

# **THE ROLE OF PROBIOTICS IN *HELICOBACTER PYLORI* INFECTION**

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*To Markku, Ansuliina, and Ilmari*

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I **Mylyluoma E**, Veijola L, Ahlroos T, Tynkkynen S, Kankuri E, Vapaatalo H, Rautelin H, Korpela R. Probiotic supplementation improves tolerance to *Helicobacter pylori* eradication therapy – a placebo-controlled, double-blind randomized pilot study. *Alimentary Pharmacology & Therapeutics*, 2005; 21:1263-72.
- II **Mylyluoma E\***, Ahlroos T\*, Veijola L, Rautelin H, Tynkkynen S, Korpela R. Effects of anti-*Helicobacter pylori* treatment and probiotic supplementation on intestinal microbiota. *International Journal of Antimicrobial Agents*, 2007;29:66-72. \* equally contributed.
- III **Mylyluoma E**, Kajander K, Mikkola H, Kyrönpalo S, Rasmussen M, Kankuri E, Sipponen P, Vapaatalo H, Korpela R. Probiotic intervention decreases serum gastrin-17 in *Helicobacter pylori* infection. *Digestive and Liver Disease*, in press.
- IV **Mylyluoma E**, Ahonen AM, Korpela R, Vapaatalo H, Kankuri E. Probiotic combination conceals strain-specific anti-inflammatory effects in *Helicobacter pylori* -infected Caco-2 cells. *Clinical and Vaccine Immunology*, submitted for publication.

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# MAIN ABBREVIATIONS

Bb12	<i>Bifidobacterium lactis</i> Bb12
Bb99	<i>Bifidobacterium breve</i> Bb99
CFU	colony forming unit
CI	confidence intervals
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescent <i>in situ</i> hybridization
G	gastrin
<i>H. pylori</i>	<i>Helicobacter pylori</i>
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
LAB	lactic acid bacteria
LC705	<i>Lactobacillus rhamnosus</i> Lc705
LDH	lactate dehydrogenase
LGG	<i>Lactobacillus rhamnosus</i> GG
LTB <sub>4</sub>	leukotriene B <sub>4</sub>
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	pepsinogen
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PJS	<i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS
PPI	proton pump inhibitor
rRNA	ribosomal RNA
TER	transepithelial electrical resistance
TLR	Toll-like receptor
TNF	tumor necrosis factor
UBT	urea breath test



# ABSTRACT

*Helicobacter pylori* (*H. pylori*) infection is a major cause of chronic gastritis and peptic ulcer disease, and it is also designated as a class-I carcinogen for stomach cancer. The role of probiotics in the treatment of gastrointestinal infections is increasingly documented as an alternative or complement to antibiotics, with the potential to decrease the use of antibiotics or reduce their adverse effects.

These studies were conducted to investigate the role of probiotics in the treatment of *H. pylori* infection. Various aspects included: an investigation of the effects of a probiotic combination consisting of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Bifidobacterium breve* Bb99 or *B. lactis* Bb12 as a supplementation to *H. pylori* eradication therapy, with special reference to tolerability, effectiveness, and microbiota alterations following the treatment; discovering the role of probiotics *in vivo* with *H. pylori* infected and uninfected patients, as well as with an *in vitro* model of *H. pylori* infection.

The probiotic combination therapy was able to reduce significantly the total symptom score, which takes into account both the frequency and the severity of the adverse effects, during the eradication treatment. The supplementation did not improve the success of the eradication treatment significantly, though some difference was seen in the eradication percentages (91% vs. 79%). The quantities of predominant bacterial groups were altered significantly following the triple treatment. Probiotics slightly counteracted the effects of anti-*H. pylori* treatment, monitored as significantly less alterations in the total numbers of aerobes and lactobacilli / enterococci group bacteria.

After probiotic intervention, *L. rhamnosus* GG adhered to a minority of the patients' upper gastrointestinal mucosa, but all of the probiotics survived well through the gastrointestinal tract transit with and without antimicrobial treatment. Probiotic intervention decreased gastrin-17 levels in *H. pylori* infected patients and appeared to decrease the <sup>13</sup>C-urea breath test values. In *in vitro* Caco-2 cell line experiments, probiotics inhibited *H. pylori* adhesion to intestinal epithelial cells.

Both *L. rhamnosus* strains, *P. freudenreichii* ssp. *shermanii* JS and the combination inhibited the *H. pylori*-induced acute cell leakage. Simultaneously, both *L. rhamnosus* strains and the combination transiently improved the epithelial barrier function. The pro-inflammatory effects prevailed when the probiotics were used in combination.

According to this series of studies, probiotic combination could have some potential in reducing adverse effects induced by *H. pylori* eradication treatment and beneficial effects on *H. pylori* infected subjects.

# INTRODUCTION

*Helicobacter pylori* (*H. pylori*) infection is recognized as a causative agent for acute and chronic gastritis and as a predisposing factor to peptic ulcer disease, gastric cancer and gastric lymphoma. This important discovery, rewarded with the 2005 Nobel Prize in Medicine, has changed gastroenterological practice worldwide since, after its discovery, many gastroduodenal diseases became curable infectious diseases. The prevalence of *H. pylori* infection in the adult population of industrialized countries is estimated to be at 20–50%, and in developing countries, the rate is as high as 80% (for review, see Go 2002). The rate of carriage increases according to age group. Infection is usually acquired during childhood and persists lifelong if not treated specifically.

Only a combination of antimicrobials can be used *in vivo* to eradicate *H. pylori*, and none of the antimicrobials is effective enough to eliminate *H. pylori* when given as monotherapy (for review, see Kusters *et al.* 2006). The first-line recommended eradication treatment of *H. pylori* consists of a combination of two antimicrobials and an acid-suppressive drug (Malfertheiner *et al.* 2002). However, the triple treatment has many shortcomings, such as several adverse effects possibly leading to discontinuation of the treatment (Deltenre *et al.* 1998), and limited efficacy particularly because of antimicrobial resistance (for review, see Gerrits *et al.* 2006).

In recent years, the development of alternative anti-*H. pylori* treatments has been actively pursued, and investigations have been carried out to define components that could be used either as monotherapy or synergistically in combination with antimicrobials thus resulting in more effective anti-*H. pylori* therapy or alternative ways of controlling *H. pylori* infection. These novel treatments could potentially reduce the costs related to the treatment of *H. pylori* associated diseases. Promising results have been obtained in initial studies with several probiotic strains (for reviews, see Hamilton-Miller 2003, Gotteland *et al.* 2006), but there are still many open questions. As defined by FAO/WHO (2002), probiotics are live microorganisms

that, when administered in adequate amounts, confer a health benefit on the host (such as *Lactobacillus* spp. and *Bifidobacterium* spp.).

The present study aims to investigate the effects of probiotics in patients receiving the recommended eradication treatment for *H. pylori* infection, as well as the effects on the stomach and intestine in untreated *H. pylori*-positive patients, and to evaluate the characteristics of individual probiotics or the probiotic combination in an *in vitro* model of *H. pylori* infection.

# REVIEW OF THE LITERATURE

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## 1. GASTROINTESTINAL DEFENCES AGAINST PATHOGENS

Natural gastrointestinal defences against pathogens can be theoretically divided into three levels of responses (for reviews, see McCracken and Lorenz 2001, Bourlioux *et al.* 2003). The host's microbiota provides the first level of defence against pathogens by preventing them from developing in the gastrointestinal tract, for instance, by protecting the mucosa against colonization of pathogenic microorganisms. The intestinal epithelium constitutes a second level of defence and a tight barrier against pathogens by the combined effect of the mucus layer and the epithelial cells themselves. The immune system, which constitutes the third defensive barrier against pathogenic microorganisms, can be divided into two lines of defence against pathogens: innate and adaptive immunity.

### 1.1 GUT MICROBIOTA

#### Composition and diversity of gut microbiota

The human microbiota is considered to be an enormously diverse and complex ecosystem affected by host cells, ingested food and microbes (for review, see Zoetendal *et al.* 2006). In the past, it was commonly believed that over 400 species compose this microbiota. However, it is nowadays estimated that more than 1,000

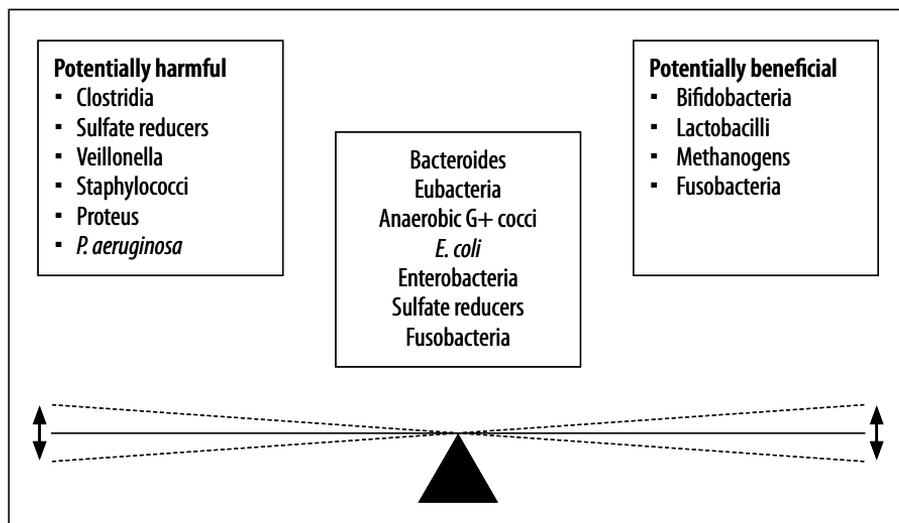
species are present in the gut (Bäckhed *et al.* 2005). Previously, traditional methods using culturing have been used as the gold standard for investigating bacteria. However, use of 16S ribosomal RNA (rRNA) and genome based approaches has revealed further information about microbiota. The new culture-independent techniques, such as sequencing of cloned 16S rRNA gene amplicons, 16S rRNA gene fingerprinting, quantitative fluorescent *in situ* hybridization (FISH), and quantitative polymerase chain reaction (PCR), provide more specific methods for detailed investigations at the species and strain levels (Zoetendal *et al.* 2006). Also, development of high-diversity deoxyribonucleic acid (DNA) microarrays will open new possibilities in gut microbiota research (Rajilic-Stojanovic *et al.* 2006). Thus, new microbes in the human microbiota are continuously being discovered. It has been suggested that only 20–40% of the bacteria from intestinal samples have been characterized, due to a lack of knowledge of appropriate culture conditions (Zoetendal *et al.* 1998, Suau *et al.* 1999, Hayashi *et al.* 2002).

There are very few studies on the microbiota of the stomach with culture-independent techniques, and recent evidence indicates that the stomach microbiota is also more diverse than previously thought (Hill 1985, Monstein *et al.* 2000, Peña *et al.* 2002, Roos *et al.* 2005, Bik *et al.* 2006). The most common bacterial phyla found in the stomach are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria*, and the most abundant genera found are *Helicobacter*, *Streptococcus* and *Prevotella* (Bik *et al.* 2006). Thus, even in young normochlorhydric subjects, the lumen is not bacteria free, despite pH values being most of the time below 3. However, the gastric acid is buffered during a meal, allowing microbes to proliferate. Impaired gastric acid secretion, caused, for example, by chronic atrophic gastritis, prolonged use of histamine-2 receptor antagonists or proton pump inhibitors, is associated with bacterial overgrowth in the stomach and small intestine (Hill 1985, Väkeväinen *et al.* 2000, Sanduleanu *et al.* 2001, Williams and McColl 2006).

The composition of the predominant bacterial community in the gut is reported to be host-specific and stable over time in healthy adults (for review, see Zoetendal *et al.* 2006). Only a limited fraction of bacterial phyla compose the major intestinal microbiota (Manichanh *et al.* 2006). In healthy adults, 80% of phylotypes belong to four major phylogenetic groups, which are the *Clostridium leptum*, *Clostridium coccooides*, *Bacteroides* and *Bifidobacteria* groups (Lay *et al.* 2005). However, a large fraction of dominant phylotypes is subject specific (Zoetendal *et al.* 1998, Seksik *et al.* 2003, Vanhoute *et al.* 2006). Also, recent studies have found that mucosal microbiota is stable along the distal gastrointestinal tract from ileum to rectum, but mucosa-associated microbiota is different from fecal microbiota. The difference has been estimated to be between 50–90% (Zoetendal *et al.* 2002, Lepage *et al.* 2005).

## The importance of microbes for the host

The intestinal microbiota has crucial physiological functions in the gut, such as metabolic capacity and the ability to ferment carbohydrates into short-chain fatty acids, which have been shown to stimulate the growth and well-being of the colonic mucosa and colonic motility (for reviews, see Mitsuoka 2000, O'Hara and Shanahan 2006). Studies of germ-free mice have revealed that gut microbiota plays an important role in the maturation of the immune system (for review, see Hooper *et al.* 2002). The intestine of germ-free mice has several physiological differences as compared to that of conventional mice and also a less developed immune system. Several other specific interactions between host and bacteria have also been discovered using germ-free animal models and *in vitro* cell models. These include antimicrobial peptide production, maintenance of intestinal homeostasis and development of vascular network in the villi after microbial colonization (Zoetendal *et al.* 2006). Thus host-microbe interactions can shape the immunity and maturation of the gastrointestinal tract of the host and have a further impact on the ecology of microbiota. The balance between potentially beneficial and harmful bacteria is very important (Figure 1).



**Figure 1** Potentially harmful and potentially beneficial bacteria (Modified from Bourlioux *et al.* 2003). *P.*, *Pseudomonas*; G+, Gram positive; *E.*, *Escherichia*.

## Colonization resistance

The indigenous microbiota is a natural resistance factor against potential pathogenic microorganisms and provides colonization resistance, also known as gut barrier, by controlling the growth of opportunistic microorganisms (Fons *et al.* 2000, Vollaard

and Clasener 1994). It has been suggested that commensal bacteria protect their host against microbial pathogens by interfering with their adhesion and toxic effects (for reviews, see Brook 1999, Servin *et al.* 2004).

## 1.2 GASTROINTESTINAL EPITHELIUM

The tight epithelial cell barrier forms the second line of defence between the gut luminal contents and the host. Epithelial cells lining the gastrointestinal tract are able to respond to infection by initiating either nonspecific or specific host-defence response (for reviews, see Kagnoff and Eckmann 1997, Strober 1998). Bacterial adhesion to the host cell or recognition by the host cell is often an essential first stage in the disease process.

A wide range of gastrointestinal cell surface constituents, such as several glycoconjugates, can serve as receptors for bacterial adherence (Servin and Coconnier 2003, Pretzer *et al.* 2005). Furthermore, epithelial cells express constitutively host pattern recognition receptors, such as Toll-like receptors (TLR). These are a family of transmembrane receptors that recognize repetitive patterns, i.e. the pathogen-associated molecular patterns present in diverse microbes, including gram-positive and gram-negative bacteria (for reviews, see Bäckhed and Hornef 2003, Takeda *et al.* 2003). TLRs are also found on innate immune cells, such as dendritic cells and macrophages (Vinderola *et al.* 2005). TLR4 recognizes lipopolysaccharide and gram-negative bacteria, while TLR2 recognizes a variety of microbial components, such as peptidoglycan and lipoteichoic acids, from gram-positive bacteria (Abreu 2003, Matsuguchi *et al.* 2003, Takeda *et al.* 2003). Also, several other TLRs with specific actions are known, such as TLR5, which responds to the bacterial flagella (Rhee *et al.* 2005), and TLR9, which is activated by bacterially derived short DNA fragments containing CpG sequences (Pedersen *et al.* 2005). Other known recognition receptors are nucleotide-binding oligomerization domain proteins, which recognize both gram-positive and gram-negative bacteria. They are located in cell cytoplasm and are implicated in the induction of defensins.

Increased epithelial barrier permeability is frequently associated with gastrointestinal disorders contributing to both disease onset and persistence (for reviews, see Lu and Walker 2001, Berkes 2003). The gatekeeper of the paracellular pathway is the tight junction, which is an apically located cell-cell junction between epithelial cells. The tight junction permits the passage of small molecules, such as ions, while restricting the movement of large molecules, such as antigens and microorganisms, which can cause inflammation. The integral membrane protein family, which are mainly claudins, occluding and zonula occludens 1, are implicated in the formation of the paracellular channels (Berkes *et al.* 2003).

The permeability of the tight junction to ions can be assessed by transepithelial electrical measurements and by paracellular ion flux assays for major extracellular ions, such as Na<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup>.

Epithelial cells are also involved in a wide range of mucosal immune and inflammatory responses together with dendritic cells, macrophages, neutrophils and lymphocytes. Epithelial cells can secrete pro- and anti-inflammatory cytokines and chemokines, such as tumor necrosis factor (TNF) - $\alpha$ , interleukin (IL) -2, IL-6, and IL-8, which either diminish or stimulate response or provide feedback (Goodrich and McGee 1998). IL-8, a C-X-C chemokine that is transcriptionally regulated by nuclear factor-kappa B, shows potent chemotactic activity for neutrophils. Intestinal epithelial cells also secrete many other mediators involved in immune responses to potentially pathogenic microorganisms, including antimicrobial peptides, such as defensins and mucins (for review, see Servin 2004). The immunoinflammatory reaction is highly important in eliminating pathogens, but this reaction must be controlled to avoid the risk of a more widespread inflammation. Microbes differ in terms of their ability to induce inflammatory response. The commensal microbiota produces a very mild inflammation response and is thus tolerated by the mucosa, while modified microbiota induces a more marked response (for review, see O'Hara and Shanahan 2006).

### 1.3 GUT IMMUNE SYSTEM

The third level of defence is the immune system, which is crucial for humans and animals in protecting the host against invading pathogens. Bacteria are the main source of antigenic materials, and the gut microbiota is the most important stimulant of the body's immunological defence (Bourlioux *et al.* 2003). The immune system comprises a complex array of interacting mechanisms. It consists of local immune tissue (mucosa associated lymphoid tissue) and the systemic immune system (in blood, liver, spleen and bone marrow). Both components can be further theoretically divided into two types of response: innate (nonspecific) and adaptive (specific) immune response (for reviews, see Borregaard *et al.* 2000, Janeway and Medzhitov 2002). Specific immune response is usually induced by direct contact, despite an intact epithelial barrier, between the lymphoid tissue and the potentially pathogenic macromolecules or microorganisms in the intestinal lumen.

Innate or "natural" immunity is a rapidly activated host defence that recognizes conserved microbial structures not expressed by the host and mounts a nonspecific immune response against these structures (often specific carbohydrates or lipoproteins). The activated effectors of innate immunity, such as phagocytic cells, natural killer cells, and the complement system, are able to destroy the invader

(Suffredini *et al.* 1999, Borregaard *et al.* 2000, Janeway and Medzhitov 2002). Innate immunity also includes acid in the stomach, lysozyme, lactoferrin and antimicrobial molecules (Borregaard *et al.* 2000). Intracellular pathogens, like viruses, are killed by natural killer cells.

The adaptive immune system is activated by the infection if the innate immune system is insufficient (Borregaard *et al.* 2000). The adaptive immune system is a more specific and powerful tool against pathogens, but the primary response mounts slower than in innate immunity. Crucially, adaptive immunity develops a memory, which enables a rapid and effective response in a reinfection. Adaptive immunity recognizes antigenic structures (often peptides), not expressed in the host, as non-self. Antigens are presented to the effector cells of the adaptive immunity by antigen presenting cells. Furthermore, intestinal dendritic cells can directly sample the contents of the gut lumen by extending dendrites between epithelial cells.

Although innate and adaptive immunity represent two separate arms of immunity, a close relationship exists between them (Palucka and Banchereau 1999, Bourlioux *et al.* 2003). The initiation and direction (cellular or humoral) of adaptive immunity is influenced by innate immunity, which regulates its direction via cytokines, T and B cell co-stimulatory mechanisms and antigen presentation. Furthermore, pattern recognition receptors, such as TLRs, in epithelial cells, dendritic cells and macrophages are important in bridging the innate and adaptive immune responses (for review, see Abreu and Arditi 2004). Continuous formation of immunoglobulin (Ig) A in plasma cells in the lamina propria also plays an essential role in the protective function of the mucosa (Goodrich and McGee 1998). This IgA is transported to the luminal side of the mucosa and released into the bowel as secretory IgA, where it is able to neutralize potentially pathogenic bacteria and viruses.

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**To summarize**, the host's microbiota protects the mucosa against colonization and invasion by pathogenic microorganisms (colonization resistance). If this normal microbial habitat is damaged, ecological niches are created for pathogens. The intestinal epithelium constitutes a tight barrier against pathogens that interact with commensal microbiota. Pathogens also crosstalk with the epithelium and modify epithelial responses, e.g. to enhance their penetration across the epithelial barrier. The immune system constitutes the more specific defence against pathogenic bacteria. The innate immunity reacts immediately but unspecifically. The adaptive immunity takes longer but is specific and keeps memory of previous aggressions. A mild inflammatory state is necessary to keep the defence enabled, but the system must remain balanced.

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## 2. *HELICOBACTER PYLORI* INFECTION

### 2.1 GENERAL

*Helicobacter pylori* (*H. pylori*) was isolated from gastric mucosa and bacteriologically identified in 1982 (Warren and Marshall 1983). Since then, it has been reported that ulcer recurrence rates have decreased after the eradication of *H. pylori* from the stomach of peptic ulcer patients (Marshall *et al.* 1988, Hantschel *et al.* 1993). It has been established that *H. pylori* infection is a major cause of chronic gastritis and peptic ulcer disease. *H. pylori* was designated a class-I (definite) carcinogen for stomach cancer in 1994 after epidemiological investigation by the International Agency for Research on Cancer (IARC), a subordinate organization of the World Health Organization (IARC 1994). Furthermore, association of primary malignant gastric lymphoma with *H. pylori* has been reported in a large-scale cohort study (Parsonnet *et al.* 1994). However, the role of *H. pylori* as an obligate pathogen has been questioned, and it has been proposed that *Helicobacter* strains could be part of the indigenous microbiota of the human stomach and that *H. pylori* could have both pathogenic and symbiotic features (for reviews, see Blaser 1999, 2006).

### 2.2 EPIDEMIOLOGY AND TRANSMISSION

Approximately half of the world's population is infected with *H. pylori* and the prevalence shows large geographical variations (for review, see Go 2002). The infection is generally acquired during childhood and usually persists indefinitely if left untreated. It has been suggested that *H. pylori* infection rates are associated with age, ethnicity, socio-economic status, sanitary environments and lifestyle. In Finland, the prevalence of *H. pylori* infection in children is 6–10% (Ashorn *et al.* 1995, Rehnberg-Laiho *et al.* 1998) and in adults 12–70% (Rehnberg-Laiho *et al.* 2001). Prevalence rates generally remain stable in each birth cohort, and thus the higher prevalence of infection among the elderly reflects a birth cohort effect with higher infection rates in the past (for review, see Rautelin and Kosunen 2004). The main reservoir of *H. pylori* is the human stomach with the most likely mode of transmission being person to person (for review, see Vaira *et al.* 2001). Recent epidemiological study suggests that infected mothers are the main source of *H. pylori* infection in their children (Weyermann *et al.* 2006).

## 2.3 MICROBIOLOGY AND PATHOGENESIS

The genus *Helicobacter* belongs to a subdivision of *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*, and consists of over 20 recognized species which are all microaerophilic organisms (for review, see Kusters *et al.* 2006). Most of the helicobacter species are catalase and oxidase positive, and many but not all species are also urease positive. They can be subdivided into two major lineages: the gastric *Helicobacter* species and the enterohepatic (nongastric) *Helicobacter* species. *H. pylori* is a gram-negative bacterium which usually appears spiral-shaped or as a rod with several flagella (Kusters *et al.* 2006). *H. pylori* is genetically heterogeneous, suggesting a lack of clonality. This results in every *H. pylori* positive subject carrying a distinct strain, although differences within relatives may be small (Logan and Walker 2001).

The pathogenetic mechanisms underlying *H. pylori* infection are not completely understood. *H. pylori* is sheltered from gastric acidity in the mucus layer. The majority of *H. pylori* in colonized hosts are free-living, but approximately 20% bind to gastric epithelial cells (for review, see Peek and Crabtree 2006). Colonization of the gastric epithelium by *H. pylori* is specific *in vivo* and when *H. pylori* is found in the duodenum, it overlays gastric metaplasia (Steer 1984). Colonization of the gastric mucosa by *H. pylori* evokes local inflammatory responses, which result in further mucosal injury but are not able to clear the infection (for reviews, see Algood and Cover 2006, Kusters *et al.* 2006). *H. pylori* infection thus escapes the natural gastrointestinal defences, which allows it to induce chronic infection. It has been suggested that the differences in the outcome of the disease are due to various virulence factors present in different *H. pylori* strains. Virulence factors help bacteria to invade the host, cause disease and evade host defences. Several virulence factors of *H. pylori*, such as production of urease, a vacuolating cytotoxin, and the cytotoxin-associated gene A encoded protein, are associated with injury to the gastric epithelium (Dunn *et al.* 1997, Atherton 1998).

*H. pylori* induces a strong inflammatory response in the gastric mucosa and results in the expression of a wide spectrum of cytokines, chemokines and eicosanoids such as interleukin-8 (IL-8), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) (for reviews, see Bodger and Crabtree 1998, Nauman and Crabtree 2004, Kusters *et al.* 2006). Released from the epithelial cells, these potent pro-inflammatory mediators promote inflammation and tissue damage locally as well as induce migration and activation of neutrophils, macrophages, lymphocytes and plasma cells to the site of infection. Closely associated with inflammation and cancer (Prescott and Fitzpatrick 2000), the production of PGE<sub>2</sub> and LTB<sub>4</sub> has been shown to be induced in the mucosa of *H. pylori* positive patients (Wakabayashi *et al.* 1998). Moreover, *H. pylori* can activate epithelial cell release of arachidonic acid,

required for the production of eicosanoids (Pomorski *et al.* 2001). In accordance with promoting chronic inflammation and development of malignancy, *H. pylori* infection also disturbs epithelial barrier functions (Papini E *et al.* 1998, Terres *et al.* 1998, Pelicic *et al.* 1999) and induces epithelial cell damage (Pai *et al.* 1999). The presence of both local and circulating antibodies can regularly be demonstrated in infected patients. All the machinery needed for immune defence thus seems to be present, but still, spontaneous recovery is rare. In the absence of treatment, chronic *H. pylori* infection persists for years (Algood and Cover 2006).

*H. pylori* modulates also the endocrine and physiological functions of the stomach (Blaser 2006). Studies have shown that serum pepsinogen (PG) I, sPGII and gastrin (G)-17 levels are high in the presence of *H. pylori* infection related to non-atrophic chronic gastritis and that the sPGI, sPGII and sG-17 concentrations are found to decrease significantly after a successful *H. pylori* eradication (Wagner *et al.* 1994, Plebani *et al.* 1996, Perez-Paramo *et al.* 1997, Sokic-Milutinovic *et al.* 2005). The determination of sPGI and sPGII, sG-17 and IgG anti-*H. pylori* antibodies has been proposed as a series of non-invasive markers that reflect both the morphological and functional status of the gastric mucosa. PGI and II are both precursors of pepsin. PGI is a specific marker of corpus secretion capacity, and PGII is strongly influenced by gastric inflammation. These precursors are secreted into the gastric lumen, and thus only a minimal quantity is measurable from the blood. Gastrin is a peptide hormone, produced by endocrine cells in the antrum of the stomach, that stimulates acid secretion in the gastric corpus (for review, see Plebani 1993). It has been also suggested that *H. pylori* affects gastric hormones that have a role in energy homeostasis, such as leptin and ghrelin (Blaser 2006).

## 2.4 DIAGNOSIS

*H. pylori* infection can be confirmed by invasive and/or noninvasive methods. Invasive tests require esophageal gastroduodenal endoscopy. During endoscopy, biopsy specimens of the stomach and duodenum are obtained, and the diagnosis of *H. pylori* is generally made by urease testing, histology and/or culture. All of these biopsy-based methods are unpleasant for patients, carry a small but definite risk of complications and are subject to sampling error since infection is patchy (for reviews see, Logan and Walker 2001, Gatta *et al.* 2003). Non-invasive tests are based on peripheral samples, and the most commonly used ones are the urea breath test, fecal antigen tests and serology. There is no single golden standard for the diagnosis of *H. pylori*. In research settings, a combination of at least two methods is often applied, as compared to clinical practice, where it is common to use a single test (Kusters *et al.* 2006). Several other tests, such as whole-blood rapid tests, saliva

and urine antibody tests, and PCR, are also available, both from biopsy and from stool samples (for reviews, see Gatta *et al.* 2003, Krogfelt *et al.* 2005).

### Common invasive methods

It was noted shortly after its initial isolation that *H. pylori* (as well as the other gastric helicobacters) produced high amounts of urease (Warren and Marshall 1983). Rapid urease tests detect this enzyme directly in biopsy samples colonized with helicobacters (Krogfelt *et al.* 2005). The tests usually contain 10% urea (either in agar, solution or tablet form) and phenol red as an indicator. As the urease enzyme of the gastric helicobacter hydrolyzes urea, the pH rises and a color change occurs; a positive result can be recorded in minutes or hours.

For histology, two antral and two corpus biopsies are recommended to avoid sampling error (Price 1991). The sensitivity of the histological test depends mainly on the experience of the pathologist. The sensitivities and specificities usually achieved by histology are both above 95% (Kusters *et al.* 2006). The benefit of histological detection of *H. pylori* is that it provides histological data on inflammation and atrophy, and it also allows the classification of possible gastroduodenal lesions and reveals premalignant alterations in the mucosa (Vaira *et al.* 2002).

The culturing of helicobacters *in vitro* is very demanding and special culture conditions are necessary for it to succeed. These include a microaerobic atmosphere (oxygen level of 5–7%) with high humidity, an incubation temperature of 37°C, and a rich growth medium (Goodwin and Armstrong 1990, Dunn *et al.* 1997). The benefit of culturing is that it enables antibiotic susceptibility testing of the strain involved (Krogfelt *et al.* 2005).

### Common non-invasive methods

In urea breath test (UBT), the patient is given an oral preparation of either nonradioisotope carbon-13- (<sup>13</sup>C-) labeled urea, or radioactive isotope carbon-14- (<sup>14</sup>C) labeled urea (Gatta *et al.* 2003). In the presence of a *H. pylori* infection, bacterial urease metabolizes the urea to produce labeled carbon dioxide and ammonia. The labeled carbon diffuses into the bloodstream and is excreted by the lungs. This labeled carbon dioxide can then be measured in the patient's breath to determine the presence of *H. pylori*. The <sup>13</sup>C-labeled urea is detected by mass spectrometry and the <sup>14</sup>C-labeled urea by liquid scintillation. UBT is indicated for the initial diagnosis of *H. pylori* infection and for follow-up of eradication therapy. The sensitivity and specificity of UBT are above 95% (Kusters *et al.* 2006). False negatives can result from acid suppression with proton pump inhibitors; therefore, acid suppression treatment should be withheld for two weeks prior to testing. In

addition, retesting for confirmation of eradication should be done at least four weeks after the completion of therapy.

Serological assays measure specific *H. pylori* IgG and IgA antibodies that can determine if an individual has been infected. The sensitivity and specificity of these assays generally range between 80 and 90%, depending on the assay used (Kusters *et al.* 2006). In Finland, very high sensitivities (97–100%) and specificities (95–99%) have been obtained for detecting *H. pylori* antibody titers of the IgG class (Rautelin and Kosunen 2004). Serological tests are unreliable indicators of *H. pylori* status if a pretreatment serum sample is not available to run in parallel.

Stool antigen testing identifies active infection and has a sensitivity and specificity above 90% (Kusters *et al.* 2006). In the stool antigen test, a simple enzyme-linked immunosorbent assay (ELISA) method is used to detect the presence of *H. pylori* antigens shed in the feces (Gatta *et al.* 2003). The principle of the stool antigen test is that a polyclonal or monoclonal antibody to *H. pylori* is adsorbed to microwells. Diluted patient samples are added to the wells and any *H. pylori* in the fecal sample is bound to the adsorbed antibody. A second *H. pylori* antibody conjugated to peroxidase is added and binds to *H. pylori*. After unbound material is washed off, a substrate is added that reacts with bound peroxidase enzyme to produce a yellow color, the intensity of which can be measured to estimate *H. pylori* levels.

## **2.5 CURRENT TREATMENT OF *H. PYLORI* INFECTION**

The spontaneous decline in the prevalence of *H. pylori* infection in developed countries to 10%–15% allows the remaining nonmalignant gastroduodenal diseases associated with infection to be addressed with antimicrobial treatment (Table 1). Triple therapy, which combines a proton pump inhibitor (PPI) or ranitidine bismuth citrate with two antibiotics (clarithromycin and amoxicillin), is the current standard of therapy for eradicating *H. pylori* in Europe (Malfertheiner *et al.* 2002). In Finland, the recommendations follow these guidelines (Finnish Gastroenterological Association 2002). A meta-regression analysis of 74 reported studies using amoxicillin and clarithromycin plus omeprazole in the eradication of *H. pylori* in adults found an eradication rate of 82% (Schmid *et al.* 1999). Rescue therapies after the failure of first-line and second-line therapies are available, but the selection of proper antimicrobial therapy should be made on a case-by-case basis and with the help of susceptibility test results (Rautelin and Kosunen 2004). However, treatment of infection is challenged by, for example, the rapid rate with which the bacteria acquire resistance to the drugs, poor compliance, an excessively high bacteria load, impaired mucosal immunity, early re-infection and the presence

of intracellular bacteria (for review, see Megraud and Lamouliatte 2003). Thus, there is still a need for new compounds that are specific for *H. pylori*.

**Table 1.** Suggested therapeutic regimens for eradication of *H. pylori* infection (modified from Finnish Gastroenterological Association 2002, Malfertheiner *et al.* 2002)

<b>First-line therapy (7 days)</b>
PPI standard dose twice daily (or ranitidine bismuth citrate 400 mg twice daily) clarithromycin 500 mg twice daily + amoxicillin 1 g twice daily
<b>Second-line therapy (7 days)</b>
PPI standard dose twice daily metronidazole 400 mg thrice daily + tetracycline 500 mg 4 times daily ranitidine bismuth citrate 400 mg twice daily

### Effect of amoxicillin and clarithromycin on the ecological balance of gut microbiota

Administration of antimicrobial agents disturbs the ecological balance between the host and the normal microbiota (for review, see Sullivan *et al.* 2001). A well balanced microbiota prevent establishment of resistant microbial strains. Disturbances in the microbiota depend on the properties of the agents as well as on their absorption, route of elimination, and possible enzymatic inactivation and/or binding to fecal material. Antimicrobial agents used in eradication of *H. pylori* have been found to cause several disadvantages for the ecological balance of gastrointestinal microbiota.

**Amoxicillin** is a broad spectrum penicillin derivate. The primary mechanism of the action of amoxicillin is to prevent bacterial cell wall synthesis. Several clinical studies with amoxicillin supplementation have revealed significant increases in the number of enterobacteria and the emergence of resistant enterobacteria strains (Brismar *et al.* 1991, Floor *et al.* 1994, Stark *et al.* 1996, Adamsson *et al.* 1999). The dosages have varied between 750 and 2000 mg/day for seven to fourteen days. More recently, Lode *et al.* (2001) have found that amoxicillin administration in healthy subjects induced greater counts of enterococci and *Escherichia coli* strains and decreased the number of lactobacilli, bifidobacteria and clostridia. In addition, the minimal inhibitory concentration for enterococci was found to increase.

**Clarithromycin** belongs to the group of macrolide antimicrobial agents. The absorption of macrolides is incomplete, and thus high fecal concentrations have a strong impact on the balance of the microbiota. Enterobacteria are resistant to macrolides, which induces overgrowth of extremely resistant strains. The effect of clarithromycin on the composition on intestinal microbiota has been studied in only a few clinical trials (Brismar *et al.* 1991, Edlund *et al.* 2000, Edlund and Nord

2000). The dosage regimens have been from 500 to 1000 mg/day for seven to ten days. These studies clearly demonstrate that marked reduction in the number of lactobacilli, bifidobacteria and bacteroides is evident in the anaerobic microbiota. Reduction in enterobacterial group bacteria and increased numbers of new resistant enterobacteria and enterococci strains have also been observed after treatment.

### Proton pump inhibitors

Proton pump inhibitors (PPIs) inhibit H<sup>+</sup>/K<sup>+</sup>-ATPase via covalent binding to cysteine residues of the proton pump, and they remain the most effective available therapy to control gastric acid secretion (for review, see Sachs *et al.* 2006). Omeprazole was the first clinically used PPI. Others available include lansoprazole, pantoprazole, rabeprazole and tenatoprazole. The main difference between the PPIs is the duration of inhibition of gastric acid secretion. However, this has not affected *H. pylori* eradication treatment efficacy when systematically compared (for review, see Bazzoli *et al.* 2002). Omeprazole and lansoprazole remain reference PPIs in first-line eradication treatment of *H. pylori* infection.

PPIs have shown an excellent safety profile, but there have been concerns that the suppression of gastric acid might alter the gastric and duodenal microbiota, which could lead to gastric cancer, enteric or other infections and malabsorptions (for review, see Williams and McColl 2006). It has been demonstrated that elevation of intragastric pH by antisecretory drugs increases bacterial growth both in mucosal biopsies and in gastric juice. Moreover, it has been found that *H. pylori* infected patients have greater increases of gastric pH during PPI treatment than non-infected patients (Williams and McColl 2006). The overall evidence indicates that PPIs are linked with an increased risk of enteric infections (for review, see Williams and McColl 2006) and the production of carcinogenic acetaldehyde in the upper gastrointestinal tract (Väkeväinen *et al.* 2000). However, there are no convincing results showing production of nitrosamines or interference with intestinal digestive processes (Williams and McColl 2006).

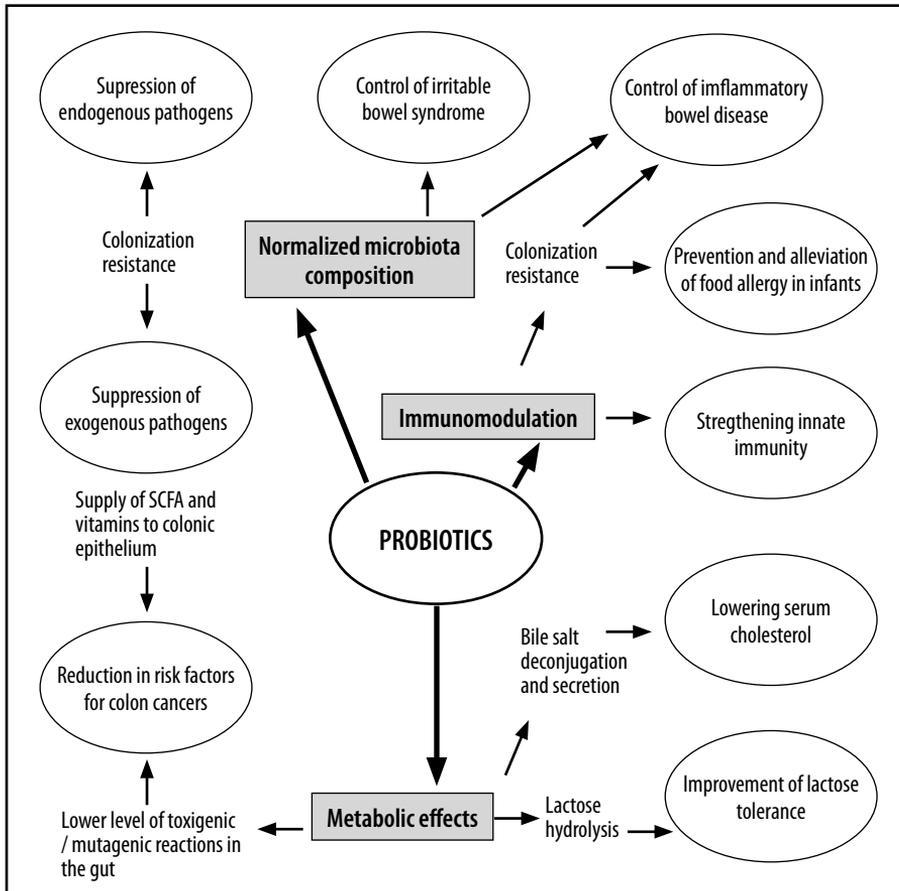
# 3. PROBIOTICS IN *HELICOBACTER PYLORI* INFECTION

## 3.1 OVERVIEW OF PROBIOTICS

Fermented dairy products and vegetables have been used for thousands of years. As early as 1907, Nobelist Elie Metchnikoff attributed the longevity of Bulgarian peasants to their consumption of fermented milk products (Metchnikoff 1907). He suggested that regular consumption of dairy yogurt may suppress “putrefactive” bacteria in the colon. Since then, several definitions have been used to describe these probiotics, such as substances that are produced by one microorganism and stimulate the growth of other microorganisms (Lilly and Stillwell 1965), live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance (Fuller 1989), and more recently, live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2002).

The most commonly used organisms in probiotic products belongs to *Lactobacillus* sp., and *Bifidobacterium* sp. (Saxelin *et al.* 2005). Other organisms have also been used including *Bacillus* sp., and yeast such as *Saccharomyces boulardii*. Probiotic products are commercially available in different formulations with and without prebiotics such as fructo- and galacto-oligosaccharides. The concentration of probiotics in research trials and in food or other products varies greatly, and there are no international standards regarding the levels of bacteria required (for review, see Parvez *et al.* 2006).

Several proposed health effects of probiotics are summarized in Figure 2. The primary clinical interest in the application of probiotics has been in the prevention and treatment of gastrointestinal infections and diseases (Parvez *et al.* 2006). The use of probiotics for controlling chronic gastrointestinal inflammatory diseases, such as ulcerative colitis and pouchitis, has also received considerable attention. Moreover, the consumption of probiotics has been linked to improvement of a wide variety of health conditions, including lactose intolerance, high cholesterol and rheumatoid arthritis. Also, there is evidence of beneficial effects of probiotics with respect to the development of dental caries, allergy and cancer (Parvez *et al.* 2006). The general mechanisms by which probiotics may have an effect can be divided into three broad categories: normalization of microbiota, modulation of immune response, and metabolic functions. However, the molecular details behind these mechanisms remain mostly unknown (Marco *et al.* 2006).



**Figure 2** Summary of various health effects of probiotics (modified from Parvez 2006).  
SCFA, short-chain fatty acid

### 3.2 CLINICAL STUDIES

The role of probiotics in the treatment of *H. pylori* infection is increasingly documented as a complement or alternative to antibiotics, and thus having the potential to decrease the use of antibiotics.

**Probiotics as a complement to antibiotics** may have the potential to reduce the adverse events of triple anti-*Helicobacter* treatment and to improve the eradication rate (Table 2). The first study provided evidence that *L. acidophilus* LB improved the eradication rate significantly in the probiotic group (Canducci *et al.* 2000). However, in this study the supplementation did not alleviate the adverse effects of the anti-*Helicobacter* treatment. In contrast, Armuzzi *et al.* (2001a, 2001b) reported in two separate studies that *L. rhamnosus* GG was able to reduce the occurrence of adverse effects, such as diarrhea, taste disturbance, nausea and bloating. The latter of these two studies was conducted in double-blinded fashion. Moreover, Sheu *et al.* (2002) reported in an open and uncontrolled trial that the *L. acidophilus* La5 and

*B. lactis* Bb12 containing yogurt (AB-yogurt) was able to increase the eradication rate and also decrease several side-effects of the triple therapy. Administration of *L. rhamnosus* GG, *Saccharomyces boulardii* or combination of *L. acidophilus* and *B. lactis* for two weeks also decreased adverse events during the triple treatment (Cremonini *et al.* 2002). However, the effect of probiotic supplementation seemed independent of the probiotic species used. Tursi *et al.* (2004) also recently found that a 10-day quadruple anti-*Helicobacter* therapy with *L. casei* ssp. *casei* DG supplementation significantly increased the eradication rate in patients after failure of first-line eradication treatment. Similarly, *L. acidophilus* La5 combined with *B. lactis* Bb12 improved the second-line rescue therapy in patients with *H. pylori* resistance (Sheu *et al.* 2006). There are two studies conducted with dyspeptic children. In the first, *L. casei* DN-114 001 containing fermented milk product was effective in increasing the eradication rate of standard triple treatment (Sykora *et al.* 2005). More recently, *L. reuteri* was found to alleviate eradication treatment associated adverse effects, but it was not able to increase the eradication rate (Lionetti *et al.* 2006).

There are not many studies on the attenuation of microbiota disturbances with probiotics following an anti-*Helicobacter* triple treatment. In a pilot study, Madden *et al.* (2005) found that probiotic combination including two strains of *L. acidophilus* (CLT60 and CUL21) and two strains of *B. bifidum* (CUL17 and *B. bifidum* Rhodia) stabilized the number of facultative anaerobes. Later the same probiotic product was able to reduce the amount of antibiotic resistance among enterococci and reduce the disruption of the enterobacterial component in the re-growth population (Plummer *et al.* 2005). However, despite the probiotic supplementation, the microbiota in both studies was susceptible to the effects of the antibiotics administered to eradicate *H. pylori*.

**Table 2.** Clinical trials using probiotics as a complement during *H. pylori* eradication treatment.

Patient group	Study design	Eradication therapy	Probiotic strain(s)	Product, dose, time	Results	Reference
120 dyspeptic adults	O, R	CA + rabeprazole	<i>L. acidophilus</i> LB	Capsule, inactivated bacteria, $1.5 \times 10^{10}$ CFU/day, 10 days	Eradication rate ↑ Adverse effects ↔	Canducci <i>et al.</i> 2000
60 asymptomatic adults	O, R	CT + pantoprazole	<i>L. rhamnosus</i> GG	Freeze-dried powder, $1.2 \times 10^{10}$ CFU/day, 14 days	Eradication rate ↔ Adverse effects ↓	Armuzzi <i>et al.</i> 2001a
120 asymptomatic adults	DBPC,R	CT + pantoprazole	<i>L. rhamnosus</i> GG	Freeze-dried powder, $1.2 \times 10^{10}$ CFU/day, 14 days	Eradication rate ↔ Adverse effects ↓	Armuzzi <i>et al.</i> 2001b
160 dyspeptic adults	O, R	CA + lansoprazole	<i>L. acidophilus</i> La5 and <i>B. lactis</i> Bb12	Yoghurt, $1 \times 10^{10}$ CFU/day, 4 weeks	Eradication rate ↑ Adverse effects ↓	Sheu <i>et al.</i> 2002
85 asymptomatic adults	DBPC,R	CT + rabeprazole	1. <i>L. rhamnosus</i> GG, 2. <i>Saccharomyces boulardii</i> , 3. <i>L. acidophilus</i> La5 and <i>B. lactis</i> Bb12	Freeze-dried powder, $1-1.5 \times 10^{10}$ CFU/day, 2 weeks	Eradication rate ↔ Adverse effects ↓ by all probiotics	Cremonini <i>et al.</i> 2002
70 dyspeptic adults with resistant <i>H. pylori</i>	R	AT+ ranitidine bismuth citrate+esomeprazole or pantoprazole	<i>L. casei</i> ssp. <i>casei</i> DG	Capsule, $1.6 \times 10^{10}$ CFU/day, 10 days	Eradication rate ↔ Adverse effects ↓	Tursi <i>et al.</i> 2004
86 dyspeptic children	DBPC,R	CA + omeprazole	<i>L. casei</i> DN-114 001	Fermented milk, $1 \times 10^{10}$ CFU/day, 2 weeks	Eradication rate ↑ Adverse effects ↔	Sykora <i>et al.</i> 2005
40 dyspeptic children	DBPC,R	A + omeprazole 5 days following CT + omeprazole 5 days	<i>L. reuteri</i> ATCC 55730	Capsule, $1 \times 10^8$ CFU/day, 20 days	Eradication rate ↔ Adverse effects ↓	Lionetti <i>et al.</i> 2006
138 dyspeptic adults with resistant <i>H. pylori</i>	O, R	AM + bismuth citrate+omeprazole	<i>L. acidophilus</i> La5 and <i>B. lactis</i> Bb12	Yogurt, $4 \times 10^{10}$ CFU/day, 4 weeks before eradication treatment	Urease activity ↓ during pretreatment, Eradication rate ↑ Adverse effects ↓	Sheu <i>et al.</i> 2006

O, open; R, randomized; DBPC, double-blind placebo-controlled; C, clarithromycin; A, amoxicillin; T, tinidazole; M, metronidazole; CFU, colony forming units; ↑, increase; ↓, decrease; ↔, no effect

**Probiotics as an alternative to antimicrobials** have also been the focus of several studies (Table 3). Administration of culture supernatant or fermented milk containing the strain of *L. acidophilus* La1 decreased *H. pylori* urease activity, measured by <sup>13</sup>C-UBT in adults (Michetti *et al.* 1999) and in children (Cruchet *et al.* 2003), and also in two other trials by histological analysis (Felley *et al.* 2001, Pantoflickova *et al.* 2003). Furthermore, in the latter two studies, a decrease in *H. pylori* infection-associated inflammation was evident. However, the regular intake of *L. acidophilus* (*johnsonii*) La1 did not eradicate *H. pylori* in any of the studies. Sakamoto *et al.* (2001) found *L. gasseri* OLL2716 to be effective in suppression of *H. pylori* and reduction in gastric mucosal inflammation as measured by <sup>13</sup>C-UBT and assays of serum pepsinogen I. In their study, 31 subjects with *H. pylori* infection ingested yogurt containing *L. gasseri* daily for an eight-week period. *L. casei* was also shown to inhibit *H. pylori* growth and to reduce <sup>13</sup>C-UBT values (Cats *et al.* 2003). Similar effects on growth of *H. pylori* were reported for yogurt containing *L. acidophilus* La5 and *B. lactis* Bb12 and consumed for 6 weeks by 59 human volunteers (Wang *et al.* 2004). However, not all clinical trials have shown effectiveness. In one open study, 27 *H. pylori* infected volunteers received yogurt containing three *Lactobacillus* spp. and one commercial starter culture for one month (Wendakoon *et al.* 2002). At the end of the trial <sup>13</sup>C-UBT values remained positive in 26 of the 27 subjects. However, this study used strains that were not proven probiotics.

Studies on the effects of synbiotics (probiotics combined with prebiotics) on *H. pylori* infection are very scarce, and to my knowledge, no clinical studies on prebiotics exist. A randomized, open, eight-week study investigated the effects of *L. acidophilus* LB in comparison with antibiotics and with the synbiotic combination of probiotic yeast *Saccharomyces boulardii* with inulin (Gotteland *et al.* 2005). The eradication rate was slightly better in the *S. boulardii* combined with inulin study group.

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**To summarize**, these observations suggest that consumption of certain strains of probiotics may be useful in combating *H. pylori* infection as a complement to the first-line or the second-line eradication therapy. Generally, complete eradication of *H. pylori* without anti-helicobacter therapy has not succeeded. However, regular consumption of probiotic products with specific strains as an alternative to antibiotics may have some potential in the suppression of *H. pylori* infection.

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**Table 3.** Clinical trials using probiotics in the treatment of *H. pylori* infection.

Patients	Design	Probiotic strain(s)	Product, dose, time	Results	Reference
20 asymptomatic adults	R, DB, PC	<i>L. acidophilus (johnsonii)</i> La1	Culture supernatant + Omeprazole, dose NA, 14 days	Eradication ↔, Urease activity ↓, <i>H. pylori</i> colonization ↔, Inflammation and gastritis ↔	Michetti <i>et al.</i> 1999
52 asymptomatic adults	R, DB, PC	<i>L. acidophilus (johnsonii)</i> La1	Acidified milk + clarithromycin, dose NA, 3 weeks	Eradication ↔, Urease activity ↓, <i>H. pylori</i> colonization ↓, Inflammation and gastritis ↓	Felley <i>et al.</i> 2001
31 asymptomatic adults	PC	<i>L. gasseri</i> 0LL2716	Yogurt, 1.8-2.5 x 10 <sup>9</sup> CFU/day 8 weeks	Serum pepsinogen I / II ratio ↑, Serum pepsinogen ↓, Urease activity ↓	Sakamoto <i>et al.</i> 2001
27 asymptomatic adults	O	<i>L. casei</i> 03, <i>L. acidophilus</i> 2412 and <i>L. acidophilus</i> ACD1	Yogurt, 2.8 x 10 <sup>11</sup> CFU/day, 30 days	Urease activity ↔	Wendakoon <i>et al.</i> 2002
236 asymptomatic children	DB, PC	Living and heat killed <i>L. acidophilus</i> La1 or <i>L. paracasei</i> ST1	Fermented milk products, 1x10 <sup>10</sup> CFU/day, 4 weeks	Eradication ↔, Urease activity ↓ by live La1	Cruchet <i>et al.</i> 2003
50 asymptomatic adults	DB, PC	<i>L. acidophilus (johnsonii)</i> La1	Acidified milk, 1.25 x 10 <sup>9-10</sup> CFU/day, 16 weeks	Eradication ↔, <i>H. pylori</i> colonization ↓, Inflammation ↓ and gastritis ↔	Pantoflickova <i>et al.</i> 2003
20 asymptomatic adults, 6 adults in control group	O, C	<i>L. casei</i> Shirota	Milk based drink, 1.95 x 10 <sup>10</sup> CFU/day, 3 weeks	Eradication ↔, Urease activity tended to ↓	Cats <i>et al.</i> 2003
70 dyspeptic adults, endoscopy for 14 subjects	O, C	<i>L. acidophilus</i> La5 and <i>B. lactis</i> Bb12	Yogurt, 1x10 <sup>10</sup> CFU/day, 4 weeks	Eradication ↔, Urease activity ↓, Gastritis and <i>H. pylori</i> colonization ↓	Wang <i>et al.</i> 2004
254 asymptomatic children	O, R	<i>L. acidophilus</i> LB or <i>Saccharomyces boulardii</i> with inulin	Capsule or sachet, LB 1x10 <sup>10</sup> CFU/day, <i>S. boulardii</i> 500 mg + 10 g inulin/day, 8 weeks	Eradication ↓ <i>S. boulardii</i> with inulin more effective than <i>L. acidophilus</i> LB	Gotteland <i>et al.</i> 2005

R, randomized; DB, double-blind; PC, placebo-controlled; O, open; *L.*, *Lactobacillus*; CFU, colony forming units; NA, not available; ↑, increase; ↓, decrease; ↔, no effect

### 3.3 EXPERIMENTAL STUDIES

Various probiotics have shown favorable effects in animal models of *H. pylori* infection (Table 4). The first two studies presented a highly protective and therapeutic effect of oral administration of *L. salivarius* on *H. pylori* infected gnotobiotics (animals that have been raised in germ-free environments, or contain only specific germs) BALB/c mice model (Kabir *et al.* 1997, Aiba *et al.* 1998). Similarly, Coconnier *et al.* (1998) reported that *L. acidophilus* strain LB was able to protect against *H. pylori* infection in conventional mice. Inhibition of stomach colonization of *H. felis* SC1 (a bacterium closely related to *H. pylori*) was observed and no evidence of gastric histopathological lesions was found. Recently, probiotic combination containing *L. acidophilus* R0052 and *L. rhamnosus* R0011 reduced the effects of *H. pylori* infection in a C57BL/6 mice model of infection through reducing *H. pylori* colonization and alleviating *H. pylori*-induced inflammation of the stomach (Johnson-Henry *et al.* 2004). Also, the same probiotic preparation has proven effective in a Mongolian gerbil model of *H. pylori* infection via its attenuating effect on the *H. pylori* colonization, the mucosal inflammation, and the impairment of the gastrin-somatostatin link (Brzozowski *et al.* 2006). Studies by Sgouras *et al.* (2004, 2005) in a C57BL/6 mice model demonstrated that *L. casei* strain Shirota and *L. johnsonii* La1, both administered in drinking water, attenuated *H. pylori* infection-induced gastric mucosa inflammation. However, only *L. casei* strain Shirota was able to down-regulate the colonization of *H. pylori* to gastric mucosa. Moreover, *L. gasseri* was found to decrease clarithromycin resistant *H. pylori* colonization (Ushiyama *et al.* 2003).

Development of an effective vaccine is also an interesting area in the prevention of *H. pylori* infection. However, the ability of recombinant *Lactobacillus* or other probiotics to be used as an antigen-delivery vehicle to induce protective immune responses has rarely been studied. In the study by Lee *et al.* (2001), *Lactococcus lactis* producing cytoplasmic urease B was shown to be unable to induce protection against *H. pylori* in a mouse model. In contrast, a recombinant *L. plantarum* strain producing *H. pylori* urease B subunit was found to successfully induce a partial mucosal protection against *Helicobacter* (Corthésy *et al.* 2005).

**Table 4.** Probiotics used in experimental *H. pylori* infection.

Model	Study protocol	Probiotic strain(s)	Dose, time	Results	Reference
Gnotobiotic BALB/c mice	Prevention and treatment	<i>L. salivarius</i> (WB 1004) <i>L. brevis</i> (WB1005)	10 <sup>9</sup> CFU once for 1 wk before or 4 weeks after <i>H. pylori</i> infection	Only <i>L. salivarius</i> effective Anti- <i>H. pylori</i> IgG titers ↓ <i>H. pylori</i> colonization ↓	Kabir <i>et al.</i> 1997
Gnotobiotic BALB/c mice	Treatment	<i>L. salivarius</i> (WB1004) <i>L. acidophilus</i> (ATCC 393) <i>L. casei</i> (ATCC 4356)	10 <sup>9</sup> CFU, first week 3 times, then once per week for 3 weeks	Only <i>L. salivarius</i> effective Anti- <i>H. pylori</i> IgG titers ↓	Alba <i>et al.</i> 1998
Conventional BALB/c mice	Treatment	<i>L. acidophilus</i> strain LB	5x10 <sup>8</sup> CFU for 7 days	<i>H. felis</i> colonization ↓ Gastric inflammation ↓	Coconnier <i>et al.</i> 1998
Gnotobiotic BALB/c mice	Treatment	<i>L. gasser</i> OLL2716	10 <sup>9</sup> CFU once per week, 4 weeks	<i>H. pylori</i> colonization ↓	Ushiyama <i>et al.</i> 2003
C57BL/6 mice	Prevention and treatment	<i>L. acidophilus</i> R0052 and <i>L. rhamnosus</i> R0011	10 <sup>9</sup> CFU/ml ( <i>ad libitum</i> ), 7 days prior <i>H. pylori</i> infection and 49 days after	<i>H. pylori</i> growth ↓ Gastric inflammation ↓ Apoptosis ↔	Johnson-Henry <i>et al.</i> 2004
C57BL/6 mice	Treatment	<i>L. casei</i> strain Shirota	10 <sup>8</sup> CFU/ml ( <i>ad libitum</i> ), 9 months	Chronic gastritis ↓ Anti- <i>H. pylori</i> IgG titers ↓ <i>H. pylori</i> colonization ↓	Sgouras <i>et al.</i> 2004
C57BL/6 mice	Treatment	<i>L. johnsonii</i> La1 <i>L. amylovorus</i> CDE 471 <i>L. acidophilus</i> IBB 801	1.5 x 10 <sup>8</sup> CFU /daily, 3 months 2.1 x 10 <sup>8</sup> CFU /daily, 3 months 4.6 x 10 <sup>8</sup> CFU /daily, 3 months	Chronic gastritis ↓ Anti- <i>H. pylori</i> IgG titers ↓ <i>H. pylori</i> colonization ↔	Sgouras <i>et al.</i> 2005
Mongolian gerbil	Prevention and treatment	<i>L. acidophilus</i> R0052 and <i>L. rhamnosus</i> R0011	2x 10 <sup>9</sup> CFU 4 hours before <i>H. pylori</i> and daily for 2 weeks	Chronic gastritis ↓ Gastric acid output ↔ Gastrin ↓	Brzozowski <i>et al.</i> 2006

*L.*, *Lactobacillus*; CFU, colony forming units; IgG, immunoglobulin G; ↑, increase; ↓, decrease; ↔, no effect

### 3.4 POSSIBLE MECHANISMS OF PROBIOTIC ACTION

The mechanisms of probiotic action on *H. pylori* infection are unclear, but there are a number of proposed or hypothesized possibilities from *in vitro* studies of host intestinal epithelial or immune cell responses to probiotic strains. A summary of these possible mechanisms is provided in Figure 3 and described here in more detail.

Generally, probiotics such as lactic acid bacteria and bifidobacteria are able to produce organic acids, hydrogen peroxide and carbon dioxide to inhibit potential pathogens. In addition, many probiotics have been found to produce more defined antimicrobial substances (for review, see Servin 2004). Coconnier *et al.* (1998) found that the anti-*Helicobacter* substance(s) in the *L. acidophilus* LB strain were different from lactic acid. Also, *L. johnsonii* La1, shown to be beneficial in several clinical and experimental studies in treatment of *H. pylori* infection, has been found to release non-bacteriocin antimicrobial substances (Bernet-Camard *et al.* 1997). Furthermore, some *Bifidobacterium* strains have been found to release heat-stable proteinaceous antimicrobial compounds against *H. pylori in vitro* (Collado *et al.* 2005).

The anti-infective activity of probiotics may also partly be due to coaggregation with pathogens (Cesena *et al.* 2001), whereby pathogens are exposed to high doses of potential growth-inhibiting factors produced by probiotics. One mechanism proposed recently is that the *L. johnsonii* La1 expresses cell-surface associated La1GroEL protein, and its recombinant variant, expressed in *Escherichia coli*, is able to induce aggregation of *H. pylori*, but not of other intestinal pathogens. The *L. johnsonii* La1 was also shown to have pro-inflammatory activity, thus favoring the activation of intestinal immunological defences (Bergonzelli *et al.* 2006). Adhesion of pathogens can also be inhibited by steric hindrance, where a large number of beneficial bacteria may cover receptor sites in a non-specific manner, or by competing for specific carbohydrate receptors that would otherwise be available to pathogens. Several probiotic species, such as *L. salivarius*, *L. gasseri* and *L. acidophilus*, have shown growth inhibition or anti-adhesion capacity against *H. pylori* in a gastric epithelial cell model (Midolo *et al.* 1995, Coconnier *et al.* 1998, Lorca *et al.* 2001, Mukai *et al.* 2002, Nam *et al.* 2002, Sgouras *et al.* 2004, Tsai *et al.* 2004).

Mukai *et al.* (2002) have also examined competition in the binding of nine *L. reuteri* strains and *H. pylori* to gangliotetraosylceramide (Asialo-GM1) and sulfatide, which are putative glycolipid receptor molecules of *H. pylori*, and identified a possible sulfatide and Asialo-GM1 binding protein of the two *L. reuteri* strains (JCM1081 and TM105). Moreover, several probiotics are able to inhibit adhesion of pathogenic microorganisms by modifying the glycosylation state of the receptor

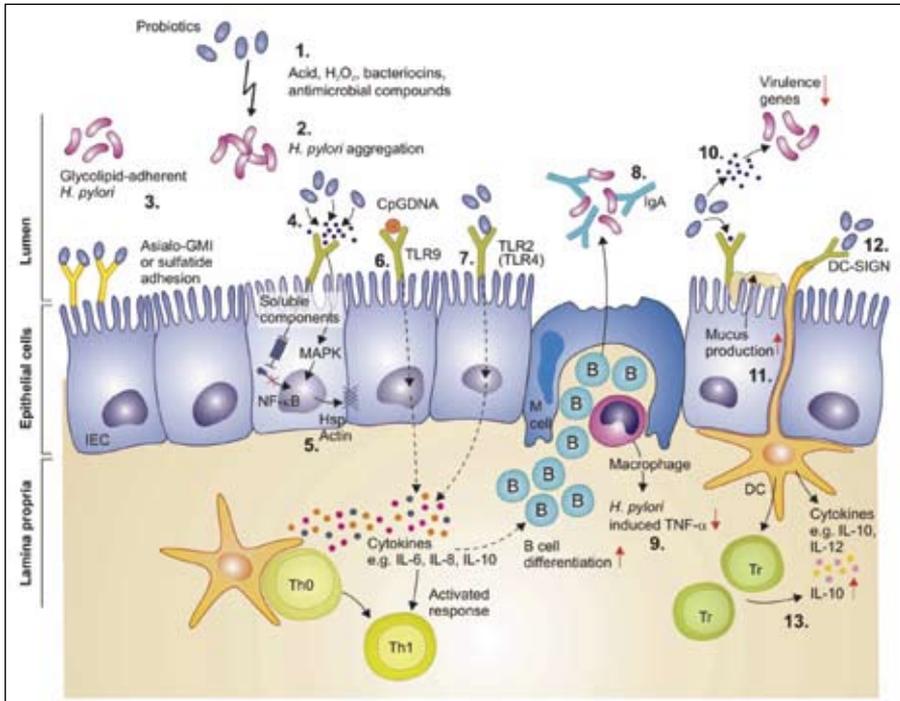
in epithelial cells using soluble factor(s) excreted by the probiotics (for review see, Servin 2004).

Several probiotic bacteria have been shown to prevent and repair mucosal damage by inhibiting damage to tight junction proteins (Montalto *et al.*, 2004). The probiotic strains prevented the pathogen-associated disruption of the cytoskeletal and tight junction proteins in the epithelial cells, thus improving the mucosal barrier function and preventing failure in the secretion of electrolytes (Resta-Lenert and Barrett 2002, 2003). Regular ingestion of live *L. rhamnosus* GG protected the integrity of gastric mucosa, as evaluated by the sucrose permeability test, against alterations by indomethacin (Gotteland *et al.* 2001), thus suggesting at least transient residence in the human stomach and functional effectivity. Probiotic combination of VSL#3, which is a mixture of eight different strains (*L. acidophilus*, *L. delbrueckii* ssp. *bulgarus*, *L. casei*, *L. plantarum*, *Bifidobacterium longum*, *B. infantis*, *B. breve* and *Streptococcus thermophilus*), and soluble factors of *L. rhamnosus* GG were able to induce specific heat shock proteins, known for their ability to maintain actin cytoskeleton integrity (Petrof *et al.* 2004, Tao *et al.* 2006). Further, a novel mechanism of maintaining barrier function was identified by Yan and Polk (2002). These investigators showed that *L. rhamnosus* GG was able to prevent cytokine-induced apoptosis in intestinal epithelial cell models through the inhibition of a TNF-induced activation of the proapoptotic p38/mitogen-activated protein kinase.

Several reports suggest that probiotics are able to differentially modulate innate immune responses in both anti-inflammatory and pro-inflammatory directions. Probiotic bacteria can bind to recognition receptors, such as TLRs expressed on the surface of epithelial cells, and thus trigger a cascade of immunological defence mechanisms (for review, see Saxelin *et al.* 2005, Sartor 2005). TLR4 recognizes lipopolysaccharide and gram-negative bacteria, while TLR2 recognizes a variety of microbial components, such as peptidoglycan and lipoteichoic acids, from gram-positive bacteria (for review, see Abreu 2003, Matsuguchi *et al.* 2003, Takeda *et al.* 2003). Oral CpG delivery was found to reduce significantly *H. pylori* colonization in a mouse model of *H. pylori* infection (Raghavan *et al.* 2003). Thus bifidobacteria, which generally contain DNA with high GC contents and hence a high fraction of unmethylated CpG sequences, could affect the immune system by interacting with TLR9, triggering the production of pro-inflammatory cytokines and promoting T helper cell -1 response to reduce *H. pylori* colonization (Saxelin *et al.* 2005). The possible mechanism underlying this effect, when regarding concomitant CpG administration, is enhanced mucosal cytokine production and immune stimulation.

Also, enhancement of secretory IgA production on intestinal epithelium may have a role in pathogenic bacterial defence (Viljanen *et al.* 2005c). Furthermore, it was shown recently that *L. rhamnosus* GG is able to antagonize *H. pylori* induced TNF- $\alpha$  production by murine macrophages *in vitro* by a contact-independent mechanism (Peña *et al.* 2003). Probiotics could down-regulate virulence genes in pathogenic bacteria. Furthermore, mucins, high-molecular-weight glycoproteins secreted by epithelial cells, may also bind to pathogens, thereby inhibiting their adherence to epithelial cells. Moreover, release of gastric mucin is found to be down-regulated by *H. pylori* (Byrd *et al.* 2000). Probiotics could thus interfere with the actions of *H. pylori*, since several strains, for example *Lactobacillus plantarum* 299V and *L. rhamnosus* GG, have induced mucin gene expression (Mack *et al.* 1999, Mattar *et al.* 2002, Mack *et al.* 2003).

Various microorganisms have been described as binding the dendritic cell (DC) receptor specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), including *H. pylori*. It has been suggested that distinct ligands from different microorganisms show different functional effects. Ligation of DC-SIGN by *H. pylori* induces increased levels of IL-10 in human DCs and a shift toward Th2 development (Bergman *et al.* 2004). In contrast, probiotics, specifically *L. reuteri* and *L. casei*, do not induce increased levels of IL-10 from DCs. Probiotics were also found to induce regulatory T cell development through modulation of DC function (Smits *et al.* 2005). It is currently unclear which different signals are transduced by DC-SIGN ligation. Probiotic bacteria such as *L. rhamnosus* induced maturation of dendritic cells, but resulted in a different cytokine profile than pathogenic bacteria (Braat *et al.* 2004). The probiotic product VSL#3 upregulated IL-10 and down-regulated IL-12 production by dendritic cells derived from human blood and the lamina propria. The main effect of this combination is found to come from bifidobacteria (Hart *et al.* 2004). Also, the *Bifidobacterium* strain's DNA from the same combination, VSL#3, is effective in enhancing the production of IL-1 and IL-10 (Lammers *et al.* 2003).



**Figure 3** Schematic presentation of possible mechanisms of probiotics to interfere with *H. pylori* induced functional effects (modified from Marco *et al.* 2006). Probiotics potentially interfere with *H. pylori* by production of antimicrobial substances (1), inducing aggregation (2) and competing with host-cell-binding sites (3). They could modulate inflammatory responses (4) and strengthen the mucosal barrier (5). Probiotics with a high fraction of CpG sequences can possibly reduce *H. pylori* colonization (6). Differential modulation of cytokine production recognised by Toll-like receptors (TLR) (7), enhancement of secretory immunoglobulin A (Ig) A production (8) and decreased tumor necrosis factor (TNF)- $\alpha$  production by macrophages could have a role in *H. pylori* infection (9). Probiotics could down-regulate the production of virulence factors of *H. pylori* (10) and induce mucin release from epithelial cells (11). They could compete with *H. pylori* in binding to dendritic cell (DC) receptor specific intercellular adhesion molecule 3-grabbing nonintegrin (SIGN) (12) and modulate DC function and regulatory T (Tr) cell development (13). B, B cell (plasma cell); DC, dendritic cell; Hsp, heat shock protein; IL, interleukin; NF, nuclear factor; Th, T helper cell

### 3.5 SAFETY ASPECTS

Data on the safety of probiotics suggest that probiotic therapy is generally considered safe (Boyle 2006, Hammerman *et al.* 2006). Lactobacilli and bifidobacteria are part of normal gastrointestinal microbiota, and systemic infections with these bacteria may thus occur naturally, unrelated to the ingestion of probiotics. Accordingly, increased probiotic use of *L. rhamnosus* GG in Finland has not led to an increase in *Lactobacillus* bacteremia (Salminen *et al.* 2002) and is also regarded as safe in immunocompromised HIV-infected patients (Salminen *et al.* 2004). Bifidobacteria are found to be even safer because no cases of sepsis related to probiotic ingestions have been reported. Propionibacteria are also regarded as

safe to use, mainly because of their long history of safe use in Emmental cheese manufacturing. *P. freudenreichii* ssp. *shermanii* has consequently been proposed for “Qualified Presumption of Safety” status by the European Food Safety Authority (EFSA 2007).

Safety considerations regarding antimicrobial resistance are also recognized among probiotics. There are questions about the possibility of resistance transfer both from probiotics to pathogenic bacteria and from commensal microbiota to probiotics (for review, see Courvalin *et al.* 2006). Probiotics strains are generally susceptible to the majority of antibiotics, but a recent clinical study shows that they can survive gastrointestinal transit quite well during antibiotic treatment (Saarela *et al.* 2007). However, this did not lead to transfer of resistance genes from original microbiota to the ingested probiotics. In any case, different strains of probiotics have different safety profiles, which should be taken into account, and generalizations concerning all probiotics should be avoided (Hammermann *et al.* 2006).

# AIMS OF THE STUDY

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The aim of the present study was to discover the role of probiotics in the treatment of *Helicobacter pylori* infection and to evaluate the mechanisms of action.

The specific aims of this study were:

- To clarify the importance of probiotics as an adjuvant to the current recommended treatment of *H. pylori* infection (Study I).
- To investigate the effects of *H. pylori* eradication treatment and probiotics on intestinal microbiota alterations during the treatment of *H. pylori* infected subjects (Study II).
- To find out the recovery and the effects of probiotics in gastric and duodenal mucosa in *H. pylori* infected and uninfected patients (Study III)
- To characterize the actions of probiotics in *H. pylori* infected intestinal epithelial cells (Study IV).



# SUBJECTS, MATERIALS AND METHODS

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The subjects, materials and methods are described in detail in the original studies. A summary of the subjects is shown in Table 5.

## 1. STUDY DESIGNS AND SUBJECTS

Studies I and II were conducted in a randomized, double-blind, placebo-controlled design. All participants received a seven-day triple therapy [lansoprazole (30 mg), clarithromycin (500 mg) and amoxicillin (1 g) twice daily; Helipak K, Orion Pharma, Espoo, Finland] and were randomized to receive either a milk-based drink containing a total of  $1 \times 10^9$  colony-forming units (CFU) / ml of probiotics or the same drink without the probiotics. Randomization was carried out according to a computer-generated blocked randomization list using a block size of four. The subjects consumed the drink twice a day during the eradication treatment and once a day for the following three weeks. For one month before the study and during the intervention (4 weeks) and the follow-up period (6 weeks), all other probiotic products were forbidden. Exclusion criteria were antibiotic treatment during the previous two months, the use of H<sub>2</sub>-receptor antagonists, bismuth or proton pump inhibitors (PPI) during the previous two weeks, *H. pylori* eradication treatment during the previous five years, use of probiotic products during the previous month, gastric surgery, any diagnosed chronic gastrointestinal disease, allergies to drugs used in the study, medication for fungal infection, and pregnancy or lactation.

A paired study design in which each subject served as his/her own control was used in Study III. The study interventions lasted a total of 56 days (8 weeks)  $\pm$  3 days. All patients underwent gastroduodenoscopy, and biopsy samples were taken by an experienced endoscopist. The probiotic preparation intake was started on the morning after the first endoscopic examination, and the last probiotic drink was taken on the evening of the day before the second endoscopic examination. The volunteers fasted over the night preceding each gastroduodenoscopy. Exclusion criteria were upper gastrointestinal tract lesions, a history of upper gastrointestinal surgery, chronic severe atrophy in the gastric or duodenal mucosa and were otherwise similar to Studies I and II. All the subjects were asked to abstain from commercial products containing probiotic microorganisms two weeks prior to the intervention and during the intervention.

**Table 5.** A summary of the subjects.

Study	n	Gender male/female	Age, y mean (range)	Description of study groups
I <i>H. pylori</i> infected	47	18/29	56 (24–69)	<i>H. pylori</i> infected with triple eradication treatment
II Total	58	18/40		<i>H. pylori</i> infected with triple eradication treatment and uninfected controls
<i>H. pylori</i> infected	39	15/24	57 (34–69)	
Uninfected controls	19	3/16	44 (26–64)	
III Total	13	1/12		<i>H. pylori</i> infected and uninfected patients referred for gastroduodenoscopy
<i>H. pylori</i> infected	7	1/6	51 (40–69)	
Uninfected controls	6	0/6	48 (41–58)	

## 2. ETHICS

All subjects gave their written informed consent (Studies I, II, III). The Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa approved the protocols for Studies I and II. The Ethics Committee of the Pirkanmaa Hospital District (Tampere University Hospital, Tampere, Finland) and the research licensing committee at the City of Tampere approved Study III.

### 3. PROBIOTIC PRODUCTS AND BACTERIAL STRAINS

#### Probiotic products (Studies I, II, III)

The probiotic products consisted of four different strains: *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7076), and *Bifidobacterium breve* Bb99 in Studies I and II or *B. lactis* Bb12 in Study III (Chr. Hansen, Horsholm, Denmark). This probiotic combination was used because LGG has been found beneficial as a supplement in *H. pylori* eradication treatment (Armuzzi *et al.* 2001a, 2001b), and LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) have shown anti-pathogenic effects (Suomalainen and Mäyrä-Mäkinen 1999, Hatakka *et al.* 2007). Moreover, Bb12 has shown beneficial effects in *H. pylori* infection *in vitro* and *in vivo* (Cremonini *et al.* 2002, Wang *et al.* 2004, Sheu *et al.* 2006). In Studies I and II, two batches of the milk-based drinks were used during the clinical study to maintain product quality and to prevent loss of microbial activity in the products. In Study III, several batches of the drink were used during the study period. The daily dose of the drink was 250 ml, and the amount of each probiotic bacteria in the drink was on average  $10^7$  CFU/ml. Microbial quality of the products was regularly assessed during the interventions by microbial plating methods.

#### Bacterial cultures (Study IV)

The lyophilized *H. pylori* was grown on Brucella agar enriched with horse serum at +37°C for 4 to 6 days under microaerophilic conditions. The bacterial suspension was stored at -80°C. Before the experiments, *H. pylori* was subcultured twice on Brucella agar as described above. For the experiments, the bacteria were aseptically harvested and suspended in Dulbecco's modified Eagle's medium (DMEM) and kept under microaerophilic conditions until use. *H. pylori* concentrations were determined by plating methods.

Both *L. rhamnosus* GG (LGG) and *L. rhamnosus* LC705 (LC705) were grown in de Man, Rogosa and Sharpe broth at +37°C under aerobic conditions for 18–20 h. PJS was grown in broth for propionibacteria strains at +30°C for 48 h. *Bifidobacterium breve* Bb99 (Bb99) was grown in de Man, Rogosa and Sharpe broth enriched with 1% L-cysteine hydrochloride monohydrate under anaerobic conditions at +37°C for 24 h. *E. coli* strain DH5 $\alpha$  was grown in Luria-Bertani broth under aerobic conditions at +37°C for 18 h. All probiotic bacteria were subcultured three times before harvesting for the experiments. Harvested bacteria

were centrifuged, washed with phosphate buffered saline (PBS) and resuspended at  $10^9$  CFU/ml, as estimated by plating methods, in DMEM.

**Table 6.** Summary of bacterial strains used in study IV.

Strain	Origin
<i>L. rhamnosus</i> GG, ATCC 53103	Isolated from adult human
<i>L. rhamnosus</i> LC705, DSM 7061	Isolated from milk
<i>Propionibacterium freudenreichii</i> ssp. <i>shermanii</i> JS, DSM 7076	Isolated from cheese
<i>Bifidobacterium breve</i> Bb99, DSM 13692	Isolated from a 3-month-old breast-fed infant
<i>Helicobacter pylori</i> , NCTC 11637	Isolated from human, gastric antrum (cytotoxin-associated gene A -positive strain)
<i>Escherichia coli</i> , DH5a	General laboratory strain

## 4. METHODS

### 4.1 *H. PYLORI* INFECTION ASSESSMENT

#### <sup>13</sup>C-urea breath test (Studies I, II, III)

After an overnight fast, subjects gave duplicate breath samples before and ten minutes after swallowing the <sup>13</sup>C-labeled urea tablets with a glass of water (Diabact UBT, Diabact AB, Uppsala, Sweden). The breath samples were analyzed using isotope ratio mass spectrometry, and delta over baseline values over 4 *per mil* (‰) was considered confirmation of a positive *H. pylori* infection.

#### Serological tests (Studies I, II, III)

For each volunteer (studies I and II), rapid whole-blood tests (Pyloriset Screen II, Orion Diagnostica, Espoo, Finland, and Biocard *Helicobacter pylori* IgG, AniBiotech Ltd, Helsinki, Finland) were carried out on fingertip blood according to the manufacturers' instructions. A blood sample was obtained from each subject, and the sera were stored at -20°C until analysis. Serum samples collected at the baseline were determined by an in-house EIA, together with samples collected at 4 months after therapy, for *H. pylori* antibodies of the IgG and IgA classes. As an indicator of successful eradication therapy, antibody titers of the IgG class had fallen by more than 40% from the pre-treatment level (Studies I and II). In Study III, serum samples were stored before analysis at -20 °C for determination of IgG-*H. Pylori* levels by ELISA according to the manufacturer's instructions (Biohit

Ltd., Helsinki, Finland). The normal reference value for IgG-*H. Pylori* titers was <44 IU.

### Histology (Study III)

The presence and colonization of *H. pylori* infection was determined by modified Giemsa staining. Gastric biopsies (two from the antrum and two from the corpus) were obtained from each patient for analysis. Biopsy samples were fixed in 4% paraformaldehyde, stained with hematoxylin-eosin and Giemsa, and subsequently evaluated by an experienced pathologist.

### Biopsy-based rapid urease test (Study III)

A rapid urease test was performed according to the manufacturer's instructions (Biohit) on at least one biopsy sample from either the gastric antrum or the corpus. The biopsy sample was placed on gel containing urea and phenol red as an indicator. The color change was monitored after 2 and after 30 minutes.

## 4.2 QUESTIONNAIRES

### Treatment related symptoms (Study I)

The participants recorded their daily symptoms in a standardized questionnaire, modified from de Boer *et al.* (1996), at baseline and during the entire intervention period, five weeks in all. Baseline symptoms were recorded daily for one week before the intervention. The following symptoms were included in the total symptom score: epigastric pain, bloating, flatulence, taste disturbance, loss of appetite, nausea, vomiting, heartburn, constipation, and skin rash. The symptoms were scored from 0 to 3: absent (0), mild (1), moderate (2) and severe (3).

### Bowel function (Study I)

The participants also filled in a daily questionnaire on their bowel function (frequency and consistency). Stool consistency was graded from hard (0) to watery (5). Diarrhea was defined as at least three watery or loose stools per day for a minimum of two consecutive days.

### Probiotic drink consumption (Studies I, II, III)

Compliance regarding consumption of the study drink was evaluated by questionnaires in all clinical studies.

## 4.3 MICROBIOLOGICAL METHODS

### Molecular methods

For FISH analysis, fecal samples were diluted, homogenized and centrifuged to remove the coarse material. The supernatant was mixed 1:4 with fresh 4% paraformaldehyde and fixed. The fixed bacterial cells were collected, washed and resuspended in PBS with added 94% ethanol. The cell suspension was diluted 1:20 with hybridization buffer and the fluorescent probe was added (Table 7). Hybridization was carried out overnight; washing and filtering were performed according to Franks *et al.* (1998). Filters were mounted on a slide with AntiFade reagent and covered with a glass. Cells were counted visually with an epifluorescence microscope. DAPI dye (4',6-diamidino-2-phenylindole) was used for the detection of total bacteria counts.

**Table 7.** *Cy3-labeled oligonucleotide probes used in FISH analysis.*

Bacteria group	Probe	Reference
<i>Bacteroides fragilis</i>	Bfra602	Franks <i>et al.</i> 1998
<i>Bacteroides distasonis</i>	Bdis656	Langendijk <i>et al.</i> 1995
<i>Bifidobacteria</i>	Bif164	Franks <i>et al.</i> 1998
<i>Clostridium histolyticum</i>	Chis150	Harmsen <i>et al.</i> 1999
<i>Eubacterium rectale - Clostridium coccooides</i>	Erec482	Suau <i>et al.</i> 2001
<i>Lactobacilli and enterococci</i>	Lab158	Harmsen <i>et al.</i> 1999
<i>Faecalibacterium prausnitzii</i>	Fprau0645	Suau <i>et al.</i> 2001

The concentration of LGG in the feces was analyzed with real-time quantitative PCR (Study II). A strain-specific quantitative PCR method was used for LGG using LightCycler FRET technique (Halme *et al.* 2002). DNA for PCR was isolated from the feces using a Wizard® Genomic DNA purification kit following the manufacturer's instructions (Promega, Madison, WI, USA), except that lysozyme and mutanolysin were used during the 1 h incubation at +37°C. The standard DNAs for quantification were isolated from the dilutions of the known concentrations (CFU/ml) of LGG. The concentration of LGG in the culture was determined by plating, and a tenfold dilution series was made for DNA isolation. The isolation steps were the same for the standards and for the fecal DNAs.

For quantification of the PJS strains, fecal samples were melted and ten-fold dilutions were made in Wilkins-Chalgren broth (study II). The dilutions were plated on buffered propionibacterium agar and incubated anaerobically at +30°C for seven days. Typical PJS-like colonies, light yellow in color, were counted and the cell morphology was verified by microscopy. Isolates were further identified

by biochemical tests (nitrate reduction and lactose fermentation) and random amplification of polymorphic DNA analysis. PCR amplification was performed with a DyNAzyme® DNA Polymerase kit (Finnzyme, Espoo, Finland) according to the instructions of the manufacturer. PCR was performed straight from a fresh colony. PJS was used as a positive control in all the analyses. The preliminary count of the amount of PJS strains was corrected according to RAPD-verified PJS isolates.

In Study III, a strain-specific real-time PCR assay was developed for quantification of each strain using a LightCycler FRET technique similarly to that in Study II (Halme *et al.* 2002, Mikkola *et al.* 2006), except that the concentration of Bb12-strain was determined with a *B. lactis*-specific primer pair (Ventura *et al.* 2001) with the SYBR Green and melting curve analysis method. Each volunteer's two biopsy samples from the same locus were combined and immersed, vortexed vigorously and cooled on ice. Bacterial genomic DNA was extracted from the biopsy and fecal samples, and pure cultures were isolated with the Wizard® genomic DNA purification kit (Promega) following the manufacturer's instructions. In addition, lactic acid bacteria (LAB) of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* were analyzed with group-specific primers using SYBR Green in real-time PCR to confirm successful DNA isolation (Walter *et al.* 2001). The standard curves used for quantification of the strains consisted of a series of 10-fold dilutions of the target species genomic DNA.

## Culture methods

For microbial populations analyzed by cultivation (Study II), the fecal samples were thawed in an anaerobic chamber and diluted 1:10 with Wilkins-Chalgren medium. The mixture was homogenized and dilutions were plated on agar plates. Total aerobic bacteria were cultivated on BHI agar and anaerobic bacteria on the same agar reduced before used. Enterobacteria were grown on Violet red bile glucose agar. YGC agar was used for yeast and molds. Propionic acid bacteria were grown on buffered propionic agar.

## 4.4 MORPHOLOGICAL AND FUNCTIONAL STATUS OF THE GASTRODUODENAL MUCOSA

### Histological evaluation of gastric and duodenal biopsies

Six gastric biopsies (two from the antrum, two from the corpus and two from the duodenum) were obtained from each patient for histological analysis. Biopsy samples were fixed in 4% paraformaldehyde, stained with hematoxylin-eosin and

subsequently evaluated by an experienced pathologist. The degree of inflammation present in the histological specimens was classified according to the updated Sydney system (Dixon *et al.* 1996). A grading scale from 0 to 3 (none, mild, moderate, or severe) was assigned for four histological variables: chronic inflammation (mononuclear cell infiltration), activity (polymorphonuclear neutrophil infiltration), intestinal metaplasia, and glandular atrophy for the antrum and corpus separately.

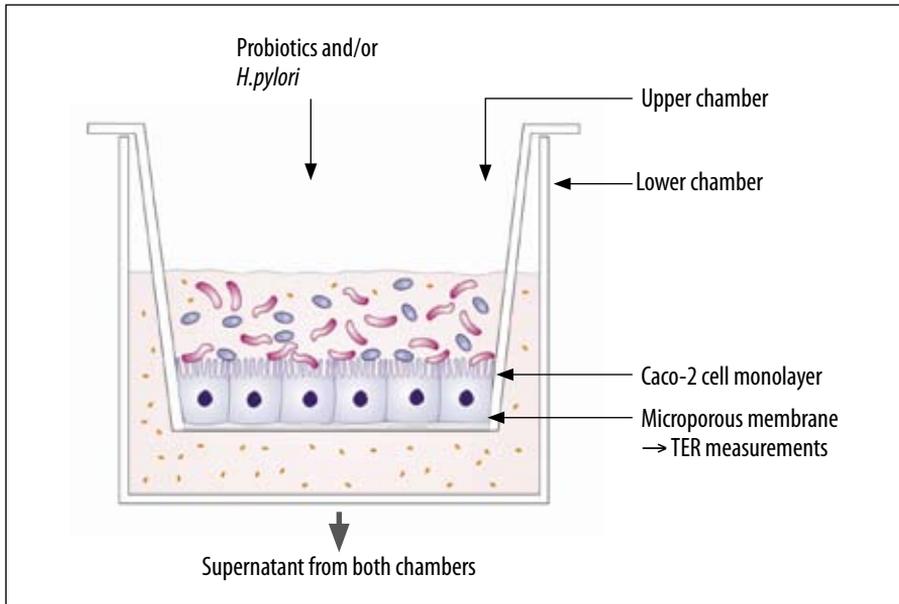
### Determination of serum pepsinogens and gastrin-17

Serum samples were stored before analysis at  $-20^{\circ}\text{C}$  for determination of serum pepsinogen (sPG) I, sPGII and gastrin-17 levels by ELISA, according to the manufacturer's instructions (Biohit). Normal reference values were as follows: sPGI: 25–100  $\mu\text{g/l}$ ; sPGII: 2–10  $\mu\text{g/l}$ ; and sG-17: 2.5–7.5  $\text{pmol/l}$ .

## 4.5 CELL CULTURE METHODS

### Caco-2 cell culture

Caco-2 cells (HTB 37) obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured at  $+37^{\circ}\text{C}$  in DMEM supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and antibiotics (penicillin G 100 U/ml, amphotericin B 250 ng/ml, and streptomycin 100  $\mu\text{g/ml}$ , GIBCO). Cells were seeded ( $1 \times 10^5$  cells/ $\text{cm}^2$ ) on microporous inserts as presented in Figure 4 (Transwell, Corning Costar, Corning, NY, USA) or on 12-well plates (Corning Costar). Confluent monolayers were differentiated for 15 to 21 days and were cultured in medium without antibiotics for 24 h before each experiment.



**Figure 4** Schematic presentation of cell insert model to study interaction of probiotics, *H. pylori* and epithelial cells. TER, Transepithelial electrical resistance

### *H. pylori* adhesion

Adhesion of *H. pylori* was analyzed using a modified version of the method used by Nozawa *et al.* (2002). Briefly, Caco-2 cells were differentiated on standard 96-well plates (Nalge Nunc International, Naperville, IL, USA) at an initial density of  $1 \times 10^5$  cells/cm<sup>2</sup>. Cell monolayers were pretreated with the individual probiotics or their combination at desired concentration in fresh culture medium at +37°C for 1 h. Culture medium was then replaced with a 100 µl aliquot of *H. pylori* at desired concentrations in fresh culture medium. The plates were incubated at +37°C for 90 min and washed twice with PBS to remove the non-adherent *H. pylori*. Cells with adherent *H. pylori* were fixed and after three washes with PBS, 70 µl of rabbit anti-*H. pylori* antibody (Dako A/S, Glostrup, Denmark) in PBS (1:30) was added to each well. After one hour incubation at room temperature and three washes with 1% bovine serum albumin-PBS, 70 µl of secondary antibody (Alexa Fluor 488, goat anti-rabbit IgG, Molecular Probes, Eugene, OR, USA) in PBS (1:500) was added to each well. The plates were incubated for 1 h protected from light, then washed four times with PBS and measured for fluorescence with a Victor<sup>2</sup> multilabel counter 1420 (PerkinElmer, Boston, MA, USA) using excitation and emission wavelengths of 485 nm and 535 nm, respectively.

## Epithelial cell integrity

Bacteria were added in various concentrations to the apical compartments of cell-culture inserts, and the cultures were incubated at +37°C for 42 h. Transepithelial electrical resistance (TER), as an index of epithelial integrity, was measured with the EVOM™ Epithelial Voltometer using “chopstick” electrodes (World Precision Instruments, Stevenage, UK). TER across monolayers was measured at the indicated time points during 42 h incubation. Measurements are expressed in  $\Omega/\text{cm}^2$  after subtracting the mean resistance of cell-free inserts. Prior to experimentation, the Caco-2 monolayers were differentiated for 21 days to acquire a mean baseline TER of 950  $\Omega/\text{cm}^2$ .

## Measurement of epithelial cell leakage and apoptosis

Lactate dehydrogenase (LDH) release indicating cell membrane damage and caspase-3 indicating apoptosis were measured at 8 and 24 h after *H. pylori* infection. Release of LDH into the culture medium was quantified using a kit from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture supernatants were collected at 8 or 24 h after *H. pylori* infection, centrifuged to remove particulate matter, and assayed according to the manufacturer’s instructions. Activation of caspase-3 was measured with a kit from Molecular Probes. Cells from 12-well experiments were lysed and assayed according to the manufacturer’s instructions. Fluorescence intensities were measured at excitation and emission wavelengths of 355 nm and 460 nm, respectively, after one hour incubation at room temperature.

## Cytokine and eicosanoid measurements

Interleukins IL-8 and IL-10, and PGE<sub>2</sub> and LTB<sub>4</sub> were analyzed from culture supernatants by enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer’s instructions. The assays’ detection limits were 1 pg/ml for the IL-8 and IL-10 ELISAs (both from CLB, Sanquin, Amsterdam, The Netherlands), and 15 pg/ml and 6 pg/ml for the PGE<sub>2</sub> and LTB<sub>4</sub> assays (both from Cayman Chemical, Ann Arbor, MI, USA), respectively.

## 4.6 STATISTICAL ANALYSIS

In Study I, the results are expressed as means, 95 percent confidence intervals (CI), medians or interquartile ranges (IQR). Statistical comparison was made using the t test, the Mann-Whitney U test or the Hodges-Lehmann estimation of the shift of medians. Median regression analysis (also known as the least absolute value model) was used to estimate the difference between adjusted medians with the baseline

value as a covariate. Measures with a discrete distribution are expressed as counts (%) and analyzed by the chi-square test, Fischer's exact test or the Fisher-Freeman-Halton test. Hochberg's adjustments were performed to correct significance levels for the multiple tests.

In Studies II and III, Wilcoxon's signed rank test for paired samples was used for the comparison of alterations among groups. Two-way Student's t test was used in Study II to analyze the microbial results between the placebo and probiotic groups. Logarithmic transformation was performed when appropriate. A p value <0.05 was considered significant. In Study IV, statistical differences were analyzed by one-way analyses of variance (ANOVA) and Bonferroni multiple comparisons testing with p values <0.05 considered significant. GraphPad Prism version 3.0 (GraphPad, SanDiego, CA, USA) was used for statistical analyses of the data in Studies II-IV.



# RESULTS

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The main results of Studies I-IV are presented in the following chapters.

## 1. EFFECTS OF PROBIOTICS ON THE TOLERABILITY AND EFFICACY OF *H. PYLORI* ERADICATION TREATMENT

The probiotic combination therapy significantly reduced the total symptom score during *H. pylori* eradication treatment. During the eradication week, the baseline-adjusted median symptom score decreased in the probiotic group, whereas an increase was seen in the placebo group, -3 (95% CI: -7 to 0) vs. 3 (95% CI: -1 to 6) respectively,  $p = 0.038$  between the two groups. The median total symptom score was 22 (IQR 15, 34) in the probiotic group and 32 (IQR 13, 46) in the placebo group. As to individual symptoms, major differences in epigastric pain and bloating were observed between the groups.

The probiotic combination therapy did not significantly increase the eradication rate of the treatment even though the eradication percentages appeared to differ. Eradication efficacy was evaluated at four weeks ( $^{13}\text{C}$ -UBT) and at four months (EIA serology) after completion of the course of triple therapy. *H. pylori* eradication was verified by both  $^{13}\text{C}$ -UBT and serology in 21 out of 23 subjects [91% (95% CI: 72 to 99)] in the probiotic therapy group and 19 out of 24 [79% (95% CI: 56 to 93)] in the placebo group ( $p = 0.42$ ).

No significant differences were observed in defecation frequencies nor in diarrhea between the placebo group and the probiotic group.

To prove the viability and survival of the probiotic bacteria during antibiotic treatment and to test patient compliance objectively, concentrations of two probiotic bacteria (LGG and PJS) were analyzed from fecal samples. Despite the triple treatment for *H. pylori* eradication, the mean fecal concentration of LGG increased in the probiotic therapy group by 2.1 log<sub>10</sub> CFU/g (p < 0.001) and attained a maximal increase of 2.35 log<sub>10</sub> CFU/g (p < 0.001) by the end of the intervention compared to the placebo group. The mean fecal concentration of PJS showed an even greater increase, of 3.7 log<sub>10</sub> CFU/g (p < 0.001) after the first week, and a 2.8 log<sub>10</sub> CFU/g (p < 0.001) increase at the end of the trial, compared to the placebo group.

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**To sum up**, the probiotic combination was able to reduce the subjects' total adverse symptoms during the eradication week. The fecal recovery of LGG and PJS showed that these probiotics are able to survive gastrointestinal transit during intense antimicrobial treatment for *H. pylori* eradication.

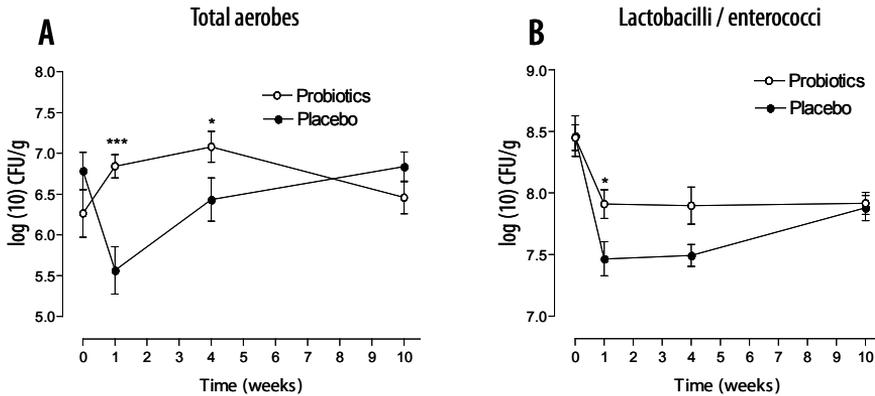
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## 2. EFFECTS OF PROBIOTICS ON MICROBIOTA FOLLOWING *H. PYLORI* ERADICATION TREATMENT

During eradication treatment, the total bacterial counts decreased significantly in the fecal samples of both the placebo and the probiotic group. The counts of bifidobacteria, lactobacilli/enterococci and clostridia also decreased significantly. Furthermore, the fecal species *F. prausnitzii* and *E. rectale* – *C. coccooides* and the total anaerobe population were strongly suppressed in both study groups. In contrast to the placebo group, where aerobic bacteria significantly decreased after antimicrobial treatment, the number of total aerobes clearly increased during the treatment and intervention when compared to the baseline in the probiotic group.

The ratio of anaerobes/aerobes was significantly increased in the placebo group during the eradication treatment. In the probiotic group, there was no major change in the ratio of anaerobes/aerobes during the trial. An increase was found in the number and prevalence of fecal yeasts in both groups after *H. pylori* eradication treatment.

In the post-eradication period, the counts of bifidobacteria and lactobacilli/enterococci groups were clearly lower at end of the intervention than at the baseline. When the bacterial populations in the two groups were compared, the number of total aerobes and lactobacilli/enterococci between Weeks 1 to 4 was found to be significantly higher in the probiotic group than in the placebo group (Figure 5, A and B). After the antimicrobial treatment, a significant decrease was also found in the numbers of *Enterobacteriaceae* in both study groups.



**Figure 5** Fecal concentrations (CFU/g feces) of total aerobes (A) and lactobacilli/enterobacteria group bacteria (B) in the two study groups. Values are expressed as mean ( $\pm$  SEM),  $n = 19-20$ /group. \*  $p < 0.05$  and \*\*\*  $p < 0.001$  when compared to the placebo group.

There were no major differences in the microbiota of *H. pylori* infected patients and healthy control volunteers. Only the number of total anaerobes and clostridia were slightly, but significantly lower in *H. pylori* infected patients.

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**To summarize**, significant reductions in the amount of total bacteria, bifidobacteria, lactobacillus/enterococcus group bacteria, bacteroides, *C. histolyticum* group, *E. rectale - C. coccoides* group, *F. prausnitzii*, total anaerobes and enterobacteria were seen in the fecal samples of both the placebo and probiotic group during the anti-*H. pylori* treatment and in the post-treatment period. Probiotic combination slightly stabilized the microbial disturbances during *H. pylori* eradication treatment since the number of lactobacilli/enterococci alterations decreased and the total amount of aerobes increased.

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### 3. EFFECTS OF PROBIOTICS IN UNTREATED *H. PYLORI* INFECTED PATIENTS

Before probiotic ingestion, at the baseline, a detectable level of LGG was found in the fecal samples of eight of the 12 subjects. LC705 was detected in five, PJS in two and Bb12 in three of the subjects. The amount of all four probiotics in feces increased significantly from baseline values during the intervention in all patients.

The recovery of individual probiotic strains and lactic acid bacteria in gastric and duodenal mucosal biopsies was analyzed from a total of 72 samples. At the baseline, LGG was detected in the duodenum of one of the 12 subjects. At the end of the intervention, three of the subjects were found to have LGG: two in the corpus and one in the duodenum. Other probiotic strains, LC705, PJS and Bb12, were not found in any of the biopsy samples before or after the intervention.

Histologically chronic inflammation in the antrum and corpus level remained the same in all patients during the intervention. In contrast, inflammation activity decreased in two subjects' antrums and also in one subject's corpus, but these effects did not achieve statistical significance. All *H. pylori* uninfected patients had healthy, non-inflamed gastric and duodenal mucosa.

During the eight week probiotic treatment, the <sup>13</sup>C-UBT values appeared to decrease between baseline and Week 8 ( $p=0.063$ ). Mean values for baseline and after probiotic treatment were 22.3 (range 13.8–44.5) and 16.3 (range 6.2–34.1), respectively.

The effect of the probiotic combination intervention on several non-invasive gastric serum markers was determined. Serum gastrin-17 decreased significantly from the baseline level in the *H. pylori* infected group ( $p=0.046$ ). Other markers studied, e.g. pepsinogens I, II and the pepsinogen I/II ratio, remained stable throughout the intervention. Marked differences were found between *H. pylori* positive and *H. pylori* negative subjects in all serum markers evaluated.

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**To sum up**, the individual bacterial strains in the probiotic combination, when administered twice daily for 8 weeks, could be recovered in all of the subjects' fecal samples. Of the probiotics used, LGG showed marginal ability to adhere to the upper gastrointestinal tract mucosa. Furthermore, the probiotic intervention decreased serum gastrin-17 levels and appeared to lower <sup>13</sup>C-UBT values.

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## 4. CHARACTERISTICS OF PROBIOTICS IN *H. PYLORI* INFECTED EPITHELIAL CELLS

A summary of the *in vitro* results is shown in Table 8.

### Adhesion

LGG inhibited *H. pylori* adhesion by half already at the concentration of  $10^7$  CFU/ml. At this concentration, none of the other probiotics studied inhibited *H. pylori* adherence. LC705, Bb99, and the combination were effective at one order of magnitude greater concentrations, whereas PJS inhibited *H. pylori* adherence only with the highest concentration of  $10^9$  CFU/ml. Maximal inhibitory effects were seen with  $10^9$  CFU/ml concentrations of LGG and PJS.

### Barrier function

When *H. pylori*-infected epithelial cells were pretreated with LGG, LC705, or the combination, an increase in transepithelial resistance (TER) ( $p < 0.001$ ) was observed during the first 8 h, suggesting barrier function enhancement. After 18 h of incubation, however, this effect was counteracted with a TER decline and the potentiation of *H. pylori*-induced barrier deterioration.

*H. pylori* increased LDH release from epithelial cells when measured at 8 h, suggesting induction of acute cell membrane leakage. Pretreatment with LGG, LC705, PJS, or the combination counteracted this *H. pylori*-induced effect. At 8 h of incubation, no significant effects on caspase-3 activity were observed, suggesting that neither *H. pylori* nor the probiotic pretreatments promoted acute apoptotic effects. After 24 h of incubation, probiotic bacteria and their combination potentiated LDH-release from *H. pylori*-infected Caco-2 cells, suggesting that the probiotics induced infected cells to cause membrane damage.

### Immunoinflammatory responses

Pretreatment with LGG, LC705, or PJS inhibited IL-8 secretion from *H. pylori* infected cells, but had no effect in uninfected cells. In contrast, Bb99 and the combination of probiotics induced a massive increase in IL-8 secretion as compared to the control cell monolayer or to the *H. pylori* infected monolayer. The Bb99 strain also substantially potentiated IL-8 release from *H. pylori* infected cells. Of all probiotic strains, only the effect of Bb99, although diminished, persisted when the probiotics were used as a combination. A weak measurable release of IL-10 was detected after the cells' exposure to *H. pylori* (1.9 pg/ml at concentration  $10^8$  CFU/ml and 2.0 pg/ml at  $10^7$  CFU/ml) and also after exposure to Bb99-pretreated

*H. pylori*-infected cell monolayer (2.6 pg/ml at concentration  $10^8$  and 3.5 pg/ml at concentration  $10^7$ ). This anti-inflammatory effect was lost when the probiotics were used as a combination.

*H. pylori* dose-dependently increased PGE<sub>2</sub> and LTB<sub>4</sub> release from Caco-2 cells. Treatment of *H. pylori*-uninfected cells with LGG, LC705, and PJS also induced the production of PGE<sub>2</sub>. This effect was not seen with Bb99 or the combination. However, pretreatment of *H. pylori*-infected cells with LC705, PJS, or Bb99 attenuated *H. pylori*-induced PGE<sub>2</sub> production. An opposite and enhancing effect was observed with LGG and the combination treatment.

A decrease in the release of *H. pylori*-induced chemotactic proinflammatory LTB<sub>4</sub> was evident by pretreatment with LC705. In contrast, Bb99 produced a 1.7-fold increase and the combination pretreatments a 3.5-fold increase in LTB<sub>4</sub> release from cells infected with the lower concentration of *H. pylori*. The anti-inflammatory effect of LC705 was lost when the probiotics were combined, and the combination persistently increased LTB<sub>4</sub> release also from cells infected with the higher concentration of *H. pylori*. Probiotics had no effect on uninfected cells' LTB<sub>4</sub> release.

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**To sum up**, all strains and their combination inhibited adherence of *H. pylori* to Caco-2 cells. The acute *H. pylori*-induced membrane damage was alleviated by LGG, LC705, PJS and the combination. This was in accordance with the epithelial cell barrier strengthening effect found with LGG, LC705 and the combination. Strain-specific pro-inflammatory effects were shown to dominate over the anti-inflammatory components when probiotic bacteria were combined.

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**Table 8.** Summary of the effects of probiotics in *H. pylori* infection in vitro.

Measurement	H. pylori		LGG		LC705		PJS		Bb99		Combination	
	Acute	Delayed	Acute	Delayed	Acute	Delayed	Acute	Delayed	Acute	Delayed	Acute	Delayed
Adhesion	100%		-		-		-		-		-	
Transepthelial resistance	↓	↓	+	-	+	-	0	0	0	0	+	-
Lactate dehydrogenase	↑	(↑)	-	+	-	+	-	+	0	+	-	+
Caspase-3	↔	↔	0	-	0	-	0	0	0	0	0	-
Interleukin-8		↑		-		-		-		+		+
Prostaglandin E <sub>2</sub>		↑		+		-		-		-		+
Leukotriene B <sub>4</sub>		↑		0		-		0		+		+

LGG, *L. rhamnosus* GG; LC705, *L. rhamnosus* LC705; PJS, *P. freudenreichii* ssp. *shermanii* Js; Bb99, *B. breve* Bb99.

The effects of *H. pylori* are measured against uninfected control Caco-2 cells: ↑, increased activity; ↓, decreased activity; ↔, no effect on the activity; ( ), slight. The effects of probiotics are measured against *H. pylori* infected Caco-2 cells: +, increased activity; -, decreased activity; 0, no effect on the activity.



# DISCUSSION

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During *H. pylori* eradication treatment, many patients experience antibiotic-associated adverse events, such as bloating, epigastric pain and diarrhea. Even if the short duration of the treatment helps to prevent most discontinuations, the usual eradication rates in clinical trials remain at approximately 80%. Previous studies investigating anti-*H. pylori* regimens have revealed that the gut microbiota is significantly altered during eradication treatment, and specifically the concentrations of lactobacilli and bifidobacteria decrease substantially.

As probiotic bacteria have been shown to have beneficial effects on several disorders of the gastrointestinal tract, it was investigated whether a probiotic mixture of four strains (*L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* JS and *B. breve* 99 or *B. lactis* Bb12) would be able to alleviate the adverse effects associated with anti-*Helicobacter* treatment and increase the efficacy of the eradication treatment. Furthermore, the mechanisms of action by which probiotics provide protection from *H. pylori* infection or promote therapy still remain more or less obscure. Thus, the effects of probiotics on upper gastrointestinal tract mucosa were assessed in *H. pylori* infected and uninfected patients, and the cross-talk between intestinal epithelial cells, probiotic bacteria and *H. pylori* was investigated in an *in vitro* model of infection.

# 1. METHODOLOGICAL ASPECTS

## Selection of the probiotic strains

The justification for the use of the chosen probiotic strains and combination may be questioned. However, two earlier studies by Armuzzi *et al.* (2001a, 2001b) have found that LGG is effective in decreasing the adverse effects related to *H. pylori* eradication treatment, but does not increase the *H. pylori* eradication rate. Also, it was shown that *L. rhamnosus* GG is able to antagonize *H. pylori* induced TNF- $\alpha$  production by murine macrophages *in vitro* (Peña *et al.* 2003). *L. rhamnosus* LC705 (LC705) and *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) have been shown to antagonize yeasts in the manufacture of sour milk products (Suomalainen and Mäyrä-Mäkinen 1999). Furthermore, the combination of LGG, LC705 and PJS has reduced the prevalence of oral *Candida* infection in the elderly (Hatakka *et al.* 2007). *B. lactis* Bb12 combined with *L. acidophilus* La5 have turned out to be beneficial in *H. pylori* infection with and without anti-helicobacter treatment (Cremonini *et al.* 2002, Wang *et al.* 2004, Sheu *et al.* 2006). Furthermore, the combination of probiotics have been observed to be effective in the treatment of irritable bowel syndrome (IBS) in two double-blind, randomized, placebo-controlled long-term clinical studies (Kajander *et al.* 2005, Kajander *et al.* 2006). Moreover, it has been demonstrated that this probiotic combination and LGG have opposite immunological effects in infants with atopic eczema-dermatitis syndrome (Pohjavuori *et al.* 2004, Viljanen *et al.* 2005a, 2005b).

## Assessment of *H. pylori* infection status

There is no single standard for the assessment of *H. pylori* infection status. Therefore, the presence of *H. pylori* infection was evaluated by several methods in the present study. In Study I, the invasive methods were not possible since volunteers did not have indications for gastroscopy, and thus non-invasive urea breath test (UBT) served as the standard. The limitation of this UBT method is that the gastric microbiota of some subjects may also produce urease without any *H. pylori* infection. To increase the reliability of the diagnoses, all positive screening results were confirmed also with an in-house EIA-based serological method that is well-validated locally (Rautelin and Kosunen 2004). The same subjects' blood samples before and after therapy were run in parallel. In the third study, *H. pylori* infection was identified by both invasive methods (histology and rapid biopsy tests) and non-invasive ones (UBT and serology).

## Evaluation of adverse events

A standardized and validated questionnaire, modified from de Boer *et al.* (1996), was used to evaluate adverse effects in Study I. In this questionnaire, 10 treatment-related symptoms were systematically recorded daily for one week before the eradication treatment (baseline), during the treatment (eradication week) and also for three weeks thereafter (follow-up intervention). Correct diary completion was checked at the study meetings after baseline, during the intervention and at the end of the intervention. Diarrhea was evaluated using the criteria recommended by the World Health Organization. In order to gain a solid understanding of “background” symptoms, an evaluation period for baseline symptoms prior to the study was considered important because participants commonly have, for example, questionnaire-induced greater subjective awareness of symptoms. Most of the previous studies have not considered the symptoms before treatment, and thus all of the symptoms recorded during or after treatment were regarded as adverse events in those studies (Canduzzi *et al.* 2000, Armuzzi *et al.* 2001a, 2001b, Sheu *et al.* 2002, Cremonini *et al.* 2002, Tursi *et al.* 2004, Sykora *et al.* 2005). However, part of these baseline symptoms could be related to an ongoing *H. pylori* infection or undiagnosed dyspepsia. In any case, this fact does not significantly interfere with the conclusions drawn from our study since there were no significant differences in baseline symptom scores between the two study groups and all analyses were made with baseline as a covariate. We also used median values instead of means to better accommodate for the small sample size and rather skewed data.

## Microbiological analysis

There are several methodological difficulties in investigating intestinal microbiota alterations. The traditional fecal culture method has been regarded as the standard for studying bacteria. However, microbes may fail to grow on artificial agar, the selectivity of culture media used may be poor, microbes may even prevent the growth of others, or some species may be misidentified (for review, see Tannock 2001). To obtain a more accurate microbial profile, we have used novel molecular techniques based on FISH and quantitative PCR, which provide rapid quantitative and qualitative information on the composition of the intestinal microbiota, in addition to the traditional culture methods (for review, see Zoetendal *et al.* 2006).

## Survival of probiotics

To investigate survival of the probiotic bacteria during antibiotic treatment and probiotic intervention, and to test patient compliance objectively, concentrations of two probiotic bacteria (LGG and PJS) were analyzed from fecal samples in Study

I and all four individual strains in Study III. The amount of probiotic bacterial strains recovery from feces increased markedly following the probiotic intervention in both Studies I and III, indicating excellent survival of gastrointestinal transit and excellent patient compliance during the studies.

LGG was the only probiotic strain recovered from biopsies, which is consistent with several *in vitro* studies indicating that LGG is a very well adhering strain (Tuomola and Salminen 1998, Tuomola *et al.* 2000). Host genetic factors and the original bacterial community adapted to this upper gastrointestinal niche (Bik *et al.* 2006) probably explain why only a minority of the patients had detectable levels of LGG in the biopsies.

## Epithelial cell model

The relevance of the choice of a single intestinal cell line, Caco-2, for studying the effects of probiotics in *H. pylori* infection may be questioned, and this selection may to some extent limit the drawing of conclusions. However, none of the gastric epithelial cell lines available to date (e.g. AGS, MKN-28) are able to grow as a tightly sealed polarized monolayer. Therefore, the Caco-2 cell line which displays a well organized brush border with good transepithelial resistance and a capacity to release several inflammatory mediators upon treatment with *H. pylori* (Hidalgo *et al.* 1989, Kim *et al.* 2002) was chosen. Also, it provides a suitable and highly cited method for studying the effects of infectious agents as well as probiotic bacteria (for reviews, see Servin and Coconnier 2003). Moreover, a single well-characterized experimental gut epithelial cell model was chosen to enable unequivocal head-to-head comparison of probiotics' effects on the level of cell responses to infection with a live pathogen, *H. pylori*. Comparison of responses by different cell models was not the aim of this study.

## 2. MAIN RESULTS

### 2.1 EFFECTS OF PROBIOTICS ON THE ERADICATION TREATMENT OF *H. PYLORI*

Generally 20–30% of first-line anti-*H. pylori* treatments fail (Fischbach *et al.* 2002), and one of the important causes for the failure in clinical practice is the high prevalence of adverse effects (Deltenre *et al.* 1998). In the present study, the probiotic combination was effective in alleviating the adverse effects of *H. pylori* eradication treatment. The tolerability of the treatment was assessed primarily by total symptom score, which takes into account both the frequency and the

severity of the symptoms with baseline symptoms as a covariate. The hypothesized mechanisms of probiotics in the attenuation of symptoms are largely unknown. It can be speculated that probiotic supplementation may resist alterations of microbiota or diminish the antibiotic-induced overgrowth of potentially harmful microorganisms. In support of this hypothesis, probiotic combination stabilized disturbances in *Lactobacillus* and *Enterococcus* group bacteria and the total aerobes following *H. pylori* eradication treatment. Furthermore, probiotics, specifically LGG and PJS, were able to survive in the gastrointestinal tract during eradication treatment. It may be that these particular species are, to some extent, resistant to antimicrobial therapy, because concentrations of lactobacilli and bifidobacteria in general have been shown to decrease during the eradication of *H. pylori* (Bühling *et al.* 2001, Madden *et al.* 2005, Plummer *et al.* 2005) Thus, the stabilization of the microbiota by probiotic therapy could explain, at least in part, the alleviation of symptoms in the present study.

The eradication rate, exceeding 90% in the probiotics group, is considered high. However, the small sample size of Study I does not allow one to draw firm conclusions, although our results are in line with previous studies with other probiotics (for reviews, see Hamilton-Miller 2003, Gotteland *et al.* 2005). It is of interest that while the *H. pylori* infection of two volunteers receiving probiotics in this study was not successfully eradicated, their <sup>13</sup>C-UBT value decreased to borderline levels. In the placebo group, none of the five treatment failures had borderline results from <sup>13</sup>C-UBT. <sup>13</sup>C-UBT values have been reported to correlate positively with *H. pylori* colonization (Perri *et al.* 1998, Zagari *et al.* 2005). This suggests that probiotic therapy could have a colonization-lowering effect on *H. pylori* infection during eradication treatment as measured by <sup>13</sup>C-UBT. This finding is also supported by the results from Study III, since probiotic intervention appeared to decrease bacterial load when administered to untreated patients.

Although the numbers of bifidobacteria, lactobacilli and enterococci, bacteroides and *F. prausnitzii* increased after the cessation of the triple therapy, they did not reach baseline levels even after the 9-week follow-up period. These quite long-term disturbances are partly in accordance with earlier studies of anti-*H. pylori* treatment with clarithromycin and metronidazole, showing that the amounts of lactobacilli and bifidobacteria increase but do not reach the baseline level 4 weeks after the treatment (Bühling *et al.* 2001). It can be speculated that long-term changes in the microbiota could contribute to the long-term persistence of resistant enterococcal populations after anti-*Helicobacter* treatment as demonstrated by others; these resistant bacteria groups could serve as reservoirs of resistance genes (Sjölund *et al.* 2003).

## 2.2 EFFECTS AND CHARACTERISTIC OF PROBIOTICS IN *H. PYLORI* INFECTION

Probiotic intervention decreased gastrin-17 levels in *H. pylori* infected patients. *H. pylori* infection is associated with increased basal and stimulated plasma gastrin concentrations and acid output, especially in patients with duodenal ulcers (Schubert 1999). Elevations in gastrin-17 levels have also been associated with an increased risk for gastric atrophy and cancer (Fox and Wang 2002). The exact mechanisms mediating the effects are not known, but it is suggested that both bacteria products and the inflammatory infiltrate are able to stimulate gastrin and acid secretion (Schubert 1999). Our study is the first, as far as we know, to show that probiotics decrease serum gastrin-17. Furthermore, probiotic intervention appeared to reduce <sup>13</sup>C-UBT values. The density of *H. pylori* in mucosa is strongly related to the gastric inflammation and secretion capacity (Di Mario *et al.* 2006), and the <sup>13</sup>C-UBT is an indirect indicator of *H. pylori* density in gastric mucosa (Perri *et al.* 1998, Zagari *et al.* 2005). A moderate reduction in values (approximately 27%), as shown in the present study, suggests that the total amount of *Helicobacter* in the stomach decreases during the intervention. Support for this conclusion is found in some recent studies in which certain probiotic strains or combinations were seen to reduce *H. pylori* density (Michetti *et al.* 1999, Sakamoto *et al.* 2001, Cruchet *et al.* 2003, Wang *et al.* 2004, Sykora *et al.* 2005). Our results are also consistent with these findings, indicating that probiotics are not able to eradicate *H. pylori* infection.

The mechanisms behind the decreased gastrin-17 seem to be unrelated to the adherence of probiotics in the gastric mucosa. Several probiotic strains have been shown to decrease IL-8 and TNF- $\alpha$  (Morita *et al.* 2002, Peña *et al.* 2003, Zhang *et al.* 2005), and this could hypothetically be one mechanism in which probiotics interfere with gastrin-17 release, since during *H. pylori*-induced inflammation these mediators have been implicated in increasing gastrin production from G cells (for review, see Walter 2006). However, based on the present study, a more logical explanation for the reduced gastrin-17 after probiotic consumption could be the decreased *Helicobacter* amount in the stomach as indicated by <sup>13</sup>C-UBT measurements, and this could possibly lead to decreased release of pro-inflammatory cytokines in the stomach.

After probiotic intervention, *L. rhamnosus* GG adhered to a minority of patients' upper gastrointestinal mucosas, but all probiotics survived well through the gastrointestinal tract transit. This is supported by the *in vitro* study (IV) since probiotics affected barrier function and immunoinflammatory mediators upon *H. pylori* infection by mechanisms unrelated to their ability to inhibit pathogen adhesion. Recent results indicate that beneficial effects of probiotics could be, at

least partially, mediated by their own DNA rather than their ability to colonize the gut (for review, see Mottet and Michetti 2005). On the other hand, adhesion of probiotics at the target sites would possibly result in an enhanced exposure to probiotics at the place of action, perhaps achieving the desired responses, such as immune modulation at a lower dosage.

Probiotics exerted strain-dependent biphasic effects on the barrier function of *H. pylori* infected Caco-2 cells. In the present study, the monolayer's resistance, TER, as an indicator of epithelial barrier function, was acutely tightened by the two *Lactobacillus rhamnosus* strains (LGG and LC705), whereas after longer exposure, these treatments potentiated the decline in barrier function in *H. pylori* infected epithelial cells. In conjunction with measuring transepithelial resistance, we also evaluated the morphological integrity of all cell layers, apoptosis and cell leakage. No cell detachment indicating necrosis was observed. In accordance with TER results, we found that both of the *Lactobacillus* strains studied, LGG and LC705, inhibited significantly caspase-3 when cells were infected with *H. pylori*. Similar acute enhancement has been reported for the probiotic mixture VSL#3 (Otte *et al.* 2004), and a decreased integrity of the epithelial cell monolayer under inflammatory conditions has been reported for probiotics (Menard *et al.* 2004). Our results show a time-dependent dual effect of probiotics on intestinal barrier function and thus present a novel rationale to explain the varying outcomes of previous reports.

For evaluation of the effects of probiotics on the different immunoinflammatory responses, we measured the release of IL-8, IL-10, PGE<sub>2</sub>, and LTB<sub>4</sub> from the epithelial cells *in vitro*. Both the *Lactobacillus* strains (LGG and LC705) and the propionibacteria PJS were able to reduce *H. pylori*-induced IL-8 production. These results are in accordance with reports on non-pathogenic bacteria suppressing IL-8 production in colon epithelial cells stimulated with TNF- $\alpha$  (Hidalgo *et al.* 1989, Kim *et al.* 2002, Otte *et al.* 2004).

*H. pylori* dose-dependently increased PGE<sub>2</sub> and LTB<sub>4</sub> release from epithelial cells. The present findings support the role of intestinal PGE<sub>2</sub> and LTB<sub>4</sub> in mediating mucosal inflammation in *H. pylori* infection. LC705, PJS, and Bb99 were able to suppress the PGE<sub>2</sub> release induced by *H. pylori*, despite the fact that LC705 and PJS alone activated PGE<sub>2</sub> production. Pretreatment with LC705 evidently reduced the *H. pylori*-induced release of chemotactic pro-inflammatory LTB<sub>4</sub>. In contrast, LTB<sub>4</sub> release from cells infected with the lower concentration of *H. pylori* was increased 1.7-fold with Bb99 and 3.5-fold with the combination pretreatments. Probiotics had no effect on uninfected cells' LTB<sub>4</sub> release.

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**Taken together**, the present results suggest some degree of beneficial effect of this probiotic combination on *H. pylori* infected subjects, at least in terms of decreasing gastrin-17 levels. Moreover, these results show that probiotics exert their effects via mechanisms unrelated to their adherence to the gastric mucosa. The combination does not necessarily provide additional immunomodulatory benefit due to the varying and counterbalancing effects of the individual components. However, the immune-stimulatory effects of the probiotic combination may have beneficial indirect effects on *H. pylori* infected patients, since the persistence of the *H. pylori* infection suggests that the host immune response is not effective in eliminating the infection.

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# CONCLUSION AND FUTURE PROSPECTS

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The present study investigated the effects of a probiotic combination supplementing recommended *H. pylori* eradication treatment and probiotic intervention in *H. pylori* infected and uninfected patients. Furthermore, the effects of individual probiotics and the combination were characterized using an *in vitro* model of *H. pylori* infection.

In conclusion, the probiotic combination alleviated the adverse events associated with anti-*H. pylori* therapy following eradication treatment and stabilized the microbiota. The multispecies probiotic combination evidently had some degree of beneficial effect on *H. pylori* infected subjects, at least in terms of decreasing gastrin-17 levels, possibly by decreasing the *H. pylori* load in the stomach, as indicated by <sup>13</sup>C-UBT measurements. The results suggest that the regular intake of probiotics does not eradicate *H. pylori*, but could be considered as an adjuvant to the conventional antibiotic therapy of *H. pylori* infection. Probiotics exert their effects via mechanisms unrelated to their adherence to the gastric mucosa, and thus the interference exerted by this probiotic combination on *H. pylori* could be due to the probiotics' indirect immunomodulating properties.

In order to further evaluate the value of this probiotic combination in *H. pylori* infection, studies comprising larger numbers of patients are necessary. Cutting-edge approaches, such as DNA microarray, would also be of interest to define the effects of anti-*Helicobacter pylori* treatment and probiotics on gut microbiota and to create molecular maps of host-pathogen interactions. The goal of the research should be to provide *H. pylori* infection eradication treatment with optimal efficacy and tolerability to avoid excess use of antimicrobials and to offer alternative ways of controlling *H. pylori* infection if eradication treatment is not recommended.

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Töölö, May 2007

A handwritten signature in black ink, appearing to read 'Eveliina Myllyluoma'. The signature is fluid and cursive, with a large initial 'E'.

*Eveliina Myllyluoma*

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