On Lactobacillus plantarum 299v, bacterial translocation and intestinal permeability.

Mangell, Peter

2007

On *Lactobacillus plantarum* 299v, Bacterial Translocation and Intestinal Permeability

Peter Mangell

Faculty of Medicine
Lund University

Malmö 2007

ON *LACTOBACILLUS PLANTARUM* 299V,
BACTERIAL TRANSLOCATION AND INTESTINAL PERMEABILITY

On *Lactobacillus plantarum* 299v, Bacterial Translocation and Intestinal Permeability

Experimental and Clinical Studies

Peter Mangell

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Kirurgiska klinikens föreläsningssal, Universitetssjukhuset MAS, Malmö, ingång 42, torsdagen den 8 februari 2007, kl 9.00.

Fakultetsopponent: Docent Johan D Söderholm,
Kirurgiska kliniken, Linköping

FACULTY OF MEDICINE
Lund University

Lund 2007
Department of Surgery, Malmö University Hospital

Organization
LUND UNIVERSITY
Department of Surgery,
Malmö University Hospital

Document name
DOCTORAL DISSERTATION

Date of issue
February 8, 2007

Sponsoring organization

Author(s)
Peter Mangell

Title and subtitle
On *Lactobacillus plantarum* 299v, Bacterial Translocation and Intestinal Permeability.
Experimental and Clinical Studies.

Abstract
The gastrointestinal tract harbours a huge load of bacteria which exerts important biological functions to maintain health. In certain conditions the relation between commensal bacteria and pathogens may be set off balance, with an increase in number of pathogens and risk of bacterial translocation (BT). Disease and trauma may also render the intestinal barrier dysfunctional, inducing BT and increased permeability. Probiotic bacteria, *i.e.* living bacteria which, when ingested, exerts beneficial effects on the host, have been found to stabilize the intestinal mucosa and affect the intestinal microflora. The aim of this thesis was to study the effects of *Lactobacillus plantarum* 299v in relation to intestinal permeability and BT.

In study I L. plantarum 299v normalized *E. coli*-induced permeability in distal ileum of rats. This effect was achieved with a more continuous supply of *L. plantarum* 299v, rather than intermittent or acute pretreatment.

Study II showed that pretreatment with *L. plantarum* 299v prevented BT in rats rendered septic with LPS. The protective effect seemed to be dependent on the ability of *L. plantarum* 299v to adhere to intestinal mucosa, indicating competitive inhibition as a possible mechanism behind prevention of translocation. Moreover, treatment with prebiotics, without a probioticum, did not prevent translocation.
In study III LPS-induced intestinal barrier dysfunction on rats was found to be regulated by P-selectin-dependent leukocyte recruitment. With anti-P-selectin antibody intestinal permeability was attenuated, as well as leukocyte rolling and adhesion. This indicates anti-P-selectin treatment as a mean of ameliorating barrier dysfunction in sepsis.

Finally, in study IV, it was found that high doses of *L. plantarum* 299v given to patients undergoing colon surgery were not able to reduce translocation of selected enteric bacteria but seemed to have a stimulatory effect on bacterial load in the colon. Further, *L. plantarum* 299v could be given to patients with malignancy without risk of tumour proliferation.

Taken together, the findings herein indicates a potential of *L. plantarum* 299v pretreatment in reducing BT and intestinal permeability and warrants further studies in the clinical setting.

Key words:
Lactobacillus plantarum 299v, intestinal microflora, bacterial translocation, permeability, leukocyte recruitment, probiotics, P-selectin, colon resection

Classification system and/or index terms (if any):

Supplementary bibliographical information:

Language
English

ISSN and key title: 1652-8220

ISBN 91-85559-85-7

Recipient's notes

Number of pages 104

Price:

Security classification

To my family

Science without religion is lame,
religion without science is blind.
Albert Einstein

Tutor	Professor Bengt Jeppsson
Assistant tutor	Associate professor Henrik Thorlacius

Peter Mangell 2007
e-mail: peter.mangell@med.lu.se

Be careful about reading healh books.
You may die of a misprint. *Mark Twain*

Layout & typography: Maria Näslund/Formfaktorn
Printed by Lidbergs Grafiska AB, Skurup, 2007

ISBN 91-85559-85-7
ISSN 1652-8220

Contents

List of Publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I **Mangell P,** Nejdfors P, Wang M, Ahrné S, Weström B, Thorlacius H, Jeppson B. *Lactobacillus plantarum* 299v Inhibits *Escherichia coli*-Induced Intestinal Permeability. *Dig Dis Sci* 2002;47:511–16

II **Mangell P**, Lennernäs P, Wang M, Olsson C, Ahrné S, Molin G, Thorlacius H, Jeppsson B. Adhesive Capability of *Lactobacillus plantarum* 299v is Important for Preventing Bacterial Translocation in Endotoxaemic Rats. *APMIS* 2006;Sep 114(9):611–8

III **Mangell P**, Mihaescu A, Wang Y, Schramm R, Jeppsson B, Thorlacius H. Critical Role of P-selectin-dependent Leukocyte Recruitment in Endotoxin-induced Intestinal Barrier Dysfunction in Mice. Accepted *Infl Res* 2006

IV **Mangell P**, Thorlacius H, Syk I, Ahrné S, Molin G, Olsson C, Jeppsson B. *L. plantarum* 299v to Patients Undergoing Colon Resection – A Randomised Placebo-controlled Study. Manuscript.

Papers reprinted with kind permissions from Blackwell Publishing, Springer Science and Business Media and Birkhäuser Verlag, Basel.

Abbreviations

AJ	adherence junction
AJC	apical junctional complex
BT	bacterial translocation
CD	Crohn´s disease
CFU	colony-forming units
Da	Dalton
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic Escherichia coli
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
i.p.	intraperitoneal
i.v.	intravenous
IFN-γ	interferon gamma
IgA	immunoglobulin A
IL	interleukin
KC	cytokine-induced neutrophil chemoattractant
LPS	lipopolysaccharide
MIP-2	macrophage inflammatory protein – 2
MLCK	myosin light chain kinase
MLN	mesenteric lymph node
MNL	mononuclear leukocyte
MPO	myeloperoxidase
MW	molecular weight
NF-κB	nuclear factor kappa B
PMNL	polymorphonuclear leukocyte
PPB	potentially pathogenic bacteria
s.c.	subcutaneous
SCFA	short-chain fatty acid
TJ	tight junction
TNF-α	tumour necrosis factor alfa
UC	ulcerative colitis

Introduction

Intestinal microflora

The gastrointestinal (GI) tract has several important functions. It has to absorb nutrients to support life, but at the same time function as a barrier against bacteria and toxins that may constitute a threat to health and even life. Located within the intestine is also the largest immunological tissue in the body, the gut-associated lymphoid tissue (GALT) (Shanahan 1994). The microflora which is contained within the lumen of the GI tract is essential for the intestinal functions and therefore plays an important role in health and disease.

Within the human body reside 10^{14} bacteria, ten times more than eukaryotic cells, *i.e.* 90% of the cells in our body are bacteria (Luckey 1972). While some bacteria are found on the skin, in the respiratory tract and vagina, the vast majority is located within the GI tract. These bacteria form a complex ecosystem that influences the host and is a prerequisite for health and normal physiology.

At birth the GI tract is sterile but soon afterwards an increasing amount of bacteria colonise the intestine. Conventional cultures have primarily found bifidobacteria in early infancy, especially with breast-fed infants (Fanaro *et al.* 2003). However, DNA-based fingerprinting techniques revealed *Enterobacteriaceae* and *Bacteroides* soon after birth, with increasing number of clostridia and bifidobacteria after weaning (Wang *et al.* 2004). The flora will then remain rather stable, except for changes later in life towards more enterobacteria (Mueller *et al.* 2006).

The flora differs in composition and concentration along the GI tract (Holzapfel *et al.* 1998; Simon *et al.* 1986). Thus, due to low pH, the stomach contains few bacteria (10^2 bacteria per ml gastric content) dominated by *Streptococcus*, *Staphylococcus* and lactobacilli (Wang *et al.* 2005). The concentration of bacteria in the jejunum remains low due to inhibitory action of bile and pancreatic juices. In the ileum, the microbiota gradually begins to take on the characteristics of the colonic flora and there is an increase in luminal pH. As a result, anaerobic Gram negative bacteria and *Enterobacteriaceae* become established and the concentration of bacteria increases to 10^8 per ml content (Evaldson *et al.* 1982). The concentration and diversity of bacteria are the greatest in the colon due to its higher pH, abundance of nutrients and lesser peristalsis. Here 10^{12} organisms per gram faeces are found (Simon *et al.* 1986). The microorganisms consist mainly of obligate anaerobes such as *Bacteroides, Eubacterium,* bifidobacteria and clostridia. Facultative anaerobes, *e.g. Escherichia coli*, are also present (Wang *et al.* 2005).

The human colon can be considered a continuously running fermentor where biological processes important for health and well-being take place. By fermentation of non-degradable carbohydrates, such as non-starch polysaccharides and oligosaccharides, intestinal flora produces short-chain fatty acids (SCFA), mainly butyrate, propionate and acetate. These are then metabolised by the colonic epithelium, liver and peripheral tissue, respectively (Cummings *et al.* 1996; Wong *et al.* 2006). In fact, the epithelium in the colon receives 70% of its energy from the lumen (Roediger 1995). The bacteria also have a role in the synthesis of vitamin B and K (Conly *et al.* 1994) and both potentially pathogenic bacteria (PPB) and commensals influence the immune system (Kelly *et al.* 2005). Further, bacterial enzymes, such as β-glucosidase and β-glucuronidase, can hydrolyse different glucosides and glucuronidases, forming toxic and carcinogenic substances and convert primary bile acids to secondary ones that are mutagenic (Simon *et al.* 1986). Moreover, the indigenous microflora is important in the defence against exogenous pathogens by colonization resistance (Vollaard *et al.* 1994).

Despite the fact that more than 100 different species have been characterised in faeces, the number of yet undefined species is thought to be more than 400 (Holzapfel *et al.* 1998). Therefore, much is still unknown about the composition of the intestinal microflora, the physiological processes in which they are involved and the influence the intestinal bacteria have on the homeostasis of the body (Eckburg *et al.* 2005). We need better tools to study the bacterial flora and PCR techniques with 16S rRNA gene sequencing may be a useful one in this respect. However, more studies utilizing this technique are required.

A well balanced diversity of the intestinal microflora is an important aspect of health. The indigenous microflora contains a wide variety of bacteria, from PPB, *e.g. Enterobacteriaceae* and certain clostridia, such as *Clostridium perfringens* and *C. difficile*, to those with health promoting properties, such as bifidobacteria and lactobacilli. This may mirror a selection of bacteria favourable for the host. In the healthy state, PPB's are kept under control by the non-pathogenic flora, so called colonization resistance. Diversity is, however, challenged, for example in critical illness (Marshall *et al.* 1993), inflammatory bowel disease (Ott *et al.* 2004) and with antibiotic therapy. When such changes occur, it affects the host through the intestinal flora's interplay with the body's immune system and through bacterial translocation (BT). The reduced diversity often results in a greater niche for PPB's, further fuelling the disease, which may result in a vicious circle. In this situation, the idea of supplementation with certain bacteria that might counterbalance the expanding PPB's and re-establish diversity and thereby health, have been put forward (Salminen *et al.* 1996; Sartor 2004). The concept of ingestion of fermented food products containing health promoting bacteria was presented already in the end of the 19th century by Metchnikoff (Metchnikoff 1908), but with increasing knowledge of the intestinal microflora, and the processes taking place therein, the last decades have brought about a renewed interest in probiotic bacteria, *e.g.* lactobacilli and bifidobacteria (Dunne *et al.* 1999; Fuller 1991; Servin 2004).

Probiotics

General aspects

Probiotic bacteria are defined as living organisms which, when ingested, exert beneficial effects on the host (Schrezenmeir *et al.* 2001). Species often used as probiotics belong to the genera *Lactobacillus* (*e.g. L. paracasei*, *L. rhamnosus*, *L. johnsonii*, *L. plantarum, L. reuteri* and *L. acidophilus*), *Bifidobacterium* (*e.g. B. longum*, *B. animalis*) or the yeast *Saccharomyces boulardii* (Herek *et al.* 2004; Holzapfel *et al.* 1998). Others are *Streptococcus thermophilus* (Resta-Lenert *et al.* 2003) and *E. coli* strain Nissle (Sartor 2004). Combinations of several probiotic bacteria have also been used (Gionchetti *et al.* 2000). Besides probiotic bacteria, prebiotics are often used. Prebiotics are defined as dietary non-digestible carbohydrates with the ability to selectively stimulate growth of one or a limited number of bacteria, often lactobacilli and bifidobacteria, and thereby contribute to enhancement of the intestinal barrier function (Schrezenmeir *et al.* 2001). This definition is very close to that of dietary fiber, the major difference being the controlling effect on the colonic microflora. Thus, fructooligosaccharides and inulin are examples of prebiotics, while β-glucans in for example oat, is a dietary fiber without particular selective effects on the microflora. Symbiotics are combinations of pre- and probiotics.

Probiotic bacteria to be used should be resistant to gastric acid, bile and pancreatic juices, survive the transit through the intestine, have the ability to adhere to the intestinal mucosa of the host and colonise the intestine, be nontoxic and nonpathogenic, easy to culture and preferably of human origin when used in humans (Dunne *et al.* 1999; Salminen *et al.* 1996). The ability of probiotics to affect the host beneficial-

ly is claimed to be through properties such as production of antimicrobial substances (Coconnier *et al.* 1997; Servin 2004), modulation of the intestinal microflora composition by lowering of pH through production of SCFA (Cummings *et al.* 1996; Wong *et al.* 2006), induction of mucin-production (Mattar *et al.* 2002), immunostimulation (Miettinen *et al.* 1996; Roller *et al.* 2004), colonization resistance by competitive inhibition of other microbes on the intestinal mucosa (Gueimonde *et al.* 2006) and production of enzymes and anticarcinogenic substances (Gallaher *et al.* 1999). Different effects have been found in animal and human studies. Thus, in animal *in vivo* studies, probiotics have been found to reduce BT (Adawi *et al.* 2001), attenuate liver injury (Kasravi *et al.* 1997) and colitis (Mao *et al.* 1996a), reduce tumour incidence (McIntosh *et al.* 1999) and restore intestinal integrity in IL-10 gene-deficient mice (Madsen *et al.* 2001). In humans, effects with probiotics have been found in prevention of pouchitis (Gionchetti *et al.* 2000; Gionchetti *et al.* 2004), reduction in septic complications requiring surgical intervention in acute pancreatitis (Olah *et al.* 2002) and reduced postoperative septic complications after major abdominal surgery (Rayes *et al.* 2002a) as well as after liver transplantation (Rayes *et al.* 2002b). Probiotics have also been successfully used in treating rota virus infections in children (Majamaa *et al.* 1995; Weizman *et al.* 2005) and seem promising in preventing relapse in *Clostridium* enterocolitis (Wullt *et al.* 2003). Further, probiotics seem to prevent antibiotic associated diarrhoea (D'Souza *et al.* 2002) as well as to reduce the cholesterol and fibrinogen levels in smokers (Bukowska *et al.* 1998). Finally, a probiotic given to heavy smokers has been found to lower blood pressure and reduce fibrinogen levels, a risk factor for cardiovascular disease (Naruszewicz *et al.* 2002). All these effects are claimed with different probiotics or combinations of them, with different doses and length of treatment.

Lactobacillus plantarum 299v

The species *L. plantarum* is a Gram positive microaerofilic facultative heterofermentative rod, being able to use carbohydrates for fermentation to lactic acid, acetic acid and CO_2. *L. plantarum* is often found in several plant (Randazzo *et al.* 2004; Spano *et al.* 2004) and dairy (Manolopoulou *et al.* 2003) products. With consumption of lactic acid fermented products, large amounts of *L. plantarum* are also ingested. The strain *L. plantarum* 299v (DSM 9843) was originally isolated from the human intestinal mucosa (Molin *et al.* 1993) and has since then been used in animal and human studies to test its probiotic potential.

Several animal experiments have been designed to study the effect of *L. plantarum* 299v on colitis and intestinal permeability. In rats with methotrexate-induced colitis, *L. plantarum* 299v was found to reduce BT and permeability while re-establishing microecology and attenuating the mucosal injury (Mao *et al.* 1996a; Mao *et al.* 1997). In the same experimental model *L. plantarum* 299v increased secretory IgA in the colon, indicating an immunomodulatory function (Mao *et al.* 1996b). This effect on intestinal immunity was also highlighted on germ-free mice with IL-10 deficiency, where pretreatment with *L. plantarum* 299v before colonization with specific pathogen free bacteria attenuated colitis (Schultz *et al.* 2002). The ability of *L. plantarum* 299v to affect the concentration of PPB in the intestine was studied on a model with colitis induced by dextran sulphate sodium (DSS), where a reduced BT was found, possibly due to a decrease in viable count of *Enterobacteriaceae* (Osman *et al.* 2004). Moreover, a decrease in *Enterobacteriaceae* in colon, concomitant with reduced BT and decreased hepatocellular damage was shown in rats where *L. plantarum* 299v was given rectally for eight days before induction of liver injury (Adawi *et al.* 1997). Further, competition for colonisation was studied on gnotobiotic mice, where *L. plantarum* 299v

was found to decrease the concentration of *E. coli* in small intestine and caecum, but this effect did not remain for more than five weeks (Herias *et al.* 1999). However, co-colonisation resulted in higher levels of serum IgA and the influence of *L. plantarum* 299v on the intestinal immunity was further indicated by an increase in the density of $CD25^+$ cells in lamina propria of the intestine (Herias *et al.* 1999).

Possible mechanisms behind the abovementioned effects have been explored in several *in vitro* studies. Thus, *L. plantarum* 299v was found to prevent attachment of enteropathogenic *E. coli* to HT-29 cells, while increasing mRNA expression of MUC2 and MUC 3, the two predominant mucins in colon (Mack *et al.* 1999; Mattar *et al.* 2002) resulting in an extracellular production of mucins (Mack *et al.* 2003). Besides a potential effect of *L. plantarum* 299v in preventing attachment of microbes through strengthening of the mucus layer covering the intestinal mucosa, another way could be through competition for receptors. This was studied by Adlerberth *et al.* where *L. plantarum* 299v was tested for adherence to HT-29 cells and found to carry a mannose-specific adhesin (Adlerberth *et al.* 1996). Many strains of *E. coli* also carry mannose-specific adhesins (Abraham *et al.* 1985) and this may be a possible mechanism behind the competitive inhibition exerted by *L. plantarum* 299v. This is further emphasized in a study where *L. plantarum* 299v reduced enteropathogenic *E. coli* (EPEC)-induced migration of neutrophils across T-84 cell monolayers, while a solution in which *L. plantarum* 299v had grown was not able to achieve this effect (Michail *et al.* 2003).

Several studies have also been performed on humans. *L. plantarum* 299v is a common colonizer on human mucosa of rectum (Johansson *et al.* 1993) and many of the isolates were found to adhere to HT-29 cells in a mannose-sensitive manner, indicating the presence of such a mannose-containing receptor on the intestinal mucosa (Ahrné *et al.* 1998). Intake of *L. plantarum* 299v resulted in an increase in lactobacilli and bifidobacteria in faeces while sulphite-reducing clostridia were reduced (Johansson *et al.* 1998), demonstrating the ability of the bacterium to pass through the GI tract and exert a biological effect. The survival of orally provided *L. plantarum* 299v to healthy individuals was confirmed by Goossens *et al.* but the increase in concentration of lactobacilli in faeces disappeared quickly after cessation of feeding (Goossens *et al.* 2003). No effects were seen on counts of other bacteria, enzyme activity or concentration of SCFA (Goossens *et al.* 2003; Goossens *et al.* 2005). This is in contrast to the previously mentioned study, where increased fermentation by *L. plantarum* 299v was indicated through increased concentrations of acetic acid and propionic acid in faeces (Johansson *et al.* 1998).

In the clinical situation varying effects have been found with administration of the probioticum. *L. plantarum* 299, which is another *L. plantarum* strain but with high genomic similarity to *L. plantarum* 299v, reduced postoperative complications in liver transplant patients (Rayes *et al.* 2002b) and reduced the incidence of septic complications in patients with pancreatitis (Olah *et al.* 2002). Heat-killed *L. plantarum* 299 did not achieve these effects. However, in a study on general surgery patients, *L. plantarum* 299v could not prevent BT, gastric colonisation or postoperative septic complications (McNaught *et al.* 2002). The general surgery patients were pre-treated with the probioticum for a considerable time before surgery, in contrast to the liver transplant patients who were given only postoperative treatment. Pretreatment, with the possibility of lactobacilli to colonise the intestine, would supposedly increase the chance of any positive effects. The reason why this was not the case might be due to the severity of the systemic disease, *i.e.* the liver transplant patients were more affected by their disease and therefore favourable results were easier to detect. This possibility is further emphasized in another study by Rayes *et al.* where the positive effect of *L. plan-*

tarum 299 was primarily seen among the most severely ill patients (Rayes *et al.* 2002a).

The immunomodulatory influence of *L. plantarum* 299v found in animal studies have been more difficult to detect in humans studies. In a study by Woodcock *et al.* (2004) no effect on plasma cells, mucus IgA, IgM-positive cells or IgA-positive cells in lamina propria of small intestine was found after preoperative intake of the probioticum, and the authors concluded that *L. plantarum* 299v does not enhance the function of the GALT (Woodcock *et al.* 2004). However, the study subjects were few and the intake of the probioticum low. Moreover, *L. plantarum* 299v given to critically ill patients attenuated IL-6 levels after 14 days of treatment, indicating an ability to modulate the inflammatory response (McNaught *et al.* 2005).

Several different probiotic bacteria have been studied in varying experimental and clinical situations. Results from animal studies are encouraging and the concept of treating disease, and preventing complications when doing so, with probiotic bacteria is appealing. Still, much has to be elucidated concerning the potential effect of individual strains, prevention of translocation of PPB, attenuation of permeability defects and strengthening of the intestinal barrier.

Intestinal barrier

Structure

The intestine has an area of approximately 300 m^2 and is the largest surface between the human body and the surrounding environment. The intestine has to absorb water and nutrients, and at the same time prevent intraluminal bacteria, antigens, toxins and other noxious components in the lumen to pass into the body (Snoeck *et al.* 2005). To achieve this, a complex physical barrier separates the intestinal lumen from the body. (Fig. 1) This consists of an unstirred water layer, a mucous layer, the epithelium with its hydrophobicity, tight junctions (TJ) and the endothelium. When one or several parts of the barrier fail, disease ensues (Farhadi *et al.* 2003).

The unstirred water layer is rate limiting in the transport of many nutrients and pharmacological substances, but its role in permeability remains unknown.

The intestinal mucosa is covered by a mucus layer. Its hydrophobic characteristic is important in the protection of the epithelium from physical and chemical injury (Maxson *et al.* 1994). The mucus is also a strong diffusion barrier. Furthermore, it prevents bacterial adherence to the mucosa by trapping both bacteria and secretory IgA attached to bacteria, which are then transported distally with the peristalsis and excreted (Albanese *et al.* 1994; Macpherson *et al.* 2001).

The mucus membrane consists of several different cells. The most abundant are the enterocytes (Snoeck *et al.* 2005) which arise from stem cells in the crypts of the mucosa. With maturation, they move up towards the villi where they have absorptive functions but also restrict passage of compounds from the lumen. They are polarized with microvilli on the apical, luminal side, further increasing the absorptive area of the intestine. From the stem cells arise also goblet cells which are spread among the enterocytes and produce mucus. Further, Paneth cells are located deep in the crypts of the small intestine, providing a defence against microbes by secreting antimicrobial molecules such as defensins, lysozymes and phopholipas A into the lumen (Bry *et al.* 1994). M-cells are found in the follicle-associated epithelium of the Peyer's patch of the small intestine, especially distally. They are specialized in the uptake of micro- and macromolecules from the lumen of the intestine and deliver the antigens via transcytosis to antigen presenting cells and lymphocytes located in a pocket-like structure on the basolateral side and further to the lymphoid tissue underlying the mucosa (Gullberg *et al.* 2006). M-cells differ from normal enterocytes in that they do not have microvilli on their apical surface, but broader microfolds that give the cell its name.

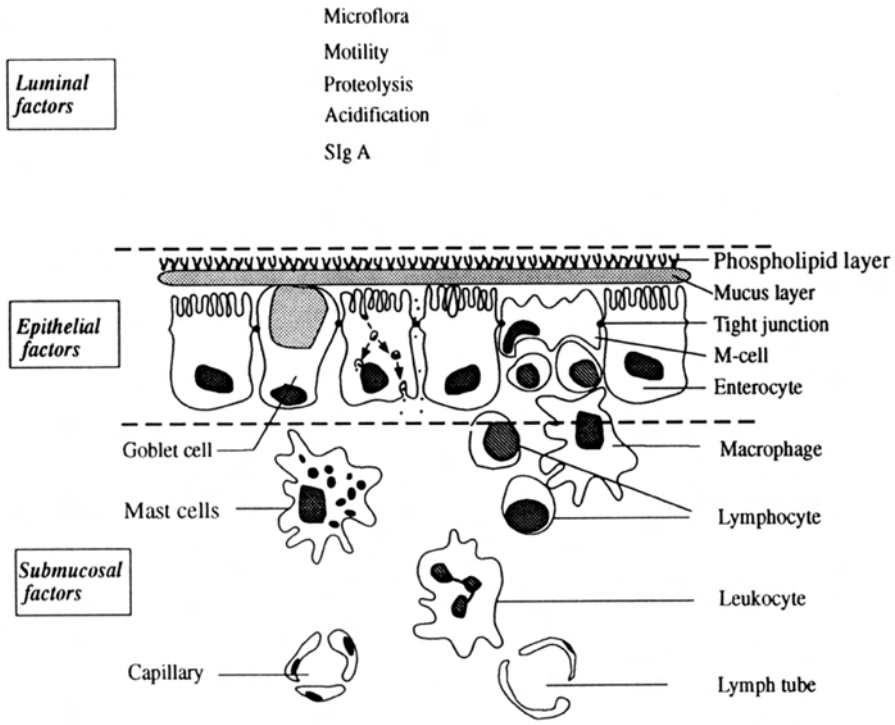

Fig. 1. Schematic presentation of gastrointestinal barrier.

The filamentous brush border glycocalyx, an extracellular polysaccharide layer found throughout the intestine attached to enterocytes, is much thinner or absent on M-cells, facilitating antigen uptake. M-cells are exploited by several pathogens, including *Shigella flexneri* and *Salmonella typhimurium*, as a way to penetrate the intestinal epithelium (Jensen *et al.* 1998; Lu *et al.* 2001). Factors promoting the differentiation of M cells have yet to be elucidated, but they are thought to develop in response to signals from immune cells found in the developing Peyer's patch.

Besides the cellular components of the mucosa, epithelial barrier function is regulated by the apical junctional complex (AJC), consisting of tight junctions (TJ) and adherence junctions (AJ). TJ is closest to the lumen and made up of fibrils that cross between the plasma membrane of adjoining cells (Nusrat *et al.* 2000). The fibrils consist of the membrane proteins occludin and claudin which are in contact with the intracellular proteins ZO 1–3 (Balda *et al.* 2000). AJ is located further towards the basolateral membrane and is built up by the transmembrane protein E-cadherin. AJ is responsible for cell-cell contact and forms a complex with the TJ (Balda *et al.* 2003).

The TJ complex is linked to the actin microfilaments making up the cytoskeleton (Kucharzik *et al.* 2001). Myosin movement along actin filaments are regulated by myosin light chain kinase (MLCK) (Moriez *et al.* 2005). Phosphorylation of MLCK causes increased perijunctional contraction of actin and thereby increase paracellular permeability (Moriez *et al.* 2005). TJ has both a fence-function, maintaining apical-to-ba-

solateral polarity by inhibiting diffusion of lipophilic molecules, and a gate-function by regulating the passage of ions and molecules through the paracellular pathway.

Besides the physical components of the intestinal barrier, the intestinal wall contains as much as 70% of the body's immune system, the GALT (Shanahan 1994), which constitute an important barrier against exogenous (*e.g.* dietary) and endogenous (bacteria) antigens. The barrier defence consists of Peyer's patches, lamina propria, intraepithelial lymphocytes and mesenteric lymph nodes (MLN). These compartments contain immunocompetent cells with different functions, *e.g.* macrophages (phagocytosis), dendritic cells (antigen presentation), B-lymphocytes (production of antibodies against soluble antigens) and T-lymphocytes (cell-mediated immune response), responding to luminal antigens (Acheson *et al.* 2004).

Normal function

The cellular components building the intestinal epithelium and the TJ's between the cells constitute a barrier against the intestinal lumen. The normal function of the barrier is to facilitate the uptake of water, ions, nutrients and a controlled amount of antigens (Acheson *et al.* 2004). The route across the barrier is either paracellular (between the cells) or transcellular (across the plasma membrane of the cells). Intestinal permeability denotes the unmediated passage of molecules greater than 150 Daltons across the mucosal barrier, while mucosal permeability implies passage of smaller molecules or ions (Travis *et al.* 1992).

The transcellular route is used by small and medium-sized molecules, mainly lipophilic ones, due to the nature of the phospholipid membrane of the cells. An absorptive process of nutrients occurs at the microvilli of the apical membrane and discharge at the basolateral side of the enterocytes. Meanwhile electrolytes are either absorbed or secreted. When an electrically charged ion is transported transcellularly, electroneutrality is maintained by passing of an opposite charged ion through the TJ's (Sun *et al.* 1998).

The paracellular route is the main path for small and medium-sized water-soluble compounds across the intestinal barrier. This occurs through the TJ's which are more numerous but smaller at the tip of the villi, compared to the fewer but broader ones in the crypts (Hollander 1999).

Macromolecules are transported across the intestinal barrier either through receptor-mediated endocytosis, non-selective pinocytosis or direct penetration of the cell membrane. In the pathogenesis of *e.g.* inflammatory disorders of the intestine, paracellular permeability to macromolecules plays an important role (Bruewer *et al.* 2006).

Normal function of the barrier requires normal blood flow to the intestine, adequate access to nutrients, a balanced luminal microflora and a well-functioning immune system, constantly scanning the mucosal barrier for pathogens, while simultaneously collecting small amounts of antigens to stay active and updated. All these requirements are, surprisingly enough, at work most of the time. However, in certain conditions one or several of these factors are set off balance, causing the intestinal barrier to become dysfunctional, which may lead to increased permeability to antigens and translocation of bacteria.

Barrier dysfunction

Altered intestinal permeability is an important part of the pathogenesis in several critical conditions. Patients with sepsis (Johnston *et al.* 1996) or major trauma (Pape *et al.* 1994) develop increased permeability, which may initiate or promote multiorgan failure in the early phase of the trauma, but also predispose to significant infections later in the clinical course, as seen with burn injuries (LeVoyer *et al.* 1992).

Changes in permeability are thought to be

involved in the pathogenesis of inflammatory bowel disease (Nejdfors *et al.* 1998; Söderholm *et al.* 1999). In Crohn's disease (CD), barrier function in the follicle-associated epithelium is reduced allowing passage of intraluminal bacteria and antigens (Gullberg *et al.* 2006). This occurs both across antigen-sampling M-cells in the follicle-associated epithelium and by down-regulation of E-cadherin in AJ (Karayiannakis *et al.* 1998), indicating an increased permeability across the TJ complex. The increased permeability may be an early event in CD, perhaps genetically induced and promoted by the environmental factors such as intestinal bacteria or other antigens. In contrast, the permeability changes seen in ulcerative colitis (UC) is presumably secondary to the inflammatory process (Schmitz *et al.* 1999b). The proinflammatory cytokines IFN-γ and TNF-α have been found to increase permeability in cell monolayers (Bruewer *et al.* 2003; Schmitz *et al.* 1999a) and are thought to be involved in the increased permeability seen in IBD through endocytosis of TJ proteins (Ivanov *et al.* 2004).

Cytotoxic agents used in oncology treatment commonly create adverse reactions, such as mucositis and enteritis. Due to the nature of the drugs given, there is a morphological and functional damage to the cells, creating a weakened intestinal barrier, both on the cellular level and on TJ (Carneiro-Filho *et al.* 2004; Keefe *et al.* 1997). This leads not only to increased passage of antigens and bacteria across the intestinal barrier, but also increased passage of water and electrolytes into the lumen, causing diarrhoea. Radiotherapy, likewise, alters the morphology of the intestinal epithelium. Thus, permeability has been shown to increase in rectal mucosa following radiotherapy, indicating a barrier damage to the mucosa (Nejdfors *et al.* 2000).

The intestinal barrier function may also be weakened by changes in the intestinal gut flora. Under normal conditions, PPB are kept under control by factors such as diet, pH, peristalsis, immune competence and the intestinal microflora itself, competing for nutrients. However, modification of one or several of these factors may lead to overgrowth of pathogens, leading to mucosal damage and increased permeability (Deitch *et al.* 2004). One example is the non-invasive EPEC which induces diarrhoea by increasing paracellular permeability. This is done through redistribution of occludin in the TJ and is dependent on *E. coli* secreted protein (Shifflett *et al.* 2005). However, the increase in paracellular permeability induced by EPEC can be diminished by the probiotics *L. casei* (Parassol *et al.* 2005) or *L. plantarum* 299v (Michail *et al.* 2003). While the diminishing effect on permeability with EPEC-infected T84 cells was equal whether the probioticum was given before or after infection, other studies have shown a preventive, rather than therapeutic, effect with probiotics (Michail *et al.* 2003; Resta-Lenert *et al.* 2003).

The increased permeability, whether due to destruction of epithelium or breakdown of TJ, opens up the interior milieu of the body. This sets off an immunologic reaction which either succeeds in resisting the invasion of antigens, or the body is invaded by antigens, bacteria, toxins and other noxious substances from the intestinal lumen, leading to local or systemic disease. Even when the invasion is halted, side effects of the immunologic reactions may create disease. Therefore, it is of interest to prevent, rather than to treat, breakdown of the intestinal barrier in situations where it is challenged.

Bacterial translocation

Despite multiple physical and physiological components, bacteria sometimes manage to breach the intestinal wall, leading to BT, which is defined as the passage of bacteria, or endotoxins produced by Gram negative bacteria, to usually sterile locations in the body (Berg *et al.* 1979)(Fig. 2). There are three primary mechanisms promoting BT: intestinal bacterial overgrowth, increased intestinal permeability and

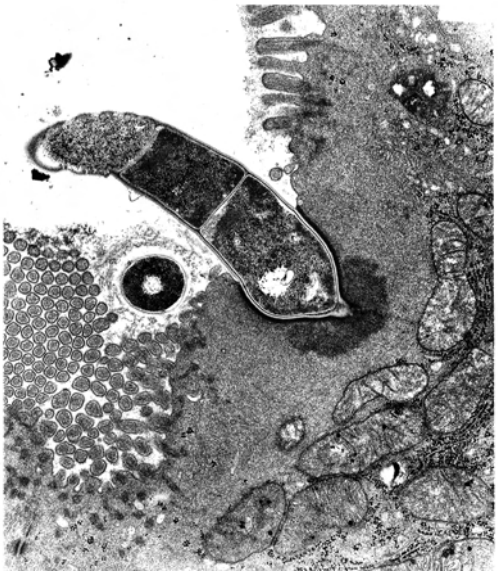

Fig. 2. Translocation in rat distal ileum. Transmission electron microscope x 15000.

host immunodeficiency (Berg 1999). The first mechanism, bacterial overgrowth, is the most effective one. Several experimental studies have shown a correlation between the concentration of bacteria in the intestine, especially the caecum, and BT to MLN (Katayama *et al.* 1997; Mao *et al.* 1997; Wang *et al.* 2001a). Further spread, beyond the MLN, often requires some grade of immunodeficiency (Berg 1999). However, primary insufficiency in sIgA-production by B-cells, or an attenuated T-cell response to bacterial invasion, also leads to the spread of intestinal bacteria to extraintestinal sites. In endotoxaemia, BT occurs as a result of the mucosal damage inflicted, separating epithelial cells and increasing permeability (Garcia *et al.* 2001). In hemorrhagic shock, the reduced tissue perfusion due to hypotension damages the intestinal mucosa, thus promoting BT (Nettelbladt *et al.* 1998).

BT has clearly been shown to occur in animal models (Adawi *et al.* 2001; Goldman *et al.* 2001; Mao *et al.* 1997). Also in humans BT is found. Thus, in general surgery BT to MLN occurs in 10–20% of the patients (MacFie *et al.* 1999; MacFie *et al.* 2006; Sedman *et al.* 1994). Emergency surgery and total parental nutrition are found to predispose to BT (MacFie *et al.* 2006) and with intestinal obstruction as many as 60% of the patients have positive MLN (Sagar *et al.* 1995). There seems to be a correlation between the finding of culture-positive MLN's, as a sign of BT, and septic morbidity. Patients in whom BT is found have a higher incidence of septic complications (MacFie *et al.* 1999; MacFie *et al.* 2006). However, the clinical importance of BT has been more difficult to confirm (Lemaire *et al.* 1997; MacFie 2004). It might be that a trauma or disease process initiates an inflammatory response with concomitant injury to the intestinal barrier. If this is followed by a second insult, such as surgery or continuing disease, BT is prone to occur. Thus, BT might be a promoter, rather than initiator, of systemic inflammatory response syndrome (SIRS) in a situation when immune response is already exhausted, leading to septic morbidity. In such situations it would seem rational to strengthen the intestinal barrier and limit bacterial overgrowth early in the inflammatory phase with the intention to prevent secondary BT.

Efforts have been made to prevent or attenuate BT. Nitric oxide inhibition (Unno *et al.* 1997), fiber (Spaeth *et al.* 1995) and anti-TNF-α (Goldman *et al.* 2001) are examples of substances that have shown an effect on the magnitude of BT. Bowel decontamination is used to prevent BT but it seems preferable to keep the intestinal microflora, mucosa and immunological competence stable. Thus, by stimulating GI motility, preventing bacterial overgrowth and maintaining the intestinal barrier intact, BT can be prevented or at least attenuated. Probiotics might have these effects (Besselink *et al.* 2005). Promising results have come from animal studies where probiotic bacteria have been found to prevent BT in Caco2-cells (Mattar *et al.* 2002),

in rats with liver injury (Adawi *et al.* 1997; Wang *et al.* 2001b) or enterocolitis (Mao *et al.* 1996a) and after liver- and colon resection (Seehofer *et al.* 2004). The prevention of BT in humans has, however, been more difficult to achieve (Anderson *et al.* 2004; McNaught *et al.* 2002).

Several mechanisms may be involved in the potentially protective effects of lactobacilli in BT. Previous studies have shown that *L. plantarum* 299v has adhesive capabilities on colon cells (Adlerberth *et al.* 1996), but it is not known whether this is of any clinical significance in preventing BT. This stresses the need for further studies *in vivo* to elucidate the importance of adhesion of probiotics in preventing BT, but also studies aiming to clarify the ability to prevent BT in the clinical situation.

Lipopolysaccharide (LPS)

In the concept of BT is also included translocation of endotoxin (lipopolysaccharide, LPS), which is the principal component of Gram negative bacterial cell membrane (Helander *et al.* 1999). LPS consists of lipid A, a hydrophobic component which anchors the molecule to the cell membrane, a polysaccharide in the middle and a hydrophilic oligosaccharide which is projecting into the extracellular space. Lipid A varies between different bacterial species, accounting for the specificity of different LPS (Helander *et al.* 1999) and is essential for endotoxin-induced BT (Deitch *et al.* 1989). A low dose of LPS is probably favourable to the host by priming the inflammatory response, resulting in a rapid elimination of hostile microorganisms (Abdul-Hai *et al.* 2006). But with an increased translocation of LPS, as well as translocation of viable or nonviable Gram negative bacteria, classical symptoms of septicaemia are elicited.

LPS forms a complex with LPS-binding protein (LBP) which binds to CD14 on macrophages (Helander *et al.* 1999). Transcriptional activation goes through the transmembrane signalling protein Toll-like receptor 4 (TLR4) which induce cell-activation through NF-κB pathways (Chow *et al.* 1999). The resulting response is production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α. Thus, TNF-α added to Caco2-cells exposed to enteric bacteria and hypoxia increases apoptosis and intestinal permeability, an effect abrogated by anti-TNF-α (Diebel *et al.* 2005). Moreover, IFN-γ acted synergistically with TNF-α on the intestinal epithelium (Schmitz *et al.* 1999a). The mechanism behind the change in permeability seems to be an altered function of TJ (Schmitz *et al.* 1999a). Besides macrophages, LPS activates monocytes and endothelial cells, leading to the release of cytokines as mentioned above. These pro-inflammatory cytokines may up-regulate the expression of endothelial adhesion molecules and chemokines. However, whether there is a relation between the increased permeability and the LPS-induced cellular recruitment is not known.

Leukocyte recruitment

The recruitment of leukocytes to extravascular sites is central in inflammation. The process involves several interacting factors on leukocytes and endothelium (Fig. 3), the most important being the selectins and integrins (Thorlacius 2004).

Leukocyte recruitment is a multi-step process (Butcher 1991; Carlos *et al.* 1994), initiated by a rolling adhesive phase, where leukocytes are displaced laterally in the laminar blood flow and their velocity gradually reduced, allowing for increased time of interaction between the leukocyte and the endothelium. If the slowly rolling leukocyte does not detect any further signals, it will be released back into the circulation. However, if the leukocytes are exposed to chemoattractants on the endothelial cells, there will be a gradual change from reversible rolling to firm adhesion. This is mediated by upregulation of β_2-integrins on the leukocyte surface, interacting with receptors on the endothelium. The last step in leukocyte recruitment is the transmigration

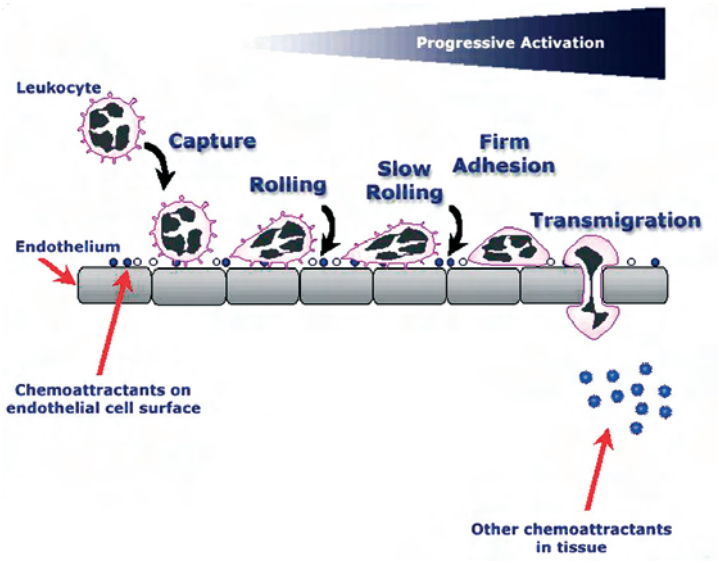

Fig. 3. Leukocyte recruitment in a postcapillary venule. The extravasation of circulating leukocytes during inflammation is a sequence of events including capture and activation, rolling along the endothelium, adhesion and finally transmigration between endothelial cells to the extravascular tissue.
(Modified with kind permission from Prof K Ley, University of Virginia, USA).

through the endothelium to the inflammatory process in the extravascular tissue.

Among the family of adhesion molecules, P-selectin is the most important in regulating rolling (Riaz *et al.* 2002). P-selectin is found in α-granulae of platelets and also in Weibel Palade bodies of endothelial cells (McEver *et al.* 1989). When stimulated, preformed P-selectin is released within minutes. A second peak occurs several hours later and is of greater magnitude. This release of P-selectin is regulated by transcription and induced by factors such as LPS and TNF-α (Thorlacius 2004).

Involved in the arrest of the rolling leukocyte are also chemokines, a family of chemoattractants that have pro-inflammatory functions. The chemokines are structurally divided into two main groups, CXC and CC (Mantovani 1999). CXC chemokines, of which IL-8 is one, are potent chemoattractants for neutrophils. The murine equivalents to IL-8 is macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC). CC chemokines may attract T-lymphocytes, macrophages and eosinophils.

Increased intestinal permeability is seen with LPS-induced sepsis (Garcia *et al.* 2001; O'Dwyer *et al.* 1988) and several studies have reported that pro-inflammatory substances may increase the permeability of epithelial monolayers *in vitro* (Diebel *et al.* 2005; Schmitz *et al.* 1999a). However, these studies, by being done *in vitro*, lack the presence of leukocytes, which have a central role in inflammation. Therefore, one of the aims of this work was to elucidate the role of leukocytes in intestinal permeability induced by LPS.

Aims

The main aim of this thesis was to study the effect of *L. plantarum* 299v on gut function in health and disease. This was done in four papers with the specific aims to:

- study the ability of *L. plantarum* 299v to reduce intestinal permeability induced by a strain of *E. coli* (paper I),
- study the ability of *L. plantarum* 299v to prevent BT in sepsis (paper II) and
- determine whether such a preventive effect is dependent on the adhesive capability of *L. plantarum* 299v (paper II),
- investigate the role of leukocytes and P-selectin in sepsis-induced permeability in the intestinal barrier dysfunction associated with sepsis (paper III),
- investigate whether oral treatment with *L. plantarum* 299v to patients undergoing colon resection can affect the load of some potentially pathogenic bacteria in the intestine and prevent BT (paper IV)

Material

Animals

Rats (study I–II)

In study I and II male Sprague-Dawley (Möllegaard, Skensved, Denmark) rats weighing 321–456 g were used. They were maintained in a 12-hr light-dark cycle at 20 ± 2°C and a relative humidity of 50 ± 10% for at least one week before starting the experiment. The rats had access to rat chow (B&K Universal, Sollentuna, Sweden) and water *ad libitum*. The rats were temporarily sedated with CO_2 for intraperitoneal injections in study II. For harvest of intestine for Ussing chamber experiments in study I and intestine, MLN's and liver for culture in study II, diethyl ether was used for anesthesia.

Mice (study III)

Male C57BL/6 mice (Taconic Europa, Ry, Denmark) weighing between 20–22 g were maintained at 12 h dark and 12 h light cycles and had free access to standard pellet food (R3, Lactamin AB, Kimstad, Sweden) and water *ad libitum*. Anesthesia was achieved by intraperitoneal (i.p.) injection of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel Switzerland) and 2.5 mg xylazine (Jansen Pharmaceutica, Beerse, Belgium) per 100 g body weight.

Patients (study IV)

Patients of both sexes referred for colon surgery were eligible for entry into the study. They were enrolled into the study at the first visit to the Department of Surgery outpatient clinic. The study was double blinded, randomized and placebo controlled. Exclusion criteria were planned rectal surgery, history of endocarditis, congenital or acquired heart valve disease or inability to follow the preoperative study protocol at home. Randomization was achieved by randomly linking the patient number to either 299v or placebo group. Patients in the treatment group (299v group) were given sealed containers of an oatmeal based drink containing 10^9 colony forming units (CFU)/ml of *L. plantarum* 299v (Probi, Lund, Sweden). Patients in the control group (placebo group) were given the same oatmeal based drink, but without the bacteria added. Patients, medical staff and study personnel were blinded as to which group the patients were randomized to. All patients presented written consent, signed after written and oral information. Pre-, peri- and postoperative measures, *i.e.* bowel preparation, antithrombotic prophylaxis, perioperative antibiotics, anaesthesia and postoperative care were performed according to routines at the department.

Ethics

All animal studies were approved by the Animal Ethics Committee of Lund University. The patient study was approved by the Ethics Committee of Lund University and was performed according to the Declaration of Helsinki.

Marker molecules

[^{14}C]mannitol (MW 182 Da) was used in paper I. The passage of this hydrophilic marker across the mucosa is not clearly defined, but both transcellular permeation through small pores in the enterocytes, and paracellular via tight junctions, have been suggested (Krugliak *et al.* 1994; Travis *et al.* 1992).

In study III sodium fluorescein (MW 376 Da) was used. It is generally held as a marker of paracellular permeability (Bernkop-Schnurch *et al.* 2004).

Bacterial strains

L. plantarum 299v (DSM 9843) was used in studies I, II and IV. In study I and II, the bacterium was mixed with an oatmeal based drink containing 18.5 g oatmeal based per 100 g drink (Molin *et al.* 1992) and given either through a gastric tube or mixed with the drinking water.

In study II one group of rats were fed bacteria in drinking water as mentioned above, while one group received *L. plantarum* 299v-adh$^-$, a strain that originates from *L. plantarum* 299v but has lost the ability to adhere to HT-29 cells (Mack *et al.* 2003). When given in the drinking water, this bacterium was also mixed with an oatmeal based drink as mentioned above.

In study IV the subjects in the 299v-group received 100 ml of the oatmeal based drink containing *L. plantarum* 299v in the same concentration daily, starting eight days before surgery and resuming intake from the first until the sixth postoperative day. The placebo group received the oatmeal based drink without any bacteria added.

E. coli F131 (kindly provided by I Alderberth, Dept of Clinical Immunology, Göteborg University, Göteborg, Sweden) was used in study I.

Methods

Experimental protocols

This chapter briefly describes the experimental procedures in the studies presented. Detailed information is found in the different papers.

Study I

Rats were divided into three groups and pretreated for one week with either regular feeding only; addition of oatmeal based drink through tube feeding twice daily; or addition of oatmeal based drink mixed into the drinking water. The oatmeal contained 10^9 CFU *L. plantarum* 299v per ml. All rats had free access to standard rat chow. Under anesthesia, 25 cm of distal ileum was harvested, carefully cleared from mesentery, divided and placed in Ussing chambers for permeability studies.

Study II

Rats were divided into five groups. Group 1 and 2 were negative and positive control respectively and received regular drinking water. Group 3 received oatmeal based drink mixed into the drinking water, while group 4 received the same oatmeal based drink mixed with 10^9 CFU *L. plantarum* 299v per ml in the drinking water. Finally, in group 5 the bacteria were changed to a strain which had lost its ability to adhere to HT-29 cells (Mack *et al.* 2003), *L. plantarum* 299v-adh$^-$. All rats had free access to standard rat chow. Following one week pretreatment, the rats were sedated and given an intraperitoneal injection of LPS from *E. coli* serotype O111:B4 (Sigma, Stockholm, Sweden). 24 hours later the rats were anaesthetized and MLN, liver and distal ileum harvested.

Study III

Mice were treated with either 0.3 ml sterile saline i.p. (negative control); 2 mg LPS from *E. coli* serotype O111:B4 (Sigma Chemical Co, St Louis, MO, USA) per 100 g body weight i.p. (positive control); 40 μg of an isotype-matched rat antibody IgG (clone R3-34, BD Biosciences Pharmingen, San Diego, CA, USA) dissolved in sterile saline to a total volume of 0.2 ml intravenously (i.v.) by a lateral tail vein injection, immediately followed by i.p. administration of LPS (control antibody); or 40 μg of a monoclonal antibody against mouse P-selectin (clone RB40.34, BD Biosciences Pharmingen) dissolved in sterile saline to a total volume of 0.2 ml i.v. immediately followed by LPS (anti-P-selectin antibody). All animals had free access to regular mouse chow and water before and after treatment. Permeability studies and intravital microscopy were conducted 18 h after LPS injection.

Study IV

Starting in the morning eight days before surgery, and continuing daily until admitted into the hospital, each of the 72 patients included in the study drank 100 ml of the oatmeal based drink to which they were randomized (Fig. 4). In the 299v group (treatment group) this corresponds to a daily intake of 10^{11} CFU of *L. plantarum* 299v. After preoperative enema, an extra dose of the study preparation was given the night before surgery, adding up to a total of nine doses before surgery. The first postoperative morning, consumption was resumed and continued for five days. Intake of the study preparation was documented by the patient at home. After admission, intake of study preparation, postoperative passage of gas and stool, as well as fluid intake, solid food intake and days on epidural analgesia were registered by the nursing staff.

Rectal swabs for bacterial culture were taken at time of inclusion in the study, the day before surgery, corresponding to eight days intake of the study preparation, postoperative day six as well as six weeks and six months after surgery.

Mucosal biopsies at the 10 cm level of the dorsal rectum were taken through a rectoscope at time of inclusion, the day before surgery and six weeks postoperatively. Further, a mucosal biopsy was taken in the distal end of the resected colonic specimen at surgery.

Immediately after laparotomy a MLN at the

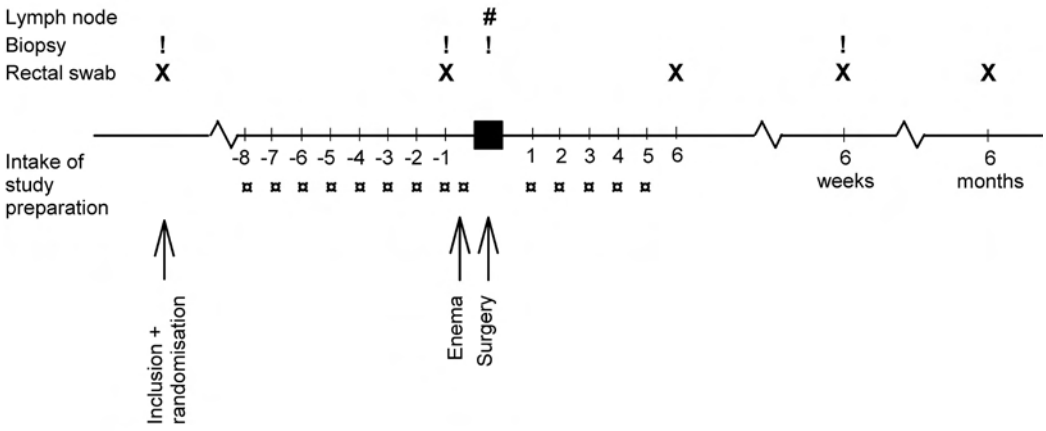

Fig. 4. Flow chart of experimental setup in study IV.

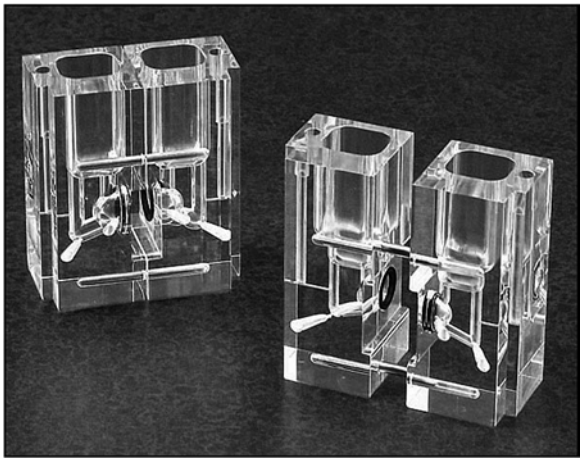

Fig. 5. Modified Ussing diffusion chamber. An insert with the intestinal segment to be studied is mounted between the two chambers. One of the chambers is filled with buffer solution containing a permeability marker. The other chamber is filled with only buffer solution from which the marker is retrieved according to its permeation.

ileocaecal junction was harvested, cleared of visible fat and placed in a sterile tube with buffer solution. The tube was snap frozen in liquid nitrogen and stored in –70°C for later analysis.

Ussing chamber experiments

Distal ileum of rats (study I) and mice (study III) were harvested, longitudinally opened along the mesenteric border, rinsed and mounted in Ussing chambers (Grass *et al.* 1988; Ussing *et al.* 1951) with an exposed area of 1.78 cm^2 for rats (Precision Instrument Design, Los Altos, CA, USA) and 0.25 cm^2 for mice (Harvard Apparatus, Holliston, MA, USA) (Fig. 5). The chambers were placed in heat blocks and each half chamber filled with Krebs' buffer solution.

In study I the solution in the mucosal (donor) reservoir was replaced with buffer containing [^{14}C]mannitol (182 Da, 0.031 μCi/ml, DuPont, Dreieich, Germany). Further, either *L.plantarum* 299v, *E. coli*, a mixture of both bacteria or phosphate-buffered saline (PBS) was added to sets of Ussing chambers on the mucosal side. One ml samples were then taken at 20, 40, 60 and 120 min from the serosal (recipient) reservoir and replaced with Krebs' buffer solution.

In study III the buffer solution in the mucosal (donor) reservoir was instead substituted with sodium fluorescein (Na-FITC, MW 376 Da, Sigma Chemical Co, St Louis, MO, USA). The chambers were covered to protect from light and after 60 min specimens were taken for spectrofluorometry from the serosal (recipient) reservoirs.

Marker molecule analysis

The amount of radiolabelled mannitol passing across the distal ileum of rats (study I) was determined in a beta counter (LKB, Bromma, Sweden) after mixing the samples with 5 ml scintillation cocktail (Ready Safe, Beckman, Fullerton, CA, USA).

Spectrofluorometry (SpectaMax Gemini, Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 485 nm and emission wavelength of 525 nm was used for analysis of the amount of sodium fluorescein crossing the mounted ileum of mice (study III). Known amount of sodium fluorescein was used to make a standard curve from which the amount of sodium fluorescein passage was determined.

Electrical measurements

In paper I electrical potential difference (PD) was measured at t = 0, 20, 40, 60 and 120 min using Ag-AgCl electrodes embedded in KCl agar and connected to a millivoltmeter (Millicell-ERS, Stockhomlm, Sweden). PD measurement was done at t = 0 and 60 min in study III. PD < 3.0 mV at t = 0 excluded that specimen from the experiment.

Bacterial translocation

Bacterial culturing (study II)

Samples from MLN and liver were placed in an ultrasonic bath (Millipore, Sundbyberg, Sweden) for two minutes and vortexed on a Chiltern (Thera-Glas, Gothenburg, Sweden) for two minutes. Viable counts were obtained by placing one ml of each suspended tissue sample on brain heart infusion agar (BHI; Difco Laboratories, Detroit, MI, USA) and incubated aerobically and anaerobically (Gas Pack System, Gas Pack, Beckton Dickenson Microbiology Systems, Cockeysville, MD, USA) at 37°C for three days, thus yielding total aerobic and anaerobic counts, respectively. The number of colonies formed on each plate was counted and the result expressed as CFU per gram tissue.

PCR (study IV)

The MLN's harvested from the ileocaecal junction as described above were thawed and cut into smaller pieces. A single piece was transferred to a 1.5 ml tube with 190 μl buffer G2 (DNA Tissue Kit, Qiagen, Hilden, Germany) and 10 μl of Proteinase K (Qiagen). Sterile glass beads were added and the cells were lysed at 56°C for 3–4 hours in a shaking water bath. Tubes were cooled on ice and shaken for 30 min at 4°C to disintegrate all bacteria. After centrifugation at 300 x g for one minute, the solution was transferred to a Qiagen sample tube and total DNA extracted by using Biorobot EZ1 (Qiagen) according to manufacturer's instructions. The DNA was eluted in 200 μl.

Primer sequences and PCR conditions are detailed in paper IV. PCR products were run on 1.5% agarose gel and stained with ethidium bromide. Gels were photographed by digital camera in UV-light. Samples showing a band on the gel of correct size were subjected to a second PCR for confirmation of positive results.

Intestinal microflora

In study II samples of rat distal ileum were placed in ultrasonic bath and thereafter vortexed as described above. Culturing was done on violet bile glucose agar (VRBG; Oxoid, Hampshire, England) incubated aerobically at 37°C for 24 h (*Enterobacteriaceae)* and on Rogosa agar (Oxoid) incubated anaerobically at 37°C for 72 h (lactobacilli).

Flow cytometry

In study II both *L. plantarum* 299v and *L. plantarum* 299v-adh$^-$ were used, the later being a strain that has lost its ability to adhere to HT-29 cells (Mack *et al.* 2003). To verify the adhesive capability of the two strains, flow cytometry was used. The bacteria were grown overnight on Rogosa agar for viable count. After reculturing on Lactobacillus Carrying Media (LCM) for 20 h, the bacteria were harvested, centrifuged, washed and stained with carboxyfluorescein diacetate (CFDA-SF; Molecular Probes Inc, Eugene, OR, USA). HT-29 cells were grown to confluence, trypsinated and seeded into microtiter plates and incubated for two days. CFDA-stained bacteria were added to the HT-29 cells and incubated for one hour. After fixation with paraformaldehyde, flow cytometry was performed on a Coulter® EPICS® XL Flowcytometer (Coulter Corp, Miami, FLA, USA).

Intravital microscopy

For the study of leukocyte rolling and adhesion, intravital microscopy of distal ileum was performed (study III). A catheter was inserted in the jugular vein of the anaesthetized mouse for administration of the marker solutions FITC-dextran (blood perfusion; MW 150 000; Sigma Chemical Co, St Louis, MO, USA) and rhodamine 6G (labeling of leukocytes; MW 479; Sigma Chemical). Through a midline incision, distal ileum was exteriorized and the mouse was put under an inverted Olympus microscope (IX70, Olympus Optical Co, GmgH, Hamburg, Germany). The image was recorded on videotape for later off-line evaluation. Analysis of rolling and adhesion was made in 3–6 distal ileum venules with an inner diameter of 25–50 μm and with stable blood flow. Blood perfusion was studied by illumination with blue light (excitation wavelength 490 nm, emission wavelength 510 nm) and leukocytes by illumination with green fluorescent light (excitation wavelength 530 nm, emission wavelength 560 nm). Leukocyte rolling was determined by counting the number of leukocytes passing a reference point in the venule during a 20 sec observation period and expressed as cells/min. Firm adhesion of leukocytes was determined by counting the number of leukocytes adhering and remaining stationary along a 100 μm segment of the venular endothelium for 20 sec. The results are expressed as cells/mm venule length. Blood flow velocity was analysed off-line by means of a computer image analysis programme (CapImage, Zeintl, Heidelberg, Germany). The velocity is expressed as mm/sec and wall shear rate calculated from the formula wall shear rate = 8([velocity/1.6]/venular diameter) (House *et al.* 1987).

Enzyme-linked immunosorbant assay (ELISA)

In study III distal ileum tissue concentrations of the chemokines MIP-2 and KC were determined by the use of ELISA. After rinsing, a segment of distal ileum was weighed and put in PBS containing 1% PEST (penicillin and streptomycin) (Gibco Invitrogen, Carlsbad, CA, USA) and 0.1 mg/ml amphotericin B (Fungizone, Bristol Myers Squibb, NY, NY, USA) for 60 min. The tissue was then incubated in Dulbecco's modified Eagle's medium solution containing 10% fetal calf serum, 1% PEST and amphotericin B for 24 h in 37°C on a 12-well plate. The medium was harvested, centrifuged for 10 min at 3000 rpm and the supernatant was collected and frozen in –20°C . Analysis was made using a quantitative sandwich enzyme immunoassay technique with polyclonal antibodies specific for murine MIP-2 and KC (R&D Systems, Minneapolis, MN, USA). The minimum detectable amount of protein in these kits is 1.5 pg/ml. Each sample was analysed in duplicates and optical density read at 450 nm (Milenia Kinetic Analyzer, DPC, Los Angeles, CA, USA).

Myeloperoxidase activity (MPO)

Segments of distal ileum from mice (study III) were harvested, weighed and homogenized in hexadecyltrimethylammonium bromide (Sigma Chemical, St Louis, MO, USA) after which MPO was extracted and purified. Absorbance was measured in a spectrophotometer at 450 nm. MPO activity was calculated from a standard curve and corrected for sample weight.

Systemic leukocyte count

Peripheral leukocytes were collected from mice (study III), counted in Burker chambers and differentiated in the microscope as mononuclear (MNL) or polymorphonuclear (PMNL) leukocytes.

Cell proliferation

Formaldehyde fixed and paraffin embedded sections of tumour and normal tissues from patients in study IV were studied for cell proliferation, using the proliferation marker Ki-67. 32 patients in the 299v group and placebo group respectively were used. Details of the procedure are described in paper IV. After staining with anti-human Ki-67 antigen, visualisation and counterstaining, sections were analysed in a light microscope.

Statistics

In all studies, $P<0.05$ was considered statistically significant.

Study I

Data are presented as mean ± SEM. Multiple comparisons were analyzed by use of one-way ANOVA with Tukey's test *post hoc*.

Study II

Data are expressed as median (25^{th}–75^{th} percentile). The incidence of BT was compared using Fisher's Exact Test. Kruskall-Wallis ANOVA on ranks, followed by multiple comparison with Dunn's method, was used to compare number of bacteria between groups.

Study III

Data are presented as mean ± SEM. Comparison was made with one-way ANOVA followed by Holm-Sidak's method for multiple comparisons between groups. Kruskall-Wallis ANOVA on ranks, followed by multiple comparisons with Dunn's method was used for non-parametric data.

Study IV

Quantitative data are expressed as medians and interquartile range (IQR). Chi-square test was used to compare qualitative data. Non-parametric data were compared by using Wilcoxon signed rank test within groups, while Mann-Whitney rank sum test was used between groups.

Results and discussion

L. plantarum 299v inhibits intestinal permeability induced by *E. coli* (paper I).

The purpose of this study was to explore if the probiotic bacterium *L. plantarum* 299v is able to prevent *E. coli*-induced intestinal permeability.

E. coli increased mucosal-to-serosal permeability across segments of rat distal ileum mounted in Ussing chambers. However, *E. coli*-induced permeability was abolished by pretreatment with *L. plantarum* 299v *ad libitum* for one week (Fig. 6). Pretreatment with *L. plantarum* 299v through tube feeding twice daily, or adding *L. plantarum* 299v to *E. coli* in the acute situation, *i.e.* in the Ussing chamber, did not prevent the *E. coli*-induced permeability. Further, *L. plantarum* 299v had no adverse effect on tissue viability, measured as potential difference across the intestinal segments, and did not in itself increase intestinal permeability.

Altered intestinal permeability is an important patophysiological event in many critical conditions, such as trauma (Pape *et al.* 1994), thermal injuries (LeVoyer *et al.* 1992), sepsis (Johnston *et al.* 1996) and inflammatory bowel disease (Nejdfors *et al.* 1998; Söderholm *et al.* 1999). The mechanism behind the deterioration in barrier function is often multifactorial. Some of the bacteria belonging to the indigenous microflora are PPB, but are kept under

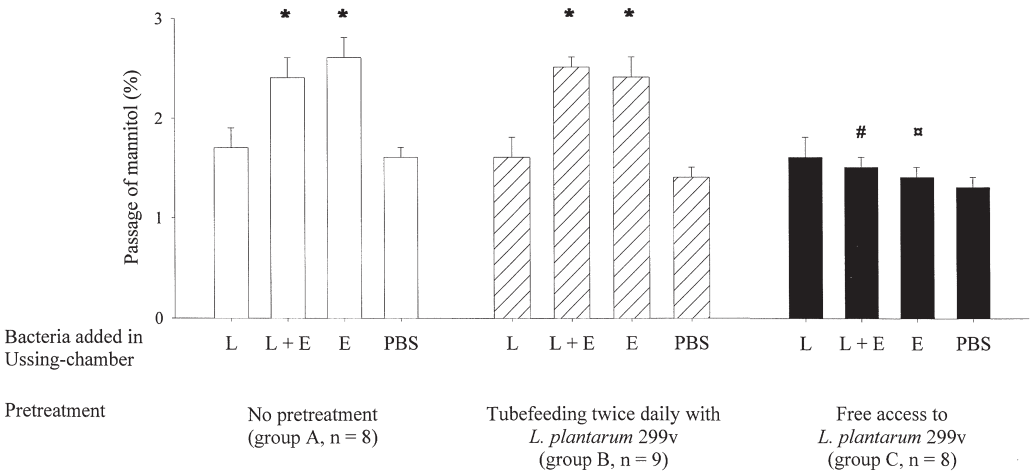

Fig. 6. Passage of mannitol expressed as percentage of initially added marker molecule across intestinal segments mounted in Ussing chambers at 120 min. Bacteria added in the Ussing chambers: L = *L. plantarum* 299v, L + E = *L. plantarum* 299v + *E. coli*, E = *E. coli* and P = phosphat-buffered saline (control). Data are mean values ± SEM.
* = $P < 0.05$ L + E and E *vs.* PBS
# = $P < 0.05$ L + E in group C *vs.* L + E group A and B
¤ = $P < 0.05$ E in group C *vs.* E in group A and B

control by the commensal bacteria. However, in situations mentioned above, or with *e.g.* antibiotic medication, cytotoxic treatment or infectious disease, the microflora is set off balance and the PPB may increase in number relative to the non-pathogenic ones. In such situations, intestinal permeability may be increased. Examples are *Salmonella typhi* and *E. coli* which have been shown to disrupt the intestinal barrier and augment permeability (Kops *et al.* 1996; Spitz *et al.* 1995).

The abrogating effect of *L. plantarum* 299v on *E. coli*-induced permeability seems to be dependent both on number of lactobacilli and the duration of pretreatment, as seen when comparing the groups receiving tube-feeding in a fixed dose and *L. plantarum* 299v added to the drinking water and given *ad libitum*. The intake of *L. plantarum* 299v is more continuous and steady when delivered through the drinking water, but in both cases, propulsion of ingested bacteria is rather quick down to the distal ileum and colon. There the transit time is slowed down and the mucosa is exposed to the bacteria for a longer time. Therefore, the way the lactobacilli are fed is probably of less importance, but by adding *L. plantarum* 299v together with oatmeal based drink to the drinking water a quantitative effect was achieved, as each rat ingested four times as many lactobacilli as when tube-fed. Further, it is less stressful for the rats to receive the study preparation through the drinking water in contrast to tube-feeding twice daily. This may also affect the intestinal permeability (Bailey *et al.* 2006; Söderholm *et al.* 2001).

The increased permeability to mannitol may be either through an enhancement of paracellular mucosal-to-serosal water flux with concomitant mannitol solvent drag, or through transcellular passive diffusion, which may be of greater

importance in the distal ileum compared to colon (Krugliak *et al.* 1994).

The possible mechanisms behind the decrease in *E. coli*-induced permeability are several. *L. plantarum* 299v supplied may create an advantageous milieu for other probiotic bacteria in the lumen, seen as an increase in concentration of lactobacilli as in previous studies on rats with enterocolitis (Mao *et al.* 1996a) and liver injury (Adawi *et al.* 1997). Together these bacteria lower the pH in the lumen which may decrease the concentration of PPB (Johansson *et al.* 1993). With an increase in number of probiotic bacteria, there is also a competition for nutrients which may further shift the bacterial balance towards fewer PPB's.

Lactobacilli given to rats with enterocolitis or liver injury were able to decrease the number of *Enterobacteriaceae* in distal ileum and colon (Adawi *et al.* 1997; Mao *et al.* 1996a). Thus, the attenuation of *E. coli*-induced permeability may be through an absolute or relative decrease in the number of *E. coli* in the intestine.

Previous studies have shown *L. plantarum* 299v to have a mannose-rich adhesion receptor for epithelial cells (Adlerberth *et al.* 1996). The receptor is similar to a receptor on most *E. coli* conferring adherence to intestinal cells (Cruz *et al.* 1994). This might be a mechanism behind the decrease in *E. coli*-induced permeability, *i.e.* competitive exclusion at binding sites on the mucosal surface. The binding and unbinding of bacteria to receptors on the epithelial cells is a continuously on-going process and as soon as the luminal availability of lactobacilli decreases, the number of lactobacilli found on the mucosa decreases quickly.

To summarize, this study shows that *E. coli* induce increased permeability across rat distal ileum. This can be prevented by pretreatment with high doses of *L. plantarum* 299v, possibly through several mechanisms, of which competitive exclusion is one. *L. plantarum* 299v itself does not induce permeability increase.

Adhesive capability of Lactobacillus plantarum 299v is important for preventing bacterial translocation in endotoxemic rats (paper II).

This study examined whether oral treatment with *L. plantarum* 299v could prevent BT to MLN in rats challenged with endotoxin LPS and if such a preventive effect was related to adhesive capacity of *L. plantarum* 299v.

It was found that one week of oral pretreatment with *L. plantarum* 299v reduced the incidence of BT to MLN and liver in rats subjected to sepsis by means of LPS (Fig. 7). This effect of the probioticum was abolished when using *L. plantarum* 299v that has lost its ability to adhere to intestinal cells. Thus, one of the properties of *L. plantarum* 299v in preventing BT seems to be dependent on adhesive capability. Flow cytometry confirmed a difference in adhesive capability to HT-29 cells between *L. plantarum* 299v and the strain originating from *L. plantarum* 299v, but which has lost its adhesive ability (Fig. 8). Further, it was found that pretreatment with oatmeal based drink did not prevent BT in endotoxaemia.

Probiotic bacteria have been used in several experimental settings to elucidate their effects in preventing disease. Both single strains and combinations, with or without addition of prebiotics, have been tried. In this study *L. plantarum* 299v was used, as it has been shown to interact with the immune system (Herias *et al.* 1999; Karlsson *et al.* 2002), increase mucin production (Mack *et al.* 1999), compete for colonization (Herias *et al.* 1999), and reduce BT (Adawi *et al.* 2001; Mao *et al.* 1996a).

The ability of *L. plantarum* 299v to adhere to the intestinal mucosa is one possible mechanism by which the bacterium prevents BT of enteric organisms as was also the case when reducing *E. coli*-induced permeability in paper I. *L. plantarum* 299v carries a mannose-specific

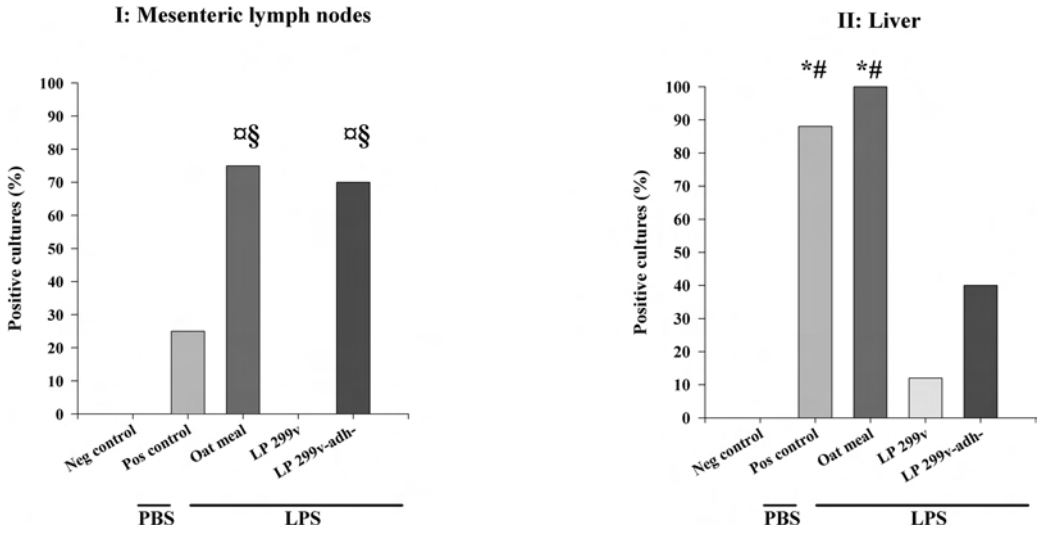

Fig. 7. Translocation to MLN And H Liver. Incidence of positive cultures (%) in
I: mesenteric lymph nodes (¤ = $P<0.01$ vs negative control and § = $P<0.01$ vs *L. plantarum* 299v)
II: liver (* = $P<0.001$ *vs* negative control and # = $P<0.05$ *vs L. plantarum* 299v).
See paper II for description of different groups.

adhesin (Adlerberth *et al.* 1996) which adhere to rat intestine (Herias *et al.* 1995) and HT-29 cells (Ahrné *et al.* 1998). Many *E. coli* express the same adhesin (Abraham *et al.* 1985). By supplying the intestinal mucosa with an excessive number of lactobacilli, there might be a competitive exclusion by these bacteria, preventing PPB to translocate. The effect of the probioticum is preventive, *i.e.* it has to be provided before the insult to the mucosa. Also, the preventive effect is temporary and a continuous supply of the probiotic bacterium is needed. Other preventive mechanisms might be induction of enterocyte mucus production (Mack *et al.* 1999) or an immunostimulating effect on sIgA production (Roller *et al.* 2004).

The oatmeal based drink given in this study increased the incidence of BT. This is contrary to some other studies (Wang *et al.* 1993) and could be due to difference in prebiotics administered. It might, however, also indicate that the usually positive, trophic effect of prebiotics can be questioned in sepsis where the integrity of the mucosa is under stress due to reduced blood flow, toxic substances and pro-inflammatory cytokines. Instead of strengthening the mucosa, the prebiotic might stimulate PPB and thus have a negative effect by promoting BT. However, if the prebiotic is given together with a probioticum, additive functions such as competitive exclusion of PPB, immunostimulation by increased production of sIgA, lowering of pH and upregulation of intestinal mucin might be able to prevent BT.

While LPS increased the number of *Enterobacteriaceae* in the distal ileum, pretreatment with *L. plantarum* 299v was not able to reduce this genera of bacteria, despite a reduction in BT. This indicates that the preventive effect of *L. plantarum* 299v is not solely by reducing the number of PPB on the mucosa, but other mechanisms, such as competitive exclusion as mentioned above, are important. It might also be that the composition of different species in the

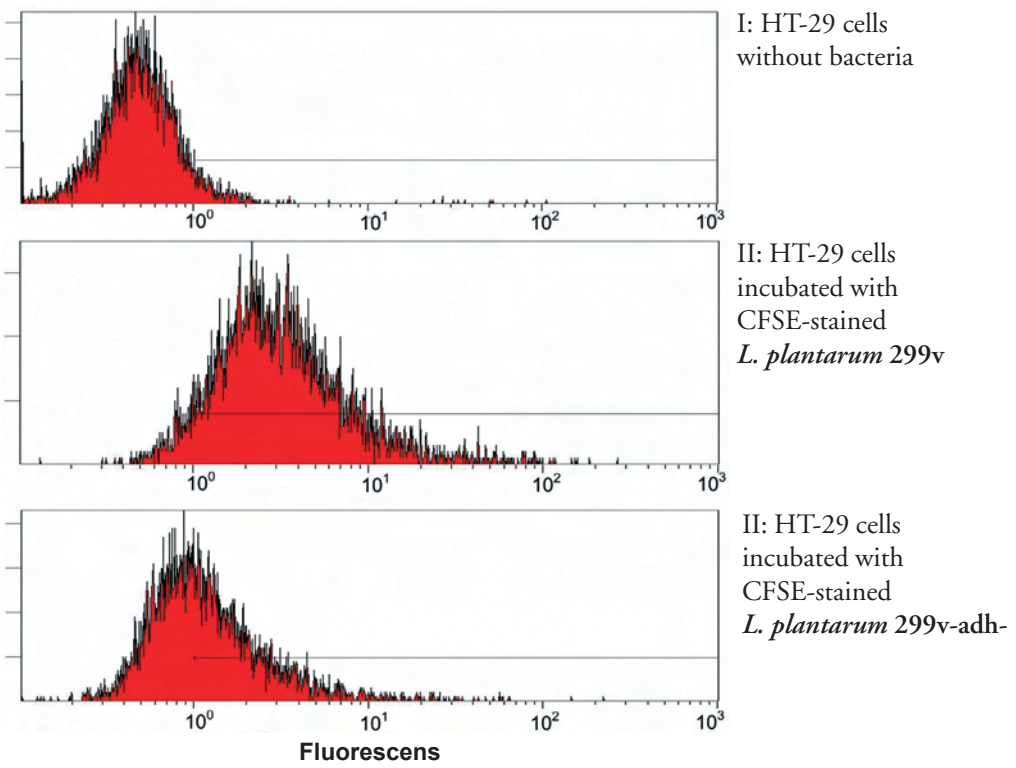

Fig. 8. Flow cytometry. *L. plantarum* 299v and *L. plantarum* 299v-adh- were stained with CFDA-SE and coincubated with HT-29 cells to study adherence. A reduced fluorescens intensity with *L. plantarum* 299v-adh$^-$ indicates lower adherence of these bacteria to HT- 29 cells
I: HT-29 cells without bacteria
II: HT-29 cells coincubated with CFDA-SE stained *L. plantarum* 299v.
III: HT-29 cells coincubated with CFDA-SE stained *L. plantarum* 299v-adh-.

Enterobacteriaceae group has changed. This was, however, not explored in this study.

Similarly, the total number of lactobacilli on the intestine did not change with either LPS or the exogenously supplied *L. plantarum* 299v. The microflora of rat intestine contains an abundance of lactobacilli and an addition of more bacteria might be difficult to detect. However, even if no quantitative difference is seen, there might be a qualitative difference, if the exogenously supplied lactobacillus replaces the endogenous bacteria on the intestinal mucosa. This could explain the effect of *L. plantarum* 299v in preventing BT when given continuously for a week before LPS-insult.

In summary, this study shows that exogenously supplied *L. plantarum* 299v prevents BT in LPS-induced sepsis and that there is a relation between adhesive capability and this preventive effect. Further, oatmeal based drink given alone in a septic state might have a negative effect regarding BT.

Critical role of P-selectin-dependent leukocyte recruitment in endotoxin-induced intestinal barrier dysfunction in mice (paper III).

The aim of this study was to define the importance of leukocyte recruitment in intestinal barrier dysfunction induced by LPS. For this purpose an antibody to P-selectin was used, as this selectin supports most rolling adhesive interactions *in vivo* (Klintman *et al.* 2004).

LPS induced an increased intestinal permeability of sodium fluorescein in the Ussing chamber (Fig. 9). This increase was abolished with anti-P-selectin but not with control-antibody. LPS did not affect the potential difference of the enterocytes. Concomitant with increased permeability, LPS increased leukocyte rolling and adhesion. These effects were attenuated when mice were pre-treated with anti-P-selectin antibody before administration of LPS. Systemic mononuclear, but not polymorphonuclear, leukocytes, were reduced in the septic state, but normalized in the mice pre-treated with anti-P-selectin

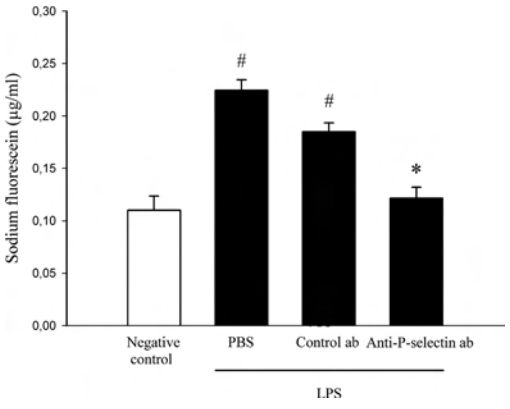

Fig. 9A

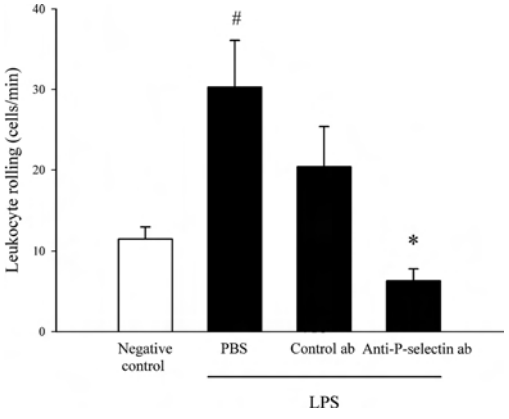

Fig. 9B

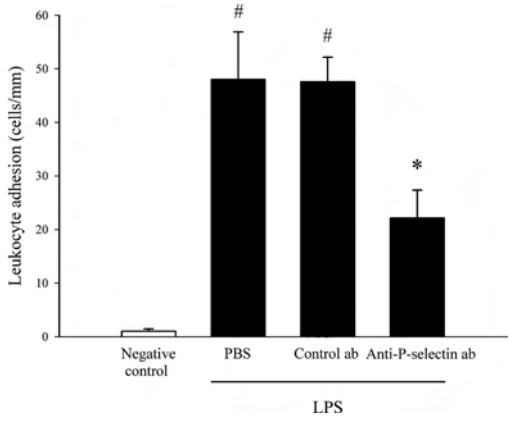

Fig. 9C

A) Mucosal-to-serosal permeability of sodium fluorescein (μg/ml) in distal ileum.
B) Leukocyte rolling (cells/min) along the endothelium of postcapillary venules in the submucosa of distal ileum was measured by use of intravital microscopy.
C) Leukocyte adhesion (cells/mm) on the endothelium of postcapillary venules in the submucosa of distal ileum was measured by use of intravital microscopy.
Mice were pre-treated with PBS, control-antibody (control ab) or anti-P-selectin antibody (anti-P-selectin ab) and then challenged with LPS (2 mg/100 g body weight) for 18 hours. Values are mean ± SEM. #$P < 0.05$ *vs* negative control and *$P < 0.05$ *vs* positive control (LPS) or control-antibody + LPS.

antibody. Intestinal levels of the CXC chemokines MIP-2 and KC increased with LPS challenge. Immunoneutralisation with anti-P-selectin antibody did not attenuate the LPS-induced mobilization of chemokines.

The findings in this study suggest a causal link between leukocyte accumulation and intestinal leakage in a state of LPS-induced sepsis. The mechanism underlying these effects might be the release of tissue damaging substances from activated leukocytes in the tissue. These include reactive oxygen species, matrix metalloproteinases, defensins and elastases (Faurschou *et al.* 2003), which may disrupt intestinal integrity and increase permeability. It has been shown that some proteases derived from neutrophils may be able to disrupt TJ's, which could explain the leukocyte-dependent barrier dysfunction in sepsis (Ginzberg *et al.* 2001). Also the leukocyte migration itself may cause physical disruption of the mucosal integrity (Nash *et al.* 1987).

In previous studies, pro-inflammatory mediators, such as MIP-2 and KC, have been found to exert powerful effects on permeability. These studies, however, were performed *in vitro* without the presence of leukocytes (Diebel *et al.* 2005; Schmitz *et al.* 1999a). Our findings that leukocytes play an important role in sepsis-associated intestinal leakage *in vivo* do not exclude the possibility that local mediators also contribute to this leakage. However, leukocyte accumulation seems to be of greater importance than the pro-inflammatory mediators in regulating LPS-induced permeability changes.

P-selectin is central in leukocyte rolling and adhesion, and thus in the subsequent tissue accumulation. The finding that anti-P-selectin antibody treatment abolished LPS-induced permeability by inhibiting leukocyte recruitment forms a possible path in abrogating the effects seen with sepsis. As of now, no such antibody is clinically available.

In summary, this study shows that leukocyte recruitment is critical in mediating sepsis-associated intestinal barrier dysfunction and that by preventing leukocyte recruitment with anti-P-selectin antibody, barrier dysfunction might be attenuated.

L. plantarum 299v to patients undergoing colon resection – a randomised placebo-controlled study (paper IV)

The aim of this study was to investigate the effect of high doses of *L. plantarum* 299v on the intestinal load of primarily *Enterobacteriaceae* and lactobacilli, as well as determine BT, measured as presence of 16S rRNA genes in MLNs on patients undergoing elective colon resection.

72 patients were randomized either to pre- and postoperative treatment with an oatmeal based drink containing a high concentration of *L. plantarum* 299v (299v group) or the same drink without bacteria (placebo group). There were no significant differences in demography, diagnosis or operative procedure between the groups. The number of postoperative complications was higher in the placebo group, but not to a statistically significant extent. The preoperative administration of *L. plantarum* 299v resulted in the same bacteria emerging on rectal swabs and mucosa biopsies from rectum, as detected with RAPD, highlighting the ability of the ingested bacteria to survive passage through the GI tract and colonize the rectal mucosa (Fig. 10). Six weeks after surgery, no ingested lactobacilli could be detected.

The intake of both study preparations resulted in increased viable counts of *Enterobacteriaceae* and Gram negative anaerobes on rectal swabs. The increase in *Enterobacteriaceae* was most pronounced in the 299v group. On mucosal biopsies, all studied bacteria increased with intake of *L. plantarum* 299v while no changes were seen in the placebo group. Postoperative rectal swabs in the 299v group showed increased vi-

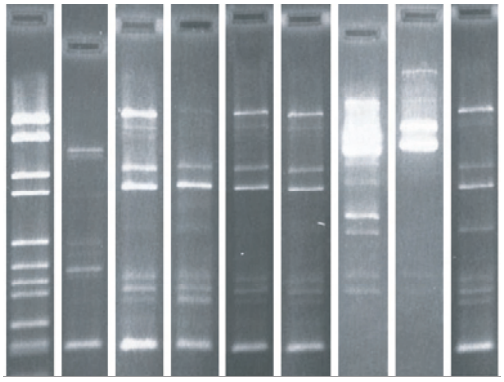

Fig. 10. Typical band patterns of RAPD-gels for bacterial colonies picked from the lactobacilli plate-count (Rogosa) from a patient randomised to the 299v group. Lane 1 shows DNA size markers and lane 9 *L.plantarum* 299v strain. Lane 2: rectal swab at inclusion (before intake of the study preparation); Lane 3: preoperative rectal swab and 4: mucosal biopsy (after one week intake of study preparation); 5: mucosal biopsy from resected colon; 6: rectal swab postoperative day 6; 7: rectal swab 6 weeks postoperative and 8: mucosal biopsy 6 months postoperative.

Table 1. 16S rRNA genes found in mesenteric lymph nodes

	299v group *n*=22	Placebo group *n*=24
Positive lymph nodes	6 (27%)	3 (13%)
Translocated bacteria:		
Lactobacillius	2	–
Clostridium perfingens group	2	–
Streptococcus	1	2
Bacterioides fragilis	1	1

able counts of all studied bacteria compared to before start of intake of the preparation, while in the placebo group this was only seen with *Enterobacteriaceae* and Gram negative anaerobes. Also, *Enterobacteriaceae* in the placebo group increased significantly compared with the preoperative value.

BT was evaluated with detection of 16S rRNA genes in MLN. Even though there was a higher number of MLN's with signs of BT in the 299v group, this difference was not statistically significant (Table 1).

L. plantarum 299v is considered to have trophic effects on the intestinal mucosa through increased production of SCFA. Thus, providing tumors with *L. plantarum* 299v might theoretically increase the risk of proliferation of tumour cells. However, Ki-67 antigen, an accepted parameter of cell proliferative activity, was not increased in tumour tissue by from patients treated with high doses of *L. plantarum* 299v (Fig. 11).

The intestinal wall, protecting the body from the luminal content of bacteria, toxins and other noxious substances, is broken in GI surgery. The more distal on the intestine this occurs, the greater is the risk of primary septic complications, due to the higher concentration of bacteria. BT occurs in 10–20% of patients undergoing GI surgery (MacFie *et al.* 1999; MacFie *et al.* 2006; Sedman *et al.* 1994) but some studies indicate that sheer manipulation of the gut may result in up to 80% BT (Reddy *et al.* 2006). The term "gut origin of sepsis" (MacFie *et al.* 1999) highlights the potential connection between BT and postoperative septic complications. Also psychological stress can induce BT, as seen in rats (Bailey *et al.* 2006; Nazli *et al.* 2004) but there are no studies on physiological stress and BT in humans.

In contrast to studies on healthy volunteers (Johansson *et al.* 1993; Johansson *et al.* 1998), *L. plantarum* 299v in this study did not reduce the concentration of *Enterobacteriaceae* and Gram negative anaerobes when given in high dose be-

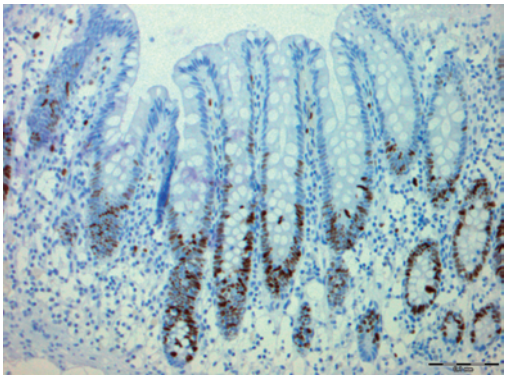

Fig. 11 A

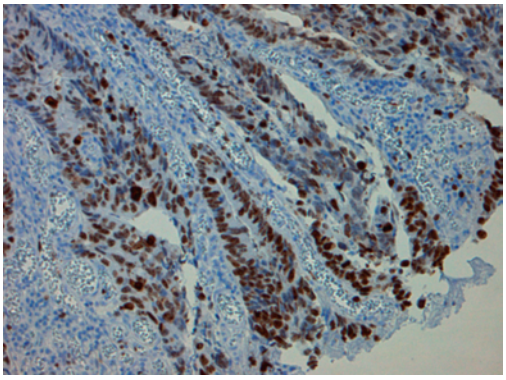

Fig. 11 B

Staining for the proliferation marker Ki-67 in A) normal mucosa and B) tumour. Ki-67 positive cells stain dark brown.

fore surgery. On one hand it is reasonable to think that probiotics to otherwise healthy individuals would not show any dramatically positive effects, *i.e.* a good microbial balance can not be further improved. If so, effects of probiotics should be easier to detect in patients with a disturbed microecology. On the other hand, it might be that patients with pathology in their colon have an imbalanced intestinal microecology due to their disease and the lactobacilli added would need more time to exert any detectable effect. If this is the case, to have an effect on PPB, perhaps the lactobacilli must be given for a longer duration than the eight days given in this study. However, in a study on patients undergoing colonoscopy, the same lactobacilli as in our study was given for twice as long, but without any effect on bacterial composition in the colon (Goossens *et al.* 2006). Thus, at present it is not known which concentration of lactobacilli is therapeutic and the duration of treatment needed to detect effects on intestinal microecology.

It could be speculated that the lack of effect when it comes to reducing PPB is due to the lactobacilli not surviving passage through the GI tract. However, by recovering *L. plantarum* 299v from the colon and identifying them with RAPD, we have shown in this study that *L. plantarum* 299v do survive passage and colonize the intestinal mucosa. Colonization is considered a prerequisite for biological effect and should be established in any study evaluating a probiotic bacterium. In some clinical studies colonization has been determined by recovering the supplied probiotic in nasogastric aspirates (McNaught *et al.* 2005). This is, however, questionable as the probiotic bacterium hardly has any effects in the stomach other than possibly displacing other bacteria there.

The intestinal microflora is considered to be rather stable during life. Small shifts between different species might occur, usually without any detectable effects. In this study, *Enterobacteriaceae* increased with the surgical trauma, but this increase was not prevented by pretreatment with *L. plantarum* 299v. A previous pilot study had shown this effect, as have animal studies (Adawi *et al.* 2001; Mao *et al.* 1996a). Again, it might be that the duration of *L. plantarum* 299v ingestion was too short. Moreover, within the family *Enterobacteriaceae* are several different genera. These might respond differently to *L. plantarum* 299v. One example is *Salmonella*

enterica subsp *enterica* to which *L. plantarum* 299v *in vitro* showed a higher inhibitory effect than to *Shigella sonni* (Hutt *et al.* 2006). A depression of one genus might enable another genus to expand, thus the total number of *Enterobacteriaceae* would remain unchanged. Only the total number of *Enterobacteriaceae* was calculated in this study, not different genera. As *E. coli* is one of the *Enterobacteriaceae* often found in septic complications after surgery, it might be of value to find a probioticum with specific effects against *E. coli*. As of now, no such probioticum is known.

The number of complications in the two study groups did not differ statistically and was at an expected level in colon surgery. It is, however, of interest to note that in the 299v group, the number of complications was half of those in the placebo group, despite a higher number of positive MLN's in the 299v group. The translocated bacteria were in two of the six cases lactobacilli. It might be that translocating lactobacilli prevents other bacteria to translocate, thus reducing the risk of septic complications. This could be achieved by competitive exclusion at receptor sites on the enterocytes, which would assume lactobacilli to be present in abundance and prior to the trauma predisposing to BT. However, some studies have shown a reduced postoperative septic complications rate even when the probiotic was given postoperatively (Rayes *et al.* 2002a; Rayes *et al.* 2002b). In these studies the greatest effect of the probiotic in reducing postoperative septic complications was in immunocompromised patients due to gastric or pancreatic malignancies or medical immunosuppression with liver transplantation, where complication rate is high and any positive effect of the probiotic is more easily detected. Here, the ability of the probiotic to prevent adhesion of PPB's is perhaps of lesser importance than the immunostimulating properties and therefore an effect is seen even when given postoperatively. However, any beneficial effect of probiotics given postoperatively seems to be reinforced when also given in the preoperative period (Sugawara *et al.* 2006).

In previous studies where *L. plantarum* 299v was given preoperatively, no effects on BT or postoperative septic complications could be seen (Anderson *et al.* 2004; McNaught *et al.* 2002). Even though the present study supports the previous one, they are not fully comparable. In one study a mixture of four different probiotics and prebiotics was used (Anderson *et al.* 2004). In the other study, the number of probiotic bacteria given was lower and placebo was not used. Further, the probiotic was reintroduced gradually after surgery and colonization in the distal GI tract was not confirmed (McNaught *et al.* 2002).

The frequency with which lactobacilli translocate to MLN in surgical patients is not previously studied. It is assumed that translocation in general occurs due to overgrowth of bacteria, a defective intestinal barrier or impaired immunological defense. The *L. plantarum* 299v given in high concentration could therefore have translocated due to overgrowth and the trauma elicited to the intestinal barrier. In this process, they might stimulate the immunological properties of the intestine, *e.g.* by activating B-cells, producing sIgA.

By using the method of 16S rRNA gene sequencing instead of conventional culturing, a higher incidence of BT would have been expected in both study groups. However, only a limited number of primers were used in the PCR in this study. Other bacteria might have translocated but would not be detected with this system.

In summary, this study on patients undergoing elective colon resections showed that high doses of *L. plantarum* 299v do not reduce viable counts of *Enterobacteriaceae* before or after surgery. Neither is BT or postoperative complications reduced by *L. plantarum* 299v in this setting. Moreover, *L. plantarum* 299v does not elicit any adverse effects when ingested in high dose by colon cancer patients.

Conclusions

- *L. plantarum* 299v protects against *E. coli*-induced intestinal permeability in rats. *L. plantarum* 299v itself does not increase intestinal permeability.
- *L. plantarum* 299v prevents BT in septic rats, which may be dependent on adhesion of the probiotic on the intestinal mucosa. Prebiotics given without probiotics do not prevent BT.
- Sepsis-associated intestinal leakage is regulated by P-selectin-dependent leukocyte recruitment. Targeting P-selectin may ameliorate gut barrier failure in sepsis.
- *L. plantarum* 299v do not reduce translocation of selected enteric bacteria in patients undergoing colon surgery, but seem to have a stimulatory effect on bacterial load in the colon.

Future aspects

Despite intense research on the GI tract, much remains to be understood. A tremendous amount of intestinal bacteria take part in biological processes which start at birth, continue without interruption throughout life and are crucial for health. The concept of the gut as an initiator or promoter of disease is only a few decades old, but is the base for several areas of interest.

L. plantarum 299v is one of many probiotic bacteria that have shown potentially beneficial effects on intestinal physiology such as mucosal immunity and permeability. Findings from animal studies have been difficult to reproduce in humans. However, *L. plantarum* 299v seems to be innocuous even when given in high concentrations. Therefore, studies with higher concentrations of bacteria, given for a longer period of time and exploring the effect on the indigenous microflora, BT and rate of complications in the clinical situation, are of great interest. Is *L. plantarum* 299v, in combination with a prebiotic, able to substitute antibiotics as prophylaxis in GI surgery? Microbial resistance to antibiotics is a global and quickly expanding problem, much due to overuse of broadspectrum compounds. This limits the therapeutic arsenal especially for immunocompromised and intensive care patients. Might prophylactic ingestion of a probiotic reduce the need of antibiotics in these settings? Can *L. plantarum* 299v promote production of SCFA to such an extent that trophic effects on the mucosa will render it more resistant to pathogenic bacteria, inflammation or trauma? Is there a place for *L. plantarum* 299v in preventing secondary manifestations of inflammatory disease, such as arthritis? *L. plantarum* 299v has immunomodulatory effects on animals, but can that also be found in humans, *e.g.* on pro- or anti-inflammatory cytokines and thus affect to progression of chronic inflammation or autoimmune disease?

The relation between leukocyte activation and intestinal barrier permeability is intriguing and opens up the possibility of stabilizing the intestinal mucosa by controlling the leukocyte accumulation. This is, however, a delicate balance between stimulating and abrogating inflammatory response.

The methods used in the present work may serve as useful tools in clarifying the considerations above.

Populärvetenskaplig sammanfattning

Tarmväggen har till uppgift att både absorbera vatten och näringsämnen till kroppen, och samtidigt utgöra en barriär mot alla de bakterier, gifter och skadliga ämnen som vi får i oss. Tarmväggen innehåller också kroppens största förråd av immunologisk vävnad, dvs de celler och ämnen som utgörs kroppens försvar. I mag-tarmkanalen, ffa i tjocktarmen, finns 100 biljoner (10^{14}) bakterier. De allra flesta bakterierna är av godo och deltar i biologiska processer viktiga för vår hälsa. I vissa tillfällen rubbas dock balansen mellan goda och skadliga bakterier. Blir de skadliga tillräckligt många, och i synnerhet om slemhinnan i tarmväggen samtidigt är skadad, sker bakteriell translokation (BT), dvs bakterierna tar sig igenom tarmväggen och in i kroppen varvid de kan utgöra ett hot mot hälsan. BT kan ske i samband med allvarlig sjukdom, svåra skador, brännskador, kirurgi, cellgiftsbehandling eller andra tillstånd med nedsatt immunförsvar. Överhuvudtaget kan tarmväggen vid sådana tillstånd uppvisa ökad permeabilitet, genomsläpplighet. Probiotiska bakterier, dvs levande bakterier som vid intag utövar gynnsamma effekter, kan bidra till att stärka tarmslemhinnan, förhindra ökad permeabilitet och återställa den normala balansen av bakterier i mag-tarmkanalen.

En av många probiotiska bakterier är *Lactobacillus plantarum* 299v. Den finns naturligt i tarmen hos människa och vid djurförsök har man funnit att den minskar leverskada och tarminflammation, stärker tarmslemhinnans skyddande slemlager och stimulerar immunförsvaret. Även när *L. plantarum* 299v ges till människa har man funnit gynnsamma effekter, t ex i form av minskad BT vid levertransplantation, minskad komplikationsrisk vid bukspottkörtelinflammation och en möjlig effekt på immunförsvaret.

Målsättningarna med detta avhandlingsarbete har varit att studera *L. plantarum* 299v´s förmåga att minska tarmens permeabilitet, minska BT och utröna om sådana skyddande effekter beror på *L. plantarum* 299v´s förmåga att fästa på tarmslemhinnan. Vidare har vi studerat vita blodkroppars betydelse för ökad permeabilitet orsakad av svår inflammation. Slutligen har *L. plantarum* 299v prövats på patienter inför tjocktarmsoperation för att studera om BT kan motverkas och förekomsten av skadliga bakterier minskas.

För tre av dessa studier användes råttor och möss. I de studier där *L. plantarum* 299v användes, tillfördes bakterien bl a genom tillsats i djurens dricksvatten. I en studie deltog 72 patienter som skulle genomgå tjocktarmsoperation. De blev lottade till att dricka antingen *L. plantarum* 299v eller placebo.

Olika metoder har användes för dessa studier, t ex Ussing kammare (tarmpermeabilitet), bakterieodling (förekomst av BT till lever och lymfkörtlar samt mängd bakterier på tarmslemhinnan), PCR (leta efter bakteriegener i lymfkörtlar), flödescytometri (cellers ljusspridning beroende på om *L. plantarum* 299v är fästa vid dem) och intravital mikroskopi (registrering av vita blodkroppars rörelse i blodkärlen på sövda möss).

Vi fann att en vanligt förekommande tarmbakterie, *E. coli*, ökar tarmens permeabilitet, men en veckas förbehandling med *L. plantarum* 299v motverkar denna ökning. *L. plantarum* 299v själv orsakade ingen ökad permeabilitet. Vid svår inflammation orsakad av ämnen från skadliga bakterier motverkar *L. plantarum* 299v BT. Denna effekt bedöms vara beroende av *L. plantarum* 299v´s förmåga att fästa vid tarmceller. Den ökade tarmpermeabilitet som orsakas av svår inflammation är beroende av att vita blodkroppar aktiveras och vandrar ut genom blodkärlens väggar, något som kan motverkas med en antikropp mot aktiverande ämnen i blodkroppar och kärlvägg. När *L. plantarum* 299v ges till patienter före och efter tjocktarmsoperation ser man ingen påverkan på BT, men *L. plantarum* 299v förefaller stimulera tarmens bakterieflora.

Fynden från dessa studier pekar på *L. planta-*

rum 299v´s förmåga att påverka tarmens slemhinna och minska riskerna för BT vid svår sjukdom och inflammation. Några säkra skyddande effekter i samband med tjocktarmsoperation gick inte att påvisa, men studien visade att *L. plantarum* 299v kan tillföras patienter i hög dos utan biverkan. I framtiden bör fortsatta studier utföras för att utvärdera möjligheten att motverka ökad tarmpermeabilitet genom att minska aktiveringen av vita blodkroppar. Dessutom bör studier med patienter göras med högre dos och längre tids behandling för att se om *L. plantarum* 299v kan motverka BT och komplikationer i samband med kirurgi på tjocktarmen.

If an expert says it can´t be done,
get another expert *Ben Gurion*

Acknowledgements

First and foremost **Ansti**, my wife, my love and my very best friend. The effort you have put into this work and all the support you always give me in each and every situation is more than anyone can wish. I look forward to many, many years of good laughs and happy moments together.

Victor and **Gabriel**, my future and guarantee for a life after this. The joy and happiness you bring is the light and spice of every day. You two make me so proud!

Suse and **Jacob**, my very dear parents, who guided me all the way and taught me the importance of family and friends.

Donald and **Riki,** my dear brothers, and their families. It's tough to be in the middle, but on the other hand, there is always someone to lean on. Thanks for letting me lean on you when needed.

My sincere gratitude to all co-workers and colleagues who have helped me during the work with this thesis. Special thanks to:

Bengt Jeppsson: my supervisor and professor, for support all the way and for never saying no (except for once!). Thanks for your impressive patience and for having confidence in me.

Henrik Thorlacius: assistant supervisor, with a great and wide mind for research and an even wider swing at the squash court.

My close friends **Ingvar Syk, Björn Sonesson, Peter Månsson, Antoni Zawadzki:** for many good moments, laughs and discussions about life (and sometimes even research). Your support, and above all, your friendship, has been invaluable.

I not only use all the brains that I have,
but all that I can borrow. *Woodrow Wilson*

All the **colleagues** at the Colorectal Unit, Malmö University Hospital: you had fantastic patience with me when I forced you to spend time taking that little lymph node and those tiny millilitres of blood during surgery. Former head of the Colorectal Unit, **Hasse Jiborn,** for being my role model of an excellent surgeon.

Ingrid Palmquist, our incredible research nurse: without you keeping track of everything (not the least me!), taking all those samples and remembering where we put it all, this would not have been. My fingers will not forget the hours we spent sorting -70°C vials.

Susanne Eiswohld: queen of the lab. Your help has been invaluable. **Diya Adawi, Nadia Osman, Alejandra Vásquez**, **Qing Liu** and others for lots of help in the lab.

My co-authors: **Pernilla Lennernäs** (many fun moments when you taught me the secrets of the Ussing chamber), **Mei Wang** (excellent mastering of the bugs), **Crister Olsson** (never ending patience with the PCR), **Yusheng Wang** (another PCR master), **René Schramm** (chief of velocity), **Andrada Mihaescu** (an enthusiast with the intravital microscope and a fantastic help in all situations).

Björn Weström: for helping me out with the Ussing chambers in the very beginning.

Siv Ahrné: for invaluable help in starting me off with this project and support all the way in handling the Lp 299v.

Göran Molin: for your vast knowledge and your capacity of always getting something good out of something not looking so good. Your help and support in getting things together has been priceless.

Nurses and **staff** at the Colorectal Unit: for taking so good care of our patients and convincing them to take that oatmeal shot every day.

All dear **patients** who had trust in me. I hope I gave you the support and comfort you needed, while you gave yourself to help science.

If I´m going to believe in something I can´t see,
I prefer wonders before bacteria. *George Bernard Shaw*

References

1. Abdul-Hai, A., Weiss, L., Slavin, S.,Or, R. (2006) Improved survival following induction of GVHD following lipopolysaccharide immunization. *Experimental Hematology* 34: 549-553.
2. Abraham, S. N., Babu, J. P., Giampapa, C. S., Hasty, D. L., Simpson, W. A.,Beachey, E. H. (1985) Protection against Escherichia coli-induced urinary tract infections with hybridoma antibodies directed against type 1 fimbriae or complementary D-mannose receptors. *Infect Immun* 48: 625-628.
3. Acheson, D. W. ,Luccioli, S. (2004) Microbial-gut interactions in health and disease. Mucosal immune responses. *Best Pract Res Clin Gastroenterol* 18: 387-404.
4. Adawi, D., Ahrné, S.,Molin, G. (2001) Effects of different probiotic strains of *Lactobacillus* and *Bifidobacterium* on bacterial translocation and liver injury in an acute liver injury model. *Int J Food Microbiol* 70: 213-220.
5. Adawi, D., Kasravi, B., Molin, G.,Jeppsson, B. (1997) Effect of Lactobacillus supplementation with and without arginine on liver damage and bacterial translocation in an acute liver injury model in the rat. *Hepatology* 25: 642-647.
6. Adlerberth, I., Ahrné, S., Jeppsson, B., Johansson, M-L., Hansson, L. Å.,Wold, A. E. (1996) A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl Environ Microbiol* 62: 2244-2251.
7. Ahrné, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. E.,Molin, G. (1998) The normal Lactobacillus flora of healthy human rectal and oral mucosa. *J Appl Microbiol* 85: 88-94.
8. Albanese, C. T., Cardona, M., Smith, S. D., Watkins, S. C., Kurkchubasche, A. G., Ulman, I., Simmons, R. L.,Rowe, M. I. (1994) Role of intestinal mucus in transepithelial passage of bacteria across the intact ileum in vitro. *Surgery* 116: 76-82.
9. Anderson, A. D. G., McNaught, C. E., Jain, P. K.,MacFie, J. (2004) Randomised clinical trial of synbiotic therapy in elective surgical patients. *Gut* 53: 241-245.
10. Bailey, M. T., Engler, H.,Sheridan, J. F. (2006) Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. *Journal of Neuroimmunology* 171: 29-37.
11. Balda, M. S. ,Matter, K. (2000) Transmembrane proteins of tight junctions. *Seminars in Cell & Developmental Biology* 11: 281-289.
12. Balda, M. S. ,Matter, K. (2003) Epithelial cell adhesion and the regulation of gene expression. *Trends in Cell Biology* 13: 310-318.
13. Berg, R. ,Garlington, A. W. (1979) Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. *Infect Immun* 23: 403-411.
14. Berg, R. D. (1999) Bacterial translocation from the gastrointestinal tract. *Adv Exp Med Biol* 473: 11-30.
15. Bernkop-Schnurch, A., Clausen, A. E,Guggi, D. (2004) The use of auxiliary agents to improve the mucosal uptake of peptides. *Medicinal Chemistry Reviews* 1: 1-10.
16. Besselink, M. G., Timmerman, H. M., van Minnen, L. P., Akkermans, L. M.,Gooszen, H. G. (2005) Prevention of Infectious Complications in Surgical Patients: Potential Role of Probiotics. *Dig Surg* 22: 234-244.
17. Bruewer, M., Luergering, A., Kucharzik, T., Parkos, C. A., Madara, J. L., Hopkins, A. M.,Nusrat, A. (2003) Proinflammatory Cytokines Disrupt Epithelial Barrier Function by Apoptosis-Independent Mechanisms. *J Immunol* 171: 6164-6172.
18. Bruewer, M. A. T. T, Samarin, S. T. A. N.,Nusrat, A. S. M. A. (2006) Inflammatory Bowel Disease and the Apical Junctional Complex. *Annals of the New York Academy of Sciences* 1072: 242-252.
19. Bry, L., Falk, P., Huttner, K., Ouellette, A., Midtvedt, T.,Gordon, J. I. (1994) Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proc Natl Acad Sci U S A* 91: 10335-10339.
20. Bukowska, H., Pieczul-Mróz, J., Jastrzebsk, K., Chelstowski, K.,Naruszewicz, M. (1998) Decrease in fibrinogen and LDL-cholesterol levels upon supplementation of diet with Lactobacillus plantrum in subjects with moderately elevated cholesterol. *Atherosclerosis* 137: 437-438.

21. Butcher, E. C. (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* 67: 1033-1036.
22. Carlos, T. M. ,Harlan, J. M. (1994) Leukocyte-endothelial adhesion molecules. *Blood* 84: 2068-2101.
23. Carneiro-Filho, B. A., Lima, I. P. F., Araujo, D. H., Cavalcante, M. C., Carvalho, G. H. P., Brito, G. A. C., Lima, V., Monteiro, S. M. N., Santos, F. N., Ribeiro, R. A.,Lima, A. A. M. (2004) Intestinal Barrier Function and Secretion in Methotrexate-Induced Rat Intestinal Mucositis. *Digestive Diseases and Sciences* 49: 65-72.
24. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J.,Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 274: 10689-10692.
25. Coconnier, M. H., Lievin, V., Bernet-Camard, M. F., Hudault, S.,Servin, A. L. (1997) Antibacterial effect of the adhering human Lactobacillus acidophilus strain LB. *Antimicrob Agents Chemother* 41: 1046-1052.
26. Conly, J. M., Stein, K., Worobetz, L.,Rutledge-Harding, S. (1994) The contribution of vitamin K2 (menaquinones) produced by the intestinal microflora to human nutritional requirements for vitamin K. *Am J Gastroenterol* 89: 915-923.
27. Cruz, N., Alvarez, X., Specian, R. D., Berg, R. D.,Deitch, E. A. (1994) Role of mucin, mannose, and beta-1 integrin receptors in Escherichia coli translocation across Caco-2 cell monolayers. *Shock* 2: 121-126.
28. Cummings, J. H. ,Macfarlane, G. T. (1996) Role of intestinal bacteria in nutrient metabolism. *JPEN J Parenter Enteral Nutr* 21: 357-365.
29. D'Souza, A. L., Rajkumar, C., Cooke, J.,Bulpitt, C. J. (2002) Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *BMJ* 324: 1361–1364
30. Deitch, E. A., Ma, W. J., Ma, L., Berg, R., Specian, R. D. (1989) Endotoxin-induced bacterial translocation: a study of mechanisms. *Surgery* 106: 292-9.
31. Deitch, E. A. M., Lu, Q., Feketkova, E., Hauser, C. J. M.,Xu, D. Z. (2004) Intestinal Bacterial Overgrowth Induces the Production of Biologically Active Intestinal Lymph. *J Trauma* 56: 105-110.
32. Diebel, L. N., Liberati, D. M., Baylor, A. E., III, Brown, W. J.,Diglio, C. A. (2005) The pivotal role of tumor necrosis factor-alpha in signaling apoptosis in intestinal epithelial cells under shock conditions. *J Trauma* 58: 995-1001.
33. Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E. M., O'Sullivan, G. C., Shanahan, F.,Collins, J. K. (1999) Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie van Leeuwenhoek* 76: 279-292.
34. Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargen, M., Gill, S. R., Nelson, K. E.,Relman, D. A. (2005) Diversity of the Human Intestinal Microbial Flora. *Science* 308: 1635-1638.
35. Evaldson, G., Heimdahl, A., Kager, L.,Nord, C. E. (1982) The normal human anaerobic microflora. *Scand J Infect Dis Suppl* 35: 9-15.
36. Fanaro, S., Chierici, R., Guerrini, P.,Vigi, V. (2003) Intestinal microflora in early infancy: composition and development. *Acta Paediatrica* 92: 48-55.
37. Farhadi, A. S. H. K, Banan, A. L. I., Fields, J. E. R. E.,Keshavarzian, A. L. I. (2003) Intestinal barrier: An interface between health and disease. *Journal of Gastroenterology and Hepatology* 18: 479-497.
38. Faurschou, M. ,Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5: 1317-1327.
39. Fuller, R. (1991) Probiotics in human medicine. *Gut* 32: 439-442.
40. Gallaher, D. D. ,Khil, J. (1999) The effect of synbiotics on colon carcinogenesis in rats. *J Nutr* 129: 1483S-1487S.
41. Garcia, Soriano F., Liaudet, L., Marton, A., Hasko, G., Batista, Lorigados C., Deitch, E. A.,Szabo, C. (2001) Inosine improves gut permeability and vascular reactivity in endotoxic shock. *Crit Care Med* 29: 703-708.
42. Ginzberg, H. H., Cherapanov, V., Dong, Q., Cantin, A., McCulloch, C. A., Shannon, P. T.,Downey, G. P. (2001) Neutrophil-mediated epithelial injury during transmigration: role of elastase. *Am J Physiol Gastrointest Liver Physiol* 281: G705-G717.

43. Gionchetti, P., Morselli, C., Rizzello, F., Romagnoli, R., Campieri, M., Poggioli, G., Laureti, S., Ugolini, F.,Pierangeli, F. (2004) Management of pouch dysfunction or pouchitis with an ileoanal pouch. *Best Practice & Research Clinical Gastroenterology* 18: 993-1006.
44. Gionchetti, P., Rizzello, F., Venturi, A., Brigidi, P., Matteuzzi, D., Bazzocchi, G., Poggioli, G., Miglioli, M.,Campieri, M. (2000) Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 119: 305-309.
45. Goldman, G., Soffer, D., Heller, L., Aderka, D., Lahat, A.,Klausner, J. M. (2001) Tumour necrosis factor mediates bacterial translocation after haemorrrhagic shock and endotoxaemia. *Eur J Surg* 167: 299-304.
46. Goossens, D., Jonkers, D., Russel, M., Stobberingh, E., Van Den Bogaard, A.,StockbrUgger, R. (2003) The effect of Lactobacillus plantarum 299v on the bacterial composition and metabolic activity in faeces of healthy volunteers: a placebo-controlled study on the onset and duration of effects. *Alimentary Pharmacology and Therapeutics* 18: 495-505.
47. Goossens, D., Jonkers, D., Russel, M., Thijs, A., Bogaard, A. van den, Stobberingh, E.,StockbrUgger, R. (2005) Survival of the probiotic, L. plantarum 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition. *Digestive and Liver Disease* 37: 44-50.
48. Goossens, D. A. M., Jonkers, D. M. A. E., Russel, M. G. V. M., Stobberingh, E. E.,Stockbrugger, R. W. (2006) The effect of a probiotic drink with Lactobacillus plantarum 299v on the bacterial composition in faeces and mucosal biopsies of rectum and ascending colon. *Alimentary Pharmacology and Therapeutics* 23: 255-263.
49. Grass, G. M. ,Sweetana, S. A. (1988) In vitro measurement of gastrointestinal tissue permeability using a new diffusion system. *Pharm Res* 5: 372-376.
50. Gueimonde, M., Jalonen, L., He, F., Hiramatsu, M.,Salminen, S. (2006) Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Research International* 39: 467-471.
51. Gullberg, E. ,Söderholm, J. D. (2006) Peyer's Patches and M Cells as Potential Sites of the Inflammatory Onset in Crohn's Disease. *Annals of the New York Academy of Sciences* 1072: 218-232.
52. Helander, I. M., Mamat, U.,Riethschel, E. T. (1999) Lipopolysaccharides. In: Encyclopedia of life sciences. John Wiley & Sons, Ltd: Chichester http://www.els.net.ludwig.lub.se/ [doi: 10.1038/npg.els.0000703]1-8.
53. Herek, O., Kara, I. G.,Kaleli, I. (2004) Effects of antibiotics and Saccharomyces boulardii on bacterial translocation in burn injury. *Surg Today* 34: 256-260.
54. Herias, M. V., Hessle, C., Telemo, E., Midtvedt, T., Hansson, L. Å.,Wold, A. E. (1999) Immunomodulatory effects of *Lactobacillus plantarum* colonizing the intestine of gnotobiotic rats. *Clin Exp Immunol* 116: 283-290.
55. Herias, M. V., Midtvedt, T., Hanson, L. A.,Wold, A. E. (1995) Role of Escherichia coli P fimbriae in intestinal colonization in gnotobiotic rats. *Infect Immun* 63: 4781-4789.
56. Hollander, D. (1999) Intestinal permeability, leaky gut, and intestinal disorders. *Curr Gastroenterol Rep* 1: 410-416.
57. Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U.,Huis in¦t Veld, J. J. J. (1998) Overview of gut flora and probiotics. *Int J Food Microbiol* 41: 85-101.
58. House, S. D. ,Lipowsky, H. H. (1987) Leukocyte-endothelium adhesion: Microhemodynamics in mesentery of the cat. *Microvascular Research* 34: 363-379.
59. Hutt, P., Shchepetova, J., Loivukene, K., Kullisaar, T.,Mikelsaar, M. (2006) Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. *J Appl Microbiol* 100: 1324-1332.
60. Ivanov, A. I., Nusrat, A.,Parkos, C. A. (2004) The epithelium in inflammatory bowel disease: potential role of endocytosis of junctional proteins in barrier disruption. Novartis Found. Symp.; 263: 115-124.
61. Jensen, V. B., Harty, J. T.,Jones, B. D. (1998) Interactions of the invasive pathogens Salmonella typhimurium, Listeria monocytogenes, and Shigella flexneri with M cells and murine Peyer's patches. *Infect Immun* 66: 3758-3766.

62. Johansson, M-L., Molin, G., Jeppsson, B., Nobaek, S., Ahrné, S.,Bengmark, S. (1993) Administration of different *Lactobacillus* strains in fermented oatmeal soup; in vivo colonization of human intestinal mucosa and effect on the indigenous flora. *Appl Environ Microbiol* 59: 15-20.
63. Johansson, M-L., Nobaek, S., Berggren, A., Nyman, M., Bjorck, I., Ahrné, S., Jeppsson, B.,Molin, G. (1998) Survival of Lactobacillus plantarum DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. *Int J Food Microbiol* 42: 29-38.
64. Johnston, J. D., Harvey, C. J.,Menzies, I. S. (1996) Gastrointestinal permeability and absorptive capacity in sepsis. *Crit Care Med* 24: 1144-1149.
65. Karayiannakis, A. J., Syrigos, K. N., Efstathiou, J., Valizadeh, A., Noda, M., Playford, R. J., Kmiot, W.,Pignatelli, M. (1998) Expression of catenins and E-cadherin during epithelial restitution in inflammatory bowel disease. *The Journal of Pathology* 185: 413-418.
66. Karlsson, H., Hessle, C.,Rudin, A. (2002) Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. *Infect Immun* 70: 6688-6696.
67. Kasravi, B., Adawi, D., Molin, G., Bengmark, S.,Jeppsson, B. (1997) Effect of oral supplementation of lactobacilli on bacterial translocation in acute liver injury induced by D-galactosamine. *J Hepatol* 26: 417-424.
68. Katayama, M., Xu, D. Z., Specian, R. D.,Deitch, E. A. (1997) Role of bacterial adherence and the mucus barrier on bacterial translocation. *Ann Surg* 225: 317-326.
69. Keefe, D. M., Cummins, A. G., Dale, B. M., Kotasek, D., Robb, T. A.,Sage, R. E. (1997) Effect of high-dose chemotherapy on intestinal permeability in humans. *Clin Sci (Lond)* 92: 385-389.
70. Kelly, D., Conway, S.,Aminov, R. (2005) Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology* 26: 326-333.
71. Klintman, D., Li, Xiang,Thorlacius, H. (2004) Important Role of P-Selectin for Leukocyte Recruitment, Hepatocellular Injury, and Apoptosis in Endotoxemic Mice. *Clin Diagn Lab Immunol* 11: 56-62.
72. Kops, S. K., Lowe, D. K., Bement, W. M.,West, A. B. (1996) Migration of *Salmonella typhi* through intestinal epithelial monolayers: an in vitro study. *Microbiol Immunol* 40: 799-811.
73. Krugliak, P., Hollander, D., Schlaepfer, C. C., Nguyen, H.,Ma, T. Y. (1994) Mechanisms and sites of mannitol permeability of small and large intestine in the rat. *Dig Dis Sci* 39: 796-801.
74. Kucharzik, T., Walsh, S. V., Chen, J., Parkos, C. A.,Nusrat, A. (2001) Neutrophil Transmigration in Inflammatory Bowel Disease Is Associated with Differential Expression of Epithelial Intercellular Junction Proteins. *Am J Pathol* 159: 2001-2009.
75. Lemaire, L. C. J. M., van Lanschot, J. J. B., Stoutenbeek, C. P., van Deventer, S. J. H., Wells, C. L.,Gouma, D. J. (1997) Bacterial translocation in multiple organ failure: cause or epiphenomenon still unproven. *Br J Surg* 84: 1340-1350.
76. LeVoyer, T., Cioffi, W. G. J.,Pratt, L. (1992) Alterations in intestinal permeability after thermal injury. *Arch Surg* 127: 26-29.
77. Lu, L. ,Walker, W. Allan. (2001) Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *Am J Clin Nutr* 73: 1124S-1130.
78. Luckey, T. D. (1972) Introduction to intestinal microecology. *Am J Clin Nutr* 25: 1292-1294.
79. MacFie, J. (2004) Current status of bacterial translocation as a cause of surgical sepsis. *Br Med Bull* 71: 1-11.
80. MacFie, J., O'Boyle, C., Mitchell, C. J., Buckley, P. M., Johnstone, D.,Sudworth, P. (1999) Gut origin of sepsis: a prospective study investigating associations between bacterial translocation, gastric microflora, and septic morbidity. *Gut* 45: 223-228.
81. MacFie, J., Reddy, B. S., Gatt, M., Jain, P. K., Sowdi, R.,Mitchell, C. J. (2006) Bacterial translocation studied in 927 patients over 13 years. *Br J Surg* 93: 87-93.
82. Mack, D. R., Ahrné, S., Hyde, L., Wei, S.,Hollingsworth, M. A. (2003) Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. *Gut* 52: 827-833.

83. Mack, D. R., Michail, S., Wei, S., McDougall, L.,Hollingsworth, M. A. (1999) Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. *Am J Physiol* 276: G941-G950.
84. Macpherson, A. J., Hunziker, L., McCoy, K.,Lamarre, A. (2001) IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes and Infection* 3: 1021-1035.
85. Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L.,de Simone, C. (2001) Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 121: 580-591.
86. Madsen, K. L., Doyle, J. S., Jewell, L. D., Tavernini, M. M.,Fedorak, R. N. (1999) *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology* 116: 1107-1114.
87. Majamaa, H., Isolauri, E., Saxelin, M.,Vesikari, T. (1995) Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. *J Pediatr Gastroenterol Nutr* 20: 333-338.
88. Manolopoulou, E., Sarantinopoulos, P., Zoidou, E., Aktypis, A., Moschopoulou, E., Kandarakis, I. G., Anifantakis, E. M. (2003) Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *International Journal of Food Microbiology* 82: 153-161.
89. Mantovani, A. (1999) The chemokine system: redundancy for robust outputs. *Immunology Today* 20: 254-257.
90. Mao, Y., Nobaek, S., Adawi, D., Molin, G.,Jeppsson, B. (1997) Comparison of the effects of different strains of *Lactobacillus* in reducing bacterial translocation on methtrexate-induced enterocolitis in rats. *Dig Surg* 14: 284-291.
91. Mao, Y., Nobaek, S., Kasravi, B., Adawi, D., Stenram, U., Molin, G.,Jeppsson, B. (1996a) The effects of Lactobacillus strains and oat fiber on methotrexate-induced enterocolitis in rats. *Gastroenterology* 111: 334-344.
92. Mao, Y., Yu, J-L., Ljungh, Å., Molin, G.,Jeppsson, B. (1996b) Intestinal immune response to oral administration of *Lactobacillus reuteri* R2LC, *Lactobacillus plantarum* DSM 9843, pectin and oatbase on methotrexate-induced enterocolitis in rats. *Microbial Ecology in Health and Disease* 9: 261-270.
93. Marshall, J. C., Christou, N. V.,Meakins, J. L. (1993) The gastrointestinal tract. The "undrained abscess" of multiple organ failure. *Ann Surg* 218: 111-119.
94. Mattar, A. F., Teitelbaum, D. H., Drongowski, R. A., Yongyi, F., Harmon, C. M.,Coran, A. G. (2002) Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. *Pediatr Surg Int* 18: 586-590.
95. Maxson, R. T., Dunlap, J. P., Tryka, F. Jackson R. J.,Smith, S. D. (1994) The role of the mucus gel layer in intestinal bacterial translocation. *J Surg Res* 57: 682-686.
96. McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L.,Bainton, D. F. (1989) GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest* 84: 92-99.
97. McIntosh, G. H., Royle, P. J.,Playne, M. J. (1999) A probiotic strain of L. acidophilus reduces DMH-induced large intestinal tumors in male Sprague-Dawley rats. *Nutr Cancer* 35: 153-159.
98. McNaught, C. E., Woodcock, N. P., Anderson, A. D. G.,MacFie, J. (2005) A prospective randomised trial of probiotics in critically ill patients. *Clin Nutrition* 24: 211-219.
99. McNaught, C. E., Woodcock, N. P., MacFie, J.,Mitchell, C. J. (2002) A prospective randomised study of the probiotic Lactobacillus plantarum 299V on indices of gut barrier function in elective surgical patients. *Gut* 51: 827-831.
100. Metchnikoff, E. (1908) The Prolongation of Life. New York, Putman Sons
101. Michail, S. ,Abernathy, F. (2003) Lactobacillus plantarum inhibits the intestinal epithelial migration of neutrophils induced by enteropathogenic Escherichia coli. *J Pediatr Gastroenterol Nutr* 36: 385-391.
102. Miettinen, M., Vuopio-Varkila, J.,Varkila, K. (1996) Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect Immun* 64: 5403-5405.

103. Molin, G., Andersson, R., Ahrné, S., Lönner, C., Marklinder, I., Johansson, M-L., Jeppsson, B.,Bengmark, S. (1992) Effect of fermented oatmeal soup on the cholesterol level and the Lactobacillus colonization of rat intestinal mucosa. *Antonie van Leeuwenhoek* 61: 167-173.
104. Molin, G., Jeppsson, B., Johansson, M-L., Ahrné, S., Nobaek, S., Ståhl, M.,Bengmark, S. (1993) Numerical taxonomy of Lactobacillus spp associated to healthy and diseased mucosa of the human intestines. *J Appl Bacteriol* 74: 314-323.
105. Moriez, R., Salvador-Cartier, C., Theodorou, V., Fioramonti, J., Eutamene, H.,Bueno, L.. (2005) Myosin Light Chain Kinase Is Involved in Lipopolysaccharide-Induced Disruption of Colonic Epithelial Barrier and Bacterial Translocation in Rats. *Am J Pathol* 167: 1071-1079.
106. Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., Cresci, A., Silvi, S., Orpianesi, C., Verdenelli, M. C., Clavel, T., Koebnick, C., Zunft, H. J., Dore, J.,Blaut, M. (2006) Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol* 72: 1027-1033.
107. Naruszewicz, M., Johansson, M. L., Zapolska-Downar, D.,Bukowska, H. (2002) Effect of Lactobacillus plantarum 299v on cardiovascular disease risk factors in smokers. *Am J Clin Nutr* 76: 1249-1255.
108. Nash, S., Stafford, J.,Madara, J. L. (1987) Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 80: 1104-1113.
109. Nazli, A., Yang, P. C., Jury, J., Howe, K., Watson, J. L., Söderholm, J. D., Sherman, P. M., Perdue, M. H.,McKay, D. M. (2004) Epithelia Under Metabolic Stress Perceive Commensal Bacteria as a Threat. *Am J Pathol* 164: 947-957.
110. Nejdfors, P., Ekelund, M., Weström, B. R., Willén, R.,Jeppsson, B. (2000) Intestinal permeability in human rectum is increased after radiation therapy. *Dis Colon Rectum* 43: 1582-1588.
111. Nejdfors, P., Wang, Q., Ekelund, M., Weström, B. R., Jansson, O.,Jeppsson, B. (1998) Increased colonic permeability in patients with ulcerative colitis: An *in vitro* study. *Scand J Gastroenterol* 33: 749-753.
112. Nettelbladt, C-G., Katouli, M., Bark, T., Svenberg, T., Möllby, R.,Ljungqvist, O. (1998) Bulking fibre prevents translocation to mesenteric lymph nodes of an efficiently translocating *Escherichia coli* strain in rats. *Clin Nutrition* 17: 185-190.
113. Nusrat, A., Turner, J. R.,Madara, J. L. (2000) Molecular Physiology and Pathophysiology of Tight Junctions: IV. Regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol* 279: G851-G857.
114. Olah, A., Belagyi, T., Issekutz, A., Gamal, M. E.,Bengmark, S. (2002) Randomized clinical trial of specific lactobacillus and fibre supplement to early enteral nutrition in patients with acute pancreatitis. *Br J Surg* 89: 1103-1107.
115. Osman, N., Adawi, D., Ahrné, S., Jeppsson, B.,Molin, G. (2004) Modulation of the effect of dextran sulfate sodium-induced acute colitis by the administration of different probiotic strains of Lactobacillus and Bifidobacterium. *Dig Dis Sci* 49: 320-327.
116. Ott, S. J., Musfeldt, M., Wenderoth, D. F., Hampe, J., Brant, O., Folsch, U. R., Timmis, K. N.,Schreiber, S. (2004) Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* 53: 685-693.
117. O´Dwyer, S. T., Michie, H. R., Ziegler, T. R., Revhaug, A., Smith, R. J.,Wilmore, D. W. (1988) A single dose of endotoxin increases intestinal permeability in healthy humans. *Arch Surg* 128: 1459-1464.
118. Pape, H. C., Dwenger, A., Regel, G., Auf´m´Kolck, M., Gollub, F., Wisner, D., Sturm, J. A.,Tscherne, H. (1994) Increased gut permeability after multiple trauma. *Br J Surg* 81: 850-852.
119. Parassol, N., Freitas, M.,Thoreux, K. (2005) Lactobacillus casei DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic Escherichia coli-infected T84 cells. *Research in Microbiology* 156: 256-263.
120. Randazzo, Cinzia L., Restuccia, Cristina, Romano, A. Daniele,Caggia, Cinzia. (2004) Lactobacillus casei, dominant species in naturally

fermented Sicilian green olives. *International Journal of Food Microbiology* 90: 9-14.
121. Rayes, N., Hansen, S., Seehofer, D., Muller, A. R., Serke, S., Bengmark, S.,Neuhaus, P. (2002a) Early enteral supply of fiber and Lactobacilli versus conventional nutrition: a controlled trial in patients with major abdominal surgery. *Nutrition* 18: 609-615.
122. Rayes, N., Seehofer, D., Hansen, S., Boucsein, K., Muller, A. R., Serke, S., Bengmark, S.,Neuhaus, P. (2002b) Early enteral supply of lactobacillus and fiber versus selective bowel decontamination: a controlled trial in liver transplant recipients. *Transplantation* 74: 123-127.
123. Reddy, B. S., Gatt, M., Sowdi, R.,MacFie, J. (2006) Surgical manipulation of the large intestine increases bacterial translocation in patients undergoing elective colorectal surgery. *Colorectal Disease* 8: 596-600.
124. Resta-Lenert, S. ,Barrett, K. E. (2003) Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). *Gut* 52: 988-997.
125. Riaz, A. A., Wan, M. X., Schaefer, T., Schramm, R., Ekberg, H., Menger, M. D., Jeppsson, B.,Thorlacius, H. (2002) Fundamental and distinct roles of P-selectin and LFA-1 in ischemia/reperfusion-induced leukocyte-endothelium interactions in the mouse colon. *Ann Surg* 236: 777-784.
126. Roediger, W. E. W. (1995) The place of SCFA´s in colonocyte metabolism in health and ulcerative colitis. The impaired colonocyte barrier.
127. Roller, M., Rechkemmer, G.,Watzl, B. (2004) Prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis modulates intestinal immune functions in rats. *J Nutr* 134: 153-156.
128. Sagar, P. M., MacFie, J., Sedman, P. C., May, J., Mancey-Jones, B.,Johnston, J. D. (1995) Intestinal obstruction promotes gut translocation of bacteria. *Dis Colon Rectum* 38: 640-644.
129. Salminen, S., Isolauri, E.,Salminen, E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: Successful strains for future challanges. *Antonie van Leeuwenhoek* 70: 347-358.
130. Sartor, R. B. (2004) Probiotic therapy of intestinal inflammation and infections. *Curr Opin Gastroenterol* 21: 44-50.
131. Schmitz, H., Barmeyer, C., Fromm, M., Runkel, N., Foss, H. D., Bentzel, C. J., Riecken, E. O.,Schulzke, J. D. (1999a) Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. *Gastroenterology* 116: 301-309.
132. Schmitz, H., Fromm, M., Bentzel, C. J., Scholz, P., Detjen, K., Mankertz, J., Bode, H., Epple, H. J., Riecken, E. O.,Schulzke, J. D. (1999b) Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. *J Cell Sci* 112 (Pt 1): 137-146.
133. Schrezenmeir, J. ,de Vrese, M. (2001) Probiotics, prebiotics and synbiotics - approaching a definition. *Am J Clin Nutr* 73(suppl): 361S-364S.
134. Schultz, M., Veltkamp, C., Dieleman, L. A., Grenther, W. B., Wyrick, P. B., Tonkonogy, S. L.,Sartor, R. B. (2002) Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm Bowel Dis* 8: 71-80.
135. Sedman, P. C., MacFie, J., Sagar, P., Mitchell, Ch. J., May, J., Mancey-Jones, B.,Johnstone, D. (1994) The prevalence of gut translocation in humans. *Gastroenterology* 107: 643-649.
136. Seehofer, D., Rayes, N., Schiller, R., Stockmann, M., Muller, A. R., Schirmeier, A., Schaeper, F., Tullius, S. G., Bengmark, S.,Neuhaus, P. (2004) Probiotics partly reverse increased bacterial translocation after simultaneous liver resection and colonic anastomosis in rats. *J Surg Res* 117: 262-271.
137. Servin, A. L. (2004) Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *Fems Microbiology Reviews* 28: 405-440.
138. Shanahan, F. (1994) The intestinal immune system. In: Johnson, L.R., Christensen, J., Jacobsen, E., Walsh, J.H. (eds.) Physiology of the Gastrointestinal Tract. 3rd ed.: 643-684. New York, Raven Press3rd: 643-684.
139. Shifflett, D. E., Clayburgh, D. R., Koutsouris, A., Turner, J. R.,Hecht, G. A. (2005) Enteropathogenic E. coli disrupts tight junction bar-

rier function and structure in vivo. *Lab Invest* 85: 1308-1324.
140. Simon, G. L. ,Gorbach, S. L. (1986) The human intestinal microflora. *Dig Dis Sci* 31: 147S-162S.
141. Snoeck, Veerle, Goddeeris, Bruno,Cox, Eric. (2005) The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes and Infection* 7: 997-1004.
142. Söderholm, J. D. ,Perdue, M. H. (2001) Stress and the Gastrointestinal Tract: II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 280: G7-13.
143. Söderholm, J. D., Holmgren Peterson, K., Olaison, G., Franzen, L. E., Weström, B. R., Magnusson, K. E., Sjodahl, R. (1999) Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease?. *Gastroenterology* 117: 65-72.
144. Spaeth, G., Gottwald, T.,Hirner, A. (1995) Fibre is an essential ingredient of enteral diets to limit bacterial translocation in rats. *Eur J Surg* 161: 513-518.
145. Spano, G., Chieppa, G., Beneduce, L.,Massa, S. (2004) Expression analysis of putative arcA, arcB and arcC genes partially cloned from Lactobacillus plantarum isolated from wine. *J Appl Microbiol* 96: 185-193.
146. Spitz, J., Yuhan, R., Koutsouris, A., Blatt, C., Alverdy, J.,Hecht, G. (1995) Enteropathogenic Escherichia coli adherence to intestinal epithelial monolayers diminshes barrier function. *Am J Physiol* 268: G374-G379.
147. Sugawara, G., Nagino, M., Nishio, H., Ebata, T., Takagi, K., Asahara, T., Nomoto, K.,Nimura, Y. (2006) Perioperative Synbiotic Treatment to Prevent Postoperative Infectious Complications in Biliary Cancer Surgery: A Randomized Controlled Trial. *Ann Surg* 244: 706-714.
148. Sun, Z., Wang, X.,Andersson, R. (1998) Role of intestinal permeability in monitoring mucosal barrier function. History, methodology, and significance of pathophysiology. *Dig Surg* 15: 386-397.
149. Thorlacius, H. (2004) Selectins as targets of inflammatory diseases. *Curr Med Chem* 3: 31-38.
150. Travis, S. ,Menzies, I. (1992) Intestinal permeability: functional assessment and significance. *Clin Sci (Colch)* 82: 471-488.
151. Unno, N., Wang, H., Menconi, M. J., Tytgat, S. H. A. J., Larkin, V., Smith, M., Morin, M. J., Chavez, A.,Hodin, R. A. (1997) Inhibition of inducible nitric oxide synthase ameliorates endotoxin-induced gut mucosal barrier dysfunction in rats. *Gastroenterology* 113: 1246-1257.
152. Ussing, H. H. ,Zerhan, K. (1951) Active transport of sodium as the source of electrical current in the short, circuited isolated frog skin. *Acta Physiol Scand* 23: 110-127.
153. Vollaard, E. J. ,Clasener, H. A. (1994) Colonization resistance. *Antimicrob Agents Chemother* 38: 409-414.
154. Wang, M., Adawi, D., Molin, G., Pettersson, B., Jeppsson, B.,Ahrné, S. (2001b) Identification of the translocating bacteria in rats with acute liver injury and their relation to the bacterial flora of the intestinal mucosa. *APMIS* 109: 551-558.
155. Wang, M., Adawi, D., Molin, G., Pettersson, B., Jeppsson, B.,Ahrné, S. (2001a) Identification of the translocating bacteria in rats with acute liver injury and their relation to the bacterial flora of the intestinal mucosa. *APMIS* 109: 551-558.
156. Wang, M., Ahrné, S., Antonsson, M.,Molin, G. (2004) T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. *Journal of Microbiological Methods* 59: 53-69.
157. Wang, M., Ahrné, S., Jeppsson, B.,Molin, G. (2005) Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS Microbiology Ecology* 54: 219-231.
158. Wang, X., Andersson, R., Soltesz, V., Guo, W.,Bengmark, S. (1993) Water-soluble ethylhydroxyethyl cellulose prevents bacterial translocation induced by major liver resection in the rat. *Ann Surg* 217: 155-167.
159. Weizman, Z., Asli, G.,Alsheikh, A. (2005) Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. *Pediatrics* 115: 5-9.
160. Wong, J. M., de, Souza R., Kendall, C. W., Emam, A.,Jenkins, D. J. (2006) Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* 40: 235-243.
161. Woodcock, N. P., McNaught, C. E., Morgan-DR, GreggKL,MacFie, J. (2004) An investiga-

tion into the effect of a probiotic on gut immune function in surgical patients. *Clin Nutr* 23: 1069-1073.
162. Wullt, M., Hageslätt, M. L.,Odenholt, I. (2003) Lactobacillus plantarum 299v for the treatment of recurrent Clostridium difficile-associated diarrhoea: a double-blind, placebo-controlled trial. *Scand J Infect Dis* 35: 365-367.

From the moment I picked up your book until I laid it down,
I was convulsed with laughter. Some day I intend reading it.

Groucho Marx