Madsen, 2001). Therefore it is reasonable to assume that supplementing the intestinal microflora with non-pathogenic strains of these species, and thereby out-competing the pathogenic bacteria of nutrients can correct the imbalance. This can be accomplished in two manners, either by supplementing a specific antagonist to a suspected pathogen for example non-invasive *E. coli* Nissle 1917 against pathogenic *E. coli* bacteria capable of invading the mucus layer and adhering to the epithelial cells. Another strategy is to add a general non-pathogenic bacterium like lactobacilli that survive passage through the stomach and can colonize the entire length of the intestine (not like *E. coli* that mainly colonizes the colon). The eukaryote *S. boulardii* is also believed to be able to bind adherent pathogens to its surface and thereby preventing them from adhering to endothelial cells (Plein and Hotz, 1993).

It is also known that mutations in CARD15 (NOD2) are associated with CD in the ileum. CARD15 is believed to be involved in production of defensins in Paneth cells. CD patients with CARD15 mutations are known to have higher concentrations of luminal bacteria especially in crypts (Sartor, 2006). Probiotics secreting substances with similar effect as defensins could therefore possibly compensate for this deficiency. *E. coli* Nissle 1917 possibly has a such effect by secreting bacteriocins or microcins and thereby inhibiting other bacteria (Rembacken *et al.*, 1999).
2. Increasing epithelial barrier function.

The barrier function of the GI is essential in protecting the gut from pathogens. Interaction between the commensal bacteria and the immune system takes place only in designated places – such as Peyer’s patches. The epithelial cells lining the gut are protected from the microflora by the mucosal layer (only certain pathogens are able to penetrate and invade the mucosal layer). If the mucus layer is compromised the second line of defence – tight junctions between the epithelial cells - will protect the lamina propria from invasion by preventing pathogens from passing via paracellular transport. Studies have shown that expression of tight junction proteins can be upregulated by secreted metabolites or direct contact with commensal bacteria. Bordin et al., have demonstrated that butyrate secreting bifidobacteria and lactobacilli can stimulate formation of tight junctions (Bordin et al., 2004). Bruewer et al., found that LGG secreted proteins (p75 and p40) could impede internalization of tight junction proteins. This effect was probably caused by countering elevated levels of proinflammatory cytokines TNF-α and INF-γ (Bruewer et al., 2003).


It has been shown that a normal microflora is essential for the development of the Gut-associated lymphoid tissue (GALT) mainly Peyer’s patches and lymphoid tissue. Germ-free mice show immunological abnormalities such as poorly formed B- and T-cell zones in Peyer’s patches, lymph nodes and spleen. The gene expression profile of epithelial cells is also different compared to normal mice (Macpherson and Harris, 2004). This indicates that the mucosal immune system needs to be exposed to microorganisms in order to develop normally. The hygiene hypothesis suggests that certain microorganisms or harmless helminths up through evolution have assumed this role of activating our immune system. This would explain the rise in autoimmune diseases (IBD, Type 1 diabetes and allergies) in the Western world, but not in third world countries where infection with microorganisms and helminths are common. This would explain why probiotics, at least in UC, seems to have an anti-inflammatory effect. By suppressing the destructive T_{H}1 response (characterizing both CD and UC) probiotics can prevent tissue damage. This can be achieved by inducing anti-inflammatory cytokines such as IL-10 and TGF-β (Guarner et al., 2006). Animal models also show a down-regulation of pro-inflammatory cytokines such as IFN-γ and TNF-α after treatment with probiotics (McCarthy et al., 2003; Sturm et al., 2005; Dalmasso et al., 2006).
This indicates that non-pathogenic microorganisms (e.g. Lactobacilli, Bifidobacteria and helminth) are perceived as “friendly” by the immune system and therefore activate T_{reg} cells secreting IL-10 and TGF-β and thereby down-regulates the T_{H1} response (Guarner et al., 2006). If further indicates that probiotics can help in obtaining homeostasis in the mucosal immune system, thereby controlling the inflammatory process of IBD. This also correlates with the efficacy of TNF-α antibodies in treatment of both CD and UC (Waller et al., 2005).

**Evaluating the etiology of UC and CD**

Experimental colitis in model animals is often used to simulate CD or UC. However, it does not show the same etiology as CD and UC. In experimental colitis the inflammation is induced artificially by substances such as dextran sulphate sodium (DSS) or 2,4,6-trinitrobenzenesulphonic acid (TNB) and when the substance is discontinued the symptoms disappear. Similarly the bacterial flora of animals (such as mice and rats) differs significantly from humans. This makes it difficult to extrapolate from animal experiments to humans and possibly explains why the promising results in animal models are difficult to reproduce in the clinical studies. It would therefore be useful with more research in probiotic action mechanisms with experiments in human models, either cell cultures or *in vivo* when possible.

We saw in the clinical trials that probiotics were beneficial against UC but not CD, at least not significantly. The reason for this could be the genetic factors in IBD. Several gene defects have been associated with CD, whereas only a few have been connected to UC. Even though genetic factors only partially explain the diseases it might play a significant role in CD. This would also explain why CD is more refractory to treatment (both conventional and with probiotics) and more often requires surgery. UC secondly is confined to the colon, which has a richer microflora; it would therefore be plausible that alterations of the microflora with probiotics would be more efficient in UC as compared to CD, which can be located over the entire gut.
11. Conclusion and future prospects

Our literature study showed that several probiotics (*E. coli* Nissle 1917, BIFICO, BFM, VSL#3, *Trichuris suis* and LGG) showed efficacy in maintaining remission in UC, but not in CD. This is probably due to the different localization of the inflammation and maybe different etiology. Numerous sources suggest that the genetic factor plays a larger role in CD than UC and that this is one of the reasons for the poor results in the clinical trials with probiotics. We believe that probiotic species used in clinical trials are well chosen but we suggest that the probiotic treatment in IBD should be given before the outbreak of the disease. Possibly by treating children genetically predisposed for IBD at an early age, preferably neonatal. This will allow the immune system to develop the necessary mechanisms to control the immune response and avoid the uncontrolled T_{H1} response characteristic for IBD. We therefore recommend further investigation in genetic markers for IBD, in order to be able to offer genetic screening of babies with a known family history of IBD.

Apart from modulating the immune response of intestinal epithelia and mucosal immune cells, probiotics have also been shown to inhibit growth of microbial pathogens. This effect has been associated with *E. coli* Nissle 1917 and with *S. boulardii*, which were the only two organisms showing an effect against CD in clinical trials, but because of the low number of subjects they were not shown to be significantly better than placebo. *E. coli* Nissle 1917 also showed efficacy in treatment of UC. Probiotics (Lactobacilli and Bifidobacteria) can increase the barrier function of the epithelial cells, thereby protecting from invading pathogens. This indicates that probiotics also can play a role in treatment of developed IBD, especially UC.

Our recommendation for the future is to pursue the possibility of neonatal treatment with probiotics. We also believe that more human trials are needed in order to understand the mechanisms of action of probiotics. The possibility of combining pre- and probiotic agents in one treatment (synbiotics) seems appealing, as the prebiotic moiety could be integrated in the patient’s diet while the administration of the probiotics could be reduced in frequency due to the prolonged survival of the beneficial bacteria. Since probiotics seem to be safe their use in treatment of IBD should be further investigated.
12. Glossary

Blinded: When one or more parties (subject, investigator and assessor) to the study are kept unaware of which treatment each subject is receiving until the end of the study.

Controlled study: A study in which a treatment group, receiving the investigational drug, is compared with a similar control group. The control group may receive no treatment, standard treatment or a placebo.

Double-blind: A study in which both subject and investigator are unaware of which treatment each subject is receiving until the end of the study.

Double-dummy: A technique for retaining the blind in a clinical study, when the two treatments cannot be made identical (for example pills versus injections). Supplies are prepared for treatment A (active and indistinguishable placebo) and for treatment B (active and indistinguishable placebo). Subjects then take two sets of treatment; either A (active) and B (placebo) or A (placebo) and B (active).

Intention-to-treat: The principle that a subject that has been allocated to a group (treatment or control) should be followed up, assessed and analyzed as a member of that group, even if he/she discontinues the treatment.

Open label study: A study in which both subject and investigator know what treatment the subject is receiving.

Pilot study: A pilot study is a small-scale study used to obtain information, and work out the logistics and management, deemed necessary for further large scale clinical studies.

Placebo-controlled: A study in which the treatment group, receiving the investigational drug, is compared with a similar control group receiving a placebo. The placebo should be an inactive product indistinguishable from the active drug.
**Randomized:** When the subjects in a study are allocated into treatment group(s) and control group in an unpredictable way; to avoid allocation of similar subjects to the same group.

**Single-blind:** A study in which one party, either the subject or the investigator, is kept unaware of which treatment is administered to the subject until the end of the study.
13. Literature


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Appendix A: Anatomy of the small and large intestine.

The small intestine

The small intestine consists of the duodenum, jejunum and ileum. The small intestine is about 6 m long where the duodenum is about 25 cm long, the jejunum is about 2.5 m long and the ileum is about 3.5 m. In the Duodenum immediately after the stomach, the low pH of about 4 restricts the bacterial flora; only acid tolerant bacteria such as lactobacilli and enterococci are the main inhabitants. As the pH is gradually raised, the microflora becomes more diverse.

![Diagram of the digestive system]

Figure A.1 The listed bacteria are the predominant species, in the small intestine.

The surface area of the duodenum is increased about 600-fold caused by circular fold in the mucosa and submucosa and by villus in the mucosa which is covered by microvilli. The jejunum and ileum are similar in structure except that a gradual decrease occurs in the number of circular folds and villi. There is also seen a gradual decrease in the diameter of the small intestine and the thickness of the intestinal wall.

The mucosal lining of the small intestine is simple columnar epithelium. Four cell types are found in the mucosa (1) absorptive cells produce digestive enzymes and absorb digested food (2) goblet cells produce protective mucus (3) granular cells (Paneth’s cells) protect the
intestinal epithelium from bacteria and (4) endocrine cells produce regulatory hormones. In the mucosa and submucosa of the ileum there are found numerous lymphatic nodules called Peyer’s patches.

**The large intestine**

The large intestine consists of the cecum, colon, rectum and anal canal. The cecum forms a blind sac at the transition of the small and large intestine. The colon with is about 1.5-1.8 m long is divided in the ascending colon, transverse colon, descending colon and sigmoid colon. In the colon were the pH is close to 7; the largest number and diversity of bacteria exists. In total about 500-1000 different species are present in the GI, in a variety of numbers; the major species are listed in figure A.2.

![Diagram of the large intestine](image)

**Figure A.2** The major bacterial species of the large intestine.

The mucosal lining of the large intestine is simple columnar epithelium and consists of numerous straight tubular glands called crypts. Three cell types are found in the mucosa (1) absorptive cells (2) goblet cells and (3) granular cells. The goblet cells are dominated in number. The mucus produced by goblet cells lubricates and protect the wall of the colon and helps faecal matter stick together.
Appendix B: The Jadad-scale.

Grading clinical trials using the Jadad-scale is performed by answering the following three questions and scoring them as described:

1. Is the study described as randomized (this includes the use of words such as randomly, random, and randomization)?

2. Is the study described as double blind?

3. Is there a description of withdrawals and dropouts?

Scoring the items:
Either give a score of 1 point for each “yes” or 0 points for each “no”. There are no in-between marks.

Give 1 additional point if: For question 1, the method to generate the sequence of randomization was described and it was appropriate (table of random numbers, computer generated, etc.)

And/or: If for question 2 the method of double blinding was described and it was appropriate (identical placebo, active placebo, etc.)

Deduct 1 point if: For question 1, the method to generate the sequence of randomization was described and it was inappropriate (patients were allocated alternately, or according to date of birth, hospital number, etc.)
And/or: For question 2, the study was described as double blind but the method of blinding was inappropriate (e.g. comparison of tablet vs. injection with no double dummy)

**Guidelines for assessment:**

1. **Randomization**
   A method to generate the sequence of randomization will be regarded as appropriate if it allowed each study participant to have the same chance of receiving each intervention and the investigators could not predict, which treatment was next. Methods of allocation using date of birth, date of admission, hospital numbers, or alternation should not be regarded as appropriate.

2. **Double blinding**
   A study must be regarded as double blinded if the word “double blind” is used. The method will be regarded as appropriate if it is stated that neither the person doing the assessment nor the study participant could identify the intervention being assessed, or if in the absence of such a statement the use of active placebos, identical placebos, or dummies is mentioned.

3. **Withdrawals and dropouts**
   Participants who were included in the study but did not complete the observation period or who were not included in the analysis must be described. The number and the reasons for withdrawal in each group must be stated. If there were no withdrawals, it should be stated in the article. If there is no statement on withdrawals, this item must be given no points.