The effect of the probiotic *L.plantarum* 299v on the intestinal flora : methodological studies in health and disease

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The effect of the probiotic
L. plantarum 299v on the intestinal flora

Methodological studies in health and disease

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**The effect of the probiotic
L. plantarum 299v on the intestinal flora**
Methodological studies in health and disease

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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Dominique Anne Marie Goossens
geboren op 5 januari 1976 te Eindhoven

Promotor:

Prof dr. R.W. Stockbrügger

Co-promotores:

Dr. D.M.A.E. Jonkers

Dr. M.G.V.M. Russel

Beoordelingscommissie:

Prof. dr. A. Schols (voorzitter)

Prof. dr. C. I. Baeten

Prof. dr. C. A. Bruggeman

Prof. dr. R-J.M. Brummer

Prof. dr. G. Molin (Lund University, Lund, Sweden)

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Chapter 1

Introduction

This chapter has been adapted from the article entitled:
“Probiotics in Gastroenterology: Indications and Future Perspectives”
D. Goossens, D. Jonkers, E. Stobberingh, A. van den Bogaard,
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Human microflora

The human microflora comprises a complex ecosystem with high concentrations and variety of bacterial species that can have an influence on the physiology of the host. The human body contains approximately 10^{14} aerobic and anaerobic bacteria that outnumber the total cells of the human body by a factor ten. These bacteria can be found on the skin, and on the mucosa of the oral cavity, upper respiratory tract, urinary tract and vagina, but the largest population inhabits the gastrointestinal tract^{1,2}. This intestinal bacterial flora is not equally distributed in the gastrointestinal tract. The stomach contains relatively small numbers of bacteria ($0-10^3$ colony forming units (CFU) per ml gastric content) because of the gastric acidity. In the lower gastrointestinal tract, there is a higher pH, lower transit time and a higher amount of carbohydrates, resulting in gradually increased numbers and variety of intestinal bacteria. The highest bacterial numbers ($10^{11}-10^{12}$ CFU per ml intestinal content) and the largest diversity are found in the colon that contains mainly obligatory anaerobic species (99.9%) such as *Bacteroides* spp., *Clostridium* spp., bifidobacteria, fusobacteria and eubacteria¹⁻⁴. More than 100 different species have been identified in faecal samples from healthy individuals, but at least 400-500 different species are thought to be present in the colonic microflora⁵.

The composition of the human intestinal flora is influenced by host factors (such as pH, transit time, bile acids, pancreatic enzymes and the mucus composition), non-host factors (such as nutrients, medication, environmental factors) and bacterial factors (such as adhesion capacity, enzymes and metabolic capacities). The intestinal bacteria can interact and these interactions could be either positive or negative by metabolic cooperation or competition, by changes in environmental pH, availability of O_2 or by the production of metabolites such as short chain fatty acids (SCFA) and antimicrobial substances⁶⁻⁸.

The gastrointestinal tract is sterile at the time of birth but the intestinal flora will be developed during and after birth by exposure to bacteria from the external world. The way of giving birth (naturally or via Caesarean section), the type of feeding (breast-fed versus bottle-fed) and contact with bacteria from the mother are examples of factors determining the composition of the intestinal flora of neonates⁹⁻¹¹. The intestinal tract of breast-fed children is considered to be mainly colonised by bifidobacteria while in bottle-fed children the numbers of bifidobacteria are lower and the numbers of enterobacteriaceae, *Bacteroides* spp. and clostridia are higher⁹. After weaning, each individual develops an own unique but complex intestinal flora (i.e., a bacterial 'fingerprint')¹². This intestinal flora is thought to be relatively stable over time: Zoetendal *et al.* reported that the faecal flora of each of four individuals was stable over a four months' time period based on molecular analyses but showed inter-individual differences in its bacterial 'fingerprints'¹³. On the other hand, Goldin *et al.* demonstrated that changing the diet (i.e., removal of red meat and adding fibres to a 'Western-

type' diet) influenced the metabolic activity of the intestinal flora, while influences on the bacterial composition have not been studied¹⁴. Moreover, the faecal intestinal composition might be affected by the physiology associated with aging¹⁵ and by the intake of medication such as antibiotics and 5-aminosalicylic acid¹⁶⁻¹⁸. These findings indicate that also environmental factors may influence the composition and/or metabolic activity of the intestinal flora.

Important functions of the intestinal flora for the host comprise the synthesis of vitamins (B and K) and the metabolism of nutrients, e.g. the production of SCFA such as acetate and butyrate by fermentation of non-digested polysaccharides. Butyrate, for example, is an important energy source for colonocytes and can change cell functioning by influencing lipogenesis, cell membrane assembly, DNA/RNA turnover and crypt cell proliferation^{19,20}. Acetate improves the microcirculation and will facilitate the growth of the small intestinal mucosa²¹. Faecal concentrations of SCFA can be influenced by changes in the composition of the intestinal flora for instance by the intake of probiotics^{22,23}.

In addition, intestinal bacteria can convert primary bile acids into mutagenic secondary ones. Bacterial enzymes such as β -glucosidase and β -glucuronidase can hydrolyse a large number of different glucosides and glucuronides, forming carcinogenic and toxic substances²⁴. These bacterial enzyme activities can be influenced by dietary factors. Goldin *et al.* demonstrated, for example, that rats, fed a high meat diet, showed a significant increase in the β -glucuronidase activity⁸. Finally, intestinal bacteria can influence the immune system such as the maturation of regulatory T cells and antigen presenting cells changing the T-helper 1/T-helper 2 balance²⁵. Intestinal bacteria can also play a role in the defence against the invasion of unwanted exogenous bacteria^{12,26} by colonisation resistance²⁷.

Probiotics

During the past few years there is growing interest in changing the intestinal bacterial flora towards a more 'beneficial' and 'healthier' composition by probiotics, characterised by high concentrations of lactobacilli and bifidobacteria and low concentrations of pathogenic bacteria such as *Bacteroides* spp. 'Probiotics' are defined by Havenaar *et al.* (1992) as "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora"²⁸. Both *Lactobacilli* spp. and *Bifidobacterium* spp. are frequently applied as probiotics, mostly given in fermented milk products or in a freeze-dried form². *Lactobacillus casei* Shirota, *Lactobacillus* GG, *Escherichia coli* and *Saccharomyces boulardii* are examples of probiotic strains frequently investigated in animal and human studies.

Next to probiotics, prebiotics and synbiotics (combinations of pre- and probiotics) are also often used. 'Prebiotics' are food components that are not digested by the host and therefore reach the colon in intact form, where they stimulate the growth of bifidobacteria and lactobacilli²⁹.

Probiotics should be of human origin, genetically stable and it is very important that they are able to survive passage through the gastrointestinal tract (such as low gastric pH and high intestinal bile acid concentrations). They should preferably be able to adhere to the intestinal mucosa so that they can colonise the host³⁰. Probiotics are considered to be safe as the strains are isolated from humans and have long been used in food fermentation³¹. The beneficial effects of probiotics are postulated to be due to modulation of the composition of the intestinal bacterial flora, adherence to the mucosa thereby competing with pathogens, changes of enzyme activities in the colon contents, influences on the immune system of the host, and changes in the availability of metabolic products such as SCFA^{2,32-34}.

Potential beneficial effects of probiotics can be expected in clinical conditions where the intestinal bacterial composition is thought to be disturbed. Although the mode of action of probiotics is still not exactly known, several clinical studies focused on the effects of probiotics in gastrointestinal disorders such as diarrhoea (rotavirus-associated and antibiotic-associated), inflammatory bowel disease, pouchitis, irritable bowel syndrome, colorectal cancer, lactose absorption, *Helicobacter pylori* infection and constipation. The following paragraphs review the main findings of clinical trials performed with probiotics in the field of gastroenterology.

Probiotics and diarrhoea

Diarrhoea can occur as a consequence of amongst others acute viral or bacterial infections or as a side effect of antibiotics. In patients with acute or antibiotic-associated diarrhoea, a disturbance of the intestinal flora has been observed¹⁸. The role of probiotics in the prevention or treatment of acute viral or bacterial and antibiotic-associated diarrhoea has been studied extensively as they may restore a balance in the composition of the intestinal flora, stimulate the immune system, and may compete with pathogens.

Acute viral or bacterial diarrhoea

A major group of interest for probiotic use is acute diarrhoea caused by rotavirus infections, which is the most common cause of acute gastro-enteritis in children. In several placebo-controlled studies an improvement of rotavirus-associated diarrhoea in children has been reported after treatment with *L. GG*, measured as reduction in duration of diarrhoea or decrease in number of bowel movements per day³⁵⁻³⁹. The beneficial effects were accompanied by an

increase of serum IgA antibody levels in these patients^{37,40} and a decrease in rotavirus shedding in the faeces^{35,41}. One study on the prophylactic effect of *L. GG* in 204 undernourished Peruvian children reported a decrease in the incidence of acute diarrhoea⁴².

The positive results of *L. GG* in acute diarrhoea cannot be extrapolated to other probiotic strains or to other causes of acute diarrhoea of viral or bacterial origin. On the other hand, beneficial effects have been reported for *S. boulardii* in the treatment of acute diarrhoea in children or adults^{43,44} and for *Lactobacillus reuteri* in children with rotavirus-associated diarrhoea³⁹. Maiamaa *et al.*⁴⁰ have also compared several probiotic strains and found a beneficial effect of *L. GG* in rotavirus gastro-enteritis but not of *Lactobacillus rhamnosis*, *Lactobacillus delbrueckii* and *Saccharomyces thermophilus*. In addition, placebo-controlled trials in children with acute diarrhoea could not demonstrate a beneficial effect of *Lactobacillus acidophilus*^{45,46}, and inconsistent results were reported for *Enterococcus* SF68 in adults with acute diarrhoea⁴⁷⁻⁴⁹.

The therapeutic effects of especially *L. GG* in rotavirus-associated diarrhoea are well established. Benefits comprise a reduction in duration of diarrhoea by one or two days compared to a median duration of diarrhoea of three days.

Antibiotic-associated diarrhoea

Diarrhoea can also occur as an adverse effect of antibiotic therapy with clinical symptoms ranging from mild diarrhoea to severe pseudomembranous colitis. The use of antibiotics can disturb the intestinal microflora and modulate the immune system directly and indirectly, thereby resulting in diarrhoea^{18,50}. Probiotics can potentially treat or prevent antibiotic-associated diarrhoea by restoring the intestinal bacterial balance and/or by modulating the immune system. Several placebo-controlled studies have demonstrated a decrease in the incidence of diarrhoea or a change in stool frequency and consistency in patients treated with probiotics in addition to the antibiotic therapy⁵²⁻⁵⁸. Frequently applied probiotic strains in these studies are *L. GG*, *L. acidophilus* and *S. boulardii*. In contrast, some others could not demonstrate a decrease in the incidence of diarrhoea⁵⁹⁻⁶¹. Most studies on probiotics in antibiotic-associated diarrhoea are placebo-controlled and performed with reasonable numbers of patients. Recently, two meta-analyses regarding the effect of probiotics in the prevention of antibiotic-associated diarrhoea have been published^{62,63}. These included nine and seven randomised placebo-controlled double-blind trials and reported an odds ratio of 0.37 (95% confidence interval, 0.26-0.53) and a relative risk of 0.40 (95% confidence interval, 0.27-0.57), respectively, in favour of probiotic over placebo treatment.

In contrast to prevention studies, a few studies have focused on the treatment of antibiotic-associated diarrhoea. In nine such patients participating in an uncontrolled study, a faecal enema of colonic bacteria resulted in normalisation of the metabolic activity of the colonic microflora and disappearance of diarrhoea within four days⁶⁴. A study enrolling 45 antibiotic-associated diarrhoea patients from 10 different centres found a significant resolution of

diarrhoea within one week in 91% of the patients treated with *E. coli* SF68 versus 73% in the placebo group⁴⁹. Also in patients with *Clostridium difficile*-associated diarrhoea, beneficial effects of probiotics on the duration or resolution of diarrhoea^{65,66} and a decrease in recurrence rate^{67,68} have been observed. However, the studies investigating the treatment of antibiotic-associated diarrhoea with probiotics were generally performed with limited numbers of patients and mostly not placebo-controlled.

The role of probiotics in the prevention of traveller's diarrhoea has also been studied extensively, but results are conflicting and vary with the probiotic strain used and with the travel destination^{69,70}.

Most studies on probiotics and diarrhoea focus on outcome parameters such as the incidence or duration of diarrhoea or stool frequency. Sometimes the presence of *Clostridium* toxins or rotavirus shedding in stool has been investigated without studying possible modes of action of probiotics such as influences on the composition and metabolic activity of the intestinal flora.

Probiotics and inflammatory bowel disease

Possible pathogenic mechanisms associated with inflammatory bowel disease (IBD), mainly ulcerative colitis (UC) and Crohn's disease (CD), are an imbalance of the intestinal bacterial flora, a defective suppressive immune response and an increased gut permeability^{71,72}. Animal models of colitis have shown that inflammation will not develop without the presence of intestinal bacteria⁷³⁻⁷⁵. Clinical studies also support a role of the intestinal flora in the pathogenesis of IBD: disease activity of CD and UC has been found to improve by faecal diversion⁷⁶, bowel rest^{77,78}, intestinal lavage^{26,77}, and antibiotics^{6,79,80}. Metronidazole, for instance, is a frequently used antibiotic in CD patients that significantly decreased the concentration of *Bacteroides* spp.⁸¹.

Although the composition of the bacterial flora seems to play a role in IBD, bacteriological findings with regard to the bacterial composition are inconsistent. Decreased numbers of lactobacilli and increased numbers of anaerobes have been found in faecal and mucosal samples of CD patients⁸²⁻⁸⁴, while Giaffer *et al.* found an increase in the number of faecal aerobes⁸⁵. In UC patients, increased numbers of *Bacteroides* spp. but decreased numbers of lactobacilli were observed in faecal samples and colonic mucosa^{83,86}. Furthermore, in some studies a decrease in the total number of obligatory anaerobic bacteria in UC patients was found, while others reported an increase in both CD and UC patients^{83,86}.

As probiotics are able to influence the intestinal flora, they are likely to have beneficial effects in IBD patients. In colitis models with genetically modified or chemically treated animals, intake of lactobacilli reduced the severity of the

colitis^{74,87,88}. Administration of *L. GG* to children with CD increased mucosal IgA levels⁸⁹, improved intestinal permeability and lowered the disease activity⁹⁰. In adults with inactive CD, the relapse rate was reduced to 6% after treatment with *S. boulardii* in combination with mesalazine versus 38% by mesalazine alone⁹¹. A reduced relapse rate was also found in active CD patients treated with prednisolon in combination with *E. coli* versus placebo (33% versus 64%, respectively)⁹². Administration of *Lactobacillus salivarius* for ten days to ten adults with active disease caused no remission, but an improvement in quality of life was reported⁹³. In contrast to the beneficial findings, no positive results were found in a placebo-controlled study in which 32 CD patients were treated with *L. GG* for 12 months after curative resection. *L. GG* did not prevent relapses and even more severe endoscopic findings were reported in the *L. GG* treated group⁹⁴.

A case report of a UC patient with active disease showed that antibiotic use followed by implantation of a 'normal colonic flora' via a faecal enema induced remission within one week⁹⁵. Other studies investigated the effect of *E. coli* Nissle versus mesalazine in the maintenance of 120, 357 and 116 inactive UC patients during twelve weeks and twelve months, respectively. All studies found a similar relapse rate in *E. coli* Nissle treated and the mesalazine treated groups and concluded that *E. coli* Nissle could be an alternative maintenance therapy⁹⁶⁻⁹⁸. A multispecies probiotic preparation (VSL#3) was also found to have potential as maintenance therapy: in an uncontrolled study, 15 of 20 UC patients remained in remission during a twelve months treatment with VSL#3 while four patients relapsed. One patient was lost to follow up⁹⁹.

So far, results of probiotic trials are encouraging although the trials mostly included limited numbers of IBD patients and a placebo group is often missing. Moreover, the influence of other medication was frequently not taken into account, and studies have often been performed with patients in remission and during a limited study period.

Probiotics and pouchitis

Pouchitis is a complication of ileal pouch reconstruction after subtotal colectomy, developing in 10-50% of the patients and most frequently observed in UC patients¹⁰⁰. In many patients, pouchitis responds to oral antibiotics such as metronidazole. In patients with pouchitis a decrease in the number of lactobacilli and bifidobacteria and an increase in the number of clostridia and aerobes has been found in faecal samples¹⁰¹. Furthermore, in patients with pouchitis an increase in bile acids and a decrease in SCFA can be measured in faecal samples with subsequent increase in pH^{101,102}.

Probiotics can change the intestinal bacterial balance in patients with pouchitis: *L. GG* has been shown to increase the concentration of lactobacilli in faecal

samples of 20 pouchitis patients after three months treatment¹⁰³. However, in this placebo-controlled study no clinical benefit could be found, as the pouchitis disease activity index did not change significantly¹⁰³. In contrast, Laake *et al.* showed a beneficial effect on the pouchitis disease activity index after four weeks treatment with *L. GG* while no effect on the histological parameters could be seen^{104,105}. Two placebo-controlled studies demonstrated that in 40 and 36 patients with chronic relapsing pouchitis, relapses occurred less frequently after nine months and one year treatment, respectively, with the multispecies probiotic VSL#3 (15% probiotic versus 100% placebo and 15% probiotic versus 94% placebo)^{106,107}. Moreover, VSL#3 has been shown to prevent the occurrence of pouchitis if taken during one year immediately after ileostomy (10% probiotic versus 40% placebo)¹⁰⁸. This delay in the first onset of pouchitis could also be seen after 3 years of *L. GG* treatment (7% probiotic versus 29% placebo)¹⁰⁹.

Other studies in ileal-anal pouch patients have been performed with prebiotics. For example, three weeks treatment with inulin in twenty patients, resulted in less *Bacteroides* spp. and an increase in butyrate concentration measured in faecal samples¹¹⁰.

The beneficial effects of prebiotics and probiotics suggest that these can play a role in the prevention of pouchitis. However, more placebo-controlled studies investigating clinical and bacteriological parameters are warranted to get further insight in the efficiency of probiotics as therapeutic option in pouchitis.

Probiotics and irritable bowel syndrome

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder with a heterogeneous pathophysiology; varying clinical symptoms such as abdominal pain, diarrhoea or constipation can be experienced. The pathophysiology remains largely unknown although abdominal motility, prior enteric infection, visceral hypersensitivity and psychosocial factors have been associated with IBS^{111,112}. Furthermore, alterations in the composition of the faecal intestinal flora have been reported compared to healthy volunteers¹¹³⁻¹¹⁵: a decrease of numbers of faecal *E. coli*, lactobacilli and bifidobacteria and an increase of other faecal aerobes^{113,114}. Moreover, an abnormal colonic fermentation, particularly an increased hydrogen production, has been found^{116,117}. Modifications of the intestinal flora of IBS patients by probiotics may improve clinical symptoms.

The therapeutic effects of *L. acidophilus*, characterised by an improvement of for instance physical state, abdominal pain and daily numbers of stool, were demonstrated in a cross-over trial with 18 IBS patients¹¹⁸. Symptomatic improvement was also found in 19 of 28 patients in an uncontrolled study after the administration of *Enterococcus faecium* PR88¹¹⁹. The administration of

Lactobacillus plantarum 299v versus placebo during four weeks decreased pain and flatulence and showed overall clinical improvement in patients with IBS^{120,121}. A recent study also demonstrated a reduction in symptoms scores such as abdominal pain, bloating and bowel motions after eight weeks treatment with *Bifidobacterium infantis* 35624 compared to treatment with *L. salivarius* UCC4331¹²². Moreover, *B. infantis* 35624 normalized the abnormal IL-10/IL-12 ratio in IBS patients¹²². In contrast, no improvement with respect to pain, urgency to defaecation and bloating was reported after the consumption of *L. GG* for eight weeks in bloating-predominant IBS patients^{123,124}.

Most studies about the efficiency of probiotics in IBS patients were double-blind and placebo-controlled. However, the inclusion criteria with regard to diarrhoea-predominant or constipation-predominant IBS differed among the studies and the patient groups were rather heterogenous. In addition, the influence of concurrent other medical treatments was mostly not taken into account and the placebo-response in these studies was found to be relatively high. So far, no conclusions can be drawn whether probiotics may benefit in certain subgroups of IBS patients like diarrhoea-predominant or constipation-predominant IBS.

Probiotics and lactose malabsorption

The prevalence of lactose malabsorption in Northern Europe is 5-20%, but in Asia the prevalence can be as high as 90-100%¹²⁵. Lactose malabsorption is characterised by the absence or decreased production of the lactose-cleaving enzyme β -galactosidase (lactase) in the mucosa of the small intestine, so that unsplit and non-absorbed lactose reaches the colon. Lactose is a fermentable substrate for the colonic flora and causes bloating, flatulence, bowel cramps, and diarrhoea as clinical symptoms possibly on the basis of dysbacteriosis. Lactose absorption may be improved by the consumption of probiotic bacteria due to microbial β -galactosidase activity, changes in colonic bacterial flora and delayed transit time in the small bowel^{126,127}.

In placebo-controlled studies, it has been shown that milk containing *L. acidophilus* improved the clinical symptoms of lactose malabsorption in lactose intolerant patients^{128,129}. However, some other studies have demonstrated that *L. acidophilus* did not improve clinical symptoms or lactose absorption according to hydrogen breath test¹³⁰⁻¹³². A recent review article concluded that probiotic bacteria may alleviate clinical symptoms caused by malabsorbed lactose¹³³ although more well-designed studies are warranted.

Probiotics and *Helicobacter pylori*

H. pylori infection is associated with gastritis, gastric and duodenal ulcers, and gastric malignancies. *In vitro*, antibacterial activities of probiotic bacterial strains such as *L. acidophilus* against *H. pylori* have been demonstrated^{134,135}. In animals infected with *H. pylori*, *L. salivarius* suppressed the colonisation and growth of this bacterium and reduced the inflammatory response in gastric epithelial cells^{136,137}. Reduction of the severity of gastritis and the *H. pylori* density in the gastric mucosa as well as decreased *H. pylori* breath test values have been observed in infected volunteers treated with probiotics such as *L. acidophilus johnsonii* and *Lactobacillus gasseri* OLL 2716^{134,138,139}, but these results could not be confirmed by others^{140,141}. Placebo-controlled studies found a reduction of the unwanted side effects of the standard triple eradication therapy for *H. pylori* infections (containing two antibiotics) if concurrently with this therapy probiotics such as *L. GG* and *S. boulardii* were given^{141,142}. Canducci *et al.* have described an improved efficacy of eradication when the standard treatment regimen was combined with a daily intake of inactivated *L. acidophilus*¹⁴³.

The results of this limited number of studies indicate a possible supporting role for probiotics when added to standard *H. pylori* eradication therapies, but no evidence is available that they can replace standard eradication therapy.

Probiotics and constipation

Probiotics may have a positive effect on intestinal transit time in constipated patients by modulating the colonic microflora. Several studies investigated the treatment of constipation with prebiotics such as inulin, oligofructose and lactulose. In addition to an improvement of constipation, these studies report an increase in numbers of faecal bifidobacteria and/or lactobacilli¹⁴⁴⁻¹⁴⁶.

A limited number of studies has been published about the effects of probiotics on constipation. An increase in defaecation frequency in constipated patients treated with lactobacilli has been observed^{129,147,148}. One group studied the combination of lactulose with *L. acidophilus* in 21 elderly patients with chronic constipation and found a significant increase in weekly bowel motion frequency¹⁴⁹, while a study including children did not see a clinical improvement of constipation after treatment with lactulose plus *L. GG*¹⁵⁰. A major drawback of these studies was that they were all open except one by Banaszekiewicz *et al.*¹⁵⁰. In contrast to the beneficial effects reported, Ling *et al.* did not find any significant effects on bowel function in constipated elderly patients treated with *L. GG* in a blinded placebo-controlled study¹⁵¹.

Probiotics and colorectal cancer

Colorectal cancer is one of the leading causes of cancer mortality in Western countries¹⁵². In women, colorectal cancer is the second most common cancer after breast cancer and in men, colorectal cancer is the third most common cancer after lung and prostate cancer¹⁵³. Epidemiological studies in for instance Finland, the Netherlands and California have demonstrated low cancer incidences in populations that consume regularly large quantities of yoghurt and/or other fermented milk products. These lower incidences were also found after adjustment for potential confounding variables such as fat and caloric intake¹⁵⁴⁻¹⁵⁷. These observations indicate that intake of lactic acid bacteria might lower the risk of colon cancer. Mechanisms by which probiotics may exert an anti-carcinogenic effect include binding of mutagenic compounds, deactivation of carcinogens, decreasing the amounts of bacterial enzymes that convert procarcinogens into carcinogens, and enhancing the host's immune response. These mechanisms have been studied *in vitro*¹⁵⁸⁻¹⁶⁰ and in experimental animals. In animals, in which colon cancer was induced by chemical carcinogens, administration of lactic acid bacteria showed a suppression in DNA damage, in the formation of aberrant crypt foci, and in tumour formation and growth¹⁶¹⁻¹⁶⁶.

Despite these encouraging results in animal models, there is no prospective evidence that probiotics reduce the colon cancer incidence in humans. So far, trials in humans only have shown that probiotics affect biomarkers for colon cancer risk such as short chain fatty acids and bacterial enzymes. Lactic acid bacteria, for example, significantly decreased faecal bacterial enzyme activities such as β -glucosidase and β -glucuronidase, involved in carcinogenesis, in healthy volunteers consuming a 'Western-type' diet^{8,14,23,167,168}. However, other studies in healthy volunteers failed to demonstrate an effect of lactic acid bacteria on these bacterial enzyme profiles^{169,170}.

Critical focus on probiotic studies

In the past decade, the interest in probiotics has grown extensively. They are commercially available, but are also increasingly used for both research and clinical purposes. Probiotics are becoming an adjuvant or alternative therapy in gastrointestinal disorders as they are considered to be safe, have minimal pathogenic potential for the host and are without side effects.

Because of their postulated modes of action it is supposed that probiotics might be especially beneficial in gastrointestinal disorders where the composition of the intestinal bacterial flora has been disturbed. However, the role of the intestinal bacterial flora in gastrointestinal disorders such as IBD, pouchitis and IBS is still not completely understood and should be investigated in controlled

trials that take into account stage and activity of the disease and side effects of concurrent medication.

An overview of the outcome of probiotic studies in gastrointestinal disorders is shown in table 1.1. The most conclusive evidence exists for the prevention and treatment of diarrhoea by *L. GG*, *L. reuteri* and *S. boulardii*. Two meta-analyses concluded that probiotics can reduce the risk of developing antibiotic-associated diarrhoea and significant benefits have also been shown in acute (rotavirus-associated) diarrhoea. A second group of patients who could benefit from probiotics are IBD patients. Animal studies have shown that probiotics decrease the severity of colitis. Lactobacilli, *E. coli* Nissle and *S. boulardii* seem to reduce relapse rates in UC and CD patients, but studies showing these effects are mostly not placebo-controlled and/or have other methodological limitations. Reduced relapse rates have also been demonstrated in pouchitis patients treated with multi-, and monospecies probiotics in placebo-controlled studies.

Furthermore, probiotic treatment may have potential in the therapy of IBS patients associated with previous enteric infections and/or a change in the intestinal flora. Although some beneficial effects of probiotic therapy have been described in these patients, also negative results have been reported and possible benefits for subgroups need to be studied. Furthermore, probiotics may have beneficial effects in the treatment of lactose malabsorption, constipation and as adjuvant in *H. pylori* eradication. Finally, probiotics can reduce the amounts of genotoxic compounds and biomarkers of colon cancer risk in the colon contents of humans. However, despite some effectiveness in the prevention of colorectal cancer in animal models, their value in preventing colorectal cancer in humans still has to be elucidated.

In general, results of studies on the effects of probiotics in gastrointestinal disorders are encouraging. However, before recommendations for therapy and/or prevention can be given, the results of well-designed blinded placebo-controlled studies with sufficient numbers of clinically well defined (sub)groups of patients have to be awaited.

Many other issues still have to be resolved including dose and duration of probiotic treatment and the onset and duration of effects after start and cessation of probiotics. It is unlikely that one single probiotic strain will be effective in all clinical indications. Differences between bacterial strains as well as the effects of multispecies versus monospecies probiotics warrant further research although so far multispecies probiotics seem to be more effective in treating gastrointestinal disorders, probably due to synergistic effects of the bacterial strains¹⁷¹. In addition, the survival of probiotic strains through the gastrointestinal tract is one of the selection criteria of probiotics, but for many probiotic strains the resistance to gastric acidity, bile acids and pancreatic acids still has to be tested.

More attention should especially be paid to the modes of action by which probiotics may exert beneficial effects. It is thought that one of the possible modes of action is a change in the intestinal bacterial composition.

Also in healthy volunteers, the modes of action of probiotics on the intestinal flora have been little investigated. Studies including healthy volunteers demonstrated a faecal increase in the number of lactobacilli after *L. plantarum* 299v, *L. casei* Shirota, *L. rhamnosus* and *L. acidophilus* consumption, respectively^{23,172-174}. However, most studies have focused on the effects of probiotics on the intestinal flora in faecal samples while it is unknown if faecal samples are representative for the bacterial composition in the mucosa. Furthermore, the composition of the intestinal flora may differ between the distal and proximal colon and rectum. Faecal samples may reflect the bacterial composition of the distal colon but will not necessarily give information of the proximal colon. Measuring the effects of probiotics in biopsy samples from different intestinal locations can be of additional value.

Moreover, the intestinal flora can also be considered as a metabolic entity and studying the influence of probiotics on the metabolic activities such as on enzyme activities and the production of SCFAs will be interesting. Changes in bacterial metabolites have been observed after probiotic consumption with *L. plantarum* 299v, *L. acidophilus* and *L. casei* Shirota in healthy volunteers such as an increase in the acetic and propionic acid concentrations and a decrease in the β -glucuronidase activity^{14,22,23}. However, these parameters have not been studied in the majority of previous studies with healthy volunteers^{23,173,175}. Measuring the effects of probiotics on the intestinal bacterial concentrations as well as on the metabolic activities would be important to get further insight in the possible working mechanism of probiotics. These effects can be studied in healthy volunteers with a 'normal' intestinal bacterial balance, as well as in gastrointestinal patients with changes in the intestinal bacterial balance.

L. plantarum 299v

A potential probiotic strain is *L. plantarum* 229v (DSM 9843), which is a Gram-positive facultative anaerobic rod and a facultative hetero-fermentative lactobacillus, able to ferment carbohydrates to for instance lactic acid, acetic acid, ethanol and CO₂. *L. plantarum* 299v has been found in meat, fish and vegetables and is the dominant species in a range of fermented foods, including sourdough, sauerkraut, green olives, natural wines and beers⁶. *L. plantarum* 299v has originally been isolated from the human intestinal mucosa of Swedish subjects¹⁷⁶.

Table 1.1 Overview of results of probiotic studies in gastrointestinal disorders.

Gastrointestinal disorder	Major study results	Remarks
Acute diarrhoea	Decreased duration of diarrhoea with 1-2 days and decreased number of bowel motions ^{35-39,41,48,49} No beneficial effect ⁴⁵⁻⁴⁷	Different results between probiotic strains Bacteriological parameters hardly studied Clinical relevance decrease of duration of diarrhoea? Well-designed placebo-controlled studies
Antibiotic-associated diarrhoea	Decreased duration and frequency of diarrhoea ^{52-58,65-68} Decreased incidence ⁵²⁻⁵⁸ No decrease of incidence ⁵⁹⁻⁶¹	Different antibiotics in one single study Bacteriological parameters hardly studied Well-designed placebo-controlled studies
Inflammatory bowel disease	Reduced relapse rates in CD patients ^{91,92} Relapse rates in UC patients comparable to mesalazine ⁹⁶⁻⁹⁸ No remission in active disease in CD patients ⁹³ No prevention of relapses in CD patients ⁹⁴ Decreased relapse rates ¹⁰⁶⁻¹⁰⁸ Improvement pouchitis disease activity index ¹⁰⁵	Mostly no placebo-controlled studies Methodological limitations in study design Bacteriological parameters hardly studied
Pouchitis	Overall clinical improvement ^{119,120} Improvement pain and flatulence ¹²⁰⁻¹²² No clinical improvement ^{123,124}	Limited number of (placebo-controlled) studies
Irritable bowel syndrome	Improvement lactose absorption ^{128,129} No improvement lactose absorption ¹³¹⁻¹³³ Decrease of side effects of eradication therapy ^{141,142} Increased eradication rates ^{134,136,139,143} No increase of eradication rates ^{140,141} Increased defaecation frequency ^{129,147-149} No significant effect on bowel function ¹⁴³	Limited number of (placebo-controlled) studies Heterogeneous patient groups Benefits for subgroups? Inconsistent results
Lactose malabsorption	Anticancer effects in animal models ¹⁶¹⁻¹⁶⁶ Beneficial influence on biomarkers for cancer risk ^{14,23,167,168} No influence on biomarkers for cancer risk ^{169,170}	Limited number of (placebo-controlled) studies No direct evidence in humans
<i>H. pylori</i> infection		
Constipation		
Colorectal cancer		

In a healthy volunteer study performed in Sweden, *L. plantarum* 299v was shown to survive passage through the gastrointestinal tract and was recovered from jejunum and rectum biopsy samples in 8 of 13 healthy volunteers 11 days after cessation of probiotic consumption¹⁷². Another Swedish study demonstrated the recovery of *L. plantarum* 299v from faeces in all of the 26 healthy volunteers after three weeks' administration and an increase in the faecal numbers of lactobacilli^{22,172}. However, these studies used a non-selective agar plate, Rogosa agar, for the culture of lactobacilli.

In vitro, *L. plantarum* 299v binds via a mannose-rich adhesion receptor and thereby can compete with *E. coli* for colonisation¹⁷⁷. Furthermore, the oral administration of *L. plantarum* 299v significantly increased the SCFA concentrations such as acetic and propionic acids in healthy volunteers after three weeks treatment²².

L. plantarum 299v has been tested in several clinical trials performed with different patient groups. Forty and 60 IBS patients received *L. plantarum* 299v or placebo during four weeks, and reported a significant improvement in abdominal pain, defaecation frequency and other IBS symptoms compared to patients treated with the placebo or inactive *L. plantarum* 299v^{120,121}. These clinical improvements could be due to an increase in colonic fermentation influencing the hydrogen production during probiotic consumption. However, no changes in gas or hydrogen production have been measured in 12 IBS patients after *L. plantarum* 299v intake¹²⁴.

The ability of *L. plantarum* 299v to prevent *Clostridium difficile*-associated diarrhoea was also tested in a placebo-controlled trial. The patients treated with the probiotic showed less clinical symptoms defined as the recurrence of diarrhoea within five to ten days after initiating treatment compared to the placebo group (4 of 11 versus 6 of 9) but the groups were too small to draw a definite conclusion¹⁷⁸. In contrast, no beneficial effect was seen in a study performed with elective surgical patients: *L. plantarum* 299v did not influence the rate of bacterial translocation, gastric colonisation or the incidence of postoperative septic morbidity¹⁷⁹. *L. plantarum* 299v was also found to be an useful protective agent in atherosclerosis of smokers as a reduction in systolic blood pressure, leptin and fibrinogen was observed after 6 weeks probiotic intake¹⁸⁰.

The effects of *L. plantarum* 299v on colitis, gut permeability and the immune system as well as on the competition of *L. plantarum* 299v with *E. coli* have been tested in several animal studies. In these experiments, *L. plantarum* 299v was found to reduce the severity of methotrexate-induced colitis in rats and in IL-10 deficient mice^{88,181}. Colonisation of germfree IL-10 deficient mice with *L. plantarum* 299v followed by the colonisation of specific bacteria, attenuated the development of colitis¹⁸². Moreover, the competition for colonisation between *L. plantarum* 299v and *E. coli* has been studied in an experiment with germ-free rats, showing lower amounts of *E. coli* in the small intestine and caecum if *L. plantarum* was given in addition to *E. coli* during five weeks¹⁸³.

Furthermore, pre-treatment of *L. plantarum* 299v protected against an *E. coli* induced increase in gut wall permeability but had no influence on the intestinal permeability if given alone¹⁸⁴.

Five weeks treatment with *L. plantarum* 299v influenced the systemic immune system beneficially by increasing the IgA antibody levels against *E. coli* in gnotobiotic rats¹⁸³. The beneficial immunomodulatory activity of *L. plantarum* 299v was also shown in an *in vitro* study demonstrating an increase in IL-10 synthesis and secretion by macrophages and T-cells derived from the inflamed colon of UC patients and incubated with *L. plantarum* 299v¹⁸⁵. Finally, *L. plantarum* 299v with an oatmeal base was more effective in reducing intestinal pathogens in methotrexate-induced enterocolitis in rats than without oats⁸⁸. For this reason, *L. plantarum* 299v was combined in a commercial available probiotic drink with an oatmeal fermented component.

L. plantarum 299v has been demonstrated to be one of the dominant lactobacillus species in humans, to survive passage through the gastrointestinal tract, to be able to adhere to the epithelium via mannose-specific receptors, to increase the faecal numbers of lactobacilli during consumption and to reduce colitis in animal models. Moreover, *L. plantarum* 299v is a probiotic strain easy to handle in the laboratory and to use in probiotic products which makes it a good candidate to study its effects on the bacterial composition and metabolic activities in healthy volunteers and in patients with gastrointestinal diseases such as UC, CD and IBS.

Aims of the studies

The main goal of this project was to investigate the effects of the probiotic *L. plantarum* 299v on the composition of the faecal and mucosal intestinal flora and metabolic activities in healthy volunteers as well as in persons with intestinal disorders. Special attention was paid to the onset and duration of the effects and to the survival of *L. plantarum* 299v through the gastrointestinal tract.

In **chapter 2** the effect of *L. plantarum* 299v on the faecal bacterial composition and metabolic activities was studied in healthy volunteers by measuring short chain fatty acids, endotoxins and enzyme activities. In this study the onset and duration of the effects after start and cessation of *L. plantarum* 299v intake have been studied.

Lowering gastric acidity may result in a better survival of the probiotic bacteria during passage through the gastrointestinal tract; a study investigating the recovery of *L. plantarum* 299v in faecal samples with and without gastric acid inhibition was performed (**chapter 3**).

As the effects of *L. plantarum* 299v are thought to be different in persons with a 'disturbed' intestinal bacterial balance, the next study focused on the effects of *L. plantarum* 299v on the faecal bacterial composition after a bowel cleansing procedure for colonoscopy. Bowel cleansing will wash out the luminal bacteria and probiotics could have a beneficial effect in the recolonisation phase after bowel cleansing. The bacterial composition has been studied using conventional culture techniques as well as the molecular technique, 16S rDNA based PCR followed by denaturation gradient gel electrophoresis. This study is presented in **chapter 4**.

The influence of *L. plantarum* 299v on the faecal as well as on the mucosal flora was studied and discussed in **chapter 5**. A secondary aim of this study was the comparison of the intestinal flora between rectal and colon ascendens biopsy samples. Therefore, patients undergoing a colonoscopy as control for colorectal polyps were included in this study.

In UC patients the intestinal flora might also be disturbed. Differences in bacterial composition between inactive UC patients and healthy volunteers were compared in **chapter 6**. Moreover, the effects of *L. plantarum* 299v on the intestinal flora in faecal and mucosal samples as well as on faecal metabolic activities were studied in inactive UC patients (**chapter 6**).

The general discussion in **chapter 7** focuses on the effects of *L. plantarum* 299v found in the healthy volunteers as well as in the patients and compares the results of the studies performed in more detail.

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Chapter 2

The effect of *L. plantarum* 299v on the bacterial composition and metabolic activity in faeces of healthy volunteers: a placebo-controlled study on the onset and duration of effects

D. Goossens, D. Jonkers, M. Russel, E. Stobberingh, A. van den Bogaard, R. Stockbrügger

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Abstract

Aim

To study the onset and duration of a possible effect of a fermented oatmeal drink containing *L. plantarum* 299v on the composition of the faecal flora of healthy volunteers in a placebo-controlled double-blind study.

Methods

Twenty-two participants consumed a fermented oatmeal drink with or without *L. plantarum* 299v for four weeks. Faecal samples were collected weekly: two samples before, four during, and four after consumption of the drinks. Several bacterial species were counted and enzyme activities, short chain fatty acid (SCFA) concentrations, endotoxin concentration and pH were determined. *L. plantarum* 299v was identified with RAPD.

Results

In contrast to the placebo group, median lactobacilli counts increased significantly from 4.2 (3.4-6.3) to 8.2 (7.3-8.5) log cfu/gram faeces ($p=0.005$) after 1 week consumption of *L. plantarum* 299v, thereafter remaining stable during the treatment period. One week after cessation, a significant decrease of lactobacilli (towards 4.4 (2.2-6.5) log CFU/gram faeces) was observed ($p=0.003$). These lactobacilli were identified as *L. plantarum* 299v. All other bacterial counts, enzyme activities, SCFA concentrations, endotoxin concentration and pH remained unchanged.

Conclusions

L. plantarum 299v intake significantly increased the number of lactobacilli in the faecal flora within one week and this effect disappeared within one week after cessation of intake. No other changes in bacterial counts and metabolic products were observed.

Introduction

In the colon 10^{11} - 10^{12} micro-organisms per gram faeces are found. It is estimated that at least 400-500 different species are present, but only about 100 species have been identified in faecal specimens from healthy individuals¹. The intestinal bacterial flora is involved in a wide variety of metabolic and immunologic processes, which can play a role in health and disease of the host². Nowadays, there is growing interest in probiotics which are defined as "mono- or mixed cultures of live micro-organisms that, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora"³. Monocultures of lactobacilli, bifidobacteria or *Saccharomyces* spp. are frequently used as probiotics but the use of multispecies products is also growing. Clinical studies have suggested that probiotics may be useful in the prevention and treatment of diarrhoea (antibiotic-associated, rotavirus, *Clostridium difficile* associated or traveller's diarrhoea), lactose-intolerance, reduction of food allergy symptoms, reduction of serum cholesterol levels and the protection against colon and bladder cancer⁴⁻⁸. Furthermore, some studies have shown that probiotics might be efficient in the treatment of inflammatory bowel disease, irritable bowel syndrome and pouchitis⁹⁻¹³.

To be effective, probiotics have to be able to survive passage through the gastrointestinal tract and to colonise the intestinal mucosa^{4,14-17}. However, there are several unresolved issues concerning the use of probiotic bacteria and their effect on the intestinal bacterial flora; the number of bacteria to be given, the use of a mono-, or multispecies probiotic preparation, the frequency and duration of administration and the duration of an effect after cessation of the consumption of a probiotic.

Some of these questions were investigated in a limited number of studies. Tannock *et al.* performed an uncontrolled study with ten healthy volunteers who consumed milk containing *Lactobacillus rhamnosus* DR20 for six months and found a change in the number of colony forming units (CFU) of lactobacilli and enterococci in the faecal samples during the consumption period¹⁸. In this study samples were collected only once per month. An increase in the number of lactobacilli was also found by Spanhaak *et al.* and Johansson *et al.*, who studied the effect of *Lactobacillus casei* shirota and *Lactobacillus plantarum* 299v, respectively, on the bacterial composition in the faeces of healthy volunteers in placebo-controlled studies. However, in these studies only one faecal sample was collected after either one and two weeks consumption of probiotic drinks^{19,20}.

Besides studying the composition of the intestinal bacterial flora, the flora can also be considered as a metabolic entity with a variety of metabolites. Short chain fatty acids are formed by fermentation of non-starch polysaccharides by colonic bacteria²¹. Endotoxins, or lipopolysaccharides, are major components of the cell wall of Gram-negative bacteria and changes in the composition of

the bacterial flora can result in a change of endotoxin concentrations. Finally, bacterial enzymes like β -glucosidase and β -glucuronidase can play a role in the conversion of pre-carcinogens into carcinogens²². Lactobacilli might provide some protection against ingested pre-carcinogens by decreasing the enzyme activity of the colonic flora²³. Furthermore, probiotics may also affect other microflora-associated characteristics like cholesterol metabolism, transformation of bilirubin, trypsin activity, degradation of β -aspartylglycine and degradation of mucin²⁴. These bacterial metabolites can be of importance for the host in both health and disease. Measurement of these metabolites can be used as indicators for changes in the composition of the intestinal bacterial flora^{23,24}.

In this placebo-controlled double-blind study, the effects of a fermented oatmeal drink with *L. plantarum* 299v on both the bacterial flora and metabolites is studied in faecal samples of healthy volunteers. Special attention is paid to the onset and duration of the effects.

Methods

Subjects

Twenty-two healthy volunteers were enrolled in this study using the following exclusion criteria: pre-existing bowel-pathology including irritable bowel syndrome, inflammatory bowel disease, diverticulitis, diarrhoea and cancer. Volunteers were not allowed to use gastric acid inhibitors, laxantia, anti-diarrhoea medication or antibiotics for at least one month before as well as during the study. Furthermore, the consumption of other probiotics or prebiotics for at least two weeks before the start of the study as well as during the study was not allowed. Participants were asked to continue their normal diet.

Study design

The study was double-blind and placebo-controlled. Total duration of the study was ten weeks: a pre-treatment period (week 1-2), a treatment period (week 3-6), and a post-treatment period (week 7-10). During the four week treatment period, participants consumed daily 200 ml (100 ml in the morning and 100 ml in the evening) of a fermented oatmeal drink with 10^9 CFU/ml *L. plantarum* 299v (probiotic group, n=11) or without *L. plantarum* 299v (placebo group, n=11). The two drinks were indistinguishable in colour, smell and taste. Ten faecal samples were collected: two just before the start of the study, four during consumption and four after consumption of the drinks. Samples were collected every Thursday ($\pm$ one day) to avoid major influences of changed dietary habits during the weekend. At the time of each faeces collection, a short questionnaire had to be completed on bowel habits (stool frequency and consistency according to the Bristol scale²⁵), side effects and compliance with

regard to the consumption of the drinks. Participants were excluded from analyses if less than 80% of the faecal samples were collected or the drinks were consumed less than six days a week based on the questionnaires.

Microbiological analyses

Faecal samples were collected in sterile containers of 20 ml, brought to the laboratory within twelve hours after defaecation and were divided into three parts. Five grams were diluted (1:4) in pepton water with cysteine (2.1 mM) and glycerol (37%) and homogenised with the ultraturax for two minutes. Cultures were performed directly and the remaining part of the material was frozen (-18°C) for analysis of enzyme activities and short chain fatty acids. Ten grams were centrifuged at 4°C for 2 hours at 25.000 *g* to obtain faecal water that was frozen at -80°C for analysis of endotoxins and determination of the pH. The remaining material was frozen immediately for future additional analyses.

Culture

Ten-fold serial dilutions of the faecal samples (10^{-1} - 10^{-6}) were made in physiological saline (0.9%) and 40 µl were inoculated, using a spiral plater, (Eddy Jet v1.2, IUL-instruments, Barcelona, Spain) on the following agar plates: Blood agar plates (Oxoid CM 271, Basingstoke, UK) for total (facultative) aerobic bacteria, Eosin-methylene blue agar plates (Oxoid CM69, Basingstoke, UK) for enterobacteriaceae, KF-streptococcus agar plates (Oxoid Cm701, Basingstoke, UK) for enterococci, Fastidious anaerobic agar plates (Lab M LabGo, Lancashire BI9, 6AU, UK) for total (facultative) anaerobic bacteria, Bile-esculine agar plates (Becton Dickinson 287920, La Pont de Claix, France) for *Bacteroides* spp., Egg yolk agar plates for spore-forming clostridia, Rogosa agar plates (Oxoid CM627, Basingstoke, UK) for lactic acid bacteria and LAMVAB (lactobacillus anaerobic MRS with vancomycin and bromocresol green) for lactobacilli²⁶.

Egg yolk agar plates were prepared with proteose peptone (46 g/l) (Oxoid L85), sodium phosphate dibasic-2-hydrate (7.2 g/l), magnesium sulphate-7-hydrate (23 mg/l), potassium phosphate (1.15 g/l), sodium chloride (2.3 g/l), glucose (2.3 g/l) and agar (23 g/l) (BBL 11849, Cockeysville, USA). Finally, hemin (5 mg/ml), vitamin K (1 mg/l), egg yolk (sterile egg yolk diluted with physiological saline 1:1) and neomycin (10 mg/ml) were added. Before inoculation, the faecal suspensions were heated at 80°C for ten min.

LAMVAB (pH 5) was prepared with MRS-broth (104.4 g/l), cysteine-HCl (0.5 g/l), bromocresol green (0.05 g/l), agar (40 g/l) and vancomycin hydrochloride (>95% purity, 2 mg/ml) according to the method described by Hartemink *et al.*²⁶.

Blood agar and Eosin-methylene blue agar plates were incubated aerobically at 37°C for 24 hours. KF- streptococcus agar was incubated aerobically at 42°C for 48 hours. Fastidious anaerobic, Bile-esculine, Egg yolk, Rogosa and LAMVAB agar plates were pre-reduced in the anaerobic cabinet for 24 hours

before inoculation and were incubated in the anaerobic cabinet at 37°C for 48 hours after inoculation.

Identification of lactobacilli

From each LAMVAB agar plate four colonies with the appearance of lactobacilli were isolated and purified on blood agar plates. Isolates were frozen at -80°C for molecular identification of *L. plantarum* 299v using randomly amplified polymorphic DNA (RAPD), eventually confirmed by restriction enzyme analyses (REA) according to the method described by Johansson *et al.*^{27,28}.

Bacterial enzyme activities

The β -glucosidase and β -glucuronidase activities were analysed aerobically. Faecal samples were diluted (1:1) in 0.1 M PBS (pH 6.8, 5-7°C), sonicated for one min. at 0°C and subsequently centrifuged at 3000 rpm during 15 min. at 4°C. The supernatant was used for the assessment of β -glucosidase and β -glucuronidase activities.

β -Glucosidase: 0.2 ml faecal supernatant was added to 0.8 ml reaction mixture (0.1 M PBS, 1 mM p-nitrophenyl β -glucopyronoside). The reaction ran for one hour at 37°C and was stopped by adding 5 ml 0.1 M NaOH per ml reaction mixture. For every sample, a control was also determined (faecal sample with reaction mixture without incubating). Readings were taken at 405 nm. Concentrations were calculated, after correction for controls, from a standard curve for p-nitrophenol and were expressed as mg p-nitrophenol released per 60 min per gram faeces²⁹.

β -Glucuronidase: 0.1 ml faecal supernatant was added to 0.9 ml reaction mixture (0.02 M PBS, 0.1 mM EDTA, 1 mM phenylphthalein β D glucuronide). The reaction ran for 30 min. at 37°C and was stopped by adding 5 ml of 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl per ml reaction mixture. For every sample, also a control was determined (faecal sample with reaction mixture without incubating). Readings were taken at 520 nm. Concentrations were calculated, after correction for controls, from a standard curve for phenylphthalein and were expressed as mg phenolphthalein per gram faeces per 30 min²⁹.

Azoreductase: The azoreductase was analysed anaerobically. Faecal samples were diluted (1:1) in pre-reduced Tris buffer (0.2 M, pH 7.8) and were vortexed and centrifuged at 7000 rpm during five min. in the anaerobic cabinet. One ml pre-reduced Tris-buffer (pH 7.8, 0.2 M) was added to one ml supernatant. Direct blue 15 solution (Sigma D2535, Steinham, Germany, final concentration 24 μ g/ml) was added. For every sample, a control without direct blue solution was determined. The samples were incubated anaerobically at 37°C for 16 hours until the dye had been decolourised. After incubation, direct blue was added to the control samples and the absorbance was read at 615 nm. The amount of dye reduced per gram faeces per 16 hours was calculated from a

standard curve generated for direct blue, after correction for controls and was expressed as µg direct blue converted per gram faeces per 16 hours³⁰.

Endotoxin assay

The endotoxin concentration was determined in faecal water using the microtechnique of the *Limulus* amoebocyte lysate test (Endosafe, end point chromogenic analysis endochrome test kit, Charles River, Kent). Pyrogen-free water was used to dilute the faecal samples, to dissolve the solutions and as negative control. The analysis was performed according to the manufacturer's instructions under pyrogen-free conditions. The range of the detectable concentration of the assay was between 0.015 and 1.2 EU/ml. The mean value of faecal endotoxin was expressed as log ng endotoxin per ml faecal water.

Short chain fatty acid assay

Short chain fatty acids (SCFA) were measured in the faeces using gas-liquid chromatography (GLC). The GLC system consisted of a DANI 8521-a gas chromatograph equipped with flame ionisation detector in conjunction with Maestro software (Chrompack, Middelburg, Holland) for calculations. The chromatographic column used, was WCOT fused silica 25m x 0.32 mm id, coated with FFAP-CB df 0.3. This column was used in an isotherm mode at 140°C and the injector and detector temperature were 270°C. The injection type was split with a ratio of 1:50. Helium was used as carrier gas, with a head pressure of 0.8 bar.

One ml HCl with heptanoic acid (internal standard, 10 mmol/l) was added to one ml of the diluted faecal samples to form free fatty acids. SCFA were extracted with two ml of diethyl ether. One µl of the diethyl ether extract was injected for measurement. Concentrations of SCFA were determined in mmol/g faeces after correction for dilution³¹.

pH

The pH was measured in faecal water using a PHM201 portable pH meter (Radiometer, Nederland BV).

Statistical analyses

Statistical evaluation of changes within groups was carried out using the non-parametric Friedman test and the Wilcoxon signed-rank test. The Mann-Whitney U-test was used for comparisons between groups. P-values were adjusted with the Bonferroni-Holm correction because of multiple comparisons. A p-value below 0.05 was considered to be statistically significant.

Results

Twenty participants completed the study (9 males, mean age 34.1 ± 12.9 / 11 females, mean age 31.9 ± 7.2). Two participants from the placebo group were excluded: one person was not able to collect 80% of the faecal samples and one person did not tolerate the fermented oatmeal drink and suffered from more than five liquid stools per day. No further side effects were reported. One probiotic and one placebo consumer mentioned a change of defaecation frequency during the consumption period, from one to two defaecations per day. However, comparing the probiotic and the placebo group no significant differences were observed in defaecation frequency and consistency (table 2.1). The compliance to the drinks was approximately 100% in both groups.

Table 2.1 Faeces frequency, consistency (mean $\pm$ SEM) before (week 1-2), during (week 3-6) and after (week 7-10) the consumption of the probiotic drink or placebo drink.

	Probiotic group			Placebo group		
	before	during	after	before	during	after
Faeces frequency	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Faeces consistency	3.4 ± 0.3	3.5 ± 0.2	3.6 ± 0.2	4.1 ± 0.3	3.7 ± 0.2	3.9 ± 0.2

Culture

During the consumption of the probiotic drink, a significant increase in the number of lactobacilli in the probiotic group was observed ($p=0.005$) (figure 2.1). The number of lactobacilli reached levels of eight log CFU per gram faeces after one week consumption of the probiotic drink and did not increase further after two, three or four weeks treatment. The number of lactobacilli decreased significantly one week after ending the consumption of the probiotic drink ($p=0.003$) (figure 2.1). During the consumption of the drinks, a significant difference could be observed between the probiotic and placebo group ($p=0.006$). Lactic acid bacteria (lactobacilli and bifidobacteria) counted on Rogosa agar plates changed in a similar way as the lactobacilli counted on LAMVAB agar plates.

L. plantarum 299v was identified during the consumption period in all participants of the probiotic group in all faecal samples during the probiotic treatment. No *L. plantarum* 299v could be recovered one week after ending the consumption of the probiotic drinks except for one person.

The number of aerobic bacteria, enterococci, enterobacteriaceae, anaerobic bacteria, *Bacteroides* spp., and spore-forming clostridia did not change in both groups (table 2.2).

Figure 2.1 Concentration of lactobacilli (log CFU/gram faeces) before (week 1-2), during (week 3-6) and after (week 7-10) the consumption of the probiotic or placebo drinks (mean $\pm$ SEM).

* $p < 0.05$ between probiotic and placebo group during the consumption of the drinks.

Table 2.2 Concentrations of bacterial strains (log CFU/gram faeces), median and ranges before (week 1-2), during (week 3-6) and after (week 7-10) the consumption of the probiotic or placebo drink.

	Probiotic group			Placebo group		
	before	during	after	before	during	after
Aerobic bacteria	6.8 (5.7-8.7)	7.1 (5.5-8.2)	7.1 (6.0-7.3)	6.9 (6.4-7.9)	6.9 (5.5-8.1)	7.4 (6.0-8.3)
Enterobacteriaceae	6.2 (0-7.2)	6.2 (2.6-7.0)	6.1 (3.5-7.2)	6.7 (4.6-8.0)	6.5 (5.1-8.1)	6.9 (3.6-8.0)
Enterococci	4.5 (0-6.1)	4.6 (2.7-3.3)	3.7 (0-6.9)	4.4 (3.0-6.4)	4.2 (3.3-5.6)	4.7 (2.6-6.5)
Anaerobic bacteria	9.8 (8.4-10.4)	9.9 (9.4-10.1)	9.9 (9.6-10.2)	9.9 (9.6-10.4)	9.8 (9.5-10.3)	9.8 (8.1-10.1)
<i>Bacteroides</i> spp.	8.0 (7.0-9.7)	8.5 (7.8-8.6)	8.3 (7.8-8.6)	8.1 (7.3-9.0)	8.4 (8.0-8.9)	8.4 (8.0-8.7)
Clostridia	4.1 (0-5.4)	4.3 (2.8-6.0)	4.6 (3.5-5.6)	4.8 (1.9-6.3)	5.0 (2.9-5.6)	4.7 (2.3-5.3)
Bifidobacteria	7.7 (2.0-9.1)	8.2 (6.4-9.0) ^a	4.9 (3.2-7.3)	7.4 (6.8-9.3)	7.6 (4.5-8.9)	5.8 (2.8-7.7)
Lactobacilli	4.2 (3.4-6.3)	8.2 (7.3-8.5) ^a	4.4 (2.2-6.5)	5.1 (3.4-7.9)	5.4 (4.2-8.4)	5.2 (1.9-7.6)

^a $p < 0.05$ between concentrations of bacteria before, during and after the consumption of the probiotic drink

Metabolic activity

β -Glucosidase and β -glucuronidase activity did not change significantly neither in the probiotic nor in the placebo group during the ten weeks study period (table 2.3).

Table 2.3 Enzyme activities (mean $\pm$ SEM) measured before (week 1-2), during (week 3-6) and after (week 7-10) the consumption of the drinks, expressed in mg/60 min/g faeces (β -glucosidase) and in mg/30 min/g faeces (β -glucuronidase).

	Probiotic group			Placebo group		
	before	during	after	before	during	after
β -glucosidase	1.71 $\pm$ 0.41	2.64 $\pm$ 0.45	2.32 $\pm$ 0.37	2.32 $\pm$ 0.51	3.19 $\pm$ 0.82	2.71 $\pm$ 0.60
β -glucuronidase	0.51 $\pm$ 0.23	0.56 $\pm$ 0.20	0.72 $\pm$ 0.21	0.69 $\pm$ 0.32	0.99 $\pm$ 0.34	0.75 $\pm$ 0.26

No significant changes could be seen in endotoxin concentration between groups and within the probiotic and placebo group (figure 2.2).

Figure 2.2 Endotoxin concentration (mean $\pm$ SEM) measured before (week 2), during (week 6) and after (week 9) the consumption of the drinks, expressed in log ng/ml faecal water.

Short chain fatty acids were analysed in all samples. Mean values of SCFA before (week 1-2), during (week 3-6) and after (weeks 7-10) treatment are given in table 2.4. Acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acid concentration did not change significantly as a consequence of the probiotic or placebo drink. Moreover, no differences were observed between week 1 and 2, between week 3-6 and between week 7-10 (data not shown). Before consumption of the drinks, there was a significant difference between probiotic and placebo group in the isocaproic acid concentration. After ending the administration of the placebo drinks the isocaproic acid concentration decreased significantly in comparison to the concentration measured before and during consumption of the placebo drinks.

Table 2.4 Short chain fatty acids concentrations (mean $\pm$ SEM) measured before, during and after the consumption of the drinks.

	Probiotic group			Placebo group		
	before	during	after	before	during	after
Acetic acid	80.0 $\pm$ 6.3	75.0 $\pm$ 7.9	82.7 $\pm$ 10.6	87.5 $\pm$ 12.7	80.5 $\pm$ 13.4	92.6 $\pm$ 12.3
Propionic acid	21.0 $\pm$ 1.5	18.8 $\pm$ 2.2	22.1 $\pm$ 3.6	24.6 $\pm$ 4.3	22.1 $\pm$ 4.2	22.4 $\pm$ 3.9
Isobutyric acid	2.9 $\pm$ 0.3	2.5 $\pm$ 0.4	2.9 $\pm$ 0.5	2.6 $\pm$ 0.4	2.6 $\pm$ 0.5	2.4 $\pm$ 0.6
Butyric acid	20.7 $\pm$ 2.9	17.0 $\pm$ 2.2	21.1 $\pm$ 3.9	24.6 $\pm$ 4.6	22.2 $\pm$ 4.9	27.6 $\pm$ 4.3
Isovaleric acid	4.0 $\pm$ 0.4	3.4 $\pm$ 0.4	4.1 $\pm$ 0.7	3.9 $\pm$ 0.7	3.2 $\pm$ 0.6	4.0 $\pm$ 1.2
Valeric acid	3.4 $\pm$ 0.3	2.9 $\pm$ 0.3	2.9 $\pm$ 0.6	3.6 $\pm$ 0.5	2.8 $\pm$ 0.4	2.7 $\pm$ 0.4
Isocaproic acid	0.8 $\pm$ 0.1 ^a	0.4 $\pm$ 0.06	0.3 $\pm$ 0.04	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	0.2 $\pm$ 0.04 ^b
Caproic acid	3.3 $\pm$ 0.5	2.2 $\pm$ 0.4	2.2 $\pm$ 0.6	2.7 $\pm$ 0.3	2.1 $\pm$ 0.4	2.6 $\pm$ 0.5

^a $p < 0.05$ significant difference in isocaproic acid concentration in the probiotic group compared to the placebo group before the consumption of the drinks; ^b $p < 0.05$ significant difference in isocaproic acid concentration in the placebo group after consumption of the drink in comparison to concentrations before and during the consumption of the drinks.

No significant differences in pH between probiotic and placebo group and within both groups were observed (figure 2.3).

Figure 2.3 pH (mean $\pm$ SEM) measured in faecal water before (week 1-2), during (week 3-6) and after (week 7-10) the consumption of probiotic and placebo drinks.

Discussion

Probiotics are advertised as treatment modality for various gastrointestinal diseases and for improving general health. However, there are still many unresolved issues concerning probiotic bacteria and their effects on the human body and intestinal flora. Intervention studies are often performed without basic biological data being obtained. The aim of this study was to investigate the onset and duration of a possible effect of *L. plantarum* 299v on both the bacterial composition and metabolic activity in healthy volunteers before starting clinical studies.

In the present study, twice daily intake of *L. plantarum* 299v (total daily dosis 10^{11} CFU) significantly increased the number of lactobacilli in the faecal flora after one week consumption, indicating that *L. plantarum* 299v is able to survive passage through the gastrointestinal tract. This increase disappeared within one week after ending the consumption of the fermented oatmeal drink with *L. plantarum* 299v, showing that *L. plantarum* 299v did not colonise the colon permanently. *L. plantarum* 299v could be recovered in the probiotic group with RAPD and REA during the consumption period, but not after ending the consumption of the probiotic drink except for one person. In a study of Johansson *et al.*, *L. plantarum* 299v was found in faecal samples of five of 24 persons eight days after ending the administration of the probiotic drink¹⁹. In addition, *L. plantarum* 299v could also be recovered from the mucosa in eight of the 13 patients, 11 days after the consumption of *L. plantarum* 299v³². Unfortunately, in these two studies samples were not collected for a longer period of time after ending the administration of *L. plantarum* 299v.

An increase in the faecal number of lactobacilli was also observed after the consumption of *L. plantarum* 299v, *L. rhamnosus* or *Lactobacillus acidophilus* for three weeks, six months and three weeks, respectively^{18,32,33}. In these

studies, with healthy volunteers, no attention was paid to the onset and duration of lactobacilli increase. However, in some patient groups several faecal samples were collected during long-term treatment and one faecal sample thereafter, demonstrating a significant increase in the number of lactobacilli 20 and 30 days after starting treatment and a significant decrease 15 and 30 days after cessation^{12,13}. The number of lactobacilli in the pre-treatment and post-treatment period in our study is relatively low in comparison with the studies performed by Johansson *et al.* and Spanhaak *et al.* but comparable with the patient studies performed by Venturi *et al.* and Gionchetti *et al.*^{12,13,19,20}. Differences in lactobacilli counts can possibly be explained by the use of a recently developed more selective agar for the culture of lactobacilli in our study: LAMVAB (lactobacillus anaerobic MRS with vancomycin and bromocresol green)²⁶. The studies mentioned above¹⁸⁻²⁰, used Rogosa agar for culture of lactobacilli. This medium is not selective for lactobacilli as growth of other acid-resistant bacteria such as bifidobacteria, corynebacteria and enterococci is not completely suppressed³⁴. Differences in basal numbers of lactobacilli might also be caused by differences in the consumption of fermented dairy products. In the present study, healthy volunteers did not consume standardised diets but were asked to continue the consumption of their normal dietary habits.

One of the possible therapeutic mechanisms of probiotics is the partial replacement of other bacteria in the intestinal lumen. The consumption of the fermented oatmeal drink with or without *L. plantarum* 299v had no influence on the numbers of other faecal aerobic and anaerobic bacteria investigated. However, in the study of Johansson *et al.* a significant decrease in the number of sulphite-reducing clostridia was found, but this decrease was present in both the probiotic and placebo group¹⁹. The number of bacteria counted in the present study, were investigated by culture techniques. As many intestinal bacteria cannot be cultured or are not yet identified, molecular methods will be of additional value in studying the effects of probiotics on the intestinal flora³⁵.

A change in the composition of the intestinal flora can be accompanied by a change in enzyme activities like β -glucosidase, β -glucuronidase and azoreductase. A decrease of these enzymes is expected to be beneficial because β -glucosidase and β -glucuronidase could produce carcinogenic end-products²². In this study with healthy volunteers, the fermented oatmeal drink with *L. plantarum* 299v had no influence on the β -glucosidase and β -glucuronidase activities. These results are in accordance with results of previous studies in which *L. rhamnosus* DR20 and *L. acidophilus* A1 had no influence on the β -glucuronidase activities^{18,36}. However, β -glucuronidase activities have been decreased as consequence of lactobacilli consumption in some other studies including healthy volunteers using *L. casei* Shirota and *L. acidophilus*^{20,22}. Furthermore, an increase in β -glucosidase activity during *L. acidophilus* A1 consumption and a decrease after *L. casei* Shirota consumption were observed^{20,36}. These differences could be explained by the use of different lactobacilli strains as probiotic. Different lactobacilli subspecies could

replace the intestinal flora, change the intestinal conditions like the pH and stimulate reactions leading to the production of potentially beneficial or damaging products in different ways³⁷. These aspects as well as the hetero versus homofermentative capacities of lactobacilli may thereby affect the SCFA concentration or enzyme activities differently.

In the study of Spanhaak *et al.* the bacterial enzyme activities were expressed in units per 10^{10} CFU²⁰. In our study the bacterial enzyme activities are expressed in mg per gram faeces per hour, which makes comparisons between the studies difficult. Calculations of the enzyme activity per mg protein to correct for metabolic changes did not result in significant differences in β -glucosidase and β -glucuronidase activities during the consumption of the fermented oatmeal drink (data not shown).

No azoreductase activity could be measured as values were often below the detection limit.

The consumption of the probiotic drink did not change the endotoxin concentrations. This result is in accordance with the finding that no changes were seen in the total counts of enterobacteriaceae because the intestinal endotoxin is mainly released by aerobic Gram-negative bacteria such as enterobacteriaceae^{38,39}.

No influence of the probiotic drinks was seen on the concentration of short chain fatty acids in faecal samples. Butyric acid is an important energy source for colonocytes and one of the effects of probiotics could be an increase in butyric acid concentration⁴⁰. However, no increase in the butyric acid concentration was found in the probiotic or placebo group. This result is in accordance with a previous animal study where no changes in SCFA concentration were seen after inoculation of *L. plantarum* 299v to germ-free mice⁴¹. A human study, in which healthy volunteers consumed *L. plantarum* 299v in a rose-hip drink with fermented oats, also showed no change in butyric acid concentration but a significant increase in the total SCFA, acetic and propionic acids concentration was found¹⁹. The concentrations described in that study were low compared to the concentrations in the present study, which might be due to different methods. A significant decrease of acetic acid and propionic acid was noticed after consumption of a fermented drink with *L. casei* Shirota²⁰. Results can be different due to the consumption of different bacterial strains. Furthermore, in that study the concentration of SCFA was determined in faecal water using a high-performance liquid chromatography, which makes a comparison between the results of the studies difficult.

We cannot explain the difference in isocaproic acid concentration before the start of the consumption of the drinks and the significant decrease of isocaproic acid concentration in the placebo group after ending the administration of the drinks.

Two patients from the placebo-group were excluded from analyses of faecal bacterial counts, SCFA, enzyme activities and endotoxin concentration

because of non-compliance. However, statistical evaluation with an intention-to-treat analysis did not change the results. In this placebo-controlled study, healthy volunteers consumed a fermented oatmeal drink with *L. plantarum* 299v resulting in increased lactobacilli counts but no effects on other bacterial counts, SCFA and metabolic activities were observed. Major changes in metabolic activities or faecal bacterial counts other than the lactobacilli increase in the probiotic group could not be observed in individuals.

These participants were assumed to have 'normal' values for enzyme activities, endotoxins, SCFA concentration and the numbers of faecal bacteria. However, in persons with intestinal inflammatory conditions like Crohn's disease (CD), ulcerative colitis (UC), pouchitis and irritable bowel syndrome (IBS), the composition of the faecal bacterial composition might be disturbed. In pouchitis and UC patients, also a deficient metabolism of butyrate has been found^{42,43}. For these reasons, it would be interesting to investigate the effect of *L. plantarum* 299v on the intestinal bacterial flora and metabolic activities in these conditions. Furthermore, some studies indicate that also clinical benefits can be expected from *L. plantarum* 299v. In UC patients and CD patients systemically circulating levels of endotoxins have found to be increased and to be correlated positively with the anatomic region and disease activity⁴⁴. *L. plantarum* 299v has a strong adhesiveness to the mucosa by mannose-containing glycoproteins, which could result in preventing *E. coli* from adhering to the mucosa and from delivering endotoxins into the body^{4,45}. In animal studies, *L. plantarum* 299v was found to reduce colitis in methotrexate-induced inflammation in rats and interleukin-10-deficient mice also suggesting a potential therapeutic role in IBD^{46,47}. Moreover, in human studies *L. plantarum* 299v was proven to decrease symptoms like pain and flatulence in IBS patients^{48,49}. However, in general the number of studies including patients is still limited and the studies often suffer from some methodological problems. Studies are needed to explore whether the effects of probiotic treatment in inflammatory disorders of the bowel, causes changes of the intestinal environment that are more evident than those seen in healthy volunteers, before studies on the clinical efficacy of probiotics are performed.

In conclusion, this study shows that the fermented oatmeal drink with *L. plantarum* 299v increases the number of lactobacilli in the faeces of healthy volunteers but has no influence on other bacterial counts or on metabolic activities. Nevertheless, studies to evaluate the effect of this probiotic drink on the faecal flora of patients, such as those with inflammatory bowel disease, are warranted. The observed effect of *L. plantarum* 299v on the intestinal flora appears already within 1 week after the start of the consumption of the probiotic drink and disappears completely 1 week after cessation of consumption of the drink.

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Chapter 3

Survival of the probiotic,
L. plantarum 299v and its effects on the
faecal bacterial flora, with and without
gastric acid inhibition

D. Goossens, D. Jonkers, M. Russel, A. Thijs, A. van den Bogaard,
E. Stobberingh, R. Stockbrügger

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Abstract

Introduction

Probiotic bacteria have to survive passage through the gastrointestinal tract. In this placebo-controlled double-blind study, the effect of *Lactobacillus plantarum* 299v on the faecal flora was studied with and without gastric acid inhibition.

Methods

Thirty-two healthy volunteers were given pantoprazole (40 mg/day) or placebo during three weeks from week 2 until week 4. In addition, from week 3 until week 4, *L. plantarum* 299v in an oatmeal fermented drink (10^8 CFU/ml) was given twice daily to both groups. From each healthy volunteer, faecal samples were collected at the end of week 1, 2, 4 and 8 (4 weeks after cessation of *L. plantarum* 299v and pantoprazole/placebo). Several aerobically and anaerobically growing bacteria were counted and short chain fatty acid concentrations were determined.

Results

In both the pantoprazole and the placebo group, median lactobacilli counts increased significantly in week 4 compared to week 1 (from log 4.5 to 8.0 CFU/g faeces in pantoprazole and from log 4.2 to 7.7 CFU/g faeces in placebo group) and decreased significantly in week 8 (to log 4.5 CFU/g faeces in pantoprazole and log 4.3 CFU/g faeces in placebo group). These lactobacilli were identified as *L. plantarum* 299v. No significant differences were observed in all other bacterial counts and short chain fatty acid concentrations.

Conclusions

The comparable increases of faecal lactobacilli counts in both the pantoprazole and the placebo treated group demonstrate that *L. plantarum* 299v survives passage through the gastrointestinal tract irrespective of gastric acidity. The increment of the intragastric pH in combination with *L. plantarum* 299v did not modulate bacterial composition and/or the production of short chain fatty acids.

Introduction

Probiotics as defined by Havenaar *et al.* are "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora"¹. Probiotics such as lactobacilli have been reported to effect positively gastrointestinal disorders such as diarrhoea (rotavirus-associated and antibiotic-associated), inflammatory bowel disease, pouchitis, irritable bowel syndrome, colorectal cancer, lactose absorption, *Helicobacter pylori* infection, and constipation and are increasingly used as a treatment modality²⁻⁹.

For therapeutic purposes, probiotics are restricted by certain selection criteria: probiotics have to be of human origin, have to be safe for the host, and genetically stable⁸. Above all, it is important that probiotics are able to survive passage through the gastrointestinal tract irrespective of gastric acidity, pancreatic enzymes and bile acids so that they may reach the ileum and colon and can colonise the intestinal mucosa¹⁰. Furthermore, as high as possible numbers of probiotic bacteria reaching the large intestine seem to be warranted, because of the complexity of the intestinal flora and the high bacterial load.

The healthy stomach contains relatively small numbers of bacteria (0-10³ colony forming units per ml gastric content) due to the intragastric acidity^{10,11}. In healthy volunteers, the median 24-hours intragastric pH is below 3 in about 80% of time¹². Most of the ingested bacteria cannot be metabolically active at pH values below 4 and 99.9% of the bacteria are killed at pH less than 4.0 within 30 minutes^{13,14}. The survival of lactobacilli in acid environments has been tested by *in vitro* studies: after four hours, *Lactobacillus* GG showed no loss of viability in gastric juice within a pH range 3.0-7.0, but there was a rapid loss in viability at pH 1.0¹⁵. *Lactobacillus plantarum* 299v was able to survive at least four hours incubation at pH 2.5 *in vitro*, but no growth or replication could occur¹⁴.

In healthy volunteers, the median 24-hours intragastric pH has been found to be 1.7¹². This may affect survival and/or metabolic activity of probiotic bacteria. An increase in the intragastric pH, for instance by use of gastric acid inhibitors, may theoretically improve the survival of lactobacilli and may increase the metabolic activities of probiotics such as the production of short chain fatty acids.

In this double-blind placebo-controlled study, the effect of *L. plantarum* 299v on the faecal flora was studied with and without gastric acid inhibition. Special attention was paid to the recovery of *L. plantarum* 299v in the faeces.

Methods

Subjects

Thirty-two healthy volunteers were enrolled in this study. Exclusion criteria were pre-existing bowel-pathology including irritable bowel syndrome, inflammatory bowel disease, diverticulitis, diarrhoea and cancer. Volunteers were not allowed to have used gastric acid inhibitors, laxantia, anti-diarrhoea medication or antibiotics for at least one month before as well as during the study. Furthermore, the consumption of other probiotics or prebiotics for at least two weeks before the start of the study as well as during the study was not allowed. Participants were asked to continue their 'normal' diet. Participants were recruited by local advertisement. The study was approved by the Medical Ethical Committee of the University Hospital Maastricht and was performed in accordance with good clinical practice and the declaration of Helsinki. All participants have given written informed consent.

Study design

The study was double-blind and placebo-controlled. Total duration of the study was eight weeks: a pre-treatment period (week 1), one week treatment with pantoprazole or placebo (week 2), two weeks treatment with pantoprazole or placebo in combination with the fermented oatmeal drink with *L. plantarum* 299v (week 3 and 4) and a four weeks post-treatment period (week 5 until 8) (figure 3.1). Volunteers were first treated with pantoprazole (40 mg/day) (n=16) or placebo (n=16) to stabilise the intragastric pH profile. Pantoprazole or placebo (indistinguishable in colour, smell and taste) were taken half an hour before breakfast.

Figure 3.1 Time schedule of the study.

During weeks 3 and 4, participants daily consumed 200 ml (100 ml in the morning and 100 ml in the evening) of a fermented oatmeal drink with *L. plantarum* 299v (10^9 CFU/ml). In the morning, the probiotic drink was consumed two hours after pantoprazole or placebo intake to minimize direct antibacterial effect of pantoprazole on the probiotic strain. Four faecal samples

were collected: at the end of week 1 (before administration), at the end of week 2 (after one week pantoprazole/placebo administration), at the end of week 4 (after three weeks pantoprazole/placebo administration and probiotic consumption) and after week 8 (four weeks after cessation pantoprazole/placebo administration and probiotic consumption) (figure 3.1). Faecal samples were collected every Thursday ($\pm$ one day) to avoid major influences of possible changed dietary habits during the weekend. At each faeces collection, a short questionnaire had to be completed on bowel habits (stool frequency and consistency according to the Bristol scale¹⁶), side effects and compliance with regard to the consumption of pantoprazole/placebo and the probiotic drinks. Participants were asked to return their study medication at the end of the study period. Participants were excluded from analyses if less than 80% of the faecal samples were collected and/or the pantoprazole/ placebo and/or probiotic drinks were consumed less than six days per week based on questionnaires and counting of the study medication.

Microbiological analyses

Faecal samples ($\pm$ 20 gram) were collected in sterile containers, kept at room temperature and brought to the laboratory within twelve hours after defaecation and were divided into three parts. Five grams were diluted (1:4) in pepton water with cysteine (2.1 mM) and glycerol (37%) and homogenised with an ultra-turrax (IKA®, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) during two minutes. Cultures were performed directly and the remaining part of the material was frozen (-18°C) for further analyses. Ten grams were centrifuged at 4°C for two hours at 25.000 *g* to obtain faecal water that was frozen at -80°C for determination of the pH. The remaining material was immediately frozen for additional analyses.

Culture

Ten-fold serial dilutions of the faecal samples were inoculated with a spiral plater (Eddy Jet v1.2, IUL-instruments, Barcelona, Spain) on selective agar plates for total aerobic bacteria, enterobacteriaceae, enterococci, total (facultative) anaerobic bacteria, *Bacteroides* spp., *Clostridia* spp., lactic acid bacteria and lactobacilli according to the method previously described¹⁷.

pH

The pH was measured in faecal water using a PHM201 portable pH meter (Radiometer Nederland BV).

Short chain fatty acids

Short chain fatty acid concentrations (acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic acid) were measured in the faeces with gas-liquid chromatography (GLC) as described elsewhere^{17,18}. SCFA were analysed in the faecal samples collected in week 1 and week 4 (before and

after probiotic consumption). Concentrations were expressed in mmol/g faeces after correction for dilution.

Statistical analyses

Statistical evaluation of changes within groups during the study period and between groups was carried out using the linear mixed model analysis with SPSS version 11.0. A p-value below 0.05 was considered to be statistically significant.

Results

Twenty-nine participants completed the study (9 males, mean age 34 years ± 13 and 20 females, mean age 26 years ± 9). Three participants were excluded: one person did not start to take the pantoprazole/ placebo for personal reasons, another person used antibiotics (flucloxacillin) during the second week of the study and the third person used antibiotics (metronidazole) in week five of the study. No side effects were reported after the treatment with pantoprazole and *L. plantarum* 299v and no significant differences in defaecation frequency and consistency of faeces were seen between or within the groups (table 3.1).

The compliance to pantoprazole was 99.0%, to the placebo 100% and to the probiotics drinks 100% and 99.5% in the pantoprazole and placebo group, respectively.

Table 3.1 Defaecation frequency (per day), consistency of faeces (median $\pm$ SEM) according to the Bristol scale in week 1 (before administration), week 2 (after one week pantoprazole/placebo administration), week 4 (after three weeks pantoprazole/placebo administration and two weeks probiotic consumption) and in the end of week 8 (four weeks after cessation pantoprazole/placebo administration and probiotic consumption) in the pantoprazole and placebo group.

	Week 1	Week 2	Week 4	Week 8
Consistency ^a				
Pantoprazole	4.0 $\pm$ 0.3	4.0 $\pm$ 0.3	3.5 $\pm$ 0.3	3.0 $\pm$ 0.3
Placebo	3.0 $\pm$ 0.3	3.0 $\pm$ 0.2	4.0 $\pm$ 0.3	3.0 $\pm$ 0.2
Frequency				
Pantoprazole	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2
Placebo	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2

^a Consistency scale from 1 (hard lumps) until 7 (watery).

Culture

The intake of pantoprazole did not influence the number of total aerobic bacteria, *Enterococcus* spp., enterobacteriaceae, total anaerobic bacteria, *Bacteroides* spp., *Clostridia* spp. and lactic acid bacteria or lactobacilli compared to the placebo group (table 3.2).

Table 3.2 Numbers of bacterial strains (log CFU/g faeces), median $\pm$ SEM in week 1 (before administration), week 2 (after one week pantoprazole/placebo administration), week 4 (after three weeks pantoprazole/placebo administration and two weeks probiotic consumption) and in the end of week 8 (four weeks after cessation pantoprazole/placebo administration and probiotic consumption) in the pantoprazole and placebo group.

	Week 1	Week 2	Week 4	Week 8
Total aerobic flora				
Pantoprazole	7.13 $\pm$ 0.27	7.75 $\pm$ 0.20	7.95 $\pm$ 0.19 ^a	7.34 $\pm$ 0.23
Placebo	7.36 $\pm$ 0.20	7.26 $\pm$ 0.15	7.82 $\pm$ 0.13 ^a	7.49 $\pm$ 0.20
<i>E. coli</i>				
Pantoprazole	6.60 $\pm$ 0.28	6.94 $\pm$ 0.32	6.49 $\pm$ 0.49	5.94 $\pm$ 0.76
Placebo	7.05 $\pm$ 0.27	6.78 $\pm$ 0.23	6.59 $\pm$ 0.20	6.68 $\pm$ 0.33
Enterococci				
Pantoprazole	4.91 $\pm$ 0.43	4.66 $\pm$ 0.43	4.89 $\pm$ 0.49	5.01 $\pm$ 0.48
Placebo	4.12 $\pm$ 0.55	4.59 $\pm$ 0.31	3.45 $\pm$ 0.54	3.67 $\pm$ 0.61
Total anaerobic flora				
Pantoprazole	9.63 $\pm$ 0.14	10.02 $\pm$ 0.08	9.90 $\pm$ 0.08	9.92 $\pm$ 0.12
Placebo	9.63 $\pm$ 0.18	9.85 $\pm$ 0.11	9.93 $\pm$ 0.09	9.99 $\pm$ 0.07
<i>Bacteroides</i> spp.				
Pantoprazole	7.59 $\pm$ 0.55	8.15 $\pm$ 0.18	8.00 $\pm$ 0.17	8.35 $\pm$ 0.11
Placebo	7.57 $\pm$ 0.28	7.21 $\pm$ 0.57	8.11 $\pm$ 0.12	8.16 $\pm$ 0.14
<i>Clostridia</i> spp.				
Pantoprazole	5.04 $\pm$ 0.18	4.83 $\pm$ 0.47	4.85 $\pm$ 0.28	4.67 $\pm$ 0.23
Placebo	4.96 $\pm$ 0.25	4.41 $\pm$ 0.19	3.54 $\pm$ 0.58	4.45 $\pm$ 0.20
Lactic acid bacteria				
Pantoprazole	6.46 $\pm$ 0.49	5.58 $\pm$ 0.26 ^b	8.02 $\pm$ 0.16 ^a	4.57 $\pm$ 0.25 ^b
Placebo	6.30 $\pm$ 0.53	4.34 $\pm$ 0.52 ^b	7.63 $\pm$ 0.19 ^a	4.03 $\pm$ 0.52 ^b
Lactobacilli				
Pantoprazole	4.50 $\pm$ 0.21	5.50 $\pm$ 0.36	8.02 $\pm$ 0.17 ^a	4.50 $\pm$ 0.28 ^c
Placebo	4.23 $\pm$ 0.48	4.17 $\pm$ 0.53	7.74 $\pm$ 0.18 ^a	4.30 $\pm$ 0.53 ^c

^a statistically significant difference ($p < 0.05$) compared to before *L. plantarum* 299v intake (week 1, 2); ^b statistically significant difference ($p < 0.05$) compared to before treatment (week 1); ^c statistically significant difference ($p < 0.05$) compared to during *L. plantarum* 299v intake (week 4)

In week 4 (after two weeks consumption of the probiotic drink in addition to the pantoprazole/placebo intake) the number of lactic acid bacteria and lactobacilli increased significantly compared to the figures before probiotic intake (week 1) in both the pantoprazole and placebo group. No significant difference could be seen between the pantoprazole and placebo group in week 4. In week 8, the number of lactic acid bacteria and lactobacilli decreased significantly and the number of lactobacilli reached pre-treatment levels (figure 3.2). A significant decrease in the number of lactic acid bacteria was observed in week 2 and 8 compared to week 1 in both the pantoprazole and placebo group. In week 4 a significant increase in the number of total aerobic bacteria was found (table 3.2).

Figure 3.2 Numbers of lactobacilli (log CFU/g faeces) (median $\pm$ SEM) at week 1 (before administration), 2 (after one week pantoprazole/placebo administration), 4 (after three weeks pantoprazole/placebo administration and probiotic consumption), and 8 (four weeks after cessation pantoprazole/placebo administration and probiotic consumption). * $p < 0.05$ in the pantoprazole and placebo group during probiotic consumption compared to before and after the consumption.

Recovery of *L. plantarum* 299v

Before probiotic intake, *L. plantarum* 299v was identified in one faecal sample in week 1 and in two faecal samples in week 2 of three different individuals. In contrast, *L. plantarum* 299v was identified in all faecal samples during probiotic consumption (week 4). Four weeks after ending probiotic consumption (week 8), no *L. plantarum* 299v could be recovered except for one person in whom of the three isolated lactobacilli, one was identified as *L. plantarum* 299v.

pH of faecal water

The pH of faecal water did not change significantly between the pantoprazole and placebo group in week 1 (6.3 and 6.7), in week 2 (6.4 and 6.7), in week 4 (6.3 and 6.5) and in week 8 (6.5 and 6.6) or within both groups.

Short chain fatty acids

No significant differences in acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids could be demonstrated in the faecal samples collected in week 1 and week 4 between and within pantoprazole and placebo group (table 3.3).

Table 3.3 Short chain fatty acids concentration (mean $\pm$ SEM), expressed in mmol/g faeces, measured in week 1 (before administration) and week 4 (after three weeks pantoprazole/placebo administration and probiotic consumption).

	Week 1	Week 4
Acetic acid		
Pantoprazole	67.2 $\pm$ 11.5	74.5 $\pm$ 10.0
Placebo	90.4 $\pm$ 19.0	101.7 $\pm$ 22.1
Propionic acid		
Pantoprazole	19.5 $\pm$ 3.7	19.8 $\pm$ 4.1
Placebo	23.2 $\pm$ 3.0	24.2 $\pm$ 4.8
Isobutyric acid		
Pantoprazole	2.5 $\pm$ 0.2	3.0 $\pm$ 0.8
Placebo	3.4 $\pm$ 0.7	3.0 $\pm$ 1.3
Butyric acid		
Pantoprazole	18.6 $\pm$ 5.1	18.4 $\pm$ 3.3
Placebo	21.0 $\pm$ 3.8	23.1 $\pm$ 7.1
Isovaleric acid		
Pantoprazole	3.2 $\pm$ 0.4	3.0 $\pm$ 0.9
Placebo	3.7 $\pm$ 0.6	3.4 $\pm$ 1.7
Valeric acid		
Pantoprazole	3.8 $\pm$ 0.9	3.6 $\pm$ 0.8
Placebo	4.0 $\pm$ 0.7	3.4 $\pm$ 1.0
Isocaproic acid		
Pantoprazole	1.3 $\pm$ 0.9	0.7 $\pm$ 0.6
Placebo	0.8 $\pm$ 0.3	0.6 $\pm$ 0.4
Caproic acid		
Pantoprazole	3.7 $\pm$ 1.5	2.3 $\pm$ 0.9
Placebo	2.9 $\pm$ 0.7	2.0 $\pm$ 0.9

Discussion

Probiotics may be of interest as adjuvant therapy in diseases like irritable bowel syndrome, diarrhoea, inflammatory bowel diseases and lactose intolerance. The aim of the present study was to investigate the effect of gastric acid inhibition on the survival of *L. plantarum* 299v in faecal samples. Moreover, the influence of gastric acid inhibition in combination with the consumption of

L. plantarum 299v on the composition of the faecal flora and metabolic activity (i.e. SCFA) was studied.

Twice daily intake of the probiotic *L. plantarum* 299v for two weeks significantly increased the number of faecal lactobacilli from log 4.4 to log 7.9 CFU/g faeces. The comparable increase of lactobacilli in the pantoprazole and placebo treated group indicates that *L. plantarum* 299v survives passage through the gastrointestinal tract very well, irrespective of gastric acidity. The increment of lactobacilli and lactic acid bacteria disappeared within four weeks after cessation of the consumption of *L. plantarum* 299v demonstrating that prolonged colonisation does not occur. The recovery of log 8 lactobacilli per gram faeces after probiotic consumption is, considering a consumption of 10^{11} lactobacilli per day and a faecal volume of 100 g per day, relatively high. Other studies with healthy volunteers also found high concentrations of lactobacilli after *L. plantarum* 299v consumption: log 8.2 CFU/g faeces and log 9.0 CFU/g faeces, respectively^{17,19}. The recovery data found in this study were similar to the results found in a placebo-controlled study performed with 21 healthy volunteers consuming *L. plantarum* 299v for four weeks¹⁷. *L. plantarum* 299v could be isolated using molecular techniques in the faecal samples collected during consumption of the probiotic drinks but could not be identified in most persons after cessation of the probiotic drinks. The high recovery of *L. plantarum* 299v during probiotic consumption in both the pantoprazole and placebo group indicates that *L. plantarum* 299v survives passage through the gastrointestinal tract.

The aerobic and anaerobic bacterial counts before probiotic intake were comparable to former studies performed with healthy volunteers^{17,19}. The significant decrease in number of lactic acid bacteria in week 2 and 8 compared to the numbers before treatment could not be explained.

The consumption of the fermented oatmeal drink with *L. plantarum* 299v had no influence on the other aerobic and anaerobic bacteria measured, with the exception of the total aerobic bacteria which numbers increased in both pantoprazole and placebo group during probiotic consumption. Such an increase after probiotic consumption was not demonstrated in the former study with healthy volunteers¹⁷ nor in a study performed by Johansson *et al.*, where 26 healthy volunteers consumed *L. plantarum* 299v (5×10^7 CFU/ml) during 21 days¹⁹. Previous observations from our group found an increase of aerobic Gram-positive cocci after long-term (at least 12 months) treatment with proton pump inhibitors. In line with these findings, pantoprazole treatment may result in a significant increase found in the pantoprazole group but the increase in the placebo group cannot be explained²⁰. In addition, during this short-term treatment, pantoprazole did not change the composition of the faecal flora.

The consumption of *L. plantarum* could increase the concentration of short chain fatty acids like acetic and propionic acid¹⁹. However, during passage through the gastrointestinal tract the gastric acidity may lower the metabolic activity (i.e. production of SCFA) of *L. plantarum* 299v. SCFA concentrations

were measured in faecal samples but no increase in SCFA production could be demonstrated in the group with gastric acid inhibition compared to the placebo group during probiotic consumption. An increase in SCFA concentrations could probably not be seen because SCFA are excreted in the faeces for only 5%, suggesting that an increase in the SCFA production may result in an increase in SCFA absorbed in the colon without an increase in faecal concentrations of SCFA²¹. Moreover, SCFA production could lower the colonic pH. However, no decrease of pH in faecal water was demonstrated in this study. SCFA concentrations were comparable to concentrations found in a previous study¹⁷. Measuring bacterial enzyme activities or for instance the production of bacteriocins could give further insight in the influence of gastric acidity on the metabolic activity of *L. plantarum* 299v.

Although the short-term use of pantoprazole did not seem to improve the survival or to increase the metabolic activity (i.e. production of SCFA) of *L. plantarum* 299v, gastric acid inhibition may be of benefit in influencing survival and metabolic activities of other bacteria used as probiotic. In a study performed by Jacobsen *et al.* 15 of the 44 lactobacilli strains tested did not survive a pH of 2.5 during four hours *in vitro*, whereas *L. plantarum* 299v showed a good survival under these circumstances²². These lactobacilli, that cannot survive a pH below 2.5, may benefit from adding gastric acid inhibitors to the probiotics to increase survival through the gastrointestinal tract. Moreover, secretions of mucus, lysozyme, bile salts and pancreatic juice in the duodenum can also influence the viability of lactobacilli^{23,24}. *L. plantarum* 299v has a relatively high resistance to bile salts¹⁴. However, Drouault *et al.* have shown that testing duodenal survival is important for selection of (future) probiotic strains²⁵.

In this study, the intragastric pH has not been measured. However, it has been demonstrated in several studies with healthy volunteers that an once-daily dose of 40 mg pantoprazole increased the intragastric pH to a median 24-hours pH above 3.0²⁶⁻²⁸. Pantoprazole was taken half an hour before breakfast because this dosing regimen results in high steady state gastric acid inhibition over the total 24-hours period²⁹. The probiotic drinks were consumed at least two hours after pantoprazole or placebo administration to avoid a direct contact between *L. plantarum* 299v and pantoprazole/placebo as proton pump inhibitors could have an antibacterial effect. Omeprazole showed a decrease in the number of active growing Gram-positive bacteria *in vitro*³⁰, pantoprazole also has an antibacterial effect *in vitro*³¹. Recently, probiotics are sometimes added to *Helicobacter pylori* eradication therapy (i.e. proton pump inhibitors) to decrease side effects. The direct effect of proton pump inhibitors on lactobacilli is not known. As faecal lactobacilli were not significantly decreased after pantoprazole compared to placebo intake, intake of pantoprazole did not seem to have an antibacterial effect on lactobacilli in the fermented oatmeal drink if an interval of at least two hours was taken into account. This lack of interaction

is important in an era where both proton pump inhibitors and probiotics are frequently used.

In conclusion, this double-blind placebo-controlled study supports the capacity of *L. plantarum* 299v to survive passage through the gastrointestinal tract in healthy volunteers irrespective of gastric acidity. Because of the documented survival of *L. plantarum* 299v *in vivo*, *L. plantarum* 299v is a good candidate to study the effect on the faecal flora and metabolic activities in clinical trials.

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Chapter 4

Can *L. plantarum* 299v change the faecal flora after bowel cleansing for colonoscopy?

D. Goossens, D. Jonkers, M. Russel, E. Vaughan, E. Stobberingh,
R. Stockbrügger

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Abstract

Introduction

The intestinal flora may be washed out after bowel cleansing and may be beneficially influenced by probiotics during recolonisation. In this randomised placebo-controlled double-blind study, the effect of bowel cleansing with or without subsequent inoculation by *L. plantarum* 299v was assessed.

Methods

Patients, who underwent bowel cleansing, were included. After colonoscopy, patients consumed a drink with or without *L. plantarum* 299v (10^{11} CFU/day). Faecal samples were collected: before bowel cleansing (week 0), the first sample after colonoscopy (week 1), after two and four weeks of consumption (week 2 and week 4), and four weeks after cessation of the drinks (week 8). Total colony forming units (CFU) per gram faeces of bacteria were counted. Denaturing gradient gel electrophoresis (DGGE) was used to analyse the dominant flora, expressed as 'similarity values'.

Results

Twenty-two patients completed the study (12 probiotic, 10 placebo). The mean concentration of lactobacilli increased ($p < 0.05$) from 4.7 ± 1.9 at week 0 to 6.5 ± 0.8 at week 2 in the probiotic group and returned back to 4.6 ± 1.4 at week 8 ($p < 0.05$). DGGE profiles demonstrated high similarity values within subjects over time: mean value $86.5\% \pm 2.6$. No significant differences in similarity values were seen after bowel cleansing (week 2) or between probiotic and placebo group.

Conclusions

Although faecal lactobacilli counts increased during probiotic consumption, no further changes in culture and DGGE results were observed. Therefore, the faecal flora can be considered as relatively stable over time: bowel cleansing or probiotic consumption had no major influence on the composition of the faecal flora.

Introduction

The human gastrointestinal tract comprises a complex ecosystem with a myriad of bacteria that probably have substantial impact on the physiology and health of the host. The highest numbers of bacteria are found in the colon containing about 10^{11} , mainly anaerobic (99.9%), bacteria per gram intestinal content. The predominant isolates found in the colon are *Bacteroides* spp., bifidobacteria, *Clostridium* spp. and eubacteria¹⁻⁴. The intestinal flora contains enzymes, and produces vitamins and short chain fatty acids from non-absorbed carbohydrates and plays a role in the defence against pathogens by influencing the barrier function of the gut, the immune system and the colonisation resistance of the intestinal tract^{2,5-7}. The intestinal flora is suspected to play a role in the pathogenesis of gastrointestinal diseases such as ulcerative colitis, Crohn's disease and irritable bowel syndrome⁸⁻¹⁰.

Each individual is thought to develop a unique, stable and complex intestinal flora (i.e. 'bacterial fingerprint')¹¹ after birth by ingestion of environmental microbes colonising the previously sterile colon¹². After two years of age, the intestinal flora has stabilised and adapted to an 'adult-like' composition influenced by genotype and environmental factors¹². High similarity values between the bacterial fingerprints of monozygotic twins have been found compared to the similarity values found in marital partners or genetically unrelated individuals. This demonstrates the important role of genetic factors influencing the composition of the intestinal flora¹³. Furthermore, environmental factors like diet and antibiotic use have been shown to influence the intestinal flora¹³⁻¹⁶. Adequate knowledge of factors influencing the composition of the intestinal flora may give further insight into the development and pathogenesis of gastrointestinal diseases and may provide opportunities to modulate the flora with the aim of therapeutic intervention.

Bowel cleansing, required as preparation for colonoscopic examinations, could be a factor influencing the future composition of the intestinal flora by elimination of the mass of luminal bacteria from the gastrointestinal tract. Bowel cleansing has been found to decrease disease activity and to induce clinical improvement in patients with Crohn's disease¹⁷⁻¹⁹. Van den Bogaard *et al.* demonstrated a reduction in aerobic as well as in anaerobic faecal bacteria after bowel cleansing and found an overgrowth of an *Escheria coli* strain after contamination of this bacterial strain indicating that the intestinal flora has a decreased resistance against the colonisation of bacteria and has to recolonise after bowel cleansing²⁰.

Probiotics, defined as "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous flora" may have a beneficial effect in this recolonisation phase²¹. Nowadays, *Lactobacillus* spp. and *Bifidobacterium* spp. are frequently used as probiotics³. One of the selection criteria for probiotics is the ability to colonise and adhere to the mucosa³. As *Lactobacillus plantarum*

299v can adhere to human mucosa cells *in vitro*, competing with the colonisation of *E. coli*, and is able to survive passage through the gastrointestinal tract, this bacterium may be a suitable candidate to influence the recolonisation of the intestinal flora after bowel cleansing²²⁻²⁴.

Changes in the composition of the intestinal flora can be studied by culture techniques. Due to insufficiently selective media, it is estimated that over 50% of the bacteria in the gastrointestinal tract have not yet been cultured and/or are not yet identified^{11,25,26}. For these reasons the intestinal flora should preferably be studied with molecular techniques²⁷. Polymerase chain reaction (PCR) in combination with denaturing gradient gel electrophoresis (DGGE) is a useful technique to study changes in the dominant individual faecal flora over time by producing bacterial fingerprints^{28,29}.

In this study the effect of bowel cleansing on the intestinal bacterial composition with and without subsequent treatment by the probiotic *L. plantarum* 299v was investigated using both culture techniques and PCR/DGGE analyses.

Materials and methods

Subjects

Twenty-three consecutive patients undergoing a bowel cleansing procedure prior to colonoscopy were enrolled in the study. Patients were not allowed to consume probiotics, prebiotics or antibiotics during the two weeks before as well as during the study. The use of medication was registered and, if clinically possible, dose and type of the medication were kept stable during the study period. Participants were asked to continue their normal dietary habits. The study was approved by the Medical Ethical Committee of the University Hospital Maastricht and was performed in accordance with the declaration of Helsinki. All participants have given written informed consent.

Study design

The study was double-blind and placebo-controlled. The patients were randomised to consume probiotic or placebo drinks. Total duration of the study was nine weeks: one week before bowel cleansing and colonoscopy (week 0), four weeks probiotic or placebo consumption after colonoscopy (week 1 until 4) and four weeks after cessation of the probiotic or placebo consumption (week 5 until 8).

Patients started with the consumption of the probiotic and placebo drinks in the evening after the colonoscopy. During week 1 until 4, patients consumed daily 200 ml (100 ml in the morning and 100 ml in the evening) of a fermented oatmeal drink with *L. plantarum* 299v (10^9 CFU/ml) (probiotic) or without

L. plantarum 299v (placebo). Probiotic and placebo drinks were indistinguishable in smell, taste and colour.

Twenty-four hours prior to the colonoscopy, the patients took the macrogol/electrolytes laxative Kleanprep® to clean the gastrointestinal lumen. After colonoscopy, the endoscopist completed a questionnaire about the effectiveness of the bowel cleansing in colon ascendens, transversum, descendens and rectum ('very good': no faeces visible, not even in the diverticula; 'good': some faeces visible without hampering the colonoscopy; 'poor': faeces visible, hampering the colonoscopy).

Five faecal samples were collected: one before bowel cleansing (week 0), the first faecal sample which could be produced after the colonoscopy (week 1), after two and four weeks of probiotic or placebo consumption (week 2 and 4) and four weeks after cessation of the drinks (week 8). Together with the faecal sampling, the patients completed a questionnaire on medication, bowel habits (defaecation frequency and faeces consistency scores from hard lumps (1) until watery diarrhoea (7) according to the Bristol scale³⁰), compliance, side-effects and changes in dietary habits. Participants were excluded from analyses if less than 85% of the faecal samples were collected and/or less than 85% of the probiotic/placebo drinks were consumed.

Microbiological analyses

Faecal samples were collected and processed as described previously³¹. Ten-fold serial dilutions of the faecal samples were made in physiological saline and 40 µl were inoculated on agar plates to culture and count total (facultative) aerobic bacteria, enterobacteriaceae, enterococci, total (facultative) anaerobic bacteria, *Bacteroides* spp., clostridia, lactic acid bacteria and lactobacilli³¹.

DNA extraction

DNA from faecal samples was isolated with the fast DNA SPIN® Kit for soil (Q-biogene, Heidelberg, Germany) with disruption of bacterial cells by highly energetic mechanical means using the FastPrep® Instrument (Q-biogene, Heidelberg, Germany). Genomic DNA was purified with a proprietary silica matrix, eliminating contaminants that inhibit reactions.

PCR

PCR was performed with a Taq polymerase kit. The primers, F-0968-GC and R-1401, used in this study amplify the V6 to V8 region of 16S rRNA. The PCR mixture contained 5 µl of 10x PCR buffer containing 15 mM MgCl₂, 3 µl of 50 mM MgCl₂, 1 µl of deoxynucleoside triphosphate preparation (10 mM), 1 µl of each primer (10 µM), 0.25 µl of Taq DNA polymerase (5 U/µl), 37.75 µl sterile Milli-Q water and 1 µl of a ten-fold diluted DNA-solution.

The following PCR program was used: pre-denaturation for 5 minutes at 94°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 20 seconds and elongation at 68°C for 45 seconds. The program ended with a

post-elongation step at 68°C for 7 minutes followed by cooling down to 4°C. The amplification of the amplicons was confirmed on ethidium-stained 1% agarose gels for 20 minutes at 100V.

DGGE analysis

PCR products were analysed on DGGE gels according to the method described by Zoetendal *et al.*¹¹ with the following modifications. The 8% polyacrylamide gels contained a urea / formamide gradient from 30% to 60%. Electrophoresis was performed in 0.5x Tris acetic acid EDTA (TAE) buffer (2.42 g tris, 570 µl acetic acid, 0.37 g EDTA) at 85 V during 16 hours using the DCode System apparatus (Bio-rad, Hercules, California). Amplicons of isolates of *L. plantarum* 299v were electrophoresed next to the PCR products of the faecal samples as well as a marker. After electrophoresis, the gels were stained as described by Sanguinetti *et al.* using the rapid silver staining procedure³² and scanned at 400 DPI. DGGE profiles were compared using the Bionumerics software version 3.0 (Applied-Maths, Sint-Martens-Latem, Belgium). The similarity between the five faecal samples of each individual was determined by calculating the similarity value using the Pearson's product-moment correlation coefficient³³.

Similarity values were called 'deviant' when the value was below the mean similarity value of the group minus one standard deviation. Similarity values at different time points were correlated with changes in type or dose of medication.

Statistical analyses

Statistical evaluation of changes in culture results between groups and within groups during the study period was carried out using the linear mixed model analysis with SPSS version 11.0. Non-parametric Wilcoxon signed-rank test and Mann-Whitney U-test with a Bonferroni-Holm correction were used for comparison of DGGE results within and between groups, respectively. A p-value below 0.05 was considered to be statistically significant.

Results

Twenty-two patients completed the study: twelve in the probiotic group (4 male, 8 female, mean age 46.5±8.7 years) and ten in the placebo group (3 male, 7 female, mean age 55.0±16.1 years). No significant differences in gender and age were seen between probiotic and placebo group. One patient (probiotic group) was excluded because he collected only three out of five faecal samples. Clinical diagnoses of patients at colonoscopy included: inflammatory bowel diseases (IBD) (n=5), irritable bowel syndrome (n=4), colorectal adenomas (n=5), diverticular disease (n=2), rectal ulcer (n=1) and ileocaecal resection (n=1). Four patients had normal findings at colonoscopy and had no

diagnoses of gastrointestinal diseases. Patients used different types of medication such as corticosteroids (n=3), 5-ASA medication (n=4), proton pump inhibitors (n=3), laxatives or anti-diarrhoea medication (n=7), and other non-gastrointestinal medication such as antihistamines, analgesis and cholesterol-lowering medication (n=17). Ten patients changed the type and two patients the dose of their gastrointestinal medication during the study period.

No significant differences were observed in defaecation frequency and faeces consistency scores between probiotic and placebo group or within both groups over time (table 4.1). Mean period of time between colonoscopy and collection of the first faecal sample produced after colonoscopy (i.e. sample of week 1) was 82 hours (range 20-146) in the probiotic group and 60 hours (range 23-120) in the placebo group and did not differ significantly ($p=0.46$).

In thirteen patients the effectiveness of the bowel cleansing was scored: two patients had a 'poor' bowel cleansing in the whole colon and rectum and five patients in some parts of the colon and rectum. The other six patients had a 'very good' or 'good' bowel cleansing procedure. Furthermore, no side effects were reported after the treatment with *L. plantarum* 299v or the placebo drink. The compliance was 96% for the probiotic drinks and 90% for the placebo drinks.

Table 4.1 Defaecation frequency (per day) and faeces consistency scores according to the Bristol scale (1:hard lumps-7:watery diarrhoea) in week 0 (before bowel cleansing), week 1 (first faecal sample after colonoscopy), week 2 (after two weeks probiotic/placebo consumption), week 4 (after four weeks probiotic/placebo consumption) and week 8 (four weeks after cessation of the probiotic/placebo consumption) in 22 patients (mean $\pm$ sd).

	Week 0	Week 1	Week 2	Week 4	Week 8
Consistency					
Probiotic	4.4 $\pm$ 1.4	4.8 $\pm$ 1.6	4.2 $\pm$ 1.3	4.2 $\pm$ 1.5	3.7 $\pm$ 1.6
Placebo	4.2 $\pm$ 1.8	4.1 $\pm$ 1.6	3.9 $\pm$ 1.3	4.1 $\pm$ 1.3	4.3 $\pm$ 1.1
Frequency					
Probiotic	1.9 $\pm$ 1.6	1.2 $\pm$ 1.6	1.7 $\pm$ 1.5	1.5 $\pm$ 0.8	1.4 $\pm$ 0.8
Placebo	0.9 $\pm$ 0.9	0.9 $\pm$ 1.1	1.3 $\pm$ 0.9	1.6 $\pm$ 0.7	1.6 $\pm$ 0.7

Culture

During the consumption of the drinks (weeks 2 and 4), a significant increase in the number of lactic acid bacteria ($p=0.008$) and lactobacilli ($p<0.005$) was observed in the probiotic group but not in the placebo group (table 4.2). The number of lactic acid bacteria ($p=0.006$) and lactobacilli ($p<0.005$) decreased significantly after cessation of the drinks (week 8) compared to the number during the consumption of the probiotic drinks (week 2 and 4). No significant differences could be observed in other aerobic and anaerobic bacterial counts before (week 0) and after bowel cleansing (week 1) and during probiotic intake (week 2 and 4) with the exception of the total number of aerobic bacteria, which

increased significantly ($p=0.001$) in the probiotic group in week 1 compared to week 0.

Table 4.2 Concentrations of bacterial strains (log CFU/g faeces) in week 0 (before bowel cleansing), week 1 (first faecal sample after colonoscopy), week 2 (after two weeks probiotic/placebo consumption), week 4 (after four weeks probiotic/placebo consumption) and week 8 (four weeks after cessation of the probiotic/placebo consumption) in 22 patients (mean $\pm$ sd).

	Week 0	Week 1	Week 2	Week 4	Week 8
Total aerobic flora					
Probiotic	6.2 $\pm$ 0.8	7.4 $\pm$ 0.7 ^a	6.4 $\pm$ 0.5	6.2 $\pm$ 1.2	6.1 $\pm$ 1.0
Placebo	7.3 $\pm$ 1.1	7.3 $\pm$ 0.9	7.1 $\pm$ 1.3	7.5 $\pm$ 1.0	6.7 $\pm$ 1.3
<i>E. coli</i>					
Probiotic	5.7 $\pm$ 1.2	6.7 $\pm$ 2.1	5.6 $\pm$ 1.7	6.0 $\pm$ 2.1	4.6 $\pm$ 2.4
Placebo	6.9 $\pm$ 1.2	6.7 $\pm$ 1.0	6.4 $\pm$ 2.3	6.6 $\pm$ 2.5	6.1 $\pm$ 2.4
Enterococci					
Probiotic	2.6 $\pm$ 1.6	3.2 $\pm$ 2.2	3.3 $\pm$ 1.6	2.7 $\pm$ 1.6	2.5 $\pm$ 1.6
Placebo	4.5 $\pm$ 1.7	4.8 $\pm$ 2.2	3.9 $\pm$ 1.9	4.1 $\pm$ 2.0	3.5 $\pm$ 1.7
Total anaerobic flora					
Probiotic	8.2 $\pm$ 1.6	8.3 $\pm$ 1.3	8.2 $\pm$ 1.0	8.5 $\pm$ 1.1	7.9 $\pm$ 1.1
Placebo	8.5 $\pm$ 1.2	8.1 $\pm$ 1.6	8.5 $\pm$ 1.3	8.8 $\pm$ 1.4	8.4 $\pm$ 1.1
<i>Bacteroides</i> spp.					
Probiotic	5.5 $\pm$ 2.2	6.4 $\pm$ 1.2	5.5 $\pm$ 1.6	4.9 $\pm$ 2.5	5.3 $\pm$ 1.8
Placebo	6.3 $\pm$ 1.4	6.7 $\pm$ 1.0	6.4 $\pm$ 2.1	6.3 $\pm$ 2.3	6.9 $\pm$ 0.8
Clostridia					
Probiotic	4.1 $\pm$ 1.4	3.7 $\pm$ 1.3	4.2 $\pm$ 0.9	4.1 $\pm$ 0.8	3.5 $\pm$ 1.3
Placebo	4.7 $\pm$ 0.4	3.9 $\pm$ 1.3	5.0 $\pm$ 0.6	4.3 $\pm$ 1.0	4.5 $\pm$ 0.8
Lactic acid bacteria					
Probiotic	5.2 $\pm$ 1.3	6.4 $\pm$ 0.9 ^a	6.4 $\pm$ 0.9 ^a	6.6 $\pm$ 0.9 ^a	5.0 $\pm$ 1.4
Placebo	4.9 $\pm$ 2.2	5.4 $\pm$ 1.3	5.7 $\pm$ 1.5	4.9 $\pm$ 2.3	4.2 $\pm$ 2.1
Lactobacilli					
Probiotic	4.7 $\pm$ 1.9	6.6 $\pm$ 1.3 ^a	6.5 $\pm$ 0.8 ^a	6.8 $\pm$ 1.0 ^a	4.6 $\pm$ 1.4
Placebo	5.0 $\pm$ 2.2	5.2 $\pm$ 1.5	4.9 $\pm$ 2.0	4.7 $\pm$ 2.0	4.4 $\pm$ 1.7

^a statistically significant difference ($p<0.05$) compared to week 0.

DGGE analyses

Faecal samples for DGGE analyses and calculations of similarity values were available for 20 patients (11 probiotic and 9 placebo group). Mean similarity values over-time were relatively high (probiotic group: 86.8 ± 3.1 and placebo group: 86.3 ± 2.2) (figure 4.1). No major differences could be observed between probiotic and placebo group or within the groups.

Figure 4.1 Mean DGGE similarity values (in percentages) in the probiotic (n=11) and placebo group (n=9). Comparisons were made between week 0 and 1 (0 vs. 1), week 1 and 2 (1 vs. 2), week 2 and 4 (2 vs. 4), week 4 and 8 (4 vs. 8) and week 0 and 8 (0 vs. 8).

Eighteen individual similarity values were lower than the mean of the total group minus the standard deviation and therefore defined as 'deviant'. These time points were checked for possible changes in type or dose of medication. No correlation could be seen between these time points and changes in medication except for one patient who stopped the use of loperamide in week 4 resulting in a 'deviant' similarity value comparing week 2 to week 4.

Figure 4.2 shows the DGGE pattern of two individual patients during the study period: one from the probiotic and one from the placebo group. Changes in individual banding patterns could be observed in both probiotic and placebo patients mainly after bowel cleansing (n=11), and returned within two weeks. For example, in the probiotic patient (figure 4.2b), some bands disappeared after bowel cleansing procedure (week 1 compared to week 0). However, in week 8 these bands were again present.

Figure 4.2 DGGE banding patterns of one patient of the placebo group (a) and one patient of the probiotic group (b). Lane 0=week 0, lane 1=week 1, lane 2=week 2, lane 4=week 4, lane 8=week 8. Arrows indicate changes in individual bands.

Discussion

In this study, pre-colonoscopy bowel cleansing procedure and subsequent consumption of *L. plantarum* 299v had no significant influence on the composition of the colonic flora based on both culture results and bacterial fingerprints of the dominant faecal flora, indicating that the composition of the faecal flora remains relatively stable over time despite these major interventions.

No changes in the faecal bacterial numbers due to bowel cleansing could be demonstrated by culture with the exception of the number of total aerobic bacteria. The effectiveness of the bowel cleansing (very good, good and poor) did not influence the culture results significantly (data not shown) and was successful in the majority of patients. The number of total aerobic bacteria increased significantly after bowel cleansing in all patients in the probiotic group. In contrast, van den Bogaard *et al.* demonstrated a reduction of log 2 to 3 in the faecal number of total aerobic bacteria and of log 4 to 5 in the number of total anaerobic bacteria after bowel cleansing²⁰. These inconsistent results might be explained by the use of different bowel cleansing procedures (saline versus Kleanprep). Moreover, van den Bogaard *et al.* collected clear rectal effluent immediately after bowel cleansing, while in the present study the first faecal sample after colonoscopy was collected with a mean period of time of 82 hours in the probiotic and 60 hours in the placebo group. This difference in faeces collection time comparing the probiotic to the placebo group could not be explained by differences in defaecation frequency or faeces consistency.

The increase in the number of total aerobic bacteria might also be caused by the consumption of the probiotic drinks. However, the increase in aerobic bacteria did not persist during the probiotic consumption and has been disappeared in week 4. In addition, previous studies performed with healthy volunteers consuming *L. plantarum* 299v for 4 and 2 weeks respectively, could not observe an increase in the number of total aerobic bacteria^{24,31}.

Furthermore, the consumption of the probiotic, *L. plantarum* 299v increased the amount of lactic acid bacteria and lactobacilli significantly with log 1.5 CFU/g faeces. These results were in accordance with the results of a previous study in which twenty healthy volunteers consumed *L. plantarum* 299v during four weeks³¹. However, the number of lactobacilli recovered in faeces during probiotic consumption was lower in this study (log 6.8 CFU/g faeces) compared to the previous one (log 8.2 CFU/g faeces) while the daily dose of *L. plantarum* 299v was the same (10^{11} CFU/day). Comparison of the pre-treatment faecal bacterial concentrations to the results found in a previous study with the healthy volunteers³¹, demonstrated lower bacterial concentrations for total aerobic bacteria, enterococci, total anaerobic bacteria, *Bacteroides* spp. and lactic acid bacteria in the present study, although similar culture methods were used in both studies. These differences could be explained by higher

defaecation frequency and faeces consistency scores found in the present study, which can result in dilution of the faecal samples and lower numbers of bacteria counted per gram faeces. These looser stool samples could be due to the inclusion of various types of patients such as IBD patients. For example, decreased numbers of lactobacilli and aerobic bacteria have been reported in IBD patients in literature^{8,34-37}. However, exclusion of the bacterial concentrations of the IBD patients did not result in an increase of bacterial concentrations.

In this study, PCR combined with DGGE analyses was found to be an useful additive tool to study the effects of bowel cleansing and probiotic consumption on the dominant faecal flora over time. The intra-individual similarity values calculated between the faecal samples obtained before and after bowel cleansing were relatively high: mean 86.5%. Zoetendal *et al.* calculated similarity values ranging from 66 to 88% comparing two faecal samples of four individuals over time in a four month period¹³. In this study, bowel cleansing did not significantly influence the overall banding patterns of the dominant flora. Although bowel cleansing was successful, it cannot be ruled out that even very small amounts of remaining bacteria can result in a recolonisation of the original flora.

The increase in the number of lactobacilli during probiotic consumption was not reflected in one single band of the molecular fingerprint as the dominant flora consists for less than 1% of lactobacilli and universal primers used for the analyses of the dominant faecal flora detect the most prevalent species, representing 90-99% of the total faecal flora³⁸.

Factors affecting the intestinal flora, except for bowel cleansing and *L. plantarum* 299v intake, were kept as stable as possible during the study period. However, medication taken in this study such as sulfasalazine and proton pump inhibitors has been demonstrated to influence the faecal bacterial composition^{16,35,39,40}. Changes in dose and type of these compounds could not be correlated to the 'deviant' similarity values found in this study except in one patient. This patient stopped the use of loperamide in week 4, resulting in changes in the banding patterns of the dominant flora and in a similarity value of 76%. Loperamide has been found to change the number of streptococci, but did not cause bacterial overgrowth in 14 children receiving the drug because of severe diarrhoea^{41,42}.

Similarity values did not change due to bowel cleansing but some changes in individual bands occurred, mainly in the first week after bowel cleansing, suggesting that bowel cleansing could have an influence on specific bacterial species. Moreover, repeated bowel cleansing procedures might result in a more prominent elimination of the bacterial mass. To get further insight into bowel cleansing as therapeutic modality in gastrointestinal diseases like IBD, influences of bowel cleansing on specific bacterial subspecies have to be studied in defined patient groups using PCR/DGGE with specific primers instead of universal primers.

This study was performed with consecutive patients undergoing a bowel cleansing procedure instead of healthy volunteers, which could have influenced culture results and DGGE banding patterns of the dominant faecal flora. However, even in this heterogeneous population, the intestinal flora remained stable over time. Unfortunately, numbers of patients with specific clinical diagnoses were too small to compare these groups statistically and in a future study more patients should be included.

Seven patients in this study were older than 60 years. As the faecal flora might change by the physiological conditions of the elderly, their flora might be different compared to the younger participants⁴³. However, no significant differences were found in the faecal samples collected before bowel cleansing of the younger (<60 years) compared to the older patients (>60 years) (data not shown).

In conclusion, drastic environmental intervention and changes in dietary factors such as bowel cleansing and probiotic consumption did not result in major changes in the dominant faecal flora. Neither did changes in type or dose of medication significantly alter the bacterial fingerprint of the flora supporting the results of previous studies, indicating that the individual faecal flora is relatively stable over time. However, some changes in single species of the dominant flora of individuals could be noticed, particularly in the early phase of bowel recolonisation.

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Chapter 5

The effect of a probiotic drink with
L. plantarum 299v on the bacterial
composition in faeces and mucosal
biopsies of rectum and ascending colon

Abstract

Background

Studies on probiotics mainly based their results on faecal samples, which may not represent the situation in the mucosa of distal and proximal colon.

Aim

In this placebo-controlled study, the effect of *L. plantarum* 299v was assessed on the bacterial composition of faecal versus mucosal samples.

Methods

Twenty-nine patients undergoing colonoscopic control for polyps consumed a drink with or without *L. plantarum* 299v (10^{11} CFU/day) for two weeks. Faecal samples were collected before and after consumption. During colonoscopy, biopsies were collected from the ascending colon and rectum. The faecal and mucosal bacterial concentrations and prevalence were determined.

Results

L. plantarum 299v significantly increased the concentration of faecal lactic acid bacteria, lactobacilli and clostridia and was identified in 2 rectal biopsies but not in the ascending colon biopsies of probiotic-treated persons. Concentrations and prevalence in ascending colon and rectum biopsies were comparable but were significantly lower compared to faecal samples.

Conclusions

After probiotic consumption, a significant increase in the faecal concentration of lactobacilli was found but concentrations were low in biopsies. The bacterial composition in biopsies of ascending colon and rectum did not differ based on culture techniques. To further elucidate the modes of action of probiotics, it might be necessary to study differences in colonisation with molecular techniques.

Introduction

Probiotics defined as "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora" could have therapeutic effects in gastrointestinal diseases such as Crohn's disease, ulcerative colitis and irritable bowel syndrome^{1,2}. However, clinical studies have often been performed without measuring (micro)biological parameters while the possible modes of action of probiotics are still unresolved. One of the potential modes of action of probiotics could be changing the intestinal bacterial composition. Probiotics were demonstrated to increase the numbers of faecal lactobacilli during consumption³⁻⁶, while a decrease in the number of enterococci and sulphate-reducing clostridia was found after *Lactobacillus rhamnosus* DR20 and *Lactobacillus plantarum* 299v intake, respectively^{3,5}. *L. plantarum* 299v has been shown to strongly adhere to the mucosa by mannose-specific binding sites *in vitro* and in gnotobiotic rats, in this last model, preventing *E. coli* from adhering^{7,8}. This lactobacillus strain has been used in the present study.

Studies investigating the effects of probiotics on the intestinal flora mainly based their results on faecal samples. However, faecal samples may not represent the composition of the intestinal flora in the mucosa of the proximal and distal colon, while investigating the effects on the mucosal flora is important as the mucosal flora has a direct interaction with the host cells. A study including only ten persons, demonstrated that the bacterial composition in faecal samples was significantly different from that of biopsy samples⁹. However, this study investigated the composition of the dominant mucosa-associated flora and the lactobacilli qualitatively by denaturing gradient gel electrophoresis (DGGE) and not quantitatively by determining bacterial concentrations. Other studies only focused on the recovery of the probiotic strain in biopsy samples and did not compare mucosal to faecal bacterial composition^{3,10,11}. To our knowledge only one study showed an alteration in the mucosal concentrations after dietary modulation: a significant increase in the mucosal concentration of lactobacilli and bifidobacteria after treatment of prebiotic carbohydrates has been found¹². However, in this study only biopsy samples were collected and comparisons between faecal and mucosal samples were not made¹². Comparisons between the mucosal and faecal flora are needed to determine if analysis of faecal samples is representative in studying effects of dietary modulations on the intestinal mucosal flora.

In this context, it is important to pay attention to the differential distribution of probiotics over the length of the large bowel. It is plausible that the composition of the mucosal bacteria in the proximal colon differs from that in the distal colon as the availability of nutrients and of bacterial fermentation products differs between these locations: short chain fatty acid concentrations are highest in ascending colon while products of protein fermentation such as ammonia as well as the pH are highest in colon descendens¹³. Limited number of studies compared the bacterial composition in the proximal colon to that of the distal

colon and concluded that there was no variation in the mucosal bacteria along the colon^{9,14,15}. However, these studies included small numbers of participants (4, 10 and 12 individuals, respectively) of whom some persons have been diagnosed with ulcerative colitis (3 of 10 and 6 of 12) which may influence the bacterial composition¹⁶⁻¹⁸. More insight in the composition of the mucosal flora in the proximal colon versus that of the rectum is important to determine if analysis of rectal biopsy samples only is representative to study the effects of probiotics on the intestinal mucosal flora.

In this placebo-controlled double-blind study, the effect of the probiotic *L. plantarum* 299v was assessed on the bacterial composition of faecal versus mucosal samples and of rectal versus ascending colon biopsy samples.

Methods

Subjects

Thirty consecutive patients were enrolled in the study undergoing a colonoscopic control because of a previous history of polyps or a positive family history of colorectal cancer. Exclusion criteria were pre-existing bowel pathology including irritable bowel syndrome, inflammatory bowel disease, cancer and bowel resection. Participants were neither allowed to use antibiotics, laxatives, probiotics and/or prebiotics during two weeks before nor during the study. Participants were asked to continue their 'normal' dietary habits. All participants have given written informed consent. The study was approved by the Medical Ethical Committee of the University Hospital Maastricht and was performed in accordance with the declaration of Helsinki.

Study design

The study was randomised, placebo-controlled and double-blind. The total duration of the study period was two weeks. During the two weeks treatment period, patients consumed 100 ml in the morning and 100 ml in the evening of an oatmeal fermented drink without (placebo) or with (probiotic) *L. plantarum* 299v (10^9 CFU/ml). Both probiotic and placebo drinks were provided by Probi AB, Lund, Sweden, who also performed the randomisation using a computer program and kept the codes until all samples were analysed. Probiotic and placebo drinks were indistinguishable in colour, smell and taste. Two faecal samples were collected: one before and one after the probiotic or placebo treatment. At the time of the collection of faecal samples, questionnaires had to be completed with questions about bowel habits (defaecation frequency and faeces consistency scores according to the Bristol scale¹⁹), compliance with regard to the consumption of the drinks and possible side effects. Patients were excluded from analyses if the drinks were consumed for less than 6 days per week based on the questionnaires.

After collection of the second faecal samples, patients started with the bowel cleansing procedure as preparation for the colonoscopy. All participants have taken a laxative containing macrogol and electrolytes (Kleanprep®) during 4.5 hours to clean the gastrointestinal lumen, 24 hours prior to colonoscopy. During the colonoscopy one biopsy sample from each rectum and ascending colon was collected with a sterile biopsy forceps.

Collection of samples

Faecal samples (± 20 gram) were collected in a sterile container and brought to the laboratory within 12 hours after defaecation. Five grams were diluted (1:4) in peptone water with cysteine (2.1 mM) and glycerol (37%) and homogenised with an ultra-turrax (IKA®, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) for one minute. The remaining undiluted part was frozen for additional analyses (-20°C).

Biopsies from ascending colon and rectum were collected in sterile 0.8% sodium chloride with cysteine-HCl (0.1%) to limit exposure to oxygen and were processed immediately after collection.

Bacteriological culture

Within 12 hours after defaecation, ten-fold serial dilutions of the faecal samples (10^{-1} - 10^{-6}) were made in sodium chloride (0.9%) and 40 μl were inoculated using a spiral plater (Eddy Jet v1.2, IUL-instruments, Barcelona, Spain) on the following agar plates: Blood agar plates (Oxoid CM 271, Basingstoke, UK) for total (facultative) aerobic bacteria, Eosin-methylene blue agar plates (Oxoid CM69, Basingstoke, UK) for enterobacteriaceae, KF-streptococcus agar plates (Oxoid Cm701, Basingstoke, UK) for enterococci, Fastidious anaerobic agar plates (Lab M LabGo, Lancashire BL9, 6AU, UK) for total (facultative) anaerobic bacteria, Bile-esculine agar plates (Becton Dickinson 287920, La Pont de Claix, France) for *Bacteroides* spp., Egg Yolk agar plates for clostridia, Rogosa agar plates (Oxoid CM627, Basingstoke, UK) for lactic acid bacteria and LAMVAB (lactobacillus anaerobic MRS with vancomycin and bromocresol green) for lactobacilli²⁰ according to the method described previously⁴.

Biopsy samples were washed three times by shaking gently in sterile 0.8% sodium chloride with cysteine-HCl (0.1%). Biopsy samples were homogenised in 0.5 ml 0.8% sodium chloride with cysteine-HCl (0.1%) (10^{-1}) by a sterile tissue grinder (No. 43950, ICN Flow Biomedicals NVSA, Asse-Relegem, Belgium). Forty μl of this solution was diluted in 0.8% sodium chloride with cysteine-HCl (0.1%) (10^{-2}). After homogenisation, 50 μl of both dilutions were inoculated on the agar plates described above.

Blood agar and eosin-methylene blue agar plates were incubated aerobically at 37°C for 24 hours. KF- streptococcus agar plates were incubated aerobically at 42°C for 48 hours. Fastidious anaerobic, Bile-esculine, Egg yolk, Rogosa and LAMVAB agar plates were incubated anaerobically at 37°C for 48 hours after inoculation. Colony forming units (CFU) were counted and expressed as log colony forming units per gram faeces (log CFU/g faeces) or per biopsy sample

(log CFU/biopsy). The prevalence of bacteria culturable in faecal and biopsy samples has been calculated. Mean mucosal bacterial concentrations were calculated if the bacteria were present in more than five biopsy samples. To compare the bacterial concentrations of faecal to mucosal samples, the CFU were expressed as log CFU per mg protein.

Identification of lactobacilli

From each LAMVAB agar plate four colonies from the outer ring with the appearance of lactobacilli were isolated and purified on blood agar plates. Isolates were frozen at -80°C for molecular identification of *L. plantarum* 299v using randomly amplified polymorphic DNA (RAPD), eventually confirmed with restriction enzyme analysis (REA) according to the method described by Johansson *et al.*^{21,22}.

Bacterial enzyme activities

The β -glucosidase and β -glucuronidase activities were analysed aerobically. Homogenised faecal samples were diluted (1:1) in 0.1 M PBS (pH 6.8, $5-7^{\circ}\text{C}$), sonicated for one minute on ice and subsequently centrifuged at 3000 rpm during 15 minutes at 4°C . The supernatant was dried under vacuum during 75 minutes (Savant speedvac plus sc 210A) and was used for the assessment of the β -glucosidase and β -glucuronidase activities as described previously⁴.

Statistical analyses

Statistical evaluation of changes within groups was carried out using the non-parametric Wilcoxon signed-rank test. The Mann-Whitney U-test was used for comparison between probiotic and placebo group. The Chi-square test was used to compare prevalence of bacteria countable in biopsy samples between probiotic and placebo group and between rectum and ascending colon biopsies. A p-value below 0.05 was considered to be statistically significant.

Results

Twenty-nine patients completed the study: 15 in the probiotic group (10 male and 5 female, mean age 57.5 ± 10.9 years) and 14 in the placebo group (6 male and 8 female, mean age 56.3 ± 10.2 years). One participant did not start with the study because of personal reasons and was excluded. After colonoscopy, 13 patients had been diagnosed with bowel abnormalities such as polyps (6 probiotic, 4 placebo), diverticula (2 probiotic) and an irritated mucosa characterised by red spots (1 placebo). Based on the questionnaires, the compliance to the consumption of the drinks was 98% in the probiotic and 99% in the placebo group. No side effects were reported during consumption of either the probiotic or placebo drinks. No significant differences in defaecation frequency or faeces consistency scores could be observed between probiotic

and placebo group before and after consumption or within groups over time (table 5.1).

Table 5.1 Defaecation frequency per day and faeces consistency scores (scale 1: hard lumps to 7: watery diarrhoea) before and after the consumption of the probiotic or placebo drink (median and range).

	Probiotic		Placebo	
	before	after	before	After
Frequency	1.0 (1-4)	1.0 (0-5)	1.5 (0-5)	2.0 (0-6)
Consistency	4.0 (2-6)	5.0 (3-7)	4.5 (1-6)	4.5 (1-6)

Effects of *L. plantarum* 299v on the lactobacilli

Before start of the study, *L. plantarum* 299v could be identified in faecal samples of two persons from the placebo group but in none from the probiotic group. After the consumption of the probiotic drinks, a significant increase in the faecal concentration of lactic acid bacteria ($p=0.009$) and lactobacilli ($p=0.002$) were observed (table 5.2) reaching levels of log 7.1 CFU/g faeces. Moreover, *L. plantarum* 299v could be identified in faecal samples of 10 out of the 11 probiotic-treated persons, in whom lactobacilli were isolated, while *L. plantarum* 299v could not be identified anymore in the placebo-treated persons. The person, in whom *L. plantarum* 299v was not isolated after probiotic consumption, did not demonstrate an increase in the faecal concentration of lactobacilli either.

Furthermore, lactobacilli could be cultured in three rectal biopsies and in two ascending colon biopsies of three probiotic-treated persons, but in none of the placebo group. Of these lactobacilli, *L. plantarum* 299v was identified in the rectal biopsies of two probiotic-treated patients but not in the ascending colon biopsies of these patients.

Effects of *L. plantarum* 299v on the bacterial composition

In addition to the lactobacilli increase, the concentration of clostridia increased significantly ($p=0.0048$) in faecal samples in the probiotic but not in the placebo group. No other changes in the bacterial concentrations of the faecal samples were observed after either probiotic or placebo consumption. Percentages of faecal samples in which pre-defined bacteria could be counted, were high and ranged from 79% to 100% (figure 5.1). Intake of *L. plantarum* 299v did not influence faecal β -glucosidase and β -glucuronidase activities significantly (table 5.3).

Table 5.2 Concentrations of bacteria (log CFU/gram faeces), before and after the consumption of the probiotic or placebo drink (mean $\pm$ SEM).

	Before	After
Total aerobic flora		
Probiotic	6.7 $\pm$ 0.3	7.1 $\pm$ 0.2
Placebo	6.7 $\pm$ 0.1	6.7 $\pm$ 0.2
<i>E. coli</i>		
Probiotic	6.0 $\pm$ 0.3	6.6 $\pm$ 0.3
Placebo	5.9 $\pm$ 0.3	6.2 $\pm$ 0.2
Enterococci		
Probiotic	4.4 $\pm$ 0.4	4.0 $\pm$ 0.5
Placebo	4.0 $\pm$ 0.3	3.7 $\pm$ 0.5
Total anaerobic flora		
Probiotic	8.6 $\pm$ 0.3	8.3 $\pm$ 0.4
Placebo	8.1 $\pm$ 0.3	8.3 $\pm$ 0.3
<i>Bacteroides</i> spp.		
Probiotic	6.1 $\pm$ 0.3	5.7 $\pm$ 0.4
Placebo	6.5 $\pm$ 0.2	6.2 $\pm$ 0.3
Clostridia		
Probiotic	4.3 $\pm$ 0.2	4.7 $\pm$ 0.2 ^a
Placebo	3.9 $\pm$ 0.3	4.3 $\pm$ 0.2
Lactic acid bacteria		
Probiotic	5.7 $\pm$ 0.4	7.1 $\pm$ 0.4 ^a
Placebo	5.2 $\pm$ 0.4	5.4 $\pm$ 0.3
Lactobacilli		
Probiotic	5.0 $\pm$ 0.4	7.1 $\pm$ 0.3 ^a
Placebo	4.8 $\pm$ 0.2	4.3 $\pm$ 0.3

^a statistically significant difference ($p < 0.05$) before and after probiotic intake.

In biopsy samples, enterococci, clostridia, lactic acid bacteria and lactobacilli were cultured in relatively few biopsies (0-31%) (figure 5.1). Percentages of biopsy samples in which bacteria were cultured, did not differ significantly between probiotic and placebo group. The mean concentrations of mucosal bacteria, calculated of the bacterial subspecies present in at least five biopsy samples, are expressed in table 5.4. A significant increase ($p = 0.019$) in total anaerobic bacteria in rectal biopsy samples between probiotic and placebo group were found after consumption of the drinks. No other significant differences were observed in bacterial concentrations between probiotic and placebo group.

Figure 5.1 Prevalence of bacteria culturable in faecal samples and rectum and ascending colon samples in the probiotic (n=15)(a) and placebo (n=14)(b) group.

Table 5.3 β -glucosidase (mg/60 min/g faeces) and β -glucuronidase (mg/30 min/g faeces) activities before and after probiotic or placebo consumption (mean $\pm$ SEM).

	Probiotic		Placebo	
	before	after	before	after
β -glucosidase	1.23 $\pm$ 0.29	0.99 $\pm$ 0.16	1.06 $\pm$ 0.27	1.23 $\pm$ 0.25
β -glucuronidase	0.86 $\pm$ 0.38	0.87 $\pm$ 0.34	0.87 $\pm$ 0.22	0.96 $\pm$ 0.30

Table 5.4 Concentrations of bacteria (log CFU/biopsy) in rectum and ascending colon biopsies after probiotic and placebo consumption (mean $\pm$ SEM).

	Rectum	Ascending colon
Total aerobic flora		
Probiotic	3.9 $\pm$ 0.3	3.9 $\pm$ 0.3
Placebo	3.7 $\pm$ 0.3	3.6 $\pm$ 0.3
<i>E. coli</i>		
Probiotic	3.8 $\pm$ 0.3	3.9 $\pm$ 0.4
Placebo	3.4 $\pm$ 0.3	4.0 $\pm$ 0.3
Total anaerobic flora		
Probiotic	4.5 $\pm$ 0.3 ^a	4.3 $\pm$ 0.3
Placebo	3.3 $\pm$ 0.3 ^a	3.9 $\pm$ 0.3
<i>Bacteroides</i> spp.		
Probiotic	2.6 $\pm$ 0.3	3.4 $\pm$ 0.4
Placebo	3.0 $\pm$ 0.3	3.4 $\pm$ 0.3

^a statistically significant difference ($p < 0.05$) between probiotic and placebo group.

Comparisons of the prevalence and the bacterial concentrations

Comparing rectum to ascending colon biopsies, prevalence and concentrations of mucosal bacteria did not differ significantly.

Comparing the prevalence of bacteria of the faecal samples to that of the rectum and the ascending colon biopsies, demonstrated that the prevalence of enterococci, clostridia, lactic acid bacteria and lactobacilli was significantly lower in the rectal as well as the ascending colon biopsies compared to that in faecal samples collected from both probiotic and placebo-treated patients.

Discussion

To our knowledge, this is the first study investigating the effects of the probiotic *L. plantarum* 299v on both the faecal and mucosal bacterial composition in order to gain further insight in the possible modes of action of probiotics. Moreover, the bacterial composition of faecal versus mucosal samples and of rectum versus ascending colon biopsy samples has been compared.

Effects of *L. plantarum* 299v on the lactobacilli

After *L. plantarum* 299v consumption, the faecal concentration of lactic acid bacteria and lactobacilli increased significantly and *L. plantarum* 299v could be recovered in faecal samples in the majority of persons from the probiotic-treated group, indicating that the probiotic strain survives passage through the gastrointestinal tract. In mucosal samples, *L. plantarum* 299v was also detected but to a minor degree. Although an *in vitro* and animal study demonstrated that *L. plantarum* 299v can adhere to epithelial cells by mannose-specific binding sites competing with *E. coli*⁷, the present study did not support an extensive colonisation of *L. plantarum* 299v in the mucosa. In

contrast, in a study performed in Sweden, *L. plantarum* 299v could be recovered from jejunum and rectum biopsies in 11 of the 13 participants, even 11 days after cessation of a 10 days treatment period¹¹. Differences in the colonisation of *L. plantarum* 299v between the Swedish and the present study might be due to differences in bowel cleansing or biopsy handling but also due to host factors (mucus composition, intake of nutrients), which could be different in the two populations. Interestingly, the probiotic strain *L. plantarum* 299v used in the studies has been isolated from Swedish persons and it is possible that the Swedish population is more susceptible for colonisation of this strain. Because of these inconsistencies in the colonisation results, the mucosal adherence of *L. plantarum* 299v needs further study.

Moreover, the increase in the concentration of faecal lactobacilli is 1 log CFU/g faeces smaller compared to previous studies with healthy volunteers, although the same daily dose (10^{11} CFU/day) has been administered^{4,23}. This difference cannot be explained by the inclusion of patients, of whom 13 patients have been diagnosed with abnormalities during the colonoscopy, as comparison of the faecal bacterial concentrations between patients with or without abnormality did not show any significant difference (data not shown). In addition, no significant difference in the defaecation frequency and faeces consistency scores could be seen comparing the patients to the healthy volunteers (data not shown). The smaller increase in lactobacilli after *L. plantarum* 299v intake might also be due to differences in age as the participants in the present study were older (mean age: 56.9 years) compared to the healthy volunteers (mean age: 34 years)⁴. However, age below 60 years is not suspected to have a major influence on the bacterial composition²⁴.

Effects of *L. plantarum* 299v on the bacterial composition

Probiotic intake led to an increase in the faecal concentration of clostridia. This increase could be beneficial if non-pathogenic clostridia subspecies increased, dominating potentially pathogenic clostridia subspecies. However, previous studies including healthy volunteers did not demonstrate changes in the concentration of clostridia after *L. plantarum* 299v intake, while a significant decrease in sulphate-reducing clostridia has been shown in one Swedish study including healthy volunteers^{3,4,23}. A recent study by Langlands *et al.* observed changes in the composition of clostridia subspecies after prebiotic intake. However, no changes in the concentration were noticed¹². Further insight is warranted in the effect of *L. plantarum* 299v on clostridia subspecies.

In the present study, *L. plantarum* 299v intake did alter the mucosal bacterial concentrations to a minor extent as the concentration of total anaerobic bacteria in the rectal biopsies increased compared to the placebo group. Unfortunately, no pre-treatment data were available due to ethical problems. In a previous study, no increase in the mucosal concentration of total anaerobic bacteria was shown after 10 days consumption of *L. plantarum* 299v¹¹. In future intervention trials, biopsies should be collected before as well as after probiotic

intake to get more insight in the possible changes of mucosal bacterial concentrations.

Comparisons of the prevalence and the bacterial concentrations

The prevalences and the bacterial concentrations detected in the biopsies from ascending colon and rectum were comparable. This indicates that in future studies culturing of rectal biopsy samples will be sufficient to study the probiotic effects on the intestinal mucosal flora. Although the mucosal concentrations found in the present study are difficult to compare to previously described concentrations, as they were expressed in different units (CFU/biopsy, per gram, or per μ l biopsy dilution), the mucosal bacterial concentrations and prevalence found in the present study seemed to be low compared to previous studies^{9,12,15}. This might be due to the bowel cleansing procedure prior to colonoscopy, to differences in handling of the biopsies and/or the use of different culture media. However, the effect of bowel cleansing is expected to be limited as in a previous study performed by our group, bowel cleansing did not significantly affect the intestinal flora of faecal samples. Moreover, Schultz *et al.* demonstrated that bacterial concentrations in rectal biopsies did not differ after bowel cleansing compared to preparation by an enema¹⁸. Comparing the effects of different bowel cleansing procedures on the faecal and mucosal flora will be interesting but is hampered as not all the previous studies described the procedure precisely^{15,17,18}.

Moreover, in the present study gently washing of the biopsies was performed to remove contaminating flora and to study the adherent mucosal flora. Previous studies showed that washing of the biopsies did not systematically result in lower bacterial concentrations and prevalence^{11,15-17}.

Mucosal concentrations might be difficult to interpret, as the localisation of mucosal bacteria is still controversial. On the one hand, bacteria are shown to be present in the epithelial cells, without having contact with the faecal stream¹⁷. On the other hand, bacteria have been observed within or at the luminal side of the mucus layer^{18,25}. In such a situation, washing and handling of the biopsies may have a major impact on the bacterial culture results.

The prevalence of bacteria found in biopsies was lower compared to those of the faecal samples, indicating that both types of samples need to be collected in studying potential effects of intervention (drug, nutrition) on the intestinal flora. Unfortunately, comparison of the bacterial concentrations of biopsies versus faecal samples was not possible, as the biopsies could not be weighed under strictly anaerobic conditions without disturbing the mucus layer. However, based on data from previous studies, the biopsy weight is expected to be around 10 mg¹⁵. If the mucosal concentrations would be expressed based on such weight, lower mucosal concentrations would be calculated compared to the faecal concentrations. This will be in accordance with results from previous studies²⁶⁻²⁸.

The bacterial concentrations described in the present study were based on culture results. However, more than 50% of the bacteria in the gastrointestinal tract cannot be cultured or are not yet identified^{5,29,30}. Polymerase chain reaction (PCR) in combination with DGGE will be an additive tool to compare the faecal and mucosal bacterial composition by producing molecular 'bacterial fingerprints' of the individual dominant flora^{31,32}. Based on DGGE results, Zoetendal *et al.* have shown that the bacterial composition found in biopsy samples collected from different locations in the colon was comparable, but significantly different from the bacterial composition found in faecal samples. This is in line with the results found in the present study⁹.

In conclusion, the present study demonstrated that the probiotic *L. plantarum* 299v survives passage through the gastrointestinal tract. Moreover, the probiotic strain colonised the colonic mucosa to a minor extent. No changes in prevalence and bacterial concentrations between rectum and ascending colon were observed, which suggests that analysis of rectal biopsies might be sufficient in future studies. Prevalence of bacteria in the biopsy samples was lower compared to that of faecal samples, indicating that collection of both samples is necessary to investigate the effects of probiotics on the bacterial composition of the large bowel. Investigating factors that influence the adherence of bacteria to the mucosa might be interesting in view of the mechanisms that modulate intestinal bacteria in health and disease.

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Chapter 6

The faecal and mucosal bacterial composition of patients with inactive UC: can it be changed by *L. plantarum* 299v?

D. Goossens, D. Jonkers, E. Stobberingh, G. Molin, S. Ahné, M. Russel, R. Stockbrügger

Submitted

Abstract

Introduction

The intestinal flora of patients with ulcerative colitis (UC) is considered to be different from that of healthy persons. Moreover, beneficial effects of probiotics have been found in UC. In this study, the faecal bacterial composition of inactive UC patients was compared to that of healthy controls, and the effects of the probiotic *L. plantarum* 299v on the faecal and mucosal bacterial composition and faecal metabolites were examined.

Methods

Bacteriological culture results of faecal samples of thirty-one inactive UC patients on a stable dose of maintenance therapy were compared to those of 20 healthy controls. In a randomised placebo-controlled study design, patients consumed a drink with or without *L. plantarum* 299v (10^{11} CFU/day) during four weeks. Faecal samples were collected before and after consumption. In a subgroup of patients, rectal biopsies were collected after consumption. Concentrations and prevalence of faecal and mucosal bacteria as well as faecal enzyme activities, short chain fatty acids (SCFA) and endotoxin concentrations were analysed. Moreover, temporal temperature gel electrophoresis (TTGE) was performed to compare the molecular 'fingerprints' of the dominant bacteria present in rectal biopsies of the probiotic versus the placebo-treated UC patients.

Results

Faecal bacterial concentrations (log CFU per mg protein) were lower in UC patients than in healthy controls, being significant for total anaerobic bacteria (7.6 ± 0.1 vs. 8.2 ± 0.1), *Bacteroides* spp. (5.1 ± 0.3 vs. 6.6 ± 0.2) and lactic acid bacteria (2.6 ± 0.3 vs. 5.5 ± 0.5). However, the prevalence of faecal bacteria was comparable in UC patients and healthy controls except for *E. coli* (70 vs. 95%, $p < 0.05$).

L. plantarum 299v intake resulted in a significant increase in the faecal concentration of lactobacilli and lactic acid bacteria and in a reduction of the mucosal prevalence of *Bacteroides* spp. However, *L. plantarum* 299v intake did not influence significantly other faecal and mucosal bacterial concentrations and mucosal bacterial 'fingerprints' nor the enzyme activities, SCFA and endotoxin concentrations.

Conclusions

Comparing UC patients to healthy controls, differences in faecal bacterial composition were observed, which could be a consequence of either the disease and/or its treatment. As intake of *L. plantarum* 299v did only partly change the bacterial composition, more studies are warranted to investigate other modes of action of probiotics such as modulation of the immune system.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon and/or rectum and belongs to the group of inflammatory bowel diseases (IBD). Although the aetiology of UC is still unknown, genetic, microbiological, immunological and environmental factors are thought to be involved in the pathogenesis¹. It is hypothesised that UC is the result of an exaggerated intestinal host response against commensal intestinal bacteria or their components². Experimental animal models demonstrated that intestinal bacteria trigger chronic intestinal inflammation as the presence of these bacteria is required for the development of spontaneous colitis in IL-10 deficient mice and in rats transgenic for the human HLA-B27 gene³⁻⁵. Furthermore, a role of intestinal bacteria in influencing colitis has been confirmed in several clinical observations: inflamed sites of the colon in UC patients comprise the highest number of bacteria, and antibiotic treatment results in decreased disease activity in acute UC patients⁶⁻⁹.

Some studies suggested that the intestinal bacterial composition of active and inactive UC patients might be different from that of healthy controls. Both an increase and a decrease in anaerobic bacterial counts (*Bacteroides* spp., lactobacilli, clostridia, eubacteria and bifidobacteria) and aerobic bacterial counts (enterobacteriaceae) have been described in faecal and mucosal samples of active UC patients compared to inactive patients and to healthy controls¹⁰⁻¹⁵. In contrast, Giaffer *et al.* did not demonstrate a difference in the concentration of faecal anaerobic bacteria comparing active to inactive UC patients¹⁶. Studies investigating the bacterial composition of UC patients differ in their design. Some studies compared the bacterial composition to controls, while others did not^{11,12}. These controls were healthy volunteers^{10,16} or non-IBD patients¹³⁻¹⁵. Several studies did not register disease activity^{14,15} or the use of the medication^{12,14,15} including antibiotics^{10,11}. Moreover, other studies did not investigate the bacterial composition quantitatively¹²⁻¹⁵.

The concentrations of some intestinal bacteria, such as *Bacteroides* spp. and enterobacteriaceae, as well as some bacterial components such as lipopolysaccharides (LPS) were higher in the mucosa of UC patients compared to healthy controls^{14,17}. These bacteria and bacterial products can translocate through the mucosa as the permeability of the mucosa in UC patients was found to be increased^{7,18}. This higher mucosal permeability may be related to a deficient butyrate oxidation¹⁹⁻²¹ and a reduced mucus production²²⁻²⁴ caused by increased bacterial enzyme activities (glucosidases and sulphatases)^{25,26}. Metabolic products such as bacterial enzymes, short chain fatty acids (SCFA) and LPS concentrations appear to be useful indicators to study changes in the bacterial composition in UC patients but are still not investigated in studies comparing the bacterial composition of UC patients to that of healthy controls.

Probiotics are defined as "mono or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous flora"²⁷. As the intestinal flora is suspected to play an important role in the pathogenesis of UC, changing this flora by probiotics may have a beneficial therapeutic effect. Three clinical studies have investigated the effect of the probiotic *Escherichia coli* strain Nissle 1917 versus mesalazine in UC patients with inactive disease and found a similar relapse rate in the treatment groups suggesting that probiotics could be an alternative maintenance therapy²⁸⁻³⁰. In an uncontrolled study, a multispecies probiotic preparation (VSL#3) was also found to have potential as maintenance therapy³¹. However, most probiotic studies only focused on clinical outcome without investigating the possible modes of action of these probiotics or suffered from methodological limitations such as low numbers of patients included, a heterogeneous population included and the lack of a randomised placebo-controlled design.

The monospecies probiotic *Lactobacillus plantarum* 299v has been found to reduce colitis in rats and IL-10 deficient mice³²⁻³⁵. In addition, several studies have shown that *L. plantarum* 299v survived passage through the gastrointestinal tract and can adhere to mucosal cells^{32,36-38}. Consequently, this bacterium is a good candidate to study the effects of a probiotic on the intestinal bacterial composition and the metabolic activity of UC patients.

In this study, the faecal bacterial composition of inactive UC patients was compared to that of healthy controls, and in these UC patients the effect of the probiotic *L. plantarum* 299v on the intestinal bacterial composition in faecal and in mucosal samples as well as on faecal metabolites was investigated by a placebo-controlled double-blind design.

Methods

Subjects

Thirty-one UC patients of the South Limburg IBD registry³⁹ were asked to participate in the study. Patients had a well-defined diagnosis of UC established by endoscopic, radiologic and/or histologic criteria and had inactive disease confirmed by a Clinical Activity Index (CAI) below 10⁴⁰. Patients known with other pre-existing bowel diseases including irritable bowel syndrome, diverticular disease, cancer and bowel resection were excluded. Only patients receiving mesalazine or sulfasalazine with or without azathioprine as maintenance therapy were included in the study. The selected patients used this medication for at least 8 weeks prior to inclusion and were on a stable dose for at least 4 weeks prior to inclusion and remained so during the study period. The use of enemas, gastric acid inhibition, laxantia or antibiotics was not allowed during the 4 weeks before inclusion as well as during the study while

the consumption of prebiotics and probiotics was not allowed for at least 2 weeks before inclusion as well as during the study period. The study was approved by the Medical Ethics Committee of the University Hospital Maastricht and was performed in accordance with the Declaration of Helsinki. All participants had given written informed consent.

Comparison of UC patients and healthy volunteers

Culture results of the faecal samples of the UC patients before start of probiotic or placebo consumption were compared to the results of a previously performed study in which 20 healthy volunteers were enrolled and in which the handling of the faecal samples and bacteriological culture methods used were similar⁴¹.

Study design

The study was randomised, placebo-controlled and double-blind with a four-week treatment period. During the treatment period, participants consumed 200 ml (100 ml in the morning and 100 ml in the evening) of an oatmeal fermented drink without (placebo) or with (probiotic) *L. plantarum* 299v (10^9 CFU/ml). The two types of drinks were indistinguishable in colour, smell and taste.

Two faecal samples were collected: one before start of treatment and one on the last day of the treatment period. At each time of faeces collection, the patients also completed questionnaires regarding the use of medication, disease activity, side effects, compliance with regard to the consumption of the drinks, and bowel habits (defaecation frequency and faeces consistency score according to the Bristol scale⁴²). All patients were asked to undergo a proctoscopy at the end of the treatment period in order to collect two rectal biopsies. A subgroup of patients ($n=13$) agreed to undergo the proctoscopy. Patients were excluded from the analyses if the drinks were consumed for less than 6 days per week based on the information of the questionnaire. Patients were asked to continue their usual dietary habits during the study period.

Microbiological analyses

Faecal samples (± 20 gram) were collected in a sterile container, brought to the laboratory within 12 hours after defaecation, and were divided into three parts. Five grams were diluted (1:4) in 20 ml peptone water with cysteine (2.1 mM) and glycerol (37%) and homogenised with an ultra-turrax (IKA®, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) for 1 minute. Half a ml was used immediately for bacteriological culture while the remaining part was frozen at -20°C for the determination of β -glucosidase and β -glucuronidase activities and SCFA concentrations. Ten grams were centrifuged at 4°C for 2 hours at 25.000 *g* to obtain faecal water, which was frozen at -80°C for determination of the endotoxin concentration. The remaining undiluted faeces was frozen at -20°C for additional analyses.

In a subgroup of patients, two rectal biopsy samples were collected during a proctoscopy. These patients had to take an enema (Klistier®, Fresenius Kabi, Bad Homburg, Germany) to clean the rectum half an hour prior to the proctoscopy. Biopsy samples were taken with a sterile biopsy forceps. Biopsy samples for culture were collected in 1 ml sterile 0.8% saline with 0.1% cysteine-HCl and were processed immediately. Biopsy samples for temporal temperature gel electrophoresis (TTGE) analysis were collected in TE-buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0) and frozen immediately at -80°C until further analysis.

Culture

Ten-fold serial dilutions of the faecal samples (10^{-1} - 10^{-6}) were made in physiological saline (0.9%) and 40 µl were inoculated using a spiral plater (Eddy Jet v1.2, IUL-instruments, Barcelona, Spain) on the following agar plates: Blood agar plates (Oxoid CM 271, Basingstoke, UK) for total (facultative) aerobic bacteria, Eosin-methylene blue agar plates (Oxoid CM69, Basingstoke, UK) for enterobacteriaceae, KF-streptococcus agar plates (Oxoid CM701, Basingstoke, UK) for enterococci, Fastidious anaerobic agar plates (Lab M LabGo, Lancashire BI9, 6AU, UK) for total (facultative) anaerobic bacteria, Bile-esculine agar plates (Becton Dickinson 287920, La Pont de Claix, France) for *Bacteroides* spp., Egg Yolk agar plates for clostridia, Rogosa agar plates (Oxoid CM627, Basingstoke, UK) for lactic acid bacteria and LAMVAB (lactobacillus anaerobic MRS with vancomycin and bromocresol green) for lactobacilli⁴³ according to the method described previously⁴¹.

Biopsy samples collected were washed three times in sterile 0.8% sodium chloride with cysteine-HCl (0.1%) and were homogenised in 0.5 ml 0.8% sodium chloride with cysteine-HCl (0.1%) using a sterile tissue grinder (10^{-1}) (No. 43950, ICN Flow Biomedicals NVSA, Asse-Relegem, Belgium). Forty µl of this solution was further diluted (10^{-2}) and 50 µl of both biopsy dilutions (10^{-1} and 10^{-2}) were inoculated on the agar plates described above using glass spatula.

Colony forming units (CFU) were counted and expressed as log CFU per mg protein (log CFU/mg protein) for the faecal samples or per biopsy sample (log CFU/biopsy).

Protein analysis

Homogenised faecal samples were diluted (1:99) in 0.1 M PBS (pH 6.8, 5-7°C) and added to BioRad Assay Protein Dye Reagent (1:1). After 30 minutes, the absorbance was read at 595 nm. Concentrations of proteins were calculated from a standard curve for proteins ranging from 0 to 120 µg/ml and were expressed, after correction for dilution, as mg protein per gram faeces.

TTGE analysis

Temporal temperature gel electrophoresis (TTGE) was performed to compare the molecular 'fingerprints' of the dominant bacteria present in rectal biopsies of the probiotic versus placebo-treated UC patients. After thawing on ice, the biopsies were centrifuged and resuspended in 10 μ l proteinase K and 190 μ l of buffer G2 (Qiagen, Hilden Germany). UV-treated glass beads were added to the biopsy samples and samples were heated at 56°C for 4 hours and subsequently shaken on a mixer for 30 minutes at 4°C. Thereafter, DNA was extracted using the BioRobot EZ1 DNA isolation kit (Qiagen, Hilden, Germany) according to the instructions by the manufacturer.

The eluted DNA was used in PCR reactions amplifying the entire 16S rRNA-gene using the primers ENV1 (5'-AGA GTT TGA TII TGG CTC AG-3') and ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3')⁴⁴. These PCR-products were used as templates in the TTGE-PCR.

For TTGE-PCR amplification, 16S rDNA primers P5 (5'-gc AAC GCG AAG AAC CTT AC-3') (gc=gc-clamp according to Heuer *et al.*⁴⁵) and P6 (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') flanking the variable V3, V4 and V5 regions were used⁴⁶. The PCR-conditions and TTGE-electrophoresis were run as previously described by Monstein *et al.*⁴⁷. The TTGE-results were compared by band based analyses using Dice coefficient and UPGMA for clustering using the computer program BioNumerics (Applied Maths, Sint-Martens-Laten, Belgium). In order to see if there were any differences between probiotic and placebo group, the similarity of the patterns were calculated numerically.

Bacterial enzyme activities

The β -glucosidase and β -glucuronidase activities were analysed aerobically. Homogenised faecal samples were diluted (1:1) in 0.1 M PBS (pH 6.8, 5-7°C), sonicated for 1 minute on ice and subsequently centrifuged at 3000 rpm during 15 minutes at 4°C. The supernatant was dried under vacuum during 75 minutes (Savant speedvac plus sc 210A) and was used for the assessment of the β -glucosidase and β -glucuronidase activities as described previously⁴¹.

Short chain fatty acid assay

Short chain fatty acid (SCFA) concentrations were measured in the faeces using gas-liquid chromatography (GLC). The GLC system consisted of a DANI 8521-a gas chromatograph equipped with flame ionisation detector in conjunction with Maestro software (Chrompack, Middelburg, Holland) for calculations. The chromatographic column used, was WCOT fused silica 25m x 0.32 mm id, coated with FFAP-CB df 0.3 and was used in an isotherm mode at 140°C. The injector and detector temperature were 270°C. The injection type was split with a ratio of 1:50. Helium was used as carrier gas, with a head pressure of 0.8 bar. One ml HCl with heptanoic acid (internal standard, 10 mmol/l) was added to 1 ml of the diluted faecal samples to form free fatty acids. SCFA were extracted with 2 ml of diethyl ether. One μ l of the diethyl

ether extract was injected for measurement. Concentrations of SCFA were determined in mmol/g faeces after correction for dilution⁴⁸.

Endotoxin assay

The bacterial endotoxin concentration (i.e. LPS concentration) was determined in faecal water using the microtechnique of the *Limulus* amoebocyte lysate test (Endosafe, end point chromogenic analysis endochrome test kit, Charles River, Kent, UK). Pyrogen-free water was used to dilute the faecal water samples, to dissolve the solutions and as negative control. The analysis was performed according to the manufacturer's instructions under pyrogen-free conditions. The range of the assay in which endotoxin concentration can be detected was between 1.25×10^{-3} and 0.1 ng/ml. The mean value of faecal endotoxin was expressed as log ng endotoxin per ml faecal water.

Statistical analyses

The non-parametric Mann-Whitney U-test was used for comparison between UC patients and healthy controls and between probiotic and placebo group while the non-parametric Wilcoxon signed-rank test was used for the statistical evaluation of changes of faecal bacterial concentrations and metabolites within groups. Mucosal bacterial concentrations of the probiotic and placebo subjects were statistically compared using the non-parametric Mann-Whitney U-test. A p-value below 0.05 was considered to be statistically significant.

Results

UC patients characteristics

Thirty-one UC patients were enrolled in the study and 27 UC patients completed the study: 13 in the probiotic (median 46 (range 35-67) yrs, 54% male) and 14 in the placebo group (median 50 (range 33-73) yrs, 64% male)

No significant differences in age and gender were observed between the probiotic and placebo group. Four patients did not complete the study (2 probiotic and 2 placebo group) due to bowel complaints. Inactive disease was present in all patients at inclusion and at the end of the study period. Thirteen patients agreed to undergo a proctoscopy for the collection of rectal biopsies: 5 in the probiotic (median 57 (range 43-67) yrs, 60% male) and 8 in the placebo group (median 50 (range 33-73) yrs, 63% male).

In the probiotic group, 11 patients used maintenance therapy for UC: sulfasalazine (n=2), mesalazine (n=7) and azathioprine in combination with sulfasalazine or mesalazine (n=2). In the placebo group, 13 patients were on maintenance therapy: sulfasalazine (n=4), mesalazine (n=5) and azathioprine in combination with sulfasalazine or mesalazine (n=4). The use of different types of maintenance therapy was equally distributed in the probiotic and the

placebo group. Moreover, dose and type of medication did not change during the study period.

Median defaecation frequency of UC patients in this study was twice per day while the median faeces consistency score was 5 according to the Bristol scale (defined as 'soft blubs') indicating that the UC patients had loose faecal samples. No significant differences were observed in defaecation frequency and faeces consistency scores between probiotic and placebo group before or after consumption or within groups over time (table 6.1).

Table 6.1 Defaecation frequency and faeces consistency scores according to the Bristol scale (from 1: hard lumps, to 7: watery diarrhoea) before and after consumption of probiotic or placebo drinks (median and range).

	Probiotic		Placebo	
	before	after	before	after
Consistency	4.0 (4-7)	4.5 (3-7)	5.0 (2-7)	5.0 (4-7)
Frequency	2.0 (1-7)	2.0 (0-4)	2.0 (0-10)	3.0 (0-6)

Baseline comparison of UC patients and healthy volunteers

Faecal culture results found in this study were compared to the results of 20 healthy volunteers included in a previous study using the same method of collection of the faecal samples and of bacterial culture⁴¹. These healthy volunteers were significantly younger compared to the UC patients (32 (20-57) versus 50 (33-73) years, $p < 0.05$) while no significant difference in gender was observed (45% versus 59% male).

The median defaecation frequency (2 (0-10) versus 1 (0-2), $p = 0.005$) and faeces consistency score (5 (2-7) versus 4 (2-6), $p = 0.002$) were significantly different in the UC patients compared to the healthy volunteers indicating that inactive UC patients had more frequent and more loose stools. To correct for the differences in faeces consistency, faecal bacterial concentrations were calculated per mg protein (table 6.2).

In the UC patients, faecal concentrations of total anaerobic bacteria, *Bacteroides* spp. and lactic acid bacteria were significantly lower compared to those of the healthy volunteers. Prevalence of bacteria in faecal samples was comparable in UC patients to healthy controls, except for *E. coli* (70 versus 95%, $p < 0.05$) (table 6.3).

Comparison of the faecal bacterial concentrations found in this study in relation to the type of maintenance medication did not show significant differences, with the exception of the concentration of enterococci, which was significantly lower in patients using mesalazine ($p = 0.032$) compared to patients not taking maintenance therapy ($\log 2.9 \pm 0.4$ versus 4.6 ± 0.7 CFU/ g faeces).

Table 6.2 Concentrations of faecal bacteria (log CFU/mg protein) in UC patients (n=31) and healthy volunteers (n=20) before probiotic or placebo consumption (mean $\pm$ SEM).

	UC patients	Healthy volunteers
Total aerobic flora	5.5 $\pm$ 0.2	5.3 $\pm$ 0.2
<i>E. coli</i>	4.3 $\pm$ 0.4	4.7 $\pm$ 0.3
Enterococci	2.8 $\pm$ 0.4	2.9 $\pm$ 0.2
Total anaerobic flora	7.6 $\pm$ 0.1 ^a	8.2 $\pm$ 0.1
<i>Bacteroides</i> spp.	5.1 $\pm$ 0.3 ^a	6.6 $\pm$ 0.2
Clostridia	2.5 $\pm$ 0.2	2.5 $\pm$ 0.3
Lactic acid bacteria	2.6 $\pm$ 0.3 ^a	5.5 $\pm$ 0.5
Lactobacilli	2.8 $\pm$ 0.3	3.5 $\pm$ 0.3

^a statistically significant difference ($p < 0.05$) between UC patients and healthy volunteers.

Table 6.3 Percentages of faecal samples of UC patients and healthy volunteers in which specific bacteria could be cultured.

	UC patients	Healthy volunteers
Total aerobic flora	97%	100%
<i>E. coli</i>	70% ^a	95%
Enterococci	77%	95%
Total anaerobic flora	100%	100%
<i>Bacteroides</i> spp.	94%	100%
Clostridia	81%	80%
Lactic acid bacteria	66%	90%
Lactobacilli	90%	100%

^a statistically significant difference ($p < 0.05$) between UC patients and healthy volunteers.

Effect of *L. plantarum* 299v on culture results

Culture results of the UC patients before and after probiotic or placebo consumption are shown in table 6.4. Before start of the treatment period, the concentration of total aerobic bacteria was significantly lower in the probiotic group compared to the placebo group while the other bacterial concentrations were comparable in both groups.

At the last day of *L. plantarum* 299v intake, a significant increase in the faecal number of lactic acid bacteria ($p = 0.012$) and lactobacilli was observed ($p = 0.005$) (table 6.4), reaching mean levels up to log 5.6 $\pm$ 0.2 CFU/mg protein. No other changes in faecal bacterial concentrations were observed after *L. plantarum* 299v or placebo intake.

The mucosal prevalence (i.e. percentage of persons with culture positive biopsies) after the treatment period is shown in figure 6.1. The prevalence of total anaerobic bacteria was 100%, while total aerobic bacteria, lactic acid bacteria, lactobacilli and *E. coli* were cultured in lower prevalences in both

groups (14-63%). Enterococci and *Bacteroides* spp. were not cultured in the probiotic-treated group while *Bacteroides* spp. was detected in 63% of the biopsy samples collected in the placebo group. Clostridia was not cultured in both the probiotic-treated and the placebo-treated group.

The mean concentrations of mucosal bacteria are shown in table 6.5. In general, the concentration of bacteria cultured from rectal biopsies was relatively low (ranging from log 1.0 to 4.7 CFU/biopsy). The mucosal concentration of total anaerobic bacteria was significantly lower in the probiotic compared to the placebo group ($p=0.019$) after consumption.

Table 6.4 Concentrations of faecal bacteria (log CFU/mg protein) before and after consumption of probiotic or placebo drinks (mean $\pm$ SEM).

	Probiotic		Placebo	
	before	after	before	after
Total aerobic flora	5.2 $\pm$ 0.3 ^a	5.9 $\pm$ 0.3	5.8 $\pm$ 0.1 ^a	5.6 $\pm$ 0.2
<i>E. coli</i>	3.6 $\pm$ 0.6	3.8 $\pm$ 0.5	4.9 $\pm$ 0.5	4.8 $\pm$ 0.5
Enterococci	2.5 $\pm$ 0.6	2.4 $\pm$ 0.5	3.0 $\pm$ 0.5	3.0 $\pm$ 0.5
Total anaerobic flora	7.5 $\pm$ 0.2	7.3 $\pm$ 0.3	7.6 $\pm$ 0.2	7.2 $\pm$ 0.5
<i>Bacteroides</i> spp.	4.8 $\pm$ 0.5	5.0 $\pm$ 0.6	5.4 $\pm$ 0.3	4.9 $\pm$ 0.5
Clostridia	2.5 $\pm$ 0.3	2.4 $\pm$ 0.4	2.4 $\pm$ 0.3	2.2 $\pm$ 0.3
Lactic acid bacteria	2.9 $\pm$ 0.5	5.5 $\pm$ 0.3 ^b	2.5 $\pm$ 0.3	4.3 $\pm$ 0.5
Lactobacilli	2.5 $\pm$ 0.4	5.6 $\pm$ 0.2 ^b	3.0 $\pm$ 0.5	3.3 $\pm$ 0.5

^a statistically significant difference ($p<0.05$) between probiotic and placebo group;

^b statistically significant difference ($p<0.05$) before versus after probiotic intake.

Figure 6.1 Percentages of biopsy samples in which specific bacteria could be cultured after probiotic and placebo consumption.

Table 6.5 Mucosal concentration of bacteria (log CFU/biopsy) after probiotic or placebo intake (mean $\pm$ SEM). n.d. means "not detected".

	Probiotic	Placebo
Total aerobic flora	2.9 $\pm$ 1.6	3.5 $\pm$ 0.8
<i>E.coli</i>	1.0	4.7 $\pm$ 0.8
Enterococci	n.d.	2.38
Total anaerobic flora	2.3 $\pm$ 0.4 ^a	4.0 $\pm$ 0.4 ^a
<i>Bacteroides</i> spp.	n.d.	3.2 $\pm$ 0.4
Clostridia	n.d.	n.d.
Lactic acid bacteria	2.0 $\pm$ 0.1	2.2 $\pm$ 0.4
Lactobacilli	2.2 $\pm$ 0.03	1.0

^a statistically significant difference ($p < 0.05$) between probiotic and placebo group.

Effect of *L. plantarum* 299v on bacterial 'fingerprints'

TTGE banding patterns were available for 12 rectal biopsy samples: 5 from the probiotic and 7 from the placebo group. No significant differences could be found in the similarity patterns comparing these two groups. The samples of the different treatment groups were intermixed and no clustering could be found per group (i.e. probiotic or placebo) (figure 6.2).

Figure 6.2 TTGE banding patterns of the dominant bacteria in rectal biopsy samples of 5 probiotic and 7 placebo-treated UC patients after clustering analysis. No clustering per treatment group was seen.

Effect of *L. plantarum* 299v on enzyme activities, short chain fatty acid and endotoxin concentrations

Faecal β -glucosidase and β -glucuronidase activities did not differ significantly before and after consumption of the drinks in either the probiotic or placebo group, nor within groups over time (table 6.6). SCFA concentrations did not change due to probiotic intake (table 6.7) and no significant differences in bacterial endotoxin concentrations were observed between and within probiotic and placebo group (figure 6.3).

Table 6.6 β -glucosidase (mg/60 min/g faeces) and β -glucuronidase (mg/30 min/g faeces) activities before and after probiotic or placebo consumption (mean $\pm$ SEM).

	Probiotic		Placebo	
	before	after	before	after
β -glucosidase	0.67 $\pm$ 0.15	0.76 $\pm$ 0.13	0.59 $\pm$ 0.11	0.59 $\pm$ 0.11
β -glucuronidase	1.47 $\pm$ 0.37	1.17 $\pm$ 0.25	0.90 $\pm$ 0.22	0.38 $\pm$ 0.11

Table 6.7 Short chain fatty acid concentrations (mmol/g faeces) measured before and after probiotic or placebo intake (mean $\pm$ SEM).

	Before	After
Acetic acid		
Probiotic	67.7 $\pm$ 7.3	85.2 $\pm$ 11.4
Placebo	66.1 $\pm$ 9.3	61.9 $\pm$ 10.6
Propionic acid		
Probiotic	20.3 $\pm$ 4.1	21.6 $\pm$ 2.4
Placebo	19.1 $\pm$ 2.4	19.4 $\pm$ 3.5
Isobutyric acid		
Probiotic	1.46 $\pm$ 0.19	1.53 $\pm$ 0.20
Placebo	1.97 $\pm$ 0.50	1.71 $\pm$ 0.30
Butyric acid		
Probiotic	17.3 $\pm$ 2.4	18.5 $\pm$ 2.5
Placebo	23.9 $\pm$ 4.92	24.8 $\pm$ 5.64
Isovaleric acid		
Probiotic	1.65 $\pm$ 0.23	1.92 $\pm$ 0.28
Placebo	1.99 $\pm$ 0.39	1.80 $\pm$ 0.43
Valeric acid		
Probiotic	2.63 $\pm$ 0.52	2.42 $\pm$ 0.29
Placebo	2.65 $\pm$ 0.48	2.18 $\pm$ 0.40
Isocaproic acid		
Probiotic	0.62 $\pm$ 0.22	0.76 $\pm$ 0.31
Placebo	0.81 $\pm$ 0.18	0.43 $\pm$ 0.03
Caproic acid		
Probiotic	1.34 $\pm$ 0.27	1.38 $\pm$ 0.30
Placebo	1.23 $\pm$ 0.11	1.64 $\pm$ 0.45

Figure 6.3 Endotoxin concentrations (log ng/ml faecal water) before and after probiotic or placebo consumption (mean $\pm$ SEM).

Discussion

In the present study, it has been demonstrated that the bacterial composition of inactive UC patients is significantly different from that of healthy volunteers, as lower bacterial concentrations of total anaerobic bacteria, *Bacteroides* spp. and lactic acid bacteria were found in the faecal samples of the UC patients.

Four-weeks treatment of the UC patients with *L. plantarum* 299v compensated the low concentration of lactobacilli and lactic acid bacteria, while no effect on other faecal and mucosal bacterial concentrations or metabolites was observed. A remarkable finding was the absence of mucosal *Bacteroides* spp. in the probiotic-treated group, while they were detected in 63% of the biopsy samples collected in the placebo group.

The lower bacterial concentrations in both faecal and mucosal samples of the UC patients compared to healthy controls, confirm the results of published studies^{10,14,15}. These differences in bacterial concentration could be due to the disease and/or of its treatment as the majority of UC patients included in this study were on maintenance therapy. Sulfasalazine, for instance, is known to decrease the concentration of *E. coli* in inactive UC patients⁴⁹. Comparison of the faecal bacterial concentrations per type of maintenance therapy in this study did not show major differences. However, the subgroups of patients using different types of maintenance therapy were small and a larger study investigating the role of maintenance therapy on the intestinal flora is warranted. Moreover, the UC patients were significantly older compared to the healthy volunteers. Exclusion of the culture results of the three UC patients

above 60 years did not influence the results (data not shown) indicating that age is not expected to influence the faecal culture results significantly.

L. plantarum 299v significantly increased the faecal concentration of lactic acid bacteria and lactobacilli but did not affect the other faecal and mucosal bacterial concentrations drastically. These findings were supported by the results of the TTGE analyses as no treatment specific patterns in the dominant bacteria could be found. The significantly lower concentration in the probiotic group of faecal total aerobic bacteria before and mucosal total anaerobic bacteria after *L. plantarum* 299v intake cannot be explained. Remarkably, there was a clear reduction in the mucosal prevalence of *Bacteroides* spp. after *L. plantarum* 299v intake. This suppression of *Bacteroides* spp. by *L. plantarum* 299v is potentially beneficial as UC patients have increased concentrations of *Bacteroides* spp. and increased antibody responses against *Bacteroides vulgatus*^{10,14,50,51}. Further investigation of possible changes in subspecies of *Bacteroides* as well as the role of host factors in the adhesion of *Bacteroides* spp. may lead to further insights in the effects of probiotic bacteria on the *Bacteroides* spp. However, a reduction in the prevalence of *Bacteroides* spp. due to *L. plantarum* 299v intake was not seen in a previous study including 29 subjects undergoing polyp control⁵². In the present study, biopsy samples were only collected after the intervention and the number of biopsies collected in the study was small, indicating that collection of more biopsy samples both before as well as after intervention is needed.

The difference in the bacterial composition in UC patients versus healthy controls can be supported by differences in endotoxin concentrations (data not shown), as endotoxin concentrations in UC patients were found to be significantly higher compared to the healthy controls⁴¹. A higher endotoxin level suggests a higher release of cell wall components of Gram-negative bacteria such as *E. coli*^{17,53,54}. However, the faecal bacterial concentration of *E. coli* was decreased in the UC patients compared to healthy controls in the present study suggesting that the increased faecal endotoxin concentration is either based on non-viable *E. coli*, or on other Gram-negative bacteria^{17,53,54}.

Comparing the bacterial composition in the mucosa between UC patients and healthy volunteers would be of additional value but for ethical and practical reasons biopsy samples were not collected in the healthy persons in the present study. However, in a previous study the mucosal bacterial composition of subjects undergoing polyp control was analysed⁵². Compared to these results, UC patients had a lower prevalence in the mucosa of bacteria, especially of *E. coli* (probiotic group 20% versus 87% and in the placebo group 43% versus 93%) indicating that also the mucosal bacterial composition is different in UC patients compared to healthy controls. An explanation for this low mucosal prevalence may be changes in the mucosa or shorter transit times in the UC patients resulting in lower colonisation rates of mucosal bacteria⁵⁵⁻⁵⁷. The present study did not demonstrate drastic changes in the bacterial composition due to *L. plantarum* 299v intake. This is in line to previous studies,

which observed only an increment in the faecal concentrations of the bacteria, which were given as probiotic strain^{31,58}. Several clinical trials showed a potential role of probiotic bacteria, for instance as maintenance therapy in IBD patients^{28-31,59}.

The effects of *L. plantarum* 299v on the immune system were not investigated in the present study, although modulation of the immune system can be another mode of action and may be of benefit for UC patients as they have an altered immune response (exaggerated T helper 2 response and overproduction of IL-4 and IL-5)^{4,60-62}. Animal studies showed the immunomodulating properties of several probiotic strains such as *L. plantarum*, *Lactobacillus salivarius* and *Bifidobacterium infantis* and a recent trial demonstrated clinical improvement of UC patients after an immunomodulating therapy by *Trichuris suis*^{32,63-69}. The immunomodulating properties of probiotics need to be studied in future.

In conclusion, this study showed that inactive UC patients differed in faecal bacterial composition from healthy controls. Intake of the probiotic *L. plantarum* 299v during four weeks significantly increased the concentration of lactobacilli. More well-designed and controlled studies are required to investigate the role of intestinal bacteria in UC and to investigate the potential of *L. plantarum* 299v as treatment. In such studies, the intake of medication has to be considered because of possible confounding. Moreover, other possible modes of action of probiotics need to be studied such as the influence on the immune system.

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Chapter 7

General discussion and conclusions

Introduction

Results of probiotic trials are encouraging for the promotion of general health but also as alternative or supplementary therapy for various gastrointestinal diseases, in which the intestinal flora may be disturbed (chapter 1). However, results of well-designed blinded placebo-controlled probiotic studies, also focusing on possible modes of actions, have to be awaited, before rational recommendations about the use of probiotics can be done (chapter 1). One of the potential modes of action is the replacement of intestinal bacteria by the probiotic strains and the modulation of the bacterial metabolic activities.

In this thesis, five double-blind placebo-controlled studies have been described, focusing on the effects of the probiotic *L. plantarum* 299v on the composition of the faecal and mucosal intestinal flora and on faecal metabolic activities, in healthy subjects with a 'normal' flora as well as in patients with intestinal disorders with a potentially disturbed flora.

Effect of *L. plantarum* 299v on faecal lactobacilli

All studies described in this thesis showed that oral application of *L. plantarum* 299v significantly increased the concentration of lactobacilli (table 7.1) and documented the good survival of *L. plantarum* 299v in the gastrointestinal tract. This survival was irrespective of gastric acidity, as pharmacological increment of the intragastric pH did not result in an increase in the faecal lactobacilli concentration (chapter 3). One week after start of the probiotic drinks, *L. plantarum* 299v could be identified in the faecal samples, paralleling the increase in the concentration of lactobacilli (chapter 2). One week after cessation of *L. plantarum* 299v, the lactobacilli concentration decreased and *L. plantarum* 299 could not be identified anymore in the faecal samples from the probiotic group demonstrating that no prolonged colonisation was achieved (chapter 2).

Table 7.1 Concentration of lactobacilli (log CFU/g faeces) before, during and after *L. plantarum* 299v intake (daily dose 10^{11} CFU) in the five studies described in this thesis (mean $\pm$ SEM). n.m. means "not measured".

	before	last day of intake	4 weeks after cessation
Chapter 2 (healthy volunteers)	4.6 $\pm$ 0.3	8.1 $\pm$ 0.1	4.4 $\pm$ 0.4
Chapter 3 (healthy volunteers+pantoprazole)	4.4 $\pm$ 0.3	7.9 $\pm$ 0.1	4.4 $\pm$ 0.3
Chapter 4 (bowel cleansing)	4.7 $\pm$ 0.5	6.8 $\pm$ 0.3	4.6 $\pm$ 0.4
Chapter 5 (controls for polyps)	5.0 $\pm$ 0.4	7.1 $\pm$ 0.3	n.m.
Chapter 6 (UC patients)	3.4 $\pm$ 0.4	7.1 $\pm$ 0.2	n.m.

The increase in lactobacilli concentration found in the five present studies ranged from log 2.1 to 3.7 CFU/g faeces and was lowest in the subjects undergoing polyp control (chapter 5) and in the patients who underwent bowel

cleansing (chapter 4) and was highest in the UC patients (chapter 6) (table 7.1). The magnitude of the increase seems to be influenced by the pre-treatment concentration: a lower pre-treatment concentration was accompanied by a higher increase. Such a low pre-treatment concentration can be caused by the disease or medication used and compensation can be beneficial, although the exact clinical importance still needs to be determined.

The magnitude of the increase in the concentration of lactobacilli was high compared to results from previous studies using *L. plantarum* 299v, *Lactobacillus rhamnosus* DR20 and *Lactobacillus casei* strain Shirota (increase of log 0.4, 0.6 and 1.3 CFU/g faeces, respectively)¹⁻³, which could be due to the probiotic strain used in the present studies and/or to the consumption of relatively high concentrations (daily dose 10¹¹ CFU).

The present studies demonstrated that *L. plantarum* 299v could not be identified anymore in faecal samples collected after cessation of the probiotic drinks. In contrast, a study performed in Sweden showed that *L. plantarum* 299v was still present in faecal samples in five of 26 healthy volunteers one week after cessation¹. The prolonged survival of *L. plantarum* 299v in the Swedish study might be due to a higher susceptibility to colonise *L. plantarum* 299v in Swedes, as the probiotic strain originally was isolated from this population. Host factors such as the mucus composition may also play a role in the colonisation. In addition, isolation of lactobacilli from faecal samples differed between the present studies and the Swedish one as in the present studies four lactobacilli have been isolated from the outer ring of the LAMVAB plates after inoculation with a spiral plater representing the dominant lactobacilli flora, while in the Swedish study lactobacilli with the appearance of *L. plantarum* 299v have been isolated from Rogosa agar plates¹.

Effect of *L. plantarum* 299v on other faecal bacteria

Intake of *L. plantarum* 299v did not influence other faecal bacteria with the exception of small increases (<log 1 CFU/g faeces) in total aerobic bacteria (chapter 3) and clostridia (chapter 5). An explanation for these differences could not be found. However, the clinical importance of these small changes is questionable, especially as the variation of the conventional culture method is log 0.5 CFU/g faeces.

In line with the culture results, no changes in the molecular 'fingerprints' based on the denaturing gradient gel electrophoresis (DGGE) analyses of the dominant faecal flora could be seen due to *L. plantarum* 299v intake or to bowel cleansing, indicating that the intestinal flora can be considered as relatively stable. In contrast, studies described in the literature observed changes in the composition of the intestinal flora after the intake of antibiotics, sulfasalazine or after major changes in diet (such as a switch to a vegetarian diet)⁴⁻⁷. Recently, a study demonstrated that intake of the antibiotic amoxycillin

drastically affected the composition of the dominant faecal flora⁸. These changes in the bacterial composition might be due to selective suppression of bacteria versus total non-selective washing out such as bowel cleansing.

Effect of *L. plantarum* 299v on mucosal bacteria

The consumption of *L. plantarum* 299v did not significantly influence the concentration or prevalence of lactobacilli in biopsies (chapter 5, 6). *L. plantarum* 299v could not be identified in the colon ascendens biopsies and only in two of the 15 rectum biopsies collected in the subjects undergoing polyp control (chapter 5), indicating that *L. plantarum* 299v did only colonise the mucosa of these patients to a minor degree. It has been shown in *in vitro* studies and in gnotobiotic rats that *L. plantarum* 299v competes with *E. coli* for mannose-specific receptors for adherence^{9,10}. However, a decrease in the mucosal concentration of *E. coli* was not seen in the present studies (chapter 5, 6).

Remarkably, *L. plantarum* 299v decreased the prevalence of *Bacteroides* spp. in the mucosa of UC patients (chapter 6). This decrease can be beneficial, as UC patients are known to have enhanced *Bacteroides* spp. concentrations¹¹⁻¹³. However, this decrease was not seen in the subjects undergoing polyp control or in faecal samples (chapter 5). Investigation of the effect of *L. plantarum* 299v intake on the subspecies of *Bacteroides* is needed.

Furthermore, the intake of *L. plantarum* 299v did not change other mucosal bacterial prevalences or concentrations and molecular 'fingerprints' of the dominant bacteria in rectal biopsies, except for the concentration of total anaerobic bacteria, that became significantly higher in the subjects undergoing polyp control (chapter 5) and significantly lower in the UC patients after *L. plantarum* 299v intake (chapter 6). This difference may be due to differences in bowel cleansing (total bowel cleansing (chapter 5) versus enema (chapter 6)). However, it has been demonstrated in chapter 4 that bowel cleansing did not change the faecal bacterial composition significantly based on culture as well as on molecular results.

Comparing the mucosal prevalence and concentrations after *L. plantarum* 299v intake in subjects undergoing polyp control versus UC patients (chapter 5, 6) showed that both prevalence and concentrations of most bacterial species tend to be lower in the UC patients (figure 7.1). This lower mucosal prevalence found, may be caused by differences in the UC patients in the mucus composition, the metabolic activity or the intestinal transit time¹⁴⁻¹⁶. A thinner mucus layer has been reported in UC patients possibly resulting in less adherence sites for bacteria^{17,18}. However, bacterial prevalences and concentrations found in chapter 5 and 6 have not been compared statistically as the number of collected biopsies was small.

Figure 7.1 Prevalence of mucosal bacteria in rectal biopsies collected from subjects undergoing polyp control (chapter 5) and inactive UC patients (chapter 6)

Lower bacterial prevalences were found in biopsy samples compared to faecal samples supporting the idea that host and/or bacterial factors are responsible for a selective adherence of bacteria to the mucosa. More insight into this selection process will be interesting in view of future attempts to modulate the intestinal flora. These differences between mucosal and faecal samples indicate that an analysis of both types of sample is needed to give further insight in the effects of probiotics on the bacterial composition (chapter 5, 6).

The study described in chapter 5 demonstrated that the bacterial composition of colon ascendens biopsies is comparable to rectum biopsies indicating that analysis of rectum biopsies seems to be sufficient to study effects of probiotics on the intestinal mucosal flora in a non-inflamed colon. The collection of rectum biopsies only is more preferable because of practical and ethical reasons and because intensive bowel cleansing is not needed prior to biopsy collection. However, the comparison of the bacterial composition in colon ascendens and rectum biopsies in the present studies has only been based on culture results and molecular analyses might be of additional value.

It is difficult to compare the mucosal results of the present studies to results found in the literature, as in those studies the samples were handled and processed differently. For example, some investigations washed the biopsies^{13,19,20}, while others did not^{12,21,22}. However, comparing the culture results, washing of the biopsy samples did not systematically result in lower bacterial concentrations and prevalence. Furthermore, the bacterial concentrations have in different studies been expressed in different units: log CFU/biopsy, log CFU/g tissue, log CFU/ μ l biopsy dilution or log CFU/mg protein. Protein concentrations in biopsy samples have been analysed in the

study including subjects undergoing polyp control (chapter 5) but were below the detection level in the majority of the samples.

Effect of *L. plantarum* 299v on faecal metabolic activity

Consumption of *L. plantarum* 299v did not significantly influence β -glucosidase and β -glucuronidase activities (chapter 2, 5, 6), endotoxin concentrations (chapter 2, 6), SCFA concentrations in faeces (chapter 2, 3, 6), or the pH of faecal water (chapter 2, 3). Nor did *L. plantarum* 299v intake influence defaecation frequency and faeces consistency scores according to the Bristol scale in the five studies.

An increase in SCFA concentrations due to *L. plantarum* 299v intake could probably not be found because SCFAs are excreted in the faeces for only 5% suggesting that an increase in SCFA production may result in an increase in absorption and not in excretion²³. Furthermore, higher amounts of carbohydrates are present in the proximal compared to the distal colon, increasing the bacterial fermentation as well as the production of SCFA possibly more proximal. For this reason, measuring SCFA concentrations both proximal and distal in the mucosa will give further insight in the effects of *L. plantarum* 299v.

The SCFA concentration before treatment did not differ comparing the two healthy volunteer populations (chapter 2, 3). Unfortunately, the pre-treatment SCFA concentrations and also the enzyme activities found in the healthy volunteers could not be compared to the results found in the UC patients because of variation in the methods and the use of new equipment in the course of the studies. However, pre-treatment endotoxin concentrations found in healthy volunteers (chapter 2) could be compared to the UC patients (chapter 6) and showed a significantly higher concentration in the UC patients (UC: $\log 2.0 \pm 0.1$ versus healthy volunteers: 1.5 ± 0.03 ng/ml faecal water, $p=0.003$). This is probably caused by a higher release of cell wall components of Gram-negative bacteria.

In literature, changes in metabolic activities after probiotic intake are described in healthy volunteers such as a decrease as well as an increase in β -glucosidase activity^{2,24}, and an increase in acetic and propionic acid after probiotic consumption¹. However, some of these studies used other probiotic strains such as *L. casei* and *L. acidophilus*, which may be the reason of these different results compared to the studies described in this thesis.

In the present studies a subset of metabolites have been investigated based on results described in the literature. Measuring other microflora-associated characteristics such as cholesterol metabolism, transformation of bilirubin, trypsin activity, degradation of β -aspartylglycine and the degradation of mucin

may give further insight in the effects of *L. plantarum* 299v on the metabolic activity of the faecal flora²⁵.

Comparison of pre-treatment faecal bacterial concentrations

Faecal samples collected in the five studies were handled in a similar way and similar culture methods were used, which allows a comparison of the pre-treatment concentrations (table 7.2).

Table 7.2 Pre-treatment concentrations of bacteria (log CFU/g faeces) cultured before probiotic or placebo intake (mean $\pm$ SEM)

	Healthy volunteers (chapter 2)	Healthy volunteers + pantoprazole (chapter 3)	Bowel cleansing (chapter 4)	Controls for polyps (chapter 5)	UC patients (chapter 6)
Total aerobic flora	7.0 $\pm$ 0.2	7.2 $\pm$ 0.2 ^a	6.7 $\pm$ 0.2	6.7 $\pm$ 0.2	6.8 $\pm$ 0.2
<i>E. coli</i>	6.1 $\pm$ 0.3	6.8 $\pm$ 0.2 ^b	6.2 $\pm$ 0.3	6.0 $\pm$ 0.2	5.2 $\pm$ 0.4
Enterococci	4.4 $\pm$ 0.3 ^d	4.7 $\pm$ 0.3 ^c	3.7 $\pm$ 0.3	4.3 $\pm$ 0.2	3.8 $\pm$ 0.3
Total anaerobic flora	9.8 $\pm$ 0.1 ^e	9.6 $\pm$ 0.1 ^e	8.3 $\pm$ 0.3	8.4 $\pm$ 0.2	9.0 $\pm$ 0.1 ^g
<i>Bacteroides</i> spp.	8.2 $\pm$ 0.2 ^e	7.7 $\pm$ 0.2 ^e	5.9 $\pm$ 0.4	6.3 $\pm$ 0.2	6.4 $\pm$ 0.3
Clostridia	4.1 $\pm$ 0.3	5.0 $\pm$ 0.2 ^f	4.4 $\pm$ 0.2	4.2 $\pm$ 0.1	3.7 $\pm$ 0.2
Lactic acid bacteria	7.1 $\pm$ 0.5 ^e	6.4 $\pm$ 0.4 ^c	5.1 $\pm$ 0.3	5.5 $\pm$ 0.3	3.4 $\pm$ 0.2
Lactobacilli	5.1 $\pm$ 0.3 ^d	4.5 $\pm$ 0.2	4.9 $\pm$ 0.4 ^d	4.9 $\pm$ 0.2	4.0 $\pm$ 0.3

^a significant increase compared to controls for polyps and patients bowel cleansing

^b significant increase compared to controls for polyps and UC patients

^c significant increase compared to patients bowel cleansing and UC patients

^d significant increase compared to UC patients

^e significant increase compared to controls for polyps, patients bowel cleansing and UC patients

^f significant increase compared to healthy volunteers (chapter 2), controls for polyps, patients bowel cleansing and UC patients

^g significant increase compared to controls for polyps

Comparison of the bacterial concentrations of the two studies that included healthy volunteers demonstrated similar pre-treatment bacterial concentrations with the exception of the concentration of clostridia, which was significantly higher in the subjects who took pantoprazole or placebo in addition to the probiotic (chapter 3). These two populations are comparable concerning gender, age and bowel habits (defaecation frequency and faeces consistency scores) (chapter 2, 3). However, the studies including patients (chapter 4, 5, 6) are different compared to the healthy volunteers studies concerning age (healthy volunteers were younger) and bowel habits (patients had more loose faecal samples). Comparison of the pre-treatment bacterial concentrations between the healthy volunteers and the patient populations demonstrated that healthy volunteers have significantly higher concentrations for almost all

bacteria (table 7.2). Remarkably are the low pre-treatment bacterial concentrations found in the UC patients compared to the healthy volunteers as well as to the other patient populations. As the UC patients had significantly more loose faecal samples, the concentrations were corrected for dilution (log CFU/mg protein)(chapter 6). After this correction, the concentrations were still significantly lower, which indicates that disease and/or medication used may influence the bacterial concentrations found. More insight in the bacterial composition and the role of the intestinal flora in UC is still warranted.

The pre-treatment faecal bacterial concentrations found in the present studies including healthy volunteers were comparable to the results described in the literature¹⁻³, except for the concentration of lactobacilli, which was significantly lower in our studies^{1,2}. This lower concentration might be due to the use of a more selective LAMVAB agar plate versus the less selective Rogosa agar plate mostly used¹⁻³, as Rogosa agar plates also support the growth of other acid-resistant bacteria, such as bifidobacteria, corynebacteria and enterococci²⁶. Moreover, studies described in this thesis cultured the bacteria quantitatively using a spiral plater whilst several previous studies used semi-quantitative methods such as a standard loop method²⁷. Finally, differences in bacterial concentrations may be explained by differences in processing as faecal samples were processed within 12 hours after defaecation while in some previous studies the faecal samples were stored in the freezer.

Concluding remarks

In the present studies bacterial concentrations and prevalence have been based on culture techniques. One of the major disadvantages of this method is that not all bacteria are identified and can be cultured yet. However, conventional culture techniques are well established and frequently used to investigate the bacterial composition quantitatively, making comparisons to results described in the literature possible. The use of molecular techniques such as DGGE, fluorescent in situ hybridisation (FISH) and real-time polymerase chain reaction (PCR) are of additional value to get a more complete picture of the complex microbial ecosystem of bacteria present in the intestine. Disadvantages of these techniques are that also non-viable bacteria will be analysed and that these techniques, with the exception of real-time PCR, are not quantitative^{3,28}. Moreover, molecular techniques can be biased as not all intestinal bacteria will lyse and amplify just as easy.

The studies described in this thesis investigated the effect of a monospecies probiotic, *L. plantarum* 299v. Several studies in the literature used different monospecies or even multispecies probiotics. Probiotic strains might operate in different ways and it is unlikely that one single probiotic strain will be (equally) effective for all clinical indications. More studies are warranted to investigate

modes of action of these different bacterial strains separately or as a multispecies product.

Based on the results described in this thesis, *L. plantarum* 299v did not change the intestinal flora profoundly. Apart from affecting the intestinal flora, probiotics may modulate the immune system. Indeed, *L. plantarum* 299v was found to have immunomodulatory properties such as an increase of serum IgA against *E. coli* in gnotobiotic rats after incubation with *E. coli* and *L. plantarum* 299v^{10,29}. Moreover, *L. plantarum* 299v has been shown to reduce colitis in IL-10 deficient mice and experimental rats^{29,30} indicating that future studies should also include immunological parameters to get further insight in the immunomodulatory effects of probiotics.

General conclusions

- The recovery of *L. plantarum* 299v in faeces is high indicating that *L. plantarum* 299v survives passage through the gastrointestinal tract.
- Intake of *L. plantarum* 299v increased the concentration of lactobacilli in faeces, and has minor influence on other bacterial concentrations or metabolic activity.
- Both faecal and mucosal samples need to be collected to study the effects of *L. plantarum* 299v on the intestinal flora.
- UC patients have a significantly different bacterial composition compared to healthy controls.

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Chapter 8

Summary

Summary

This thesis describes the effects of the probiotic *L. plantarum* 299v on the composition of the faecal and mucosal intestinal flora and faecal metabolites in healthy volunteers as well as in patients with a possibly disturbed intestinal flora.

Chapter 1 critically reviews the literature about the effects of probiotics in gastrointestinal disorders such as diarrhoea, inflammatory bowel disease, pouchitis, irritable bowel syndrome, lactose malabsorption, *Helicobacter pylori* infection, constipation and colorectal cancer. The composition and functions of the human microflora as well as the environmental factors that might influence this microflora are described. The definition of probiotics and possible modes of actions are also reported. In this chapter, it is postulated that the results of probiotic studies are encouraging although more well-designed placebo-controlled studies with sufficient numbers of clinically well-defined patients are required. More attention should be paid to the onset and duration of probiotic effects, the survival of probiotic strains and the differences between probiotic strains (mono-, versus multispecies). Moreover, the effects of probiotics on the bacterial composition but also on its metabolites need to be studied to get further insight in these possible modes of action.

Finally, this chapter summarizes studies investigating the effects of *L. plantarum* 299v *in vitro*, in animal models and in human trials. It can be concluded that *L. plantarum* 299v is a good candidate to study probiotic effects as this probiotic strain can survive passage through the gastrointestinal tract and is able to adhere to the epithelium via mannose-specific receptors.

In **chapter 2** the effects of *L. plantarum* 299v on the faecal bacterial composition were investigated in healthy volunteers. The bacterial composition was studied by measuring the concentration of bacteria by culture and by measuring metabolic activities such as the enzyme activities (β -glucosidase, β -glucuronidase and azoreductase), the short chain fatty acid concentrations (SCFA), the endotoxin concentrations and the faecal pH. This placebo-controlled study also focused on the onset and the duration of the probiotic effects. In summary, *L. plantarum* 299v increased the lactobacilli concentration significantly but did not have a significant influence on the other faecal bacteria cultured, or on the metabolic activities. The increase in *L. plantarum* 299v concentration was noticed within one week after start of probiotic intake and disappeared within one week after cessation of intake.

Chapter 3 focuses on the survival of *L. plantarum* 299v through the gastrointestinal tract and describes a placebo-controlled double-blind study with healthy volunteers. These healthy persons took a proton pump inhibitor (pantoprazole) to decrease gastric acidity, or a placebo in addition to *L. plantarum* 299v intake. Decrement of the gastric acidity might result in a better survival of *L. plantarum* 299v resulting in a higher faecal lactobacilli

concentration and changes in the faecal metabolic activity (i.e. SCFA concentrations and pH). However, the increase in the lactobacilli concentration was in the pantoprazole group as high as in the placebo group and no changes in metabolic activity were seen. Lactobacilli cultured from faecal samples were identified as *L. plantarum* 299v by molecular analyses. This study demonstrates a good survival of *L. plantarum* 299v irrespective of the gastric acidity.

In **chapter 4** the results of a placebo-controlled double-blind study including patients who had to undergo a bowel cleansing procedure are presented. This study investigated the effects of the bowel cleansing on the faecal bacterial composition and paid special attention to the eventually beneficial effects of *L. plantarum* 299v in the recolonisation phase after bowel cleansing. The bacterial composition has been studied quantitatively by culture as well as by denaturing gradient gel electrophoresis producing bacterial molecular 'fingerprints' of the dominant faecal flora.

No major changes were observed in the bacterial composition after the bowel cleansing procedure and therefore it was concluded that the intestinal flora is relatively stable. Probiotic intake increased the faecal concentration of lactobacilli significantly but did not influence other bacterial concentrations or the bacterial composition based on culture results and bacterial 'fingerprints'.

As the bacterial composition found in faecal samples may not represent that of the mucosa, a placebo-controlled study was performed to compare the effects of *L. plantarum* 299v on the faecal versus mucosal bacterial composition. Moreover, as probiotic effects may differ along the gastrointestinal tract, biopsies have been collected and compared from colon ascendens and rectum (**chapter 5**). Patients who had to undergo a colonoscopic control for colorectal polyps were included and consumed *L. plantarum* 299v during two weeks prior to colonoscopy. Intake of *L. plantarum* 299v increased the lactobacilli concentration in faecal samples significantly, while *L. plantarum* 299v could be found sporadically in mucosal samples. The low mucosal prevalence of *L. plantarum* 299v, which is in contrast to a previously performed study in Sweden, needs further study to get more insight in the possible modes of action of this probiotic strain. The bacterial composition of rectum biopsies did not differ significantly from colon ascendens biopsies suggesting that in future studies, collection of rectal biopsies only may be sufficient to study effects of probiotics on the mucosal intestinal flora. However, the faecal bacterial composition differed significantly from the mucosal bacterial composition as the mucosal prevalence of several aerobic and anaerobic bacteria was significantly lower compared to that found in faecal samples. In future studies both samples need to be collected.

In **chapter 6** differences in bacterial composition between inactive UC patients on a stable dose of maintenance therapy and healthy volunteers were compared by culture. Moreover, the effects of *L. plantarum* 299v on the

intestinal flora in faecal and mucosal samples as well as on faecal metabolic activities (enzyme activities, endotoxin concentrations and SCFA concentrations) were studied in these patients. As the UC patients had significantly more loose faecal samples, the bacterial concentration was corrected by expressing the concentrations as log CFU/mg protein in faeces. Faecal bacterial concentrations of UC patients were significantly lower compared to those of the healthy controls. These differences may be due to either the disease or the intake of medication.

Besides the significant increase in the lactobacilli concentration in faecal samples after *L. plantarum* 299v intake, no other changes in the bacterial composition of faecal samples or rectal biopsies could be observed based on culture and temporal temperature gel electrophoresis. Nor did *L. plantarum* 299v intake change metabolic products in faecal samples significantly. Remarkably, *Bacteroides* spp. was not present in mucosal samples in the probiotic group while the prevalence was relatively high in the placebo group indicating that probiotic intake might reduce *Bacteroides* spp. prevalence in the mucosa of UC patients, which can be beneficial for these patients. As the intake of *L. plantarum* 299v did not profoundly change the bacterial composition, more studies are warranted to investigate other modes of action of probiotics such as modulating the immune system.

In conclusion, this thesis shows that intake of *L. plantarum* 299v increased the lactobacilli concentration of faecal samples significantly. The clinical consequence of the increase in lactobacilli requires further study. No other changes in the faecal bacterial concentrations or bacterial 'fingerprints' were seen after *L. plantarum* 299v intake. No changes in metabolic activities were measured in faecal samples, or in the bacterial composition of mucosal samples comparing probiotic to placebo group. As *L. plantarum* 299v did not change the intestinal flora in the five studies described in this thesis, the bacterial composition can be regarded as relatively stable. However, this thesis also demonstrates that the bacterial composition of UC patients is significantly different from healthy persons.

The modes of acting of *L. plantarum* 299v in the gastrointestinal tract need further study focusing on the mucosal adherence, the influences on the mucosal metabolic activities and the effects on the immune system.

Samenvatting

Samenvatting

Dit proefschrift beschrijft de effecten van het probioticum *L. plantarum* 299v op de samenstelling van de faecale en mucosale darmflora en faecale metabolieten van gezonde vrijwilligers en patiënten met een mogelijk verstoorde darmflora.

Hoofdstuk 1 geeft een kritisch overzicht van de literatuur die bekend is over de effecten van probiotica in gastro-intestinale ziekten zoals diarree, inflammatoire darmziekten, pouchitis, prikkelbare darm syndroom, lactose malabsorptie, *Helicobacter pylori* infectie, constipatie en colorectale kanker. De samenstelling en de functies van de humane microflora en de omgevingsfactoren die deze microflora kunnen beïnvloeden, worden beschreven. De definitie van probiotica en de mogelijke werkingsmechanismen daarvan komen ook aan de orde. In dit hoofdstuk wordt geconcludeerd dat de resultaten van probioticum-onderzoek bemoedigend zijn, hoewel meer goed opgezette placebo-gecontroleerde studies met voldoende aantallen goed geselecteerde patiënten noodzakelijk zijn. Daarnaast zou aandacht besteed moeten worden aan de aanvang en duur van probioticum-effecten, de overleving van probioticumstammen en het verschil in effecten van verschillende probioticumstammen (mono-, versus multispecies). Bovendien zouden de effecten van probiotica op de intestinale bacteriële samenstelling, maar ook op de metabolieten bestudeerd dienen te worden om een beter inzicht te krijgen in de mogelijke werkingsmechanismen. Dit hoofdstuk eindigt met een overzicht van *in vitro*, dier-, en humane studies die de effecten van *Lactobacillus plantarum* 299v beschrijven. Er wordt geconcludeerd dat *L. plantarum* 299v een goede kandidaat kan zijn om probioticum-effecten te bestuderen aangezien deze probioticumstam passage door het maagdarmkanaal kan overleven en in staat is om te hechten aan het epitheel via mannose-specifieke receptoren.

Hoofdstuk 2 beschrijft de effecten van *L. plantarum* 299v op de faecale bacteriële samenstelling van gezonde vrijwilligers. De bacteriële samenstelling is onderzocht door de concentraties van bacteriën vast te stellen met behulp van kweek en door de metabole activiteit te bepalen aan de hand van enzymactiviteiten (β -glucosidase, β -glucuronidase en azoreductase), alsmede concentraties van korte keten vetzuren (KKVZ) en endotoxinen en de pH in faeces. In deze placebo-gecontroleerde studie is tevens de aanvang en de duur van de effecten van het probioticum onderzocht. Samenvattend kan gezegd worden dat *L. plantarum* 299v de concentratie van lactobacilli significant laat stijgen, maar geen significant effect heeft op de overige bacteriën gekweekt in de faecesmonsters, noch op de metabole activiteiten. De stijging van de concentratie van *L. plantarum* 299v was reeds zichtbaar binnen een week na de start van probioticum inname en verdween binnen een week nadat gestopt was met het probioticum.

Hoofdstuk 3 richt zich op de overleving van *L. plantarum* 299v in het maagdarmkanaal en beschrijft een placebo-gecontroleerde studie met gezonde vrijwilligers. Deze gezonde personen namen een maagzuurremmer (pantoprazol) in om de zuurgraad in de maag te laten dalen, of een placebo naast de inname van *L. plantarum* 299v. Daling van de zuurgraad in de maag zou kunnen leiden tot een betere overleving van *L. plantarum* 299v, wat weer resulteert in een hogere concentratie van lactobacilli in de faecesmonsters en veranderingen in de metabole activiteiten (zoals KKVZ-concentraties en pH). De stijging in de concentratie van lactobacilli was echter in the pantoprazol-groep even hoog als in de placebo-groep en veranderingen in de metabole activiteiten werden niet waargenomen. Lactobacilli gekweekt uit de faecesmonsters werden geïdentificeerd als *L. plantarum* 299v met behulp van moleculaire technieken. Deze studie toont een goede overleving aan van *L. plantarum* 299v, onafhankelijk van de zuurgraad in de maag.

Hoofdstuk 4 presenteert de resultaten van een placebo-gecontroleerde dubbel-blinde studie, waarin patiënten werden geïncubeerd die een lavage dienden te ondergaan. Deze studie onderzocht de effecten van deze lavage op de bacteriesamenstelling in de faeces en onderzocht of toediening van *L. plantarum* 299v de herkolonisatie na lavage gunstig kon beïnvloeden. De bacteriesamenstelling is kwantitatief bestudeerd met behulp van kweek, maar ook met behulp van 'denaturing gradient gel electrophoresis' (DGGE), waarbij bacteriële moleculaire bandenpatronen van de dominante faecale flora worden gemaakt.

Er werden geen significante veranderingen waargenomen in de bacteriële samenstelling na de lavage en zodoende is er geconcludeerd dat de intestinale flora relatief stabiel is. Inname van probiotica verhoogde de faecale concentratie van lactobacilli significant, maar beïnvloedde de andere bacteriële concentraties of samenstelling niet.

Aangezien de bacteriële samenstelling gemeten in de faeces zou kunnen verschillen van de samenstelling gemeten in de mucosa, is een placebo-gecontroleerde studie uitgevoerd waarin de effecten van *L. plantarum* 299v op zowel de faecale als de mucosale bacteriesamenstelling werden bestudeerd (**hoofdstuk 5**). Aangezien de effecten van probiotica op verschillende plaatsen in het maagdarmkanaal zouden kunnen variëren, zijn bipten verzameld en vergeleken in zowel colon ascendens als rectum. Patiënten die een colonoscopie dienden te ondergaan ter controle voor colorectale poliepen, werden geïncubeerd en namen *L. plantarum* 299v gedurende twee weken vóór de colonoscopie. Door de inname van *L. plantarum* 299v steeg de concentratie van de lactobacilli in de faeces significant, terwijl *L. plantarum* 299v slechts sporadisch in de mucosale bipten geïdentificeerd kon worden. De lage prevalentie van *L. plantarum* 299v in de mucosa is niet in lijn met eerder onderzoek uitgevoerd in Zweden, en zal verder onderzocht dienen te worden om meer inzicht te krijgen in de mogelijke werkingsmechanismen van deze probioticumstam.

De bacteriële samenstelling van de rectumbiopen bepaald middels kweek verschildte niet significant van de samenstelling in biopen van colon ascendens, wat aantoonde dat het verzamelen van alleen rectumbiopen voldoende zou zijn om de effecten van probiotica op de mucosale intestinale flora te bestuderen. De faecale bacteriële samenstelling verschildte wel significant van de mucosale samenstelling aangezien de prevalentie van verscheidene aërobe en anaërobe bacteriën in de mucosa significant lager was dan de prevalentie in de faeces. In toekomstige studies zullen beide monsters verzameld dienen te worden.

In hoofdstuk 6 is de bacteriële samenstelling van de faeces van patiënten met colitis ulcerosa (CU) in een inactieve fase en op een stabiele onderhoudsdosering vergeleken met die van gezonde vrijwilligers met behulp van kweek. Vervolgens is in de CU patiënten het effect bestudeerd van *L. plantarum* 299v op de intestinale flora in de faeces en in de mucosa evenals op metabole activiteiten gemeten in faecesmonsters (enzymactiviteit, endotoxinen concentraties, KKVZ-concentraties). Omdat de CU patiënten minder consistente faeces hadden, werden de bacteriële concentraties gecorrigeerd voor verdunning en uitgedrukt in log kolonie vormende eenheden per mg eiwit in de faeces.

Faecale bacteriële concentraties gemeten in de CU patiënten waren significant lager in vergelijking met de concentraties gemeten in gezonde vrijwilligers. Deze verschillen kunnen veroorzaakt zijn door de ziekte of door de inname van medicijnen.

Naast de significante stijging in de concentratie van lactobacilli in de faecesmonsters, werden geen andere verschillen gemeten in de bacteriële samenstelling van faeces of biopen na de inname van *L. plantarum* 299v. Deze resultaten zijn gebaseerd op zowel kweek als op moleculaire technieken. Ook veranderde de inname van *L. plantarum* 299v de metabole activiteiten in de faeces niet.

Opvallend is dat *Bacteroides* spp. niet aanwezig waren in de mucosale monsters van de CU patiënten in de probioticum-groep, terwijl de prevalentie relatief hoog was in de placebo-groep. Dat zou erop kunnen duiden dat *L. plantarum* 299v de prevalentie van *Bacteroides* spp. in de mucosa van CU patiënten kan verlagen, wat gunstig kan zijn voor deze patiënten. Aangezien de inname van *L. plantarum* 299v geen duidelijke verandering laat zien in de bacteriële samenstelling, zijn meer studies nodig om mogelijk andere werkingsmechanismen van probiotica, zoals het beïnvloeden van het immuunsysteem, te bestuderen.

Dit proefschrift toont dat de inname van *L. plantarum* 299v de concentratie van lactobacilli in faeces van gezonde vrijwilligers als ook van mensen van wie de darmflora mogelijk verstoord is, significant verhoogt. De klinische relevantie van deze stijging dient echter nader onderzocht te worden. Inname van *L. plantarum* 299v toont geen andere veranderingen in de bacteriële concentraties of in de bacteriële bandenpatronen, gemeten in de

faecesmonsters. Bij vergelijking van de probioticum-groep met de placebo-groep werden er ook geen veranderingen gemeten in de metabole activiteiten in de faecesmonsters, of in de bacteriële samenstelling gemeten in de mucosa. Aangezien *L. plantarum* 299v de intestinale flora niet lijkt te veranderen, zoals is aangetoond in de vijf onderzoeken beschreven in dit proefschrift, kan de bacteriële samenstelling als relatief stabiel worden beschouwd. Dit proefschrift toont echter een significant verschil in de bacteriële samenstelling indien CU patiënten worden vergeleken met gezonde personen.

Het werkingsmechanisme van *L. plantarum* 299v in het maagdarmkanaal dient verder onderzocht te worden, waarbij aandacht besteed zou moeten worden aan de hechting van de probioticumstam aan de mucosa, de invloed op de metabole activiteiten in de mucosa en de effecten op het immuunsysteem.

Dankwoord

Dankwoord

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Curriculum Vitae

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Curriculum Vitae

Dominique Anne Marie Goossens is geboren op 5 januari 1976 te Eindhoven. Zij groeide op in Aalst-Waalre en behaalde in 1994 haar VWO diploma aan het Augustinianum te Eindhoven. Vervolgens startte zij met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit te Nijmegen met als afstudeerrichting Toxicologie. Zij was gedurende een half jaar studente Humane Voeding aan de Wageningen Universiteit. Zij liep stage bij de afdeling Interne Geneeskunde in het Radboud Ziekenhuis te Nijmegen en bij de vakgroep Toxicologie aan de Wageningen Universiteit en behaalde in 1999 haar doctoraal examen. In november 1999 startte zij als assistent in opleiding bij de vakgroep Interne Geneeskunde van de Universiteit Maastricht onder leiding van Prof. Dr. R. Stockbrügger, Dr. D. Jonkers en Dr. M. Russel. In haar promotie-onderzoek werkte zij nauw samen met de afdeling Medische Microbiologie. Sinds 1 september 2005 is zij werkzaam bij Numico Research B.V.