Analyzing Global Gene Expression of *Lactobacillus plantarum* in the Human Gastro-Intestinal Tract

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Outline of this thesis

There is considerable scientific and industrial interest in studying the potential of lactic acid bacteria for improving the human gastrointestinal health. This notably holds for *Lactobacillus plantarum*, representing the first *Lactobacillus* species that is completely known at the genomic sequence level (1) and has developed into a sophisticated model for exploring the molecular mechanisms underlying the targeted intestinal properties of this species of lactic acid bacteria. Apart from determining the effect of *L. plantarum* on the human host, it is of importance to understand the response of this lactic acid bacterium to the host. The research discussed in this thesis focuses on the influence of passage of the intestine on activity and gene expression of *L. plantarum*.

Chapter 1 discusses the studies of the safety and survival of *L. plantarum* in the human intestinal tract and the impact of this bacterium on the host. In addition, it provides an overview of the molecular aproaches addressing the activity of *L. plantarum* in the human gut environment.

Methods to detect, classify, and to estimate general activity of microbes in complex ecosystems are often based on ribosomal RNA (rRNA) and their coding genes as target molecules due to their universal distribution and high nucleotide sequence conservation. In **Chapter 2**, the complete genome sequences of the lactic acid bacteria, *L. plantarum, L. johnsonii* and *Lactococcus lactis* were used to compare location, sequence, organisation, and regulation of the ribosomal RNA (*rrn*) operons. Furthermore, differences in regulation between the five *L. plantarum rrn* promoters were studied.

In **Chapter 3**, fluorescent *in situ* hybridisation (FISH) with a 16S rRNA targeted oligonucleotide probe is explored as a technique to estimate the *in situ* activity of *L. plantarum* for potential application in environments such as the gastrointestinal tract.

Chapter 4 describes the development of an approach to analyse global gene expression of *L. plantarum* in the complex environment of the human gastrointestinal tract. Patients diagnosed for colon cancer received *L. plantarum* before they underwent surgery. Total RNA was isolated from the mucosa of surgically removed intestinal segments and the RNA was hybridized to a DNA microarray comprising clones covering the *L. plantarum* genome. The reproducibility, efficiency, and specificity of the arrays were determined. A comparison was made between gene expression of *L. plantarum* within the ileum and colon of a single individual and between colonic samples of different subjects.

To determine the effect of passage of the proximal intestinal tract on gene expression of *L. plantarum*, four human volunteers were subject to perfusion of a segment of the small intestine with cells of *L. plantarum* followed by analysis of RNA using DNA microarrays. The results are described in **Chapter 5** and provide a global overview of the response of *L. plantarum* to passage of the human small intestine.

In **Chapter 6**, gene expression of *L. plantarum* was assessed by quantitative reverse transcriptase-PCR following recovery from several subjects that had an ileostoma and consumed the bacteria in a milk drink. The study focused on a specific set of representative genes in *L. plantarum* to obtain an overview of its activity following ingestion and passage through the human stomach and small intestine.

In the general discussion, **Chapter 7**, comparisons are made between the applied screens that provide insight in the general response of *L. plantarum* to the human intestinal tract. An initial comparison was made between gene expression of *L. plantarum* cells in the ileum and colon and between luminal and attached cells. The main conclusions of this thesis are summarized and future perspectives are discussed.

1. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

Chapter 1

Lactobacillus plantarum - Survival, Functional and Potential Probiotic Properties in the Human Intestinal Tract

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Abstract

Lactobacillus plantarum is a versatile lactic acid bacterium that is encountered in a range of environmental niches, has a proven ability to survive gastric transit, and can colonize the intestinal tract of human and other mammals. Several studies describe the effects of *L. plantarum* consumption on human physiology. The availability of the complete genome sequence of *L. plantarum* WCFS1 makes it a suitable model to explore the molecular mechanisms underlying the targeted intestinal properties of this species. An increasing number of studies address the development of *L. plantarum* into an ingestible living vaccine. Furthermore, studies are emerging to determine the activity of *L. plantarum* in the human intestinal tract. This review discusses the studies of the safety and survival of *L. plantarum* in the human intestinal tract, the effects of this bacterium on the host and it provides an overview of the molecular studies addressing the activity of *L. plantarum* in the human gut environment.

Keywords: *Lactobacillus plantarum*, human intestinal tract, molecular analysis, physiological effects, living vaccine

Introduction

Lactic acid bacteria are Gram-positive, non-spore forming, fermentative bacteria that grow anaerobically, and are traditionally applied in the conservation of a variety of fermented food products (31). The largest group of lactic acid bacteria belongs to the genus of *Lactobacillus* that comprises more than 50 different species (64, 66). In many cases, these lactobacilli are also used as starter cultures in industrial and artisanal food fermentation since they contribute to the conservation, flavor, and texture of the fermented foods. While the fermentative conversion of sugars present in the raw materials into lactic acid is their main function, production of anti-microbial peptides, exopolysaccharides and a variety of other metabolites are other important properties (57, 65). In addition, *Lactobacillus* species are found in the gut of humans and other animals, while their numbers may vary with the animal species, the age of the host, or the location within the gut. However, only a few *Lactobacillus* species contain representatives that are both involved in traditional and industrial food fermentations and reside in the human gut. Those include *L. crispatus, L. gasseri,* and *L.*

plantarum (15) of which the phylogenetic position is depicted in Figure 1. In this review, we will focus on *L. plantarum.*

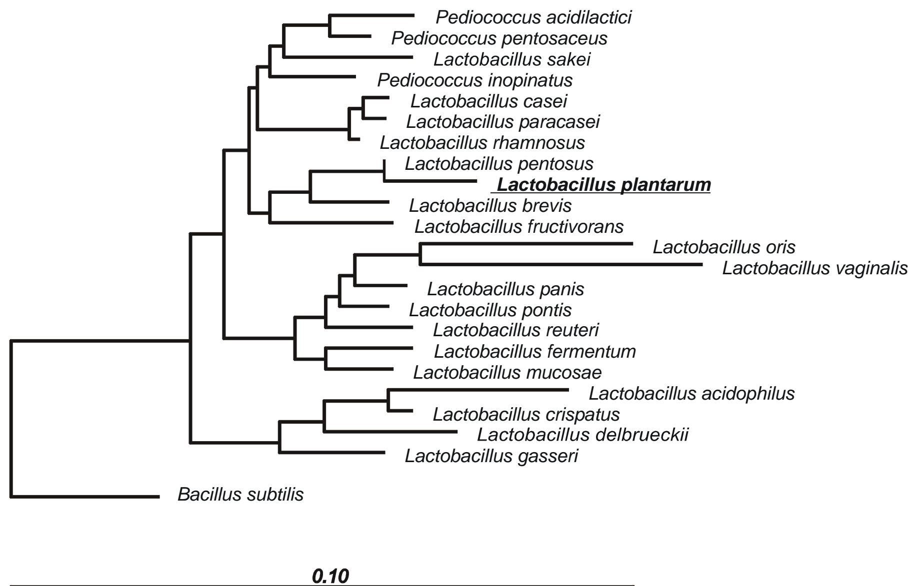

Figure 1. Neighbour-joining tree of partial 16S rDNA sequences (*E. coli* position 107-1434) showing the phylogenetic relationship of *L. plantarum* to a selected set of representative lactic acid bacteria. *Bacillus subtilis* was used to root the tree. The scale bar represents the calculated distances between the sequences.

L. plantarum is a versatile lactic acid bacterium, that is encountered in a range of environmental niches including dairy, meat and many vegetable fermentations (Table 1). Moreover, it is commonly found in the human gastrointestinal-tract (GI-tract) as described below. Furthermore, *L. plantarum* can be involved in spoilage of foods, such as meat (9), wine (8) or orange juice (5). Recently, the complete genome sequence of *L. plantarum* WCFS1, a single colony isolate of *L. plantarum* NCIMB 8826 from human saliva, has been determined and annotated (35). This analysis confirmed that *L. plantarum* has the coding capacity for the uptake and utilisation of many different sugars, uptake of peptides, and formation of most amino acids. The large number of surface-anchored proteins suggests that *L. plantarum* has the potential to associate with many different surfaces and potential substrates for growth. In addition, the relatively high number of genes encoding regulatory functions indicated the ability to adapt to many different conditions. All together this reflects the potential of *L. plantarum* to grow in a large range of environmental niches. The large number of genes encoding surface proteins (217 predicted proteins) could function in

Table 1. Food products containing *L. plantarum*

Raw material	Product	Reference
Plant products	Olives	(20, 55)
	Cocoa beans	(6)
	Cassava	(37)
	Sauerkraut	(63)
	Togwa	(34)
	Wine	(62)
Milk products	Stilton cheese	(22)
	Traditional feta cheese	(41)
	Ricotta forte cheese	(7)
Meat products	Fermented dry sausage	(17, 21, 24)
	Fermented Italian sausage	(17)

recognition of or binding to certain components in the environment, since several of those genes show homology to proteins with predicted functions like mucus-binding, aggregation-promoting, and intercellular adhesion (35). A DNA microarray based comparison between 20 strains of *L. plantarum* showed absence or presence of different DNA regions. The main differences could be found in transferable regions like prophages and IS-elements, but also in other regions that are predicted to encode for example the production of plantaricin, non-ribosomal peptides or exopolysaccharides. Very high levels of strain specific variation are encountered in a 200-kb region encoding mainly genes in sugar metabolism, which was probably acquired by horizontal gene transfer, and may represent a lifestyle adaptation island (35, 45, 61).

According to the definition of the World Health Organization, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (25). Recommended properties for a probiotic microbe include survival of the gut, persistence in the host, and proven safety for human consumption (16, 67). The claimed health effects include the reduction of gastrointestinal infection and inflammatory bowel disease risk, or modulatory effects on the immune system. However, only limited scientific support is based on validated clinical trials (60, 68). A variety of *L. plantarum* strains are presently marketed as probiotics (Table 2). This review summarizes the studies of the safety and survival of *L. plantarum* in the human GI-tract, the effects of this bacterium on the host and it provides an overview of the molecular studies addressing the activity of *L. plantarum* in the human GI-tract.

Table 2. *L. plantarum* in health products as found by the 10 highest hits in Google (www. google.com; *Lactobacillus plantarum* health products; analyzed March 2005)

Administration	**Product**	**Website**
Capsule	IFlora Acidophilus Formula	http://sedonalabs.com/products/iFlora.html
	Probiotic Eleven	http://www.greatestherbsonearth.com/nsp/probiotic_eleven.htm
	Plantadophilus	http://www.enzymeessentials.com/HTML/plantadophilus.html
	FloraFood	http://www.commercemarketplace.com/home/purprod/AIM_Products_-_WholeBody.html
	Living Vitamine C caps	http://www.monstermarketplace.com/SearchByCategory/Product/1425/Landing/457/LivingVitamin CCAPS90/266/27/82
	Udo’s Choise	http://www.florahealth.com/Flora/Home/international/Products/TG8_More.asp
	Super Detox System	http://www.vitalityatplay.com/super-detox-system.htm
Fruit drink	Proviva	http://www.proviva.co.uk; http://www.proviva.se
Drink	Lactovitale	http://www.filipinovegetarianrecipe.bravehost.com/*Lactobacillus*_plantarum/intro_lacto_pafi.htm
Powder/gel	ProBios	http://www.petfooddirect.com/store/dept.asp?dept_id=686

Safety of L. plantarum

L. plantarum has a long history of natural occurrence and safe use in a variety of food products including its well-known use in large numbers in sauerkraut and olive preparations (Table 1). Among the hundreds of reports on its safe use, there are only limited reports suggesting that some *L. plantarum* strains may be involved in infection. One of these concerns the isolation of *L. plantarum* from infective endocarditis. Those strains could *in vitro* coagulate blood by aggregation of human platelets, a possible pathogenic trait because of the danger of blood clotting (30). However, this *in vitro* trait may not be reflecting the *in vivo* danger, since a large number of lactic acid bacteria appear to share this property. Moreover, the identification of the species was based on only a limited number of tests. Recent investigations showed that the human saliva isolate *L. plantarum* NCIMB 8826 did not induce macroscopic or histological inflammation or abnormal translocation through the intestinal barrier in mice (52). On the contrary, total translocation of endogenous microbiota was reduced in mice suffering from colitis that were fed *L. plantarum* NCIMB8826 (52). After an intravenous injection of Sprague-Dawly rats with 10^8 CFU of *L. plantarum* 299v, this organism could not be recovered from the heart and blood, when the rats were sacrificed 96 h. after injection. So even if the intestinal barrier was crossed, no infection took place, showing the apparent safety of the organism (1). Moreover, a recent post-market surveillance study showed that *L. plantarum* is not found in bacteremia cases (58). Finally, various clinical studies, as described below, underline the safe use of *L. plantarum* in humans.

Survival in the human GI-tract

After oral ingestion, bacteria encounter a number of human defence systems that are associated with secretions. These include high concentrations of mucins that cover the gut, gastric acid inducing a low pH in the stomach, and bile salts secreted into the luminal content in the proximal small intestine (51). *L. plantarum* WCFS1 does not utilize mucin, although it is predicted to encode mucin binding proteins (35). In addition, different strains of *L. plantarum* were found to show a high tolerance to the consecutive exposure to hydrochloric acid (pH 2.0) and bile salts. This was observed both for strains isolated from intestinal samples and for those isolated from fermented foods (28). Of the *L. plantarum* cells 0.003 to 10% survived those conditions compared to no survival and very limited survival (0-0.001%) for *L. sakei* and *L. paracasei,* respectively (28). *L. plantarum* NCIMB 8826 also displayed

high survival *in vivo* following human ingestion (69). Survival was tested using intestinal intubation of the ileum after a single dose of 10^8 cells was given to healthy volunteers. Direct plating showed 7±2 % survival in the human ileum, which is possibly a relevant location in terms of specific gut properties of this bacterium. In contrast, *Lactococcus lactis* showed only 1±0.8 % survival and for *L. fermentum* 0.5±0.5% survival was determined in this experiment. Survival of *L. plantarum* NCIMB 8826 up to 25% compared to an inert marker was reached in the faeces after one week of daily ingestion. During this study, the strain used did not persist in these subjects, as the transit time of *L. plantarum* was the same as for an inert marker (69). However, 11 days after the end of the administration which consisted of daily simultaneous intake for ten days of different strains of lactobacilli with $5x10^8$ CFU per strain, colonisation with *L. plantarum* 299 and 299v was found in the jejunum and rectum of 85% of healthy volunteers indicating that colonisation can be person-dependent (32). In this study, colonisation with *L. plantarum* was considerable better than with *L. salivarius*, *L. reuteri*, *L. gasseri*, *L. acidophilus*, *L. casei*, and *L. agilis*, because two thirds of the strains recovered using plate-counting and confirmed with API-50CH system and restriction analysis belonged to *L. plantarum* and a mix of the other administered species accounted only for one third of the recovered strains (32).

As a common indigenous bacterium *L. plantarum* could be isolated from 1 out of 20 of the ileum and colonic samples using plate counting and phenotypical characterisation of both healthy and diseased persons (46). Nevertheless, *L. plantarum* could be isolated from the mouth or rectum of the majority of healthy subjects tested (3), indicating that the presence of *L. plantarum* differs per sampling location and is a common gut bacterium. If no *L. plantarum* was consumed, the numbers of this organism in faeces were too low to be detected, which corresponds to a viable count of less than $3.2x10^4$ CFU g^{-1} (33). Two thirds of the isolates from mouth and rectum showed mannose-inhibited adherence to the human colonic cell line HT-29 suggesting the possibility of permanent colonisation. This feature was infrequently found for other lactobacilli isolated from the gut (3). Different strains of *L. plantarum*, including 299v, indeed showed the capacity to adhere to human cells in a mannose-inhibited manner that is indicative of binding to a mannosylated cell-bound receptor (2). Competition for those receptors between *L. plantarum* and pathogenic bacteria, including *E. coli*, reduces adherence to the human cells of the latter and in this manner may protect the host from infection. By combining information from genome-wide array-based genotyping of different

L. plantarum strains with specific inactivation studies, the mannose-adhesion gene was identified (45, 54). This gene is one of the first genes to be identified that is associated with a definite probiotic effect. It is predicted to encode a large (>1000 residues) cell envelope-located protein. Its identification allows for detailed studies with deletion and overproduction strains that will allow for the construction or identification of strains that effectively exclude pathogens that contain type 1 fimbriae (54).

Effects of L. plantarum in healthy volunteers

Due to their abundance, easy growth characteristics, and human origin, various *L. plantarum* strains have been tested for health effects. Different effects were observed following consumption of significant amounts of living *L. plantarum* cells in healthy subjects (Table 3). A significant increase in the total faecal concentration of carboxylic acids (from 83 to 113 μmol per gram wet faeces), acetic acids (from 48 to 64 μmol per gram wet faeces) and propionic acid (from 11 to 17 μmol per gram wet faeces) was found in a study in which the subjects consumed *L. plantarum* 299v (=DSM 9843) daily for 3 weeks (33). This effect is probably due to an effect of *L. plantarum* on specific colonic bacteria, because *L. plantarum* produces mainly lactic acid instead of acetic acid and lacks several of the genes encoding enzymes for the production of propionic acid. Together with an increase in stool volume, a decrease in flatulation and slightly softer stools, those results indicate an altered fermentation in the colon (33).

A significant decrease in anaerobic bacterial counts (from 4.0×10^6 to 1.0×10^6 CFU g^{-1} of mucosa) and Gram-negative anaerobic counts (from 1.0×10^6 to 7.9×10^4 CFU g^{-1} of mucosa) in the rectum of healthy volunteers was found to occur with a daily intake of an oatmeal soup containing different strains of lactobacilli (32). The *Lactobacillus* strains that were mainly recovered at 1 and 11 days after the end of the trial in both the jejunum and the rectum were *L. plantarum* 299 and 299v, two very closely related strains. *L. agilis* was also recovered in high amounts after 1 day, but this was drastically reduced 11 days after ending the administration. The two *L. plantarum* strains showed survival of passage as well as a prolonged retention, indicating colonisation in the GI-tract. The reduction in Gram-negative anaerobic bacterial counts was only found 11 days after the end of the trial, suggesting that this effect takes place after a period of establishment of the lactobacilli (32). Similar reductions in Gram-negative anaerobic bacteria were obtained following *L. plantarum* E98

addition to a simulator of the human intestinal microbial ecosystem (SHIME) (4). A reduction in Gram-negative anaerobic bacteria is considered to be advantageous from a medical perspective, since those bacteria are frequently isolated from infected sites after intestinal surgery (48). In several healthy subjects who daily consumed an oatmeal soup containing different strains of lactobacilli also a reduction in *Enterobacteriaceae* (at least 1,000-fold), or sulfite-reducing clostridia (10-100 fold) could be observed (32). A significant (more than 6-fold) decrease in the carriage of enteropathogenic bacteria was also found in healthy children less than 5 years old that daily consumed an *L. plantarum* 299v fermented cereal gruel known as togwa. At the start of the experiment 38.9% of the children had enteropathogenic bacteria detected in rectal swabs, which declined to 6.1% and 6.9% after one and two weeks of togwa respectively. The effects were still detected two weeks after the consumption of togwa with only 6.5% of the children carrying enteropathogens. Several mechanisms have been proposed and included a lower transmission of enteropathogens through eating the pathogen-free togwa, an inhibitory effect in the gut through production of inhibitory factors, or a competition for specific niches and nutrients (34).

In a study with subjects with moderately elevated cholesterol, it has been shown that consumption of *L. plantarum* 299v could reduce both the LDL-cholesterol and fibrinogen levels in the blood significantly, with 9.6% ($p<0.01$) and 13.5% ($p<0.001$), respectively (14). In a study on heavy smokers, which were given twice that dose of *L. plantarum* a more pronounced effect was seen, and an 11.7% decrease in LDL-cholesterol and 21% in fibrinogen was observed. Both LDL-cholesterol and fibrinogen are independent risk factors for coronary artery disease. The suggested mechanism is an anti-inflammatory action of the propionic acid production induced by *L. plantarum*. This is also found for ibuprofen, a derivative of propionic acid (47). Consumption of *L. plantarum* indeed showed a significant increase in propionic acid in the faeces of healthy volunteers, probably by influencing other colonic bacteria (33).

Effects of L. plantarum on disease

A variety of clinical trials have been reported aimed at demonstrating an effect of consumption of *L. plantarum* on different diseases (Table 3). Considerable attention has been given to immune stimulation by *L. plantarum* strains. In one study it was shown that *L. plantarum* 299v was able to colonize children congenitally exposed to the human

Table 3. Influences of *L. plantarum* on animal models, healthy volunteers and patients as assessed by *in vitro* and *in vivo* studies.

Study	Host	Dose[1] (strain)	Subjects	Intake	Effect	Remarks
Healthy subjects						
Johansson et al., 1998	Human	$2.0x10^{10}$ (1)	26	3 weeks	Increased short-chain fatty acid content of faeces	Randomised placebo controlled double-blind study; Contribution of oatmeal on the effects is not clear
Johansson et al., 1993	Human	$5.0x10^{8}$ (1, 2)	13	10 days	Changing microbiota in ileum and rectum; Dominant recovery of *L. plantarum* (2/3 of recovered strains)	Mixed use of different strains Unclear cause-effect relation
McNaught et al., 2002	Human	$2.5x10^{10}$ (1)	129	differed	No effect on post-operative wound infection	Study not blinded and intake may differ considerably between patients
Kingamkono et al., 1999	Human	Unknown (1)	151	13 days	Up to 6 times reduction in carriage of faecal enterobacteriacea	Placebo controlled study with large number (151) of subjects, but no dose specified
Bukowska et al., 1998	Human	10^{10} (1)	30	6 weeks	Reduction in LDL-cholesterol (9.6%) and fibrinogen (13.5%)	Randomised placebo controlled double-blind study with male subjects and questionable proposed mode of action
Naruszewicz et al., 2002	Human	$2.0x10^{10}$ (1)	36	6 weeks	Reduction in LDL-cholesterol (11.7%) and fibrinogen (21.0%)	Randomised placebo controlled double-blind study
Clinical trials						
Cunningham-Rundles et al., 2000	Children exposed to HIV	Unknown (1)	18	1 month	Improved natural immune response	Placebo controlled double-blind study with unclear experimental set-up and low number of subjects
Wullt et al., 2003	Human	$5.0x10^{10}$ (1)	20	38 days	1/3 reduction in recurrence of *Clostridium difficile* associated diarrhoea	Placebo controlled double-blind study with low number of subjects; Significance unclear
Nobaek et al., 2000	Human	$2.0x10^{10}$ (1)	60	4 weeks	Reduction in symptoms IBS	Randomised placebo controlled double-blind study with subjects recording their own symptoms
Niedzielin et al., 2001	Human	$2.0x10^{10}$ (1)	40	4 weeks	Reduction in symptoms IBS	Randomised placebo controlled double-blind study with mainly female subjects and gastero-enterologists and patients recording symptoms
Sen et al., 2002	Human	$6.3x10^{9}$ (1)	12	4 weeks	No reduction in symptoms IBS	Randomised double-blind crossover study with gasteroenterologists and patients recording symptoms; low dose may reduce effects; low number of patients

Table 3. Influences of *L. plantarum o*n animal models, healthy volunteers and patients as assessed by *in vitro* and *in vivo* studies. (continued).

Study	Host	Dose[1] (strain)	Intake	Effect	Remarks
***In vitro* studies**					
Alander et. al., 1998	SHIME (GI-tract model)	$1.4x10^9$	7 days	Decrease enterobacteriaceae and clostridia and slight increase enterococci	Experiment done only once
McCracken et al., 2002	Human cell line HT-29	1 CFU 1000 cells $^{-1}$ (1)	3 hours	Increase of IL-8 mRNA in epithelial cells and down regulation of IL-8 secretion	*L. plantarum*/host-cell ratio rather unfavourable
Michail et al., 2003	T-84 cell line	10^7, 10^8, and 10^9 (1)	2.5 hours	Inhibition of EPEC induced neutrophil migration	*L. plantarum*/host-cell ratio unknown and only 10^9 cells induced effect
Mack et al., 1999	Human cell line HT-29	10^5, 10^7, 10^8 and 10^9 (1)		Inhibition of enteropathogenic *E. coli* adherence	*L. plantarum*/host-cell ratio unknown and only 10^8 cells or more induced effect
Animal models					
Perdigon et al., 1999	Mice	10^9 (3)	2, 5 or 7 days	Increase in specific and unspecific immunity	Effects were dependent on intake
Mangell et al., 2002	Rats	Differed per group (1)	1 week	Inhibition of *E. coli* induced intestinal permeability	Intake of drinking water containing *L. plantarum* was unknown
Liu et al., 2001	Rats	$2x10^9$ (1)	22 days	Reduction in side effects of external radiation on colon anastomic healing	Placebo controlled study

[1] CFU per day unless stated otherwise; strain 1 *L. plantarum* 299v;strain 2 *L. plantarum* 299; strain 3 *L. plantarum* CRL 936

immunodeficiency virus (HIV) and preliminary results showed an increase in weight and specific immune response of those children (18). However, the experimental set-up of this trial was unclear and only a low number of subjects participated. Stimulation of the immune response was also suggested by the findings that the proinflammatory cytokine TNF-α can sensitise HT-29 epithelial cells to viable *L. plantarum* 299v cells (42). While TNF-α exposure resulted in increasing production of the mRNA for the inflammatory protein, interleukin-8 (IL-8), the addition of *L. plantarum* could exert a protective effect by down-regulating IL-8 secretion from the cells. However, the mechanism behind this is not yet known (42). The mechanism for immune stimulation by *L. plantarum* was addressed in animal experiments to verify *in vivo* activity. A possible mechanism of stimulation of the immune system via oral administration of *L. plantarum* CRL 936 in mice was through stimulation of the M-cells at the Peyers patches, which both increased specific immunity by IgA^+ cells in both in the intestine and in the bronchus, and increased unspecific immunity by inducing the level of the $CD4^+$ T cells (53).

Various studies showed a protective effect of *L. plantarum* against intestinal infections (Table 3). In a small double-blind, placebo-controlled trial, *Clostridium difficile*-associated diarrhoea was found to be less recurrent in a small group of patients if antibiotics were administered in combination with *L. plantarum* 299v (4 of 11 patients instead of 6 of 9 patients in the placebo group) (70). A protective effect against *E. coli*-induced intestinal permeability in rats after mixing their drinking water with a fermented oatmeal drink, containing 10^9 CFU ml^{-1} of *L. plantarum* 299v for 1 week was also observed using Ussing chambers. In contrast to the untreated group, *E. coli* did not negatively influence the permeability of the intestinal cell wall of rats that received *L. plantarum* (40). Pre-incubation of an intestinal epithelial T-84 monolayer with *L. plantarum*, showed a reduced attachment of enteropathogenic *E. coli* and reduction in inflammatory factors, like neutrophil migration (44). However, short-term addition of *L. plantarum* did not reduce the effect of *E. coli* in similar experiments on the intestinal cells (40, 44). A possible mechanism is competitive exclusion between *L. plantarum* and *E. coli* via the mannose adhesin that can be involved in competition for the mannose-specific binding sites as described earlier (54). Another possible mechanism could involve the induction of the expression of the MUC2 and MUC3 genes in HT-29 cells and subsequently stimulating the mucin production, which could inhibit adherence of *E. coli*. This effect is induced by a component secreted in the medium, since the

cell-free supernatant of *L. plantarum* 299v showed the same effect as whole cultures (39). A candidate for this effect is the product of the *sdr* gene, that is predicted to be a 3,360-residue protein with a nearly perfect SD (Ser-Asp)-repeat. It has been suggested that glycosyltransferases could make O-linked glycosylations on the serines, producing mucin like structures, which may interact with the host cell mucins (35). This interaction may influence the expression of the MUC genes. The effect of *L. plantarum* on *Clostridium difficile* infection may have a similar mechanism involving stimulation of mucin production or another form of mannose binding.

The pathogenic mechanisms of irritable bowel syndrome (IBS) are not known yet but a major factor has been implied to be disturbance of the intestinal bacterial microbiota (50). This factor is known to be affected by the consumption of *L. plantarum,* but only few and contradicting studies have been reported with individuals suffering from IBS (Table 3). A reduction in abdominal bloating and pain of subjects suffering from IBS was found in both a Polish and a Swedish study after daily administration of 400 ml of a rose-hip drink containing *L. plantarum* 299v for four weeks. In addition, flatulence was rapidly and significantly reduced and abdominal pain was less (49, 50). However, a subsequent study did not find any effect of the daily administration of 125 ml of this rose hip drink containing *L. plantarum*, possibly due to the lower numbers of *L. plantarum* used or due to the low number of patients tested (59).

Colorectal cancer is mainly treated by surgical resection and in some cases is followed by external radiation to avoid recurrence. To reduce complications, like diarrhoea, retarded healing, and mucosal atrophy, after this treatment enteral feeding of *L. plantarum* 299v in rats was examined and found to reduce inflammatory reactions and increase healing of the wound (38)(Table 3). However, patients that were scheduled for intestinal surgery were asked to consume *L. plantarum* a week prior to the intervention. In comparison with the untreated control group no protection from wound infections were observed after intestinal surgery (43). However, the study was not blinded and *L. plantarum* intake may have differed considerably between patients.

L. plantarum as a living vaccines

The potential of living vaccines to deliver heterologous antigens to the mucosal immune system offers a number of advantages over traditional vaccination, such as non-

invasiveness and the possibility to induce both a systemic and mucosal immune response (26). This has been studied using *L. plantarum* NCIMB 8826 as a model. To determine the most appropriate manner for immunization of the host by *L. plantarum*, green fluorescent protein (GFP) was used to tag the bacterium (23). Fluorescent bacteria could be detected in the lumen mainly associated with the mucus after administration by intragastric gavage in mice. However, the *L. plantarum* cells were not found to be associated with the Peyer's patches, which may be caused by the low amount of cells administered. After nasal administration, *L. plantarum* could be visualised in the macrophages, indicating the suitability of this immunisation path (23). Another study using a non-toxic C fragment of tetanus toxin (TTFC)-producing *L. plantarum* strain for immunization of mice, indicated that both the intragastric and nasal route of administration were appropriate (56). The immune response triggered by *L. plantarum* cells excreting antigens or delivering these on the cell surface was more efficient than with antigens present in the cytoplasm (56). *L. plantarum* has a higher immunization capacity than *Lactococcus lactis,* which may indicate that persistence plays a role (26). Cell-wall mutants, lacking the alanine racemase gene (*alr*), were as persistent in the murine gut as their wild-type counterparts. However, the depletion of D-alanine in the medium showed increased membrane permeability. In addition, the cell-wall mutants were far more immunogenic than the wild-type cells (27). An *L. plantarum* strain, encoding the peptide 111-139 of Der p 1 of the house dust mite under control of the constitutive lactate dehydrogenase (*ldh*)-promoter of *L. casei*, could be used to inhibit house dust mite-specific T-cell responses in mice, indicating a possible treatment of allergic disorders (36). In conclusion, a variety of studies exploit the capacity of *L. plantarum* to secrete bioactive molecules that evoke an immune response. In general, *L. plantarum* is preferred above other food-grade lactic acid bacteria based on its convenience in production, high-level genetic accessibility and performance in the GI-tract.

The effect of conditions resembling the gut on L. plantarum

Even though much is known about the effect of *L. plantarum* on the physical condition of the host, limited knowledge is available on the reaction of the bacterium to the intestinal conditions it encounters after consumption. The determination of the complete genome sequence has expanded considerably our understanding of the possible functions of *L. plantarum*. A combination of this information with the knowledge of the behaviour of *L.*

plantarum in the gut could give a broader insight in the mechanisms behind the health promoting properties and possible further functions of this bacterium (19).

Several *in vitro* studies have been performed to detect the genes that are switched on under the conditions typical in the gut. Cloning random fragments of the *L. plantarum* WCFS1 genome upstream of a promoterless alanine racemase (*alr*) gene of *Lactococcus lactis* in a low copy number plasmid vector resulted in a plasmid library with 98% coverage of the genome (11). The plasmid library was introduced into an *L. plantarumΔalr* strain and screened for clones that could complement the D-alanine auxotroph phenotype in the presence of 0.8M NaCl. Eight clones were detected that showed significant higher *alr* production and found to contain *L. plantarum* promoters preceding genes coding for different functions such as an integral membrane protein, glycerate kinase, permease, short chain dehydrogenase, and different hypothetical proteins. Four of the promoters contained the same conserved motive, which is not found further on the chromosome, indicating a specific regulation of their genes (11). A more elaborate screen using the same *alr*-complementation approach on 0.1% porcine bile showed induction of 31 genes, including 11 membrane- and cell wall associated functions, 5 functions involved in redox reactions and also 5 regulatory factors (13). Another *in vitro* approach exploited the use of DNA microarrays and compared *L. plantarum* WCFS1 grown on MRS agar with or without 0.1% porcine bile salts (11). This global screening approach showed up regulation by bile of stress proteins, cell-envelope located proteins, and proteins involved in redox reactions (13). All three *in vitro* studies showed alterations in the cell wall, presumably to protect the cell from the harsh conditions. This is also evident from the observed altered cell morphology when *L. plantarum* is exposed to bile acids (11). The common upregulation of genes involved in redox reactions may point to different metabolic reactions under intestinal conditions. While several regulatory genes are also affected in these *in vitro* studies, it remains to be seen whether specific regulatory circuits are operating under intestinal conditions. Moreover, it is difficult to extrapolate data obtained under *in vitro* conditions to those that are really met in the intestine. Hence, approaches to study *L. plantarum* gene expression in the intestine itself are essential as discussed below.

The effect of the gut on L. plantarum

Genes of *L. plantarum* WCFS1 specifically switched on in the gut of mice have been determined using a resolvase-based *in vivo* expression technology (R-IVET) (10). These

include sugar related functions, acquisition and synthesis of amino acids, nucleotides, cofactors and vitamins (Figure 2). Also stress-related functions were found to be specifically expressed and reflecting the harsh conditions of the gut (10). Deletion mutants of those genes demonstrated a reduced survival of the GI-tract of mice indicating the importance of those genes for survival in those conditions (12). Interestingly, the *L. plantarum* genes found to be induced in the GI-tract demonstrate a large overlap to the induced genes of pathogens under the same conditions (10). To validate the R-IVET strategy, the intestinal expression of several of these genes, including several cell surface proteins, transporters, including a cellobiose PTS gene and a copper transporting ATPase gene, and genes involved in sugar metabolism, including an alcohol dehydrogenase gene and a ramnosidase gene, were analyzed by a quantitative reverse transcriptase PCR approach (Marco, M., personal communication, 2005, NIZO Food Research, Ede, The Netherlands). Insight into the promoters that are switched on in the gut can be used to construct delivery systems aimed to produce at an appropriate intestinal location enzymes, antigens or other therapeutic proteins (29). In addition, the presentation of antigens to the immune system can be improved by placing a lytic cassette under control of an intestine-specific promoter, inducing intestinal lysis of the cells (29).

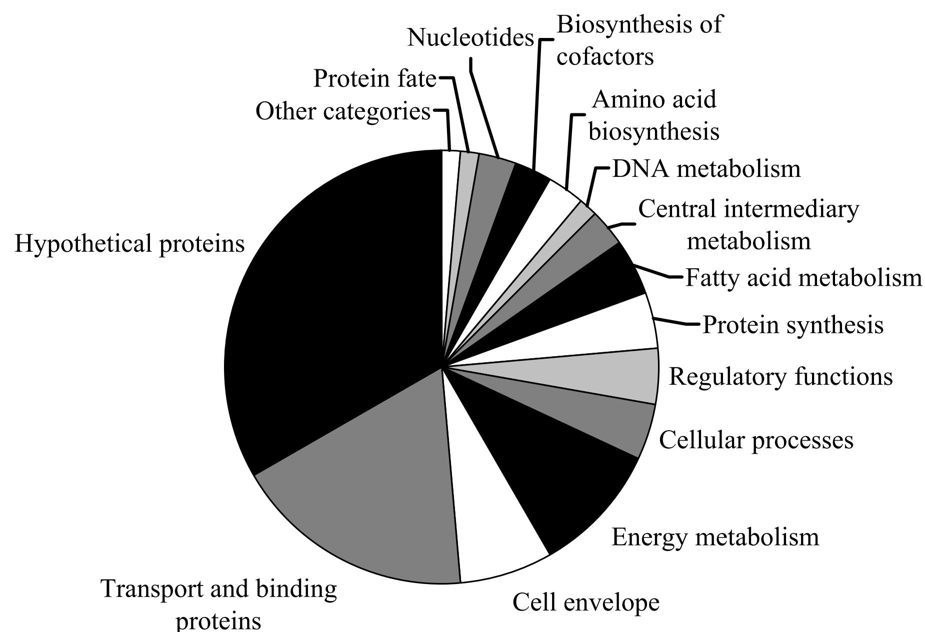

Figure 2. Functional classes of genes of *L. plantarum* identified as *in vivo* induced (*ivi*) in the gut, using R-IVET screening in a murine model (10).

While model animals such as mice are ideal systems for formulating hypotheses on microbial activity, the real answers come from experiments in human systems. Hence, various approaches have been proposed to realize this (19). In a recent study, DNA microarrays were used to monitor gene expression of *L. plantarum* in surgically removed intestinal segments of potential colon cancer patients who prior to surgery ingested a fermented oatmeal drink with *L. plantarum* 299v (10^{11} viable cells daily) or a placebo for one-week. Specific expression was observed of genes encoding sugar uptake and metabolism, amino acid biosynthesis, as well as cell division and stress related genes. These indicated survival, metabolic activity, and even growth of *L. plantarum* more or less attached to the human gut wall. In combination with clinical studies, this approach is a powerful and high-throughput tool to provide insight and new perspectives on *in vivo* host-microbe interactions (unpublished data). Even though the approaches for investigating differential gene expression in the GI-tract were different, as well as the hosts, mouse versus human, a substantial 46% of the genes revealed by R-IVET were expressed in the microarray experiments. Those genes were mainly involved in nutrient acquisition and synthesis, stress, and extracellular functions. This indicates that the GI-tract conditions of mouse and man may have similar effects on global gene expression of *L. plantarum* in the GI-tract.

Conclusions

L. plantarum has a proven ability to survive gastric transit and colonize the gut, with an apparent safety to the consumer. Many studies describe the physiological effects of consumption of *L. plantarum* on humans. However, there is a great variability in the experimental set up and quality of the studies. In some studies, consumption of *L. plantarum* showed, amongst other effects, reduction in carriage of faecal enterobacteriacea, reduction of certain risks factors for coronary artery diseases, and a dose-dependent reduction in the symptoms of IBS. The development of *L. plantarum* as a living vaccine offers a large range of therapeutic possibilities. Studying mechanisms for targeted gastrointestinal properties, like competitive adherence of *L. plantarum* to mannose-specific receptors or reduction in pathogenicity by induction of the human mucin genes, is a relatively new field of investigation. The availability of the complete genome sequence of *L. plantarum* WCFS1 makes it a suitable model to study with molecular approaches such as promoter screens, R-IVET, and DNA microarrays. This is expected to contribute to unravelling more mechanisms

behind the targeted gastrointestinal properties and possible further functions of *L. plantarum*. In addition, these molecular strategies will reveal targets for genetic screening for culture collections aiming to select strains with predictable *in situ* functional properties All this will lead to a second generation of probiotics with a scientifically proven basis for the health effect they provide.

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References

1. **Adawi, D., G. Molin, S. Ahrne, and B. Jeppsson.** 2002. Safety of the probiotic strain *Lactobacillus plantarum* DSM 9843 (=strain 299v) in an endocarditis animal model. Microbiol. Ecol. Health. Dis. **14:**50-53.

2. **Adlerberth, I., S. Ahrne, M. Johansson, G. Molin, L. Hanson, and A. Wold.** 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. Appl. Environ. Microbiol. **62:**2244-2251.

3. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

4. **Alander, M., I. De Smet, L. Nollet, W. Verstraete, A. von Wright, and T. Mattila-Sandholm.** 1999. The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Int. J. Food Microbiol. **46:**71-79.

5. **Alwazeer, D., R. Cachon, and C. Divies.** 2002. Behavior of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in fresh and thermally processed orange juice. J. Food Prot. **65:**1586-1589.

6. **Ardhana, M. M., and G. H. Fleet.** 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. **86:**87-99.

7. **Baruzzi, F., M. Morea, A. Matarante, and P. S. Cocconcelli.** 2000. Changes in the *Lactobacillus* community during Ricotta forte cheese natural fermentation. J. Appl. Microbiol. **89:**807-814.

8. **Beneduce, L., G. Spano, A. Vernile, D. Tarantino, and S. Massa.** 2004. Molecular characterization of lactic acid populations associated with wine spoilage. J. Basic. Microbiol. **44:**10-16.

9. **Borcha, E., M.L. Kant-Muermansb, and Y. Blixta.** 1997. Bacterial spoilage of meat and cured meat products. Int. J. Food Microbiol. **33:**103-120.

10. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721-5729.

11. **Bron, P. A., S. M. Hoffer, I. I. Van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-317.

12. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. *In* P. A. Bron (ed.), The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen Centre for Food Sciences, Wageningen.

13. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2005. DNA Microarray based identification of bile-responsive genes in *Lactobacillus plantarum*. J. Appl. Microbiol. in press.

14. **Bukowska, H., J. Pieczul-Mroz, M. Jastrzebska, K. Chelstowski, and M. Naruszewicz.** 1998. Decrease in fibrinogen and LDL-cholesterol levels upon supplementation of diet with *Lactobacillus plantarum* in subjects with moderately elevated cholesterol. Atherosclerosis **137:**437-438.

15. **Cataloluk, O., and B. Gogebakan.** 2004. Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. FEMS Microbiol. Lett. **236:**7-12.

16. **Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins.** 1998. Development and application of *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Bacteriol. **84:**759-768.

17. **Cocolin, L., M. Manzano, C. Cantoni, and G. Comi.** 2000. Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. Lett. Appl. Microbiol. **30:**126-129.

18. **Cunningham-Rundles, S., S. Ahrne, S. Bengmark, R. Johann-Liang, F. Marshall, L. Metakis, C. Califano, A.M. Dunn, C. Grassey, G. Hinds, and J. Cervia.** 2000. Probiotics and immune response. Am. J. Gasteroenterol. **95:**S22-S25.

19. **de Vos, W. M., P. A. Bron, and M. Kleerebezem.** 2004. Post-genomics of lactic acid bacteria and other food-grade bacteria to discover gut functionality. Curr.opinion Biotechnol. **15:**86-93.

20. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133-143.

21. **Enan, G., A. A. el-Essawy, M. Uyttendaele, and J. Debevere.** 1996b. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int J Food Microbiol. **30:**189-215.

22. **Ercolini, D., P. J. Hill, and C. E. R. Dodd.** 2003. Bacterial community structure and location in Stilton cheese. Appl. Environ. Microbiol. **69:**3540-3548.

23. **Geoffroy, M.C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. **66:**383-391.

24. **Gevers, D., M. Danielsen, G. Huys, and J. Swings.** 2003. Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl. Environ. Microbiol. **69:**1270-1275.

25. **Gilliland, S. E., L. Morelli, and G. Reid.** 2001. Presented at the Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Córdoba, Argentina. (ftp://ftp.fao.org/es/esn/food/probio_report_en.pdf)

26. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20**.

27. **Grangette, C., H. Muller-Alouf, P. Hols, D. Goudercourt, J. Delcour, M. Turneer, and A. Mercenier.** 2004. Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. Infect. Immun. **72:**2731-2737.

28. **Haller, D., H. Colbus, M. G. Ganzle, P. Scherenbacher, C. Bode, and W. P. Hammes.** 2001. Metabolic and functional properties of lactic acid bacteria in the gastro-intestinal ecosystem: a comparative *in vitro* study between bacteria of intestinal and fermented food origin. System. Appl. Microbiol. **24:**218-226.

29. **Hanniffy, S., U. Wiedermann, A. Repa, A. Mercenier, C. Daniel, J. Fioramonti, H. Tlaskolova, H. Kozakova, H. Israelsen, S. Madsen, A. Vrang, P. Hols, J. Delcour, P. Bron, M. Kleerebezem, and J. Wells.** 2004. Potential and opportunities for use of recombinant lactic acid bacteria in human health. Adv. Appl. Microbiol. **56:**1-64.

30. **Harty, D. W., H. J. Oakey, M. Patrikakis, E. B. Hume, and K. W. Knox.** 1994. Pathogenic potential of lactobacilli. Int. J. Food Microbiol. **24:**179-189.

31. **Holzapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger.** 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. **73:**365S-373.

32. **Johansson, M. L., G. Molin, B. Jeppsson, S. Nobaek, S. Ahrne, and S. Bengmark.** 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. **59:**15-20.

33. **Johansson, M. L., S. Nobaek, A. Berggren, M. Nyman, I. Bjorck, S. Ahrne, B. Jeppsson, and G. Molin.** 1998. Survival of *Lactobacillus plantarum* DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. Int. J. Food Microbiol. **42:**29-38.

34. **Kingamkono, R., E. Sjogren, and U. Svanberg.** 1999. Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels. Epidemiol. Infect. **122:**23-32.

35. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

36. **Kruisselbrink, A., M.J. Heijne Den Bak-Glashouwer, C. E. G. Havenith, J. E. R. Thole, and R. Janssen.** 2001. Recombinant *Lactobacillus plantarum* inhibits house dust mite-specific T-cell responses. Clin. Exp. Immunol. **126:**2-8.

37. **Lei, V., W. K. Amoa-Awua, and L. Brimer.** 1999. Degradation of cyanogenic glycosides by *Lactobacillus plantarum* strains from spontaneous cassava fermentation and other microorganisms. Int. J. Food Microbiol. **53:**169-184.

38. **Liu, Q., S. Nobaek, D. Adawi, Y. Mao, M. Wang, G. Molin, M. Ekelund, and B. Jeppsson.** 2001. Administration of *Lactobacillus plantarum* 299v reduces side-effects of external radiation on colon anastomotic healing in an experimental model. Colorectal Dis. **3:**245-252.

39. **Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth.** 1999. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. Am. J. Physiol. **276:**G941-950.

40. **Mangell, P., P. Nejdfors, M. Wang, S. Ahrne, B. Westrom, H. Thorlacius, and B. Jeppsson.** 2002. *Lactobacillus plantarum* 299v inhibits *Escherichia coli*-induced intestinal permeability. Digest. Dis. Sci. **47:**511-516.

41. **Manolopoulou, E., P. Sarantinopoulos, E. Zoidou, A. Aktypis, E. Moschopoulou, I. G. Kandarakis, and E. M. Anifantakis.** 2003. Evolution of microbial populations during traditional Feta cheese manufacture and ripening. Int. J. Food Microbiol. **82:**153-161.

42. **McCracken, V. J., T. Chun, M. E. Baldeon, S. Ahrne, G. Molin, R. I. Mackie, and H. R. Gaskins.** 2002. TNF-α sensitizes HT-29 colonic epithelial cells to intestinal lactobacilli. Exp. Biol. Med. **227:**665-670.

43. **McNaught, C. E., N. P. Woodcock, J. MacFie, and C. J. Mitchell.** 2002. A prospective randomised study of the probiotic *Lactobacillus plantarum* 299V on indices of gut barrier function in elective surgical patients. Gut **51:**827-831.

44. **Michail, S., and F. Abernathy.** 2003. *Lactobacillus plantarum* inhibits the intestinal epithelial migration of neutrophils induced by enteropathogenic *Escherichia coli*. J. Pediatr. Gastroenterol. Nutr. **36:**385-391.

45. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187:**6119-6127.

46. **Molin, G., B. Jeppsson, M. L. Johansson, S. Ahrne, S. Nobaek, M. Stahl, and S. Bengmark.** 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol. **74:**314-323.

47. **Naruszewicz, M., M.L. Johansson, D. Zapolska-Downar, and H. Bukowska.** 2002. Effect of *Lactobacillus plantarum* 299v on cardiovascular disease risk factors in smokers. Am. J. Clin. Nutr. **76:**1249-1255.

48. **Nichols, R. L., and J. W. Smith.** 1994. Anaerobes from a surgical perspective. Clin. Infect. Dis. **18:**S280-286.

49. **Niedzielin, K., H. Kordecki, and B. Birkenfeld.** 2001. A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299V in patients with irritable bowel syndrome. Eur. J. Gastroenterol. Hepatol. **13:**1143-1147.

50. **Nobaek, S., M.L. Johansson, G. Molin, S. Ahrne, and B. Jeppsson.** 2000. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. Am. J. Gasteroenterol. **95:**1231-1238.

51. **Ouwehand, A. C., M. Derrien, W.M. de Vos, K. Tiihonen, and N. Rautonen.** 2005. Prebiotics and other microbial substrates for gut functionality. Curr. Opin. Biotechnol.**16:**212-7.

52. **Pavan, S., P. Desreumaux, and A. Mercenier.** 2003. Use of mouse models to evaluate the persistence, safety, and immune modulation capacities of lactic acid bacteria. Clin. Diagn. Lab. Immunol. **10:**696-701.

53. **Perdigon, G., E. Vintini, S. Alvarez, M. Medina, and M. Medici.** 1999. Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. J. Dairy Sci. **82:**1108-1114.

54. **Pretzer, G., H. Snel, D. Molenaar, A. Wiersman, R. van der Meer, and M. Kleerebezem.** 2004. Identification of a mannose-specific adhesin encoding gene in *Lactobacillus plantarum*. Darmendag **Utrecht October 29th 2004**.

55. **Randazzo, C. L., C. Restuccia, A. D. Romano, and C. Caggia.** 2004. *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. Int. J. Food Microbiol. **90:**9-14.

56. **Reveneau, N., M. C. Geoffroy, C. Locht, P. Chagnaud, and A. Mercenier.** 2002. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. Vaccine **20**.

57. **Ross, P. R., S. Morgan, and C. Hill.** 2002. Preservation and fermentation: past, present and future. Int. J. Food Microbiol. **79:**3-16.

58. **Salminen, M. K., S. Tynkkynen, H. Rautelin, M. Saxelin, M. Vaara, P. Ruutu, S. Sarna, V. Valtonen, and A. Jarvinen.** 2002. *Lactobacillus* bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. Clin. Infect. Dis. **35:**1155-1160.

59. **Sen, S., M. M. Mullan, T. J. Parker, J. T. Woolner, S. A. Tarry, and J. O. Hunter.** 2002. Effect of *Lactobacillus plantarum* 299v on colonic fermentation and symptoms of irritable bowel syndrome. Digest. Dis. Sci. **47:**2615-2620.

60. **Shanahan, F.** 2004. Probiotics in inflammatory bowel disease--therapeutic rationale and role. Adv. Drug Deliv. Rev. **56:**809-818.

61. **Siezen, R. J., F. H. van Enckevort, M. Kleerebezem, and B. Teusink.** 2004. Genome data mining of lactic acid bacteria: the impact of bioinformatics. Curr. Opin. Biotechnol. **15:**105-115.

62. **Spano, G., G. Chieppa, L. Beneduce, and S. Massa.** 2004. Expression analysis of putative *arcA*, *arcB* and *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. **96:**185-93.

63. **Stamer, J. R.** 1983. Lactic acid fermentation of cabbage and cucumbers. *In* H. J. Rehm and G. Reed (ed.), Biotechnology, vol. 5. Verlag Chemie, Weinheim, Germany.

64. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. **36:**1-29.

65. **Tamime, A. Y.** 2002. Fermented milks: a historical food with modern applications--a review. Eur. J. Clin. Nutr. **56 Suppl 4:**S2-S15.

66. **Tannock, G. W.** 2004. A special fondness for lactobacilli. Appl. Environ. Microbiol. **70:**3189-3194.

67. **Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen.** 2001. Quality assurance criteria for probiotic bacteria. Am. J. Clin. Nutr. **73:**393S-398.

68. **Vaughan, E. E., B. Mollet, and W. M. de Vos.** 1999. Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel. Curr. Opin. Biotechnol. **10:**505-510.

69. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-828.

70. **Wullt, M., M.L. Johansson Hagslätt, and I. Odenholt.** 2003. *Lactobacillus plantarum* 299v for the treatment of recurrent *Clostridium difficile*-associated diarrhoea: a double-blind, placobo-controlled trail. Scand. J. Infect. Dis. **35:**365-367.

Chapter 2

Comparative and Functional Analysis of the rRNA-Operons and their tRNA Gene Complement in Different Lactic Acid Bacteria

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Abstract

The complete genome sequences of the lactic acid bacteria (LAB), *Lactobacillus plantarum, Lactococcus lactis*, and *Lactobacillus johnsonii* were used to compare location, sequence, organisation, and regulation of the ribosomal RNA (*rrn*) operons. All *rrn* operons of the examined LAB diverge from the origin of replication, which is compatible with their efficient expression. All operons show a common organisation of 5’-16S-23S-5S-3’ structure, but differ in the number, location and specificity of the tRNA genes. In the 16S-23S intergenic spacer region, two of the five *rrn* operons of *Lb. plantarum* and three of the six of *Lb. johnsonii* contain tRNA-ala and tRNA-ile genes, while *L. lactis* has a tRNA-ala gene in all six operons. The number of tRNA genes following the 5S rRNA gene ranges up to 14, 16, and 21 for *L. lactis*, *Lb. johnsonii* and *Lb. plantarum* respectively. The tRNA gene complements are similar to each other and to those of other bacteria. Micro-heterogeneity was found within the rRNA structural genes and spacer regions of each strain. In the *rrn* operon promoter regions of *Lb. plantarum* and *L. lactis* marked differences were found, while the promoter regions of *Lb. johnsonii* showed a similar tandem promoter structure in all operons. The *rrn* promoters of *L. lactis* show either a single or a tandem promoter structure. All promoters of *Lb. plantarum* contain two or three -10 and -35 regions, of which either zero to two were followed by an UP-element. The *Lb. plantarum rrnA, rrnB*, and *rrnC* promoter regions display similarity to the *rrn* promoter structure of *E. coli*. Differences in regulation between the five *Lb. plantarum* promoters were studied using a low copy promoter-probe plasmid. Taking copy number and growth rate into account, a differential expression over time was shown. Although all five *Lb. plantarum rrn* promoters are significantly different, this study shows that their activity was very similar under the circumstances tested. An active promoter was also identified within the *Lb. plantarum rrnC* operon preceding a cluster of 17 tRNA genes.

Key words: ribosomal DNA (*rrn*) operon, *Lactobacillus plantarum*, *Lactococcus lactis, Lactobacillus johnsonii,* tRNA, promoter regulation

Introduction

The use of ribosomal RNA (rRNA) and their encoding genes as target molecules has due to their universal distribution and high nucleotide sequence conservation become a widely used approach for the detection and classification of microbes (2). The number of *rrn* operons located on a genome differs considerably between species (39). It has been correlated to the rate with which microbes can respond to resource availability (22). A high number of *rrn* operons on the genome together with high expression of the rRNA genes enables a high growth rate in bacteria (1).

The upstream regions of *rrn* operons usually contain highly efficient promoters, like the *Escherichia coli rrn* promoters, the most extensively studied to date, containing four major structures that improve promoter efficiency. Firstly, *E. coli rrn* operons contain tandem promoters, i.e. two -10 and -35 recognition sequences for RNA-polymerase, which is believed to increase the transcription rate by transcribing the genes simultaneously with multiple RNA-polymerase molecules (42). Secondly, the UP-element (upstream-element), an AT-rich region upstream of the –35 region increases transcription 30 to 70 fold (34). Thirdly, a binding site for FIS-protein (factor for inversion stimulation) is located upstream of the most upstream UP-element, which can lead to a 5 to 10 fold increased transcription level (12). Finally, anti-termination-boxes at the start of the operon prevent RNA-polymerase dissociation from the DNA (16).

The genes within the *rrn* operons are in general organised in the order 5'-16S-23S-5S -3' (25). The intergenic spacer region between the 16S and 23S genes can contain between zero and two tRNA genes, usually encoding a tRNA-ile, tRNA-ala or tRNA-glu (28). In the Gram-negative bacterium *E. coli*, most tRNA genes are independently transcribed from rRNA genes (31); only one or two are situated following the *rrn* operon (29). In several Gram-positive bacteria including *Bacillus subtilis* (35), *Staphylococcus aureus* (17) and *Lactococcus lactis* (9), several tRNA genes were found downstream of the 5S rRNA gene.

Until recently, the majority of studies on rRNA operons has been focussed on differences between operons of different species. The increasing amount of complete nucleotide sequence information of bacterial genomes provides easy access to the complete *rrn* operon information allowing the study of their intra-species differences (39). In the present study, the *rrn* operons including promoter sequences of lactic acid bacteria (LAB) of which the complete genome sequence is known, namely *Lactobacillus plantarum* (23), *Lactobacillus johnsonii* (32) and *Lactococcus lactis* (6), are compared. LAB are of industrial interest as starter cultures for the

dairy industry and incorporated in functional foods, and they are often examined using molecular techniques based on ribosomal RNA (19, 24, 33). In the present study, sequence comparison of the operon structure and promoter regions within an LAB strain revealed microheterogeneity within the rRNA structural genes and spacer regions, but large variations were encountered in the rRNA promoter regions of *Lb. plantarum* and *L. lactis*. Moreover, large differences were found in the presence and type of genetically linked tRNA genes of the species examined. rRNA promoter regulation was studied in *Lb. plantarum* using a reporter gene that revealed differential expression depending on the growth phase.

Materials and Methods

The complete genomic sequences of *Lactobacillus plantarum* WCFS1 (23), *Lactobacillus johnsonii* NCC 533 (32), and *Lactococcus lactis* subsp *lactis* IL1403 (6) were used in this study. The rRNA operons within one strain were compared to each other using the ERGO Bioinformatics Suite (30). The promoter regions of the *rrn* operons were compared to each other and to well-characterised operons of other species.

Activity of the promoters of *Lb. plantarum* was tested using the low-copy promoter probe plasmid, pGKV210, which contains a promoterless chloramphenicol acetyl-transferase (CAT) reporter gene (40). All primers used in this study are shown in Table 1. A region of about 700 bp including the *rrn* promoter upstream of the operons was amplified by PCR using established protocols (36). Where necessary, smaller regions were amplified to exclude open reading frames present. Reverse primers were designed from both the predicted transcriptional start point of the operon (-)(i.e. transcription start point, Figure 2) and the start of the 16S rRNA gene (+) to investigate the influence of the first spacer including potential anti-termination boxes on transcription. The constructs were called pWUPX+ and pWUPX-, respectively, in which X represents the operon and +/- indicates presence or absence of the first spacer. pWUPX+ constructs were made of all operons. pWUPX- constructs were made only of *rrnC* and *rrnD*; due to the high activity of the *rrnA*, *rrnB*, and *rrnE* promoters, the constructs excluding the first spacer region showed instability and could not be used. *Eco*RI or *Bam*HI sites were built into the primers in order to allow cloning of the PCR products in the correct orientation preceding the CAT gene. A construct called pWUPtRNA was made of a possible promoter between the tRNA genes of *rrnC* (Figure 3).

Table 1. Primers used for PCR of promoter regions and probes used for copy number determination. Forward primers differ per operon; reverse primers are the same and either arise from the transcription start point (TSP) or from the start of the 16S rRNA gene.

Primer or probe	Sequence	Source
PromA-*Eco*RI-for	5’ TAATGAATTCGCCAATGCGTTCAGTGAATT G 3’	This study
PromB-*Eco*RI-for	5’ GTTGGAATTCTTTGAAAAACAGTCGTTG 3’	This study
PromC-*Eco*RI-for	5’ TAATGAATTCAATTTTTATCAGTTGCTAG 3’	This study
PromD-*Eco*RI-for	5’ GCGGGAATTCGACCAATCGTTAAACGTC 3’	This study
PromE-*Eco*RI-for	5’ GTAAGAATTCCCAACCAAATAAAACGAC 3’	This study
TSP-*Bam*HI-rev	5’ TCAAGGATCCAATTCCGTGTGTTAATCGC 3’	This study
16S-*Bam*HI-rev	5’ GCCAGGATCCAACTCTCAAATTAATGATGAG 3’	This study
Univ-1390-a-A-18	5’ GACGGGCGGTGTGTACAA 3’	(43)
CAT	5’ GCCTACGTTAAAGCCTGCAG 3’	This study

Plasmids were constructed in *E. coli* MC1061 using established protocols (36), and subsequently transformed into *Lb. plantarum* WCFS1 by electroporation as previously described (21). All inserts of plasmids from *Lb. plantarum* were sequenced to confirm sequence integrity. Routinely, LB medium (36) with 100 μg ml^{-1} chloramphenicol (Cm) was used to select for *E. coli* constructs containing an active promoter insert. MRS (Difco, USA) with 10 μg ml^{-1} Cm and 5 μg ml^{-1} erythromycin (Ery) was used for *Lb. plantarum* containing plasmids pWUPX- and pWUPX+. *E. coli* and *Lb. plantarum* with the promoterless plasmid were grown on 150 μg ml^{-1} and 5 μg ml^{-1} Ery, respectively. The wild-type strains were grown under the same conditions, but without antibiotics.

Differential rRNA-promoter activities of the different rRNA promoters were estimated based on a minimal inhibitory concentration (MIC) determination of Cm in triplicate. The Cm concentrations used were 2, 5 and 10 μg ml^{-1}, and 25 to 250 in steps of 25 μg ml^{-1}. In order to determine the expression of the promoters, the *Lb. plantarum* pWUPX derivatives were grown in MRS (Difco, USA) with 25 μg ml^{-1} Cm at 30°C, the OD_{600} was measured, samples were taken in different growth phases and CAT-activity was measured using a previously described spectrophotometric assay (37).

Estimation of the relative copy number of the plasmids was performed in a slot-blot experiment (36) in triplicate by hybridising 1 μg of total DNA of the different *Lb. plantarum* constructs to a plasmid-specific probe (CAT; Table 1). The probes were labelled with the DNA

5'end-labelling system (Promega, USA), and hybridisation was done in QuickHyb hybridisation buffer (Stratagene, UK) for 16 h. according to the manufacturers instructions. Washing was performed twice for 15 min at room temperature in 2xSSC with 0.1% SDS, followed by 30 min at 50°C in 1xSSC with 1% SDS. A Storm Phosphor Imager (Amersham BioSciences, Little Chalfont, U.K.) was used to measure the signal on the blot. After stripping of the blot (36), it was hybridised to a 16S specific probe (Univ-1390-a-A-18; Table 1) as described above.

All chemicals were obtained from Sigma Chemicals Co. (St. Louis, USA).

Results and Discussion

Location of the *rrn* operons

Five copies of *rrn* operons are found in the genome of *Lb. plantarum* WCFS1 (23), the same number as reported by Chevalier et al. (8) for *Lb. plantarum* strain CCM 1904 following Southern hybridisation analysis. Six copies of *rrn* operons are present in the genomes of both *Lb. johnsonii* and *L. lactis*.

The distribution of *rrn* operons over the genome of a broad range of genera is illustrated in Figure 1. Some microbes have clusters of rRNA-genes, for example *Lb. johnsonii*, *L. lactis* and *B. subtilis* (27), and in general, the rRNA genes are present on a part of the genome near the origin of replication (ori) which is compatible with their efficient expression. On average 46±22% of the *rrn* operons within one genome is located less than 10% away from the ori. However, the relatively even distribution of the *rrn* operons of *Lb. plantarum* over the genome is remarkable (Figure 1). In *Lb. plantarum* and *Staphylococcus aureus* none of the *rrn* operons is found located less than 10% away from the ori. All the operons of *Lb. plantarum*, *Lb. johnsonii* and *L. lactis* are directed away from the predicted ori, as was also described for many other bacteria.

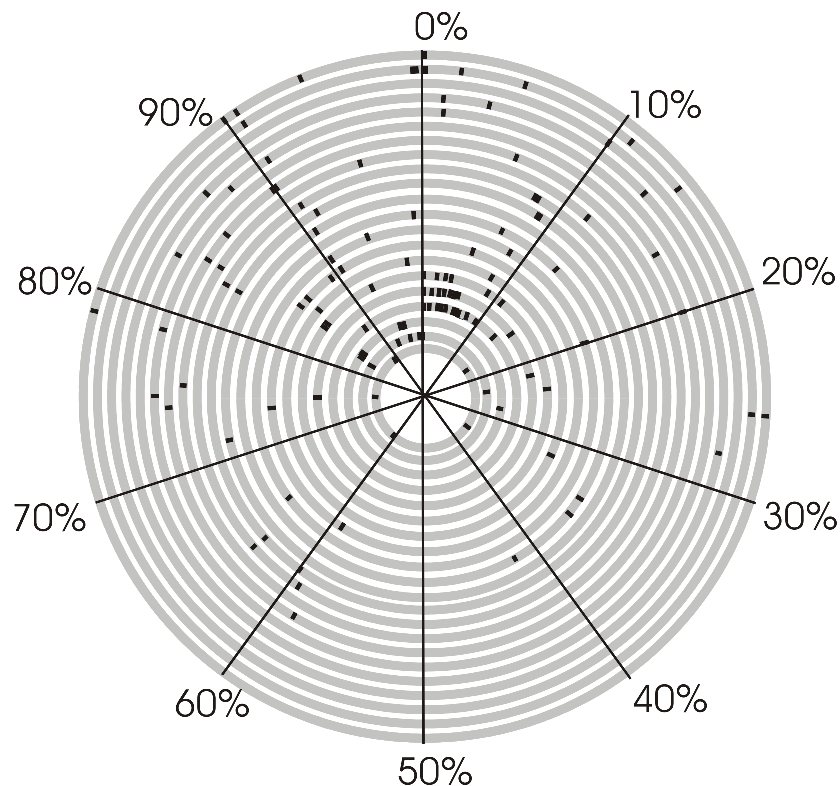

Figure 1. Location of the *rrn* operons on the genome from centre to outer ring of *Lb. plantarum* WCFS1, *L. lactis* subsp *lactis* IL1403, *Lb. johnsonii* NCC 533, *Bacillus cereus* ATCC 14579, *Bacillus subtilis*, *Clostridium perfringens*, *Enterococcus faecalis* V583, *Escherichia coli* K12, *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586, *Haemophilus influenzae* Rd, *Helicobacter pylori* 26695, *Listeria innocua* Clip11262, *Listeria monocytogenes* strain EGD-e, *Pseudomonas aeruginosa* PA01, *Salmonella typhimurium* LT2, *Staphylococcus aureus subsp. aureus* N315, *Streptococcus pneumonia* R6, *Streptococcus pyogenes* SSI-1, *Thermotoga maritima*, *Vibrio cholerae*, and *Yersinia pestis* CO92 (0%: predicted origin of replication).

Organisation and sequence comparison within the *rrn* operons

The organisation of the rRNA genes within each operon includes a 16S rRNA gene followed by an intergenic spacer region, the 23S rRNA gene, another intergenic spacer region, and the 5S rRNA gene. The organisation, length of the different genes and spacer regions are presented in Figure 2 and Table 2. The intergenic regions between 16S rRNA

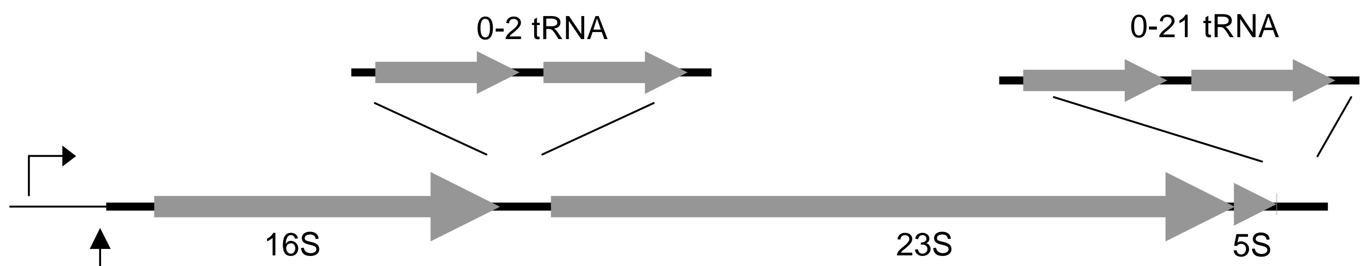

Figure 2: On scale structure of ribosomal operons of *Lb. plantarum, L. lactis,* and *Lb. johnsonii.* The intergenic spacer regions are indicated by black bars; promoter; and transcription initiation site.

genes and 23S rRNA genes of several operons of *Lb. plantarum*, *Lb. johnsonii* and *L. lactis* contain both a tRNA-ile and a tRNA-ala gene or a single tRNA-ala gene. Following the 5S rRNA genes, variable numbers (0-21) of tRNA genes are found (Figure 3), while *rrnC* of *Lb. plantarum* contains an extra 5S rRNA gene in this region. The order of rRNA genes is identical to the classic eubacterial *rrn* structure, like that of *E. coli* (7), *B. subtilis* (27) and others. The tRNA genes are present in only part of the operons and are discussed below.

Table 2. Comparison of the number of *rrn* operons, the length of the different regions and (between brackets) the single base pair differences within one strain, and the range of tRNA genes upstream of the 5S rRNA gene.

Region	*Lb. plantarum*	*L. lactis*	*Lb. johnsonii*
Number of *rrn* operons	5	6	6
Sequence preceding 16S rRNA gene	171 (0)[1]	147 (0)	135 (0)
16S rRNA gene	1569 (3)	1547 (1)	1651 (0)
Spacer 16S-23S rRNA gene	207 or 436 (1)[2]	304 (0)[2]	79 or 329 (1)[2]
23S rRNA gene	2919 (7)	2900 (2)	2977 (1)
Spacer 23S-5S rRNA gene	71 (1)	83 (0)	61 (0)
5S rRNA gene	118 (0)	116 (0)	117 (0)
Part after 5S rRNA gene	7-2097(n.r.)[3,4]	7-1327 (n.r.)[4]	97-1407 (n.r.)[4]
Range of tRNA-genes upstream of 5S gene	0-21	1-16	0-14

[1] first 47 bases differ. [2] *rrnA* and *rrnB* of *Lb. plantarum* and *rrnA*, *rrnE* and *rrnF* of *Lb. johnsonii* contain tRNA-ile and tRNA-ala genes in the intergenic regions between the 16S and 23S rRNA genes. *L. lactis* contains a tRNA-ala gene in all the intergenic regions between the 16S and 23S rRNA genes. [3] *rrnC* contains an extra 5S rRNA gene. [4] *rrnA*, *rrnB*, and *rrnC* of *Lb. plantarum*, *rrnA*, *rrnB*, *rrnE*, and *rrnF* of *L. lactis* and all *rrn* operons of *Lb. johnsonii* have tRNA genes following the 5S rRNA gene (see Figure 3), thus single base pair differences are not relevant (n.r.).

Alignment of the *rrn* operons of *Lb. plantarum*, *Lb. johnsonii*, and *L. lactis* shows almost identical rRNA coding regions within one strain (Table 2). Only the 16S rRNA gene of *rrnA* of *Lb. plantarum* contains two base substitutions in a total of 1396 nucleotides (base 224 and 672) and the last of those is shared with *rrnB*. *L. lactis* only contains one base substitution in the total 1547 nucleotides in *rrnB* (base 44). The 16S rRNA genes in *Lb. johnsonii* are identical. Similar findings were recorded for the 16S rRNA genes in 54 other bacterial genomes (10). The 23S rRNA gene of *Lb. plantarum* contains six base substitutions in different operons in the total 2746 nucleotides (base 1407, 1529, 1659, 1809, 2044, and 2626), the 23S gene of *L. lactis* contains 2 deletions of 1 base of the total 2900 nucleotides in two of the six

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rrnA Lb. plantarum     asn thr
                        |   |
rrnB Lb. johnsonii     asn
                        /
rrnE Lb. johnsonii     asn
                        /
rrnF Lb. johnsonii     asn
                        /
rrnI B. subtilis       asn thr gly arg pro ala
                        |   |
rrnF L. innocua        asn thr
                        |   |
rrnE B. halodurans     asn thr


rrnB Lb. plantarum     asn ser glu val -   -   phe -   tyr trp his gln -   cys leu
                        |   |   |   |           |       |   |   |   |       |   |
rrnA Lb. johnsonii     asn ser glu val
                        |   |   |   |
rrnD B. subtilis       asn ser glu val met asp phe thr tyr trp his gln gly cys leu -   leu
                        |   |   |   |   |   |   |       |   |   |   |   |   |   |
rrnE L. innocua        asn ser glu val met asp phe -   tyr trp his gln gly cys leu
                        |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
rrnH B. halodurans     asn ser glu val met asp phe thr tyr trp his gln gly cys leu arg


rrnC Lb. plantarum     val lys 5S  val -   lys* -   thr gly leu arg pro met -   -   ile ser met asp phe -   gly ile -   ser glu met asp
                                    |       |       |   |   |   |   |   |                       |   |       |   |       |
rrnC Lb. johnsonii     -   -   -   val -   lys leu thr gly leu arg pro met met met -   -   -   asp phe -   gly ile -   ser
                                    |       |   |   |   |   |   |   |   |   |   |               |   |       |   |       |
rrnD Lb. johnsonii     -   -   -   val -   lys leu thr gly leu arg pro met met met -   -   -   asp phe -   gly ile -   ser
                                    |       |   |   |   |   |   |   |   |   |                       |       |   |       |
rrnA L. lactis         -   -   -   val asp lys leu thr gly -   arg pro met met -   -   -   -   -   phe -   gly ile -   ser
                                    |   |   |   |   |   |   |   |   |       |                       |       |   |       |
rrnB L. lactis         -   -   -   val asp lys leu thr gly leu
                                    |       |   |       |   |
rrnB B. subtilis       -   -   -   val thr lys leu -   gly leu arg pro ala met -   ile ser met asp phe his gly ile asn ser glu
                                    |   |   |   |       |   |   |   |   |   |           |   |   |   |   |   |   |   |   |   |
rrnC L. innocua        -   -   -   val thr lys leu -   gly leu arg pro ala met met -   ser met asp phe his gly ile asn ser glu
                                    |   |   |   |       |   |   |   |   |
rrnJ B. subtilis       -   -   -   val thr lys leu -   gly leu arg pro ala
                                    |   |   |   |       |   |   |   |   |
rrnA L. innocua        -   -   -   val thr lys leu -   gly leu arg pro ala
                                    |   |   |   |       |   |   |   |   |
rrnC B. halodurans     -   -   -   val thr lys leu -   gly leu arg pro ala
```

Figure 3A. Continued on page 42

B.

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tcgagactgtcacggctca ttgttcacggctcataattaaatagtttcacaaaagttattgttgatttatgtaa
ctgagcatgatatgatgttatagttgtgttaatcgtcat gccgacttagctcagctggcagagcatctgtct
```

Figure 3. A. Comparison of tRNA genes following the 5S rRNA gene of *Lb. plantarum* WCFS1, *Lb. johnsonii*, *L. lactis*, *Bacillus subtilis*, *Listeria innocua* and *Bacillus halodurans* (| : similar tRNA gene) *Lb. plantarum* does not have any tRNA genes following *rrnD* and *rrnE,* and *L. lactis* does not have any tRNA genes downstream of *rrnC* and *rrnD*. * indicates the position of a promoter region in *rrnC* of *Lb. plantarum,* which is specified in Figure 3B (backward arrow: lys; forward arrow: thr; closed boxes: putative -10 and –35 region)

operons (base 520, and 2483) and *Lb. johnsonii* contains one extra base in *rrnE* and *rrnF* of the total 3051 nucleotides (base 9). All 5S rRNA genes are identical within *Lb. plantarum*, *L. lactis*, or *Lb. johnsonii*.

In contrast to this high degree of rDNA operon sequence conservation within a single LAB, the 23S rRNA genes in *Clostridium perfringens* are more diverse, showing differences in helix-forming regions instead of single base pair substitutions (38). Even large differences between the complete *rrn* operons within one strain can occur, like in *Thermomonospora chromogena*, which contains two types of *rrn* operons probably due to horizontal gene transfer (41).

Promoter region

Only a limited level of sequence similarity is present between the different promoter regions of the *rrn* operons of *Lb. plantarum* as is apparent from analysis of their sequence alignment (Figure 4) and structure (Figure 5). For all *Lb. plantarum rrn* promoters, –10 and –35 boxes are quite similar to the consensus sequence, and tandem and possible triple promoter structures appear to be present. The distance between the tandem promoters is 159 nucleotides for *rrnA* and *rrnB*, and 148 nucleotides in *rrnC*. Three tentative –10 and –35 regions are predicted for the promoters of *rrnD* and *rrnE*, and the distance between the triple promoters is 60 to 63 nucleotides. For the *rrnA* and *rrnB* promoters, both tandem promoters are preceded by an UP-element (90-95% similarity), that is recognised by the α-subunit of the RNA-polymerase (15). The *rrnC* promoter has a similar structure, albeit the UP-elements appear less similar to the consensus sequence or are absent. However, in the three tentative –10 and –35 regions of the promoters of *rrnD* and *rrnE* UP-elements are lacking. Both the *rrnA* and *rrnB* promoters have an identical sequence upstream of the 16S rRNA gene at the start of the operon. The region upstream of the 16S rRNA gene is similar for *rrnC*, *rrnD*, and *rrnE*.

Figure 4. Sequence alignment of the *rrn* operons of *Lb. plantarum* WCFS1 and putative features (+1 and arrow: putative transcriptional start point; shaded: -10 and –35 region; underlined: bases similar to consensus sequence of UP-element; open boxes A, B, and C: antitermination sequences)

As seen in Figure 5, the predicted structure of the *rrn* operons of *L. lactis* is also different between the different operons. A tandem promoter separated by 30 nucleotides with an AT-rich region preceding the –10 and –35 regions is found for *rrnA*, *rrnB*, *rrnD*, and *rrnF* which is different from that described by Beresford and Condon (4), who found 110 nucleotides between the tandem promoters of *L. lactis* subsp. *lactis* NCDO 712. This emphasizes the differences that can occur between different strains of the same species. *rrnC* and *rrnE* only showed one clear –10 and –35 region preceded by an AT-rich region. The AT-rich regions could have a similar function to the UP-element even though the sequence identity is only between 57 and 67% (26).

In contrast to *Lb. plantarum* and *L. lactis,* the first 117 nucleotides upstream of the start of all the *rrn* operons of *Lb. johnsonii* are identical, and all show a tandem promoter structure with 44 nucleotides between the first and the second –10 and –35 regions. Also here an AT-rich region upstream of both the –35 region is found with 76% homology to the known UP-element.

The structure of all seven promoter regions of *E. coli,* the most studied *rrn* promoter sequences, is very similar; the main sequence variations start upstream of P1, the most upstream –10 and –35 promoter region of the tandem promoters (29). However, the –10 and –35 regions found upstream of the *rrn* operons of *Lb. plantarum*, *L. lactis*, and *Lb. johnsonii* and the potential UP-elements (15) of the promoters of *Lb. plantarum* show a clear resemblance to those of *E. coli* as well as to the consensus found in several lactococcal *rrn* promoters (4, 13). Differences can be seen in the distance between the *E. coli* (42) and *Lactococcus lactis* subsp. *lactis* NCDO 712 (4)

tandem promoters, which at 110 nucleotides, is smaller than in *Lb. plantarum* (159 bases) and larger than in *Lb. johnsonii* (44 bases). In contrast to the *rrn* promoters in *E. coli*, none of the *rrn* promoters of *Lb. plantarum, L. lactis*, and *Lb. johnsonii* has a clear binding site for FIS-protein, which upon binding forms a bent DNA conformation and leads to increased transcription (20). In agreement, an ORF coding for a FIS-like protein has also not been found in the genomes of these LAB based on similarity searches. The presence of a FIS-protein coding ORF appears to be restricted to Gram-negative *Enterobacteriaceae*, like *Salmonella typhimurium*, *Haemophilus influenzae*, and *Erwinia carotovora* (3).

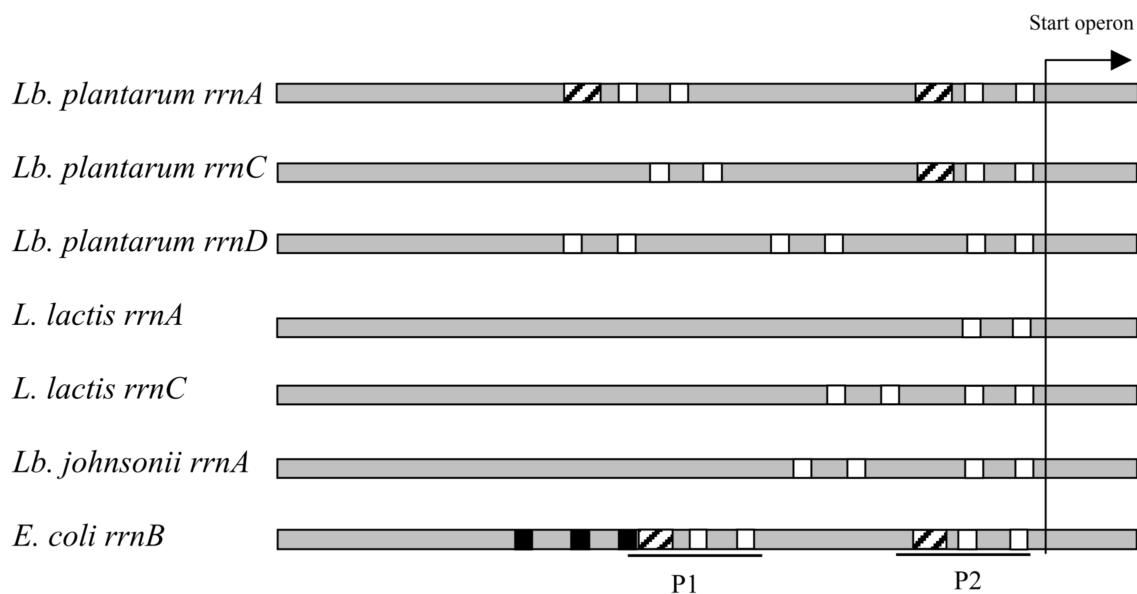

Figure 5. Structure (to scale) of the *rrn* promoters of *Lb. plantarum*, *L. lactis, Lb. johnsonii* and the typical *E. coli* promoter, *rrnB* (white boxes: -10 or –35 region; striped boxes: UP-element; black boxes: FIS-binding sites) P1 is the most upstream –10 and –35 region of the tandem promoter. P2 is the second –10 and –35 region. *Lb. plantarum rrnA* was chosen as an example for *rrnA* and *rrnB* of *Lb. plantarum; Lb. plantarum rrnD* as an example for *rrnD* and *rrnE* of *Lb. plantarum*; *L. lactis rrnA* as an example for *rrnA*, *rrnB*, *rrnD*, *rrnF* of *L. lactis*; *L. lactis rrnC* as an example for *rrnC* and *rrnE* of *L. lactis*; and *Lb. johnsonii rrnA* as example for all *rrn* operons of *Lb. johnsonii*

Anti-termination sequences in all upstream regions of the 16S rRNA gene of *Lb. plantarum*, *L. lactis*, and *Lb. johnsonii* (Box A and C; Figure 3) are almost identical to those in *E. coli* (16). The other potential anti-termination sequence (Box B) does not display high sequence similarity to the anti-termination Box B in *E. coli*. Like *E. coli* Box B, the Box B of the LAB also has a secondary loop structure; the stem consists of 6 bases and a loop of 4 to 5 bases in the LAB and 12 bases in *E. coli*. Together with the predicted ΔG of 5.9 kcal mol^{-1} in *E. coli* and between 4.2 and 6.3 kcal mol^{-1} in the LAB, it supports the existence of anti-termination activity.

Promoter activity

Based on the differences in sequence and structure found in the five *rrn* promoters of *Lb. plantarum*, it can be hypothesized that these operons are differently regulated and expressed. To evaluate this, a low-copy promoter-probe plasmid, which contains a CAT reporter gene, was used to measure the transcriptional activity of the different promoters using an MIC-analysis and CAT-assays. The constructs were called pWUPX- (pWUPC- and pWUPD-) and pWUPX+ (all operons), indicating the exclusion or inclusion of the first spacer of the operon, respectively, and in which X represents the operon from which the promoter originated. The copy number within the *Lb. plantarum* background for all promoter-reporter fusions, was assessed by dot-blot analysis, and can be considered the same based on the 95%-confidence interval except for pWUP-tRNA (data not shown). The growth rate of all clones was comparable (data not shown). The MIC-series for the different *Lb. plantarum* clones containing the pWUPX- and pWUPX+ plasmids showed a significant increase in activity when the spacer was absent in a construct (Figure 6). Note, a very low level of Cm resistance (< 10 μg ml^{-1}) was observed in the wild-type *Lb. plantarum* strain and the strain containing pGKV210; however, this was so low compared to the promoter-reporter constructs (125-150 μg ml^{-1}) that it may be considered insignificant.

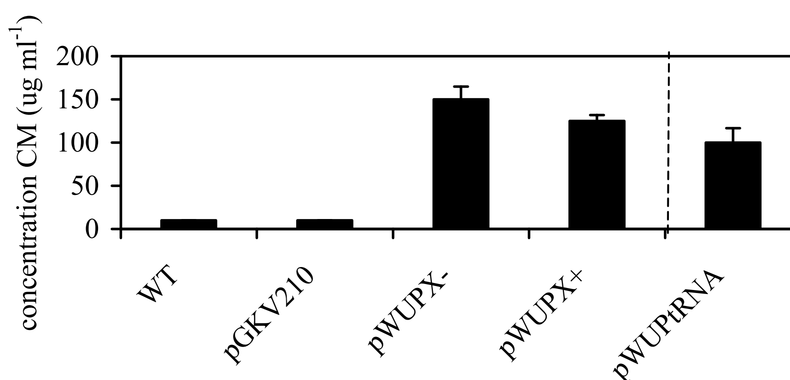

Figure 6. The chloramphenicol MIC of the *Lb. plantarum* WCFS1 clones containing the pWUP promoter reporter constructs. Similar constructs give similar values (pWUPX+: pWUPA, pWUPB, pWUPC, pWUPD, and pWUPE; pWUPX-: pWUPC and pWUPD). Comparison of pWUPtRNA to the other constructs is not possible due to differences in copy number. The experiments were performed in triplicate and the average is shown.

The CAT-assay performed on all the promoter reporter gene constructs of the different *rrn* promoters showed a differential expression between the different growth phases (Figure 7). Expression was low in the lag phase, increased during the logarithmic phase and declined in the stationary phase of growth. This was as expected, since the faster cells grow, the more ribosomes

are needed for protein synthesis as experimentally verified for *Lb. plantarum* (14). Similar to the MIC-series, the CAT-assay for the two unstable pWUPX- constructs suggested a significant decrease in promoter strength when the region preceding the mature 16S rRNA was included in the construct (data not shown). Active anti-termination boxes in the spacer preceding the 16S rRNA gene of *E. coli rrn* operons were also demonstrated previously to cause over three times down regulation when studied on a plasmid based CAT system (5).

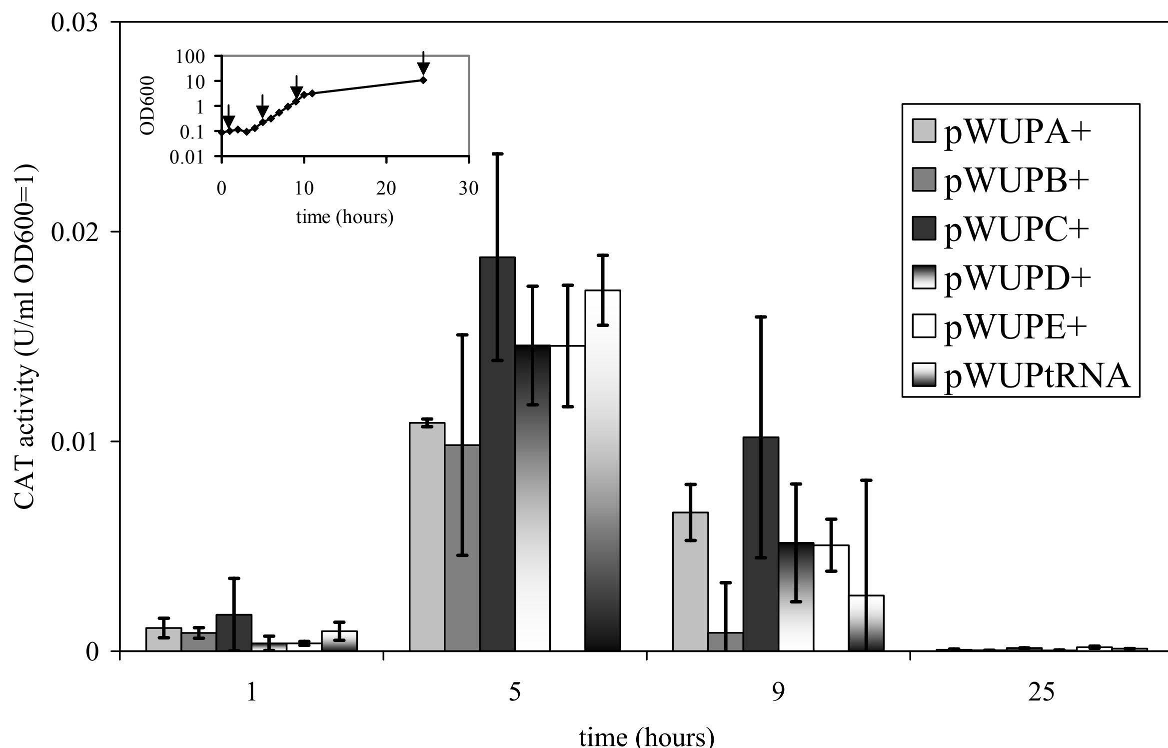

Figure 7. Comparison of expression of the different promoter regions of the rRNA operons of *Lb. plantarum* WCFS1 during different growth phases; the sampling points are indicated in the inlay. At least two experiments were performed and an average is presented.

Comparison of all the promoter clones indicated only minor differences in promoter strength in comparison to the large differences in sequence and predicted structure. The promoter structure of the *rrn* operons of *E. coli* was similar (29) and the overall activity of the seven *E. coli* promoters was also demonstrated to be very similar using promoter reporter gene (CAT) constructs (11). Although the five *Lb. plantarum rrn* promoters are significantly different, this study shows that their activity was quite similar under the conditions tested.

tRNA gene complement

The rRNA intergenic regions in *Lb. plantarum*, and *Lb. johnsonii* are highly conserved except for the presence of tRNA genes. tRNA-ile and tRNA-ala genes are found in the intergenic

spacer between the 16S and 23S rRNA genes in operon *rrnA* and *rrnB* of *Lb. plantarum* and in operon *rrnA*, *rrnE*, and *rrnF* of *Lb. johnsonii*. A tRNA-ala gene is present in the intergenic spacer between the 16S and 23S rRNA genes of all lactococcal *rrn* operons. In Gram-positive bacteria the intergenic spacer region between the 16S and 23S genes in some operons contains either a tRNA-ile or a tRNA-ala gene or both. Several *Lactobacillus* species appear to have no tRNA present in at least one of the 16S-23S spacers of their rRNA operons within one strain, as determined by PCR amplification and sequence determination of the 16S-23S intergenic spacer of five different lactobacilli (28).

The tRNA genes following the 5S rRNA gene and their genetic linkage to the *rrn* operons are presented in Figure 3. *rrnC* of *Lb. plantarum* contains an extra 5S rRNA gene that may be a duplication of the 5S rRNA gene following the 23S rRNA gene, because the sequences of the 5S and the tRNA genes following it (tRNA-val, tRNA-lys) are identical. A putative promoter is present downstream of the second 5S rRNA gene with tRNA-val and tRNA-lys of *Lb. plantarum* (Figure 3) and downstream of the tRNA-lys genes of the *rrnC* and *rrnD* operons of *Lb. johnsonii*. Interestingly, a promoter at this position is present in *B. subtilis*, allowing transcription of the downstream tRNA genes on an independent transcript. The tRNA complement can also be transcribed as part of the *rrn* operon (18). Since we were focussing on the promoter activity of the *rrn* promoters of *Lb. plantarum,* we also investigated if an active tRNA promoter was present at this position in *Lb. plantarum*. Both the MIC series (Figure 6) and the CAT-assay (Figure 7) indicated an active promoter is present between the tRNA genes following the 5S rRNA gene of *rrnC* of *Lb. plantarum*. This indicates the possibility of independent transcription of 17 of the 21 tRNA genes. pWUPtRNA showed a lower copy number than the plasmids with other promoters tested (data not shown), therefore the promoter strength of pWUPtRNA seems large compared to the other promoters.

Conservation of the tRNA complement

Up to 14, 16, and 21 tRNA genes were found following the *rrn* operons of *L. lactis*, *Lb. johnsonii*, and *Lb. plantarum* respectively. Those numbers are in the same range as the 2 to 21 tRNA genes found in *B. subtilis* (27). The order of the tRNA genes appears to be highly conserved amongst some Gram-positive bacteria (17). Clustering of high numbers of tRNA genes adjacent to rRNA genes is common in most Gram-positive bacteria (17). Gram-negative bacteria, like *E. coli*, usually transcribe their tRNA genes in much smaller transcriptional units or

as single genes, and only one or two tRNA genes follow the rRNA genes (29). The order of tRNA genes is conserved between groups of bacteria, for example, Gram negative bacteria like *E. coli*, *Salmonella typhi*, and *Haemophilus influenzae*, or Gram-positives like *B. subtilis*, *B. halodurans*, *L. innocua*, *Lb. plantarum, L. lactis,* and *Lb. johnsonii* have high similarity in their order and number of tRNA genes following the rRNA operons (Figure 3). However, there are differences in the order of tRNA genes following
different *rrn* operons within one micro-organism. The conservation in the order of tRNA genes at the 3'-end of *rrn* operons is probably due to a common ancestor in the line of evolution.

In conclusion, comparison of the *rrn* operons of three LAB, *Lb. plantarum*, *L. lactis*, and *Lb. johnsonii*, for which the total genome sequences are available, showed several similar features, in particular the order of *rrn* genes, the microheterogeneity in the genes, and the consistency in the tRNA complement, which are characteristic of the majority of *rrn* operons in bacteria described so far. However, there were considerable differences in promoter sequence and structure for *Lb. plantarum* and *L. lactis.* Differential expression over time was demonstrated for the *Lb. plantarum* promoters using promoter-reporter fusions, i.e. low expression in the lag phase, increase during the logarithmic phase and decline in the stationary phase of growth. Although the five *Lb. plantarum rrn* promoters are significantly different, their activities were found to be relatively similar under the circumstances tested, suggesting that differences in regulation either take place under specific circumstances or are not relevant. An active promoter is present within *rrnC* of *Lb. plantarum*, indicating independent transcription of the 17 tRNA genes. A correlation between the tRNA complement of the *rrn* operons within the three LAB and the *rrn* promoter structures was not detected, but possible regulation of the transcription of other tRNA gene clusters may take place on a tRNA promoter level. We propose in this paper that focussing on different rRNA operons including promoters and tRNA-complement within one strain can complement the recent knowledge about rRNA operons and improve the use of rRNA-based molecular techniques.

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References

1. **Aiyar, S. E., T. Gaal, and R. L. Gourse.** 2002. rRNA promoter activity in the fast-growing bacterium *Vibrio natriegens*. J. Bacteriol. **184:**1349-1358.

2. **Amann, R. L., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.

3. **Beach, M. B., and R. Osuna.** 1998. Identification and characterization of the *fis* operon in enteric bacteria. J. Bacteriol. **180:**5932-5946.

4. **Beresford, T., and S. Condon.** 1993. Physiological and genetic regulation of rRNA synthesis in *Lactococcus*. J. Gen. Microbiol. **139:**2009-2017.

5. **Berg, K. L., C. Squires, and C. L. Squires.** 1989. Ribosomal RNA operon anti-termination: Function of leader and spacer region box B-box A sequences and their conservation in diverse micro-organisms. J. Mol. Biol. **209:**345-358.

6. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Res. **11:**731-753.

7. **Brosius, J., T. J. Dullss, D. D. Sleeter, and H. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107-127.

8. **Chevallier, B., J. C. Hubert, and B. Kammerer.** 1994. Determination of chromosome size and number of *rrn* loci in *Lactobacillus plantarum* by pulse-field gel electrophoresis. FEMS Microbiol. Lett. **120:**51-56.

9. **Chiaruttini, C., and M. Milet.** 1993. Gene organization, primary structure and RNA processing analysis of a ribosomal RNA operon in *Lactococcus lactis*. J. Mol. Biol. **230:**57-76.

10. **Coenye, T., and P. Vandamme.** 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. FEMS Microbiol. Lett. **228:**45-49.

11. **Condon, C., J. Philips, Z.Y. Fu, C. Squires, and C. L. Squires.** 1992. Comparison of the expression of the seven ribosomal RNA operons in *Escherichia coli*. EMBO J. **11:**4175-4185.

12. **Condon, C., C. Squires, and C. L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli*. Microbiol. Rev. **59:**623-645.

13. **de Vos, W. M.** 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. **46:**281-295.

14. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2004. Optimising single cell activity assessment of *Lactobacillus plantarum* by fluorescent *in situ* hybridisation as affected by growth. J. Microbiol. Methods **59:**109-115.

15. **Estrem, S. T., T. Gaal, W. Ross, and R. L. Gourse.** 1998. Identification of an UP element consensus sequence for bacterial promoters. Proc. Natl. Acad. Sci. U.S.A. **95:**9761-9766.

16. **Gourse, R. L., H. A. de Boer, and M. Nomura.** 1986. DNA determinants of rRNA syntesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation, antitermination. Cell **44:**197-205.

17. **Green, C. J., and B. S. Vold.** 1993. *Staphylococcus aureus* has clustered tRNA genes. J. Bacteriol. **175:**5091-5096.

18. **Green, C. J., and B. S. Vold.** 1993. tRNA, tRNA processing, and aminoacetyl-tRNA synthetases. American Society for Microbiology, Washington, U.S.A.

19. **Holzapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger.** 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. **73:**365S-373.

20. **Hübner, P., and W. Arber.** 1989. Mutational analysis of a prokaryotic recombinational enhancer element with two functions. EMBO J. **8:**577-585.

21. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a Gram-positive broad-host range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

22. **Klappenbach, J. A., J. M. Dunbar, and T. M. Schmidt.** 2000. rRNA operon copy number reflects ecological strategies of bacteria. Appl. Environ. Microbiol. **66:**1328-1333.

23. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

24. **Lazzi, C., L. Rossetti, M. Zago, E. Neviani, and G. Giraffa.** 2004. Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. J. Appl. Microbiol. **96:**481-490.

25. **Liao, D.** 2000. Gene conversion drives within genic sequences: Concerted evolution of ribosomal RNA genes in bacteria and archaea. J. Mol. Evol. **51:**305-317.

26. **McCracken, A., M. S. Turner, P. Giffard, L. M. Hafner, and P. Timms.** 2000. Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. Arch. Microbiol. **173:**383-389.

27. **Moszer, I.** 1998. The complete genome of *Bacillus subtilis*: from sequence annotation to data management and analysis. FEBS Lett. **430:**28-36.

28. **Nour, M.** 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res. Microbiol. **149:**433-448.

29. **Ohnishi, M., T. Murata, K. Nakayama, S. Kuhara, M. Hattori, K. Kurokawa, T. Yasunaga, K. Yokoyama, K. Makino, H. Shinagawa, and T. Hayashi.** 2000. Comparative analysis of the whole set of rRNA operons between an enterohemorrhagic *Escherichia coli* O157:H7 Sakai strain and an *Escherichia coli* K-12 strain MG 1655. System. Appl. Microbiol. **23:**315-324.

30. **Overbeek, R., N. Larsen, T. Walunas, M. D'Souza, G. Pusch, E. Selkov, Jr, K. Liolios, V. Joukov, D. Kaznadzey, I. Anderson, A. Bhattacharyya, H. Burd, W. Gardner, P. Hanke, V. Kapatral, N. Mikhailova, O. Vasieva, A. Osterman, V. Vonstein, M. Fonstein, N. Ivanova, and N. Kyrpides.** 2003. The ERGOtm genome analysis and discovery system. Nucl. Acids. Res. **31:**164-171.

31. **Pedersen, A. G., L. J. Jensen, S. Brunak, H.-H. Staerfeldt, and D. W. Ussery.** 2000. A DNA structural atlas for *Escherichia coli*. J. Mol. Biol. **299:**907-930.

32. **Pridmore, R. D., B. Berger, F. Desiere, D. Vilanova, C. Barretto, A.-C. Pittet, M.-C. Zwahlen, M. Rouvet, E. Altermann, R. Barrangou, B. Mollet, A. Mercenier, T. Klaenhammer, F. Arigoni, and M. A. Schell.** 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc. Natl. Acad. Sci. U. S. A. **101:**2512-2517.

33. **Randazzo, C. L., S. Torriani, A. D. L. Akkermans, W. M. de Vos, and E. E. Vaughan.** 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal sicilian cheese as evaluated by 16S rRNA analysis. Appl. Environ. Microbiol. **68:**1882-1892.

34. **Ross, W., S. E. Aiyar, J. Salomon, and R. L. Gourse.** 1998. *Escherichia coli* promoters with UP elements of different strengths: modular structure of bacterial promoters. J. Bacteriol. **180:**5375-5383.

35. **Rudner, R., A. Chevrestt, S. R. Buchholz, B. Studamire, A.M. White, and E. D. Jarvis.** 1993. Two tRNA gene clusters assiociated with rRNA operons *rrnD* and *rrnE* in *Bacillus subtilis*. J. Bacteriol. **175:**503-509.

36. **Sambrook, J.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

37. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43:**737-755.

38. **Shimizu, T., S. Ohshima, K. Ohtani, K. Hosino, K. Honjo, H. Hayashi, and T. Shimizu.** 2001. Sequence heterogeneity of the ten rRNA operons in *Clostridium perfringens*. System. Appl. Microbiol. **24:**149-156.

39. **Tourova, T. P.** 2003. Copy number of ribosomal operons in prokaryotes and its effect on phylogenetic analyses. Microbiology **72:**437-452.

40. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilus* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540-542.

41. **Yap, W. H., Z. Zhang, and Y. Wang.** 1999. Distinct types of rRNA operons exist in the genome of the actinomycete *Thermonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. J. Bacteriol. **181:**5201-5209.

42. **Young, R. A., and J. A. Steitz.** 1979. Tandem promoters direct *E. coli* ribosomal RNA synthesis. Cell **17:**225-234.

43. **Zheng, D., E. W. Alm, D. A. Stahl, and L. Raskin.** 1996. Characterization of universal small-subunit rRNA hybridisation probes for quantitative molecular microbial ecology studies. Appl. Environ. Microbiol. **62:**4504-4513.

Chapter 3

Optimising Single Cell Activity Assessment of *Lactobacillus plantarum* by Fluorescent *in situ* Hybridisation as Affected by Growth

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Abstract

Fluorescent *in situ* hybridisation (FISH) with a 16S ribosomal RNA (rRNA) targeted oligonucleotide probe, Eub338, could be used to estimate the *in situ* activity of *Lactobacillus plantarum* WCFS1 in exponentially growing cells. However, *L. plantarum* is capable of growth to very high cell densities and the properties of the *L. plantarum* cell-envelope prevented effective entry of the fluorescent oligonucleotide probe into the cells at later stages of growth at high cell densities. Total rRNA measurements of cells isolated at different growth stages showed maximal amounts of RNA (8.77±0.8 fg) per cell at the early stationary phase and confirmed the effectiveness of FISH for accurate activity measurement in exponentially growing cells.

Keywords: cell density, fluorescent *in situ* hybridisation, *Lactobacillus plantarum,* single cell activity

Introduction

Fluorescent *in situ* hybridisation (FISH) is widely applied to detect, identify and enumerate bacteria and other micro-organisms, by using fluorescent oligonucleotide probes that specifically bind to the ribosomal RNA (rRNA)(3). The fluorescence intensity per cell can be used as an indicator of its overall metabolic activity, since during exponential growth at low cell densities this is coupled to efficient protein synthesis in several different bacteria and consequently linked to high numbers of ribosomes containing rRNA (5, 7, 13). A linear relationship between growth rate and total cellular RNA content was demonstrated in *Salmonella* (15). Since the ratio between rRNA and the less abundant mRNA was found to be constant, a similar linear relationship was found between growth rate and rRNA by using FISH or quantitative PCR in various bacterial species including *Escherichia coli* (16), *Staphylococcus aureus* (19) and *Lactococcus lactis* (9). Notably, the FISH method has not yet been investigated for use with cells at high densities.

To estimate the rRNA content by FISH, it is of great importance to permeabilise the cell-envelope in a controlled and reproducible way. If the cells are under- or over permeabilised, insufficient probe can enter or the rRNA may leak out, respectively, and subsequently the activity of the cell will be underestimated. Some bacteria require the activity of lytic enzymes such as lysozyme to be effectively permeabilised (2).

Lactobacillus plantarum is a versatile, Gram-positive, fermentative bacterium found in many food fermentations, and as a natural inhabitant of the human gastrointestinal tract, and whose complete genome sequence has recently become available (1, 10). *L. plantarum* can grow to high cell densities, which is a desirable property for industrial applications and which may occur in substrate-rich environments like the gastrointestinal tract.

The aim of this study was to assess the feasibility of applying FISH for estimating the *in situ* activity of *L. plantarum* cells both in low and high cell densities and to determine the changes in the amount of rRNA per cell during various growth phases.

Materials and methods

Strains and cultivation

L. plantarum WCFS1 (11) was grown at 30°C, stationary, in MRS broth (Difco, Le Pont de Claix, France). *Lactococcus lactis* MG 1363 (6), used as a control in the FISH experiments, was grown at 30°C, stationary, in M17 medium supplemented with 0.5% lactose (Difco, Le Pont de Claix, France). MRS broth was used as an optimal growth medium for *L. plantarum* since the high cell density this medium supports reflects our interest in other substrate-rich environments.

During growth the OD_{600} was also monitored and colony forming units (CFU) were determined by plating appropriate dilutions on MRS solidified with 1.5% agar followed by aerobical incubation at 30°C for 48 h.

Permeabilisation of the cells

Bacteria were harvested and washed in phosphate-buffered saline (18) before and after fixation in 4% paraformaldehyde (Merck, Darmstadt, Germany) and were used immediately or stored in equal volumes of phosphate-buffered saline and 96% ethanol at –20°C. Alternatively, cells were fixed in ice-cold 96% ethanol. Ten µl of the fixed cells were dried onto wells of gelatine-coated glass slides (ICN Biomedicals Inc., US). A variety of permeabilisation treatments of the cells on the slides were tested, all in a volume of 10 µl of permeabilising solution per well. These included lysozyme concentrations from 1-100 mg ml^{-1}, and different buffers with varying pH, namely maleic acid pH 6.5 (0.5 M lactose, 20 mM maleic acid , 20 mM $MgCl_2$); HEPES buffer pH 6.5 (15 mM Hepes, 1 mM $MgCl_2$); Tris HCl pH 7.5 (10 mM Tris, 1 mM EDTA); and Tris HCl pH 6.5 (10 mM Tris, 1 mM EDTA). The

duration of the treatment was varied from 10 min to 16 h. In addition, an enzyme permeabilisation mixture was tested containing saccharose (0.585 M), $CaCl_2$ (5 mM), sodiumtaurocholaat (0.3 mg ml^{-1}), lysozyme (5 mg ml^{-1}), pancreatic lipase (0.1 mg ml^{-1}), and mutanolysin (26 U ml^{-1}) in a buffer containing 25 mM Tris/HCl and 10 mM EDTA. All chemicals were obtained from Sigma Chemicals Co. (St. Louis, USA).

Fluorescent *in situ* hybridisation

After the permeabilisation treatments, slides were rinsed with milliQ water and the protocol for FISH was performed as previously described (2). Probe Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') (4) labelled with fluorescein (Eurogentec, Seraing, Belgium) was used for FISH (2). 4',6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St Louis, USA) was added to the washing buffer to stain all DNA present and detect all cells, whether permeable or not. The cells were detected with a Leica HC fluorescent microscope (filter I3 and A for Eub338 and DAPI, respectively) and the fluorescence intensity per cell was automatically measured using Leica Qwin software according to the manufacturer's instructions (Leica Microsystems b.v., Rijswijk, The Netherlands). Saturation of the fluorescence intensity was avoided using an appropriate closure time of the camera.

FISH was optimised by comparing the percentage of cells stained with Eub338 to DAPI, which was confirmed to stain all cells by using phase contrast microscopy. After optimisation of the protocol, the amount of fluorescence intensity was determined in cells harvested from different growth phases and compared to a reference sample. The reference sample was used to normalise between slides for differences in staining. When different permeabilisation treatments were used, the staining of the reference sample changed, and therefore fluorescence intensity values could only be compared within one slide. For comparison between samples on different slides, we could only compare the patterns of fluorescence intensity values, i.e. when there was increase, decrease or when a maximum value was reached.

Total RNA isolation

Total RNA was isolated from the *L. plantarum* cells during different growth phases in three independent experiments using the Rneasy® kit following the manufacturer's instruction (Qiagen Sciences, Maryland, USA). The total RNA concentration was determined by spectophotometric analysis at 260 and 280 nm (17). Cell disruption was done by bead

beating (mini-beadbeater; Biospec products, Bartlesville, USA) 3 times for 1.5 min and cooling on ice between the treatments. Plate counting and microscopy confirmed total lysis of the cells.

Results and discussion

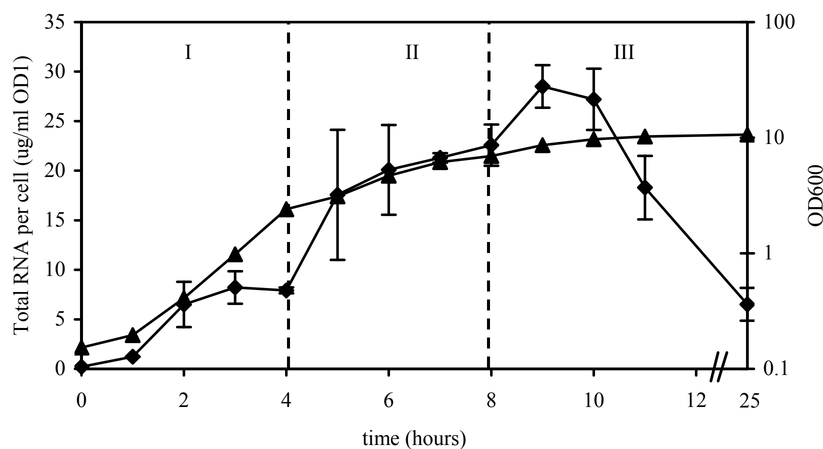
Strategy to permeabilise cells at different cell densities

While gentle or even no lysozyme treatment was sufficient to permeabilise cells of lactic acid bacteria such as *L. acidophilus* (9) and *Lactococcus lactis* (data not shown), permeabilisation of cells of *L. plantarum* was found to require more intense treatments (data not shown). Hence, we started to optimise the permeabilisation of *L. plantarum* during growth by determining FISH-fluorescence intensity of the Eub338 probe and DAPI, and total RNA amounts.

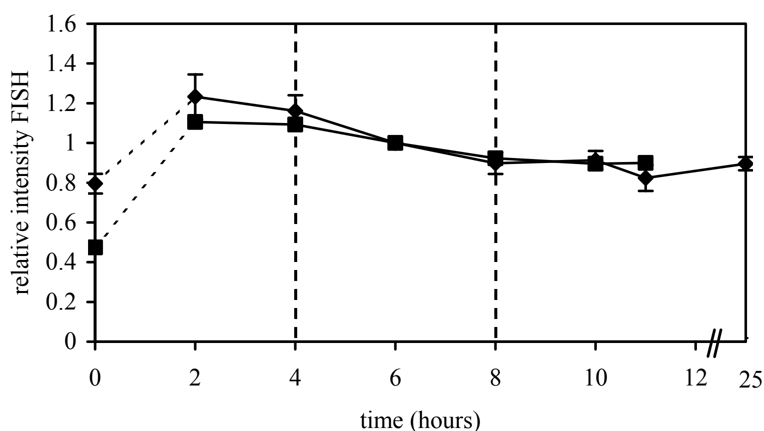
L. plantarum is capable of rapid growth to high densities of more than 10^{10} cells per millilitre of conventional buffered broth media. In all growth phases, the number of CFU's obtained by plating equalled that of cells observed following DAPI staining, indicating that all *L. plantarum* cells were viable. Following exponential growth with a specific growth rate of 0.68 h^{-1} at a density of approximately OD_{600} of 3 (phase I), a second exponential growth phase is entered with a specific growth rate of 0.20 h^{-1} (phase II) before the stationary phase (phase III) is reached (Figure 1A). The different growth phases were reproducibly observed, even in diluted MRS (data not shown) and had considerable effects on the permeabilisation of the cell-envelope.

Optimising cell envelope permeabilization

When FISH was applied to phase I, II, and III, 50% of the cells fluoresced brightly when a paraformaldehyde treatment was used followed by an overnight storage in ethanol (data not shown). The lysozyme concentrations had different effects on the cells. Cells in phase I could be efficiently permeabilised by incubation with high concentrations of lysozyme (100 mg ml^{-1}). However, with cells at high densities in phase II and III the same lysozyme treatment allowed only about 25% of all cells to be stained by the FISH probe compared to DAPI (Figure 2A).



A.



B.

Figure 1. Comparison of fluorescence intensity and total RNA measurements during different growth phases of *L. plantarum* WCFS1. A) Measurements of total RNA per OD_{600} by total RNA isolation (indicated by diamonds) and growth of *L. plantarum* WCFS1 in MRS at 30°C over time (indicated by triangles); phase I: first exponential growth phase, phase II: second exponential growth phase, phase III: stationary phase; B) Measurements of rRNA by relative fluorescence intensity by FISH over time. The lines indicate different lengths of lysozyme treatments (1h indicated by diamonds; 2h indicated by squares). The broken lines indicate the difference between the just transferred stationary cells to exponentially growing cells.

Also the composition of the buffer and its pH had a large influence on the permeabilization by lysozyme. Incubation in a Tris/HCl buffer at pH 7.5, HEPES at pH 6.5, and maleic acid buffer at pH 6.5 failed to give effective permeabilisation since only 20-30% of the cells were found to be hybridising to the Eub338-probe. In Tris/HCl buffer at pH 6.5, lysozyme treatment appeared to be most effective, and subsequently this buffer was routinely used (Figure 2B).

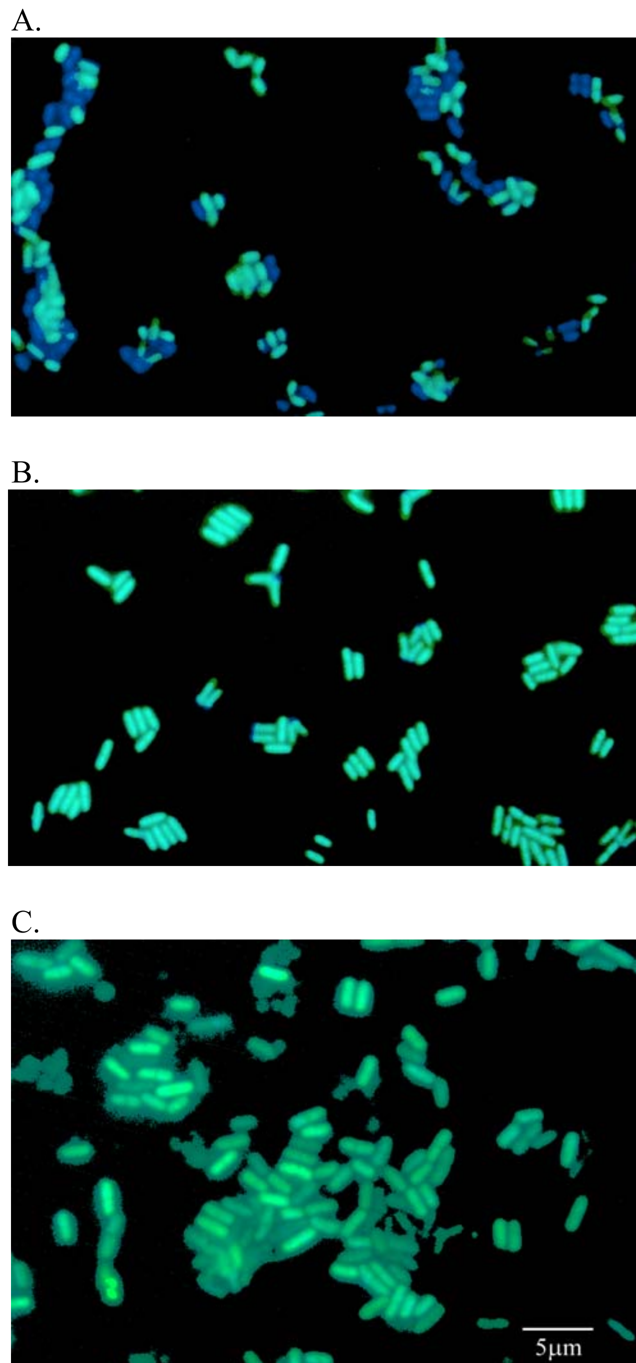


Figure 2. Exemplary analyses of variable permeabilisation efficiencies measured with *L. plantarum* WCFS1 cells hybridised with fluorescein-labelled Eub338 and stained with DAPI. All cells are stained blue with DAPI; only those cells, which hybridised with the Eub338 probe are green. A) Part of the stationary phase population is stained after permeabilisation with 100 mg ml^{-1} lysozyme in Tris/HCl buffer at pH 7.5 for 1 h; B) Exponential cells permeabilised in Tris/HCl buffer at pH 6.5 with 100 mg ml^{-1} lysozyme and a treatment time of 1 h. all appeared stained; C) Diffuse appearance of stationary phase cells permeabilised in Tris/HCl buffer at pH 7.5 with 100 mg ml^{-1} lysozyme for 16 h.

The different durations of lysozyme treatment showed that a common incubation time of 10 min was insufficient to permeabilise *L. plantarum*. However, following an overnight incubation with lysozyme, all the cells were found to hybridise to the Eub338 probe, thus demonstrating the possibility to permeabilise all cells. The disadvantage was that the cells often displayed a diffuse appearance suggesting loss of cell structure and leakage of cell content including rRNA (Figure 2C). The most optimal lysozyme treatment for both exponential and stationary phase cells was determined to be 1 h, allowing the staining of all cells with both DAPI and Eub338 but lacking the appearance of diffuse cells. The enzyme mix, as described before, failed to improve the quality of the permeability. The final buffer of choice was a Tris/HCl buffer at pH 6.5 with 100 mg ml^{-1} lysozyme and a treatment time of 1 h, which resulted in effective permeabilisation of phase I, II, and III cells. The permeabilisation procedure described above was applied to cells harvested at various stages of growth and FISH was subsequently performed (Figure 1A).

Total amount of RNA per cell

Total RNA was extracted at regular time intervals and a maximum amount of 28.5±0.9 µg RNA was found per ml of early stationary phase cells (phase III) of OD_{600} 1(Figure 1A). Based on the cell counts this represents 8.77±0.8 fg of total RNA per cell. This is in the same order of magnitude as found for *E. coli* and *Rickettsia powazekii*, respectively 5.6 and 71.2 fg per cell (14). An increasing concentration of total RNA per cell was observed during both exponential phases of growth and early stationary growth followed by a rapid decline in the late stationary phase (Figure 1A). Just after entering phase I, phase II, and phase III, a remarkable increase in total amount of RNA was detected. Thus, increasing amounts of rRNA were formed upon change of the growth phase. This may reflect adaptation to new conditions representative for the growth phase, including the adaptation of the overall protein repertoire expressed. Such adaptation would require increased protein synthesis and hence an increased amount of rRNA.

The growth phase in which the maximal RNA content per cell is observed appears to differ depending on the bacterial species analysed. *E. coli* showed a maximum amount of 16S rRNA at the beginning of exponential growth (16), while early-stationary phase cells of *Listeria monocytogenes* contained maximal rRNA amounts (12). Thus the Gram-positive bacteria, *L. plantarum* and *Listeria monocytogenes*, show a similar development in time of the amount of rRNA per cell.

Single cell activity assessment using FISH

During the FISH experiments, it was noticed that the populations of cells in phases II and III gave uneven fluorescence. Single colony isolates behaved in the same manner indicating that genetic heterogeneity in the *L. plantarum* culture was not the reason for uneven hybridisation. Since all cells could be stained by a 16 h. lysozyme treatment in earlier experiments (Figure 2C), inefficient permeability was likely to be the cause. Only the brightly stained cells were taken into account for calculations in further FISH experiments to determine fluorescence intensity per cell.

The differences in quantity of RNA per cell as measured by total RNA isolation were similar to that obtained by FISH for a 1h lysozyme treatment but only for the first phase of growth (Figure 1B). The fluorescence intensity increased for the first two h. and then essentially levelled off as was also observed for the amount of total RNA (Figure 1A and B). The measurements at higher cell densities, in phases II and III, generated a reasonable amount of fluorescence intensity. However, comparison to the major differences found by total RNA isolation, proved this method to be inadequate to estimate the rRNA concentration, and so the activity of *L. plantarum*. Based on experiments described earlier, ineffective permeabilisation was presumably responsible for the contrasting results. To overcome this, attempts to improve the permeability by increasing the length of time of lysozyme treatment on cells in all growth phases were performed (Figure 1B). One, two, three and six hours of lysozyme treatment revealed a consistent correlation between fluorescence intensity and the total amount of RNA in phase I. However, these procedure adaptations failed to give good permeabilisation of phase II and III cells (Figure 1B; data not shown). The difficulties encountered in permeabilisation of the cells are probably caused by a change in the cell envelope during phase II, which prepares the cells for survival under stationary phase conditions.

Consequently, the method developed is useful for activity assessment of pure cultures and complex ecosystems, provided low cell densities are expected. The fact that *L. plantarum* has a particularly tough cell wall was suggested both by its high survival of the upper part of the GI-tract following human ingestion as determined by ileal intubation (20), and by difficulties encountered to visualise green fluorescent protein (GFP) production in *L. plantarum* (8).

We conclude that the maximum amount of ribosomal RNA per cell is present at the start of the stationary growth phase (phase III) and that FISH can be used to estimate the *in situ* activity of *L. plantarum* during the first exponential growth phase (phase I). However, at high cell densities permeabilisation of *L. plantarum* cells becomes inconsistent and is not suited for activity determination.

References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Amann, R. I.** 1996. *In situ* identification of micro-organisms by whole cell hybridisation with rRNA-targeted nucleic acid probes, p. 3.3.6/1-3.3.6/15. *In* A. D. L. Akkermans, J. D. v. Elsas, and F. J. d. Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht.

3. **Amann, R. I., B. Zarda, D. A. Stahl, and K.H. Schleifer.** 1992. Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **58:**3007-3011.

4. **Amman, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytrometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

5. **Binder, B. J., and Y. C. Liu.** 1998. Growth rate regulation of rRNA content of a marine *Synechococcus* (Cyanobacterium) strain. Appl. Environ. Microbiol. **64:**3346-3351.

6. **Gasson, M. J.** 1983. Plasmid components of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1-9.

7. **Gausing, K.** 1977. Regulation of ribosome production in *Escherichia coli*: Synthesis an stability of ribosomal RNA and of ribosomal protein messenger RNA at different growth rates. J. Mol. Biol. **115:**335-354.

8. **Geoffroy, M.C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. **66:**383-391.

9. **Hansen, M. C., A. K. Nielsen, S. Molin, K. Hammer, and M. Kilstrup.** 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: Fluorescent *in situ* rRNA hybridisation permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. **183:**4747-4751.

10. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing, Gram-positive rods, p. 1229. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams and Wilkins, Baltimore, U.S.A.

11. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

12. **Milner, M. G., J. R. Saunders, and A. J. McCarthy.** 2001. Relationship between nucleic acid ratios and growth in *Listeria monocytogenes*. Microbiology **147:**2689-2696.

13. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: A molecular approach, 4th ed. Sinauer Associates Inc., Sunderland.

14. **Pang, H., and H. H. Winkler.** 1994. The concentrations of stable RNA and ribosomes in *Rickettsia prowazekii*. Mol. Microbiol. **12:**115-120.

15. **Rosset, R., J. Julien, and R. Monier.** 1966. Ribonucleic acid composition of bacteria as a function of growth rate. J. Mol. Biol. **18:**308-320.

16. **Ruimy, R., V. Breitmayer, V. Boivin, and R. Christen.** 1994. Assesment of the state of activity of individual bacterial cells by hybridization with a ribosomal RNA targeted fluorescently labelled oligonucleotidec probe. FEMS Microbiol. Ecol. **15:**207-214.

17. **Sambrook, J.** 1989. Quantification of DNA and RNA. Appendix E, pg. 5 *In* E. F. Fritsch and T. Maniatis (ed.), Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

18. **Sambrook, J.** 1989. Preparation of reagents and buffers used in molecular cloning, p. Appendix B, pg.12. *In* E. F. Fritsch and T. Maniatis (ed.), Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

19. **Van de Casteele, S. J., W. E. Peetermans, R. Merckx, and J. van Eldere.** 2001. Quantification of expression of *Staphylococcus epidermis* housekeeping genes with Taqman quantitative PCR during *in vitro* growth under different conditions. J. Bacteriol. **183:**7094-7101.

20. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-828.

Chapter 4

Transcript Profiling Reveals Global Gene Expression of *Lactobacillus plantarum* in the Human Gastrointestinal Tract

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Abstract

The human intestinal tract represents a dynamic ecosystem comprising various habitats each with a niche-specific microbiota. To investigate the effect of the complex human intestinal mucosa on a single commensal microbial species, we analyzed the *in vivo* global gene expression of *Lactobacillus plantarum*. Prior to surgery, three patients diagnosed with colon cancer ingested for one week a fermented oatmeal drink with probiotic *L. plantarum* 299v (10^{11} viable cells daily), and a fourth patient consumed a placebo without bacteria. Total RNA was isolated from the mucosa of surgically-removed intestinal segments of normal tissue, and hybridized to a DNA microarray comprising clones covering the *L. plantarum* genome. The reproducibility, efficiency, and specificity of the microarrays were confirmed by several approaches including quantitative reverse transcriptase PCR coupled to sequence analysis. The presence of the microbe in the samples was confirmed in at least a 100-fold higher concentration in the subjects consuming *L. plantarum* than in the placebo. The ingested *L. plantarum* cells were metabolically active in all subjects as demonstrated by the detection of about 10% expressed genes by the DNA microarrays. Genes were detected for all functional classes. The differences in *L. plantarum* gene expression between the colons of three individuals were larger than the differences between the ileum and colon of a single individual. To our knowledge, this is the first report of the global gene expression analysis *in vivo* of commensal or ingested bacteria in association with the human intestinal mucosa.

Keywords: *Lactobacillus plantarum*, DNA microarray, transcriptional profiling, mucosa, human intestine

Introduction

The human being and his intestinal microorganisms live in symbiosis having co-evolved over millions of years. The intestinal microbiota contribute significantly to host nutrition, immunity, intestinal epithelial development, and regulation of energy storage (3, 8). Ecological approaches, especially 16S rRNA gene sequencing, have revealed the substantial diversity of the largely undescribed human microbiota (11, 37). Recent analysis of the genome sequences of human intestinal lactobacilli including probiotic members (2, 18, 28) is starting to shed light on the molecular mechanisms underlying intestinal residence as well as host-microbe interactions. Ingested probiotic microorganisms are considered to offer a variety

of benefits to health and well being (29, 32). An ingested microbe encounters various ecological niches where it adapts to unusual conditions that include high acidity and osmolarity, anaerobiosis, and large changes in nutrient availability. Currently, the activity of commensal microbiota, pathogenic or ingested probiotic microbes *in vivo* in the complex human intestinal ecosystem has not been determined. Methods for analysis of bacterial gene expression *in vivo* include promoter-reporter gene constructs (9, 10, 25) and the recombinant *in vivo* expression technology (R-IVET; 5, 15, 20, 22, 38), but the use of genetically modified microbes has so far been restricted to animals. R-IVET has been used to determine genes specifically switched on in the intestinal tract, such as those involved in virulence in pathogens or persistence of commensal bacteria (5, 15, 20, 22, 38). Similarly, DNA microarrays were used to analyze the global transcriptional response of the pathogenic *Campylobacter jejuni* inoculated into the rabbit ileal loop model (34). The *C. jejuni* cells had adapted to an anaerobic, nutrient-poor intestinal environment, and in particular appeared to undergo extensive remodeling of the cell envelope. Microarray analysis of the human commensal *Bacteroides thetaiotaomicron* in the intestine of mono-associated mice revealed their preference for scavenging dietary derived polysaccharides and secondly host mucus glycans (33). Quantitative reverse transcriptase (qRT)-PCR of bacterial genes has been applied to human intestinal samples (12, 30) but is relatively low throughput.

Lactobacillus plantarum is a Gram-positive, lactic acid bacterium found in a range of dairy, meat, and plant fermentations, and is a natural inhabitant of the human gastrointestinal tract (1, 17). The intestinal-isolate *L. plantarum* strain 299v is marketed as a probiotic, especially since its consumption during a short period was found to alleviate irritable bowel syndrome (24). The genome sequence of a human pharyngeal isolate, *L. plantarum* WCFS1 (18), has allowed the design of specific DNA microarrays for use in transcript profiling of this species. Comparison between different strains of *L. plantarum*, including 299v, using DNA microarrays revealed high similarity percentage in genes involved in biosynthesis or degradation of structural components, like proteins, lipids and DNA, and differences in genes encoding sugar metabolism (23). In this study we compared the global gene expression of *L. plantarum* in the human intestinal tract following excision of intestinal segments from patients that prior to surgery had daily ingested *L. plantarum* and contrasted these with gut samples obtained from a patient on a placebo. The findings revealed differential gene expression between the ileum and colon, and between colonic mucosa of different subjects,

and signified genes likely to be important to the ingested organisms successful passage and residence in the human intestine. To our knowledge this is the first report of monitoring gene expression using DNA microarrays of a commensal, and probiotic, microbe in the complex human intestinal microbial ecosystem.

Material and Methods

Biopsy samples

During a randomised, double blind, placebo-controlled study, volunteers tentatively diagnosed with colon cancer ingested a fermented oatmeal drink with 10^{11} colony forming units of *L. plantarum* 299v per day for one week prior to surgery. The intestinal samples comprised normal tissue of segments of the ileum and sigmoid colon from a 45 year old male (A); a transverse colon segment from a 70 year old male (B); and ascending and transverse colon segments from 85 (C) and 65 year old female (D) patients, respectively. The latter person ingested a placebo product containing oatmeal but without strain 299v. The patients will be referred to as persons A, B, C, and D, respectively. The study was approved by the Humans Ethics Committee at Lund University. The intestinal segments were cut open immediately after surgical removal, the unaffected part of the upper mucosal layer with mucus and bacteria (approximately 3 g) was excised, snap frozen in liquid nitrogen and stored at -80°C until use.

RNA and DNA isolation

The frozen biopsies with associated bacterial cells (in 1-g segments) were disrupted using a bead beater (BiospecProducts inc., Bartlesville, USA) by shaking at least 5 times for 1.5 min at 4°C and placed on ice in between. Total RNA was isolated using Macaloid clay as described previously (21). The RNA was further cleaned according to the Rneasy® cleaning protocol including a DNase-treatment (Qiagen Sciences, Maryland, USA). Pure culture *L. plantarum* 299v DNA was isolated using the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, USA) following the manufacturer's instructions, except that the bead-beating step was performed as for the RNA-isolation.

Detection of *L. plantarum* in the biopsies.

The rRNA of all biopsy samples were reverse transcribed into cDNA with the reverse primers 1401 rev or Lac2 (14) for the total bacterial community 16S rRNA and *Lactobacillus*

specific 16S rRNA amplification, respectively. DNA was isolated from the biopsy samples as described above. Both the DNA and RT-PCR products were analyzed by PCR and denaturating gradient gel electrophoresis (DGGE) (14, 39-41). Sequencing confirmed that the 16S rRNA gene amplicon at this position originated from *L. plantarum*.

Transcript profiling.

Labeling and hybridization of RNA and DNA to a clone based-*L. plantarum* WCFS1 microarray (23, 36), with about 85% coverage of the genome on 3713 clones, was performed as previously described (23, 36). Total genomic DNA (2μg) of *L. plantarum* 299v labeled with cy3 and an aliquot of RNA containing 25 μg or 150 μg labeled with cy5, were simultaneously hybridized to the same microarray. Different amounts of RNA were applied because the quantity of total RNA in the mucosa originating from *L. plantarum* could vary, and due to the risk of limiting *L. plantarum* RNA.

Reproducibility of the microarrays was confirmed by comparing hybridization of different amounts of biopsy RNA from the same person to the target DNA on the microarray. To rule out the influence of eukaryotic RNA on the hybridization signal, total RNA (from person B's biopsy sample) was isolated from intestinal mucosal cells of which the upper bacteria-containing mucus layer was removed, and analyzed by microarray. An RNA sample of the same mucosal cells spiked with *L. plantarum* 299v was compared to pure culture RNA to eliminate any influences of the biopsy sample on the RNA isolation procedure. Two DNA microarrays were hybridized with 150 μg of the RNA of biopsy material of person D who consumed the placebo drink to confirm that RNA of a similar sample without additional *L. plantarum* showed no hybridization.

Analysis of the microarrays.

The mRNA used in these experiments was often limiting. Consequently, the pattern of hybridized genomic DNA of *L. plantarum* 299v was chosen as the common reference for all microarrays. The microarrays were normalized by expressing, for each spot, the RNA signal relative to the genomic DNA signal.

Overlap of clones present on the DNA microarray resulted in redundancy and this was used as a control for consistency of the microarray. A few of the clones on the microarray contained one or more of the 246 genes which are encoded in the *L. plantarum* WCFS1

genome, but not on *L. plantarum* 299v (23). The overall low signal intensity due to limited amounts of mRNA induced mainly small absolute differences in RNA signal intensity between hybridized clones. Accordingly hybridized signals that showed an intensity, which was at least two times higher than the background for both RNA and DNA, were considered "positive" and included in further analysis. As expected, this criterion excluded almost all (85%) of the few signals obtained for *L. plantarum* WCFS1-specific genes that were absent in *L. plantarum* 299v. Signals that were a result of hybridization with a clone fragment containing a gene not present on *L. plantarum* 299v (23) were also excluded. Comparison of any two microarrays was performed following normalization and the exclusion criteria as described above. The same number of positive signals was selected between any two microarrays; the positive signals on the microarray showing the lowest number of positive signals were compared to the same number of signals choosing those signals with the highest intensity. This resulted in a comparison of 641 genes between the ileal and colon samples of person A, and about 977 different genes for the comparison between the colonic samples of the three different individuals (Figure 2). By excluding genes under the cut-off value, certain genes whose expression differed only slightly were determined to be positive in one sample but discarded for another. Therefore, we focused on comparing genes in pathways or operons of which several genes were expressed.

Quantitative reverse transcriptase-PCR and sequence analysis of PCR products.

To verify the specificity and intensity of the microarray results, the expression of selected genes was determined by qRT-PCR and several of the resulting RT-PCR products were sequenced. The primers are listed in Table 1. A second DNase treatment was performed on all RNA samples to remove residual DNA as described above followed by an RT-step using Superscript III (Invitrogen, Breda, the Netherlands) and the reverse primer (Table 1). The products were quantified in an ABI prism 7700 real-time PCR apparatus (Taqman) using cybrgreen (Sybr green PCR master mix, Applied Biosystems, Warrington, UK). All results were normalized to the 16S rRNA of *L. plantarum* and compared to gene expression in the colon of person A (26). The values presented are the average of 3 experiments.

DNA fragments (approximately 600 bp) obtained by RT-PCR with the primers from Table 1 of the cellobiose PTS gene (lp_1164), and the glucose-6-phosphate 1-dehydrogenase gene (lp_2681), originating from *L. plantarum* in the large intestine, and the fructokinase gene

(lp_3637), from *L. plantarum* in the ileum of person A were sequenced. The qRT-PCR reaction products (approximately 50 bp) of the cell surface protein precursor gene (lp_0800) and the putative α-L-rhamnosidase gene (lp_3473) were also sequenced which confirmed specificity of the qRT-PCR primers.

Table 1. Primers used for RT-PCR unless stated that it includes 600bp .

Lp_ number	Gene	Forward primer	Reverse primer
	16S rRNA (6)	TGA TCC TGG CTC AGG ACG AA	TGC AAG CAC CAA TCA ATA CCA
lp_537	L-lactate dehydrogenase	TGA TCC TCG TTC CGT YGA TG	CCG ATG GTT GCA GTT GAG TAA G
lp_800	cell surface protein precursor	CGA TTA ATG CGG CAA CAA CA	CCG GTT GTT CAG CCT TTG AG
lp_1164	cellobiose PTS, EIIC	GGG CAT CTT CCT CGC ACT AT	TCG ATC TCC TGG TGG ATG TGT
lp_1164	cellobiose PTS, EIIC (600 bp)	GAT GGT CTT TCT ATT GTT GGC	GCT AAA GCA GCA CCG TGA ATC
lp_1898	6-phosphofructokinase	TCC AGG GAC GAT CGA TAA TGA	GCT TGC ACG TTG GTG TTG AC
lp_2502	glucose-6-phosphate isomerase	CCG GAT CTA TGC CAC AAC TG	TGA AAC GAC CAC CAA CAT CA
lp_2681	glucose-6-phosphate 1-dehydrogenase	GGA ACA ACC GTT ACA TCG ACA	GCA AGG CAC CGC TGT TAT C
lp_2681	glucose-6-phosphate 1-dehydrogenase (600 bp)	GGA ATC ATT ATG GAA CAA CCG	GTC ATT AGC TCG GAC GTC TTC
lp_3055	copper transporting ATPase	CGC ACT TGT GAC CAC TTT CG	TTC CGC TTC CTT GGC TTG TA
lp_3473	α-L-rhamnosidase (putative)	CAA CCA CGC TGA CGT TAC CA	CCG TGA CCA CTG GAT TGC TA
lp_3637	fructokinase	TGG AAT TAA GCA CGG CAC TG	GTG TAG CGC TGC CGT TAG AA
lp_3637	Fructokinase (600 bp)	GAA GCG AAT TCC AAC TTT AAC	CTG ATC AAA GTA CTG GCG AAC

Results and Discussion

L. plantarum in biopsies.

We determined the global gene expression of ingested *L. plantarum* cells in different locations of the human intestine using clone-based DNA microarrays. To achieve this we first confirmed the presence of the microbe in biopsy samples of three patients tentatively diagnosed with colon cancer, who ingested for one week prior to surgery a fermented oatmeal drink with *L. plantarum* 299v. The *Lactobacillus*-specific DGGE profiles of the biopsy DNA

and RNA indicated the presence and activity of *L. plantarum* species in all samples (Figure 1). Sequencing of the 16S rRNA gene amplicon migrating to the *L. plantarum* position on the DGGE gel in the ileum of subject A and the colon of subject D confirmed that these originated from *L. plantarum* (Figure 1). Although *L. plantarum*-derived 16S rRNA amplicons were barely detected in the total community (Figure 1A), they were clearly present in the *Lactobacillus* group-specific profiles (Figure 1B). While subject D (placebo) appeared to contain an *L. plantarum*-like amplicon (Figure 1B), quantitative RT-PCR showed that *L. plantarum* was present in at least a 100-fold lower concentration than in the other subjects (data not shown).

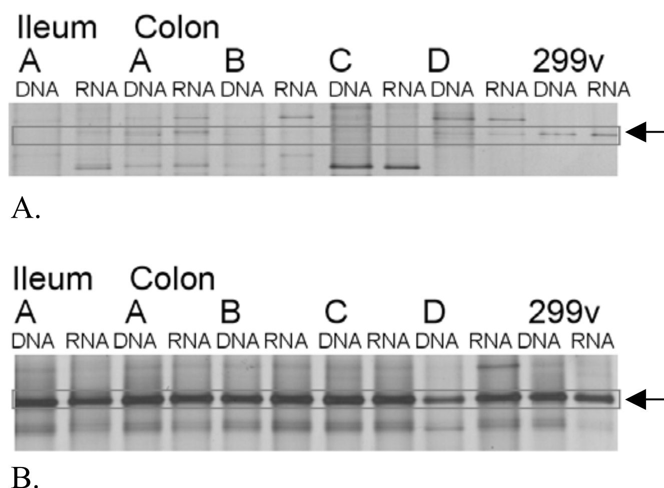

Figure 1. PCR-DGGE of the dominant bacterial community (A) and the *Lactobacillus* population (B) of intestinal mucosal samples of subjects A, B, C and D. Subjects A, B, and C consumed *L. plantarum*, and D consumed the placebo. The *L. plantarum* specific band is enclosed in a box with an arrow and was confirmed by sequence analysis of the bands from the ileum and colon of subjects A and D, respectively. Note that the detection of 16S rDNA indicates the presence of a microorganism, while 16S rRNA indicates their activity.

In vivo expression profiling validation.

The *L. plantarum* genes detected by the DNA microarrays were clearly expressed in the mucosa. In some instances, the level of detection will be influenced by the efficiency of cDNA hybridization and the RNA turnover and stability. However, in most cases, detection will depend on high-level expression in the intestine of the subjects. To date, expression profiling experiments have been limited mainly to environments with one species. To evaluate the reproducibility of the microarrays for detecting global gene expression of *L. plantarum* in the human mucosa, the number of clones positive on the microarrays following hybridization

with 25 and 150 µg RNA was compared to the total number of positive signals on the microarray containing 25 µg RNA. The reproducibility of the same clones being positive between microarrays hybridized with 25 µg and 150 µg of total RNA was between 99.6% for microarrays with high signal intensity and 73.5% for the microarrays with low signal intensity (on which < 10% of the clones gave a positive signal). Differences in signal intensity comparing 25 µg and 150 µg hybridized cDNA were less than the expected six times difference, thus, hybridization of *L. plantarum* appeared less effective when large amounts of total cDNA were hybridized. The signal intensity of the positive clones showed a Pearson correlation coefficient of 0.87 for ln(RNA signal/DNA signal). In conclusion, this confirms the reproducibility of the DNA microarrays.

The duplicate microarray of person D, who did not consume *L. plantarum,* gave either none or 27 (0.7%) positive signals of the total 3713 clones indicating the low amount of *L. plantarum* species present in this sample as confirmed by qRT-PCR. Furthermore, it demonstrated that eukaryotic and other prokaryotic RNA did not interfere with the signal and validates the specificity of the microarray for *L. plantarum*. When evaluating the influence of human cells obtained from the *L. plantarum*-consuming subject B on the signal of the microarrays, only 46 (1.2 %) positive signals of the total 3713 clones on the microarray were found to hybridize with RNA of human mucosal cells of which the bacteria had been removed. This minor signal may have been caused by a few *L. plantarum* cells left on the eukaryotic cells after removing the mucus, but essentially demonstrated that the influence of the eukaryotic cells on the total signal of the microarrays was insignificant. Similarly, the expression pattern of RNA from a pure culture of *L. plantarum* 299v spiked onto the human mucosa was not significantly different than that obtained from the pure culture. Thus, the presence of human tissue did not influence the RNA isolation method or the expression pattern observed for *L. plantarum* 299v. The cross-hybridization of other prokaryotes to the microarray was also negligible as demonstrated by similar levels of expression in qRT-PCR and microarray analysis and is discussed below.

To support the specificity of the DNA microarray for *L. plantarum* genes, we sequenced approximately 600 bp RT-PCR products of different genes. Both the mRNA amplicon of the cellobiose PTS gene, and of the glucose-6-phosphate 1-dehydrogenase gene as expressed in the large intestine of person A were identical to the pure culture and the published sequence (18). The sequence of the amplicon of the fructokinase gene as expressed

in the small intestine of person A was identical to that of the pure *L. plantarum* 299v culture. It showed three mismatches to *L. plantarum* WCFS1 and represents single nucleotide polymorphisms between strain WCFS1 and 299v. Sequence identity in combination with the similar levels of expression determined by qRT-PCR and DNA microarrays supports specificity of the DNA microarray for *L. plantarum* genes.

Gene expression of *L. plantarum* in the intestine.

The *L. plantarum* cells recovered from the mucosa were metabolically active in subjects A, B and C as demonstrated (according to the stringent criteria outlined in the methods) by the detection of 262 to 1325 of the total 3687 genes by the DNA microarrays. The *L. plantarum* genes expressed at detectable levels from the colonic and ileal mucosa of the three patients were organized in functional classes. The common genes expressed in *L. plantarum* in all colonic biopsy samples are presented in Table 2. The genes and pathways described below were selected because they comprised either several genes in one pathway, several genes within an operon, or several clones containing the same gene. The functions of the genes are assumed based on the current annotation for *L. plantarum* WCFS1 (18).

Genes expressed at a detectable level could be assigned to all functional classes (18). Comparison of the gene expression of *L. plantarum* in the colon of the three persons showed that only 33 genes were expressed in all colonic samples and 380 (10%) of the total 3687 genes in two of the three samples (Figure 2). The chance of 33 overlapping genes is higher than that expected from random overlap as determined using a Poison distribution ($p=4x10^{-5}$).

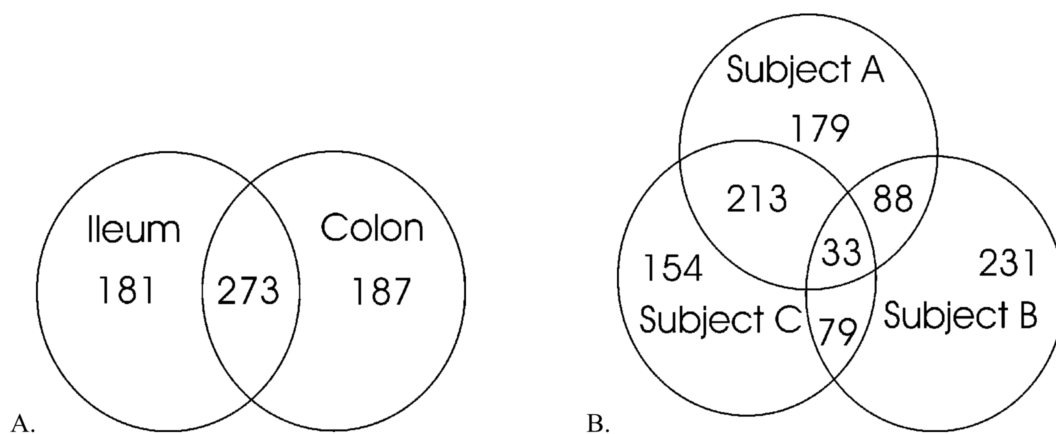

Figure 2. Number of well expressed genes of *L. plantarum* compared between (A) the ileum and colon of person A, and (B) colonic samples of three different individuals.

Table 2. Genes expressed in *L. plantarum* in all colonic biopsy samples. Genes discussed in the text are in bold.

main functional class	sub functional class	Product	lp_number
Amino acid biosynthesis	**Glutamate family**	**aspartate racemase**	lp_1523
Biosynthesis of cofactors	Glutathione, thioredoxin, and glutaredoxin	glutamate--cysteine ligase	lp_2336
Cell envelope	**Cell surface proteins: lipoproteins**	**lipoprotein precursor**	lp_0932
	Cell surface proteins: other	**cell surface hydrolase (putative)**	lp_1165
	Cell surface proteins: other	**adherence protein**	lp_1793
	Teichoic acid biosynthesis	**D-alanyl carrier protein DltC**	lp_1406
Cellular processes	**DNA Transformation and competence**	**competence protein**	lp_2226
DNA metabolism	DNA replication recombination, and repair	DNA gyrase, B subunit	lp_0006
	DNA replication recombination, and repair	DNA-directed DNA polymerase III, gamma/tau subunit	lp_0698
Energy metabolism	**Fermentation**	**alcohol dehydrogenase**	lp_1665
	Sugars	**α-mannosidase (putative)**	lp_3631
	TCA cycle	**pyruvate carboxylase**	lp_2136
Hypothetical proteins	Conserved: membrane proteins	integral membrane protein	lp_1978
	Conserved: other	No product defined	lp_0139
	Conserved: putative function	ATPase of the PilT family	lp_0607
Protein fate	Degradation of proteins, peptides, and glycopeptides	Xaa-Pro dipeptidyl-peptidase	lp_0857
	Protein modification and repair	protein-tyrosine phosphatase	lp_0069
Protein synthesis	**tRNA aminoacylation**	**serine--tRNA ligase**	lp_0501
Purines, pyrimidines, nucleosides and nucleotides	Pyrimidine ribonucleotide biosynthesis	dihydroorotate oxidase	lp_2699
Regulatory functions	MarR-family regulators	transcription regulator	lp_1795
Transcription	Degradation of RNA	ribonuclease HII	lp_1853
Transport and binding proteins	Cations	cation transporting P-type ATPase	lp_3398
	Multidrug resistance	ABC transporter	lp_1909-10
	PTS systems	phosphoenolpyruvate--protein phosphatase	lp_1274
	PTS systems	**cellobiose PTS, EIIA**	lp_2780
	Unknown substrate	cation efflux protein (putative)	lp_0085
	Unknown substrate	transport protein	lp_0280
	Unknown substrate	transport protein	lp_0894
	Unknown substrate	ABC transporter	lp_1791-2
	Unknown substrate	ABC transporter, permease protein	lp_1957
	Unknown substrate	ABC transporter, ATP-binding and permease protein	lp_3648

Heterolactic fermentation in the colon.

Many genes predicted to be responsible for energy generation were detected, in particular the pyruvate dehydrogenase complex and phosphoglycerate mutase, both enzymes of the glycolytic pathway. The genes encoding part of the pyruvate dehydrogenase complex which catalyzes the formation of acetyl-coenzyme A from pyruvate and acetaldehyde were detected in 2 of 3 colon samples. Several genes predicted to encode enzymes for the pentose phosphate (pentose-P) pathway were detected, notably glucose-6 phosphate-1-dehydrogenase, the key enzyme of the pentose-P pathway in 2 of 3 colonic samples, and other pentose-P genes, such as phosphogluconase deyhydrogenase, transketolase and transaldolase. The detection of genes of both pathways indicates, in addition to energy, the requirements of *L. plantarum* for reducing agents, nucleotide formation, and reducing equivalents for synthetic purposes (4). The gene annotated to encode the phosphoketolase enzyme that converts xylulose-5-P generated by the pentose-P pathway to acetyl-P was detected in one of the colonic samples. It is possible that in the colon the latter is converted to ethanol by alcohol dehydrogenase, since a predicted gene encoding this function was expressed in all three colonic mucosa samples. The putative gene for α-acetolactate decarboxylase which converts α-acetolactate to acetoin was expressed in the colons of subjects A and C. A relatively large number of genes coding for pyruvate-dissipating enzymes predicted to catalyze the production of other metabolites, such as acetate, ethanol, acetoin, and 2,3-butanediol have been reported in the *L. plantarum* WCFS1 genome sequence (18). Mixed acid fermentation is associated with carbon-limiting conditions, and the detectable expression of a variety of these predicted mixed acid fermentation genes highlights their role in adaptation of *L. plantarum* to the intestinal tract.

Uptake of cellobiose, a polysaccharide originating from plant cell walls, was suggested by detection of gene expression of the predicted cellobiose-PTS-system, including the clustered -EIIA and EIIB genes, in *L. plantarum* from all colon samples. However, different orphan genes encoding a cellobiose PTS- EIIC subunit were expressed in the different subjects. In *Listeria monocytogenes* (19) a role in host specific signaling was established for some of the orphan cellobiose PTS-EIIC subunits. Moreover, in *L. plantarum* a reduced survival in the intestine of mice of a cellobiose PTS-EIIC (lp_1164)-replacement mutant emphasizes the importance of this gene (7). In subjects B and C, expression of genes annotated as maltose ABC-transporter, and PTS-systems for fructose and galactitol suggested

uptake of these sugars. Various predicted α-glycosidase genes were expressed in all subjects, suggesting that internalized sucrose was hydrolyzed via fructose and glucose and that starch was being converted to glucose. Remarkably, a putative gene for α-mannosidase, an enzyme that could liberate mannose from various glycoproteins and polysaccharides found in the cell envelope of other microbes, was expressed in all samples. Expression of many predicted sugar uptake and metabolic genes were subject-dependent e.g. the genes encoding sucrose, mannitol, and mannose PTS systems were detected in only a single subject. The differences in gene expression of *L. plantarum* between the individuals suggests that this microbe can access and utilize a variety of different carbon sources available from the host or from carbohydrates consumed which are not digested by the host. Notably, the expression of several *C. jejuni* genes within the rabbit ileal loop model was found to be highly variable between individual rabbits (34) indicating subject-dependent specific expression in the intestine.

Macromolecular synthetic pathways.

In all persons numerous genes annotated as ribosomal proteins were expressed, and combined with the expression of many tRNA-ligase genes, including ligases for serine, leucine, cysteine, glutamate, phenylalanine, glycine, arginine, and proline, suggested constant protein synthesis. Some indication of the key amino acid requirements and conversions in the human intestine for survival of *L. plantarum* could be inferred from the gene expression. The predicted gene encoding pyruvate carboxylase, which converts pyruvate to oxaloacetate, an essential precursor of aspartate, was found to be expressed in all three colonic samples. In all subjects, aspartate appeared to be transformed via homoserine to lysine using amongst others the genes encoding homoserine dehydrogenase, and dihydrodipicolinate reductase. The conformation could be interconverted between L- and D-aspartate in all colonic samples as indicated by the detection of an aspartate racemase gene. Expression of putative genes for indole-3-glycerol-phosphate synthase, anthranilate phosphoribosyltransferase, phosphorybosylanthranilate isomerase, and tryptophan synthase, involved in L-tryptophan synthesis, was detected in person A and C. Also in persons A and C, genes encoding a branched chain fatty acids ABC-transporter were expressed indicating uptake of those fatty acids from the environment. Expression of genes for other amino acid biosynthetic pathways was not detected. Possibly, those amino acids could be assimilated from the intestinal environment, or gene expression was below the detection limit of the microarray analysis.

Different gene expression profiles for amino acid biosynthesis pathways between the persons may have been caused by differences in peptides and proteins available from the host self or their diet.

Cell envelope reinforcement and stress.

Many genes annotated to encode stress proteins were expressed, in particular various predicted heat shock proteins and an alkaline shock protein (lp_0930). Remarkably, the latter gene was strongly down regulated in *L. plantarum* WCFS1 mutated in the *lam* quorum sensing system involved in regulation of adherence, and that is homologous to the *Staphylococcus aureus agr*-system involved in biofilm formation (36). Thus, the putative alkaline shock protein may have a role in adherence or biofilm formation in the intestine. Furthermore, the putative genes for a family of four Mub proteins (lp_1229, a mannose-specific adhesion (27); lp_1643; lp_3114) believed to be involved in adhesion were detected in several persons, as well as a transcriptional regulator *aapR* that is co-transcribed with another one of these *mub* genes, *aapA* (35). The adhesion function of Mub proteins has been demonstrated for other lactobacilli besides the AapA protein of *L. plantarum* (31). In addition, numerous other genes encoding cell wall precursors were found to be expressed, of which lp_0932 (a predicted lipoprotein) and lp_1165 (predicted cell surface hydrolase) were expressed in all biopsy samples. Another predicted cell surface protein encoded by gene lp_1793 with an N-terminal sequence homologous to the fibronectin-binding protein family domain (FbpA), involved in adherence for *Staphylococcus aureus* (16), was detected in all three colonic samples. The expression of this considerable array of potential cell surface adherence proteins by *L. plantarum* may be necessary for adhesion to food particles or epithelial cells within the intestine.

In persons A and C, expression of the putative gene for acetylCoA carboxylase was detected, which converts pyruvate via acetyl-coenzyme A to intermediates for fatty acids that are likely intended for incorporation in the cell wall. The predicted gene for D-alanyl carrier protein DltC that transforms D-alanine to O-D- alanyl-poly ribitol phosphate was detected. The latter product is incorporated into lipoteichoic acid which is an integral part of the cell wall. In fact the composition of the lipoteichoic, specifically the D-alanine content, has been shown to modulate proinflammatory or antiinflammatory immune responses (13). Overall, the expression of stress-induced genes together with genes coding for cell wall precursors or

biosynthetic enzymes suggest that it may be challenging for the ingested *L. plantarum* to adapt to the intestinal conditions, and one consequence of this may be reinforcement of the cell wall for protective purposes.

Comparison of *L. plantarum* gene expression in the ileum versus colon

To distinguish location specific gene expression, we compared an ileal and colonic biopsy sample of one subject. Comparison of *L. plantarum* gene expression between the ileum and the colon of person A revealed an overlap of about one-third (273 genes) for the expressed genes between both samples (Figure 2). This is significantly higher than if calculated from a hypergeometric distribution (p=0). The majority of these genes encoded metabolic functions in pathways including glycolysis, purine and pyrimidine metabolism, cell division, and conversion of pyruvate via intermediates acetyl-CoA and malonyl-CoA for fatty acid biosynthesis.

Gene expression detected in the ileum of subject A alone included putative genes for glycogen phosphorylase, that converts starch to glycogen; α-amylase, that degrades starch to dextrins and oligosaccharides; and glucose-1-phosphate adenylyltransferase and starch (bacterial glycogen) synthase that synthesize glycogen from glucose. Apparently, *L. plantarum* was generating energy storage molecules while residing in (or during transit through) the ileum. Interestingly, a putative β-galactosidase, a key gene for lactose metabolism, was only expressed in the ileum, suggesting that small sugar moieties are internalized by *L. plantarum* in the ileum.

Differential gene expression among the tRNA ligase genes that couple amino acids to specific tRNAs were apparent: for example, those for methionine and aspargine were detected in *L. plantarum* in the small intestine, while aminoacylation genes encoding coupling of leucine, cysteine, arginine, tyrosine and phenylalanine to tRNA were expressed in the colon. This suggests that there may have been differences in the availability or requirement of amino acids in the different parts of the intestine. It is noteworthy that more genes encoding ribosomal proteins were detected in the colon than in the ileum signifying either more growth or increased maintenance to adapt to the colonic environment. Interestingly, the putative fibronectin-binding protein encoded by gene lp_1793, which was detected in all colonic samples, was not detected in the ileum. It is possible that this protein may be induced only under colonic conditions.

Confirmation of the microarray data by real time qRT-PCR

All selected genes detected by DNA microarray were confirmed to be well expressed by qRT-PCR (Table 3). This includes L-lactate dehydrogenase (lp_0537) and 6-phosphofructokinase (lp_1898), fructokinase (lp_3637), the glycolytic enzyme glucose-6-phosphate isomerase (lp_2502), and the pentose-P pathway glucose-6-phosphate 1-dehydrogenase (lp_2681) genes. qRT-PCR confirmed the trend in differential gene expression observed with the microarray analysis, a quantitative difference in the fold change was observed between these two technologies. In combination with the specificity of the (RT)-PCR products, this demonstrates that cross-hybridization of other prokaryotes to the microarray was negligible.

Table 3. qRT-PCR results of *L. plantarum* genes in human mucosa normalized to the 16S rRNA gene and to the colon of subject A, which is appointed 1 for all genes.

		Ileum	Colon	
		A	B	C
lp_537	L-lactate dehydrogenase	1,20	31,17	2,13
lp_800	cell surface protein precursor	30,72	27,55	12,63
lp_1164	cellobiose PTS, EIIC	14,56	15,54	11,97
lp_1898	6-phosphofructokinase	6,50	31,94	10,31
lp_2502	glucose-6-phosphate isomerase	6,48	2,36	3,14
lp_2681	glucose-6-phosphate 1-dehydrogenase	0,79	1,70	2,31
lp_3055	copper transporting ATPase	298,18	9,35	12,39
lp_3473	α-L-rhamnosidase	91,12	11,69	64,99
lp_3637	fructokinase	18,66	9,33	13,79

Several genes induced by *L. plantarum* in the mouse intestine, as shown by R-IVET (5), including a predicted cellobiose PTS- EIIC subunit (lp_1164), a large cell surface protein precursor (lp_0800), copper transporting ATPase (copA, lp_3055), and α-L-rhamnosidase (lp_3473) were also demonstrated to be expressed by *L. plantarum* 299v in the human intestine by qRT-PCR. A remarkable difference between gene expression in the colon and the ileum was the high expression of the *copA* gene in the latter, which is supported by the microarray data. The exact function of the *copA* gene has not yet been elucidated but has been suggested to be involved in copper acquisition, high copper concentration tolerance or another

function involved in persistence and survival of the gastrointestinal tract (7). Even though the approaches for investigating differential gene expression in the intestine were different, as well as the hosts, mouse versus human, a substantial 46% of the genes revealed by R-IVET were expressed in the microarray experiments. Those genes were mainly involved in nutrient acquisition and synthesis, stress, and extracellular functions. Since those genes are specifically switched on in the intestine of mice and expressed in the human intestine, it suggests intestine specific gene expression of *L. plantarum*. In addition, it indicates that the intestinal conditions of mouse and man may have similar effects on global gene expression of *L. plantarum* in the intestine.

Conclusions

DNA microarrays have been applied to determine the gene expression of a commensal bacterium *L. plantarum* that has probiotic properties, in the complex intestinal background of the human being. To our knowledge, this is also the first report of global gene expression analysis of a specific microbe in a natural *in vivo* microbial ecosystem. The gene expression pattern detected suggested a substantial degree of adaptation by the ingested microbe to the intestinal environment, as well as high metabolic activity of the microbe. Importantly, the present study generates significant leads for further investigation of relevant groups of genes for successful passage and residence of the ingested microbe in the intestine. In combination with clinical studies, this approach is a powerful and high-throughput tool to provide insight and new perspectives on *in vivo* host-microbe interactions.

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References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Altermann, E., W. M. Russell, M. A. Azcarate-Peril, R. Barrangou, B. L. Buck, O. McAuliffe, N. Souther, A. Dobson, T. Duong, M. Callanan, S. Lick, A. Hamrick, R. Cano, and T. R. Klaenhammer.** 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc. Natl. Acad. Sci. U. S. A. **:**0409188102.

3. **Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-1920.

4. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2001. 5th ed. W.H. Freeman and company, New York.

5. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721-5729.

6. **Bron, P. A., M. Marco, S. M. Hoffer, E. Van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J. Bacteriol. **186:**7829-7835.

7. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. *In* P. A. Bron (ed.), The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen Centre for Food Sciences, Wageningen.

8. **Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1996. A model of host-microbial interactions in an open mammalian ecosystem. Science **273:**1380-1383.

9. **Casula, G., and S. M. Cutting.** 2002. *Bacillus* probiotics: Spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. **68:**2344-2352.

10. **Corthier, G., C. Delorme, S. D. Ehrlich, and P. Renault.** 1998. Use of luciferase genes as biosensors to study bacterial physiology in the digestive tract. Appl. Environ. Microbiol. **64:**2721-2722.

11. **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman.** 2005. Diversity of the human intestinal microbial flora. Science **308:**1635-1638.

12. **Fitzsimons, N. A., A. D. L. Akkermans, W. M. de Vos, and E. E. Vaughan.** 2003. Bacterial gene expression detected in human faeces by reverse transcription-PCR. J. Microbiol. Methods **55:**133-140.

13. **Grangette, C., S. Nutten, E. Palumbo, S. Morath, C. Herman, J. Dewulf, B. Pot, T. Hartung, P. Hols, and A. Mercenier.** 2005. From The Cover: Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc. Natl. Acad. Sci. U. S. A. **102:**10321-10326.

14. **Heilig, H. G., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-23.

15. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by *in vivo* gene expression. Proc. Natl. Acad. Sci. U. S. A. **94:**934–939.

16. **Jonsson, K., C. Signas, H. P. Muller, and M. Lindberg.** 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. Eur. J. Biochem. **202:**1041-1048.

17. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing, Gram-positive rods, p. 1229. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams and Wilkins, Baltimore, U.S.A.

18. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

19. **Kreft, J., and J. A. Vazquez-Boland.** 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. **291:**145-57.

20. **Kretschmar, M., A. Felk, P. Staib, M. Schaller, D. Hess, M. Callapina, J. Morschhauser, W. Schafer, H. C. Korting, H. Hof, B. Hube, and T. Nichterlein.** 2002. Individual acid aspartic proteinases (Saps) 1-6 of *Candida albicans* are not essential for invasion and colonization of the gastrointestinal tract in mice. Microb. Pathog. **32:**61-70.

21. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos.** 1993. Characterizion of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281-291.

22. **Lowe, A. M., D. T. Beattie, and R. L. Deresiewicz.** 1998. Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol. Microbiol. **27:**967.

23. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187:**6119-27.

24. **Molin, G.** 2001. Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. Am. J. Clin. Nutr. **73 (suppl):**380S-385S.

25. **Oozeer, R., N. Goupil-Feuillerat, C. A. Alpert, M. van de Guchte, J. Anba, J. Mengaud, and G. Corthier.** 2002. *Lactobacillus casei* is able to survive and initiate protein synthesis during its transit in the digestive tract of human flora-associated mice. Appl. Environ. Microbiol. **68:**3570-3574.

26. **Pfaffl, M. W.** 2001. A new mathematical method for relative quantification in real time RT-PCR. Nucl. Acids. Res. **29:**2002-2007.

27. **Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P. A. Bron, J. Lambert, W. M. de Vos, R. van der Meer, M. A. Smits, and M. Kleerebezem.** 2005. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J. Bacteriol. **187:**6128-36.

28. **Pridmore, R. D., B. Berger, F. Desiere, D. Vilanova, C. Barretto, A.-C. Pittet, M.-C. Zwahlen, M. Rouvet, E. Altermann, R. Barrangou, B. Mollet, A. Mercenier, T. Klaenhammer, F. Arigoni, and M. A. Schell.** 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc. Natl. Acad. Sci. U. S. A. **101:**2512-2517.

29. **Reid, G., M. E. Sanders, H. R. Gaskins, G. R. Gibson, A. Mercenier, R. Rastall, M. Roberfroid, I. Rowland, C. Cherbut, and T. R. Klaenhammer.** 2003. New scientific paradigms for probiotics and prebiotics. J. Clin. Gastroenterol. **37:**105-18.

30. **Rokbi, B., D. Seguin, B. Guy, V. Mazarin, E. Vidor, F. Mion, M. Cadoz, and M.J. Quentin-Millet.** 2001. Assessment of *Helicobacter pylori* gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. Infect. Immun. **69:**4759-4766.

31. **Roos, S., and H. Jonsson.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology **148:**433-442.

32. **Saxelin, M., S. Tynkkynen, T. Mattila-Sandholm, and W. M. de Vos.** 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol. **16:**204-211.

33. **Sonnenburg, J. L., J. Xu, D. D. Leip, C.-H. Chen, B. P. Westover, J. Weatherford, J. D. Buhler, and J. I. Gordon.** 2005. Glycan foraging *in vivo* by an intestine-adapted bacterial symbiont. Science **307:**1955-1959.

34. **Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect. Immun. **73:**1797-1810.

35. **Sturme, M. H. J., T. Fujii, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. A regulator containing an AraC-type helix-turn-helix domain is involved in regultion of cell surface properties in *Lactobacillus plantarum*. *In* M. H. J. Sturme (ed.), Analysis of quorum sensing regulatory systems in the human isolate *Lactobacillus plantarum*. Wageningen Centre for Food Sciences, Wageningen.

36. **Sturme, M. H. J., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. An *agr*-like two component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J. Bacteriol. **187:** 5224-5236

37. **Vaughan, E. E., H. G. Heilig, K. Ben-Amor, and W. M. de Vos.** 2005. Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol. Rev. **29:**477-90.

38. **Walter, J., N. C. K. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. **69:**2044-2051.

39. **Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes.** 2001. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and Denaturing Gradient Gel Electrophoresis. Appl. Environ. Microbiol. **67:**2578-2585.

40. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, A. G. M. de Visser, and W. M. de Vos.** 2001. the host genotype affects the bacterial community in the human gastrointestinal tract. Micr. Ecol. Health. Dis. **13:**129-134.

41. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. de Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854-3859.

Chapter 5

Global Transcript Profiling of Gene Expression of *Lactobacillus plantarum* after Perfusion in the Human Small Intestine

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Abstract

Although various physiological effects of *Lactobacillus plantarum* on the host have been studied, only limited knowledge is available on the effect of the host on this and other intestinal bacteria. To determine the effect of passage of the proximal intestinal tract on global gene expression of *L. plantarum*, four human volunteers were subject to small intestinal perfusion with *L. plantarum* cells. Subsequent analysis of the transcriptional response using DNA microarrays in comparison with that of control incubations indicated that *L. plantarum* was metabolically active following passage of a 40-cm segment of the small intestine as deduced from the expression of genes involved in cell division and protein synthesis. However, also an osmotic shock response was observed as indicated by up-regulation of genes predicted to be involved in ion transport, and uptake of compatible solutes such as betaine, carnitine or choline. This was most probably induced by bile salts present in the small intestine. Finally, the expression of various genes predicted to code for cell envelope proteins was altered. While some of these may be involved in strengthening of the cell envelope, others may be implicated in specific or general interactions with the host. In conclusion, the global analysis of RNA dynamics of *L. plantarum* within the host provides new and unprecedented insight in the response of this intestinal bacterium to passage of a segment of the human small intestine and indicates that this approach is valuable for establishing the function of bacteria that colonize the human intestine and affect health and disease.

Keywords: *Lactobacillus plantarum,* perfusion, human small intestine, global gene expression

Introduction

The presence of a variety of nutrients and the changing conditions throughout the gastrointestinal (GI)-tract, including differences in pH, oxygen concentration, and secretory components, give rise to a large and diverse microbial community (29, 31). This microbiota has a major impact on the host' health with numerous functions, such as nutrient bioconversion, stimulation of innate and adaptive immunity, and intestinal epithelial development and energy storage (2, 19, 25). Lactic acid bacteria are considered to be an important group of the microbiota in the upper GI-tract that is involved in the health-stimulating processes within the host (11, 34, 37).

The Gram-positive *Lactobacillus plantarum* is a versatile lactic acid bacterium that is a natural inhabitant of the human GI-tract and can be found in a range of other habitats (1, 20). The complete genome sequence of *L. plantarum* WCFS1 (22) has confirmed the potential of this human isolate to grow in a large range of environmental niches and allowed the design of specific DNA microarrays for use in transcript profiling and comparative genomics of this species (26, 33). Products containing *L. plantarum* are marketed as probiotics as several physiological effects of consumption of *L. plantarum* on the host have been documented (13, 30). These include the apparent reduction in certain risk factors for coronary artery diseases, symptoms of irritable bowel syndrome and carriage of faecal enterobacteriacea (13). However, only limited knowledge is available on gene expression of *L. plantarum* in the host. Several approaches based on promoter induction have revealed genes that are up regulated under bile or salt stress (6, 7) or upon persistence in the GI-tract of mice (5). Knowledge of specific gene expression of *L. plantarum* along the GI-tract will not only contribute to our understanding of host-microbe interactions but also can be applied to its further development as a delivery system to evoke a specific host response (15-17).

Perfusion of the GI-tract has been recently described in various studies to determine intestinal absorption or efflux of water, the uptake of drugs and iron-induced intestinal oxidative stress *in vivo* in humans (27, 35). In addition, biopsies can be taken before and after the treatment to evaluate effects on human gene expression. In this study, we applied the perfusion technique to evaluate the effect of passage of the small intestine of *L. plantarum* in healthy volunteers and used DNA microarrays to determine the dynamics of RNA isolated from the perfusion suspension in comparison with a control incubation. The results indicate that the *L. plantarum* cells were metabolically active in all four subjects and that the common responses included metabolic functions, osmotic stress and changes in the cell envelope that may point to specific interactions with the host.

Material and Methods

Bacterial growth.

A single colony inoculate of *L. plantarum* WCFS1 was grown anaerobically, at 30°C for 17 hours in MRS. The *L. plantarum* cells were washed three times at room temperature with a physiological salt (PS) solution and after the last washing step resuspended in a PS solution containing 1% glucose to obtain a final concentration of 10^9 cells ml^{-1}. The solution was warmed to 37°C and used immediately for perfusion.

Human volunteers.

A 40-cm segment of the proximal small intestine of four healthy volunteers, subjects A, B, C, and D, was perfused using a double lumen perfusion catheter constructed in such a way that a solution could be injected at 10-cm distal from the stomach and luminal fluid samples were collected 40 cm further down the intestine. Through the perfusion port, a PS solution containing 1% glucose was infused at 10 ml min^{-1} during 180 minutes. This period was necessary to reach steady state conditions (35). Subsequently, a perfusion liquid containing *L. plantarum* cells as described above was injected during 60 minutes at 10 ml min^{-1} and perfused cells were collected on ice prior to further processing (see below). The continuous flow ensured a similar time of contact of the cells with the lumen of about 10 minutes. To assess differences in gene expression of *L. plantarum* in different intervals, RNA was extracted from the cells obtained each 15 minutes from individual A. A sample of *L. plantarum* cells was also incubated under the same conditions in PS solution containing 1% glucose at 37°C for 60 minutes to exclude all effects not induced by the intestine (control experiment).

The study protocol was approved by the Ethics committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Sample preparation.

Samples (100 ml) of the initial cells prior to the perfusion, cells from the control experiment that were incubated but not exposed to the intestine, and cells obtained after the perfusion were kept on ice, collected by centrifugation, and were stored at -80°C until further use. Total RNA was isolated from the frozen cells using Macaloid clay as described previously (23). The bacterial cells were disrupted using a bead beater (BiospecProducts inc., Bartlesville, USA) by shaking at least 5 times for 1.5 minutes and cooled on ice in between. The RNA was

cleaned according to the Rneasy® cleaning protocol including a DNase step (Qiagen Sciences, Maryland, USA).

DNA microarrays and normalisation.

Total RNA (25 μg) of the *L. plantarum* cells obtained before perfusion was fluorescently labeled with Cy3 and that obtained after the control incubation or during the perfusions with Cy5, during reverse transcription as previously described (29). Subsequently, 10 μg aliquots of these two-color labeled RNAs were hybridized to a clone-based *L. plantarum* array with 85% coverage of the genome as previously described (33). The DNA microarrays were analyzed using a Scan Array Express 4000 scanner (Perkin Elmer) and the data were corrected for the background and subject to a lowess normalisation as described previously (38).

Statistical analysis.

Initially, the significance of the perfusion interval was assessed using Pearson correlation test ($p<0.01$). In addition, genes that were differentially regulated between subjects were identified by comparing the variance of M (log^2(cy5signal/cy3signal)) of the four time intervals within one subject and the variance of M between the four subjects using an F-test ($p<0.01$). Finally, significant up- or down-regulation of the genes that were similarly regulated in all four subjects was determined using a two-sided one-sample t-test on M ($p<0.01$). Comparisons were made between clones containing the same gene to determine the consistency of regulation. Only genes consistently expressed in all subjects and affected more than 2-fold in expression are described here and the levels of regulation provided are a weighted average of all clones containing the gene. The functions of the *L. plantarum* genes were predicted based on the current annotation for *L. plantarum* WCFS1 (22).

Gene regulation induced by the experimental set-up, rather than passage of the small intestine, was determined by comparing DNA microarray profiles of *L. plantarum* cells as injected into the intestine with cells that were kept under similar experimental conditions but without injection in the intestine. The 12 genes of which regulation was more than 2-fold induced by the experimental set-up were excluded from the analysis.

Quantitative reverse transcriptase PCR.

To verify the specificity and intensity of the DNA microarray results, quantitative reverse transcriptase PCR (qRT-PCR) was performed on all samples. A second DNase treatment was done as described above followed by an RT-step using Superscript III (Invitrogen, Breda, the Netherlands) and the reverse primer (12). The products were quantified in an ABI prism 7700 real-time PCR apparatus (Taqman) using cybrgreen (Sybr green PCR master mix, Applied Biosystems, Warrington, UK). As a control, pure RNA was tested which gave no signal, confirming the absence of DNA. All results were normalized to the amount of 16S ribosomal RNA (rRNA) of *L. plantarum* and compared to gene expression in the initial cells using the $2^{-\Delta\Delta Ct}$ method (24). The values presented are the average of 3 experiments.

Results and discussion

Specific global transcriptional response of *L. plantarum* upon intestinal perfusion

To determine the effect of passage of a segment of the small intestine on gene expression of *L. plantarum*, an intestinal perfusion experiment that involved orogastrically positioning of a double-lumen perfusion tube into the proximal small intestine of 4 healthy volunteers, was conducted. Via this tube a *L. plantarum*-containing cell suspension was injected distal from the stomach and collected in the jejunum. From this constant flow, the cells were collected and used to isolate RNA for labelling and hybridization to the *L. plantarum* DNA microarray. The global transcriptional responses of *L. plantarum* in the samples obtained after 15, 30, 45 and 60 minutes of perfusion in subject A were compared to each other and were found to be highly similar. Only 9 genes showed some expression difference and only 2 of these were affected more than two-fold, namely the diaminopimelate epimerase gene (lp_2185) predicted to be involved in aspartate biosynthesis and a gene encoding a hypothetical protein (lp_0862). The extremely low number of genes that show differential regulation over time indicates that the effect of the small intestine on gene expression of *L. plantarum* is consistent and hardly changes during the incubation period within a subject.

Subsequently, the global transcriptional response of *L. plantarum* was determined after the complete period of perfusion of the four different subjects and carefully compared to each other. From the total of 2819 *L. plantarum* genes on the array, the vast majority (89%; i.e. 2521 genes) was found to be similarly affected in the four different subjects. These included 117 genes that showed a more than 2-fold up- or down-regulation (Table 1). As it could be

speculated that this specific expression modulation could be induced by the experimental set-up rather than the intestinal passage, the global transcriptional response was also determined of *L. plantarum* cells that were not perfused but kept under similar experimental conditions. As during this treatment a reduction of the pH occurred, probably due to the metabolism of glucose to lactic acid, a general acid stress response was expected, but only 12 of the 117 genes affected by the intestinal passage were found to be affected more than 2-fold in this control experiment. As a consequence, these genes were excluded from further analysis of the intestinal response of *L. plantarum*. Most of the remaining 105 genes that were significantly and reproducibly affected during passage of *L. plantarum* in the intestine were found to be up regulated while only 2 were found to be decreased in expression (Table 1). The products of these genes are predicted to be present in all functional classes but mainly belong to the transport and binding as well as cell envelope proteins, as will be discussed below (Figure 1).

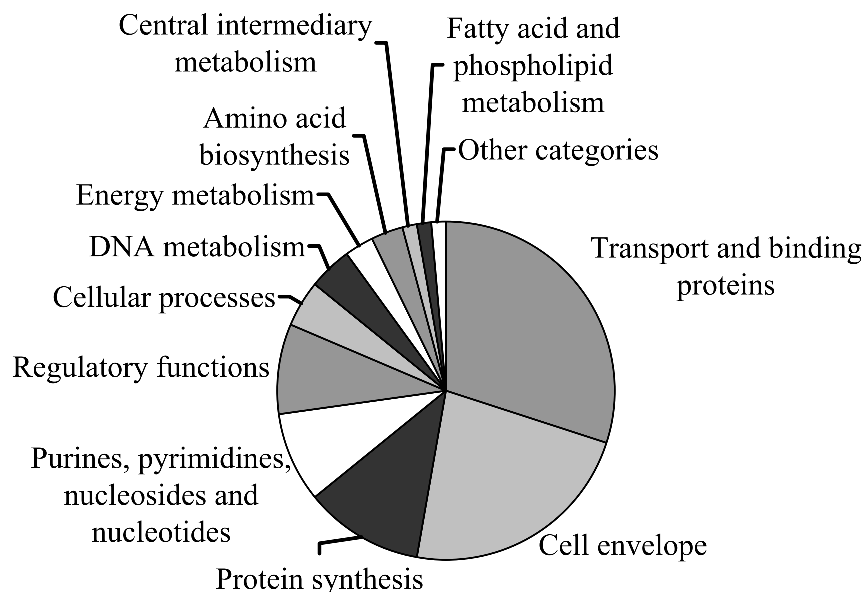

Figure 1. Division of the up- or down regulated genes over the different categories for genes similarly regulated in all subjects

Table 1. *L. plantarum* genes with a similar level of regulation in the different subjects as detected using DNA microarrays following the perfusion experiment. The 33 hypothetical proteins, which were altered, are not mentioned.

Gene category	Gene number	Product	Fold regulation
Amino acid biosynthesis	lp_2033	shikimate kinase	6.46
	lp_2790	phosphoglycerate dehydrogenase	11.32
Cell envelope	lp_0300	membrane-bound protease; CAAX family	7.22
	lp_0514	sortase	2.12
	lp_1446	extracellular protein	2.23
	lp_1447	cell surface protein precursor	2.46
	lp_1448	cell surface protein precursor	2.27
	lp_1449-50	extracellular protein	2.40, 2.37
	lp_1524	glycosyltransferase	2.51
	lp_2845	extracellular protein	2.59
	lp_3014-15	extracellular protein	6.63, 6.09
	lp_3050	extracellular protein	2.11
	lp_3254	effector of murein hydrolase (putative)	4.48
	lp_3412-14	extracellular protein	2.26, 3.20, 3.27
Cellular processes	lp_0129	small heat shock protein	0.44
	lp_0930[1]	alkaline shock protein	2.02
	lp_2192[1]	cell division protein (putative)	2.87
Central intermediary metabolism	lp_3220	α-glucosidase	2.21
DNA metabolism	lp_0006[1]	DNA gyrase; B subunit	2.00
	lp_1879	DNA-binding protein II	2.49
	lp_3293	DNA-3-methyladenine glycosylase I	2.18
Energy metabolism	lp_0952	fumarate reductase; flavoprotein subunit precursor	2.01
	lp_3487	aldose 1-epimerase	2.47
Fatty acid and phospholipid metabolism	lp_3362	choloylglycine hydrolase	3.08
Other categories	lp_1767	Prophage lysin	2.08
Protein synthesis	lp_0799[1]	SSRA RNA binding protein	2.42
	lp_0963[1]	pseudouridylate synthase	2.02
	lp_1515	translation initiation factor IF-3	2.88
	lp_1516-17	ribosomal protein L35, L20	2.88, 2.88
	lp_1558[1]	phenylalanine-tRNA ligase. α chain	2.84
	lp_1624	ribosomal protein L28	2.02

Gene category	**Gene number**	**Product**	**Fold regulation**
Protein synthesis	lp_1965[1]	glycine-tRNA ligase; α chain	2.60
Purines, pyrimidines,	lp_0693[1]	ribonucleoside-diphosphate reductase; α chain	2.32
nucleosides and nucleotides	lp_0694	glutaredoxin-like protein NrdH	2.35
	lp_2699[1]	dihydroorotate oxidase	3.24
	lp_2700[1]	carbamoyl-phosphate synthase; pyrimidine-specific; large chain	2.42
	lp_2723	amidophosphoribosyltransferase precursor	2.68
	lp_2724	phosphoribosylformylglycinamidine synthase II	2.30
	lp_2825	purine nucleosidase	2.34
Regulatory functions	lp_0965	transcription regulator	2.10
	lp_1267	transcription regulator (putative)	2.45
	lp_1911	transcription regulator (putative)	2.37
	lp_2651	transcription regulator	2.50
	lp_2708[1]	purine transport regulator	2.68
	lp_3488	galactose operon repressor	2.25
Transport and binding proteins	lp_0295	transport protein	2.06
	lp_0367-68[1]	glycine betaine/carnitine/choline ABC transporter	2.35, 2.27
	lp_0848	transport protein	2.06
	lp_0881-82[1]	glutamine ABC transporter	2.16, 2.06
	lp_1262	oligopeptide ABC transporter; permease protein	4.09
	lp_1265	oligopeptide ABC transporter; ATP-binding protein	2.11
	lp_1722	amino acid transport protein	2.50
	lp_1744-45	amino acid ABC transporter	2.38, 2.74
	lp_2240	amino acid transport protein	5.25
	lp_2649-50	N-acetylgalactosamine PTS; EIIC, B	2.10, 2.50
	lp_2710	purine transport protein	4.70
	lp_2739-40	ABC transporter	2.19, 2.17
	lp_2823[2]	ABC transporter; ATP-binding protein	6.43
	lp_2833	transport protein	0.44
	lp_2966	multidrug ABC transporter	3.51
	lp_3055	copper transporting ATPase	8.13
	lp_3363	copper transporting ATPase	3.50
	lp_3596	sugar transport protein	2.76

[1] Genes were probably more up regulated in the small intestine compared to the experimental set-up without injection in the small intestine; [2] Genes were probably more down regulated in the small intestine compared to the experimental set-up without injection in the small intestine

Subject-dependent regulation of *L. plantarum* genes

Only 298 of the 2819 genes (11%) were differentially regulated between the different subjects. Of those genes, 97 were on average more than 2-fold up- or down regulated (Table 2). These genes that are differentially regulated between subjects may reflect a subject-dependent adaptation of *L. plantarum* to the individual GI-tract conditions. Several of these are affected significantly in 3 out of the 4 subjects and those are also discussed below.

Table 2. *L. plantarum* genes with different levels of regulation in the different subjects as detected using DNA microarrays following the perfusion experiment. The 32 hypothetical proteins, which were altered, are not mentioned.

Gene category	Gene number	product	subject A	subject B	subject C	subject D
Amino acid biosynthesis	lp_0204	phosphoserine aminotransferase	3.08	0.93	1.83	7.10
	lp_2034	prephenate dehydrogenase	1.19	2.04	1.04	21.63
	lp_2035	3-phosphoshikimate 1-carboxyvinyltransferase	1.34	1.52	1.18	19.85
	lp_2037	chorismate synthase	1.46	1.05	1.08	7.84
	lp_2185[1]	diaminopimelate epimerase	2.01	0.64	1.47	4.53
Biosynthesis of cofactors. prosthetic groups. and carriers	lp_2788	2-dehydropantoate 2-reductase	1.25	36.65	1.40	1.71
Cell envelope	lp_0301	membrane-bound protease; CAAX family	6.25	1.00	2.62	4.92
	lp_0302	extracellular protein	23.78	1.26	9.34	21.21
	lp_1070	lipoprotein precursor	0.84	7.41	1.10	0.55
	lp_2162	extracellular protein; gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	6.34	0.99	3.49	7.99
	lp_2196	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	3.20	0.98	1.87	4.60
	lp_2197	UDP-N-acetylmuramoylalanine--D-glutamate ligase	2.72	0.96	1.70	3.36
	lp_2199	phospho-N-acetylmuramoyl-pentapeptide-transferase	2.24	0.90	1.53	3.34
	lp_2645[1]	muramidase	2.01	1.94	1.34	3.41
Cellular processes	lp_1747	nucleotide-binding protein; universal stress protein UspA family	1.99	1.07	1.32	13.49
	lp_1846	ATP-dependent protease HslV	2.26	1.15	1.44	3.74

Gene category	Gene number	product	subject A	subject B	subject C	subject D
Cellular processes	lp_1848	glucose inhibited division protein GidC	2.06	1.10	1.32	5.50
	lp_2189	cell division initiation protein DivIVA	2.60	0.84	1.64	4.93
	lp_2190[1]	cell division protein (putative)	2.47	0.92	1.79	5.11
	lp_2191[1]	cell division protein (putative)	2.35	0.97	1.81	5.40
	lp_2193	cell division protein FtsZ	2.60	1.10	1.73	5.22
	lp_2194	cell division protein FtsA	2.30	1.17	1.80	4.26
	lp_2195	cell division initiation protein FtsQ	2.49	0.82	1.84	4.53
Central intermediary metabolism	lp_0467	UDP-N-acetylglucosamine pyrophosphorylase	1.01	0.74	1.30	8.05
Energy metabolism	lp_2151[1]	pyruvate dehydrogenase complex; E3 component; dihydrolipoamide dehydrogenase	0.68	6.77	0.52	1.09
	lp_2152[1]	pyruvate dehydrogenase complex; E2 component; dihydrolipoamide S-acetyltransferase	0.60	9.02	0.50	1.19
	lp_2154[1]	pyruvate dehydrogenase complex; E1 component; α subunit	1.03	7.47	0.87	1.89
	lp_2776[2]	D-serine dehydratase	0.45	0.80	0.54	0.16
	lp_3662[1]	bifunctional protein: alcohol dehydrogenase; acetaldehyde dehydrogenase	0.49	14.48	0.45	2.78
Fatty acid and phospholipid metabolism	lp_0371[1]	glycerol-3-phosphate dehydrogenase	0.78	6.17	0.84	1.26
	lp_3536[2]	choloylglycine hydrolase	0.23	0.92	0.56	0.22
Other categories	lp_0796	carboxylesterase	2.67	1.41	2.17	5.20
	lp_1847	integrase/recombinase	2.13	1.17	1.39	5.27
Protein fate	lp_0795	preprotein translocase; SecG subunit	2.50	1.18	1.94	4.76
	lp_2305	zinc-dependent proteinase	1.88	0.80	1.16	5.16
Protein synthesis	lp_1025[1]	ribosomal protein S12	1.65	0.59	1.75	4.38
	lp_1026[1]	ribosomal protein S7	1.75	0.61	1.88	4.20
	lp_1559[1]	phenylalanine--tRNA ligase; β chain	3.14	0.99	2.56	4.75
	lp_1980[1]	aspartate--tRNA ligase	2.34	0.66	2.16	3.60
	lp_1981[1]	histidine--tRNA ligase	2.52	1.03	2.34	4.55
	lp_2187[1]	isoleucine--tRNA ligase	1.79	0.69	1.55	4.61
	lp_1639	tRNA (guanine-N1-)-methyltransferase	2.58	0.93	1.85	2.83
Purines, pyrimidines, nucleosides and nucleotides	lp_1289	purine/pyrimidine phosphoribosyltransferase (putative)	1.14	0.57	1.62	10.15
	lp_2698	orotidine-5'-phosphate decarboxylase	2.77	0.35	3.10	4.34

Gene category	Gene number	product	subject A	subject B	subject C	subject D
Purines, pyrimidines, nucleosides and nucleotides	lp_2721[1]	phosphoribosylglycinamide formyltransferase	1.12	0.32	1.52	6.03
	lp_2722[1]	phosphoribosylformylglycinamidine cyclo-ligase	0.97	0.34	1.53	6.16
	lp_2725	phosphoribosylformylglycinamidine synthase I	0.64	0.47	1.22	14.78
	lp_2726	conserved purine biosynthesis cluster protein	0.99	0.54	1.73	17.17
	lp_2727	phosphoribosylaminoimidazole-succinocarboxamide synthase	0.84	0.51	1.55	18.43
	lp_2728	phosphoribosylaminoimidazole carboxylase; ATPase subunit	0.49	0.49	1.00	13.31
	lp_3269	adenylosuccinate lyase	1.04	0.51	1.61	19.91
	lp_3270	adenylosuccinate synthase	1.45	0.55	1.80	12.31
Regulatory functions	lp_3633	transcription regulator	1.26	5.80	0.63	0.70
Transcription	lp_0797[1]	ribonuclease R	2.52	1.39	2.13	4.22
	lp_1151	RNA methyltransferase	1.26	0.97	1.14	4.77
Transport and binding proteins	lp_0175	maltose/maltodextrin ABC transporter; substrate binding protein	0.36	6.95	0.47	0.47
	lp_0525	potassium uptake protein	3.13	0.64	2.03	13.44
	lp_0991	multidrug transport protein	2.21	1.14	5.53	2.13
	lp_1263	oligopeptide ABC transporter; permease	1.98	0.96	0.85	11.42
	lp_1264	oligopeptide ABC transporter; ATP-binding	1.74	0.85	0.86	8.92
	lp_1746	amino acid ABC transporter; substrate binding	1.79	1.06	1.37	9.59
	lp_2038	transport protein	1.51	1.78	1.44	11.16
	lp_2789	transport protein	0.92	79.69	1.03	1.06
	lp_3288[1]	cation efflux protein	0.60	5.34	1.06	1.31
	lp_3327	cadmium-/zinc-/cobalt- transporting ATPase	1.26	4.20	1.12	1.67

[1] Genes were probably more up regulated in the small intestine compared to the experimental set-up without injection in the small intestine; [2] Genes were probably more down regulated in the small intestine compared to the experimental set-up without injection in the small intestine

L. plantarum is metabolically active during perfusion

Expression of *L. plantarum* genes involved in general metabolic functions such as cell division, purine and pyrimidine metabolism, and protein synthesis, reflect the overall activity of the cells. A series of 6 genes (*divIVA* and *fts* genes; Lp_2189-2195) that appeared to form an operon and predicted to code for cell division were up regulated in two of the four subjects

(Table 2). An increase in the formation of DNA in all subjects is suggested by increased expression of several genes involved in the purine and pyrimidine metabolism, including the genes annotated to encode for dihydroorotate oxidase, amidophosphoribosyltransferase precursor, and ribonucleoside-diphosphate reductase (Table 1). In addition, a purine transport regulator and a purine transport protein were 2.7- and 4.7- fold up regulated respectively suggesting purine uptake from the environment, possibly from lysed human or bacterial cells. The purine transport regulator is probably even more up regulated as the control experiment in which *L. plantarum* cells were not perfused in the small intestine caused a 3-fold down-regulation of the gene (data not shown). From the up regulated genes, 12 % were found to be involved in purine and pyrimidine metabolism, compared to 4 % in the control experiment (data not shown). This supports the cell division of *L. plantarum* in the human intestine, which presumably aids adaptation to and survival in the intestinal conditions.

Various genes predicted to be involved in protein synthesis and coding for proteins such as translation initiation factor IF-3 and different tRNA ligases (specific for phenylalanine and glycine, and to a lesser extent aspartate, histidine and isoleucine) were up regulated (Tables 1 and 2). This suggests increased protein turnover or biosynthesis to adapt to intestinal conditions. In addition, genes likely to be involved in uptake of amino acids, like ABC transporters proteins for amino acid, specifically glutamine, or oligopeptide and amino acid transporters were up regulated. It is interesting to notice that approximately 11% of the regulated genes belonged to the protein synthesis genes, whereas in the control experimental set-up, without injection in the intestine, less than 4 % of the up regulated genes were involved in protein synthesis (data not shown). Several genes annotated to be involved in amino acid biosynthesis, including those for shikimate kinase and phosphoglycerate dehydrogenase (SerA), were also up regulated (Table 1). The *serA* gene appeared to show the highest intestinal induction of more than tenfold within the perfused samples. Remarkably, SerA is the key enzyme in serine biosynthesis and it is known that many of the *L. plantarum* cell-envelope proteins are highly enriched in serine residues. Genes annotated to encode for ribosomal proteins were also found to be up regulated indicating an increase in ribosomal RNA. Overall, this suggests an increased protein synthesis for *L. plantarum* cells, which has been related to growth or adaptation to a new situation (14).

Osmotic stress response induced in the small intestine

Several genes involved in cellular adaptation were differentially regulated during the perfusion process. The predicted choloylglycine hydrolase (lp_3362) gene of *L. plantarum*, which may decrease the strong acidic properties of bile salts (10), was 3.1-fold up regulated. However, another predicted choloylglycine hydrolase (lp_3536) was more than 2.0 times down regulated, which was even more significant considering that this gene was up regulated in the control experiment in which *L. plantarum* cells were not perfused in the small intestine (data not shown). These appear to be specific responses to the intestinal conditions. The exact function of the two distinct predicted choloylglycine hydrolase genes is not known, but they could have different specificities for certain bile acids. In *E. coli* extrusion of bile salts from the cell by multidrug resistance transporters has been determined (28), and a similar mechanism has been suggested for lactic acid bacteria (36). Up-regulation of a gene encoding a predicted multidrug transporter (lp_0991) (Table 2) may be involved in protecting *L. plantarum* by excreting the bile salts out of the cell.

The bile salts released into the small intestine may have caused an increase in osmolarity. Up-regulation of two adjacent genes that could encode a glycine betaine/carnitine/choline ABC transporter (lp_0367-0368) suggested an osmotic stress response that would reduce the cell-turgor (36). Concomitant, up-regulation of a gene annotated to be involved in potassium uptake suggested a build-up of cations in the cell to restore the turgor. In *Bacillus subtilis*, an influx of potassium ions was seen in reaction to osmotic stress (21). In addition, several genes that may encode stress proteins were up regulated, including a nucleotide binding protein, an alkaline shock protein and the ATP-dependent protease HsIV. The gene putatively encoding a small heat shock protein was down regulated suggesting that heat shock took place during processing of the initial cells.

Two predicted copper transporting ATPase (*copA*) genes were up regulated in all subjects and specifically that coding for Lp_3055 was found to be strongly up regulated (Table 1). This *copA* gene has been suggested to be involved in either copper acquisition or high copper concentration tolerance (18). The importance of expression of the *copA* (lp_3055) gene for *L. plantarum* in the GI-tract is supported by previous studies demonstrating induction in the GI-tract of mice (5, 18). The *copA* gene appears to be involved in persistence and survival of *L. plantarum* in the GI-tract (8), nevertheless the exact function has not yet been elucidated.

Cell envelope changes of *L. plantarum* as adaptation to small intestine

As a reaction to passage of a segment of the human small intestine, the genes involved in peptidoglycan biosynthesis of *L. plantarum*, including the genes annotated as phospho-N-acetylmuramoyl-pentapeptide-transferase, UDP-N-acetylmuramoylalanine-D-glutamate ligase, and UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase, were up regulated in three of the four subjects indicating an increase in strengthening or reconstruction of the cell wall for protection against the intestinal conditions (Table 2).

Changes in the cell envelope were also visible with up to 24 times up-regulation of several genes encoding different predicted cell surface proteins of which several demonstrated subject-dependent regulation (Tables 1 and 2). The exact function of the genes is not known. One of these, lp_1447, is predicted to contain a sortase target for anchoring it to the cell envelope (4). The putative operons lp_3412-3414 and lp_1447-1450 encode extracellular protein clusters of which the speculative function involves complex carbohydrate recognition and/or degradation. Remarkably, the *L. plantarum* sortase gene (lp_0514) was also found to be increased in expression upon ileal perfusion, indicative of an increased efficiency of cell-envelope decoration by extracellular proteins. Moreover, several extracellular proteins (Lp_0302, Lp_3014, Lp_3015 and Lp_3050) contain a LysM domain, which can be found in a variety of enzymes involved in bacterial cell wall degradation. This domain may have a general peptidoglycan binding function (3). A previous study that analyzed the responses of *L. plantarum* to bile stress have indicated that major changes can be observed in the appearance of the cell wall upon bile stress, including very rough surface properties instead of the smooth surface seen in non-stressed cells (7). Moreover, both genetic screens and transcriptome profiling studies have shown that these changing physiological properties of the bacterial cell wall are accompanied by major changes in expression of genes encoding cell-envelope related protein functions, including up-regulation of Lp_3014 (7, 9). Finally, a gene predicted to code for a phage lysine is upregulated in the intestine and may point to induction of cell lysis.

Quantitative real time PCR confirms DNA microarray results

The DNA microarray results were confirmed by the qRT-PCR experiments. These showed that catabolic genes such as those coding for lactate dehydrogenase (1.3±0.5 versus 1.6 ±1.0) or glucose-6-phosphate 1-dehydrogenase (0.8±0.1 versus 0.8±0.4) involved in glycolysis

and the pentose phosphate pathway, respectively, were not affected significantly. However, the *copA* gene was on average 30-fold up regulated, which is somewhat higher than the array data indicated (8.1±3.2). While the qRT-PCR confirmed the trend in differential gene expression observed with the DNA microarray analysis, a quantitative difference in the fold change was observed between these two technologies (32, 33).

Conclusion

L. plantarum cells that passed a segment of the small intestine in healthy individuals retained metabolic activity, as can be deduced from up-regulation of genes annotated to code for cell division and protein synthesis. An osmotic shock response, probably induced by the presence of bile salts, was demonstrated by up-regulation of genes predicted to be involved in ion import, in glycine, betaine, carnitine or choline uptake and in degradation of bile salts. In addition, strengthening of the cell envelope and specific interactions with the host were indicated by the up-regulation of many genes predicted to encode cell-envelope proteins. All together, gene regulation as demonstrated using the perfusion technique provides a new insight in the reaction of *L. plantarum* to passage of a segment of the human small intestine and established the method as valuable for research into the functionality of microbes in the human small intestine.

References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-1920.

3. **Bateman, A., and M. Bycroft.** 2000. The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). J. Mol. Biol. **299:**1113-9.

4. **Boekhorst, J., M. W. H. J. de Been, M. Kleerebezem, and R. J. Siezen.** 2005. Genome-wide detection and analysis of cell wall-bound proteins with LPxTG-like sorting motifs. J. Bacteriol. **187:**4928-4934.

5. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721-5729.

6. **Bron, P. A., S. M. Hoffer, I. I. Van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-317.

7. **Bron, P. A., M. Marco, S. M. Hoffer, E. Van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J. Bacteriol. **186:**7829-7835.

8. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. *In* P. A. Bron (ed.), The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen Centre for Food Sciences, Wageningen.

9. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2005. DNA micro-array based identification of bile-responsive genes in *Lactobacillus plantarum*. J. Appl. Microbiol. in press.

10. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol. **79:**292-301.

11. **de Vos, W. M., P. A. Bron, and M. Kleerebezem.** 2004. Post-genomics of lactic acid bacteria and other food-grade bacteria to discover gut functionality. Curr. Opin. Biotechnol. **15:**86-93.

12. **de Vries, M. C., M. Kleerebezem, P. Mangell, S. Ahrne, M. Douwe, E. E. Vaughan, and W. M. de Vos.** 2005. Transcript profiling reveals global gene expression of *Lactobacillus plantarum* in the human gastrointestinal tract. *In* M. C. de Vries (ed.), Analyzing global gene expression of *Lactobacillus plantarum* in the human gastro-intestinal tract. Wageningen Centre for Food Sciences, Wageningen.

13. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. *Lactobacillus plantarum* - Survival, functional and potential probiotic properties in the human intestinal tract. Int. Dairy J. in press.

14. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2004. Optimising single cell activity assessment of *Lactobacillus plantarum* by fluorescent *in situ* hybridisation as affected by growth. J. Microbiol. Methods **59:**109-115.

15. **Grangette, C., H. Muller-Alouf, P. Hols, D. Goudercourt, J. Delcour, M. Turneer, and A. Mercenier.** 2004. Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. Infect. Immun. **72:**2731-2737.

16. **Grangette, C., S. Nutten, E. Palumbo, S. Morath, C. Herman, J. Dewulf, B. Pot, T. Hartung, P. Hols, and A. Mercenier.** 2005. From The Cover: Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc. Natl. Acad. Sci. U. S. A. **102:**10321-10326.

17. **Hanniffy, S., U. Wiedermann, A. Repa, A. Mercenier, C. Daniel, J. Fioramonti, H. Tlaskolova, H. Kozakova, H. Israelsen, S. Madsen, A. Vrang, P. Hols, J. Delcour, P. Bron, M. Kleerebezem, and J. Wells.** 2004. Potential and opportunities for use of recombinant lactic acid bacteria in human health. Adv. Appl. Microbiol. **56:**1-64.

18. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by *in vivo* gene expression. Proc. Natl. Acad. Sci. U. S. A. **94:**934-939.

19. **Hooper, L. V.** 2004. Bacterial contributions to mammalian gut development. Trends Microbiol. **12:**129-134.

20. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing, Gram-positive rods, p. 1229. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams and Wilkins, Baltimore, U.S.A.

21. **Kempf, B., and E. Bremer.** 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch. Microbiol. **170:**319-330.

22. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

23. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos.** 1993. Characterizion of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281-291.

24. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods **25:**402-408.

25. **Mackie, R. I., A. Sghir, and H. R. Gaskins.** 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. **69(suppl):**1035S-1045S.

26. **Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem.** 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. J. Bacteriol. **187:**6119-27.

27. **Mourad, F. H.** 2004. Animal and human models for studying effects of drugs on intestinal fluid transport *in vivo*. J. Pharmacol. Toxicol. Methods **50:**3-12.

28. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J. Bacteriol. **183:**5803-5812.

29. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107-133.

30. **Saxelin, M., S. Tynkkynen, T. Mattila-Sandholm, and W. M. de Vos.** 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol. **16:**204-211.

31. **Sonnenburg, J. L., L. T. Angenent, and J. I. Gordon.** 2004. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol. **5:**569-573.

32. **Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect. Immun. **73:**1797-1810.

33. **Sturme, M. H. J., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. An *agr*-like two component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J. Bacteriol. **187:** 5224-5235.

34. **Tannock, G. W.** 1995. Normal microflora: An introduction to microbes inhabiting the human body. Chapman & Hall, London.

35. **Troost, F. J., W. H. M. Saris, G. R. M. M. Haenen, A. Bast, and R.J. M. Brummer.** 2003. New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. Am. J. Physiol. Gastrointest. Liver Physiol. **285:**G354-359.

36. **van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin.** 2002. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek **82:**187-216.

37. **Vaughan, E. E., M. C. de Vries, E. G. Zoetendal, K. Ben-Amor, A. D. L. Akkermans, and W. M. de Vos.** 2002. The Intestinal LABs. Antonie van Leeuwenhoek **82:**341-352.

38. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucl. Acids Res. **30:**e15.

Chapter 6

Specific Gene Expression of *Lactobacillus plantarum* in the Ileum of Ileostomy Patients as Determined by Quantitative Reverse Transcriptase PCR

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Abstract

Gene expression of *Lactobacillus plantarum* WCFS1 was assessed by quantitative reverse transcriptase-PCR on RNA templates isolated from ileostoma effluent of three subjects who consumed these bacteria in a milk-based drink. The study focused on a specific set of representative genes in *L. plantarum* WCFS1 to obtain an overview of their expression level following ingestion and passage through the human upper gastrointestinal tract and small intestine relative to their expression level in the cells consumed by the subjects. Target gene selection was based on: (1) observed to be induced or highly expressed during intestinal passage in earlier human and mice studies; (2) predicted to code for general metabolic and energy-generating activities; and (3) anticipated to show functionality in the small intestine.

Clearly, the *L. plantarum* cells were metabolically active as indicated by induction of genes for protein synthesis (*fusA2*), amino acid biosynthesis (*argG*), and sugar uptake (*sack2*) and energy (*pfk, pgi*) and pyruvate dissipation (*ldh, adh1*) metabolism. No differential expression was observed for several specific cell surface proteins (lp_0800 and lp_2940) or bile salt hydrolase (*bsh1*). In contrast, a potential adherence protein (lp_1793), α-L-rhamnosidase (*ram2*), which is an enzyme for hydrolysis of rhamnosides and releasing L-rhamnose from various substrates, and a bacteriocin precursor (*plnE*) were clearly up regulated. This study provides insight on the adaptation of *L. plantarum* upon ingestion to the human small intestinal environment.

Keywords: gene regulation, quantitative reverse transcriptase PCR, human small intestine, *Lactobacillus plantarum*

Introduction

The human gastrointestinal (GI)-tract comprises a range of different habitats associated with mucosa, mucus, and particle surfaces in the lumen, which are continuously changing in response to dietary or environmental factors (41, 43). Distinct microbial communities are present in the different parts of the GI-tract, including small and large intestine (48, 49). The microbiota perform essential functions for the host, including nutrient conversion and uptake, modulation of host immunity, angiogenesis (44) and intestinal epithelial development (22), activity and homeostasis (4, 37).

The lactic acid bacterium *Lactobacillus plantarum* can be found as a natural inhabitant of the human GI-tract and in a range of other habitats, including some dairy, meat, and many plant fermentations (1, 24). Some *L. plantarum* strains are marketed as probiotics for their potential positive effects on the consumers health (16). The complete genome sequence of the human isolate *L. plantarum* WCFS1 has recently been determined and predicts the potential of *L. plantarum* to grow in a large range of environmental niches (25). In addition, the availability of the genome sequence enabled the application of different molecular screening techniques, including DNA microarrays and the design of gene-specific oligonucleotide primers for transcript quantification by quantitative reverse transcriptase (qRT)-PCR (9, 15).

Limited knowledge is available about the molecular responses of ingested microbes upon passage of the human stomach, small intestine and colon. The small intestine comprises the duodenum, jejunum and ileum, in that order. Because of the technical and logistical difficulties of performing human volunteer studies, most information available for GI–tract-microbe interactions comes from microbial responses to *in vitro* conditions and animal (primarily rodent) model systems. For example, promoter-trapping screens and transcriptome analyses using DNA microarrays have revealed *L. plantarum* WCFS1 genes that are regulated by bile-stress, a condition likely relevant to GI-tract environments (7, 9). Recombinant *in vivo* expression technology (R-IVET) has been used to identify *L. plantarum* and *L. reuteri* genes that were specifically switched on in the GI-tract of conventionally raised and *Lactobacillus*-free mice, respectively (5, 51). Subsequent deletion studies showed that several of the so called *L. plantarum ivi*-genes were found to be essential for survival during passage through the murine GI-tract (8). Those results indicate the importance of intestinal gene expression as is supported by the observation that *L. casei* is capable of initiating *de novo* protein synthesis upon passage of the GI-tract of mice (35).

In addition to the analysis of ingested lactobacilli, transcript profiling of the human commensal *Bacteroides thetaiotaomicron* in the mono-species associated murine GI-tract demonstrated that the microbes selectively induced enzymes for metabolism of dietary polysaccharides, and upon their limitation, shifted its glycan foraging behavior to the host intestinal mucus as a nutritive source (51). The only human *in vivo* study so far involved global transcript profiling of mucosa-associated *L. plantarum* in an ileal and three colonic human biopsies (15). This study revealed that the ingested *L. plantarum* was metabolically active in the GI-tract and exhibited a substantial degree of adaptation to the human intestine.

qRT-PCR is a sensitive technique to quantify mRNA with a broad dynamic range and excellent accuracy (11). While commonly used for examining gene expression levels in human and murine cells, there are presently only a few reports in which this technique has been applied to the study of bacteria *in situ* (42). With regard to monitoring microbial gene expression in the GI tract, qRT-PCR has been applied to the pathogenic species *Mycobacterium avium* subspecies *paratuberculosis* to study its response to oxidative stress in the ileum of goats and a cow, and to compare gene expression of *Helicobacter pylori* between the human and murine stomach (20, 40).

In the present study, we focused on the expression of a particular set of genes of the commensal bacterium *L. plantarum* WCFS1 in the ileostomy effluent of several subjects that had ingested a *L. plantarum*-containing product. The ileostoma provides access to samples from the human small intestine, a location suggested to harbor a predominance of lactobacilli, as well as represents the site of their presumed primary influence on the host (37, 48). qRT-PCR was sensitive enough to detect gene expression despite dilution of the microbes upon consumption in food and intestinal passage, and demonstrated metabolic activity and adaptation of *L. plantarum* to the human small intestine.

Material and methods

Bacterial growth

L. plantarum WCFS1 was grown anaerobically, at 30°C in MRS (Oxoid, Basingstoke, UK) for 17 h. to reach the stationary phase. The cells were harvested and washed three times in physiological salt solution (B.Braun Melsungen AG, Melsungen, Germany). After the last washing step, a portion of these *L. plantarum* cells were immediately frozen and stored at -80°C until RNA isolation. Aliquots of the remaining cells were resuspended in 300 ml of a commercially available flavored milk product (Melk met Smaak, Campina, Woerden, The Netherlands) at room temperature to a final concentration of 1.2 - 1.4x10^8 CFU ml^{-1}, and immediately provided to the human volunteers for consumption.

Ileostoma samples

Three human volunteers with an ileostoma participated in this study. All subjects had a colectomy performed at least 3 years ago and were healthy aside from the ileostoma. After an overnight fast, the subjects drank 300 ml of flavored milk containing the *L. plantarum* WCFS1 cells, and 4 h. later the subjects consumed a standardized lunch. From the moment of

ingesting the drink, ileal effluent was collected from the stoma bags of the subjects at 1-h intervals for the subsequent 6 h. The samples were immediately frozen on dry ice and stored at -80°C. The amount of sample differed from 10 to 100 g between patients and between time points. Subject A participated in this study on a separate day from subjects B and C and received a different preparation of the *L. plantarum* milk mixture.

Fluorescent *in situ* hybridisation (FISH)

FISH was used to determine which ileostomy effluent samples in the stoma bags contained the highest level of *L. plantarum*. Approximately 0.5 g of ileostomy effluent sample was used for FISH as previously described (17). Probe Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') (2) labeled with fluorescein (Eurogentec, Seraing, Belgium) and a *L. plantarum* specific probe (5'-CCAATCAATACCAGAGTTCG-3') labeled with cy3 (13, 21) were used for FISH. 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, USA) was added to the washing buffer to stain the DNA of all cells present.

RNA isolation

The frozen ileal samples and *L. plantarum* cells initially prepared for consumption were diluted 1:2 in methanol buffer (36) and vortexed with glass beads (0.1mm Zirconia-silica beads, BiospecProducts inc., Bartlesville, USA) to remove the bacteria from the particulate matter. A short spin (30 s; 400 g) was applied to remove the large particles. The bacterial cells were harvested by centrifugation at 7.000 g for 7 min. The bacterial cells were disrupted using a bead beater (BiospecProducts inc.) by shaking at least 5 times for 1.5 min with intermediate cooling on ice. Total RNA was isolated using Macaloid clay as described previously (27). The RNA was cleaned according to the Rneasy® cleaning protocol including a DNase step (Qiagen Sciences, Maryland, USA). On all samples a second DNase treatment was performed and the RNA quality and purity were checked on agarose gels and spectrophotometrically.

Quantitative reverse transcriptase-PCR

Primers for RT-PCR of *L. plantarum* genes were obtained from literature or designed and are presented in Table 1. Specificity was determined by checking the primers in databases, by performing melting curves on the RT-PCR products, and ultimately by sequencing the RT-PCR products generated by the primers. Efficiency of the primers was

assessed using a dilution series of pure *L. plantarum* DNA. The 16S ribosomal RNA (rRNA) primers were tested on different related species and confirmed to be specific (data not shown).

Superscript III (Invitrogen, Breda, the Netherlands) and the reverse primer (Table 1) were used on 2 μg of total RNA in the RT-reactions. The resulting RT-products (cDNAs) (1 ng of total RNA for mRNA and 0.1 pg of total RNA for 16S rRNA) were quantified in triplicate in an ABI prism 7700 real-time PCR apparatus (Taqman) using cybrgreen (Sybr green PCR master mix, Applied Biosystems, Warrington, UK). Transcripts could be detected for all genes in all samples. A reaction without RT-step of all samples was included as a control, which did not give any signal, confirming the absence of DNA. All results were normalized to the *L. plantarum* 16S rRNA level and compared to gene expression in the initially consumed cells according to the $2^{-\Delta\Delta Ct}$ method (28). The 16S rRNA was chosen for normalization, based upon its reliability in previous studies (11, 29).

Table 1. Primers used for qRT-PCR

Gene name	**Gene number**	**Forward primer**	**Reverse primer**	**ref**
16S rRNA		TGA TCC TGG CTC AGG ACG AA	TGC AAG CAC CAA TCA ATA CCA	(7)
adh1	lp_1665	GAT GTC ATT GTG CGC ATC GT	GGC AGC CTT GTC ATC ACC AT	
argG	lp_0775	GCT CTT GCA CCG GAT ATC AA	TTT CTT CTT CCC GTG ACC AGT	(7)
bsh1	lp_3536	GGA ATG GGC GGA CTA GGA TT	CTC TGT CTG CAT CGA CAA CGA	
copA	lp_3055	CGC ACT TGT GAC CAC TTT CG	TTC CGC TTC CTT GGC TTG TA	(15)
fusA2	lp_1027	CCA TGA TGG TGC TTC ACA A	TCG TGG CAG CAG AGG TAA TG	(7)
gpd	lp_2681	GGA ACA ACC GTT ACA TCG ACA	GCA AGG CAC CGC TGT TAT C	(15)
hpk10	lp_3088	TTC AAC AAC AAC GCC TGC AA	GAA ACC GGC GCA ATT CAT TA	
ldh	lp_0537	TGA TCC TCG TTC CGT YGA TG	CCG ATG GTT GCA GTT GAG TAA G	(15)
lp_0237	lp_0237	CTA CTG ATA TGG TTG TCG GGA ATT A	ACG GGT GCG TAG AAG AAG C	(7)
lp_0800	lp_0800	CGA TTA ATG CGG CAA CAA CA	CCG GTT GTT CAG CCT TTG AG	(15)
lp_1603	lp_1603	TGG TTC AGT CGT TGC CCT AA	AAC AGC AGG ATC ACG GAC AA	
lp_1793	lp_1793	CTC ACA GAA GCC GCG AAT CT	CGG CGA ACT TGG ACG TAA TC	
lp_2940	lp_2940	ATG GCA CGG TCA GTT TAG CA	TTG CAC CGC TTG TGT TAC CT	
pcrA	lp_1144	AGG AGG TCT GGG TCT CAA CG	AAG GTC CGT TGC TCG CTA GT	
pfk	lp_1898	TCC AGG GAC GAT CGA TAA TGA	GCT TGC ACG TTG GTG TTG AC	(15)
pgi	lp_2502	CCG GAT CTA TGC CAC AAC TG	TGA AAC GAC CAC CAA CAT CA	(15)
plnE	lp_0422	TGA GAA GTT ACA ATA TTC CAG GTT G	TTG CAT CAA CAA CAT GTC GAA	
pts14C	lp_1164	GGG CAT CTT CCT CGC ACT AT	TCG ATC TCC TGG TGG ATG TGT	(15)
ram2	lp_3473	CAA CCA CGC TGA CGT TAC CA	CCG TGA CCA CTG GAT TGC TA	(15)
sacK2	lp_3637	TGG AAT TAA GCA CGG CAC TG	GTG TAG CGC TGC CGT TAG AA	(15)

Results and discussion

L. plantarum transit time and cell recovery

Three subjects with an ileostoma consumed *L. plantarum* cells in a milk-based flavored drink and the ileal effluent was subsequently collected. FISH analysis using *L. plantarum* specific and universal 16S rRNA probes was performed on all samples to determine the recovery of *L. plantarum* cells and to estimate the transit time of *L. plantarum*. Transit time of food depends on several factors such as gastric emptying rates, gut motility and the physiology of the intestine of the host, and food intake. The FISH analysis revealed that the highest number of *L. plantarum* cells could be found in the ileostoma effluent samples collected between four to six hours after intake, correlating to the 4.1 ± 2 h. marker transit time as determined previously for ileostomy patients (46). Furthermore, enumeration of the cells with FISH indicated that the samples with highest levels of *L. plantarum* contained 3-7×10^7 cells, which was between 4 and 23% of the total bacterial community detected with the universal FISH probes. The efficiency of FISH for detection of *L. plantarum* is growth phase dependent (17), and not all cells may have been detected here following transit. Moreover, the ingested *L. plantarum* cells were distributed over multiple samples as a consequence of dilution and possible interaction with the host. This explains why the recovered cells in the samples with the highest level of *L. plantarum* still represented only 0.1-1% of the total number of ingested cells. This is equivalent to approximately 1000 fold fewer cells than ingested caused by spreading of the initial cells over multiple samples, the detection method and limited survival of the stomach (50). Total RNA isolations from the samples yielded between 4 and 50 µg of RNA from the total bacterial community. While this low amount of RNA did not allow adequate global transcript analysis by DNA microarrays it sufficed to perform gene-specific transcript quantification by qRT-PCR.

Gene selection and qRT-PCR normalization methods

In order to determine the potentially diverse responses of *L. plantarum* in the GI-tract, a selection was made of 20 genes of which expression was followed by qRT-PCR. These genes were selected based on the following criteria: (1) observed to be induced or highly expressed during intestinal passage in earlier human and mice studies; (2) predicted to code for general metabolic and energy-generating activities; and (3) anticipated to show

functionality in the small intestine. The selected genes grouped into functional classes based on their anticipated expression products are listed in Table 2.

Table 2. Genes of *L. plantarum* used for qRT-PCR with references to former expression in intestinal samples and other remarks about their significance/relevance. The R-IVET screen detected genes specifically switched on in the GI-tract of mice following recovery from the feces (5); the transcript profiling was performed on *L. plantarum* cells on one human ileal and three colonic mucosa (15); and microarrays also showed the *in vitro* bile response of *L. plantarum (9)*

Gene	Name	Functional class	Expression or relevance
adh1	Alcohol dehydrogenase	Fermentation	All 3 colon samples (15)
argG	argininosuccinate synthase	Regulatory functions	Ileum and colon of different persons; R-IVET (5, 9, 15)
bsh1	choloylglycine hydrolase	Bile acid biosynthesis and degradation	Expression expected to be induced by bile
copA	copper transporting ATPase	Transport and binding proteins	Ileum and colon of 1 person; R-IVET (5, 15)
fusA2	elongation factor G	Protein synthesis	Ileum and colon of 1 person (15)
gpd	glucose-6-phosphate 1-dehydrogenase	Pentose phosphate pathway	Colon of 2 persons (15)
hpk10	histidine protein kinase; sensor protein	Amino acid biosynthesis	Colon of 2 persons (15)
ldh	L-lactate dehydrogenase	Fermentation, housekeeping gene	Constitutive expression expected
lp_0237	integral membrane protein	Cell envelope	R-IVET (5)
lp_0800	cell surface protein precursor	Cell envelope	Ileum and colon of different persons; R-IVET (5, 15)
lp_1603	hemolysin homolog	Cell killing and immunity	Colon of 1 person; R-IVET (5)
lp_1793	adherence protein	Cell envelope	All colon samples (15)
lp_2940	cell surface protein precursor	Cell envelope	Ileum; R-IVET (5, 15)
pcrA	ATP-dependent DNA helicase PcrA	DNA metabolism	Ileum; R-IVET (5, 15)
pfk	6-phosphofructokinase	Glycolysis	Ileum and colon of 1 person; R-IVET (5, 15)
pgi	glucose-6-phosphate isomerase	Energy metabolism (Sugars)	Ileum and colon of 1 person (15)
plnE	bacteriocin precursor peptide E (putative)	Antimicrobial production and resistance	Colonisation factor; Potentially promotes colonisation resistance
pts14C	cellobiose PTS, EIIC	Transport and binding proteins	Ileum; R-IVET (5, 15)
ram2	α-L-rhamnosidase (putative)	Central intermediary metabolism	Colon of 2 persons; R-IVET (5, 15)
sacK2	Fructokinase	Energy metabolism (Sugars)	Ileum and colon of 1 person (15)

In order to differentially quantify the transcript levels of the *L. plantarum* genes in the ileostoma samples, these were compared to those found in the *L. plantarum* cells that were administered to the subjects. The latter cells were obtained from stationary phase cultures grown in MRS and provide a reference level for comparative purpose. To compensate for the relative amount of *L. plantarum* RNA as a fraction of the total microbial RNA isolated from the ileostoma effluent, the expression values were first normalized to that observed for the *L. plantarum* 16S rRNA. The 16S rRNA was selected because it is abundant, the least subject to large fluctuations and most commonly used to normalize for the total number of cells (11, 29, 40).

Metabolic gene regulation

Regulation of the genes described above following ingestion of *L. plantarum* by three ileostomy patients was assessed using qRT-PCR (Figure 1). Notably, most genes demonstrate a similar trend of differential regulation, even though samples were from different people, consuming *L. plantarum* on different days (sample A). Representative housekeeping functions, of which expression was expected, are encoded for by the ATP-dependent DNA helicase (*pcrA*) gene involved in DNA replication (12) and the elongation factor G (*fusA2*) gene involved in protein synthesis (Figure 1A). Indeed, *pcrA*, displayed up-regulation to various levels depending on the person with an average of 5 times up-regulation in the ileostomy effluent samples compared to the consumed cells. Previously, expression of the *L. plantarum pcrA* gene has been observed in an ileal biopsy sample of human origin (15), while its promoter has been identified as being activated by 0.8 M NaCl in a genetic screening study (6). It is very unlikely that the high levels of NaCl or high osmolarity that induce *pcrA* expression *in vitro* are reached in the small intestine of the subjects tested, suggesting that other environmental factors are involved in *in situ* activation of this gene and its variation of expression between individuals (Figure 1A). In addition, the *fusA2* housekeeping gene potentially encoding a translation elongation factor (39, 52) demonstrated 4 times up-regulation in ileostomy samples compared to the consumed stationary phase cells indicating an increased protein synthesis and re-initiation of the cell metabolism in the intestine. The *fusA2* gene was also observed to be well expressed in *L. plantarum* cells attached to the mucosa of the ileum (15). Initiation of new protein synthesis has also been confirmed for *L. casei* in the digestive tract of human microflora-associated mice (34, 35).

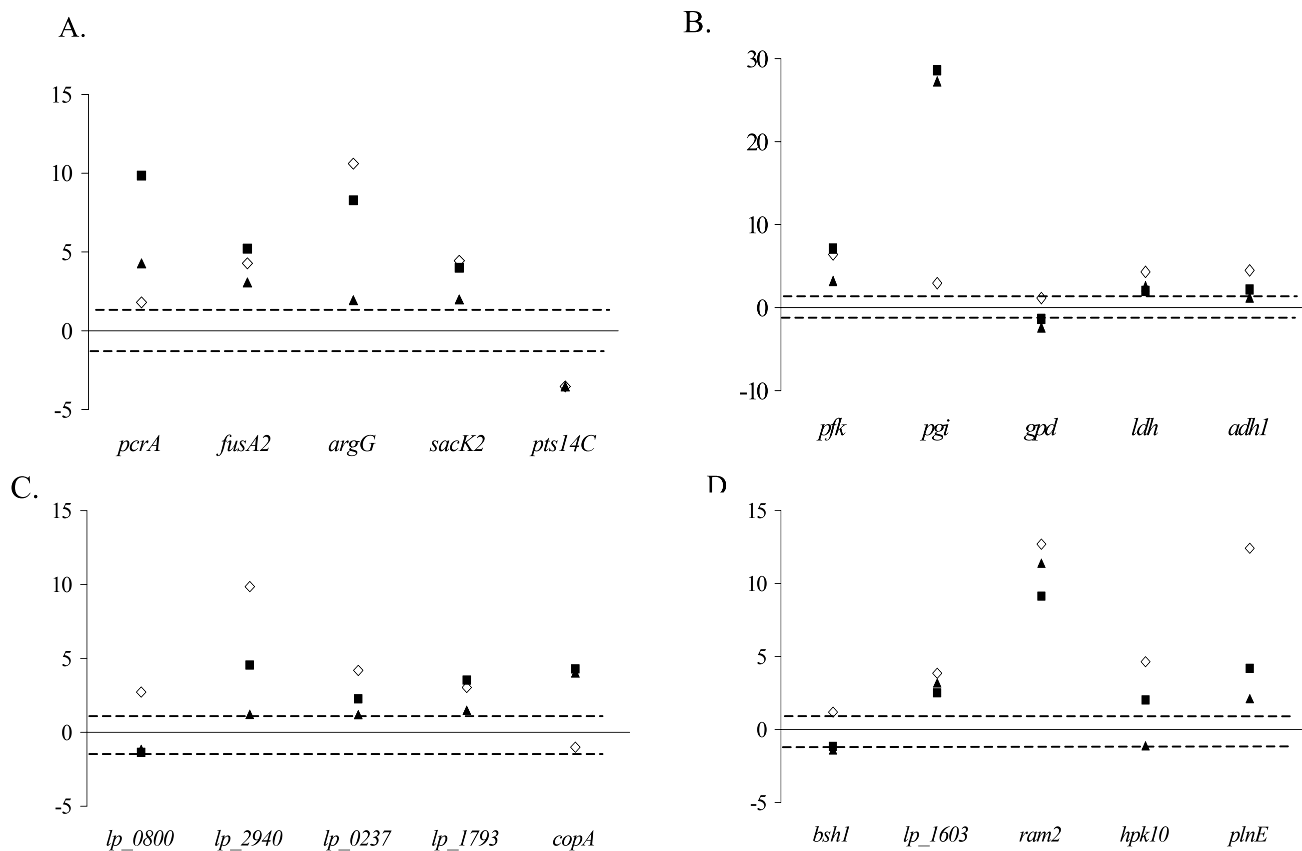

Figure 1. Fold-regulation of different genes of *L. plantarum* in ileostomy effluent samples compared to the ingested cells. A) metabolic functions, B) energy metabolism C) cell wall related genes, and D) genes potentially involved in adaptation of *L. plantarum* to the ileum. Subject A (open diamonds), B (closed squares) and C (closed triangles). The fold change is indicated on the Y-axis. Positioning of individual points between the two dotted lines implies that there was no change in gene expression between the original samples and the ileostoma samples. Note that subject A received a different batch of milk containing *L. plantarum* than subjects B and C.

Increased protein synthesis in *L. plantarum* upon human ingestion was further supported by the 2 to 10 times up-regulation of the argininosuccinate synthase (*argG*) gene, likely involved in arginine synthesis, in the ileostomy effluent samples as compared to the initial cells (Figure 1A). Previously, the *L. plantarum argG* gene has been found to be induced by exposure to bile stress *in vitro* (7), was identified as *in vivo* induced during mouse GI-tract transit (5), and also appeared to be expressed *in vivo* in the human intestine (15). Overall, up-regulation of *fusA2* and *argG* by *L. plantarum* in the ileum indicates the importance of an active amino acid biosynthesis machinery in the human intestine which is likely due to re-initiation of the consumed stationary cells and adaptation to novel conditions (17), possibly including a bile mediated response.

Energy metabolism

The necessary energy for survival of *L. plantarum* in the ileum could be obtained from different sugars and made accessible through the glycolysis or pentose phosphate pathways. Key enzymes in the glycolytic pathway include phosphofructokinase (*pfk*) and glucose-6-phosphate isomerase (*pgi*). qRT-PCR revealed that these genes were 6 and 20 times up regulated in the ileostomy samples on average, respectively (Figure 1B). Expression of the predicted glucose-6-phosphate 1-dehydrogenase (*gpd*) gene, encoding a key enzyme in the pentose-phosphate pathway, appeared to remain constant *in situ*. The large variation between *pgi* relative expression levels observed in different ileostoma samples is most likely due to a higher *pgi* activity in the batch of *L. plantarum* cells ingested by subject A as compared to the batch consumed by subjects B and C (data not shown). Overall, these results indicate that energy is being generated for cellular processes to re-initiate the consumed stationary cells in the ileum.

The glycolytic pathway generates pyruvate as an end-product, which in lactic acid bacteria is typically converted to lactic acid via lactate dehydrogenase (*ldh*). The *ldh* gene expression level appeared 2- to 4- fold higher in the ileostomy effluent samples (Figure 1B) relative to the administered cells. Analogously, the predicted alcohol dehydrogenase gene (*adh1*) that converts acetaldehyde to ethanol, demonstrated either unaltered or 4-fold higher transcript levels *in situ* (Figure 1B). Notably, expression of the *adh1* gene has previously been established in human intestinal biopsy samples (15). Taken together, these observations support re-initiation of *L. plantarum* growth and energy metabolism *in situ* in the human small intestine as compared to *in vitro* grown stationary phase cells.

Metabolism of specific sugars was also followed by focusing on fructokinase (*sacK2*) and a gene of the cellobiose PTS system (*pts14C*). Both genes are associated with the metabolism of specific sugars, and appeared 3 times up- and 4 times down regulated in the ileostomy effluent samples, respectively (Figure 1A). This suggests an altered carbohydrate utilization repertoire of *L. plantarum* in the human ileum as compared to MRS *in vitro* cultures, which might relate to the composition of the flavored milk-drink that contained mainly lactose. Fructokinase gene expression has been previously detected in *L. plantarum* cells associated with human mucosal ileal tissue (15), which is in good agreement with our current findings. The predicted cellobiose PTS, EIIC (*pts14C*) gene, which does not form part of a cellobiose PTS gene cluster on the *L. plantarum* genome, is likely involved in cellobiose

degradation. However, in *Listeria monocytogenes* a role in host-specific signaling has been established for several of the orphan cellobiose PTS-EIIC subunits (26). R-IVET demonstrated induced expression of the *L. plantarum pts14C* gene in the intestine of mice (5). Moreover, the reduced survival in the GI-tract of mice of an *L. plantarum* cellobiose PTS-EIIC (lp_1164)-replacement mutant emphasizes the importance of this gene in the GI-tract (8). Recent data from human ileal biopsy samples that contain *L. plantarum* cells support that lp_1164 is actively expressed in the human small intestine. However, in the ileal effluent samples a 4-fold down-regulation of the gene was demonstrated. The contradicting observations described here, could be related to dietary variations between human subjects or might relate to host-derived individual variations in intestinal environment. Alternatively, since all expression data in the ileal effluent samples are normalized to those of the MRS-grown cells, this could be a result of specific up-regulation of the *pts14C* gene in the stationary phase.

Previous studies have shown that expression of a predicted α-L-rhamnosidase encoding gene (Figure 1D) was induced in the GI-tracts of mice (5). Therefore, the expression level of this gene was investigated in the ileostoma effluent samples used here. Intriguingly, high level expression of this α-L-rhamnosidase gene could be established in the ileostoma effluents, supporting conservation of bacterial responses to intestinal conditions in different host organisms. While rhamnose is an energy-providing carbon source for *L. plantarum*, the source of rhamnose for the transiting *L. plantarum* is unclear. Plant cell walls are enriched for rhamnose. However, only limited amounts of plant material would be expected to be present after overnight fasting before administering the *L. plantarum*-containing drink to the subjects and there would have been insufficient time for components of the lunch to reach the end of the ileum in order to provide plant material. An alternative explanation for this dramatic increase in rhamnosidase gene expression levels could be the influence of rhamnose-containing exopolysaccharides present on the resident GI tract bacteria (19, 47), which will be discussed in more detail below.

Gene expression of cell wall related genes

Several cell surface proteins were tested in the ileostoma samples because expression had been demonstrated in the murine intestine and in a human ileal mucosal biopsy sample as determined with R-IVET and DNA microarrays, respectively (5, 15).

However, of those cell surface proteins, lp_0800 and lp_2940, and the integral membrane protein (lp_0237) demonstrated no substantial variation in gene expression between the original samples and the ileostoma fluid. The large variation in expression of these genes observed in individual subjects suggests a subject-dependent response (Figure 1C), and could possibly explain the differences in the results obtained for the ileostoma samples compared to the ileal mucosal biopsy sample. Interestingly, up to 3 times up-regulation in the ileostomy effluent samples was observed for a protein encoded by gene lp_1793 with an N-terminal sequence homologous to the fibronectin-binding protein family domain (FbpA) (Figure 1C). Previously, a similar domain found in a protein of *Staphylococcus aureus* (23) was found to be involved in binding to host epithelial cells. High transcript levels of this gene were also detected in colonic mucosal biopsies of several patients (15).

The importance of the copper transporting protein (*copA*) gene for *L. plantarum* in the GI-tract was anticipated from previous studies that showed high expression in different mucosal samples from a patient (15), induction in the GI-tract of mice (5), and reduced survival of a *copA*-replacement mutant of *L. plantarum* in the GI-tract of mice (8). The *copA* gene expression was constant between the consumed and ileostoma sample in subject A and 4 times up regulated in both subjects B and C (Figure 1C). As described for *pgi*, the *copA* expression in the initial samples was different which will have influenced the latter results (data not shown). The potential *copA* gene has been suggested to be involved in either copper acquisition or high copper concentration tolerance. Although, the exact function of this gene has not yet been elucidated, involvement in persistence and survival of the GI-tract can not be ruled out (8).

Genes potentially impacting on adaptation to GI-tract

Several genes were expected to be specifically regulated for adaptation of *L. plantarum* to the human intestine either due to their functionality or results of earlier screens. The choloyglycine hydrolase (*bsh1*), which hydrolyses bile salts, demonstrated no modulation (Figure 1C). This is in contrast to earlier findings where this gene was 5-fold up regulated when *L. plantarum* was grown on 0.1% porcine bile salts (9). While bile salt hydrolase activity could be seen as an effective response of a bacterium to decrease the strong acidic properties of bile salts and reduce the energy-burden, the deconjugated counterpart is more toxic (14). The lack of transcriptional regulation observed here could be due to different

properties of human and porcine bile salts or a reduced influence of bile at the end of the ileum.

The hemolysin homolog lp_1603, which has very high homology to a RNA-methylase gene, was 3 times up regulated in the ileostomy effluent samples (Figure 1C). The gene contains an RNA-binding domain, which amongst others is found in stress proteins, as well as an Fts-J like methyltransferase that protects the 23S rRNA upon heat shock (10). The changing conditions throughout the ileum are likely responsible for up-regulation of stress proteins such as lp_1603, as induction of this gene was also seen in a R-IVET screen (5).

The 11 times up-regulation of the expression of the potential α-L-rhamnosidase gene (Figure 1D) could have another influence. Degradation of the exocellular rhamnolipids of other bacteria could be a favorable property, because rhamnolipids of various bacterial species such as *Pseudomonas aeruginosa* (31) and *Burkholderia pseudomallei* have been described as virulence factors with a probable detergent like action. Reduction of the possibilities of other bacteria in the GI-tract would favor growth of *L. plantarum.*

The *Lactobacillus agr*-like module (*lam*) system in *L. plantarum* demonstrates growth-phase dependent gene-expression and influences expression of genes encoding cell-surface proteins and polysaccharides and regulates adherence (45). The histidine kinase gene (*hpk*10) transcript, which is annotated as part of a polycistronic *agr*-operon involved in quorum sensing regulation appeared to be up regulated in two of the three ileostomy effluent samples (Figure 1D). Perhaps the cell density of *L. plantarum* did not exceed the density dependent level for inducing *hpk10* transcription in person C or other environmental factors required to induced this system were absent (33).

Remarkably the putative plantaricin antimicrobial peptide precursor (*plnE*) gene transcript appeared to be present at high levels (2-12 fold higher than control condition) in cells in the ileostoma effluent (Figure 1D). PlnE is expected to form an active bacteriocin together with PlnF (3). The bacteriocin acts on related bacteria including other lactobacilli and pediococci, by forming α-helical pore structures in the membrane inducing a cation flow (3, 32). The production of plantaricin is cell-density dependent and is regulated by an *agr*-like two-component regulator that is activated at high cell densities (18, 38) and by the presence of other Gram-positive bacteria (30). None of the previous *L. plantarum in situ* gene expression studies has revealed an increase in the expression of genes involved in plantaricin production, probably because the experimental set-up was unsuited to detect this (5). Bacterocin

production in the human GI-tract could aid colonization and survival of *L. plantarum* by providing a competitive advantage in this niche.

Conclusions

The present study focused on a specific set of genes in *L. plantarum* WCFS1 to obtain an overview of its activity following ingestion and passage through the upper gastrointestinal tract and the human small intestine. Clearly, the *L. plantarum* cells were metabolically active as indicated by induced expression of genes involved in protein synthesis, an active amino acid biosynthesis machinery, uptake of different sugars and metabolism for energy to eventually lactate and ethanol production. While no differential expression was observed for some cell surface protein genes nor for the bile salt hydrolase gene, a potential adherence protein was up regulated. Induction of the α-L-rhamnosidase (*ram2*) gene and the bacteriocin precursor (*plnE*) gene might suggest possible mechanisms for beneficial targeted GI-tract properties of *L. plantarum*. Highly induced promoters such as those for *ram2* and *plnE* may support application of *L. plantarum* as a vector for therapeutic delivery of molecules with activity targeted to the ileum.

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References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Amman, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

3. **Anderssen, E. L., D. B. Diep, I. F. Nes, V. G. H. Eijsink, and J. Nissen-Meyer.** 1998. Antagonistic activity of *Lactobacillus plantarum* C11: Two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. Appl. Environ. Microbiol. **64:**2269-2272.

4. **Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-1920.

5. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721-5729.

6. **Bron, P. A., S. M. Hoffer, I. I. Van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-317.

7. **Bron, P. A., M. Marco, S. M. Hoffer, E. Van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J. Bacteriol. **186:**7829-7835.

8. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. *In* P. A. Bron (ed.), The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen Centre for Food Sciences, Wageningen.

9. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2005. DNA micro-array based identification of bile-responsive genes in *Lactobacillus plantarum*. J. Appl. Microbiol. in press.

10. **Bugl, H., E. B. Fauman, B. L. Staker, F. Zheng, S. R. Kushner, M. A. Saper, J. C. Bardwell, and U. Jakob.** 2000. RNA methylation under heat shock control. Mol. Cell. **6:**349-360.

11. **Bustin, S. A.** 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. **29:**23-39.

12. **Caruthers, J. M., and D. B. McKay.** 2002. Helicase structure and mechanism. Curr. Opin. Struc. Biol. **12:**123-133.

13. **Cohen, D.** 2004.

14. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol. **79:**292-301.

15. **de Vries, M. C., M. Kleerebezem, P. Mangell, S. Ahrne, M. Douwe, E. E. Vaughan, and W. M. de Vos.** 2005. Transcript profiling reveals global gene expression of *Lactobacillus plantarum* in the human gastrointestinal tract. *In* M. C. de Vries (ed.), Analyzing global gene expression of *Lactobacillus plantarum* in the human gastro-intestinal tract. Wageningen Centre for Food Sciences, Wageningen.

16. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. *Lactobacillus plantarum* - Survival, functional and potential probiotic properties in the human intestinal tract. Int. Dairy J. in press.

17. **de Vries, M. C., E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2004. Optimising single cell activity assessment of *Lactobacillus plantarum* by fluorescent *in situ* hybridisation as affected by growth. J. Microbiol. Methods **59:**109-115.

18. **Diep, D., L. Havarstein, and I. Nes.** 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J. Bacteriol. **178:**4472-4483.

19. **Goh, K. K., D. R. Haisman, and H. Singh.** 2005. Development of an improved procedure for isolation and purification of exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483. Appl. Microbiol. Biotechnol. **67:**202-8.

20. **Granger, K., R. J. Moore, J. K. Davies, J. A. Vaughan, P. L. Stiles, D. J. Stewart, and M. L. Tizard.** 2004. Recovery of *Mycobacterium avium* subspecies *paratuberculosis* from the natural host for the extraction and analysis *in vivo*-derived RNA. J. Microbiol. Methods **57:**241-9.

21. **Hensiek, R., G. Krupp, and E. Stackebrandt.** 1992. Development of diagnostic oligonucleotide probes for four *Lactobacillus* species occuring in the intestinal tract. System. Appl. Microbiol. **1992:**123-28.

22. **Hooper, L. V.** 2004. Bacterial contributions to mammalian gut development. Trends Microbiol. **12:**129-134.

23. **Jonsson, K., C. Signas, H. P. Muller, and M. Lindberg.** 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. Eur. J. Biochem. **202:**1041-1048.

24. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing, Gram-positive rods, p. 1229. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams and Wilkins, Baltimore, U.S.A.

25. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

26. **Kreft, J., and J. A. Vazquez-Boland.** 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. **291:**145-57.

27. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos.** 1993. Characterizion of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis* requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281-291.

28. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods **25:**402-408.

29. **Lovenklev, M., I. Artin, O. Hagberg, E. Borch, E. Holst, and P. Radstrom.** 2004. Quantitative interaction effects of carbon dioxide, sodium chloride, and sodium nitrite on neurotoxin gene expression in nonproteolytic *Clostridium botulinum* type B. Appl. Environ. Microbiol. **70:**2928-2934.

30. **Maldonado, A., R. Jimenez-Diaz, and J. L. Ruiz-Barba.** 2004. Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific gram-positive bacteria is mediated by an autoinduction mechanism. J. Bacteriol. **186:**1556-64.

31. **McClure, C. D., and N. L. Schiller.** 1992. Effects of *Pseudomonas aeruginosa* rhamnolipids on human monocyte-derived macrophages. J. Leukoc. Biol. **51:**97-102.

32. **Moll, G. N., E. van den Akker, H. H. Hauge, J. Nissen-Meyer, I. F. Nes, W. N. Konings, and A. J. Driessen.** 1999. Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. J. Bacteriol. **181:**4848-52.

33. **Novick, R. P., and D. Jiang.** 2003. The staphylococcal *saeRS* system coordinates environmental signals with *agr* quorum sensing. Microbiology **149:**2709-2717.

34. **Oozeer, R., J. P. Furet, N. Goupil-Feuillerat, J. Anba, J. Mengaud, and G. Corthier.** 2005. Differential activities of four *Lactobacillus casei* promoters during bacterial transit through the gastrointestinal tracts of human-microbiota-associated mice. Appl. Environ. Microbiol. **71:**1356-1363.

35. **Oozeer, R., N. Goupil-Feuillerat, C. A. Alpert, M. van de Guchte, J. Anba, J. Mengaud, and G. Corthier.** 2002. *Lactobacillus casei* is able to survive and initiate protein synthesis during its transit in the digestive tract of human flora-associated mice. Appl. Environ. Microbiol. **68:**3570-3574.

36. **Pieterse, B., R. H. Jellema, and M. J. van der Werf.** 2005. Quenching of microbial samples for increased reliability of microarray data. J. Microbiol. Methods.in press

37. **Reuter, G.** 2001. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol **2:**43-53.

38. **Risøen, P. A., L. S. Håvarstein, D. B. Diep, and I. F. Nes.** 1998. Identification of the DNA-binding sites for two response regulators involved in control of bacteriocin synthesis in *Lactobacillus plantarum* C11. Mol. Genet. Genomics **259:**224-232.

39. **Rodnina, M. V., A. Savelsbergh, and W. Wintermeyer.** 1999. Dynamics of translation on the ribosome: molecular mechanics of translocation. FEMS Microbiol. Rev. **23:**317-333.

40. **Rokbi, B., D. Seguin, B. Guy, V. Mazarin, E. Vidor, F. Mion, M. Cadoz, and M.J. Quentin-Millet.** 2001. Assessment of *Helicobacter pylori* gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. Infect. Immun. **69:**4759-4766.

41. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107-133.

42. **Sharkey, F. H., I. M. Banat, and R. Marchant.** 2004. Detection and quantification of gene expression in environmental bacteriology. Appl. Environ. Microbiol. **70:**3795-3806.

43. **Sonnenburg, J. L., L. T. Angenent, and J. I. Gordon.** 2004. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol. **5:**569-573.

44. **Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon.** 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc. Natl. Acad. Sci. U. S. A. **99:**15451-15455.

45. **Sturme, M. H. J., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. An *agr*-like two component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J. Bacteriol. **187:** 5224-5235.

46. **Takesue, Y., Y. Sakashita, S. Akagi, Y. Murakami, H. Ohge, Y. Imamura, Y. Horikawa, and T. Yokoyama.** 2001. Gut transit time after ileal pouch-anal anastomosis using a radiopaque marker. Dis. Colon. Rectum. **44:**1808-1813.

47. **Tallon, R., P. Bressollier, and M. C. Urdaci.** 2003. Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. Res. Microbiol. **154:**705-12.

48. **Tannock, G. W.** 1995. Normal microflora: An introduction to microbes inhabiting the human body. Chapman & Hall, London.

49. **Vaughan, E. E., H. G. Heilig, K. Ben-Amor, and W. M. de Vos.** 2005. Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol. Rev. **29:**477-90.

50. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-828.

51. **Walter, J., N. C. K. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. **69:**2044-2051.

52. **Wilson, K. S., and H. F. Noller.** 1998. Molecular movement inside the translational engine. Cell **92:**337-349.

Chapter 7

General Discussion and Future Perspectives

Introduction

The human gastrointestinal (GI)-tract represents a dynamic ecosystem comprising various habitats each with a niche-specific microbiota. The Gram-positive lactic acid bacteria (LAB) are considered to constitute a significant part of the microbiota in the upper GI-tract and to be involved in the health-stimulating processes within the host (9, 41, 44). Despite a widespread commercial interest, the mechanisms underlying the health-stimulating properties of LAB are scarcely known. The objective of the research described in this thesis was to elucidate the response of intestinal species of LAB from human origin during transit of the human GI-tract.

Lactobacillus plantarum is a natural inhabitant of the human GI-tract, and, due to its versatile nature, can be found in a range of other habitats as well such as plant material, especially fermented sauerkraut and olives (1, 11, 23, 37). The complete genome sequence of *L. plantarum* WCFS1, a human isolate, has been determined (25), which facilitates the use of this strain as model to study its behaviour upon intestinal transit with molecular techniques including global transcript profiling and quantitative reverse transcriptase PCR (qRT-PCR). The results of these studies are discussed here in the perspective of recent literature, and attempts are made to provide an overall picture of the behaviour of *L. plantarum* upon transit of the GI-tract.

Ribosomal RNA characterisation and activity estimation

Many methods to detect, classify, and to estimate general activity of microbes in complex ecosystems are targeting ribosomal RNA (rRNA) and their coding genes as these show an universal distribution and high nucleotide sequence conservation. In a study aimed to provide basic information on these stable RNA genes (**Chapter 2**), the location, sequence, organisation, and regulation of the rRNA operons of the LAB *L. plantarum, L. johnsonii* and *Lactococcus lactis* were compared based on their complete genome sequences. While the rRNA operons per genome vary in number, they all share a common operon organisation with order 5’-16S-23S-5S-3’. Some micro-heterogeneity was observed within the rRNA structural genes as were differences in the number, location and specificity of the tRNA genes. Between the rRNA promoter regions of *L. plantarum* and *Lactococcus lactis* marked differences were found, while the promoter regions of *L. johnsonii* showed a similar promoter structure in all operons. Although the five *L. plantarum* rRNA promoters were found to be significantly

different in their sequence, their activity was very similar under the circumstances tested. A differential expression over time was demonstrated for all operons. Similar observations were recently reported for the rRNA promoters of *Mycobacterium fortuitum* and *M. chelonae* that not only showed a differential expression over time but also an operon-specific expression (30). Basic information on the sequence, expression and regulation of rRNA and tRNA genes within one strain can improve the use of stable RNA-based molecular techniques by more specific primer development and more precise activity assessment. The analysis of the *L. plantarum* RNA complement was instrumental for the development of 16S rRNA PCR-primers and fluorescent *in situ* hybridisation (FISH)-probes that were used for the research described in this thesis.

The feasibility of using an rRNA-based FISH approach to estimate the overall *in situ* activity of *L. plantarum* in the human GI-tract was assessed (**Chapter 3**). FISH could be used to estimate the *in situ* activity of *L. plantarum* WCFS1 in exponentially growing cells. However, *L. plantarum* is capable of growth to very high cell densities up to 10^{10} cells per ml in a rich medium and the properties of its cell-envelope prevented effective entry of the fluorescent oligonucleotide probe into the cells at later stages of growth at high densities. In the GI-tract, the growth phase of *L. plantarum* is not known and possibly differs between cells. It may be influenced by the way *L. plantarum* is ingested and its location in the GI-tract, but also host-factors and diet will play a role. While the developed method could be applied for in detection of *L. plantarum* in intestinal samples, this excluded FISH as a functional method to assess the overall *in situ* activity. Since at that time the analysis of *L. plantarum* using functional and comparative genomics started, further research activities focused on the specific gene expression in the human GI-tract using DNA microarrays and qRT-PCR.

Models and method development for human intestinal samples

Different models can be applied to investigate gene expression of a commensal bacterium in the intestine, including *ex vivo* and *in vitro* methods, animal models, and human samples. Advantages of cell lines and animal models are the larger availability of samples and the accessibility of the models; samples are easy to obtain; and, in animals, samples can be taken from all the different locations of the intestine. In addition, variables, including food intake, age, and genetic variation, are easier kept constant in animal models. Testing of genetically modified or pathogenic bacteria has so far been restricted to animals. However,

one of the disadvantages of *in vitro* and animal models is that bacterial gene expression may differ from the human situation (22, 32). Consequently, in order to complement work performed on animals, we developed a method to analyze the *in vivo* global gene expression of *L. plantarum* in the complex background of the human GI-tract. We applied this method to patient material, namely human mucosal biopsies of colon cancer patients (**Chapter 4**) and to healthy individuals using perfusion samples (**Chapter 5**) and ileostoma effluent (**Chapter 6)** (Table 1). The studies and their most prominent results focusing on different locations of the GI-tract are summarized below.

Table 1. Difference between the method to assess gene expression in the biopsy, perfusion and ileostomy samples

	Biopsy samples	**Perfusion samples**	**Ileostoma effluent**
***L. plantarum* strain**	299v	WCFS1	WCFS1
Dose	10^{11} viable cells	$6x10^{11}$ viable cells	10^{10} viable cells
Intake	Daily 1 week	Via perfusion solution	Single dose
Location in intestine	Colon and ileum	Proximal small intestine	Lower ileum
Position in intestine	Associated with mucosa	Luminal content	Luminal content
Time interaction	Days	About 10 minutes	4-5 hours
Contact stomach	Yes	No	Yes
DNA microarray	Yes	Yes	No
QRT-PCR	Yes	Yes	Yes
Number of genes examined	2503	2784	21
Results compared to	DNA	Expression in initial cells	Expression in initial cells

In an approach aimed to analyse gene expression in the human GI-tract (**Chapter 4**; Table 1), three patients diagnosed with colon cancer ingested prior to surgery a fermented oatmeal drink with *L. plantarum*, and a fourth patient consumed a placebo without bacteria. The used *L. plantarum* strain 299v is closely related to *L. plantarum* WCFS1 and commercially available as probiotic in a fruit drink (**Chapter 1**). The global transcriptional response of *L. plantarum* cells associated with the human mucosa or mucus layer was determined using DNA microarrays in colonic biopsies of three subjects and in an ileal and colonic biopsy within a single individual. The relatively low amount of *L. plantarum* 299v RNA in the total isolated RNA made it necessary to develop a novel way of analyzing the

results; only signals on the DNA microarray above the detection limit were considered positive. Consequently, in most cases detection of such signal was the result of high expression of the gene. However, factors such as turnover time and stability of the specific mRNA, or the efficiency of the cDNA hybridization could also influence signal strength.

The perfusion technique has been successfully applied to the small intestinal tract for various studies including determining intestinal absorption or efflux of water, the uptake of drugs, and iron-induced intestinal oxidative stress (31, 42). In a study addressing gene expression in the upper GI-tract (**Chapter 5**; Table 1), we applied the perfusion technique to evaluate the effect of passage of the small intestine of *L. plantarum* in healthy volunteers. This involved orogastrically positioning of a double-lumen perfusion tube into the proximal small intestine of the volunteers. Via this tube a cell suspension containing *L. plantarum* cells was injected after the stomach and collected in the jejunum. The global transcriptional response of *L. plantarum* was determined with RNA isolated from the perfusion suspension using DNA microarrays, and was compared to gene expression of cells before perfusion and to cells under similar experimental conditions except passage of the small intestine.

In another study, the attention was focused on the transcriptional response of *L. plantarum* in the lower ileum, a site in the GI-tract that is not amenable to analysis in healthy individuals (**Chapter 6**; Table 1). Hence use was made of subjects with an ileostoma who consumed a single dose of *L. plantarum* WCFS1 cells in a milk drink and gene expression was assessed by qRT-PCR of recovered RNA. As the *L. plantarum* WCFS1inoculum was diluted in the ileostomy samples due to consumption of food and intestinal passage, only a low amount of RNA could be recovered. While, this amount of RNA was too little for adequate global transcript analysis by DNA microarrays, it was sufficient for directed gene expression analysis by qRT-PCR. Gene expression before and after consumption was compared. Genes to be analyzed by qRT-PCR included: (1) genes demonstrating specific gene expression of *L. plantarum* in the intestine of humans and mice in earlier studies (2) general metabolic genes indicative of metabolic and energy generating activities of *L. plantarum*; and (3) anticipated functionality in the small intestinal environment.

All the applied methods described in this thesis viewed gene expression of *L. plantarum* in the human GI-tract from a different perspective. In the following sections, a comparison is made between the results obtained from the different sampling locations, analytic approaches and interventions. The intestinal gene expression of *L. plantarum* cells

will be discussed with specific attention for their location at different parts of the GI-tract, luminal or mucosa-association, or subject-dependent variation. In addition, a comparison between *L. plantarum* gene expression in the human studies and animal models is performed.

Intestine-specific gene expression of L. plantarum

Several groups of genes can be expected to behave differently in the intestine than in a culturing medium. The expected adaptation of *L. plantarum*-cells to the human intestine could involve genes belonging to different functional categories, including nutrient uptake and processing, both of sugars and amino acids, because of the different availability in the culturing medium and in the intestine. In addition, changes in the cell envelope can be expected as adaptation to the relatively hostile conditions that are met during intestinal passage, including gastric acid, and bile stress. Alternatively, cells may secrete molecules that interact with the host cells or the myriad of other microorganisms present in the intestine. These include cell wall components, cell envelope and other secreted proteins, or signalling and other functional peptides. In this context it is important to note that the *L. plantarum* genome predicts the presence of more than 200 secreted proteins (25). To coordinate all this, changes in regulatory systems are to be expected. However, it is known that the expression of bacterial regulator genes may be only slightly regulated if at all, since in many cases control is extorted by effector molecules. Hence, notably changes in the expression of structural genes are to be expected and these have been demonstrated to occur as described in this thesis.

Significant expression of a specific gene in all biopsy, perfusion and ileostomy samples would suggest that expression of that gene is specific for the (human) intestine. However, none of the examined genes demonstrated this behaviour. This may be due to the differences between the applied methods (Table 1), including the different methods of data generation and analysis. The glucose used in the perfusion study made comparison of the sugar metabolism impossible.

When the comparison is limited to genes that are significantly expressed in two or more intestinal samples and are up regulated in the perfusion samples and are, if determined, up regulated in two or more ileostomy samples, a total of 46 genes are recognized (Table 2). Those genes were mainly involved in protein synthesis, including ribosomal proteins and tRNA ligases, in transport and binding proteins, including a predicted amino acid ABC-transporter and a sugar transporting protein, and in the cell envelope, including several

Table 2. Genes expressed in three or more human screens, excluding the hypothetical genes

Functional class	**Product**	**Gene**
Biosynthesis of cofactors, prosthetic groups, and carriers	2-dehydropantoate 2-reductase	lp_2788
Cell envelope	lipoprotein precursor	lp_1070
	extracellular protein	lp_1450
	glycosyltransferase	lp_1524
	UDP-N-acetylmuramoylalanine--D-glutamate ligase	lp_2197
	phospho-N-acetylmuramoyl-pentapeptide-transferase	lp_2199
	extracellular protein	lp_3412
Cellular processes	small heat shock protein	lp_0129
	alkaline shock protein	lp_0930
	cell division protein (putative)	lp_2191-2
	cell division protein FtsZ	lp_2193
DNA metabolism	DNA gyrase, B subunit	lp_0006
	DNA-3-methyladenine glycosylase I	lp_3293
Energy metabolism	pyruvate dehydrogenase complex, E3 and E2 component;	lp_2151-2
Fatty acid and phospholipid metabolism	glycerol-3-phosphate dehydrogenase	lp_0371
Protein synthesis	pseudouridylate synthase	lp_0963
	translation initiation factor IF-3	lp_1515
	ribosomal protein L35, L20	lp_1516-7
	phenylalanine--tRNA ligase, β chain	lp_1559
	ribosomal protein L28	lp_1624
	glycine--tRNA ligase, α chain	lp_1965
Purines, pyrimidines, nucleosides and nucleotides	orotidine-5'-phosphate decarboxylase	lp_2698
	dihydroorotate oxidase	lp_2699
	phosphoribosylformylglycinamidine cyclo-ligase	lp_2722
	amidophosphoribosyltransferase precursor	lp_2723
	phosphoribosylformylglycinamidine synthase II	lp_2724
Transcription	RNA methyltransferase	lp_1151
Transport and binding proteins	glycine betaine/carnitine/choline ABC transporter	lp_0367-8
	glutamine ABC transporter, substrate binding protein	lp_0881
	amino acid ABC transporter	lp_1744-5
	purine transport protein	lp_2710
	copper transporting ATPase	lp_3055
	cadmium-/zinc-/cobalt- transporting ATPase	lp_3327
	sugar transport protein	lp_3596

predicted secreted proteins. This indicates that *L. plantarum* is metabolically active in all subjects and adapting to the environment. A similar up-regulation of the genes encoding protein synthesis was demonstrated for *Campylobacter jejuni* in a rabbit ileal loop model (39), suggesting this is a general feature of bacteria active in the intestine.

An interesting transport gene to mention is the putative copper transporting ATPase, encoded by the *L. plantarum copA* gene, which was found to be expressed or induced in one ileal and one colonic biopsy sample, all perfusion samples and two of the three-ileostomy samples. This copper transporting protein is suggested to be involved in either copper acquisition or high copper concentration tolerance (19). A possibility that cannot be excluded is that the *L. plantarum copA* gene is involved in the transport of a compound - other than copper - that is essential for survival or persistence in the intestine. Remarkably, the *copA* gene was also found to be specifically induced in the intestine of mice (6). This would point to a conserved response of *L. plantarum* in mouse and man. The insertional inactivation of the *copA* gene was subsequently found to reduce the competition of the resulting strain in a mouse model, indicating the impact of the *copA* gene (5)). However, the exact mechanism by which *copA* mediates the survival or persistence in mice has not yet been elucidated.

Subject-specific gene expression of L. plantarum

Several *L. plantarum* genes were found to be differentially expressed in the different subjects. This subject-specific gene expression of *L. plantarum* was demonstrated for genes that were expressed in only one of the three individuals of which a biopsy sample was examined (**Chapter 4**). In addition, genes differentially regulated between four subjects during the perfusion process (**Chapter 5**) and, if applicable, differentially regulated between three ileostoma samples (**Chapter 6**) demonstrated subject-dependent gene expression. Apart from trivial causes, such as different sampling locations and approaches, this is to be expected as the intestinal response of *L. plantarum* is the result of a complex sequence of events subject to biological variation. Moreover, the selected individuals have different genetic background, different physiology, including the amount of gastric acid production, bowel movement, differences in mucus, and differences in microbiota (46, 47). Finally, diet, age or sex was not standardized between the selected subjects. As a consequence, the genes that are expressed differentially in the various subjects are predicted to code for proteins belonging to many functional categories (Figure 1). The differences in expression of genes annotated to encode

transport and binding proteins were essentially involved in sugar uptake, including PTS-systems for cellobiose, sucrose and trehalose. This suggests that availability of sugars in the human GI-tract of the various individuals was different. In addition, subject-dependent expression of genes predicted to encode transformation of pyruvate to acetyl-CoA, including the formate C-acetyltransferase, pyruvate dehydrogenase complex, and the pyruvate carboxylase genes was observed, possibly reflecting different oxygen concentrations that are known to affect pyruvate catabolism in lactic acid bacteria (10).

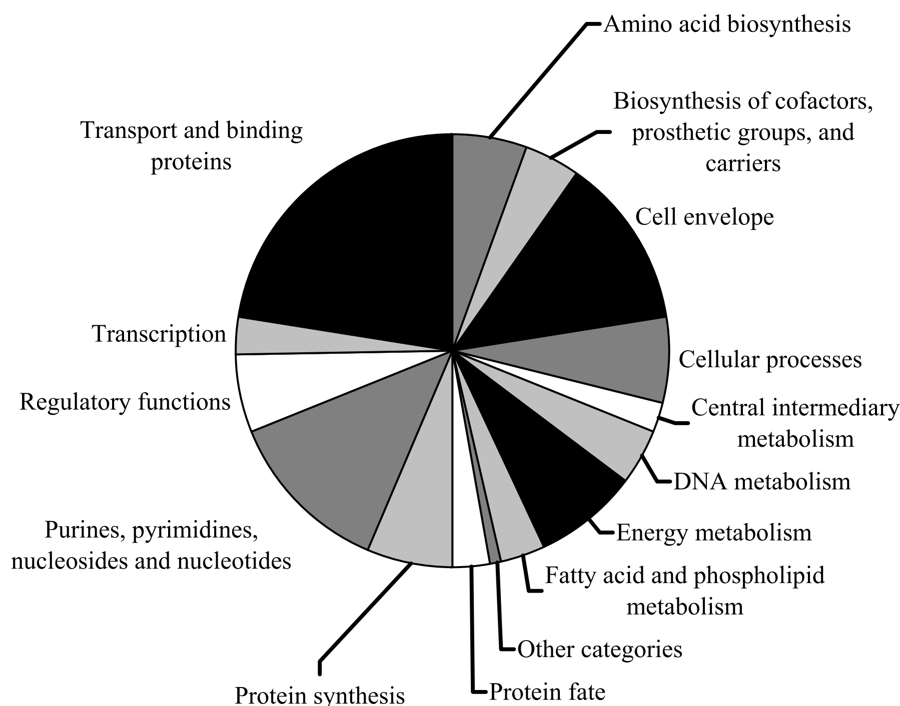

Figure 1. Subject-dependent regulated *L. plantarum* genes divided in functional categories.

For the cell envelope, specific adaptation to the different subjects could be concluded from different expression of the genes predicted to be involved in fatty acid and teichoic acid biosynthesis, and in cell envelope-located or other extracellular proteins. This was observed in all human samples for *L. plantarum*, and was also reported for *Campylobacter jejuni* in five different rabbits using a rabbit ileal loop method (39). It demonstrates that adaptation of the cell envelope is not only a general reaction of *L. plantarum* to the GI-tract, but is adjusted to the specific subject-dependent intestinal environment.

Differences in gene expression in ileum versus colon

From one individual that had consumed *L. plantarum*, biopsies of both ileum and colon were obtained (**Chapter 4**). The availability of these samples provided the unique opportunity to compare gene expression at the same time in *L. plantarum* present at those different locations without the bias that is observed when different individuals are compared (see above). This comparison provided unprecedented insight in the spatial expression of *L. plantarum*. Apart from this clean and bias-free case, all other comparisons that address differences in *L. plantarum* expression at different locations, such as comparisons between biopsy, the perfusion (**Chapter 5**) and, if applicable, ileostomy samples (**Chapter 6**), are very difficult. This can be attributed to the differences in experimental set-up (Table 1), including the different ways of data generation and analysis and the glucose used during perfusion that prevented comparison of the sugar metabolism. In addition, in the colon only *L. plantarum* cells associated with the intestinal cell wall were investigated, while in the ileum both associated and luminal cells were assessed. The absence of contact with the stomach during perfusion most probably influences gene expression, and will provide another vision on gene expression in the small intestine than in the ileal biopsy samples and in ileostoma effluent. Furthermore, the low number of genes detected in all ileal samples (6 in total), made comparison between ileum and colon complicated.

Most genes detected in the ileum were predicted to encode proteins secreted via a sec-dependent pathway, including two extracellular proteins and two cell surface protein precursors. In the colon, a large part of the expressed genes were annotated to be involved in transport and binding, including ABC-transporters and different PTS systems including fructose and galactitol, as well as in protein fate, including genes encoding peptidases, and protein synthesis. This suggests that in the ileum *L. plantarum* genes are expressed that are involved in interactions with the host or other microbes, while in the colon notably genes involved in the uptake of different components are expressed.

Interestingly, the gene encoding a 360-residue extracellular protein annotated as the putative competence protein Lp_2226 was expressed in all colonic biopsy samples but not in any of the ileal samples. The significance of this is not known, since the set of competence genes in *L. plantarum* WCFS1 appears incomplete in the genome sequence so it seems unlikely that it could become naturally competent. As the annotated function of this gene is rather speculative, a putative other function could be the scavenging of nucleic acids.

Differences between luminal and associated L. plantarum cells

A comparison was performed to examine which genes were significantly expressed or up regulated in *L. plantarum* when associated with the intestinal mucosa or mucus as opposed to the cells present in the lumen. To achieve this, gene expression of *L. plantarum* cells isolated from the intestinal mucosa or mucus using biopsy samples (**Chapter 4**) was compared to the up regulated genes found for the cells that were perfused (**Chapter 5**) as well as the cells that were isolated from the ileostomy fluid (**Chapter 6**). As described above the *L. plantarum* cells associated with the intestinal mucosa were only available for the colon, while both associated and luminal cells were obtained and assessed for the ileum. Another factor to take into account is that during perfusion the *L. plantarum* cells have not been in contact with the stomach, and although *L. plantarum* can survive gastric acid (14), it most probably influences gene expression.

The *L. plantarum* cells associated with the intestinal mucosa demonstrated a high expression of the gene annotated to encode glycogen phosphorylase, indicating uptake of starch. In addition, expression of the predicted alcohol dehydrogenase gene, involved in ethanol production and the predicted pyruvate carboxylase gene for the production of oxaloacetate was demonstrated in the associated cells.

Next to genes predicted to encode glycolytic enzymes, including the 6-phosphofructokinase gene, several genes predicted to encode enzymes for the pentose phosphate (pentose-P) pathway were detected in the associated cells, including glucose-6 phosphate-1-dehydrogenase, the key enzyme of the pentose-P pathway and a predicted transketolase gene. More ATP is formed from the same amount of sugar during glycolysis than in the pentose-P pathway. The main functions of the pentose-P pathway, which reduces C5 sugars, are to create energy and essential precursors such as ribose-phosphate for nucleotide formation, and generate reducing equivalents such as NADPH, to use for synthetic purposes (3). The detection of the expression of genes involved in both pathways in the associated cells indicates that, in addition to energy, *L. plantarum* requires reducing agents and building blocks for nucleic acids or has the need to reduce C5 sugars. The latter could be caused by a reduced access to C6 sugars, which could already be consumed in the lumen. In contrast, in the luminal cells *L. plantarum* genes were expressed that are annotated to encode the pyruvate dehydrogenase complex, indicating an active glycolysis and the use of pyruvate for the production of acetyl-CoA, but no pentose-P pathway.

In the associated cells, acetyl-CoA may be utilized to form fatty acids as indicated by expression of the predicted acetyl-CoA carboxylase gene. These fatty acids may be used to reinforce the cell wall of associated *L. plantarum* cells to enhance long-term survival of the cells in the mucus.

Many differences were found between associated and luminal cells for the use of amino acids. In the luminal cells expression of the tRNA-ligase genes for isoleucine, histidine and aspartate was observed, whereas in the attached cells tRNA-ligase expression for glutamate, argentine, serine, lysine, cysteine, tyrosine and alanine was found. This suggests a different availability or demand of amino acids in the lumen compared to the associated cells, which could be expected from the different microbiota present in the different niches, consuming and producing different substances. In addition, the presence of mucus can shield off substances from the lumen and visa versa.

Comparison to genes regulated in a *lamA*-mutant, in which biofilm formation is decreased, demonstrates a typical behaviour (40). Up or down regulated genes were only found in the biopsy samples. None of the genes regulated during perfusion or in the ileostoma samples were influenced in the *lamA*-mutant. This indicates that only associated cells express genes involved in biofilm formation, suggesting biofilm formation on the intestinal mucosa or mucus.

Similarities between microbial gene expression in animals and in humans

In an attempt to validate the animal models, a comparison between various reported animal- and human trials is made here. Even though the approaches for investigating differential gene expression in the GI-tract were different, as were the hosts, mouse versus human, approximately half (46%) of the *L. plantarum* genes induced in the mouse GI-tract, as assessed by R-IVET, are expressed in the human microarray experiments on biopsies (4)(**Chapter 4;** Figure 2). In both the ileum and colon of subject A, 18 and 19 of the R-IVET genes (26 and 25%) respectively are substantially expressed while for subjects B and C these are only 10 and 14 (14 and 20%), respectively (Figure 2A, B, C, and D). For the three perfusion samples there are 6 of the 69 genes detected with R-IVET similar (8.7%) in genes detected with the R-IVET method (Figure 2E) of which, as expected, all are up regulated (**Chapter 5**). For the ileostomy samples the similarity is as high as 50% (up-regulation of 5 of

the 10 genes determined in the ileostomy samples) (Figure 2F), but those genes were selected amongst other reasons for their expression in the R-IVET screen (**Chapter 6**). Only few of the genes differentially regulated between subjects were also found in mice using the R-IVET approach, namely genes predicted to encode a sucrose PTS, EIIBCA, a multidrug transport protein, and a gene involved in biotin synthesis. This suggests that genes detected with R-IVET demonstrated a general, subject independent, adaptation to the GI-tract. The genes responding similarly in the different screens and hosts are presented in Table 3. Those genes were mainly involved in nutrient acquisition and synthesis, stress, and extracellular functions. This is interesting because it indicates that the GI-tract conditions of mouse and man have similar effects on ingested *L. plantarum* expression.

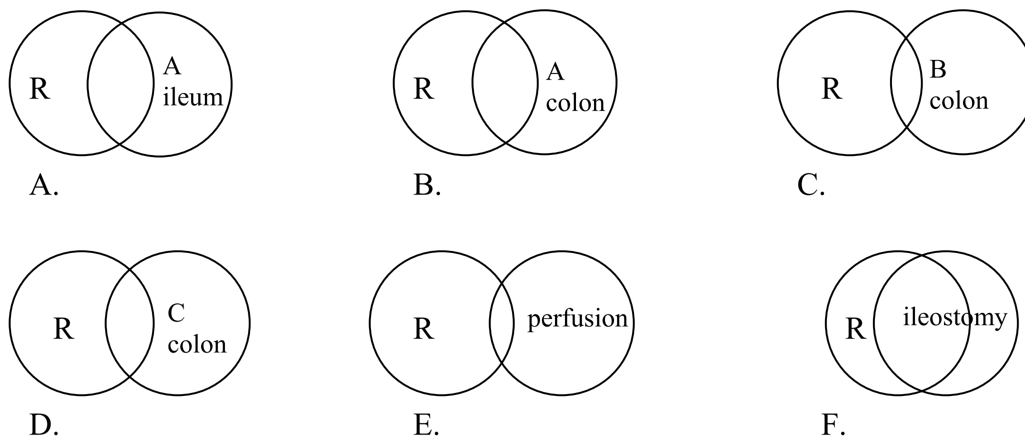

Figure 2. Venn diagrams of the overlap between the genes induced in the GI-tract of mice as determined with R-IVET (R) and A. genes expressed in the biopsy of ileal subject A; B. genes expressed in the biopsy of colonic subject A; C. genes expressed in the biopsy of colonic subject B; D. genes expressed in the biopsy of colonic subject C; E. genes up regulated during perfusion in the human small intestine and F. genes up regulated in ileostomy patients.

Genes detected in R-IVET and three or more of the other screens included: argininosuccinate synthase, involved in amino acid biosynthesis of the glutamate family; cell surface protein precursor lp_0800; glycine cleavage system, h. protein, to use fatty acids as an energy source (45); copper transporting ATPase; and a orphan cellobiose PTS, EIIC gene (Table 3). The argininosuccinate synthase, copper transporting ATPase, and orphan cellobiose PTS, EIIC genes have been described in interactions of a pathogenic bacterium with a host (6, 12, 19, 24, 29). This indicates the importance of those genes for survival in the GI-tract rather

than pathogenicity. In addition, the present human studies highlight the application potential of these genes as their promoters could be applied to drive expression of a functional antigen or other substance specifically in the GI-tract.

Table 3. R-IVET genes expressed in human screens

Functional class	Product	Gene	Ileal biopsy[1]	Colonic biopsy[1]	Perfusion[2]	Ileostomy[2]
Amino acid biosynthesis	argininosuccinate synthase	lp_0775	x	xx		+
Biosynthesis of cofactors	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor	lp_0854		x		
	bifunctional protein: riboflavin kinase; FMN adenylyltransferase	lp_2031	x	xx		
Cell envelope	extracellular protein	lp_0141	x	x		
	cell surface protein precursor	lp_0800	x	xx		0
	cell surface protein, ErfK family	lp_1403		xx		
	cell surface protein precursor	lp_2940	x			0
Cellular processes	immunity protein PlnI, membrane-bound protease CAAX family	lp_0419		x		
	hemolysin homolog	lp_1603		x		+
Central intermediary metabolism	galactoside O-acetyltransferase	lp_0393		x		
	α-L-rhamnosidase (putative)	lp_3473		xx		+
DNA metabolism	DNA-directed DNA polymerase III, gamma/tau subunit	lp_0698	x	xxx		
Energy metabolism	glycine cleavage system, h. protein	lp_0305	x	xx	+	
	6-phospho-β-glucosidase	lp_3526		x		
	bifunctional protein: alcohol and acetaldehyde dehydrogenase	lp_3662		x		
Fatty acid and phospholipid metabolism	geranyltranstransferase	lp_1602		x		
Other categories	integrase/recombinase	lp_1874			+	
Protein fate	dipeptidase	lp_0228	x	xx		
	serine/threonine protein kinase	lp_3176		x		
Protein synthesis	pseudouridylate synthase	lp_1319	x			
Regulatory functions	transcription regulator	lp_3646	x			
Transport and binding proteins	sucrose PTS, EIIBCA	lp_0185		x		
	transport protein	lp_0394		xx		

Functional class	Product	Gene	Ileal biopsy[1]	Colonic biopsy[1]	Perfusion[2]	Ileostomy[2]
Transport and binding proteins	cellobiose PTS, EIIC	lp_1164	x	xx		+
	copper transporting ATPase	lp_3055	x	x	+	0[3]
	transport protein	lp_3281	x			
Hypothetical proteins	cation efflux protein	lp_3288	x		+	
	multidrug transport protein	lp_3303	x			
	No product defined	lp_0139	x	xxx		
	oxidoreductase	lp_0190	x			
	No product defined	lp_2713		x		
	No product defined	lp_3057			+	
	secreted protein (putative)	lp_3058		x	+	
	esterase (putative)	lp_3312	x	x		

[1]Genes expressed in one (x), two (xx) or three (xxx) colonic biopsy samples; [2] Genes up regulated (+), down regulated (-), detected, but not differentially regulated (0); [3] Levels of gene expression differed between the initial cells

An experiment in mono-associated mice with *Bacteroides thetaiotaomicon* demonstrated an increase in expression of genes involved in carbohydrate transport and metabolism and a decrease in amino acid transport and metabolism compared to *in vitro* grown cultures (36). This experiment is difficult to compare to the experiments discussed here, because in the most comparable experiment, the perfusion experiment, glucose is added and, consequently, the carbohydrate metabolism cannot be compared. In the different experiments in humans, we determined differences in amino acid metabolism between initial cells and cells after perfusion or from the ileostoma. However, in the mouse experiment most genes were down regulated as in the human experiments most genes were up regulated. This could be a difference between the hosts, but more likely it is caused by the experimental set-up. Large shifts in expression of genes encoding extracellular proteins were demonstrated both in the *B. thetaiotaomicon*-colonization study and in the human studies described here.

Some striking similarities were observed between gene expression of *L. plantarum* in the human perfusion model and *Campylobacter jejuni* in the rabbit intestinal loop model (39). The genes involved in stress, like heat shock proteins and a multidrug efflux pump possibly contributing to bile resistance, were up regulated both in the rabbit intestinal loop model and the human perfusion model. Genes involved in peptidoglycan biosynthesis were subject-

dependent regulated both in humans and in rabbits, indicating similarities between gene expression in the intestine of different bacteria and different hosts.

Future perspectives

The commensal microbes in the GI-tract influence the host in various ways, including in nutrient conversion and uptake, modulation of host immunity, angiogenesis and intestinal epithelial development, activity and homeostasis (2, 20, 28, 34, 38). A great variety of mechanisms are known by which the microbes interact with the host. One way for interaction between host and microbes is recognition of the commensal microbiota by Toll-like receptors (TLRs) which establishes intestinal homeostasis (34). A reduction in the D-alanine content of the lipoteichoic acids on the *L. plantarum* cell envelope by deletion of the *dlt*-gene induces a TLR2-dependent reduction in the secretion of proinflammatory cytokines, including IL-12 and TNF (8, 17). Communication has also been established through specific chemical signals as determined for *Bacteroides thetaiotaomicron* via L-fuculose (21). The latter could play a role in host-microbe communication either by interacting directly with the intestinal epithelium, or by becoming a substrate or inducer for another metabolic pathway that produces a signalling molecule (21). Probably, more forms of interaction will be present in the GI-tract.

To understand the communication thoroughly, the global gene expression of both the host and the bacterial side need to be characterized. In combination with the analysis of the impact of the bacterium on the host, the approaches described in this thesis can provide a basis for understanding on how host and microbes influence each other in health and disease. This could be applied in various ways. An obvious one is by influencing the microbiota through food, for example via consumption of prebiotics, or by the addition of bacteria with the desired properties, such as probiotics. Selection of prebiotics or other food components can be targeted more selectively to stimulate a certain (group of) bacteria. Selection of probiotics include, until now, survival of the gastrointestinal tract, persistence in the host, and proven safety for human consumption (7, 43). Recently, several potential mechanisms have been established *in vitro*, including competitive adherence of *L. plantarum* to mannose-specific receptors or reduction in pathogenicity by induction of the human mucin genes (27, 33). Insight in the mechanisms of host-microbe interactions *in vivo* will make it possible to extend the selection criteria with the desired functioning in the GI-tract.

Understanding specific gene expression of the microbiota at a certain location in the GI-tract can be applied for the use as living vaccine or as a delivery vehicle that performs a certain function on a specific target site (16, 18). The potential of living vaccines to deliver heterologous antigens to the mucosal immune system offers a number of advantages over traditional vaccination, such as non-invasiveness and the possibility to induce both a systemic and mucosal immune response (15). In general, *L. plantarum* is preferred above other food-grade lactic acid bacteria based on its convenience in production, high-level genetic accessibility and performance in the GI-tract. The most appropriate manner for administration of *L. plantarum* has been investigated in mouse models (13, 35). An *L. plantarum* strain, encoding the peptide 111-139 of Der p 1 of the house dust mite under control of the constitutive lactate dehydrogenase (*ldh*)-promoter of *L. casei*, could be used to inhibit house dust mite-specific T-cell responses in mice, indicating a possible treatment of allergic disorders (26). To deliver an antigen at the most effective location, that protein could be produced by induced expression of the desired gene on that location. Knowing the location of expression of specific *L. plantarum* genes in the intestine, and thus which promoters are switched on, can improve the delivery on the intended location. Understanding the influence of the GI-tract, both on gene expression and the physiology of *L. plantarum*, can be exploited to develop an appropriate way of ingestion, like in a liquid formula or a capsule.

Conclusions

A variety of methods were developed to assess the activity of *L. plantarum* in the human GI-tract. FISH was found to be useful for estimating the *in situ* activity of *L. plantarum* during the initial exponential growth phase. However, at high cell densities it is not suited for activity determination. Methods for global gene expression, including DNA microarrays and qRT-PCR, could be applied to different intestinal samples to obtain specific information of *L. plantarum* on adaptation to the human GI-tract. In combination with clinical studies, the approaches constitute powerful and high-throughput tools to provide insight and new perspectives on *in vivo* host-microbe interactions.

Bibliography

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-1920.

3. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2001. 5th ed. W.H. Freeman and company, New York.

4. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721-5729.

5. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. *In* P. A. Bron (ed.), The molecular response of *Lactobacillus plantarum* to intestinal passage and conditions. Wageningen Centre for Food Sciences, Wageningen.

6. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18:**671-83.

7. **Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins.** 1998. Development and application of *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Bacteriol. **84:**759-768.

8. **de Vos, W. M.** 2005. Lipotechoic acid in lactobacilli: D-Alanine makes the difference. Proc. Natl. Acad. Sci. U. S. A. **102:**10763-10764.

9. **de Vos, W. M., P. A. Bron, and M. Kleerebezem.** 2004. Post-genomics of lactic acid bacteria and other food-grade bacteria to discover gut functionality. Curr. Opin. Biotechnol. **15:**86-93.

10. **de Vos, W. M., and J. Hugenholtz.** 2004. Engineering metabolic highways in lactococci and other lactic acid bacteria. Trends Biotechnol. **22:**72-79.

11. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133-143.

12. **Gahan, C. G., and C. Hill.** 2000. The use of listeriolysin to identify *in vivo* induced genes in the Gram-positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **36:**498-507.

13. **Geoffroy, M.C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. **66:**383-391.

14. **Goossens, D., D. Jonkers, M. Russel, A. Thijs, A. van den Bogaard, E. Stobberingh, and R. Stockbrugger.** 2005. Survival of the probiotic, *L. plantarum* 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition. Dig. Liver. Dis. **37:**44-50.

15. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20**.

16. **Grangette, C., H. Muller-Alouf, P. Hols, D. Goudercourt, J. Delcour, M. Turneer, and A. Mercenier.** 2004. Enhanced mucosal delivery of antigen with cell wall mutants of lactic acid bacteria. Infect. Immun. **72:**2731-2737.

17. **Grangette, C., S. Nutten, E. Palumbo, S. Morath, C. Herman, J. Dewulf, B. Pot, T. Hartung, P. Hols, and A. Mercenier.** 2005. From The Cover: Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc. Natl. Acad. Sci. U. S. A. **102:**10321-10326.

18. **Hanniffy, S., U. Wiedermann, A. Repa, A. Mercenier, C. Daniel, J. Fioramonti, H. Tlaskolova, H. Kozakova, H. Israelsen, S. Madsen, A. Vrang, P. Hols, J. Delcour, P. Bron, M. Kleerebezem, and J. Wells.** 2004. Potential and opportunities for use of recombinant lactic acid bacteria in human health. Adv. Appl. Microbiol. **56:**1-64.

19. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by *in vivo* gene expression. Proc. Natl. Acad. Sci. U. S. A. **94:**934-939.

20. **Hooper, L. V.** 2004. Bacterial contributions to mammalian gut development. Trends Microbiol. **12:**129-134.

21. **Hooper, L. V., J. Xu, P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. U. S. A. **96:**9833-9838.

22. **Hurley, B. P., and B. A. McCormick.** 2003. Translating tissue culture results into animal models: the case of *Salmonella typhimurium*. Trends Microbiol. **11:**562-9.

23. **Kandler, O., and N. Weiss.** 1986. Regular, nonsporing, Gram-positive rods, p. 1229. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology, vol. 2. Williams and Wilkins, Baltimore, U.S.A.

24. **Kilic, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao.** 1999. Streptococcal reporter gene-fusion vector for identification of *in vivo* expressed genes. Plasmid **42:**67-72.

25. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

26. **Kruisselbrink, A., M.J. Heijne Den Bak-Glashouwer, C. E. G. Havenith, J. E. R. Thole, and R. Janssen.** 2001. Recombinant *Lactobacillus plantarum* inhibits house dust mite-specific T-cell responses. Clin. Exp. Immunol. **126:**2-8.

27. **Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth.** 1999. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing intestinal mucin gene expression. Am. J. Physiol. **276:**G941-950.

28. **Mackie, R. I., A. Sghir, and H. R. Gaskins.** 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. **69(suppl):**1035S-1045S.

29. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assesment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Gen. **34:**139-164.

30. **Menendez, M. d. C., M. J. Rebollo, M. d. C. Nunez, R. A. Cox, and M. J. Garcia.** 2005. Analysis of the precursor rRNA fractions of rapidly growing mycobacteria: Quantification by methods that include the use of a promoter (*rrnA* P1) as a novel standard. J. Bacteriol. **187:**534-543.

31. **Mourad, F. H.** 2004. Animal and human models for studying effects of drugs on intestinal fluid transport *in vivo*. J. Pharmacol. Toxicol. Methods **50:**3-12.

32. **Pragman, A. A., and P. M. Schlievert.** 2004. Virulence regulation in *Staphylococcus aureus*: the need for *in vivo* analysis of virulence factor regulation. FEMS Immunol. Med. Microbiol. **42:**147-54.

33. **Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P. A. Bron, J. Lambert, W. M. de Vos, R. van der Meer, M. A. Smits, and M. Kleerebezem.** 2005. Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. J. Bacteriol. **187:**6128-36.

34. **Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov.** 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. Cell **118:**229-241.

35. **Reveneau, N., M. C. Geoffroy, C. Locht, P. Chagnaud, and A. Mercenier.** 2002. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. Vaccine **20**.

36. **Sonnenburg, J. L., J. Xu, D. D. Leip, C.H. Chen, B. P. Westover, J. Weatherford, J. D. Buhler, and J. I. Gordon.** 2005. Glycan foraging *in vivo* by an intestine-adapted bacterial symbiont. Science **307:**1955-1959.

37. **Stamer, J. R.** 1983. Lactic acid fermentation of cabbage and cucumbers. *In* H. J. Rehm and G. Reed (ed.), Biotechnology, vol. 5. Verlag Chemie, Weinheim, Germany.

38. **Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon.** 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc. Natl. Acad. Sci. U. S. A. **99:**15451-15455.

39. **Stintzi, A., D. Marlow, K. Palyada, H. Naikare, R. Panciera, L. Whitworth, and C. Clarke.** 2005. Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. Infect. Immun. **73:**1797-1810.

40. **Sturme, M. H. J., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos.** 2005. An *agr*-like two component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J. Bacteriol. **187:** 5224-5235.

41. **Tannock, G. W.** 1995. Normal microflora: An introduction to microbes inhabiting the human body. Chapman & Hall, London.

42. **Troost, F. J., W. H. M. Saris, G. R. M. M. Haenen, A. Bast, and R.J. M. Brummer.** 2003. New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. Am. J. Physiol. Gastrointest. Liver Physiol. **285:**G354-359.

43. **Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen.** 2001. Quality assurance criteria for probiotic bacteria. Am. J. Clin. Nutr. **73:**393S-398.

44. **Vaughan, E. E., M. C. de Vries, E. G. Zoetendal, K. Ben-Amor, A. D. L. Akkermans, and W. M. de Vos.** 2002. The Intestinal LABs. Antonie van Leeuwenhoek **82:**341-352.

45. **Vereecke, D., K. Cornelis, W. Temmerman, M. Holsters, and K. Goethals.** 2002. Versatile persistence pathways for pathogens of animals and plants. Trends Microbiol **10:**485-8.

46. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Micr. Ecol. Health. Dis. **13:**129-134.

47. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. de Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854-3859.

Summary

The human gastrointestinal (GI)-tract represents a dynamic ecosystem comprising various habitats each with niche-specific microbial communites, collectively called microbiota. Lactic acid bacteria (LAB) are considered to be a large group of the microbiota in the upper GI-tract that is involved in health-stimulating processes within the host. The lactic acid bacterium *Lactobacillus plantarum* is one of the most versatile and flexible LAB that is encountered in a range of environmental niches, has a proven ability to survive gastric transit, and can colonize the GI-tract of human and other mammals. Several studies describe the effects of *L. plantarum* consumption on the human physiology and health. The complete genome sequence of *L. plantarum* WCFS1 makes it a suitable model to study with molecular techniques like fluorescent *in situ* hybridization (FISH), quantitative reverse transcriptase PCR (qRT-PCR), and DNA microarrays to contribute to unraveling the mechanisms underlying the GI targeted properties of *L. plantarum* (Chapter 1).

Methods for the detection, classification, and to estimate general activity of microbes in complex ecosystems generally use ribosomal RNA (rRNA) and their coding genes as target molecules due to their universal distribution and high nucleotide sequence conservation. In **Chapter 2**, the complete genome sequences of the LAB, *L. plantarum, L. johnsonii*, and *Lactococcus lactis* were used to compare location, sequence, organisation, and regulation of the rRNA operons. All operons demonstrate a common organization with the order 5'-16S-23S-5S-3', but differ in the number, location and specificity of the tRNA genes. Micro-heterogeneity was found within the rRNA structural genes and spacer regions of each strain. However, in the rRNA operon promoter regions of *L. plantarum* and *Lactococcus lactis* marked differences were observed, while the promoter regions of *L. johnsonii* showed a similar promoter structure in all operons. Although all five *L. plantarum* rRNA promoters are significantly different, this study demonstrates that their activities were very similar under the conditions tested.

The feasibility of using the rRNA-based FISH method to estimate the overall *in situ* activity of *L. plantarum* in the human GI-tract was assessed (**Chapter 3**). FISH could be used to estimate the *in situ* activity of *L. plantarum* WCFS1 in exponentially growing cells. However, *L. plantarum* is capable of growth to very high cell densities and the properties of the *L. plantarum* cell-envelope prevented effective entry of the fluorescent oligonucleotide probe into the cells at later stages of growth when high cell densities are reached. Hence, FISH is not appropriate for use in the GI-tract as the growth phase of *L. plantarum* may vary

considerably and differ between cells, as influenced by way of ingestion, and location in the GI-tract.

The focus of this research shifted to analyzing specific gene expression of *L. plantarum* in the human GI-tract using DNA microarrays and qRT-PCR. Gene expression of *L. plantarum* associated with human mucosa was determined using DNA microarrays in three colonic biopsies and in the ileal and colonic biopsy within a single person (**Chapter 4**). The ingested *L. plantarum* cells were metabolically active in all screens and in all subjects, as demonstrated by the detection of about 10% expressed genes by the DNA microarrays. Genes were detected for all functional classes. The differences in *L. plantarum* gene expression between the colons of three individuals were larger than the differences between the ileum and colon of a single individual. This is the first report of global gene expression analysis of an ingested microbe in the human intestinal mucosal.

To evaluate the effect of passage of the proximal small intestine on gene expression of *L. plantarum*, the perfusion technique was conducted in healthy human volunteers (**Chapter 5**). Subsequently, RNA isolated from the perfusion suspension containing *L. plantarum* was hybridized to DNA microarrays. Passage of the small intestine left *L. plantarum* metabolically active as can be seen from up-regulation of genes involved in cell division, and protein synthesis. However, an osmotic shock response was observed, most probably induced by bile salts and including up-regulation of genes involved in ion transport, and glycine, betaine, carnitine, or choline uptake. In addition, expression of many genes involved in the cell envelope was altered, indicating a strengthening of the cell envelope and pointing to specific interactions with the host and further microbiota.

In addition, qRT-PCR established gene expression of *L. plantarum* from the low amount of RNA recovered from three subjects having an ileostoma who consumed the bacteria in a milk drink (**Chapter 6**). In the ileostomy samples the *L. plantarum* cells were metabolically active as indicated by induced expression of genes predicted to be involved in protein synthesis, an active amino acid biosynthesis machinery, and sugars uptake and degradation for energy to eventually lactate and ethanol production. Genes encoding a potential adherence protein, and α-L-rhamnosidase, which is an enzyme for hydrolysis of rhamnosides and releasing L-rhamnose from various substrates, and a bacteriocin precursor were clearly up regulated.

All screens were compared to demonstrate subject-specific expression, differences between gene expression of *L. plantarum* in the ileum and colon and between luminal cells and cells associated with mucosal cells or mucus (**Chapter 7**). In addition, a comparison between bacterial gene expression in humans and animals (mice and rabbits) indicated a large similarity between the gene expression in those different hosts.

In combination with clinical studies, the approaches for gene expression described in this thesis are powerful and high-throughput tools to provide insight and new perspective on *in vivo* host-microbe interactions. This is expected to lead to new generations of probiotics with a scientifically proven basis for their health effects.

Samenvatting

Het maagdarmkanaal van de mens herbergt een dynamisch ecosysteem dat bestaat uit verschillende habitats met elk een eigen gemeenschap van bacteriën. Melkzuurbacteriën worden hierbinnen beschouwd als een belangrijke groep die betrokken is bij het bevorderen van de gezondheid van de gastheer. De veelzijdige en flexibele melkzuurbacterie *Lactobacillus plantarum*, die op veel plaatsen in de natuur voorkomt, overleeft het maagzuur en kan zich permanent vestigen in het maagdarmkanaal van mensen en andere zoogdieren. Verschillende studies beschrijven het effect van consumptie van *L. plantarum* op de humane fysiologie (hoofdstuk 1).

In deze thesis wordt de kennis over de complete genoomsequentie van *L. plantarum* WCFS1 stam gebruikt om deze bacterie met moleculaire technieken zoals fluorescente *in situ* hybridisatie (FISH), kwantitatieve reverse transcriptase PCR (qRT-PCR) en DNA microarrays, te bestuderen. Hiermee kunnen mechanismen worden opgehelderd die de eigenschappen van *L. plantarum* in het maagdarmkanaal verklaren (**hoofdstuk 1**).

Methoden voor de detectie, classificering en algemene activiteitsbepaling van bacteriën in een complex ecosysteem, zoals FISH, maken over het algemeen gebruik van ribosomaal RNA (rRNA) en de genen die hiervoor coderen vanwege hun universele voorkomen en de grote overeenkomst in de nucleotidevolgorde. In **hoofdstuk 2** zijn de complete genoomsequenties van *L. plantarum*, *L. johnsonii* en *Lactococcus lactis* gebruikt om de locatie, volgorde, organisatie en regulatie van de rRNA operons te vergelijken. Alle operons lieten een gebruikelijke volgorde van 5'-16S-23S-5S-3' zien, maar verschilden in aantal, locatie en specificiteit van de tRNA genen. In elke stam waren de verschillen tussen de structurele genen en spacers van de rRNA operons miniem. De promoter regio's van *L. johnsonii*, die de expressie van de operons reguleren, verschilden nauwelijks van elkaar. In de rRNA promoter regio's van *L. plantarum* en *Lactococcus lactis* werden echter aanzienlijke verschillen aangetroffen. Ondanks de significante verschillen tussen de vijf promoter regio's van *L. plantarum*, kwam hun activiteit onder de geteste omstandigheden overeen.

Er is onderzoek gedaan naar de mogelijkheid om FISH te gebruiken om de algehele activiteit van *L. plantarum* in het maagdarmkanaal te bepalen (**hoofdstuk 3**). FISH kon worden gebruikt om de *in situ* activiteit te bepalen in exponentieel groeiende *L. plantarum* cellen. *L. plantarum* kan echter tot zeer hoge celdichtheid groeien en bij latere groeifases verhinderen de eigenschappen van de celwand het effectief binnendringen van de cel door de oligonucleotide probe. FISH is niet geschikt voor gebruik in het maagdarmkanaal omdat de

groeifase waarin *L. plantarum* verkeert niet bekend is. Bovendien zou de groeifase kunnen verschillen tussen cellen, manier van consumptie en locatie in het maagdarmkanaal.

Hierdoor verschoof de nadruk van rRNA-gebaseerde methoden naar specifieke gen expressie van *L. plantarum*, wat is bepaald in verschillende humane darmmonsters door middel van DNA microarrays en qRT-PCR. Gen expressie van *L. plantarum* cellen gehecht aan de humane darmwand is onderzocht met DNA microarrays in darmkanker patiënten (**Hoofdstuk 4**). Dikke darm biopsies van drie personen en een dunne en een dikke darm biopsie van één persoon, die deze bacterie gedurende een week voor de operatie geconsumeerd hadden, werden gebruikt. De geconsumeerde *L. plantarum* cellen waren metabolisch actief in alle personen, zoals blijkt uit detectie van ongeveer 10% tot expressie komende genen op DNA microarrays. Deze genen werden gevonden in alle functionele klassen. Het verschil in gen expressie van *L. plantarum* in de dikke darm van drie verschillende personen was groter dan het verschil in gen expressie tussen de dunne en dikke darm van één persoon.

Om het effect van passage van de dunne darm op gen expressie van *L. plantarum* te evalueren werd de perfusietechniek gebruikt, waarbij een *L. plantarum* cel suspensie werd geïnjecteerd vlak na de maag en 40 cm later weer werd opgepompt (**Hoofdstuk 5**). Het uit de perfusiesuspensie geïsoleerde RNA werd met behulp van DNA microarrays vergeleken met RNA van cellen vóór perfusie en van cellen die onder de zelfde experimentele omstandigheden behandeld zijn, behalve dat deze de dunne darm niet gepasseerd zijn. Tijdens passage van de dunne darm was *L. plantarum* metabolisch actief wat blijkt uit toegenomen expressie van genen betrokken bij celdeling en eiwitsynthese. Een verandering in osmolariteit van de omgeving, waarschijnlijk veroorzaakt door galzouten, kon worden afgeleid uit toegenomen expressie van de genen betrokken bij ion-transport en glycine-, betaine-, carnitine- en/of cholineopname. Van veel genen die betrokken zijn bij de vorming van de celwand, verschilde de expressie voor en na perfusie, wat wees op versteviging van de celwand en interactie met de gastheer en andere bacteriën.

qRT-PCR is gebruikt om de gen expressie van *L. plantarum* te bepalen uit de kleine hoeveelheden RNA, die gewonnen konden worden uit ileostoma monsters van drie personen (**Hoofdstuk 6**). Deze personen hadden de bacteriën in een melkdrank geconsumeerd. In de ileostoma monsters werd metabolische activiteit van de cellen duidelijk door toegenomen expressie van genen, die waarschijnlijk coderen voor eiwitsynthese, aminozuur synthese en

opname en afbraak van suikers. Bovendien is in de ileostoma monsters de expressie toegenomen van genen die coderen voor de omzetting van suikers tot energie en uiteindelijk melkzuur en alcohol. Het α-L-rhamnosidase gen, een bacteriocine precursor gen en een gen dat codeert voor een eiwit dat mogelijk betrokken is bij hechting aan de darmwand lieten een duidelijke toename in transcriptie zien.

De resultaten van de verschillende methoden zijn vergeleken om persoonspecifieke gen expressie van *L. plantarum* te bepalen (**Hoofdstuk 7**). Bovendien zijn verschillen tussen gen expressie van *L. plantarum* op verschillende locaties in de darm bepaald, zowel tussen dunne en dikke darm als tussen het lumen en cellen gehecht aan de darmwand. Verder vertoonde bacteriële genexpressie in mensen en verschillende dieren (muis en konijn) een grote overeenkomst onafhankelijk van de gastheer.

De hiervoor beschreven benaderingen zijn, in combinatie met klinische studies, krachtige en high-throughput methoden om inzicht te verwerven in de relatie tussen bacteriën en hun gastheer. Bovendien kan dit leiden tot een tweede generatie probiotica met een gedegen wetenschappelijke basis voor de gezondheidseffecten die zij veroorzaken.

Nawoord

Na vijf jaar intensief bezig te zijn geweest met *Lactobacillus plantarum* in de menselijke darm is dit proefschrift af. Het is een leuke, interesante en leerzame tijd geweest. Het was leerzaam omdat ik me heb kunnen verdiepen in de moleculaire biologie, in nieuwe technieken en omdat ik me verder heb kunnen ontwikkelen in de wetenschap. Het was interessant omdat *L. plantarum* langzaam zijn geheimen prijs geeft, ik veel verschillende mensen heb leren kennen en het werk op verschillende locaties heb kunnen uitvoeren. En het was vooral een leuke tijd met leuke collega's, zowel bij de moleculaire ecologie (moleco) groep als binnen C001 en C007 van het WCFS.

Aan het leerzame aspect hebben met name drie mensen bijgedragen, mijn co-promotoren Elaine Vaughan en Michiel Kleerebezem en mijn promotor Willem de Vos. Elaine, jou enthousiasme en onze vruchtbare discussies waren zeer motiverend en hebben geholpen om de (technische) problemen op te lossen. Ook heeft onze samenwerking mijn vaardigheid in het engels sterk verbeterd. Michiel, jij hebt me de mogelijkheid gegeven mijn onderzoek daar uit te voeren waar het het makkelijkste was, bij microbiology, TNO, NIZO Food Research of zelfs in Lund, Zweden. Samen met je nuttige adviezen en andere kijk op mijn resultaten heeft dat ervoor gezorgd dat ik me kon ontwikkelen tot een alround wetenschapper. Willem, je interesse voor mijn onderzoek, de korte gesprekken op de gang over of er al wat meer bekend was en de uitgebreide discussies over hoe we het meeste uit de data konden krijgen hebben in belangrijke mate bijgedragen aan dit proefschrift.

I would like to thank the whole moleco group for the wonderfull time we had, with trips to Texel, Bulgaria and the Efteling, Fryday-afternoon drinks in "de Vlaam" en sharing the ups and downs of research. I like to thank especially my students Janneke, Andrea, Yuan and Angelique for their contribution to this thesis. I would also like to express my gratitude to Toshio for doing the last experiments for me, which make the perfusion story a lot stronger. Binnen de moleco's zijn er nog een aantal mensen die ik met name wil noemen. Antoon, in het begin van mijn project nog onze werkgroepleider, heeft me het zetje in de rug gegeven om me vertrouwd te maken met de moleculaire biologie en mijn plaats binnen moleco te vinden. Mark en Kees, met wie ik ongeveer tegelijk ben begonnen en veel gezellige en wetenschappelijke discussies heb gevoerd. Hans, die ervoor zorgt dat het lab blijft draaien en altijd voor ons klaar staat, en Wilma en Ineke voor hun nuttige adviezen. Verder zijn er nog mijn kamergenoten Arjan (door gebrek aan ruimte letterlijk mijn backup), Erwin, eerst als begeleider van mijn afstudeervak en later als kamergenoot, Kaouther met wie ik samen de

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Via het WCFS heb ik niet alleen bij het laboratorium voor microbiologie gewerkt, maar ook bij NIZO Food Research en TNO Voeding. Vooral bij het NIZO heb ik veel tijd doorgebracht. Iedereen was bereid me weer op te komen halen bij de receptie als ik niet door mocht lopen. Met name wil ik Maria en Marc bedanken voor hun hulp met kwantitatieve reverse transcriptase PCR en de DNA microarrays.

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Er is meer in de wereld dan alleen onderzoek, gelukkig had ik mijn vrienden en familie om me daaraan te herinneren. Joanne, Judith en Monique, ik ben blij dat jullie met je ervaring in de wetenschap en binnen de universiteit hielpen met relativeren en dat jullie al mijn verhalen over het onderzoek aan hebben gehoord. Ernst en Rolf, eerst als huisgenoot en later wat meer op afstand, ben ik blij dat jullie meegeleefd hebben en dat gezellige weekenden op haarweg 111 de nodige afleiding hebben gegeven.

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Maaike

List of Publications

1. **Vogelaar, J.C/T., J.B. van Lier, B. Klapwijk, M.C. de Vries, and G. Lettinga**. 2002. Assessment of effluent turbidity in mesophilic and thermophilic sludge reactors – origin of effluent colloidal material. Appl. Microbiol. Biotechnol. 59:105-111.

2. **Vaughan, E.E., M.C. de Vries, E.G. Zoetendal, K. Ben-Amor, A.D.L. Akkermans, and W.M. de Vos**. 2002. The intestinal LABs. Antonie van Leeuwenhoek 82:341-352.

3. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O.P. Kuipers, R. Leer, R. Tarchini, S.A. Peters, H.M. Sandbrink, M.W.E.J. Fiers, W. Stiekema, R.M.K. Lankhorst, P.A. Bron, S.M. Hoffer, M.N.N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W.M. de Vos, R.J. Siezen,** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci.U. S. A. 100, 1990-1995

4. **M.C. de Vries, E.E. Vaughan, M.Kleerebezem, and W.M. de Vos**, 2004. Optimising single cell activity assessment of *Lactobacillus plantarum* by fluorescent in situ hybridisation as affected by growth, J. Microbiol. Meth., 59:109-115.

5. **R. te Biezebeke, R. Boesten, E.S. Klaassen, C.C.G.M. Booijijnk, M.C. de Vries, M. Derrien, D.P.A. Cohen, F. Schuren, E.E. Vaughan, M. Kleerebezem, W.M. de Vos**, 2004. Microbial functionality in the human gastrointestinal tract, Microbes Environ., 19, 276-280

6. **F.A.M. de Bok, H.J.M. Harmsen, C.M. Plugge, M.C. de Vries, A.D.L. Akkermans, W.M. de Vos and A.J.M. Stams**, 2005. The first true obligately syntrophic propionate-oxidizing bacterium, *Pelotomaculum schinkii* sp. Nov., co-cultured with *Methanospirillum hungatei*, and emended description of the genus *Pelotomaculum*, Int. J. Syst. Evol. Microbiol., 55: 1697-1703

7. **M.C. de Vries, R.J. Siezen, J.G.E. Wijman, Y. Zhao, M. Kleerebezem, W.M. de Vos, E.E. Vaughan**, 2005. Comparative and functional analysis of the rRNA-operons and their tRNA gene complement in different lactic acid bacteria, System. Appl. Microbiol., in press

8. **M.C. de Vries, E.E. Vaughan, M. Kleerebezem and W.M. de Vos**, 2005. *Lactobacillus plantarum* - Survival, functional and potential probiotic properties in the human intestinal tract, Int. Dairy J., in press

9. **M.C. de Vries, M.L. Marco, M. Kleerebezem, P. Mangell, S. Ahrne, D. Molenaar, W.M. de Vos, and E.E. Vaughan**, 2006. Transcript profiling reveals global gene expression of *Lactobacillus plantarum* in the human gastrointestinal tract, submitted for publication

10. **M.C. de Vries, F.J. Troost, T. Fujii, E.E. Vaughan, D. Molenaar, R.-J. Brummer, M. Kleerebezem, and W.M. de Vos**, 2006. Global transcript profiling of gene expression of *Lactobacillus plantarum* after perfusion in the human small intestine, manuscript in preparation

11. **M.C. de Vries, M.L. Marco, F.J. Troost, R.-J. Brummer, M. Kleerebezem, W.M. de Vos, and E.E. Vaughan**, 2006. Specific gene expression of *Lactobacillus plantarum* in the ileum of ileostomy patients as determined by quantitative Reverse Transcriptase PCR, manuscript in preparation

Curriculum Vitae

Maaike Catherine de Vries werd geboren op 23 januari 1975 in 's Hertogenbosch. Haar lagere school is zij begonnen in Halle-Zoersel, Belgie, en heeft zij afgemaakt in Voorschoten, Nederland. In 1993 behaalde zij het VWO-diploma aan het Vlietland college in Leiden. In datzelfde jaar, begon zij met de studie Levensmiddelentechnologie aan de Landbouw Universiteit in Wageningen. Tijdens deze studie ontstond haar interesse voor de microbiologie. Zij deed een afstudeeronderzoek naar de toxineproductie van *Staphylococcus aureus* en naar de invloed van het immuunsysteem op de darmflora van muizen. Haar stage deed zij bij Unilever Research Colworth, UK. Na haar afstuderen op 25 juni 1999, hield zij zich met verschillende dingen bezig, totdat zij in mei 2000 begon aan als AIO voor het Wageningen Centre for Food Sciences met als werklocatie het "Laboratorium voor Microbiologie" aan Wageningen Universiteit. Hier deed zij onderzoek naar *Lactobacillus plantarum* in de humane darm wat resulteerde in dit proefschrift. Een deel van het onderzoek werd gedaan in samenwerking met Lund University, Zweden. Het onderzoek werd uitgevoerd onder begeleiding van Prof. Dr. Willem M. de Vos, Dr. Elaine E. Vaughan, en Dr. Michiel Kleerebezem.

Voor het training en supervisie plan binnen de onderzoekschool VLAG zijn onder andere de volgende cursussen, congressen en andere activiteiten gevolgd:

Activiteit	**Jaar**
Winterschool Bioinformatics	2000
Ecofysiology of the GI-tract	2000
Supervision of Undergraduates	2001
Radio-activity course 5B	2001
Scientific writing	2001-2002
LAB7	2002
AIO-trip Japan	2004
3 Visits to Lund University	2002-2003

Vanaf december 2005 is Maaike de Vries werkzaam bij het Centrum voor Infectieziektenbestrijding van het RIVM als microbioloog/onderzoeker.

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