Functional analysis of *Lactobacillus plantarum* WCFS1:
a proteomic approach

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Abstract

Lactic Acid Bacteria (LAB) are involved in the production of fermented foods whereby they improve the preservation properties as well as impart characteristic flavors and textures. Specific *Lactobacillus* strains are marketed as probiotics because of potential health benefits. However, the molecular mechanisms underlying much of these beneficial effects have yet to be established. In this thesis, a proteomic approach was developed to investigate the functionality of *Lactobacillus plantarum* WCFS1 both under laboratory conditions and the human intestinal tract. Proteomics was chosen as proteins present in bacteria isolated from fecal samples will likely reflect the proteins that are produced during transit of the colon. Initially, two-dimensional (2-DE) gel electrophoresis of *L. plantarum* WCFS1, whose genome has been sequenced, was used to investigate the dynamics of the proteome from the cytosolic fraction isolated from mid- and late-log, early- and late-stationary phase cells grown in a laboratory medium. Almost 200 protein spots were identified by MALDI-TOF mass spectrometry revealing differentially regulated proteins for each growth phase, and a proteome reference map was constructed to facilitate further studies. Parallel analysis by proteomics and transcriptomics was performed for various growth phases of *L. plantarum* WCFS1 and an isogenic *ccpA* (regulator of carbon catabolite repression) mutant, showing a considerable (70-80%) correlation throughout all growth phases. Genes with deviating transcript and protein levels mainly classified among purine and pyrimidines biosynthesis, energy metabolism, and stress proteins. Next, methods for specifically labeling *L. plantarum* and for sorting and isolation from human intestinal samples for proteomic analysis were investigated. An oligonucleotide probe was validated for application in Fluorescent *In Situ* Hybridization (FISH) for *L. plantarum* against numerous other LAB and 40 bacterial species commonly found in the human intestine. The FISH probe was successfully applied to label *L. plantarum* cells from ileal effluent and fecal samples. An innovative approach involving an extraction method based on immunomagnetic beads using polyclonal antibody against *L. plantarum* WCFS1 with subsequent proteomic analysis was subsequently developed. In the enriched fraction from a human intestinal sample, *L. plantarum* accounted for 86% of the total bacterial cells based on quantitative PCR using specific primers. The proteome of *L. plantarum* cells isolated by the extraction method from the ileal effluent of a subject carrying an ileostoma that had been fed with *L. plantarum* WCFS1 was visualized on a 2-DE gel. A number of proteins could be identified using the proteome reference map involved in carbohydrate metabolism, stress adaptation and translation, confirming the metabolic activity of the cell, and two of these proteins were confirmed by MALDI-TOF analysis. Glycolytic proteins previously shown to be involved in host-microbe interactions/adherence in other gram-positive bacteria were detected in the 2-DE gels, and these were also detected in cell membrane fractions. Overall, this thesis lays the foundation for application of proteomics to study the functionality of *L. plantarum* cells from human intestinal samples.

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Chapter 1

General Introduction
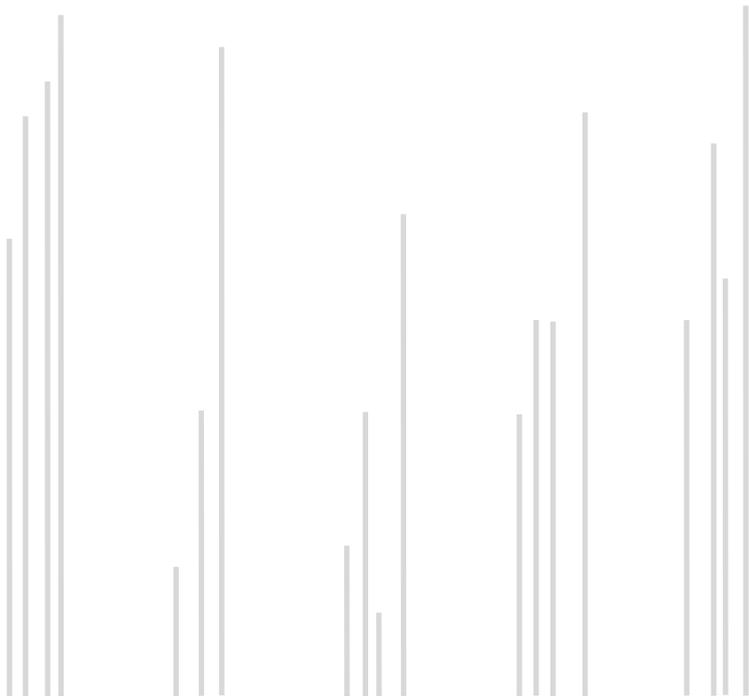
1.1 Introduction

Lactic Acid Bacteria (LAB) are involved in the production of fermented foods all over the world. They improve the preservation properties as well as impart characteristic flavors and textures to the food products. Apart from their well-established role as starter bacteria in industrial dairy fermentations, they are also used for pickling of vegetables, sourdough production, making of wine, and the curing of fish, meats and sausages. The common feature of these bacteria is the ability to ferment sugars to lactic acid but many other by-products such as acetate, ethanol, acetoin, carbon dioxide and formate can be produced (107).
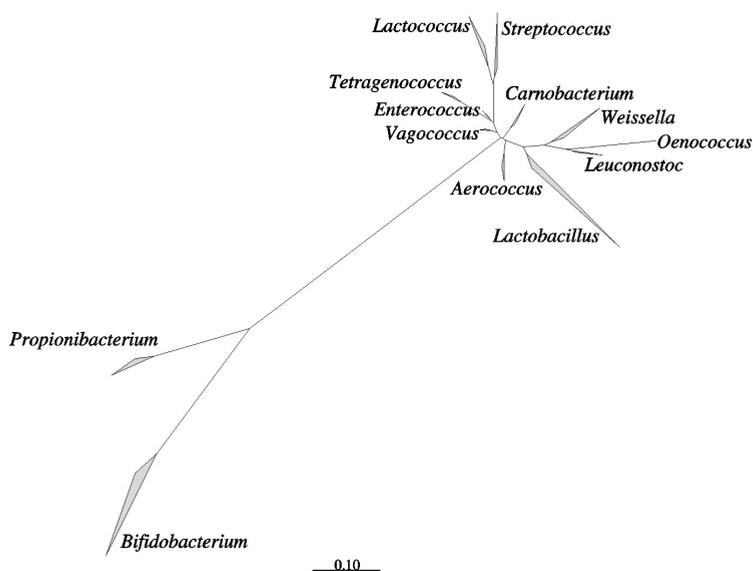
Figure 1. A 16S ribosomal RNA-based phylogenetic tree showing the diversity of the major groups of lactic acid bacteria. The position of the *Bifidobacterium* and *Propionibacterium* species that have a high DNA G+C content are also indicated. The reference bar indicates 10% sequence divergence.

The LAB are gram-positive, non-motile, non-spore forming bacteria, and have rod- or coccus like morphology. They contain genomic DNA with a relatively low DNA G+C content and are part of the Firmicutes division (44). The LAB group includes the following genera of bacteria: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weisella* (218) (Fig. 1). LAB are widespread in nature being found in plant material or dairy niches. Furthermore, they are also

present in our digestive system as commensal bacteria, implying that they are naturally associated with the human gastrointestinal tract. The human gastrointestinal tract is colonized from birth by a very high density of microorganisms with bacterial numbers increasing progressively from the stomach to the colon. The empty stomach and duodenum contain less than 10^3 bacterial cells per gram of contents, while in the small intestine the numbers increase from 10^4 to 10^7 per gram at the end of the ileum. In the large intestine the microbial community reaches densities of up to 10^{12} bacterial cells per gram (88, 260). The commensal microbiota has a profound impact on human physiology and health and contributes to resistance to infections, development and modulation of the immune response, and intestinal epithelial proliferation and differentiation (167, 201, 260). Furthermore, beneficial health effects are being claimed upon consumption of specific LAB strains, notably *Lactobacillus* spp., that are marketed as probiotics (55). There is increasing scientific support, derived from double-blind and placebo-controlled clinical intervention studies, for the probiotic function of a selected number of single LAB strains or mixtures of different LAB (77, 193, 200). However, the molecular mechanisms underlying the beneficial effects have yet to be established and hence this is an active area for research aimed at addressing both the activity of the LAB after consumption and determining the response of the host (48, 55, 160).

Any intestinal microbe, whether endogenous or derived from an ingested food, is confronted with the various conditions in the different niches in the intestine, including exposure to gastric acid and bile salts, peristalsis, and competition for space and nutrients amongst others (21). Since LAB are commonly used as starter cultures in food fermentation, some of them may be recovered from intestinal or fecal samples although many do not effectively survive passage. This was first established for *Lactococcus lactis,* a normal cheese starter, that in low numbers was found to survive intestinal transit (128). Several studies have described the isolation of members of the *Lactobacillus* genus from the human gastrointestinal tract, including *Lactobacillus acidophilus, L. casei, L. fermentum, L. gasseri, L. mucosae, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. ruminis,* and *L. salivarius* (190). Food-associated lactobacilli may also be detected including *L. delbrueckii, L. helveticus,* and *L. sakei.* Moreover, culture-independent studies have indicated that there are also new and not-yet cultured *Lactobacillus* spp. to be

recovered from different sites of the human intestine (98, 240). The lactobacilli are predominantly found in the small intestine where they are estimated to reach a significant fraction of the total population (21-74 %) (95). However, they are less dominant in the colon where they rarely make up more than 1% of the total bacterial community based on 16S ribosomal RNA (rRNA)-based approaches (95, 119).

The species *Lactobacillus plantarum* is a natural inhabitant of the human intestine and is predominantly found in the small intestine varying from 10^6 to 10^8 colony forming units per gram of mucosal biopsies along the human GI tract (4, 196). Furthermore, the species has been isolated from a variety of plant materials including cabbage, olives and vegetables (138, 140, 256). Several *L. plantarum* strains are marketed as probiotics, including *L. plantarum* strain 299v that is claimed to provide protection against intestinal infections, prevents diarrhea and alleviate symptoms of irritable bowel syndrome (55). Interestingly, *L. plantarum* strains have been demonstrated to modulate the adaptive immune response of humans (84, 178, 206, 233). In this thesis, the *L. plantarum* strain WCFS1 is used as a model organism for investigation of its behavior in the human intestinal tract using a proteomic approach. This *L. plantarum* strain was isolated from the human gastrointestinal tract, and can survive passage through the intestine of both men and mice (29, 241). It was demonstrated that *L. plantarum* induces specific genes *in vivo* using the recombination-based *in vivo* expression technology (R-IVET) in the mouse intestine and many of these genes displayed specific and differential responses at various sites along the intestinal tract (29, 159). Moreover, transcript profiling of the related *L. plantarum* 299v strain in the human intestinal tract revealed completely different gene expression compared to an *in vitro* laboratory grown culture in MRS medium (54). Furthermore, the host response to this bacterium has been investigated recently at the transcriptional level by following long-term exposure in germ-free mice and short-term transit in the upper part of the ileum of man (58, 233).

L. plantarum is an oxygen tolerant, heterofermentative species, and can grow to high densities which can be desirable for industrial purposes. In 2003, the complete genome of *L. plantarum* WCFS1 has been sequenced (126) and 3052

proteins have been predicted which would be present in either the cytosolic, membrane-bound or secreted proteins fraction (Table 1).

Table 1. Number and main functions of the proteins that are present in the cytolitic/soluble and membrane bound/secreted fractions of the predicted proteome of *L. plantarum* WCFS1. This includes 598 hypothetical proteins, the function of which is unknown.

Proteome fraction	No of Proteins	Main functions
Cytosolic/soluble	2830	Metabolic processes, cell growth and death; translation; transcription
Secreted/Membrane anchor	222	Membrane transport; cell surface proteins

Based on this proteome, an *in silico* prediction of a two-dimensional electrophoresis (2-DE) gel can be made which is presented in Fig. 2. It is likely that the experimental determined 2-DE proteome differs from this predicted one as cell-envelope-bound and secreted proteins (Table 1) as well as insoluble proteins are usually removed during the preparation of cell-free extracts.
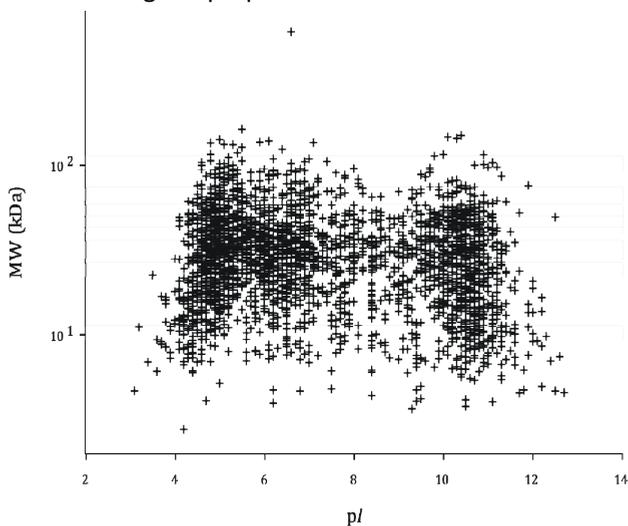
Figure 2. An *in silico* 2-DE gel of *L. plantarum* WCFS1. The 3.3-Mb genome has been sequenced and predicted to encode more than 3053 proteins (126). By calculating the molecular weight (MW) and the corresponding iso-electric point p*I* of each predicted protein an *in silico* prediction of a 2-DE gel can be made. These values have been calculated with the GCG Winconsin software packages Isoelectric and PeptideSort, respectively, obtained from Accelrys (San Diego, CA, USA).

As indicated above, there is considerable interest in analyzing the function of *L. plantarum* in the human intestinal tract and its interactions with the host (160). Previous studies have shown that the expression of particular genes classified in several functional classes is induced during passage of the gastro-intestinal tract of men and mice (Table 2) (29, 53, 159). While some of these data derive from a genetic method (R-IVET), which monitors events in the past and the present (29) most of the other studies are based on global transcriptomics and reverse-transcription (RT) PCR that require bacterial mRNA isolation from the biopsies or intestinal samples (53, 159).

Table 2. The number of genes belonging to several functional classes that have found to be induced during passage of the GI tract of men (53) or mice (29, 159).

Functional Classes	R-IVET	Transcriptomics	Quantitative RT PCR
Amino acid biosynthesis	3	11	3
Carbohydrate metabolism	3	8	2
Cell division		1	
Cell envelope	4	24	4
Cofactors metabolism	2		1
Lipid metabolism	2	3	1
Miscellaneous	20	8	5
Nucleotide Metabolism	1	15	
Protein Folding		3	
Replication and Repair	2	10	2
Stress		3	1
Transcription factor	1	2	2
Transcription regulator		5	
Translation	2	14	1
Transport	8	31	2
Total proteins	48	138	24

Due to the instability of bacterial mRNA - average half-life of several min (20) - sampling methods for transcriptomics and RT-PCR should be fast. This is technically difficult, the more so as the samples of interest derive from within the human intestinal tract. These biopsies are difficult to obtain as ethical aspects as

well as various medical expertises are involved. Moreover, the sampling and recovery of the biopsies take time as they are directly derived from human subjects. As a consequence, proteomics may have an advantage above transcriptomics and other mRNA-based approaches, as proteins are considerably more stable than mRNA and show an average half-life of 60 min during growth and various kinds of starvation (172). Moreover, the proteome of fecal samples may reflect the activity further up in the intestinal tract as the transition time in the large intestine can be as small as 1.5 h (see Fig. 3). Furthermore, the proteome of micro-organisms ingested and obtained in ileostomy effluent from colectomy patients should indicate the impact of the small intestinal tract conditions on those cells.
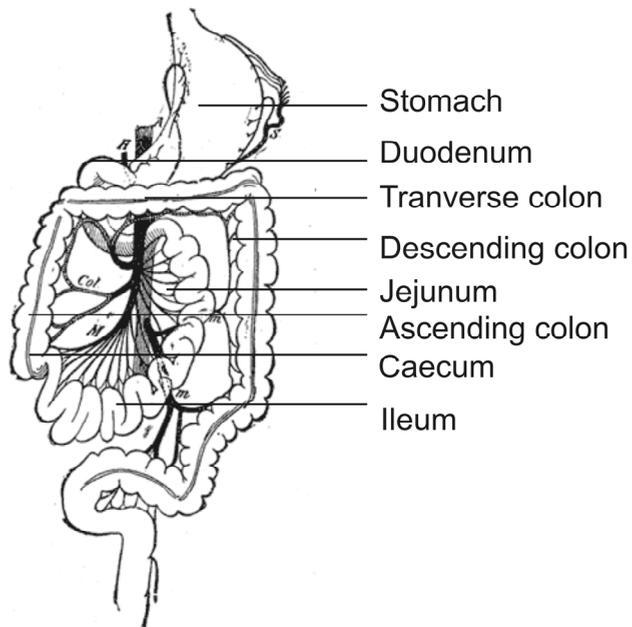
Figure 3. A schematic view of the lower part of the human gastrointestinal tract. The size of the different anatomic regions varies as does the transition time that in the stomach is between 0.8 – 1.6 h; in the small intestine (jejunum, ileum and cecum) varies between 3.3 - 3.7 h; and in the large intestine (ascending, transverse and descending colon) varies between 1.5 – 6.5 h for patients with IBS and some healthy subjects (91).

Based on the considerations described above, it is reasonable to assume that the proteins present in *L. plantarum* cells isolated from fecal samples will reflect the

proteins that were produced during transit of the colon. To determine the proteins produced by *L. plantarum* isolated from the human intestinal tract, the proteome of *L. plantarum* WCFS1 was investigated using a proteomics approach. Apart from demonstrating the feasibility of this global approach to study the function of intestinal bacteria, these studies were also initiated as they may contribute to a further understanding of the interaction between *L. plantarum* and the human host.

1.2 Outline of this thesis

Recently, transcriptional and genetic studies have been reported that showed differential gene expression of *L. plantarum* during passage of the intestinal tract of mice (29, 159). Part of these observations was confirmed in study addressing the global transcriptional response of *L. plantarum* in three human subjects (53). While highly relevant, novel and revealing, these latter human studies are anecdotal and limited to hospitalized subjects. Hence, it was aimed to develop a more generic approach to study the global response of *L. plantarum* in the intestinal tract. As motivated above, a proteomics approach was selected and developed to investigate the activity and function of *L. plantarum* WCFS1 both under laboratory conditions and the human intestinal tract.

In **Chapter 2**, the proteomics technology is explained, including advantages and disadvantages of this technique as well as the comparison to the transcriptomics technology. Furthermore, recent proteomics studies performed with LAB are reviewed, including the use of proteomics in analyzing the stress response to various conditions that LAB may experience in the GIT. Finally, the potential of metaproteomics to study the functionality of the gut microbiota is addressed.

In **Chapter 3**, a 2-DE proteome reference map is described of *L. plantarum* WCFS1 grown until the late-stationary growth phase. Furthermore, the dynamics of the proteome were analyzed during growth and revealed that specific sets of proteins were regulated in each growth phase.

Chapter 4 describes the analysis by, integration of two major –omics technologies, transcriptomics and proteomics, of *L. plantarum* WCFS1 and an isogenic CcpA-negative mutant. It has been shown using the R-IVET approach that the genes involved in the phosphotransferase systems for sugar uptake are induced during passage through the gastrointestinal tract of mice (29).

Components of these systems are activators of the CcpA regulator. Furthermore, the differences between both -omics approaches are discussed.

Chapter 5 aims to describe the evaluation of a fluorescent *in situ* hybridization (FISH)-based method targeting the 16S rRNA of *L. plantarum*. The feasibility of using FISH in combination with flow cytometric separation in order to sort the fluorescently-labeled *L. plantarum* from other unlabeled bacteria in the intestinal samples is discussed.

Chapter 6 provides an alternative approach to isolate specific bacteria based on immunomagnetic separation using *L. plantarum*-specific antibodies. The specificity of this separation was analyzed using quantitative PCR. This approach has been applied to an ileostomy sample to provide insight into the proteome of *L. plantarum*. By using the proteome reference map, a variety of *L. plantarum* proteins that are produced under intestinal conditions could be identified.

In **Chapter 7**, general conclusions are drawn based on the research described in this thesis. Future perspectives in this research field and the high throughput methodology of proteomics are discussed.

Chapter 2

A Proteomic Approach to Study Lactic Acid Bacteria

DAVID PA COHEN, ELAINE E VAUGHAN, WILLEM M DE VOS

AND ERWIN G ZOETENDAL
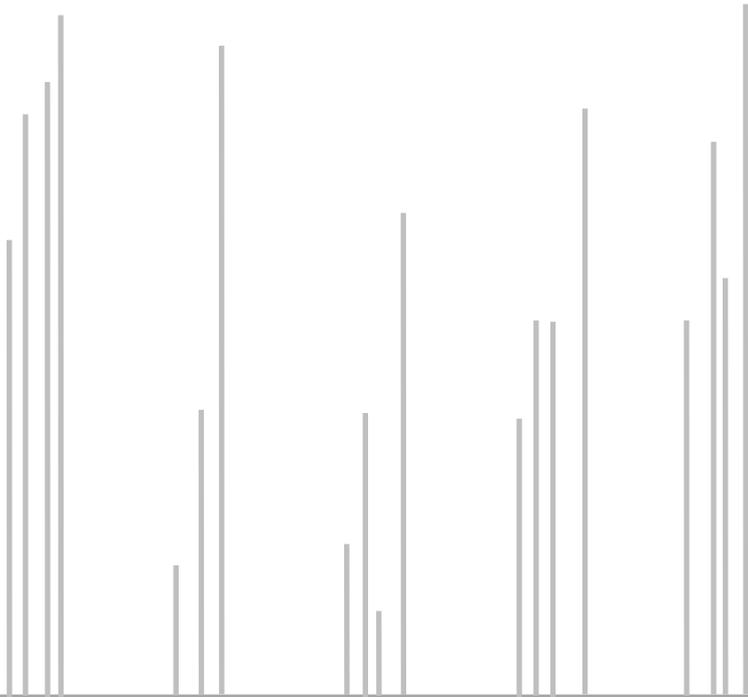
2.1 Introduction

Biology aims to describe, understand and predict the functionality of living cells, tissues, organisms, and ecosystems. While biological studies for a long time have been based on a reductionistic approach, in recent years more global approaches have been developed. In a reductionistic approach, a complex biological system is analyzed by reducing it into smaller subunits, making it easier to study its properties (199). However, analyzing a single trait (gene, protein or metabolic process), will not automatically lead to a complete understanding of a living organism (220). High-throughput approaches that allow simultaneous investigation of more than one parameter will ultimately lead to better understanding of the organism's behavior. Examples of such high-throughput approaches are genomics, transcriptomics, proteomics, and metabolomics. The suffix –`omics' refers to the study of aggregates of an entity, group, or mass, like genome, proteome, transcriptome, or metabolome (199). Genomics is the (systematic) study of the (entire) genome of an organism, which provides information on its genetic potential. On the other hand, transcriptomics, proteomics, and metabolomics focus on the total gene expression, protein synthesis or metabolite production, respectively, of the organism under certain conditions and therefore reflect its activity.

In this chapter, we focus on the application of proteomics in microbiological research. Special attention is given to the lactic acid bacterial (LAB), including members of the genera *Lactobacillus,* which are natural inhabitants of the human gastrointestinal (GI) tract, that are marketed as probiotics, and are important in the food industry because of their long history of safe use as starter cultures for industrial food fermentations.

LAB are ubiquitous bacteria that inhabit a wide variety of habitats, which include the GI tract of animals and decomposing plants, and they are traditionally used in the manufacture of fermented and functional foods (66, 68, 256). Amongst the various LAB, notably *Lactobacillus* strains, are also marketed as probiotics for which they are often claimed to have a beneficial effect on gut functionality (168).

LAB consist of a heterogenous group of bacteria, including the genera *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus,* and *Weissella,* which all share the common property that they are able to produce lactic acid by fermenting a wide variety of carbon sources (26). Furthermore, they

are gram-positive, non-motile, non-spore forming and have rod or coccus morphology. Among the LAB, *Lactobacillus* are represented by 32 species that are found as members of the commensal microbiota of humans (190), being attached to or closely associated with body surfaces covered by epithelial cells (232). Like other commensal bacteria, these LAB can contribute to regulate host processes such as nutrition and development, to immune modulation, functionally regulating both health and disease (104).

As LAB have a wide adaptation capacity in order to survive under a variety of stress conditions that they often undergo in these ecosystems and hence the secrets of these survival strategies may be reflected in their proteomes. Proteomics has been used to investigate the functionality of lactic acid bacteria during preparation or fermentation of food, or their response towards certain stress conditions that they may encounter during passage of the human GIT, like bile salt and acid stress. Proteomic approaches make it possible to gain insight into the relative abundance of proteins during a certain condition and with this knowledge it might be possible to predict which proteins of LAB are involved in survival under harsh conditions such as acid and bile salt stress. This can be of high importance for some members of the LAB group, which have been claimed to have a potential health effect on the human host when ingested. By knowing the relative abundance of the proteins during acid shock, a similar proteome can be expected when the same organism is exposed to gastric acid during passage through the humane GI tract. Following an introduction into the developments of proteomics, we describe below the major findings that were obtained by studying the proteome of LAB, especially under the acid and bile salt stress conditions as indicated above.

2.2 Genomics, Transcriptomics and Proteomics

During the past decades, the determination of genome sequences of a wide variety of organisms has increased explosively. Because of their relatively small size, most sequenced genomes derive from microbial species, and up to now, 536 bacterial genomes (http://www.ncbi.nlm.nih.gov/genomes/static/eub_g) and 42 genomes from Archaea (http://www.ncbi.nlm.nih.gov/genomes/static/a_g.html) have been fully sequenced. This genomic explosion has resulted in a new research area, termed genomics, which is the study of the entire genome of an organism.

So far, complete genome sequences of a dozen LAB used in the food industry or marketed as probiotic cultures have been reported (Table 1).

Table 1: Published genome sequences of the LAB and their size.

Organism	Size (Mbp) genome	Reference
Lactobacillus acidophilus	2	(7)
Lactobacillus brevis	2.35	(153)
Lactobacillus casei	2.93	(153)
Lactobacillus delbrueckii subsp. *bulgaricus*	1.86/1.9	(153, 235)
Lactobacillus gasseri	1.9	(153)
Lactobacillus johnsonii	2	(188)
Lactobacillus plantarum	3.35	(126)
Lactobacillus reuteri	2	(47)
Lactobacillus sakei subsp. *sakei*	1.88	(38)
Lactobacillus salivarius subsp. *salivarius*	2.13/2.5	(41)
Lactococcus lactis subsp. *cremoris*	2.56	(153, 247)
Lactococcus lactis subsp. *lactis*	2.4	(25)
Leuconostoc mesenteroides subsp. *mesenteroides*	2.04	(153)

Since more and more genomic sequences are being unraveled, comparative genomics can give insight into phylogenetic and functional diversity of these bacteria to reconstruct ancestral gene sets as well as gene losses and gains. Comparative genomics may also contribute to provide insight into the genetic organization, dynamics and control, and significantly advance the predicted functions of LAB in the GI tract (7, 153). Genomics forms the basis for functional or post-genomic studies, such as transcriptomics and proteomics (Fig. 1). The subset of genes transcribed in an organism is called the transcriptome, which is the (dynamic) link between genome, protein, and cellular phenotype (210). Transcriptomic approaches allow the expression of the genome to be studied and have been successful in determination of changes in gene expression using specifically designed micro-arrays (31, 183). Although transcriptomics have been used for functional analysis and proven to be fast, easy to apply, and generate a massive amount of data, there are several drawbacks of this approach. Transcriptomics data represent snapshots of the present as they monitor the

mRNA concentration and because of the fast turnover rate of the mRNA of prokaryotes, sampling methods can be limiting to obtain high quality of mRNA needed for micro-array hybridization. Furthermore, environmental contamination of the samples can affect the quality of the micro-array hybridization (258). On the other hand, because the concentration range of the mRNA is not as wide as that of proteins, the detection and quantification of mRNA is easier.

Genomics — Gene

Analysis of organism's (whole) genome to understand function and structure.

Transcriptomics — RNA

Analysis of genome/gene expression or transcripts, giving insight in the functionality of an organism.

Proteomics — Protein

Analysis of proteins to study functionality of an organism.
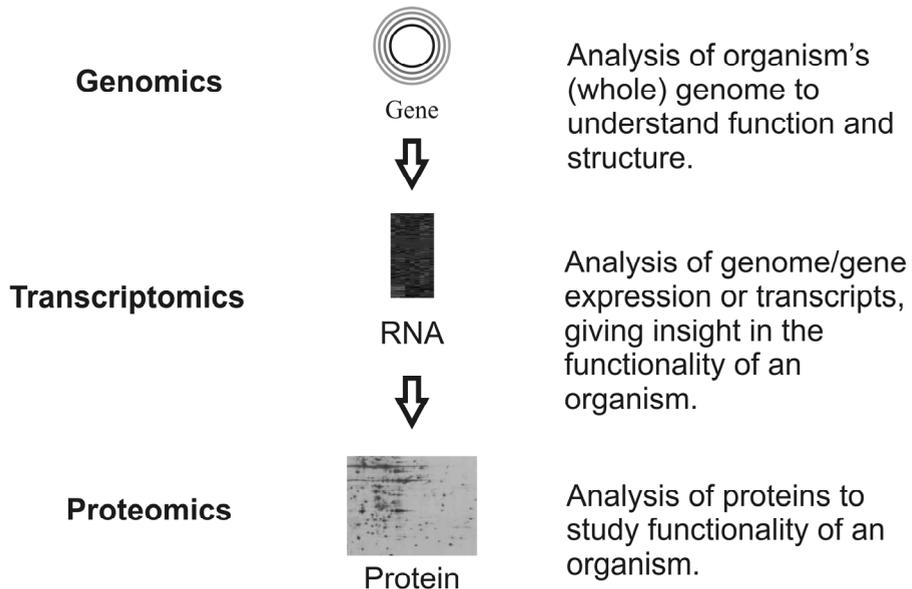
Figure 1. Schematic overview of different –omics techniques. The different techniques analyze at different levels the functionality of an organism by extrapolating to phenotype, adaptation, or fitness.

Consequently, the detection of all mRNA molecules can be achieved more easily and therefore transcriptomics is believed to give a more comprehensive view of gene expression of the whole genome in comparison to other 'omics' technologies (210).

Proteomics is a very powerful tool to study cellular functionality because of the direct link between the phenotype of an organism and its proteins. Fitness and adaptation to the environment is related directly to protein activity and protein abundance rather than the quantity of mRNA molecules (71). Transcriptomics cannot accurately predict the abundance of proteins because protein concentration depends on the rates of protein synthesis and degradation. Another important phenomenon that cannot be extrapolated from transcriptomics data is

the post-translational modification (PTM) of proteins like phosphorylation, glycosylation and acetylation. PTMs affect the physicochemical properties of proteins, dictation of protein conformation, cellular location, macromolecular interactions and activities. These depend on the cell type, the tissue and the environmental conditions. Although investigating these PTMs remains a challenge, several effective proteomic strategies addressing PTMs already have been developed (113).

Thus, not only the concentration of proteins but also PTMs have major impact on many cellular processes. Hence, simple deduction from mRNA transcript analysis to quantitative analysis of protein production is insufficient (89). This pleas for using the proteome as a basis to study functionality in relation to phenotype, adaptation, and fitness while keeping in mind that proteins are the major contributors to functionality and that they are in fact the one and only end-products of gene expression.

2.3 From Protein to Proteomics

The chemical analysis of proteins and their functions started to evolve around 1940 (74). This resulted in the development of protein (bio)chemistry, which is an example of a reductionistic approach as it provides the link between activity of a pure protein and the gene that encodes it (179). The subsequent development of analytical protein chemistry resulted in technical improvements of protein separation and the sensitivity of methods for identifying proteins. A major technical breakthrough, was the Edman degradation for the identification of the N-terminal sequence of proteins and this could be used for identification of proteins isolated from 1-dimensional and 2-dimensional electrophoresis (1-DE and 2-DE, respectively) (2, 85). Initially this method had a poor sensitivity and was slow, but further optimization allowed the identification of micrograms of proteins separated by isoelectric focusing in immobilized pH gradients (3).

Simultaneously, the number of databases containing (partially) protein and/or gene sequences expanded and therefore the identification of proteins by Edman degradation and subsequent database comparison has become more accurate and reliable (179). Meanwhile other methods were investigated to speed up the identification of proteins as the Edman degradation was still relatively slow. Mass spectrometry (MS) was already used intensively for identification of small

molecules and by the late 1980's two methods were developed making it possible to analyze peptides and proteins by MS with high sensitivity and without too much fragmentation of the molecule. These included matrix-assisted laser desorption/ionization (MALDI) and electronspray ionization (ESI). Because of their success to ionize large molecules, which is essential for MS, robust ESI and MALDI mass spectrometers became commercially available, enhancing the protein chemistry community. MS was much more sensitive than Edman degradation method, and it has been estimated that nowadays MS allows identification of proteins in the femtomolar range (85).

From 1993 on, several groups were able to correlate the data obtained from MS with protein sequence databases (11) and in 1994 it was possible to use the fragment spectrum as specific identifier of a peptide (67). This methodology is still used and is known as peptide mass fingerprinting. In the same year during the first 2-DE meeting in Sienna, Italy, the term proteome was coined, and the process of studying the proteome became known as "proteomics" (179). Nowadays, the term proteomics can be defined as "*the large-scale characterization of the entire protein complement of a cell line, tissue, or organism.*" (85).

2.4 Proteomics

Several techniques can be used to characterize the proteome. All consist of two main steps: separation of the protein from the mixture and identification of target proteins. In principle, there are two mainstream separation methods used in proteomics: gel-based and non-gel based approaches. For the gel-based method, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used for separation of protein mixtures. For the non-gel based approach, a chromatography-based method is applied to separate proteins from the mixture.

2.4.1 Gel based methods

In many cases 1-DE by SDS-PAGE is used for resolving relatively simple protein mixtures obtained after purification for the desired protein fraction. With this method the proteins will be only separated based on molecular weight (MW). The obtained protein bands can be easily excised from the gel and identified with MS. The major advantages of this method are its speed, simplicity and reproducibility

but in case of a more complex protein mixture, this method is not desirable because of the limited resolution.

To increase the separation resolution of proteins, separation in two dimensions (2-DE) was developed. 2-DE is an established and robust technique that was first developed by O'Farell in 1940 and its development is still ongoing. For highly complex protein mixtures, such as crude cell lysate, 2-DE has the advantage that this method separates on two dimensions: the first dimension is on isoelectric point or isoelectric focusing of the proteins (IEF) and the second on MW. Consequently, the resolving power is increased dramatically. Hundreds of different proteins can be separated individually and visualized by 2-DE gel and with thorough analysis of the gel up- and down-regulation of proteins including those that have undergone PTM can be detected. A widely applied workflow for gel-based proteomics is depicted in Figure 2.

By applying different pH ranges in the first dimension, one can zoom in on an area of interest. Using a pH range between 4 and 7, only proteins that have an isoelectric point between 4 and 7 will be separated and visualized on the gel. The use of a smaller pH range when performing isoelectric focusing results in an increase in the resolving power of the gel. By simply choosing the desired pH range, a greater separation of a portion of the complex protein mixture can be performed in this way.

Increasing the gel size in the second dimension significantly enhanced the resolution, in this context that is the number of protein spots per gel, as demonstrated by Inagaki and colleagues (109): a gel (93 cm x 103 cm), composed of several long-gels (dimension 24 cm x 70 cm x 1 mm) showed more than 11,000 protein spots that are produced in a dynamic range from $1-10^5$ in cells versus a standard (18 cm x 20 cm) gel display of about 800 spots with silver staining. Thus by increasing the resolution (gel size) a larger part of the proteome can be visualized and analyzed.
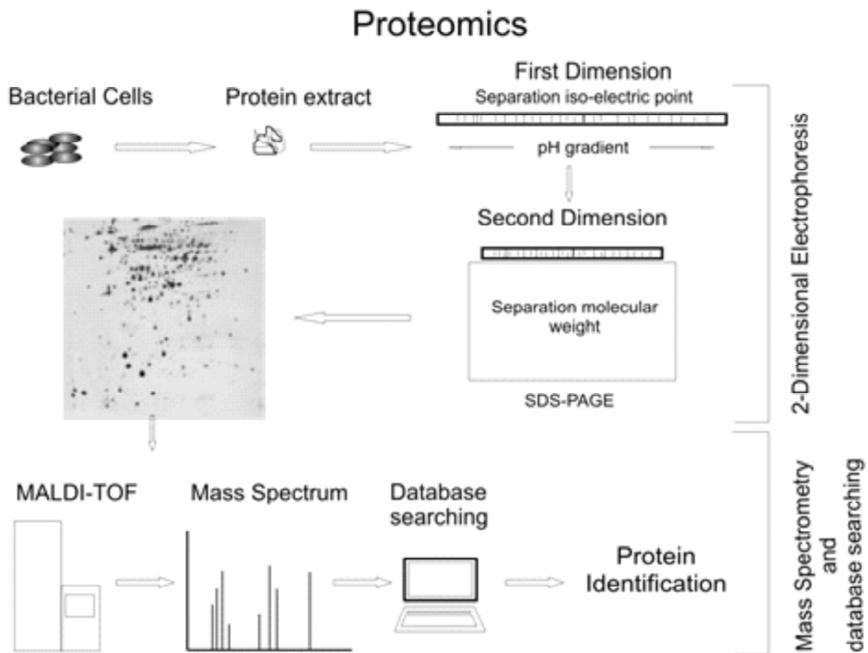
Figure 2. The gel-based proteomic method can be divided into two main divisions, separation of protein complex and identification of the proteins, respectively. The separation of the proteins is based on the protein's iso-electric point and the molecular weight. Identification of the proteins is commonly achieved using MALDI-TOF mass spectrometry and with the mass fingerprints obtained identifications can be made using a database.

The next step following separation of proteins for a gel-based approach is the visualization of the protein spots in the gels. There are many methods available but one should be chosen based on the intended use of the visualized samples. For quantification of protein spots the used detection method should have a wide dynamic range and linear correlation between the amount of protein and the intensity of the staining (250). Another demand for the detection method is that it should be compatible with MS in case of desired identification of the target spot. Staining methods that are commonly used include Coomassie Brilliant Blue-based stain, silver-based staining, staining by fluorescent dyes, and auto-radiography. These staining methods have all their advantages and disadvantages (see Table 2) and their choice, therefore, depends on the study design.

Table 2. Different staining methods used to visualize the proteins in a two-dimensional electrophoresis gel. All these methods are compatible with mass spectrometry for identification of proteins.

Staining Method	Detection limit	Advantage	Disadvantages	Reference
Coomassie Brilliant Blue	0.1 ug	Very easy to apply	Not sensitive	(246)
Fluorescent labeling	1 ng	Sensitive	Requires laser scanner	(234)
Fluorescent stains	2-8 ng	Easy to apply	Expensive	(246)
Negative Stains	15 ng	Fast and easy	Not suitable for quantification	(163)
Radioactive labeling	< 1 pg	Sensitive	More difficult to handle	(250)
Silver Staining	0.05-0.2 ng	Sensitive	Small dynamic range	(115)
Stable Isotope labeling	6-8 ng	Sensitive	Need living cells	(198)

2.4.2 Matrix Associated Laser Desorption/Ionization Time Of Flight MS

After visualization of the protein spots in the gels, identification of the protein (of interest) spots is generally desired. In 1989 fast atom bombardment to ionize peptides had evolved and the new method of peptide mass fingerprinting for faster identification of proteins was presented (101). When a peptide/protein is digested with a known enzyme, the sizes of the resulting fragments can be predicted, forming a fingerprint for that protein. With the arrival of MALDI-Time Of Flight (TOF) and ESI-MS (see table 3A&B) techniques it was possible to ionize fragments larger then 20 kDa. In case of MALDI, a laser beam is adsorbed by the samples imbedded in a matrix, giving sufficient energy to form ions. The generated ions have a low charge state (*m/z*) leading to a low complex spectra when mixtures of proteins and peptides are used (90). Furthermore MALDI-TOF can be automated resulting in high throughput proteomics.

Table 3. List of ionization methods (A) and available mass spectrometers (B) that are commonly used in proteomics. These instruments can be used as a single apparatus for mass spectrometry or combination of mass spectrometers for tandem MS/MS analyses.

A

Ionization Method	Short Description	Reference
Matrix Associated Laser Desorption/Ionisation	The energy of the laser will ionize the sample	(226)
Electrons Spray Ionisation	Analyte exists as ion in solution and leaves charged capillary as aerosol	(72)
Desorption electron Spray ionisation	Collision of charged particles on surface of compound produces gaseous ions	(224)
Fast atom bombardment	sample is ionized by bombardment of atoms from inert gas	(15)

B

Mass spectrometer	Short Description	Reference
Time of Flight	Measures the time of ions to travel from the sample to the detector	(254)
Quadrupole	Filters ions based on mass-to-charge ration by selectively stabilizing or destabilizing ions	(157)
Quadrupole ion trap	Same principle as "normal" quadrupole but ions are trapped and sequentially ejected	(131)
Linear quadrupole ion trap	Similar to quadrupole ion trap but traps ions in a two-dimensional quadrupole field	(37)
Fourier transform ion cyclotron resonance	Detects the current produced by ions traveling cyclotronic in magnetic field	(161, 162)
Orbitrap	Ions are trapped electrostatically and orbit around a central electrode	(106)

2.5 Non-gel based methods

The main advantage of non-gel based technique is that no gel is used, making it more suitable for the use of hydrophobic and basic proteins because the resolution of these proteins is rather poor in gels. Moreover, this non-gel based technique can be automated leading to high throughput analysis. Using non-gel based methods, protein mixtures are digested in solution with several specific proteases, including trypsin, generating an assortment of different peptides. These peptides are analyzed by liquid chromatography (LC), using a reverse phase capillary column, coupled with nano-ESI-MS. The dynamic range of this analytic technique is similar to that of the gel-based 2-DE. Currently, this non-gel based method is still developing to increase the separation efficiency by including more

columns. However, protein quantification and detection of post-translational modification are poorly detected with this method (198).

Another non-gel based chromatography technique is the use of a Surface Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF MS) (227). The chromatography is performed on a solid-phase affinity surface. The surface is designed to have affinity for proteins based on chemical (anionic, cationic, hydrophilic, etc) or biochemical (antibody, receptor, enzyme, etc) properties. In the case of a surface coated with antibody or receptor or other biochemical agents, specific proteins will bind to the solid-phase extractor. The captured proteins will be detected by laser/desorption ionization time-of-flight mass spectrometry and these proteins will be displayed as a series of peaks. The generated output shows the relative abundance versus the MW of detected proteins and this can be visualized in a simulated 1-DE gel using multivariate analyses and artificial neural networks (87). Thus the end-result of SELDI-TOF MS is a list of molecular weights of proteins whose relative abundance differs significantly between the analyzed samples. The next step is to actually identify the proteins that are differentially produced. This is achieved by purification of the proteins that matches the molecular weight that is differentially produced in the SELDI-TOF MS analysis. After purification of the desired protein, standard MS-based techniques like peptide mass fingerprint or tandem MS can be applied to identify the protein. SELDI-TOF MS is a powerful method, which is used regularly for diagnostic purposes, such as identifying biomarkers produced by specific tissues/organisms.

2.6 Tandem MS/MS

In certain cases, peptide mass fingerprinting is not sufficient to identify the peptide digest. This can be due to the low amount of the peptide of interest in the mixture, the unknown origin of the protein or the fact that the protein has never been discovered or characterized before. Tandem mass spectrometry can select, isolate and sequence a single peptide ion in the presence of other detected peptides (180). By *de novo* sequencing of the peptide, the amino acid of the peptide will allow *in silico* analysis of the newly identified protein. Furthermore, tandem MS/MS allows the identification of PTM of the molecule (12). Tandem mass spectrometry is in principle sequential mass analyses by different mass

spectrometers. For the ionization of the peptide, ESI is commonly used because it generates multiple charged ions of peptide fragments. Furthermore, for larger molecules, multiple charging by ESI facilitates ion dissociation and more fragmentation of the peptide as compared to singly charged ions generated by MALDI ionization. In the first mass spectrometer, the different ions are selected based on their *m/z* value. The selected ions are brought into a collision chamber where the target ions are fragmented by collision. In the second mass spectrometer the fragments are further analyzed.

There are many combinations of tandem MS/MS instruments (Table 3B) possible and the most recent commercially available instrument is the Orbitrap mass spectrometer. It is transform ion cyclotron resonance mass spectrometer, it has high sensitivity, mass accuracy but in addition it has a good dynamic range (106).

2.7 State of the Art of Proteomics of Lactic Acid Bacteria

In this section, we highlight the major results that were obtained by studying the proteome of LAB under various conditions. Two separate proteomic research strategies have been applied frequently to LAB. These include the construction or systematic indexing of proteins, and, analyzing the response of these bacteria towards a certain stress inducer inducing changes in the proteome investigated by proteomics approach.

2.8 Proteome Reference Map

Systematic identification of proteins during a specific condition will result in the construction of a map containing a part of the proteome. This map provides insight into protein production during specific conditions such as adaptation, stress and growth, and this will eventually provide fundamental knowledge of the metabolic state in which a bacterium currently exists. Furthermore this map is a powerful tool for fast indexing of known proteins in a gel for future experiments. Predicted proteins without assigned function or hypothetical proteins can be traced back on a proteome reference map, confirming the presence of those proteins. In addition, the identification of proteins that are not predicted during genomics analysis may be discovered by a proteomics approach as was the case for four proteins in *Mycoplasma pneumonia* (194).

The first proteome reference map (PRM) for a LAB was initiated for *Lactobacillus bulgaricus* (40). More than 700 proteins from the proteome of *Lactobacillus bulgaricus*, after culturing until the exponential growth phase in a chemically defined medium MPL, were visualized in gel, however, no identification of proteins was performed (143). Recently, from the proteome of *Lactobacillus sp.* 30a and *Lactobacillus. sp.* W53, two strains isolated from amine-contaminated wine, 22 spots (Table 4) were identified in the pH range 4 to 7 after culturing in MRS medium until exponential or early stationary growth phases (181). A more extensive PRM of *Lactobacillus plantarum* strain WCFS1 cytosolic fraction isolated from mid- and late-log, early- and late-stationary phase cells grown on MRS was constructed, and around 200 spots (Table 4) were identified using a pH range between 3 and 10 (45). More recently, the proteomes of two other *L. plantarum* strains were investigated using the pH range 4-7 and in total, 231 proteins were identified and indexed (129). Furthermore, 39 and 64 proteins spots were found to be differentially regulated during growth of the REB1 and MLBPL1 strains, respectively. The combination of these two studies will give extensive information about the proteome of *L. plantarum* in terms of a more global (pH 3-10) and detailed (pH 4-7) view.

The PRM for the *Lactococcus lactis* NCDO763 proteome was isolated after growing the cells in a defined medium (114) until mid-exponential phase (8). Using a pH range between 4 and 7, about 400 spots were visualized and 18 spots were identified by MALDI-TOF or N-terminal (protein) sequencing. By focusing in on more specific areas (pH ranges 4-7 and 4.5-5) of the proteome of *Lactococcus lactis*, about 800 spots were visualized and of those 330 spots (Table 4) have been identified (81). The obtained PRM was applied to identify differentially produced proteins when *Lactococcus lactis* was grown on different media including synthetic medium M17 with lactose, skim milk microfiltrate, and milk. Thus the acidic part of the proteome of *Lactococcus lactis* has been very effectively visualized. Numerous

Table 4. Proteome reference map of Lactic Acid Bacteria. The identified proteins visualized on the proteome map have been indexed into functional classes.

Functional class	Lactobacillus plantarum (45, 129)	Lactococcus lactis NCDO763 (13, 81)	Lactococcus lactis (62)	Lactobacillus sp 30a & w53 (181)	Lactobacillus delbrueckii (143)
Amino Acid metabolism	64	22			
Carbohydrate Metabolism	97	5		12	
Cell envelope			5		
Cell growth and Death	7		10		
Cofactors metabolism	1				
Energy metabolism	9			3	
Fatty Acid metabolism	7	2	4		
Folate metabolism		2			
Folding	8				
Membrane transport	12	8	6	4	
Miscellaneous	58	8	6	4	
Nucleotide metabolism	49	15	2		
Replication and Repair	10	1	4		
Signal transduction	3				
Stress proteins	20				3
Transcription	9	2	3		
Translation	25	2	47		
Unknown function	29	9	20		

enzymes were identified especially enzymes involved in glycolysis, but also glutamine synthetase, an essential enzyme needed for development of *Lactococcus lactis* in dairy products, was identified.

A PRM of the alkaline part of the proteome of *Lactococcus lactis* ranging from pH 6 to 12 has also been constructed, more than 200 proteins spots were observed and from those spots 152 proteins have been identified (62). Because the alkaline part of the proteome contains mainly hydrophobic proteins, no proteins associated with glycolysis were identified but most of the identified proteins were involved in translation or their function could not yet be predicted (Table 4).

The proteins identified in the PRMs of the various *Lactobacillus* and *Lactococcus* spp. can be indexed systematically into functional groups according to

the KEGG database (123). By comparative analysis of these PRMs, certain processes were demonstrated to be present despite the different culture media, physiological growth phase of the bacteria and the involved species, indicating the necessity of those processes. However, the major advantage of the PRM is that it can be applied to future experiments, facilitating fast identification of proteins. This was demonstrated with the analysis of the proteome during different growth phases of *Lactobacillus plantarum* WCFS1 in MRS medium (45). Proteins that were differentially regulated during early-log, mid-log, early-, and late stationary phase of growth were initially identified via the PRM and the majority of the remaining differentially produced proteins were identified with MS. It was shown that for each growth phase a different set of proteins were differentially produced: during mid-log phase the highest priority was to achieve energy; during late-log phase biosynthesis of many macromolecules and cell division proteins became predominant; during early-stationary phase alternative pathways to generate pyruvate became more important; and during the late-stationary phase strengthening of the cell wall occurred as well as an increase in stress proteins to encounter unfavorable conditions were a key activity.

In conclusion, systematic analyses of the proteomes of *Lactococcus lactis* and various *Lactobacillus* spp. have unraveled a part of the basic metabolism of these organisms. Construction of a PRM and subsequent monitoring of the proteome dynamics is a more fundamental approach of proteomics to study basic concepts such as focusing on the metabolic activity during growth in a standardized medium. A different approach is to study the differential regulation of the proteome of an organism to several (stress) conditions by analysis of the regulation of proteins during exposure or adaptation to certain stimuli, and this will be reviewed below.

2.9 Stress Adaptation/Response

Much research on the stress response of LAB has been performed to investigate the changes of the produced proteome during harsh environments that the bacteria are likely to encounter during passage of the GIT and food processing. Notable conditions are gastric and other organic acids as well bile salt secretion and other enzymatic fluids in the duodenum. Acidic environments can also be encountered during food preparation or preservation. LAB metabolize the carbon

source to lactic acid during growth; in order to survive the decrease in pH, these bacteria show an inducible mechanism called the acid tolerance response (ATR), which was first reported and described in *Lactococcus lactis* (32, 75). The ATR was induced by challenging cells of *Lactococcus lactis* initially in a mildly acidic environment prior to a more severe acid concentration. Analyses of the 2-DE gels revealed that in *Lactococcus lactis* several functional classes were induced by acid stress namely carbohydrate metabolism, translation or amino acid metabolism, and pH homeostasis and stress response (Table 5). The proteins belonging to the latter can also be associated with heat shock, oxidative, osmotic, cold shock and DNA repair, and may be a homologue of LuxS (75). The *ahpC* and *sodA* genes coding for alkyl hydroperoxide reductase and superoxide dismutase, respectively, were also shown to be involved in oxidative stress by proteomic approaches. Low pH also induced LuxS, which is a component of the auto-inducible-mediated bacterial intercellular communication (203). DnaK and GroEL, being induced during acid stress, are typical heat shock proteins involved in refolding of proteins during heat stress and have been demonstrated to be induced in lactobacilli during heat stress as well (51, 60). The up-regulation of these proteins during acid stress has been confirmed in *Lactococcus lactis* MG1363 by proteomics (Table 5) (32). In *Lactococcus lactis* CNRZ 157, it has been demonstrated by both proteomics and transcriptomics that DnaK was induced in the stationary phase due to increased lactic acid concentration (Table 5) (136). However, besides the obvious stress proteins also proteins involved in metabolic pathways were shown to be differentially regulated by adaptation to acid stress. Remarkably, low pH induced a switch to alternative carbon sources other than glucose and the formation of acids to neutral compounds was up-regulated. The genes coding for the proteins involved in the ATR - as mentioned above - have been shown to be up-regulated by transcriptomics (32). Thus, not only the production of stress proteins in order to counteract the low pH but also active changing of the metabolism to production of neutral end products in respect to pH compounds is occurring during ATR.

An ATR has also been reported for *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* spp. *bulgaricus.* For these microorganisms it was demonstrated using 2-DE that GroES/EL and DnaK proteins were up-regulated during exposure to acidic conditions (Table 5) (111, 143). Furthermore, transcriptomic analysis of the acid response of *Lactobacillus plantarum* WCFS1

showed a decrease in the transcription ratio of the gene coding for GroEL suppressor protein SugE (183), confirming that relative concentration of the stress protein GroES during acid stress increased. During acid adaptation it was confirmed that the heat shock proteins DnaK and GroEL were induced in *P. freudenreichii* together with increased RecR and RepB proteins (111). In *Bacillus subtilis* the RecR is involved in DNA recombination which can be induced by bile salts (6). In *E. coli*, these genes are involved in DNA repair and replication, respectively, and were shown to be induced by entering the stationary phase, during starvation, by mutagenic agents and bile salts (19, 175).

After passage of the stomach, the next severe condition encountered is bile in the small intestine, which the LAB must survive, in order to transit or colonize the intestine. The heat shock protein DnaK and another typical heat shock protein Hsp20 were found to be induced when *P. freudenreichii* was exposed to bile salts. So far, only the proteome of *P. freudenreichii* has been investigated during bile salt stress (141). Analysis of the gels revealed 24 distinct protein spots that were associated with protein protection/degradation, heat shock, oxidative stress, signal sensing and transduction, and an alternative sigma factor. These results suggest that during bile salt adaptation a general stress and a signal sensing responses are induced. When *P. freudenreichii* was subjected to acid, heat and bile salt stress, 6 proteins were found to be induced in common from which three proteins could be identified as DnaK, ClpB, and SodA. It was suggested that *P. freudenreichii* has the ability to acquire homologues tolerance to different forms of stresses (142).

Table 5. Stress response of Lactic Acid Bacteria. The proteins produced by LAB during different forms of stresses.

	Acid			Heat		
L. lactis (32, 75)	*P. freudenreichii* (111, 141)	*L. delbrueckii* (143)	*L. helveticus* (60)	*L. plantarum* (51)	*P. freudenreichii* (10, 141)	*L. lactis* (93)
AhpC	BCCP	DnaK	DnaK	CspC	ClpA,B	GroEL
ArcA,B	EF-Tu	GroEL	Thioredoxin reductase	DbpII	DnaK	GroES
AtpA,F	Enolase	GroES	TuaH	DnaK	GroEL	
BusAA	GroEL		Acetyltransferase	GroEL	SSB	
Cfa	GroES		DbpII	Ribosomal proteins		
ClpB,C,E,P	Malate dehydrogenase		Dps	Trigger factor		
CspE	RecR		Enolase			
DnaK	RepB		GapDH			
EraL			GroEL			
GadB			Hsp20			
GroEL			Pyruvate kinase			
GrpE			Ribosomal proteins			
Hpr						
LuxS						
RecA						
SodA						
Tig						
Tpx						
YxbE						

Table 5 (continued)

Oxidative Stess	HHP	Bile Salt	Cold/NaCl shock
L. lactis (242)	*L. sanfrancisensis* (63, 105)	*P. freudenreichii* (141, 142)	*L. sakei* (158)
Alkylhyldroperoxide reductase	ClpL	ClpB	Asp-23
Malonyl-CoA acyl carrier protein	CspE	Cysteine synthase	GapDH
NADH oxidase	DnaK	DnaK	MsrA
β-phosphoglucomutase	EF-Tu	Hsp20	Ohr
Pyrroline-5-carboxylate reductase	GMP synthase	OppD	PFK
Acetyl-CoA acetyltransferase	GroEL	Oxidoreductase	Usp
Pyruvate dehydrogenase	Maltose hydrolase	Signal transducer	
Transcription elongation factor	RbsK	SodA	
GapDH	Thioredoxin reductase		
SodA	YudG		

After passage of the stomach, the next severe condition encountered is bile in the small intestine, which the LAB must survive, in order to transit or colonize the intestine. The heat shock protein DnaK and another typical heat shock protein Hsp20 were found to be induced when *P. freudenreichii* was exposed to bile salts. So far, only the proteome of *P. freudenreichii* has been investigated during bile salt stress (141). Analysis of the gels revealed 24 distinct protein spots that were associated with protein protection/degradation, heat shock, oxidative stress, signal sensing and transduction, and an alternative sigma factor. These results suggest that during bile salt adaptation a general stress and a signal sensing responses are induced. When *P. freudenreichii* was subjected to acid, heat and bile salt stress, 6 proteins were found to be induced in common from which three proteins could be identified as DnaK, ClpB, and SodA. It was suggested that *P. freudenreichii* has the ability to acquire homologues tolerance to different forms of stresses (142).

For *Lactobacillus plantarum* no proteomics has been performed to investigate the effect of bile salts. However, transcriptomics is available and shows that some contradictions between *Lactobacillus plantarum* and *P. freudenreichii* are present. The homologue RecF in *Lactobacillus plantarum* has been down-regulated upon

bile exposure (31) while it has been shown by proteomics that this protein was up-regulated in *P. freudenreichii* as mentioned above. Other responses to stress have been observed as well including induced amino acid metabolism and pH homeostasis (31). After passage of the small intestine increased activity of the genes coding for proteins involved in amino acid metabolism and genes coding for stress proteins including a small heat shock protein and alkaline shock protein were up-regulated in expression due to acidic conditions in the small intestine (53).

The key role players in stress adaptation investigated by proteomics are DnaK, GroEL/ES, SodA, and the Clp protein family. These proteins are being up-regulated under various conditions as described before in this review. Especially the proteins GroEL/ES, DnaK have been found to be produced after exposing to acid, heat, and high hydrostatic pressure (Table 4). The evidence of a potential cross-over protection towards different kinds of stresses have been shown in *Lactococcus lactis* (94, 176) and *P. freudenreichii* (142). Exposure to heat, acid and UV-radiation led to the up-regulation of the GroEL and GroES proteins although quantitative analysis of the 2-DE gels indicated that the strength of induction is dependent of the kind of stress stimulus; GroES has a 12-fold up-regulation during heat stress but only a 3.8-fold up-regulation was observed after exposure to acid, pH 5.5 (94). However, in *P. freudenreichii* cross-protection from acid pretreatment to heat stress did not occur and the acid pretreatment sensitized the cells to bile salt stress (142). This suggests that cross-protection is not reciprocal; bile salt adaptation led to protection against acid and heat but adaptation to acid did not lead to protection against bile salt or heat stress. Furthermore, this indicates that cross-protection differs in organisms or may not occur at all. In spite of the fact that the stress proteins are highly conserved, their regulation may vary in different organisms.

2.10 Regulation of Stress Proteins

The regulation of the stress response by LAB to several stressors can be divided into two main groups: One is the regulation of proteins by a specific stress factor. The second one is the regulation of a set of proteins that are induced as a "non-specific" response to a certain stimulus, meaning that those proteins are not known to be stress proteins e.g. glycolytic proteins. Indeed proteins known to be

regulated by the general stress response regulator, σ^B, have been visualized by proteomics during several different stress inducers. The σ^B–dependent genes are induced by heat, ethanol, acid, bile salt stress, carbon starvation, and in the presence of phosphate or oxygen (63, 215). Proteomics has shown that the heat shock proteins DnaK, GroEL/ES are an example of stress-specific response and are normally induced during heat shock. The genes *dnaK* and *groE,* coding for Dna and GroES/EL, respectively, are controlled by the HrcA-CIRCE repression system which in turn can be controlled by GroEL/ES (236). The Clp protein family was shown by proteomics to be induced during different stress stimuli.
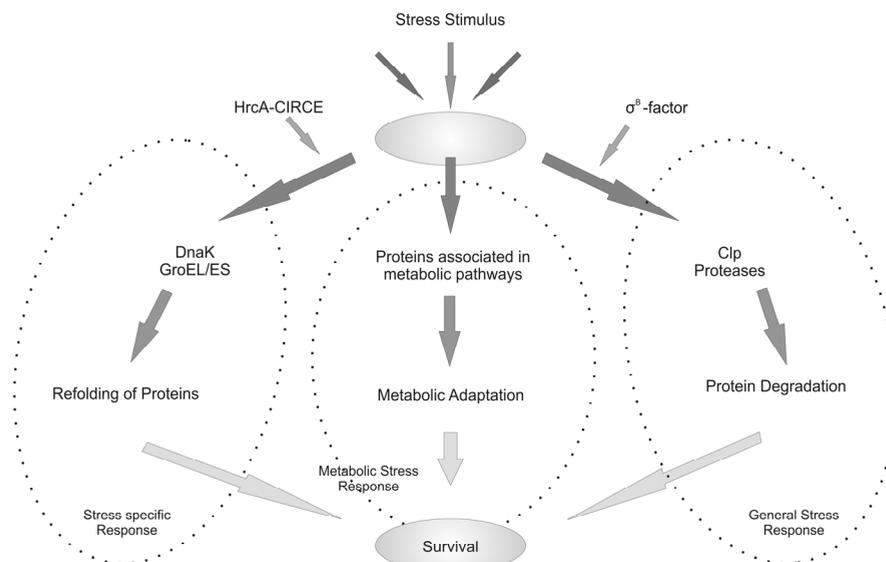
Figure 3. A schematic overview of stress regulation in LAB. Proteins within the dashed circles are associated with several stress responses visualized with two-dimensional electrophoresis. DnaK and GroES/EL are considered to be induced during specific stresses while Clp is a general stress protein. The third group consists of proteins found to be differentially regulated during stress, changing the metabolism of the bacterium towards adaptation of the stress inducer.

Clp proteins are ATP-dependent proteases involved in the repair or protein degradation under normal and stress conditions, and they proteins are under control of the stress response regulator σ^B (63). Although the stress proteins are highly conserved and the responses are similar in the different organisms, the regulation of specific (stress) proteins is still complex towards a stress stimulus.

This is especially the case when up-regulation of proteins that are not obviously associated with stress response, but involved in metabolic pathways occurs due to a certain stress inducer. This indicates that the presence of known stress proteins alone is not sufficient enough to respond to the stress and certainly not to survive for longer periods during unfavorable condition. The glycolytic proteins glyceraldehyde-3-phosphate dehydrogenase (GapDH), 6-phosphate-fructokinase (PFK), and enolase (Eno) show a higher abundance in the proteomic gels during heat and cold shock and oxidative stress, and acid stress, respectively (see Table 3).

Upon cold shock in *Lactococcus lactis* the production of CcpA and HPr was 2/3-fold up-regulated (255). The *pfk* gene is under control of the *las* operon and this operon is subjected to catabolite activation by CcpA-HPr(Ser-P), but it was shown in *Lactococcus lactis* that increased CcpA and HPr did not lead to higher abundance of PFK or GapDH (255) and it was proposed that other factors than CcpA-HPr alone is needed to regulate cold shock and glycolysis. This together indicates that investigation of a few proteins (reductionism) alone is not sufficient to predict the metabolic behavior of LAB during stress and it is expected that a proteomics approach will give a more holistic view of the proteome during (harsh) conditions.

2.11 Metaproteomics

Proteomics is a high-through put methodology, not meaning that this method is fast and simple but it gives a more complete understanding/overview of the changes that are occurring in an organism's proteome during a specific condition/environment than when looking at a single protein change. Up to now, single organisms and monocellular tissues have been investigated using proteomics and this has provided novel insight into relatively simple ecosystems. However, analyzing the proteome of bacteria cultivated under controlled conditions will not result in the understanding of their function in an ecosystem. In fact, studying the details of a single bacterium from an ecosystem is comparable to studying a single protein from a proteome and therefore studying a single bacterium can be seen again as a form of reductionism when the complete ecosystem is considered. Studying samples not from a single organism but from an (complex) ecosystem will provide a better overview of metabolic networks and interactions between different organisms and their environment. The new term

metaproteomics has been proposed by Wilmes and Bond and can be defined as large-scale characterization of the entire protein complement of environmental microbiota at a given point in time (253).

Up to now, a few articles have described the application of metaproteomics for analyzing mixed community of bacteria in sludge, Chesapeake Bay, natural microbial biofilm, and functionality of microbiota in human infant GI tract (120, 125, 191, 253). It was demonstrated that that application of 2-DE of the metaproteome of the very complex human infant GI tract ecosystem is applicable; more than 200 protein spots were visualized on gel and 21 proteins spots were differentially regulated in time (125). However, only one protein spot was identified as transaldolase, which is used in the pentose phosphate pathway to obtain D-fructose-6P molecule and NAD(P)H (KEGG database; (123)). The latter is used to provide the proton motive force for the synthesis of ATP (76). The low number of protein spots identified from the metaproteome of the human infant GIT is in accordance with the metaproteomics of the Chesapeake Bay. This study yielded significant identifications of seven proteins visualized on 2-DE gels (120). However, proteomics of a natural microbial biofilm present in a natural acid mine drainage yielded identification of 5994 proteins corresponding to 2003 unique proteins (191). The success of this study is based on a slightly different approach that will be described below.

Although 2-DE gels and the use of 2D nano-LC have been established to separate the complex protein mixture, simple identification of the proteins by mass fingerprint is unlikely. It is suggested that greater than 97% amino acid sequence identity is required to provide a positive, statistically relevant match with when searching with MALDI-TOF MS data (120). To overcome this problem tandem mass spectrometry can be used to identify protein spots by performing *de novo* peptide sequencing but still not all obtained amino acid sequences will give a high statistically relevant identification of the protein. Complementary analysis of the p*I* and molecular weight of the identified protein with *de novo* sequencing can give a good idea of the identity of the protein of interest. The differentially produced transaldolase, which has the highest probability to be derived from *Bifidobacterium infantis* in the human infant GI tract analyzed by metaproteomics, has been identified using this combination of methodology (125).

A good basis for studying the metaproteome of the human GI tract is to obtain a metagenomic dataset from which proteins can be predicted to be produced. The predicted proteins can then be used for the construction of a new (protein) database which can be used for the identification of proteins analyzed by metaproteomics. This methodology has been demonstrated to be applicable. A metagenomic database of a biofilm has been analyzed and from this database a new protein database has been constructed containing 12,148 proteins and this protein database has been used to identify two-dimensional nano-LC tandem mass spectrometry (191).
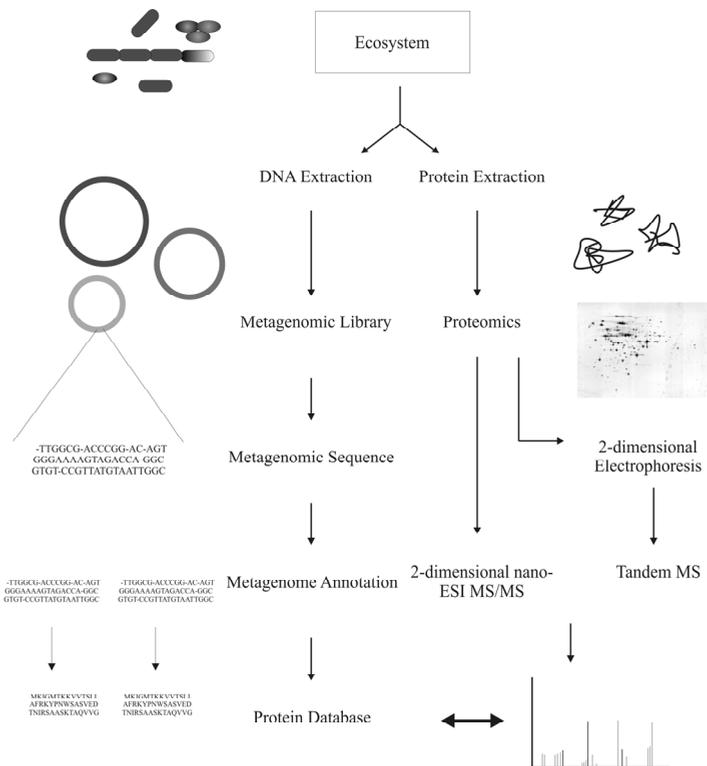
Figure 4. A schematic diagram combines metagenomic and metaproteomic dataset to address gut functionality of Lactic Acid Bacteria. With metagenomics identification of bacterial species is feasible, furthermore protein predictions of active genes of the present bacterial species will facilitate identifications of proteins during metaproteomics.

The combination of a metagenomics combined with metaproteomics datasets has been shown to be successful and this approach can and should be used for future experiments to address the functionality of LAB in gut health. Moreover, to have detailed insight in the interaction between LAB and epithelial cells in the intestine, genomic datasets of the eukaryotic cells and of the bacterial community should be combined into one database from which proteins can be predicted to be produced upon interaction. Ultimately metaproteomics from both eukaryotic and prokaryotic cells can be applied simultaneously to obtain the highly appreciated insight into the interaction between microbes and man for better understanding of the possible health effect of microbes on the human GI tract.

2.12 Future perspectives

To investigate the functionality of LAB that can have a health effect on the human host, analysis of the proteome using 2-DE may provide insights into the metabolic activity and regulation, survival, and possible interaction between microbe and host as proteins are the dynamic link between genotype and phenotype. To have a more global view how the ecosystem, in which the microbe of interest is surrounded, interacts with the microbe, metaproteomics is able to elucidate the question. A possible workflow for metaproteomics is depicted in figure 4. By establishing a specific database containing the prediction of the produced proteins of that particular ecosystem based on metagenomic data containing genes that have been found to be expressed by the microbiota in the gut, will give rise to more identification of protein spots. The first metagenomic libraries from the human GIT have been described (80, 116, 156) and provided already a wealth of information about the genetic diversity within the human GI tract microbiota. Moreover, transcriptomics analysis of the human GIT microbiota are also underway and indicated that genes involved in carbohydrate transport and metabolism are dominantly expressed, especially in the small intestine (27).

Ultimately, complete integration of all –omics techniques is needed to fully understand the complex behavior of an organism in a complex ecosystem, because organisms are much more than the sum of their parts. This will lead to high dimensional biology, using high-throughput methodologies and the integration of these technique is called systems biology; capturing experimentally derived data from genomics, transcriptomics, proteomics, metabolomics, secretomics, etc. and

combine with theoretical models to predict the behavior of the cell or organism (199). Indeed, integration of transcriptomics and proteomics data is ongoing and in the course of the holistic view of an ecosystem meta-genomics, -transcriptomics, and –proteomics should therefore be combined to have a complete portrait of how different organisms interact and in this way gut functionality and health effects can be monitored accurately.

Chapter 3

Proteomic Analysis of Log to Stationary Growth Phase *Lactobacillus plantarum* cells and a 2-DE Database

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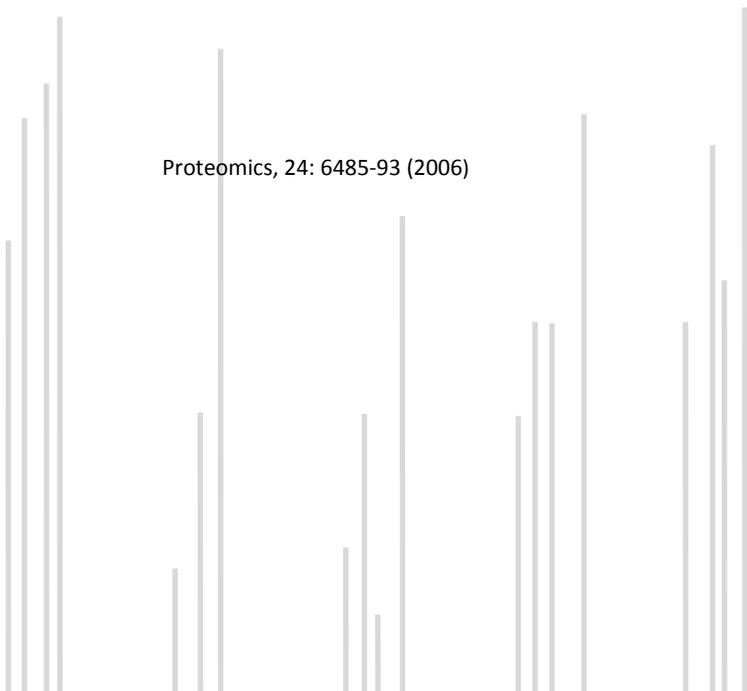
Abstract

Lactobacillus plantarum is part of the natural microbiota of many food fermentations as well as the human gastro-intestinal tract. The cytosolic fraction of the proteome of *L. plantarum* WCFS1, whose genome has been sequenced, was studied. 2-DE was used to investigate the proteins from the cytosolic fraction isolated from mid- and late-log, early- and late-stationary phase cells to generate reference maps of different growth conditions offering more knowledge of the metabolic behavior of this bacterium. From this fraction, a total of 200 protein spots were identified by MALDI-MS and a proteome production map was constructed to facilitate further studies such as detection of suitable biomarkers for specific growth conditions. More than half (57%) of the identified proteins were predicted to be involved in metabolic pathways of the bacterium. The protein profile changed during the growth of the bacteria such that 29% of the identified proteins involved in anabolic pathways were at least twofold up-regulated throughout the mid- and late-exponential and early-stationary phases. In the late-stationary phase, six proteins involved in stress or with a potential role for survival during starvation were up-regulated significantly.

3.1 Introduction

One of the few *Lactobacillus* species that is also a natural inhabitant of the human gastro-intestinal tract is *L. plantarum*. *L. plantarum* is a versatile, gram-positive, fermentative bacterium that can be found in a range of habitats, including dairy, meat, and many plant fermentations. Thus, the *L. plantarum* strains are able to adapt to different environmental conditions (4, 55, 121). Furthermore, *L. plantarum* has the advantage that it can grow to high cell densities which is desirable for industrial applications. *L. plantarum* strain WCFS1 is a single colony isolate from the human pharyngeal strain NCIMB8826 (96) and has been demonstrated to survive the gastro-intestinal tract of men (241) and mice (29). A variety of *L. plantarum* has been described to have beneficial effects on the host and have been marketed as a probiotic (55).

For several years, much research has been done on the mechanisms of adaptation and survival of *L. plantarum* under various environmental conditions (29, 223). The complete genome of *Lactobacillus plantarum* WCFS1 has been sequenced (126). Analysis of the sequence predicted 3052 proteins from which 209 proteins are located extracellularly. The genome sequence allowed the design of specific DNA microarrays for use in transcript profiling of this species (31, 222). Recently, the global gene expression of *L. plantarum* has been investigated to obtain a greater insight into the inhibitory effects of lactic acid on growth of this organism, the major end product of fermentation (183). Although transcriptomics provides detailed information about global gene expression under a certain condition, the short half life of the mRNA can be a limiting factor for certain studies. Proteins have the advantage of greater stability. In particular the high turnover rate of mRNA allows only the use of biopsies for analysis of bacterial behavior in the intestinal tract, but since proteins are more stable in time, the far more accessible fecal samples can be used. Furthermore, post-transcriptional regulations at the protein level may be investigated.

In this study, a proteome reference map of cytosolic proteins from *L. plantarum* WCFS1 was generated, for the purpose of aiding physiological investigations involved in environmental adaptation to conditions such as food-processing stress or passage of the intestinal tract. Furthermore, proteome maps of the cytosolic fraction of different growth conditions of *L. plantarum,* were made to give a global picture of the relative abundance of proteins for during growth

phases in the selective medium for lactobacilli. The proteome maps were used to indicate the dynamics of the proteome of *L. plantarum* WCFS1 during growth. The difference in protein concentration measured in this study is not solely due to increased or decreased protein production, but can be caused by other processes such as protein turnover rate and to protein modification and translocation. Consequently, this has been designated as the relative abundance of proteins (35).

3.2 Material and Methods

3.2.1 Microorganism and growth condition

The microorganism used for these experiments was *L. plantarum* WCFS1, a single-colony isolate from *L. plantarum* NCIMB8826, and was obtained from NIZO Food Research in Ede, The Netherlands. The complete genome sequence of this organism is available (126); (GenBank, AL935263). *L. plantarum* WCFS1 was cultured in 10 ml MRS (155) medium (Difco, Surrey, U.K.) at 37°C. For generation of proteome maps, precultured cells in stationary phase were used to inoculate a fresh culture, which was incubated under the same growth conditions. Samples were taken at OD_{600} of 0.5, 1.6, 4.7, and 8.0. These OD_{600}'s corresponded to mid-log, late-log, early-stationary and late-stationary phase growth, and to 1.2×10^8, 5.0×10^8, 1.4×10^9 and 3.7×10^9 CFU/ml respectively. The samples were aliquoted and used for protein extraction from different cellular fractions as described below.

3.2.2 Preparation of cytosolic protein fraction

L. plantarum WCFS1 cells were harvested and washed with PBS and double distilled water (ddH_2O), respectively, and centrifuged at 500 x *g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in buffer containing 8 M urea (Bio-Rad, Hercules, CA, USA), 2% w/v CHAPS (Sigma, St Louis, MO, USA) 65 mM DTT (Sigma), 0.5% v/v IPG buffer (pH 3-10 NL; Amersham Bioscience, Uppsala, Sweden). The bacteria were lysed mechanically by beating with zirconium beads (diam. 0.8 mm) using a minibeat beater (Biospec Products, Bartlesville, OK, USA) for 5 x 1 min with 1 min intervals on ice. The cell debris was removed by centrifugation and the supernatant was collected and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was collected and stored at -80°C until use.

3.2.3 SDS-PAGE

Prior to gel electrophoresis, the protein concentration was determined using a Bradford-based DC-protein assay (Bio-Rad), using BSA as the standard. SDS-PAGE was used to confirm protein quality of all protein extracts (135). Briefly, 20 µg of protein was loaded on a 12.5% SDS-PAGE gel that was run constantly at a voltage of 110 V until the bromophenol blue front had run off. Afterwards, the gels were stained with silver nitrate according to Shevchenko *et al.* (207). Gel images were obtained by using a GS-800 calibrated densitometer (Bio-Rad).

3.2.4 Iso-electric focusing and two-dimensional electrophoresis

L. plantarum WCFS1 cytosolic proteins were first separated by IEF. Proteins (60 µg) were loaded on Immobiline Dry Strips (pH range 3-10, non-linear, 24 cm long; Amersham). IEF was performed on an IPGphor electrophoresis unit (Amersham), operating at a constant temperature of 20°C. The strips, containing the samples, were first actively rehydrated at 30 volts for 12 h. The IEF program was as follows: 250 V for 1h, 500 V for 1 h, 1000 V for 1 h, 1,000-8,000 V for 2 h and 8,000 V for 52,000 Vh. Prior to running the second dimension, the strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% DTT and for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% iodoacetamide. The strips were placed on 12.5% SDS-PAGE gels and covered with 0.5% agarose containing a trace of bromophenol blue. The gels were run at a constant voltage of 200 V in a Dodeca Cell system (Bio-Rad) until the marker dye had reached the base of the gel. The gels were stained with silver nitrate as described above. The images were obtained with a GS-800 calibrated densitometer (Bio-Rad).

3.2.5 Protein identification

Image analysis was performed using the PDQuest software (version 7.2) (Bio-Rad). Four gels were produced for every growth condition and spots present on at least 3 gels per growth condition were used for comparison of the obtained spots. Proteins were considered differentially produced when spot intensities passed the threshold of at least a 2-fold difference in up- or down-regulation in combination with a student's t-test using a statistical 95% reliability score. Protein bands and spots were excised manually from the gels and subjected to in-gel tryptic digestion according to Bouwman *et al.* (28). The peptide mass fingerprints of the digests

were obtained using matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS; Waters, Manchester, UK) with the settings as described previously (28). The Mascot search engine (http://matrixscience.com) was used to search for peptide mass lists from the obtained spectra against the NCBInr database (http://www.ncbi.nih.gov/), with the following settings: carbamidomethylation was set as fixed modification and oxidation of methionine as optional. The mass tolerance was set to 100 ppm. No restrictions with respect to protein molecular mass and p*I* were made.

3.3 Results and Discussion

A proteome map of *L. plantarum* WCFS1 was constructed which can be used as a reference map for studying proteome dynamics of *L. plantarum* under different conditions. The map has been used initially in this study to investigate the proteome of *L. plantarum* WCFS1 during growth.

3.3.1 Generation of a 2-DE reference map from cytosolic *L. plantarum* proteins

The cytosolic protein fraction of *L. plantarum* WCFS1 having reached the stationary phase was used to construct an initial 2-DE reference map of the proteome of *L. plantarum* grown in standard medium. Using a standard medium facilitates the comparative analysis of the proteome when different conditions are used.

A substantial part of the proteome of the bacterium could be visualized by using a pH range of the IPG strips from 3 to 10 (non-linear). 2-DE followed by silver staining revealed approximately 500 spots (Fig. 1). A total of 190 well-separated and prominent spots were subjected to mass spectrometry and finally 123 proteins were identified by peptide mass fingerprinting. The latter are indicated on the 2-DE protein reference map with an identification number (Fig. 1). The protein names and specific information on each of the proteins, whose function has been annotated in the database (126), are listed in Table 1. The proteins have been classified into 14 groups based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database (122). Those proteins that could not be classified clearly were grouped together as miscellaneous. The majority of the proteins were involved in metabolic pathways (39.6%) especially carbohydrate and energy metabolism. A

further group of 14 proteins coded by potential open reading frames (ORFs) in the *L. plantarum* genome but without assigned function were also identified. Thus, on this initial 2-DE reference map 25% of the proteins from 2-DE gel spots were identified and belong to the *L. plantarum* WCFS1 genome and by extrapolation this corresponds to 3.3% coverage of the genome. This percentage is in good accordance with previously published bacterial proteome maps that varied between 2-12% (18, 43, 197).
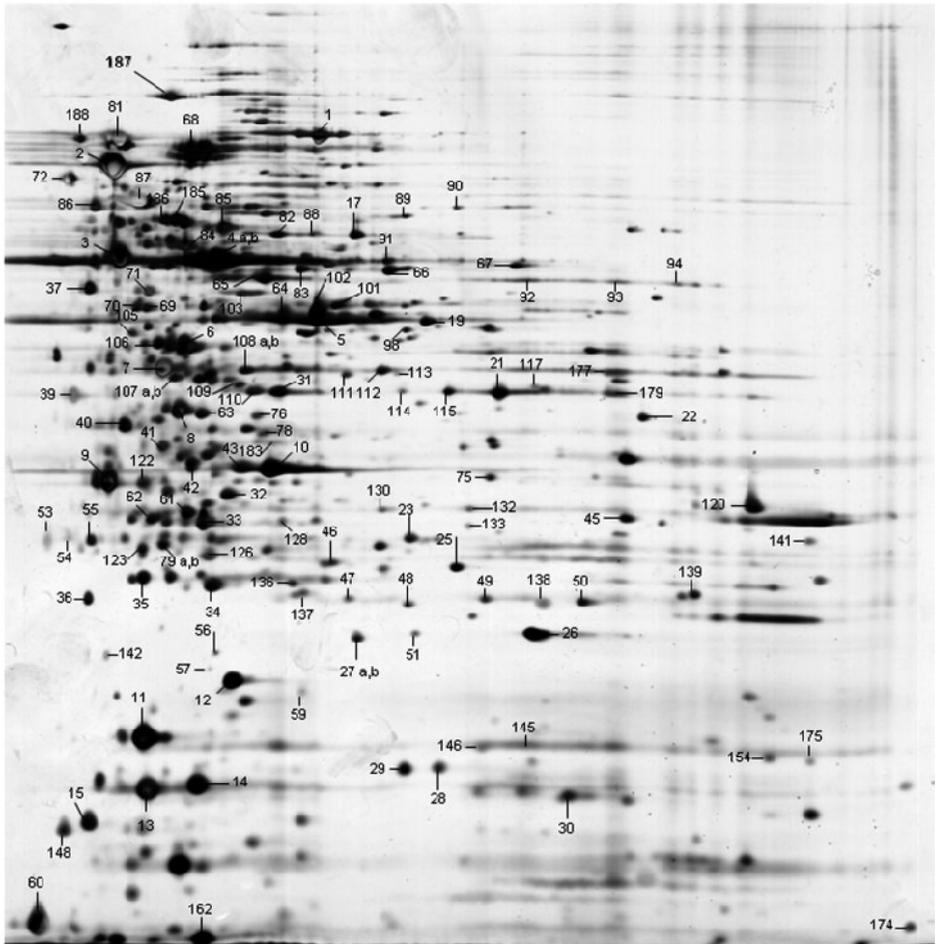
Figure 1. A 2-DE proteome initial reference map of *Lactobacillus plantarum* WCFS1 grown in MRS medium at 37ºC at cell density of OD$_{600}$ 8.0 with a pH interval between 3 and 10 (non-linear). The identified proteins are indicated by numbers and the names are listed in Table 1.

3.3.2 Proteome dynamics during *L. plantarum* growth

The proteomic approach was used to follow the changes of the *L. plantarum* proteome during growth and into stationary phase, offering a dynamic view of different processes during the growth of the bacterium. Protein extracts were prepared from cells in the mid- and late-log, and early- and late stationary phases of growth. *L. plantarum* WCFS1 showed a diminished growth between the late-log and early stationary phase (Fig. 2).
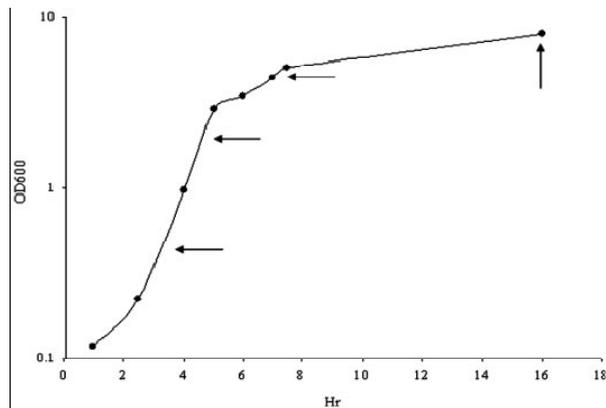
Figure 2. Growth curve of *Lactobacillus plantarum* WCFS1 in MRS medium at 37ºC. Protein samples were taken at the four different time points indicated by the arrows: OD_{600} 0.5, 1.6, 4.7 and 8.0.

It was previously reported that *L. plantarum* WCFS1 exhibits two consecutive growth phases in MRS medium, and the second phase with a lower doubling time occurs approximately between OD_{600} 3.0 and the stationary phase (56). Consequently, the late-log, and early- stationary phases were also investigated on the protein level to gain more insight in this phenomenon. Representative 2-DE gels of the 4 different growth phases are presented in Figure 3 A-D. These maps show a moderate change in the proteome between the log and stationary phases. Detailed analysis of the images revealed the highest numbers of detected spots in the late-log and the early stationary phases, 600 ± 73.8 and 597 ± 68.3, respectively. The gels of the early-log and late-stationary phases showed 532 ± 58.4 and 510 ± 30.4, respectively. Further analysis showed that 154 protein spots were at least 2-fold up- or down-regulated during growth.
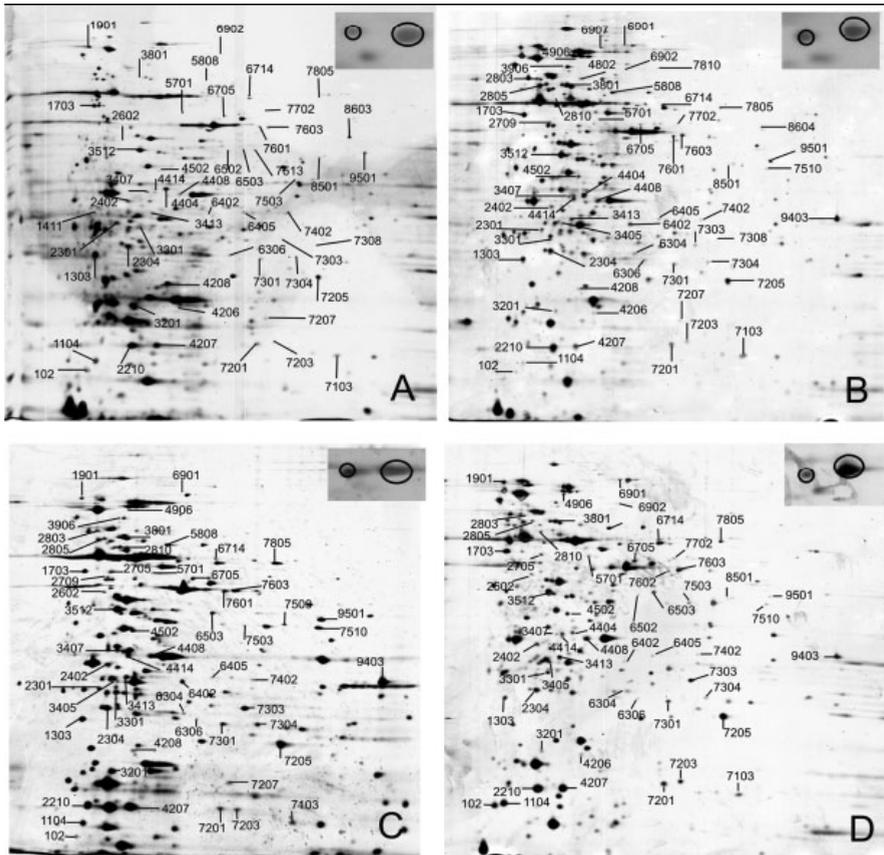
Figure 3. Silver stained 2-DE gels of *Lactobacillus plantarum* WCFS1 grown in MRS medium at 37ºC at 4 different points during growth. Protein samples were taken at OD_{600} 0.5 (A), 1.6 (B), 4.7 (C) and 8.0 (D). A sample area of spots having a difference in relative abundance has been enclosed in the box and is enlarged. The encircled spots were identified as cystathionine ß-lyase and aspartate semialdehyde-dehydrogenase, respectively with MALDI-TOF MS.

A substantial number of proteins (approximately 400) present on the 2-DE gels did not show a significant change in relative abundance during growth. Based on the results of the initial proteome reference map, these proteins were mainly associated with translation, transcription and carbohydrate metabolism, and presumably do not change in time as a constant level of activity is needed for cell survival at all times.

A total of 154 protein spots showed a differential regulation pattern (at least 2-fold up- or down-regulation) during the different growth conditions. Firstly, 41 of

these differentially regulated proteins spots were identified using the initial proteome reference map, based on their p*I* and molecular weight. Another 96 regulated unidentified protein spots was subjected to MALDI-TOF MS for identification. An additional set of in total 70 proteins could be identified which was a good efficiency for a silver stained gel (137). The (predicted) functions of all the identified proteins that showed difference in relative abundance during the various growth phases are listed in Table 2. Based on their relative abundance patterns these proteins could be clustered into 12 different groups (Table 2). Representative proteins of each group are illustrated in Fig. 4.

Figure 4. A representative of each group (1-10) with its specific differential 2-fold increased or decreased abundance pattern of protein spots during growth of *Lactobacillus plantarum* WCSF1.

A total of 154 protein spots showed a differential regulation pattern (at least 2-fold up- or down-regulation) during the different growth conditions. Firstly, 41 of these differentially regulated proteins spots were identified using the initial proteome reference map, based on their p*I* and molecular weight. Another 96 regulated unidentified protein spots was subjected to MALDI-TOF MS for identification. An additional set of in total 70 proteins could be identified which was a good efficiency for a silver stained gel (137). The (predicted) functions of all

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3.3.3 Soluble proteins produced from the mid-log to early-stationary phases

Proteins that differed in their relative abundance during growth could be assigned to groups that showed the same regulation pattern. Proteins belonging to groups 1, 2, 3 and 4 (Table 2) showed a higher relative abundance during the log phase (and in some instances in the early stationary phase), but showed a strong decrease in abundance during the late-stationary phase. The majority of these proteins were involved in (cellular) energy metabolism, translation of RNA to protein, as well as protein, lipid and nucleotide biosynthesis. Proteins involved in energy generation, such as glucokinase and phosphoglycerate mutase, were significantly up-regulated ($\geq$ 2-fold) during the log phase. The putative transcriptional regulator lp_3416, also up-regulated, was located upstream of the gene coding for phosphoenolpyruvate carboxy kinase and was therefore also predicted to be involved in energy metabolism. As expected, the majority of the proteins that showed differences in relative abundance during the log phases were involved in the generation of sufficient energy for growth.

Throughout growth, the relative abundance of the antimicrobial plantaricin biosynthesis protein PlnX's (group 2; Table 2) was increased and decreased in the late-stationary phase. The production of plantaricins is controlled by a three-component regulatory system consisting of a membrane-associated histidine protein kinase, response regulator, and a peptide pheromone with bacteriocin-like properties (127). Maldonado *et al.* (154) demonstrated in *L. plantarum* strain NC8 an auto-inducible mechanism such that the pheromone or plantaricin controls its own regulation. The organizational structure of the gene cluster coding for plantaricins and the regulatory system of *L. plantarum* WCFS1 showed high similarity with the strains mentioned above. Our data suggested indeed that reduced abundance in the stationary phase led to decreased induction of the plantaricin.

A large number of the proteins of groups 3 and 4 (Table 2) that showed a higher relative abundance during the log phase and the early-stationary phase included amino acid metabolism and protein biosynthesis genes. Some proteins involved in cell division (lp_3199; lp_2193) and DNA replication and repair (lp_1976) were also induced during these growth phases. Notably, several enzymes showing a high relative abundance in the late-log phase were involved in lipid metabolism. ORFs lp_1680 and lp_1681 (group 4) code for enzymes belonging to the fatty acid biosynthesis pathway that is the main route for the formation of membrane phospholipid acyl chains in bacteria (251). The ORF lp_3174, predicted to encode the enzyme cyclopropane-fatty-acyl-phospholipid (CFA) synthase showed a similar regulation pattern: greater abundance during mid- and late-log phase. This enzyme catalyzes the methylation of the unsaturated moieties of phospholipids which reside in the hydrophobic interior of the phospholipid bilayer. The physiological effect of this phenomenon is not completely understood. In *Escherichia coli*, this enzyme was shown to increase in concentration during the log to stationary phase transition, and was regulated by a double promoter and an unstable CFA synthase enzyme (229, 244). This enzyme was also induced during acid stress in *L. lactis* and it was proposed that its activity was reflecting a general stress response (32).

Proteins of groups 5 and 6 (Table 2) showed the highest relative abundance during the late-log or early-stationary phases. Among these, were proteins predicted to be involved in protein degradation (lp_1321) and folding (lp_2231 / *ppiB*). In addition, proteins involved in cell division and DNA repair featured strong induction during the early stationary phase. In contrast to the mid- and late-log phases (groups 1, 2 and 4) described above, proteins involved in energy metabolic pathways showed reduced relative abundance during the early stationary phase.

In conclusion, the most important reaction occurring during the log phase was generating sufficient energy, while in the late-log phase the energy metabolic pathways decreased and the biosynthesis of proteins and cell division became more important. Thus, the metabolism of the cells had shifted from energy generation pathways in the log phases to synthesis of macromolecules in the stationary phase. Furthermore, reinforcement of the cell membrane appeared to occur during the late-log to early stationary phase based on induction of enzymes involved in fatty acid biosynthesis, needed for the formation of phospholipids.

3.3.4 Proteome changes during the early and late stationary phase

The proteins described to be abundant or highly abundant in the mid- and late-log phases above were at very low levels in the stationary phase, indicating that growth arrest had occurred. Proteins of groups 8 and 9 (Table 2) essentially increased in relative abundance throughout the log phase and showed highest abundance in the early- and late-stationary phases. Proteins in group 10 (Table 2) were highly abundant in the late-stationary phase alone. These groups included a number of proteins, transporters and enzymes predicted to be involved in the uptake (mannose/glucose PTS EIIAB; lp_0575) and conversion of the carbohydrates mannose, (lp_2384), mannitol, and the amino sugar glucosamine (glucosamine-6-phosphate isomerase) into fructose-6-phosphate. Thus all these sugars might be used as a carbon source for cellular physiology. In the log phase fructose-6-phosphate was formed by the action of the enzyme glucokinase (group 1) that converted glucose from the MRS growth medium into the glucose-6-phosphate intermediate of the glycolytic pathway; this enzyme was repressed in the late-stationary phase. The utilization of mannose, mannitol and glucosamine in the early and late stationary phases suggested that *L. plantarum* was switching from the use of glucose as a carbon source to alternative sources and pathways, which could be explained by the decreasing glucose content of the medium at the later growth or stationary phases.

Protein levels for substrate specific proteins (EIIAB; lp_0575) belonging to the mannose/glucose phosphotransferase system (PTS) complex increased substantially in the stationary phase compared to the log phase (group 9 and 10; Table 2). Interestingly, two different iso-forms of the latter presumably due to post-translational modification were identified at two different locations on the gels (spots no. 7503 and 7507; Fig 3A-D). The exact modification is unclear though it is plausible that phosphorylation of the initial molecule has occurred. The addition of a phosphate group is likely, because the EnzymeIIAB components belonging to the Phophoenolpyruvate Transport System (PTS) phosphorylate the sugar substrate during transport into the cell (245).

PTS transport systems are energetically efficient as the phosphorylated substrate can directly enter glycolysis saving ATP in contrast to ABC transporters

which require an extra ATP. The former is advantageous under limiting energy conditions such as in the stationary phase of growth (228). In accordance, a putative amino acid ABC transporter (lp_2823) was significantly reduced in abundance during the late stationary phase (group 3; spot no. 7402).

A response regulator (*rrp11*; lp_3191) showed a high relative abundance only in the stationary phase. The *rrp11* gene is located together with a histidine kinase gene (*hpk11*), upstream of the *dacA1* gene that encodes a penicillin-binding protein (PBP) with DD-carboxypeptidase activity. It is likely that *rrp11* and *hpk11* are members of a two-component regulatory signal transduction system involved in the regulation of the downstream *dacA1* gene (222). This enzyme has been demonstrated to modulate the peptidoglycan cross linking of the cell wall in *E. coli* (185, 204), and the DD-carboxypeptidases were shown to be involved in the maintenance of cell shape (173). The regulation of PBP-proteins in lactobacilli is unclear (184). In this study, the induction of this putative system suggests that structural cell wall changes may be occurring during late-stationary phase in order to reinforce the cell wall for protective purposes.

Galactokinase and UDP-glucose 4-epimerase were increased in relative abundance during the late-log phase and stationary phase (group 9). Both enzymes belong to the Leloir pathway (86); galactokinase is involved in the formation of galactose to glucose-1P, and UDP-glucose 4-epimerase is responsible for the reversible conversion of UDP-glucose to UDP-galactose. Both UDP-sugars are precursors for the synthesis of exopolysaccharides (EPS) and other cell-surface associated sugars (52, 57, 192). However, *L. plantarum* WCFS1 lacks the *eps* genes for the regulation, polymerization and exporting of the exopolysaccharides (126). Interestingly, a disruption of *Streptococcus thermophilus pbp2b* gene coding for a PBP-protein blocked the EPS production of this LAB (219). Stingele and colleagues (219) suggested that peptidoglycan and EPS synthesis are interconnected pathways; the conversion of glucose-1P to dTDP-rhamnose can be performed by the enzymes coded by the genes *rfbABCD*. It was shown that the lactobacilli species possess rhamnose structures in cell wall polysaccharides (130, 219). Thus, the increased abundance of both the putative regulator of the *dacA1* gene described above and the *gal* genes in the stationary phase might be linked to each other and play a role in strengthening the cell wall.

The pyruvate oxidase enzyme (*poxB* gene; lp_0852; group 10) was mainly induced in the late stationary phase. This enzyme is responsible for the conversion of pyruvate into acetyl phosphate which in turn is metabolized to acetate and excreted into the medium. It was shown by Northern Blotting that in *L. plantarum* Lp80 the *poxB* gene was down regulated in the presence of glucose, and during the early stage of the stationary phase *poxB* expression was maximal (147). In our study during the exponential phase the abundance of pyruvate oxidase is decreased and is increased in the stationary phase. The latter can be explained by the coupling between acetate and ATP production (147); production of acetate is accompanied with increased ATP production which appears to be required during the stationary phase.

A number of proteins implicated in a stress response were induced in the stationary phase, including the stress-induced DNA binding protein (lp_3128), heat shock protein (GrpE; lp_2028), catalase and endopeptidase ClpP at two different spots (lp_0786; spot 2304 and 4408). The protein localized at spot number 2304 is probably a monomer (observed MW 20 kDa) and the other spot might be a trimer of the ClpAP complex – alternatively, it may represent ClpP in complex with another molecule, generating the observed MW of 33 kDa (39, 186).

Both the diminishing nutrients and high concentration of lactic acid in the medium would cause stress especially in the late stationary phase. The stress-induced DNA binding protein has a conserved DPS (DNA Protecting protein under Starved conditions) domain. DPS is a protein that initially was found in *E. coli* to crystallize in starved cells to protect DNA against oxidative stress (195). Cross protection between the responses to starvation and oxidative stress has been described for various bacteria (124, 165). As a result of this cross protection against starvation, also catalase was found to be increased during the stationary phase, which would facilitate the tolerance of *L. plantarum* WCFS1 to possible oxidative stress and reducing lipid oxidation (174). In support of the latter, up-regulation of catalase in *L. plantarum* WCFS1 in response to lactic acid stress was demonstrated by a transcriptomic approach (183).

In conclusion, during the early and late stationary phases, the use of glucose as the main carbon source and energy generation via glycolysis was replaced by alternative pathways for carbohydrate metabolism such as switching from glycolysis to the Leloir pathway for yielding energy. In the late-stationary phase,

many stress proteins were strongly induced due to the unfavorable conditions such as high acid and diminished nutrients. Enzymes involved in the synthesis of cell wall structures were mainly in greater abundance during the late-stationary phase, presumably to strengthen the cell wall and to maintain the bacterial morphology.

3.4 Concluding remarks

A database of intracellular soluble proteins of *L. plantarum* WCFS1 has been established using a proteomic approach. A reference proteome map was generated using data from the stationary phase of *L. plantarum*. The dynamic proteome map was established with data from growth phases namely, mid-log, late-log, early- and late-stationary phases of growth, including time points representing the 2 consecutive growth phases of this strain in MRS. Overall this provided detailed insight into metabolic pathways and their regulation in time during growth of *L. plantarum* WCFS1 under controlled conditions.

The most important reactions occurring during the log phase were metabolic pathways for generating sufficient energy. Interestingly, in the late-log phase and early-stationary phase, thus the period of reduced growth rate on MRS, more protein spots were present on the 2-DE gels compared to the mid-log and late-stationary phases. Thus the highest rate of biosynthesis of proteins was during the late-log and early-stationary phase and this may play a role in the overall lower growth rate during this period. In the late-log phase the biosynthesis of proteins and cell division pathways, i.e. synthesis of macromolecules, increased. Furthermore, strengthening of the cell membrane appeared to occur during the late-log to early stationary phase, based on greater abundance of enzymes involved in fatty acid biosynthesis, needed for the formation of phospholipids. During the early- and late- stationary phases, the lower levels of glucose in the medium provoked alternative pathways for carbohydrate metabolism as the main carbon source for energy generation. Proteins predicted to be involved in cell wall structures such as UDP-sugars and PBP-protein were increased in relative abundance at the beginning of the early-stationary phase at the cost of the glucose-1 phosphate pool, which would result in less availability of glucose-1 phosphate for glycolysis. Overall, the energy required for induction of alternative pathways, and alterations of the cell wall, possibly in preparation for the stationary

phase, may cause the reduced growth rate around late-log to early stationary phases. It was previously reported that diminished growth prior to the stationary phase coincided with alteration of the permeability of the cell envelope (56).

In the late-stationary phase, many stress proteins were strongly induced due to the harsher conditions such as high acid and reduced nutrient levels. Enzymes involved in the synthesis of cell wall structures showed mainly a higher relative abundance during the late-stationary phase, presumably to strengthen the cell wall and to maintain bacterial morphology at low pH and high lactate conditions.

The 2-DE reference map and the dynamics of the proteome of *L. plantarum* can facilitate further studies and information for industrial applications about the activity and metabolic processes of the cells under various conditions, such as those in food preparations and passage of the intestinal tract.

Acknowledgements

Table 1. *L. plantarum* WCFS1 identified proteins spots localized on the 2-DE maps (Fig. 1 and 3). The cytosolic proteins are listed according to their function classification.

Spot no	ORF[a]	Gene	Function	MW[b]	pI[c]	Score[d]	Sequence coverage	Matched peptides	2-DE map[e]
Carbohydrate Metabolism									
3	lp_0792	enoA1	Phosphopyruvate hydratase	48057	4.6	185	40	13	Fig.. 2
4b*	lp_0790	pgk	Phosphoglycerate kinase	42770	5.04	105	37	10	Fig. 2
5	lp_0789	gapB	Glyceraldehyde 3-phosphate dehydrogenase	37158	5.3	68	47	11	Fig. 2
6	lp_2057	ldhD	D-lactate dehydrogenase	37158	4.89	100	27	13	Fig. 2
7	lp_0537	ldhL1	L-lactate dehydrogenase	34242	4.8	133	41	11	Fig. 2
9	lp_0791	tpiA	Triosephosphate isomerase	27126	4.62	160	53	14	Fig. 2
10	lp_0330	fba	Fructose-bisphosphate aldolase	31016	5.07	64	20	5	Fig. 2
33	lp_3170	pmg9	Phosphoglycerate mutase	26071	4.94	80	33	6	Fig. 2
61	lp_0226	gnp	Glucosamin-6-phosphate isomerase	25922	4.92	86	37	7	Fig. 2
62	lp_0602	rpiA1	Ribose 5-phosphate epimerase	24627	4.79	81	27	6	Fig. 2
64	lp_0789	gapB	Glyceraldehyde 3-phosphate dehydrogenase	36644	5.3	146	55	14	Fig. 2
68	lp_1897	pyk	Pyruvate kinase	62941	4.99	145	24	11	Fig. 2
71	lp_3482	galK	Galactokinase	42847	4.83	82	18	8	Fig. 2
76	lp_2349	hicD3	L-2-hydroxyisocaproated dehydrogenase	32680	5.16	77	18	4	Fig. 2
84	lp_2502	pgi	Glucose-6-phosphate isomerase	49816	4.96	116	28	11	Fig. 2
85	lp_1541	gnd2	6-phosphogluconate dehydrogenase (decarboxylating	53063	5.04	142	23	10	Fig. 2
101	lp_2384	pmi	Mannose-6-phospate isomerase	36041	5036	70	29	9	Fig. 2
102	lp_0789	gapB	Glyceraldehyde 3-phosphate dehydrogenase	36644	5.3	75	31	7	Fig. 2
103	lp_0233	mtlD	Mannitol-1-phosphate 5-dehydrogenase	43173	5.12	134	40	13	Fig. 2
106	lp_2057	ldhD	D-lactate dehydrogenase	37158	4.89	90	23	11	Fig. 2
107b	lp_2057	ldhD	D-lactate dehydrogenase	37158	4.89	72	23	9	Fig. 2
110	lp_1898	pfk	6-phosphofructokinase	34244	5.13	109	26	11	Fig. 2
112	lp_3481	galE4	UDP-glucose 4-epimerase	36378	5.43	157	47	16	Fig. 2

136	lp_0027	pgmB1	ß-Phosphoglucomutase		23705	5.3	76	26	6	Fig. 2
2301	lp_0792	enoA1	Phosphopyruvate hydratase		48058	4.61	70	19	7	Fig. 3
2705	lp_3482	galK	Galactokinase		42847	4.83	82	18	8	Fig. 3
3512	lp_0537	ldhL1	L-lactate dehydrogenase		34242	4.8	133	41	11	Fig. 3
4404	lp_1573	glk	Glucokinase		33883	5.09	77	29	6	Fig. 3
4906	lp_0852	pox2	Pyruvate oxidase		63600	4.94	111	14	6	Fig. 3
5701	lp_0233	mtlD	Mannitol-1-phosphate 5-dehydrogenase		43173	5.12	134	40	13	Fig. 3
5809	lp_2681	gpd	Glucose-6-phosphate 1-dehydrogenase		56751	5.22	187	36	13	Fig. 3
6705	lp_2384	pmi	Mannose-6-phosphate isomerase		36041	5.36	71	36	11	Fig. 3
7308	lp_0900	pgm3	Phosphoglycerate mutase		25831	5.71	89	35	8	Fig. 3
2810	lp_0820	glmM	Phosphoglucosamine mutase		33713	4.79	123	31	11	Fig. 3
3413	lp_0226	gnp	Glucosamine-6-phosphate isomerase		25922	4.92	86	37	7	Fig. 3
6503	lp_3481	galE4	UDP-glucose 4-epimerase		36378	5.43	152	44	10	Fig. 3

Amino Acids Metabolism

19	lp_2570	asd2	Aspartate-semialdehyde dehydrogenase		38403	5.5	115	33	9	Fig. 2
22	lp_2790	serA3	Phosphoglycerate dehydrogenase		34238	6.12	79	24	6	Fig.2
32	lp_2264	dapD	2,3,4,5-teterahydropyridine-2-carboxylate N-succinyl transferase		24537	5.02	70	27	4	Fig. 2
42	lp_1874	dapB	Dihydrodipicolinate reductase		28536	4.96	80	46	8	Fig. 2
78	lp_0853	pepR1	Prolyl aminopeptidase		34586	5.11	101	45	10	Fig. 2
82	lp_3267	gshR4	Glutathione reductase		48343	5.19	154	45	13	Fig. 2
83	lp_0369	gshR1	Glutathione reductase		47365	5.29	131	32	11	Fig. 2
98	lp_0255	metC1	Cystathionine beta-lyase		40839	5.52	79	17	6	Fig. 2
2803	lp_0959	pepD3	Dipeptidase		52560	4.71	79	13	7	Fig. 3
2805	lp_1301	metk	Methionine adenosyltransferase		42710	4.72	147	26	9	Fig. 3
3407	lp_1874	dapB	Dihydrodipicolinate reductase		28536	4.96	42	42	42	Fig. 3
4208	lp_2360		Ribosomal protein acetylating enzyme		20978	5.01	68	24	3	Fig. 3
4414	lp_1874	dapB	Dihydrodipicolinate reductase		28536	4.96	78	29	5	Fig. 3
5808	lp_3267	gshR4	Glutathione reductase		48343	5.19	154	45	13	Fig. 3

6902	lp_3578	kat	Catalase	55294	5.31	93	17	7	Fig. 3
6907	lp_2048	proS	Proline--tRNA ligase	63534	5.16	116	19	9	Fig. 3
7601	lp_0255	metC1	Cystathionine beta-lyase	40839	5.52	70	17	6	Fig. 3
7603	lp_2570	asd2	Aspartate-semialdehyde dehydrogenase	38403	5.5	115	33	9	Fig. 3
7702	lp_0979	thrA1	Aspartate kinase	42782	5.47	84	21	6	Fig. 3
9501	lp_0256	cysK	Cysteine synthase	32184	6.09	80	43	8	Fig. 3

Energy Metabolism

27a	lp_2367	atpH	H(+)-transporting 2 sector ATPase, delta subunit	20009	5.45	179	69	14	Fig. 2
37	lp_1837	ppaC	Inorganic pyrophosphatase	33713	4.5	72	15	4	Fig. 2
66	lp_0313	ndh1	NADH dehydrogenase	43932	5.43	100	30	9	Fig. 2
86	lp_2364	atpD	H(+)-transporting 2-sector ATPase, beta subunit	50800	4.58	118	25	11	Fig. 2
185	lp_2366	atpA	H(+)-transporting two-sector ATPase, alpha subunit	54546	4.95	101	13	5	Fig. 2
186	lp_2366	atpA	H(+)-transporting two-sector ATPase, alpha subunit	54546	4.95	72	10	5	Fig. 2
1703	lp_1837	ppaC	Inorganic pyrophosphatase	33713	4.5	72	15	4	Fig. 3
3801	lp_2366	atpA	H-transporting 2-sector ATPase, alpha subunit	54546	4.95	72	10	5	Fig. 3
6714	lp_0313	ndh1	NADH dehydrogenase	43934	5.43	88	25	7	Fig. 3

Fatty Acid Metabolism

65	lp_1675	fabF	3-oxoacyl-[acyl-carrier protein] synthase II	42593	5.21	103	30	11	Fig. 2
120	lp_1681	fabI	Enoyl-[acyl-carrier protein] reductase (NADH)	26978	6.9	106	44	9	Fig. 2
6402	lp_1680	accA2	Acetyl-CoA carboxylase	36378	5.34	84	33	5	Fig. 3
8603	lp_3174	cfa2	Cyclopropane-fatty-acyl-phospholipid synthase	45440	6.03	68	17	5	Fig. 3
9403	lp_1681	fab1	Enoyl-(acyl carrier protein) reductase	26980	6.9	73	44	9	Fig. 3

Nucleotide Metabolism

25	lp_2697	pyrE	Orotate phosphoribosyltransferase	22691	5.64	70	23	5	Fig.2
26a	lp_2704	pyrR1	Pyrimidine operon regulator	19812	5.64	89	50	9	Fig.2
40	lp_0363		Purine nucleosidase	33091	4.72	71	22	5	Fig. 2
56	lp_2086	apt	Adenine phosphoribosyltransferase	18938	5.08	70	45	5	Fig. 2

67	lp_2702	pyrC	Dihydroorotase	45638	5.72	95	27	8	Fig. 2
92	lp_2702	pyrC	Dihydroorotase	45638	5.72	95	34	9	Fig. 2
113	lp_1870	thyA	Thymidylate synthase	36105	5.52	70	27	7	Fig. 2
126	lp_1058	adk	Adenylate kinase	24356	5.41	78	29	5	Fig. 2
179	lp_2703	pyrB	Aspartate carbamoyltransferase	34689	6.06	104	30	8	Fig. 2
3906	lp_0914	guaA	GMP synthase (glutamine-hydrolysing)	57592	4.97	85	27	14	Fig. 3
7301	lp_0363		Purine nucleosidase	33091	4.72	71	22	5	Fig. 3
7303	lp_2697	pyrE	Orotate phosphoribosyltransferase	22691	5.64	81	29	5	Fig. 3
7510	lp_2703	pyrB	Aspartate carbamoyltransferase	34689	6.06	104	30	8	Fig. 3
7805	lp_2702	pyrC	Dihydroorotase	45638	5.72	95	34	9	Fig. 3

Glycan Biosynthesis/Amino Acids Metabolism

89	lp_0977	murE1	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	60530	5.39	85	20	9	Fig. 2
91	lp_1462	murC	UDP-N-acetylmuramate--alanine ligase	48720	5.49	206	39	16	Fig. 2

Metabolism of Cofactors and Vitamins

7810	lp_1005	als	Acetolactate synthase	61194	5.47	74	10	5	Fig. 3

Membrane Transport

21	lp_0575	pts9AB	Mannose PTS EIIAB	35261	5.75	173	37	13	Fig. 2
23	lp_0878	glnQ2	Glutamine ABC transporter	27283	5.47	74	22	4	Fig.2
114	lp_0575	pts9AB	Mannose PTS EIIAB	35261	5.75	87	18	7	Fig. 2
115	lp_0575	pts9AB	Mannose PTS EIIAB	35261	5.75	126	29	10	Fig. 2
7402	lp_2823		ABC transporters - General	24876	5.74	103	29	7	Fig. 3
7503	lp_0575	pts9AB	Mannose PTS, EIIAB	35261	5.75	126	29	10	Fig. 3
7507	lp_0575	pts9AB	Mannose PTS EIIAB	35261	5.75	173	37	13	Fig.3

Signal Transduction

123	lp_3191	rrp11	Response regulator	25764	4.83	77	24	5	Fig. 2

183	lp_0036	rrp1	Response regulator	27044	5.2	81	24	5	Fig. 2
3405	lp_3191	rrp11	Response regulator	25764	4.83	77	24	5	Fig. 3

Cell Growth and Death

39	lp_2189	divIVA	Cell division initiation protein DivIVA	26158	4.49	84	40	6	Fig. 2
72	lp_2193	ftsZ	Cell division protein FtsZ	44986	4.44	75	27	12	Fig. 2
107a	lp_2319	mreB1	Cell shape determining protein MreB	35294	4.79	66	24	7	Fig. 2
130	lp_3199	parA	Chromosome partitioning protein, membrane-associated ATPase	27883	5.45	69	20	5	Fig. 2
3301	lp_2193	ftsZ	Cell division protein FtsZ	44986	4.44	75	23	8	Fig. 3
6405	lp_3199	parA	Chromosome partitioning protein	27883	5.45	69	20	5	Fig. 3
4802	lp_2194	ftsA	Cell division protein FtsA	48352	5.03	75	18	6	Fig. 3

Translation

4a	lp_2119	tuf	Elongation factor Tu	43350	4.95	123	40	12	Fig. 2
8	lp_2054	tsf	Elongation factor TS	31653	4.91	150	56	15	Fig. 2
27b	lp_2211	cspR	rRNA methyltransferase	19569	5.36	70	39	6	Fig. 2
34	lp_0737		Ribosomal protein S30EA	21712	5.31	71	22	5	Fig. 2
60	lp_0622	rplL	Ribosomal protein L12/L7	12584	4.3	115	62	5	Fig. 2
87	lp_1882	rspA	Ribosomal protein S1	47131	4.79	184	39	14	Fig. 2
93	lp_2119	tuf	Elongation factor Tu	43350	4.95	145	47	12	Fig. 2
94	lp_2119	tuf	Elongation factor Tu	43350	4.95	116	26	9	Fig. 2
108	lp_2055	rspB	30S ribosomal protein S2	30207	5.23	72	25	7	Fig. 2
138	lp_2052	frr	Ribosome recycling factor	20581	5.85	85	44	6	Fig. 2
145	lp_1077	rplM	Ribosomal protein L13	16159	9.6	82	46	5	Fig. 2
177	lp_2055	rpsB	Ribosomal protein S2	30207	5.23	65	18	5	Fig. 2
1411	lp_1391	argS	Arginine--tRNA ligase	62870	5.29	71	13	7	Fig. 3
6304	lp_0737		Ribosomal protein S30EA	21712	5.31	87	29	6	Fig. 3

Transcription

49	lp_1153		Transcription regulator	21804	5.72	74	37	6	Fig. 2
69	lp_1062	rpoA	DNA-directed RNA polymerase, alpha subunit	34935	4.8	127	22	9	Fig. 2
70	lp_1062	rpoA	DNA-directed RNA polymerase, alpha subunit	34935	4.8	91	26	8	Fig. 2
75	lp_0996		Metal-dependent regulator	23804	5.9	67	35	5	Fig. 2
141	lp_0563		Transcription regulator	26694	7.79	74	35	7	Fig. 2
142	lp_1563	greA2	Transcription elongation factor GreA	17903	4.61	100	56	7	Fig. 2

Replication & Repair

117	lp_1976	nfo	Deoxyribonuclease IV	33046	5.81	95	23	6	Fig. 2
148	lp_3128		Stress induced DNA binding protein	18010	4.7	77	39	5	Fig. 2
2301	lp_0002	dnaN	DNA-directed DNA polymerase III, beta chain	41495	4.67	89	22	8	Fig. 3
8501	lp_1976	nfo	Endonuclease IV	33046	5.81	95	23	6	Fig. 3

Folding

2			GroEL	57402	4.69	302	55	26	Fig. 2
81	lp_2027	dnaK	Heat shock protein DnaK	66689	4.68	86	15	7	Fig. 2
122	lp_2028	grpE	Heat shock protein GrpE	21430	4.81	132	37	8	Fig. 2
162	lp_0727		GroES co-chaperonin	10286	4.95	70	25	3	Fig. 2
2402	lp_2028	grpE	Heat shock protein GrpE	21430	4.81	132	37	8	Fig. 3

Stress Proteins

11	lp_0930	asp2	Alkaline shock protein	17556	5.11	56	30	9	Fig. 2
13	lp_0929	asp1	Alkaline shock protein	15833	4.92	49	33	5	Fig. 2
14	lp_3352	hsp3	Small heat shock protein 19.5	16662	5	106	89	12	Fig. 2
15	lp_0291	hsp1	Small heat shock protein	15989	4.53	76	48	8	Fig. 2

28	lp_1163		Nucleotide binding protein, universal stress protein UspA family	15838	5.52	68	47	3	Fig. 2
102	lp_3128		Stress induced DNA binding protein	18010	4.7	75	31	7	Fig. 3
1104			Small heat shock protein	15989	4.53	76	48	8	Fig. 3
2210	lp_0929	asp1	Alkaline shock protein	15833	4.92	49	33	5	Fig. 3
4207	lp_3352	hsp3	Small heat shock protein	16662	5	106	89	12	Fig. 3
7203	lp_1163		Nucleotide-binding protein, universal stress protein UspA family	15838	5.52	68	47	3	Fig. 3

Hypothetical Proteins

26a	lp_2340	Hypothetical protein lp_2340	17597	5.91	121	68	12	Fig. 2
29	lp_2652	Hypothetical protein lp_2652	16965	5.57	73	52	7	Fig. 2
30	lp_2877	Hypothetical protein lp_2877	16276	5.93	100	34	7	Fig. 2
46	lp_2690	Hypothetical protein lp_2690	23239	5.38	84	25	5	Fig.2
47	lp_1531	Hypothetical protein lp_1531	22957	5.43	111	34	6	Fig.2
51	lp_2340	Hypothetical protein lp_2340	17597	5.91	72	43	4	Fig. 2
53	lp_2260	Hypothetical protein lp_2260	18034	4.28	88	45	5	Fig. 2
57	lp_2260	Hypothetical protein lp_2260	18034	4.28	148	54	8	Fig.2
105	lp_1843	Hypothetical protein lp_1843	32711	4.71	70	18	8	Fig. 2
133	lp_0117	Hypothetical protein lp_0117	26453	5.76	92	39	7	Fig. 2
146	lp_2786	Hypothetical protein lp_2786	17508	5.75	85	48	4	Fig. 2
154	lp_2753	Hypothetical protein lp_2753	18263	7.88	93	24	5	Fig. 2
174	lp_2179	Hypothetical protein lp_2179	12603	8.89	67	23	3	Fig. 2
175	lp_2753	Hypothetical protein lp_2753	18263	7.88	89	24	5	Fig. 2
2602	lp_1843	Hypothetical protein lp_1843	32711	4.71	70	18	8	Fig. 3
3201	lp_1747	Hypothetical protein lp_1747	18104	4.74	68	23	3	Fig. 3
4206	lp_1872	Hypothetical protein lp_1872	17149	5.1	70	41	5	Fig. 3
7103	lp_2877	Hypothetical protein Lp_2877	16276	5.93	100	34	7	Fig. 3

| 7201 | lp_2652 | | Hypothetical protein lp_2652 | 16965 | 5.57 | 73 | 52 | 7 | Fig. 3 |
| 7205 | lp_2340 | | Hypothetical protein lp_2340 | 17597 | 5.91 | 121 | 68 | 12 | Fig. 3 |

Miscellaneous

1	lp_3583	clpL	ATP-depent Clp protease, ATP-binding subunit ClpL	77729	5.44	219	46	40	Fig. 2
17	lp_2544	npr2	NADH peroxidase	48283	5.36	108	29	12	Fig. 2
31	lp_3403		Oxidoreductase	31667	5.16	69	26	7	Fig. 2
35	lp_0786	clpP	Endopeptidase Clp, proteolytic subunit	21516	4.87	71	46	7	Fig. 2
36	lp_2231c	ppiB	Peptidylprolyl isomerase	21059	4.53	72	26	5	Fig. 2
41	lp_3045		Short-chain dehydrogenase/oxidoreductase	31609	4.85	75	21	7	Fig. 2
45	lp_2631		Lipase/esterase (putative)	27324	6.4	72	22	5	Fig.2
49	lp_1153		Transcription regulator (putative)	21804	5.72	74	37	6	Fig. 2
59	lp_0244		Oxidoreductase (putative)	19620	5.29	97	42	5	Fig. 2
63	lp_0137		Oxidoreductase	31871	5.03	100	31	8	Fig. 2
72	lp_1321	pepV	Dipeptidase	50767	4.47	66	21	10	Fig. 2
88	lp_2544	npr2	NADH peroxidase	48283	5.36	70	20	8	Fig. 2
90	lp_2843	tagE5	Poly(glycerol-phophate) alpha-glucosyltransferase	57692	5.62	146	21	9	Fig. 2
109	lp_3403		Oxidoreductase	31667	5.16	77	20	5	Fig. 2
111	lp_3100		Oxidoreductase	35479	5.39	82	23	6	Fig. 2
128	lp_3355		Oxidoreductase (putative)	23529	5.23	81	32	6	Fig. 2
137	lp_1292		Acetyltransferase (putative)	19224	5.33	77	41	5	Fig. 2
139	lp_2267	xtp1	Xanthosine triphosphate pyrophosphatase	21777	6.43	81	35	6	Fig. 2
1303	lp_2231c	ppiB	Peptidylprolyl isomerase	21059	4.53	72	26	5	Fig. 3
1901	lp_2118	tig	Trigger factor	49406	4.5	65	10	6	Fig. 3
2304	lp_0786	clpP	Endopeptidase Clp, proteolytic subunit	21516	4.87	71	46	7	Fig. 3
2709	lp_2451		Prophage Lp2 protein 6	40646	4.77	78	19	6	Fig. 3
4502	lp_0137		Oxidoreductase	31871	5.03	100	31	8	Fig. 3
6201	lp_0244		Oxidoreductase (putative)	19620	5.29	97	42	5	Fig. 3

6301	lp_1292		Acetyltransferase (putative)	19224	5.33	77	41	5	Fig. 3
6502	lp_3100		Oxidoreductase	35479	5.39	73	24	5	Fig. 3
6901	lp_3583	clpL	ATP-dependent Clp protease, ATP-binding subunit ClpL	77729	5.44	146	20	10	Fig. 3
7207	lp_3416		Transcription regulator (putative)	19720	5.72	66	27	5	Fig. 3
7304	lp_1153		Transcription regulator (putative)	31609	4.85	74	37	6	Fig. 3
7513	lp_0430	plnX	Plantaricin biosynthesis protein PlnX (putative)	8838	6.15	74	61	4	Fig. 3

(a) ORF: open reading frame. (b) MW, molecular weight of the proteins. (c) Iso-electric point. (d) Mascot Mowse Score. (e) Localization of the identified spots. (*) Spot containing two different proteins both functions have been depicted as (a) or (b), respectively.

Table 2. *L. plantarum* WCFS1 protein spots that are 2-fold or higher increased or decreased during different growth phases. The proteins have been listed according to their regulation pattern during growth.

Spot no	Function	ORF[a]	Gene	Functional Class	0.5[b] mean[c]	CV[d]	1.6 mean	CV	4.7 mean	CV	8 mean	CV	0.5	1.6	4.7	8.0 fold-change[€]
	High abundance during log-phase															
1[f] 4404	Glucokinase	lp_1573	glk	Carbohydrate Metabolism	2469	25.4	1513.7	94.6	N.D.	0	1234.3	39.8	2.0	1.2	N.D.	1.0
7304	Transcription regulator (putative)	lp_1153		Miscellaneous	1256	80.5	1141.6	60.7	1063.6	38.8	323.8	37.7	3.9	3.5	3.3	1.0
2 3512	L-lactate dehydrogenase	lp_0537	ldhL1	Carbohydrate Metabolism	5713.7	28.7	5890.3	12.5	4085.8	61.9	1512.3	34.7	3.8	3.9	2.7	1.0
7308	Phosphoglycerate mutase	lp_0900	pgm3	Carbohydrate Metabolism	89.1	48.7	94.2	30.3	72.2	80.6	19.2	53.4	4.6	4.9	3.8	1.0
7207	Transcription regulator (putative)	lp_3416		Miscellaneous	520.3	53.9	538.1	59.2	462.1	82.5	N.D.	0				N.D.
7513	Plantaricin biosynthesis protein PlnX (putative)	lp_0430	plnX	Miscellaneous	239.5	31.3	214.8	38.2	334.7	48.3	52.7	42.1	4.5	4.1	6.4	1.0
6301	Acetyltransferase (putative)	lp_1292		Miscellaneous	1293.9	35.6	1203.8	28.6	1594.9	8.8	608	53	2.1	2.0	2.6	1.0
6201	Oxidoreductase (putative)	lp_0244		Miscellaneous	116.3	13.8	841.2	43.9	1158.3	32.5	302.2	57.1	0.4	2.8	3.8	1.0
3 3407	Dihydrodipicolinate reductase	lp_1874	dapB	Amino Acids Metabolism	206.4	39.2	490.3	25.2	1022.2	24.5	623.1	54.1	0.3	0.8	1.6	1.0
4208	Ribosomal protein acetylating enzyme	lp_2360		Amino Acids Metabolism	275.1	40.6	291.1	51.6	391.5	54.1	64.3	31.5	4.3	4.5	6.1	1.0
4414	Dihydrodipicolinate reductase	lp_1874	dapB	Amino Acids Metabolism	395.1	46.9	845	28.2	1578.8	64	1021	47	0.4	0.8	1.5	1.0
7702	Aspartate kinase	lp_0979	thrA1	Amino Acids Metabolism	63.6	50.8	163	40.9	231.3	51.8	193.7	24.8	0.3	0.8	1.2	1.0
9501	Cysteine synthase	lp_0256	cysK	Amino Acids Metabolism	661.2	29.1	1603.8	21.2	2372.9	21.2	158.9	52.9	4.2	10.1	14.9	1.0
7103	Hypothetical protein lp_2877	lp_2877		Hypothetical protein	1733.6	40.5	2564.6	42.2	4442.1	40.8	1601.2	24.2	1.1	1.6	2.8	1.0
7402	ABC transporter	lp_2823		Membrane Transport	242.7	36.7	299.3	35.8	439.5	12.4	204	33.1	1.2	1.5	2.2	1.0
2304	Endopeptidase Clp, proteolytic subunit	lp_0786	clpP	Miscellaneous	2156.8	40.5	3348.8	28.9	4606.8	14.1	3303.3	28.2	0.7	1.0	1.4	1.0

	ID	Description	Locus	Gene	Category												
	7301	Purine nucleosidase	lp_0363		Nucleotide Metabolism	562.9	45.8	1087.5	44.5	1807.2	13.2	835.9	17.8	0.7	1.3	2.2	1.0
	7303	Orotate phosphoribosyltransferase	lp_2697	pyrE	Nucleotide Metabolism	175.4	44.4	597.7	46	4773.7	17.1	1984.6	10.7	0.1	0.3	2.4	1.0
	7805	Dihydroorotase	lp_2702	pyrC	Nucleotide Metabolism	59.9	43.1	660	34.4	2198.9	38.8	893.7	13.6	0.1	0.7	2.5	1.0
	8501	Deoxyribonuclease IV	lp_1976	nfo	Replication and Repair	137.6	16	166.5	22.5	164.7	44.1	67.5	29.7	2.0	2.5	2.4	1.0
	2210	Alkaline shock protein	lp_0929	asp1	Stress	6701.4	24.3	9004.5	25.6	15624	16.4	9661.2	31.5	0.7	0.9	1.6	1.0
	7203	Nucleotide binding protein	lp_1163		Stress	528.8	44.9	851.1	55.8	1967.1	29.9	1593.9	17.2	0.3	0.5	1.2	1.0
	1411	Arginine--tRNA ligase	lp_1391	argS	Translation	205.2	19.9	205.7	42.8	342.8	31.9	80	21	2.6	2.6	4.3	1.0
	6304	Ribosomal protein S30EA	lp_0737		Translation	194.3	77.8	445	30	781.9	63.9	681.6	23.6	0.3	0.7	1.1	1.0
4	2803	Dipeptidase	lp_0959	pepD3	Amino Acids Metabolism	185.2	11.9	476.2	19.3	312	38.7	157.7	34.4	1.2	3.0	2.0	1.0
	2805	Methionine adenosyltransferase	lp_1301	metk	Amino Acids Metabolism	487.1	39	1502.3	34.2	770	33.6	521.4	29.9	0.9	2.9	1.5	1.0
	7601	Cystathionine beta-lyase	lp_0255	metC1	Amino Acids Metabolism	1092.8	59.2	1389.9	45.8	972.4	36.7	190.3	25.5	5.7	7.3	5.1	1.0
	6405	Chromosome partitioning protein	lp_3199	parA	Cell Growth and Death	406.5	39.8	480	50.4	399.9	35.5	162.2	29.9	2.5	3.0	2.5	1.0
	6402	Acetyl-CoA carboxylase, subunit α	lp_1680	accA2	Fatty Acid Metabolism	126.8	30.4	1913.7	42.8	1518.8	17.7	234	52.3	0.5	8.2	6.5	1.0
	8603	Cyclopropane-fatty-acyl-phospholipid synthase	lp_3174	cfa2	Fatty Acid Metabolism	136.4	26.1	235.9	24.3	87	25.2	71.5	48.5	1.9	3.3	1.2	1.0
	9403	Enoyl-[acyl-carrier protein] reductase (NADH)	lp_1681	fab1	Fatty Acid Metabolism	283.9	17.8	5137.2	43.6	4364.5	29.5	2793.1	21.6	0.1	1.8	1.6	1.0
	2709	Prophage Lp2 protein 6	lp_2451		Miscellaneous	43.8	41.9	501.2	33.3	390.8	63.8	139.9	64.3	0.3	3.6	2.8	1.0

Relatively constant during log and stat

	ID	Description	Locus	Gene	Category												
5	2301	Phosphopyruvate hydratase	lp_0792	enoA1	Carbohydrate Metabolism	629.8	40.4	712.5	12.9	1544.5	27	489.8	35.5	1.3	1.5	3.2	1.0
	3301	Cell division protein FtsZ / dipeptidase	lp_2193	ftsZ	Cell Growth and Death	713.9	36.4	629.6	33.3	2243	31	1281.4	27.7	0.6	0.5	1.8	1.0
	7510	Aspartate carbamoyltransferase	lp_2703	pyrB	Nucleotide Metabolism	70.6	11.7	280.9	60.7	1322.7	73.2	234.4	49.6	0.3	1.2	5.6	1.0
	2301	DNA-directed DNA polymerase III, beta chain	lp_0002	dnaN	Replication and Repair	629.8	40.4	712.5	12.9	1544.5	27	489.8	35.5	1.3	1.5	3.2	1.0
6	6907	Proline--tRNA ligase	lp_2048	proS	Amino Acids Metabolism	465.4	58.4	621.8	43.6	N.D.	0	104	32.2	4.5	6.0	N.D.	1.0
	5809	Glucose-6-phosphate 1-dehydrogenase	lp_2681	gpd	Carbohydrate Metabolism	364.8	75.1	327	48.2	79.6	46.3	146.1	54.6	2.5	2.2	0.5	1.0

		Protein	lp ID	Gene	Category												
	4802	Cell division protein FtsA	lp_2194	*ftsA*	*Cell growth and Death*	266.6	57.7	607	31.6	286	26.4	302	55.1	0.9	2.0	0.9	1.0
	3801	H(+)-transporting 2 sector ATPase	lp_2366	*atpA*	*Energy Metabolism*	171.2	67.1	763.2	46.4	494	26	818.2	38	0.2	0.9	0.6	1.0
	6714	NADH dehydrogenase	lp_0313	*ndh1*	*Energy Metabolism*	1318.5	48.7	1479.2	28.1	656.5	39	949	48.5	1.4	1.6	0.7	1.0
	4206	Hypothetical protein lp_1872	lp_1872		*Hypothetical protein*	105.6	14.4	268.3	22.9	160.3	42.1	393.2	32.2	0.3	0.7	0.4	1.0
	3201	Hypothetical protein lp_1747	lp_1747		*Hypothetical protein*	1390.8	110	487.5	16.5	2553	33.8	294.2	22.8	4.7	1.7	8.7	1.0
	7810	Acetolactate synthase	lp_1005	*als*	*Cofactors & Vitamins*	55.3	62.8	129.5	34.5	31.8	23.6	75.2	41.2	0.7	1.7	0.4	1.0
	1303	Peptidylprolyl isomerase	lp_2231c	*ppiB*	*Miscellaneous*	3120.1	27.7	2864.3	19.9	4534.2	48	1559.3	30.7	2.0	1.8	2.9	1.0
	1901	Trigger factor	lp_2118	*tig*	*Miscellaneous*	1036.5	52.6	1946.9	10.1	946	43.8	1237.2	28.8	0.8	1.6	0.8	1.0
	3906	GMP synthase (glutamine-hydrolysing)	lp_0914	*guaA*	*Nucleotide Metabolism*	N.D.	0	77.4	19	19.1	56	41.8	44	N.D.	1.9	0.5	1.0
7	5808	Gluthathione reductase	lp_3267	*gshR4*	*Amino Acids Metabolism*	564	62.8	1498.9	17.2	1086.7	32.6	1757.6	22	0.3	0.9	0.6	1.0
	2602	Hypothetical protein lp_1843	lp_1843		*Hypothetical protein*	192.5	64.6	702.4	26.3	760	24.1	525.5	64.1	0.4	1.3	1.4	1.0
	7201	Hypothetical protein lp_2652	lp_2652		*Hypothetical protein*	1002	46.5	1967.7	51.1	2258.1	29.1	2015	27.1	0.5	1.0	1.1	1.0

Abundance in late-log and early-stationary phase

		Protein	lp ID	Gene	Category												
8	7603	Aspartate semialdehyde dehydrogenase	lp_2570	*asd2*	*Amino Acids Metabolism*	990	31.8	1565.8	30.4	1764.2	17	2385.7	18.8	0.4	0.7	0.7	1.0
	3413	Glucasomin-6-phosphate isomerase	lp_0226	*gnp*	*Carbohydrate Metabolism*	731	30.9	955.1	17.1	1657.3	8.2	2551.3	24.9	0.3	0.4	0.6	1.0
	5701	Mannitol-1-phosphate 5-dehydrogenase	lp_0233	*mtlD*	*Carbohydrate Metabolism*	36.6	46.7	95.8	51.9	344.3	51.4	720.2	69.1	0.1	0.1	0.5	1.0
	6705	Mannose-6-phosphate isomerase	lp_2384	*pmi*	*Carbohydrate Metabolism*	427.3	45.1	719.9	52.5	1121.3	21.8	1342.5	27.4	0.3	0.5	0.8	1.0
	2810	Phosphoglucosamine mutase	lp_0820	*glmM*	*Carbohydrate Metabolism*	N.D.	0	54.7	25.3	423.4	42.1	688.1	52.6	N.D.	0.1	0.6	1.0
	1703	Inorganic pyrophosphatase	lp_1837	*ppaC*	*Energy Metabolism*	1977.3	16.2	1532.6	35.5	2262.4	44.8	3224.9	22.5	0.6	0.5	0.7	1.0
	7205	Hypothetical protein lp_2340	lp_2340		*Hypothetical protein*	3160.1	35.5	4403.8	24.9	6528.6	42.9	6996.5	24.3	0.5	0.6	0.9	1.0
	4502	Oxidoreductase	lp_0137		*Miscellaneous*	364.5	55.1	856.1	4.5	1516.2	21	1874.1	35.4	0.2	0.5	0.8	1.0
	2402	Heat Shock protein GrpE	lp_2028	*grpE*	*Folding*	499.5	44.1	979.6	10.7	1454.1	18.9	1702.6	35.9	0.3	0.6	0.9	1.0

9	2705	Galactokinase	lp_3482	galK	Carbohydrate Metabolism	105.8	75.6	85	35.5	1093.5	6.7	974.2	51.1	0.1	0.1	1.1	1.0
	6503	UDP-glucose 4-epimerase	lp_3481	galE4	Carbohydrate Metabolism	98.8	43.9	67.3	104	1421	10.8	1107.4	36.6	0.1	0.1	1.3	1.0
	7507	Mannose PTS, EIIAB	lp_0575	pts9AB	Membrane Transport	510.4	22.2	677.9	27	1888.7	37.3	1858.7	25.6	0.3	0.4	1.0	1.0
	6502	Oxidoreductase	lp_3100		Miscellaneous	126.5	22.9	52.3	21.1	183.9	40.7	206.5	54.8	0.6	0.3	0.9	1.0
	1104	Small heat shock protein			Stress	2105.6	17.2	542.5	47.9	2350.8	14.4	4205.8	32.4	0.5	0.1	0.6	1.0
	4207	Small heat shock protein	lp_3352	hsp3	Stress	4084.5	36.9	4173.9	42.2	9412.8	20.8	8089.1	28.9	0.5	0.5	1.2	1.0
10	6902	Catalase	lp_3578	kat	Amino Acids Metabolism	86.8	84.6	130.4	67.5	55.3	0	419.5	46.4	0.2	0.3	0.1	1.0
	4906	Pyruvate oxidase	lp_0852	pox2	Carbohydrate Metabolism	106.5	67.2	243.7	5.4	69.4	77.2	444.4	47.9	0.2	0.5	0.2	1.0
	7503	Mannose PTS, EIIAB	lp_0575	pts9AB	Membrane Transport	96.7	18.4	39.8	30.7	160.6	24.2	518.3	48.4	0.2	0.1	0.3	1.0
	3405	Response regulator	lp_3191	rrp11	Signal Transduction	N.D	0	249.8	48.2	497.5	9.4	2197.9	53.8	N.D.	0.1	0.2	1.0
	6901	Clp protease, ATP-binding subunit ClpL	lp_3583	clpL	Miscellaneous	402.3	57.7	337.2	38.3	111.3	55.9	618.7	46.6	0.7	0.5	0.2	1.0
	102	Stressed induced DNA binding protein	lp_3128		Stress	648.5	45.4	196.4	31.4	451.9	29.3	2863.9	45.6	0.2	0.1	0.2	1.0

Chapter 4

Parallel Proteomics and Transcriptomics Analysis of *Lactobacillus plantarum* and an Isogenic *ccpA* Mutant

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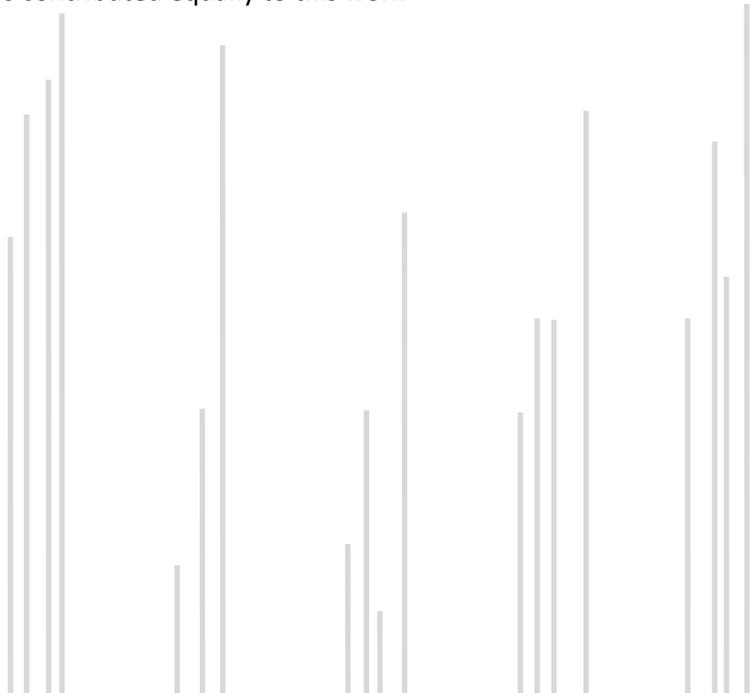
Abstract

Transcriptome and proteome data sets were generated for *Lactobacillus plantarum* WCFS1 and its isogenic *ccpA* mutant during growth, allowing comparison of the results of both –omics approaches. The *ccpA* mutant displayed the characteristic biphasic growth of the parent strain to a much lesser extent than observed for the wild -type strain, and although displaying a lower growth rate during log phase, it reached a remarkable 2-fold higher cell density in the stationary phase. The mutant also displayed reduced acidification rates, with higher pH values despite the higher cell-densities in the stationary phase, and also higher acetate levels as well as some acetoin. Pair-wise comparison of the transcriptomes of *L. plantarum* at various time points during batch-culture growth indicated differences in gene expression due to the *ccpA* mutation. These differences comprised mainly genes predicted to encode transporters, regulatory functions, and enzymes involved in energy metabolism. Comparison of these post-genomic analyses showed a 70 to 80% confirmation of proteome data by transcriptome data, independent of the growth phase or strain that was analyzed in a particular comparison. Genes with inconsistent or deviating transcript and protein levels mainly classified among purine and pyrimidines biosynthesis, energy metabolism, and stress proteins. In conclusion, this parallel transcriptome and proteome analysis illustrates the strength of combined functional post-genomic approaches to elucidate differential regulation in bacteria.

4.1 Introduction

Lactobacillus plantarum is a versatile, Gram-positive, fermentative bacterium widely found in diverse habitats including food products derived from dairy, meat, and many plant fermentations as well as the human gastrointestinal tract (55, 196). Some strains have been described to exert beneficial effects on the host and have been marketed as probiotics (55). *L. plantarum* strain WCFS1 is a single colony isolate that was obtained from the human pharyngeal strain NCIMB8826 (96) and was found to survive the gastro-intestinal tract passage of both human (241) and mice (29). This strain has been used as a model commensal or ingested bacterium to study the impact of the gastrointestinal (GI) tract environment or conditions on gene expression (29, 54, 159). Moreover, the availability of the complete genome sequence of *L. plantarum* WCFS1 has supported novel insight into this bacterium as well as facilitated the development of a transcriptomics platform (22, 126). Transcript profiling of the related *L. plantarum* 299v strain in the human intestinal tract revealed completely different gene expression compared to *in vitro* laboratory grown culture in MRS medium with a remarkable increase in the variety of carbon sources that were apparently being utilized (54).

One interesting question is the role of catabolite control protein A (CcpA) in the regulation of the many sugar degradation and other metabolic pathways in *L. plantarum* WCSF1, especially during growth or upon passage of the intestine. CcpA is the key regulator of carbon catabolite repression (CCR) in Gram-positive bacteria (99). CcpA-mediated CCR or here termed carbon catabolite control, in *Bacillus subtilis* and *Lactococcus lactis* involves the regulation of genes belonging to wide range of functional classes, confirming a global regulatory role for CcpA and growth-phase dependent variation of CcpA-mediated gene expression control (150, 169, 261). The mechanism by which CcpA represses the uptake and utilization of less-favorable sugars in the presence of favorable sugars has been studied intensively in other Gram-positive bacteria (243). CcpA is a DNA-binding protein belonging to the LacI/GalR-family of transcriptional regulators (248) and binds to a catabolite response element (*cre*) that is commonly located in the proximity of promoters, thereby blocking or enhancing transcription of downstream operons (249). The nucleotide sequence of the *cre*-site is conserved among low G+C Gram positive bacteria, allowing *in silico* prediction of CcpA-regulons via detection of putative *cre*-sites in a genome sequence (166).

Both transcriptomics and proteomics are post-genomic approaches for assessing the activity of an organism, the application of which is enabled by sequencing and annotation of genomes of numerous microorganisms. Transcriptomics addresses the global gene expression of a cell, tissue or organism by analysis of the messenger RNA (mRNA), whereas a proteomics approach investigates its protein complement. In contrast to eukaryotic cells where fundamental biological differences between transcription and translation (e.g. splicing) lead to difficulties to predict protein abundance from mRNA analysis (97), bacterial transcription is coupled to translation and correlation between mRNA and protein abundance can more easily be made (83). However, studies in which transcriptomic and proteomics data are compared are rare due to the low numbers of identified proteins or to differences in half-life of mRNA and proteins (152, 257). However, one study investigated the Gram-positive bacteria *Staphylococcus aureus*, and the data obtained by gene expression and protein analysis were found to be similar if evaluated on functional classes, but low in similarity on the single gene level (205). Despite the difficulties to correlate transcriptomics and proteomics data, integration of these data is highly desirable as it leads to a more comprehensive and complete view of cellular processes, host-microbe interaction, and genotype and phenotype correlation.

In this study, data of a combined proteomics-transcriptomics approach obtained during growth of wild-type *L. plantarum* WCFS1 and its isogenic *ccpA* mutant were compared. A proteome reference map for *L. plantarum* WCFS1 (45) was previously described and was used to identify the obtained protein spots that were found to be different in protein abundance between the wild-type and the *ccpA* mutant.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. *Escherichia coli* strain DH5α was used as a cloning host and grown aerobically in TY-medium (202). When appropriate, ampicillin (50 µg/ml) was added to the medium. *Lactobacillus plantarum* WCFS1 wild-type and its *ccpA* derivative NZ7304 (ΔCcpA) were grown in anaerobic conditions under N_2 in MRS broth (prepared without Tween 80) (155) in a non pH-controlled batch culture with stirring at 125 rpm. Glucose was added to a final concentration of 2% (w/v) and the initial pH of the medium was set at 6.5. *Lactobacillus*-growth was performed in a Biocontroller ADI 1030 (Applikon BV, Schiedam, the Netherlands). Cell growth was monitored by measuring the optical density at 600 nm in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech BV, Roosendaal, The Netherlands). Two independent biological duplicates were used.

4.2.2 DNA manipulations and gene disruption

Molecular cloning and DNA manipulations were essentially performed as described before (202). Restriction enzymes and the proof reading enzyme *Pwo* DNA polymerase were obtained from Promega (Leiden, The Netherlands). T4-ligase was obtained from Boehringer GmbH (Mannheim, Germany). Large scale plasmid DNA isolations from *E. coli* were performed using a Jet Star Maxiprep Kit (Genomed GmbH, Bad Oberhausen, Germany). Primers were purchased from Proligo France SAS (Paris, France). For construction of the *ccpA* mutagenesis plasmid pNZ7304, the up- and downstream regions of the *ccpA* gene were amplified by PCR, using *L. plantarum* WCFS1 genomic DNA as the template combined with the primers listed in Table 1. The obtained amplicons were sequentially cloned as *Eco*RI-*Bam*HI and *Pst*I-*Hind*III fragments (restriction sites introduced in the primers used) in the similarly digested mutagenesis vector pUC18-Ery (239). The resulting plasmid contains the up- and downstream regions of the *ccpA* gene flanking the erythromycin resistance cassette that can be used for selection during mutagenesis recombination (239). The resulting plasmid was transformed into *L. plantarum* by electroporation as described previously (73, 117) and primary plasmid-integrants were selected on MRS-plates with 5 µg/ml erythromycin at 37°C. The anticipated mutagenesis-plasmid integration was

checked by PCR using a universal-primer annealing in the erythromycin resistance gene and a site-specific primer annealing outside of the chromosomal region used for homologous recombination.

4.2.3 Sampling

Cells for RNA isolation were quenched in a -40ºC 60% methanol-HEPES buffer as described before (182). After sampling, cells were collected at 13000 x g at -20ºC using a Sorvall RC5B plus centrifuge (Sorvall, Newton, US). The cells were transferred with a cooled spatula to a screw-crap tube containing 500 mg zirconium beads, 500µl phenol/chloroform-mix 1:1, 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and 400 µl MRS-medium (Merck, Darmstadt, Germany), carefully avoiding the thawing of the cells. The tubes containing the cells were shaken, frozen in liquid nitrogen and stored at -80ºC. Samples for HPLC-analysis and protein isolation were taken from the culture, centrifuged in an Eppendorf table centrifuge 5417C at 20800 x g (Eppendorf, Hamburg, Germany) to separate the cells from the supernatant. The supernatant was transferred to a new tube and stored at -20ºC for further analysis.

4.2.4 RNA extraction and quality control

Cells were disrupted by bead-beating four times for 40 s at speed 4.0 using a Fastprep cell disrupter (QBiogene Inc., Cedex, France), interspersed with cooling intervals on ice. The tubes were centrifuged for 1 min at 22,800 x g (4°C) and the aqueous-phase was transferred into an Eppendorf-tube. Residual phenol traces were removed by extraction with pre-chilled chloroform. The resulting aqueous phase was mixed with an equal volume of high pure RNA isolation kit binding buffer (Roche Diagnostics, Mannheim, Germany) and applied to a RNA purification column. Further purification was performed following the protocol provided by the manufacturer, including on-column incubation with DNaseI for one hour and using a 50 µl elution volume.

Table 1. Bacterial strains, plasmids and primers used in this study.

Material	Relevant properties	Reference
Lactobacillus plantarum WCFS1	Wild-type: single colony isolate from human saliva isolate NCIMB8826	(126)
Lactobacillus plantarum NZ7304	CcpA::Ery, WCFS1 derivative	This work
Plasmids		
pUC18-Ery	Mutagenesis vector: Amp^R, Ery^R, 3.8 kb derivative of pUC19 containing 1.1 kb HinPI fragment of pIL253 carrying the Ery^R gene	(239)
pNZ7305	Amp^R, Ery^R, pNZ5318 derivative containing 1.1 kb 5'-flanking region of ccpA.	This work
pNZ7306	Amp^R, Ery^R, pNZ5318 derivative containing 1.1 kb 3'-flanking region of ccpA.	This work
pNZ7304	Amp^R, Ery^R, pNZ5318 rpoN::cat replacement derivative containing pNZ7300 and pNZ7301 derived 5'- and 3'-flanking regions of ccpA.	This work
Primers		
ccpA-upstream-5'	5'- CCGGAATTCGCCTTCCTTAGTAACGACCCC -3'	This work
ccpA-upstream-3'	5'- CGCGGATCCGGACCCAAGACAATCACGTTGACG -3'	This work
ccpA-downstream-5'	5'- AAACTGCAGCGGTTGTCTGCCAGCTAGTGACG -3'	This work
ccpA-downstream-3'	5'- CCCAAGCTTGGCCGTTGCGACCTTAGCCGGC -3'	This work
Con-ccpA-5'	5'- CGGATTATGTCGTGACGGCC -3'	This work
Con-ccpA-3	5'- GCGGTAACGTCTGTTGTAATGGC -3'	This work
Con-ery-for	5'- TAAATTTGGAAAGTTACACG-3'	This work
Con-ery-rev	5'- CACGAACCGTCTTATCTCCC-3'	This work

Amp: Ampicillin, Amp^r: Ampicillin resistant, Ery: Erythromycin, Ery^r: Erythromycin resistant, Con: control primer used for verification.

The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) according to the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labeled and used for microarray experiments.

4.2.5 cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit was used to synthesize cDNA out of 25 μg of total RNA, which was subsequently labeled according to the manufacturer's protocol (Amersham Biosciences, Amersham, UK). Subsequently, labeled cDNA solutions were concentrated in a Hetovac VR-1 (Heto Lab Equipment A/S, Birkerod, Denmark) to a final volume of 10 μl. Two individual, differentially labeled cDNAs were incubated at 95°C for 3 min, cooled down to 68°C, and mixed (final-volume 20 μl). To these mixed cDNAs 180 μl of pre-heated (68°C) Slidehyb hybridization buffer #1 (Ambion, Austin, USA) was added and the resulting solution was applied on a pre-heated slide (68°C). Slides were then hybridized at 44°C for 16 hours. Subsequently, slides were washed at 42°C, once in 1 x SSC/0.2% SDS and twice in 1 x SSC and dried by centrifugation.

4.2.6 Open reading frame-based microarray design and spotting

DNA-microarrays were prepared from PCR-derived amplicons of 2917 genes on the genome of *L. plantarum* WCFS1, (EMBL database, accession number AL935263) resulting in a coverage of 97%. Primers were designed to amplify unique regions of these genes using UniFrag and GenomePrimer (238). The optimal amplicon length was set at 750 bp unique regions within the genes to be amplified. Genes smaller then 750 bp were amplified entirely. Gene-specific primers were extended with a universal 15 bp sequence at the 5'end TGGCGCCCCTAGATG for the 5'-primers and CGCGATGCTGATTGC for the 3'-primers, to enable universal-primer based re-amplification, which was used to generate two-sided terminally aminated gene-specific amplicons. Aminated amplicons were mixed 1:1 with TeleChem spotting buffer (ArrayIT, Sunnyvale, USA) and spotted in duplicate on SMM superamine slides (ArrayIT, Sunnyvale, USA). After spotting the slides were washed and blocked with Na_2BH_4 as described previously (132). The slide-quality was checked with SpotCheck (Genetix, New Milton, UK) according to the manufacturer's protocol.

4.2.7 Scanning, data extraction, and analyses

Microarray slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and the images were analyzed with Imagene software 4.2 (BioDiscovery, El Segundo, USA). The overall mutation effect was determined by taking the genes with a significant differential expression (p-value< 0.05) throughout growth. Differential expression was determined using R (http://www.r-project.org/) and the linear models for microarray data library (Limma), which includes a LOESS fit of the data (42) and allows comparisons between many RNA targets simultaneously in arbitrary complicated designed experiments (212).

Significantly expressed genes in the different phases were determined as follows: Background-corrected spot intensities in both channels (I1 and I2) were converted to M-A coordinates, where M=log2 (I1/I2) and A=log2 (I1/I2)/2 and subsequently normalized using a LOESS fit (42), assuming that, on average, M is independent of A and centered around zero (212). Ratios of the normalized intensities were used for further analysis with a cut-off value of ^{2}log greater than 1 (which equals two times differential expression). Experiments were performed in duplicate and only genes matching the significance criteria in both experiments were taken into account.

4.2.8 Classification of genes

L. plantarum WCFS1 genes were classified based on the classification in 16 main classes proposed by Kleerebezem (126) using the latest updated version of the *Lactobacillus plantarum* WCFS1 database (https://bamics3.cmbi.kun.nl/plantdb).

4.2.9 Determination of organic compounds in the supernatant

Lactate, acetate, ethanol, and acetoin concentrations were determined using a high-performance liquid chromatography (HPLC) as described previously, with an HPX-87P anion exchange column (Bio-Rad, Inc.) using 0.01 N H2SO4 as the elution fluid (216).

4.2.10 Preparation of soluble protein fraction

Pelleted cells were washed with PBS and double distilled water (ddH$_2$O), respectively, and centrifuged at 5000 x *g* for 10 min. The supernatant was discarded and the cells were resuspended in buffer containing 8 M urea (Bio-Rad,

Hercules, CA, USA), 2% w/v CHAPS (Sigma, St Louis, MO, USA) 65 mM DTT (Sigma), 0.5% v/v IPG buffer (pH 3-10 NL; Amersham Pharmacia Biotech, Uppsala, Sweden). The bacteria were lysed mechanically by beating with zirconium beads (diameter 0.8 mm) using a mini bead-beater (Biospec Products, Bartlesville, OK, USA) for 5 x 1 min with 1 min intervals on ice. The cell debris was removed by centrifugation at 9000 x *g* and the supernatant was collected and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was collected and stored at -80°C until further use.

Prior to gel electrophoresis, the protein concentration was determined using a Bradford-based DC-protein assay (Bio-Rad), using BSA as the standard. SDS-PAGE was used to visualize the cytosolic proteins and also to confirm protein quality of all protein extracts (32). Briefly, 20 µg of protein was loaded on a 12.5% SDS-PAGE gel that was run constantly at a voltage of 110 V until the bromophenol blue front was run off. Afterwards, the gels were stained with silver nitrate according to Shevchenko *et al.* (207). Gel images were obtained by using a GS-800 calibrated densitometer (Bio-Rad).

4.2.11 Iso-electric focusing and two-dimensional electrophoresis

L. plantarum cytosolic proteins were first separated by IEF. Proteins (60 µg) were loaded on Immobiline Dry Strips (pH range 3-10, non-linear, 24 cm long; Amersham). IEF was performed on an IPGphor electrophoresis unit (Amersham), operating at a constant temperature of 20°C. The strips, containing the samples, were first actively rehydrated at 30 volts for 12 h. The IEF program was as follows: 250 V for 1h, 500 V for 1 h, 1000 V for 1 h, 1,000-8,000 V for 2 h and 8,000 V for 52,000 Vh. Prior to running the second dimension, the strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% DTT and for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% iodoacetamide. The strips were placed on 12.5% SDS-PAGE gels and covered with 0.5% agarose containing a trace of bromophenol blue. The gels were run at a constant voltage of 200 V in a Dodeca Cell system (Bio-Rad) until the marker dye had reached the base of the gel. The gels were stained with silver nitrate as described above. The images were obtained with a GS-800 calibrated densitometer (Bio-Rad).

4.2.12 Protein identification

Image analysis was performed using the PDQuest software (version 7.2) (Bio-Rad). Three gels were produced for every growth condition and were used for comparison of the obtained spots. Proteins were considered differentially produced when spot intensities passed the threshold of at least a 2-fold difference in up- or down-regulation in combination with a student's t-test using a statistical 95% reliability score. Proteins were identified using a proteome reference map of the wild-type *L. plantarum* WCFS1 (45). Using the PDQuest software, protein spots from the proteome reference map were matched with the protein gels containing protein fraction from the wild-type strain and ΔCcpA-strain used in this study.

DNA-microarrays were prepared from PCR-derived amplicons of 2917 genes on the genome of *L. plantarum* WCFS1, (EMBL database, accession number AL935263) resulting in a coverage of 97%. Primers were designed to amplify unique regions of these genes using UniFrag and GenomePrimer (238). The optimal amplicon length was set at 750 bp unique regions within the genes to be amplified. Genes smaller then 750 bp were amplified entirely. Gene-specific primers were extended with a universal 15 bp sequence at the 5'end TGGCGCCCCTAGATG for the 5'-primers and CGCGATGCTGATTGC for the 3'-primers, to enable universal-primer based re-amplification, which was used to generate two-sided terminally aminated gene-specific amplicons. Aminated amplicons were mixed 1:1 with TeleChem spotting buffer (ArrayIT, Sunnyvale, USA) and spotted in duplicate on SMM superamine slides (ArrayIT, Sunnyvale, USA). After spotting the slides were washed and blocked with Na_2BH_4 as described previously (132). The slide-quality was checked with SpotCheck (Genetix, New Milton, UK) according to the manufacturer's protocol.

4.3 Results

4.3.1 Growth and fermentation of the *ccpA*-mutant strain NZ7304

To study the function of CcpA in *L. plantarum* WCFS1, a *ccpA*-deficient strain was constructed using a double-cross-over gene replacement strategy. This strain, designated NZ7304, was grown anaerobically in a batch culture under acidifying conditions at 37°C in glucose-containing rich medium (MRS) and its growth characteristics were compared to those observed for the parental strain. As previously described (217), the wild-type strain displayed biphasic growth under these conditions, ultimately reaching an OD_{600} of approximately 4.5 and a pH of 4.0 after 10 h (Fig. 1). Growth and acidification rates were higher during the early growth phase till OD_{600} 2.0 (doubling time 0.75 hours), and decreased during later phases of growth for both wild-type and mutant (t-double 3.5 hours) (Fig.1). The NZ7304 (Δ*ccpA*) displayed the characteristic biphasic growth to a much lesser extent than observed for the wild-type strain (Fig. 1).
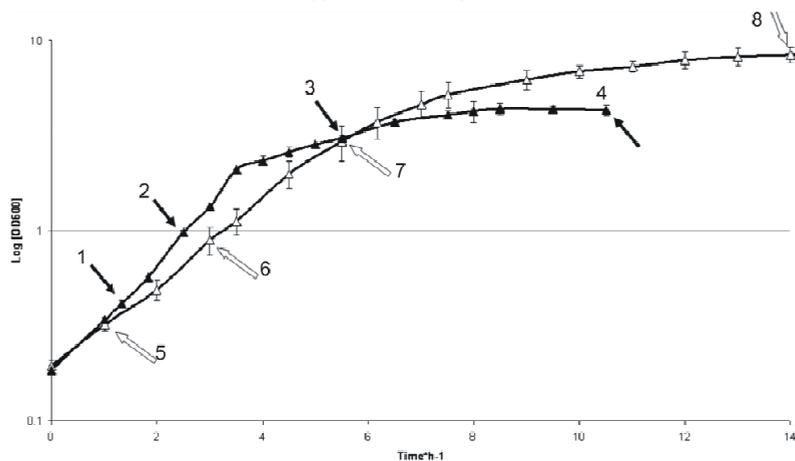
Figure 1. Anaerobic growth curves of the wild-type strain *L. plantarum* WCFS1 (filled triangles) and the CcpA-mutant strain NZ7304 (open triangles). Arrows indicate the time points at which the samples were taken for transcriptomic analysis. 1: OD_{600} 0.4 2: OD_{600} 1.0 3: OD_{600} 3.1 4: OD_{600} 4.5 5: OD_{600} 0.3 6: OD_{600} 0.9 7: OD_{600} 3.1 8: OD_{600} 8.6 At time points 2, 3, 6, 7 samples were taken for proteomic analysis. Samples for transcriptomic analysis were taken at time points 1-8. Early-log phase: 1&5 Mid-log phase: 2&6 Transition phase: 3&7 Stationary phase: 4&8, respectively.

Furthermore, the strain NZ7304 remarkably reached approximately a 2-fold higher cell density (OD_{600} 8.0) than the wild-type strain. However, the relative growth rate of NZ7304 was lower during the early growth phases, but growth continued for a

longer period to a higher density and only reduced after 8 h when cells were entering the stationary phase of growth. Interestingly, strain NZ7304 displayed reduced acidification rates as compared to the wild-type culture, and at later stages of growth (OD$_{600}$ > 2.0), the pH values were higher in the NZ7304 culture despite the higher cell-densities (data not shown). These data indicate that the correlation between cell density and acidification was altered as a consequence of the *ccpA* mutation.

Under the conditions employed here, *L. plantarum* wild-type cells are known to display a virtually homolactic fermentation pattern, producing D- and L-lactate in approximately equimolar amounts (78). The reduced acidification rate observed for the *ccpA* mutant suggests an altered or heterolactic fermentation pattern in this strain, as has also been observed for a *ccpA*-mutant derivative of the homolactic LAB *Lactococcus lactis* MG1363 (86). To investigate the postulated changes in fermentation end-products, the concentrations of different metabolic end-products in culture supernatants of *L. plantarum* wild-type and NZ7304 strains were analyzed by HPLC. The final lactate concentration that was ultimately reached when cells were in the stationary phase of growth was similar in both wild-type and mutant (Table 2). The higher cell densities reached by NZ7304 in this phase of growth indicates that the ratio between biomass and lactate production has drastically changed in this strain as compared to its parental strain. Accurate measurement of acetate concentrations formed during growth was hampered by the presence of considerable amounts of acetate in the medium used. Moreover, the presence of acetate in the native medium could inhibit the formation of this metabolite as a fermentation end-product (147). Nevertheless, acetate formation of 0.2 mM was researched during the stationary phase for the wild-type strain (Table 2). A two-fold higher acetate production, 0.46 mM, was established in the culture media of NZ7304, which appeared to be produced both during the exponential and stationary phases of growth (Table 2).

This acetate-producing phenotype has previously also been observed for wild-type *L. plantarum* cultures when grown under aerobic conditions (82). Two additional fermentation end-products were detected, i.e., ethanol and acetoin, both produced in small amounts (Table 2). Ethanol was produced in similar amounts in the wild-type and NZ7304 strains during the logarithmic phase of growth, but NZ7304 produced significantly more ethanol during the stationary phase of

growth. Acetoin was only produced by the *ccpA* mutant strain and its production was only seen during the stationary phase of growth (Table 2). Although the capacity to produce additional fermentation end-products such as succinate, formate, and 1,2-butandiol has been predicted on the basis of the *L. plantarum* genome sequence, none of these products were detected in these cultures.

Table 2: Analyses of end-fermentation products in the four growth phases of *Lactobacillus plantarum*. Values are in mM, ND is not detected.

	Early-log phase	Mid-log phase	Transition phase	Stationary phase
L. plantarum WCFS1 wild-type				
Lactate	0.22 ± 0.09	1.13 ± 0.06	5.74 ± 0.12	10.98 ± 0.56
Acetate	ND	ND	ND	0.20 ± 0.12
Acetoin	ND	ND	ND	ND
Ethanol	ND	0.09 ± 0.04	0.22 ± 0.01	0.31 ± 0.05
L. plantarum NZ7304				
Lactate	0.22 ± 0.09	1.41 ± 0.1	4.93 ± 0.08	13.97 ± 0.43
Acetate	0.03 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.46 ± 0.01
Acetoin	ND	ND	ND	0.21 ± 0.24
Ethanol	0.06 ± 0.06	0.11 ± 0.08	0.31 ± 0.08	2.00 ± 1.41

4.3.2 Comparison of transcriptome and proteome of *L. plantarum* WCFS1

In this study, transcriptomic and proteomic analyses were performed in parallel. The availability of previously determined proteome data sets (45) and here determined (Fig. 2A-D) and transcriptome data allowed us to compare the results of both –omics approaches. First, the proteomes and transcriptomes were compared of *L. plantarum* WCFS1 during different growth phases. Proteins that were identified to be differentially regulated by the proteomic analysis were analyzed for the expression of their genes in the transcriptomics analysis (Table 3). The proteomics approach detected three proteins spots which were identified as a phosphoenolpyruvate transferase system (PTS) EIIAB transport protein (Fig. 2E) indicating different isoforms of the protein.

The proteome analysis resulted in the identification of proteins which were highly produced during logarithmic growth (Table 3A) and 50% of these proteins (18 out of 36) showed comparable expression ratios in the transcriptome experiment. A total of 17 proteins were identified to be produced during the complete growth curve (Table 3B) and, within a cut-off value of two times differential expression, 88% (corresponding to 15 proteins) had a similar behavior at the gene expression level. Finally, of the proteins identified to be produced at a lower level during logarithmic growth (Table 3C) 72% (13 out of 18) displayed similar expression characteristics in the transcriptome analyses.
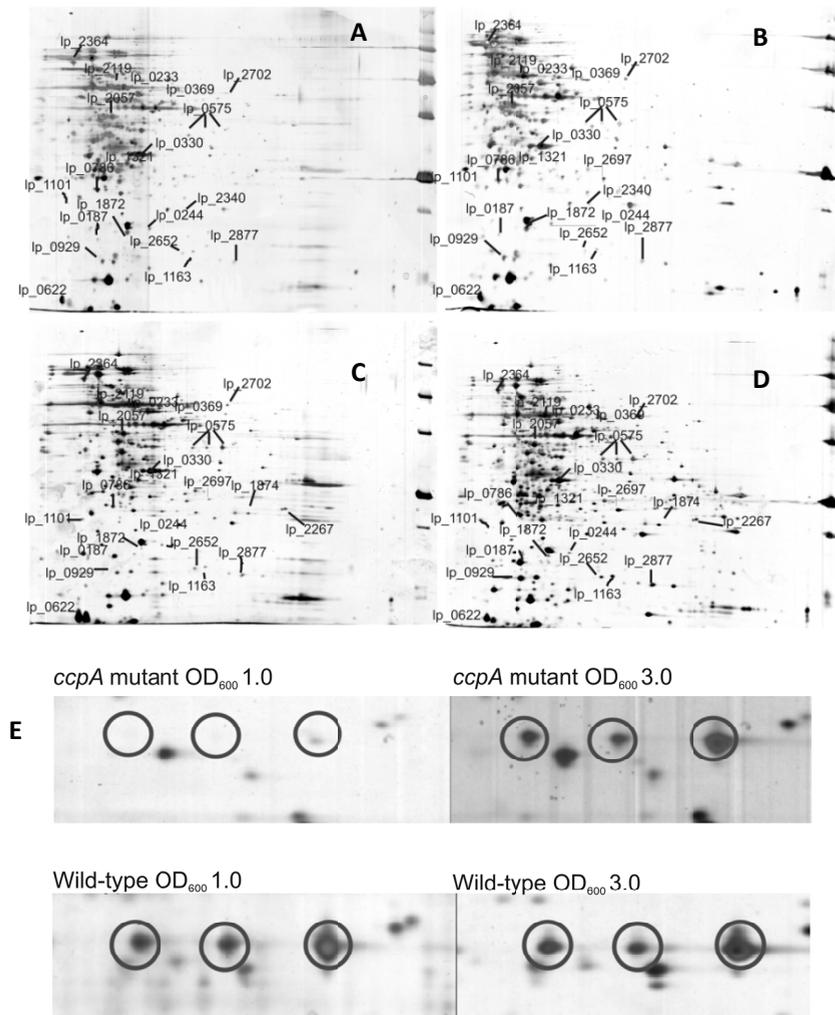
Figure 2. Two-dimensional gel electrophoresis (2-DE) gel analysis of the proteome of *Lactobacillus plantarum* strains. Panel A and B: proteome analysis of *L. plantarum* NZ7304, which has no functional gene coding for CcpA at time points OD_{600} 1.0 and 3.0, respectively. Panel C and D: proteome analysis of the wild-type strain *L. plantarum* WCFS1 at time points OD_{600} 1.0 and 3.0, respectively. Protein spots that were found to be differentially regulated between the proteomes (panels A-D) and were identified using the proteome reference map of *L. plantarum* WCFS1 (45) are indicated and the numbers correspond to the annotated open reading frames (ORFs) of *L. plantarum* WCFS1 (126).(panel E). Three isoforms of the protein PTS EIIAB are indicated on 2-DE gel (encircled)

Table 3: Comparison of the proteomic and the transcriptomic analyses throughout the different growth phases of *L. plantarum* WCFS1. Proteomics results of Cohen *et. al.* (45) were used. A: Up-regulated proteins during logarithmic growth in the proteome experiment and the regulation of their genes in the transcriptome experiment stationary phase compared to log phase. B: Proteins found to be produced in similar amounts during growth and the regulation of their gene in the transcriptome experiment stationary phase compared to log phase. C: Proteins found to be down regulated during logarithmic growth and the regulation of their gene in the transcriptome experiment stationary phase compared to log phase. Genes in bold indicate a difference between transcriptome and proteome data. P: fold-change in protein abundance detected by proteomics. T: fold-change in gene expression detected by transcriptomics

A

ORF	Name	Product	Main Class	P	T
lp_0002	dnaN	DNA polymerase III, β-chain	DNA metabolism	1.5	3.6
lp_0244	lp_0244	Oxidoreductase	Hypothetical proteins	2.84	0.6
lp_0255	metC1	Cystathionine β-lyase	Amino acid biosynthesis	7.3	1.2
lp_0363	lp_0363	Purine nucleosidase	Purines, pyrimidines, nucleosides and nucleotides	1.3	1.4
lp_0430	plnX	Plantaricin biosynthesis protein PlnX	Cellular processes	4.1	0.8
lp_0537	ldhL1	L-lactate dehydrogenase	Energy metabolism	3.9	0.9
lp_0737	lp_0737	Ribosomal protein S30EA	Protein synthesis	1.2	0.2
lp_0786	clpP	Endopeptidase Clp,	Cellular processes	1.4	0.7
lp_0792	enoA1	Phosphopyruvate hydratase	Energy metabolism	1.5	1.4
lp_0900	pgm3	Phosphoglycerate mutase	Energy metabolism	4.9	0.7
lp_0929	asp1	Alkaline shock protein	Cellular processes	1.6	0.1
lp_0959	pepD3	Dipeptidase	Protein fate	3.0	0.4
lp_1163	lp_1163	Universal stress protein UspA	Hypothetical proteins	1.2	0.2
lp_1292	lp_1292	Acetyltransferase (putative)	Hypothetical proteins	2.0	11.1
lp_1301	metK	Methionine adenosyltransferase	Central intermediary metabolism	2.9	3.4
lp_1321	pepV	Dipeptidase	Protein fate	1.8	2.3
lp_1391	argS	Arginine--tRNA ligase	Protein synthesis	2.6	1.9
lp_1573	glk	Glucokinase	Energy metabolism	1.2	14.2
lp_1680	accA2	Acetyl-CoA carboxylase	Fatty acid and phospholipid metabolism	8	20
lp_1681	fabI	Enoyl- reductase (NADH)	Fatty acid and phospholipid metabolism	1.8	0.4
lp_1874	dapB	Dihydrodipicolinate reductase	Amino acid biosynthesis	1.9	0.4
lp_1874	dapB	Dihydrodipicolinate reductase	Amino acid biosynthesis	1.9	0.4
lp_1874	dapB	Dihydrodipicolinate reductase	Amino acid biosynthesis	1.9	1.8
lp_1976	nfo	Deoxyribonuclease IV	DNA metabolism	2.5	1.9
lp_2189	divIVA	Cell division initiation protein DivIVA	Cellular processes	2.2	2.0
lp_2193	ftsZ	Cell division protein FtsZ	Cellular processes	1.8	0.7
lp_2360	lp_2360	Ribosomal protein acetylating enzyme	Protein synthesis	4.5	2.3
lp_2451	lp_2451	Prophage P2a protein 6	Other categories	3.6	0.1
lp_2697	pyrE	Orotate phosphoribosyltransferase	Purines, pyrimidines,	2.4	0.2

ORF	Name	Product	Main Class		
			nucleosides and nucleotides		
lp_2702	pyrC	Dihydroorotase	Purines, pyrimidines, nucleosides and nucleotides	2.5	0.04
lp_2703	pyrB	Aspartate carbamoyltransferase	Purines, pyrimidines, nucleosides and nucleotides	5.6	16.7
lp_2823	lp_2823	ABC transporter, ATP-binding protein	Transport and binding proteins	1.5	0.3
lp_2877	lp_2877	Universal stress protein UspA	cellular processes	1.6	3.6
lp_3045	lp_3045	Short-chain dehydrogenase/ oxidoreductase	Energy metabolism	3.5	3.8
lp_3199	parA	Chomosome partitioning protein, membrane-associated ATPase	Cellular processes	3.0	0.7
lp_3416	lp_3416	Transcription regulator (putative)	Regulatory functions	>10	

B

ORF	Name	Product	Main Class	P	T
lp_0002	dnaN	DNA polymerase III, beta chain	DNA metabolism	1.5	1.04
lp_0313	ndh1	NADH dehydrogenase	Energy metabolism	1.6	1.55
lp_0792	enoA1	Phosphopyruvate hydratase	Energy metabolism	1.5	0.87
lp_0914	guaA	GMP synthase (glutamine-hydrolysing)	Purines, pyrimidines, nucleosides and nucleotides	1.9	0.76
lp_1005	als	Acetolactate synthase	Energy metabolism	1.7	1.34
lp_1321	pepV	Dipeptidase	Protein fate	0.5	1.35
lp_1747	lp_1747	Universal stress protein UspA family	cellular processes	8.7	2.31
lp_1843	lp_1843	Unknown	Hypothetical proteins	1.7	0.64
lp_1872	lp_1872	Unknown	Hypothetical proteins	0.7	1.00
lp_2048	proS	Proline--tRNA ligase	Protein synthesis	1.3	0.70
lp_2118	tig	Trigger factor	Protein fate	1.6	0.67
lp_2193	ftsZ	Cell division protein FtsZ	Cellular processes	0.5	0.95
lp_2194	ftsA	Cell division protein FtsA	Cellular processes	2.0	0.99
lp_2366	atpA	H^+-transporting 2-sector ATPase, α-subunit	Energy metabolism	0.9	0.55
lp_2703	pyrB	Aspartate carbamoyltransferase	Purines, pyrimidines, nucleosides and nucleotides	1.2	4.72
lp_3267	gshR4	Glutathione reductase	Biosynthesis of cofactors, prosthetic groups, and carriers	0.9	1.58

C

ORF	Name	Product	Main Class	P	T
lp_0137	lp_0137	Oxidoreductase	Hypothetical proteins	2.2	8.06
lp_0226	gnp	Glucosamine-6-phosphate isomerase	Central intermediary metabolism	2.7	4.23
lp_0233	mtlD	Mannitol-1-phosphate 5-dehydrogenase	Energy metabolism	7.5	3.01
lp_0575	pts9AB	Mannose PTS, EIIAB	Transport and binding proteins	2.8	1.00
lp_0786	clpP	Endopeptidase Clp, proteolytic subunit	Cellular processes	1.9	1.38
lp_0820	glmM	Phosphoglucosamine mutase	Central intermediary metabolism	13	0.32
lp_1837	lp_1837	Inorganic pyrophosphatase	Central intermediary metabolism	2.1	0.60
lp_2028	grpE	Heat shock protein GrpE	Cellular processes	1.7	1.10
lp_2340	lp_2340	Universal stress protein UspA	cellular processes	1.6	3.46
lp_2384	pmi	Mannose-6-phosphate isomerase	Energy metabolism	1.9	1.72
lp_2570	asd2	Aspartate-semialdehyde dehydrogenase	Amino acid biosynthesis	1.5	1.26
lp_3100	lp_3100	Oxidoreductase	Hypothetical proteins	3.9	0.83
lp_3352	hsp3	Small heat shock protein	Cellular processes	1.9	4.38
lp_3481	galE4	UDP-glucose 4-epimerase	Purines, pyrimidines, nucleosides and nucleotides	17	0.89
lp_3482	galK	Galactokinase	Energy metabolism	11	0.42

4.3.3 Growth phase specific transcriptome profiling

Recent studies in *L. lactis* and *B. subtilis* showed differences in CcpA mediated control in different phases of growth. To investigate growth phase dependent *ccpA*-mediated regulation of gene expression in *L. plantarum*, transcriptomes at various time points during batch-culture growth were profiled using a full-genome amplicon-based microarray. The results obtained indicated that the expression of relatively few genes was affected by the *ccpA* mutation during all growth phases (Table 4). A total of 34 genes were expressed at a higher level in the mutant (NZ7304), indicating CcpA-dependent repression in the wild-type, while only four genes appeared to be subjected to CcpA activation as could be concluded from their higher level of expression in the wild-type strain.

In conclusion, the transcriptional analyses established that the CcpA mutation affects mainly genes encoding proteins classified among regulatory functions, energy metabolism, and transporters.

Table 4. Genes down-regulated (A) and up-regulated (B) during all growth phases in wild-type *L. plantarum* WCFS1 compared to the *ccpA*-mutant *L. plantarum* NZ7304

A

ORF	Name	Product	p-Value
Biosynthesis of cofactors, prosthetic groups, and carriers			
lp_0115	thiE	Thiamine-phosphate pyrophosphorylase	0.040
Cell envelope			
lp_1763	lp_1763	Glycosyltransferase	0.023
Central intermediary metabolism			
lp_1730	map3	Maltose phosphorylase	0.001
lp_3530	map4	Maltose phosphorylase	0.008
Energy metabolism			
lp_0849	pox1	Pyruvate oxidase	0.002
lp_0874	lp_0874	Bifunctional protein: amino acid aminotransferase; 2-hydroxyacid dehydrogenase	0.008
lp_1250	gntK	Gluconokinase	0.046
lp_1731	galM2	Aldose 1-epimerase	0.001
lp_2152	pdhC	Pyruvate dehydrogenase complex, E2 component; dihydrolipoamide S-acetyltransferase	0.001
lp_2153	pdhB	pyruvate dehydrogenase complex, E1 component, beta subunit	0.001
lp_2154	pdhA	pyruvate dehydrogenase complex, E1 component, alpha subunit	0.001
lp_3314	pflA2	formate acetyltransferase activating enzyme	0.001
lp_3487	galM3	Aldose 1-epimerase	0.001
lp_3526	pbg10	6-Phospho-beta-glucosidase	0.001
lp_3551	xpk2	Phosphoketolase	0.001
lp_3555	araD	L-Ribulose 5-phosphate 4-epimerase	0.034
lp_3607	iolE	Inositol catabolism protein IolE	0.004
Fatty acid and phospholipid metabolism			
lp_0067	bsh2	Choloylglycine hydrolase	0.002
lp_0370	glpK1	Glycerol kinase	0.001
Hypothetical proteins			
lp_0098	lp_0098	Adenylyl transferase (putative)	0.011
lp_1518	lp_1518	Unknown	0.003
lp_1566	lp_1566	Unknown	0.023
lp_2787	lp_2787	Hydrolase, HAD superfamily, Cof family	0.004

lp_2993	lp_2993	Unknown	0.002
lp_3100	lp_3100	Oxidoreductase	0.032
lp_3552	lp_3552	Unknown	0.040
Regulatory functions			
lp_0172	lp_0172	Transcription regulator	0.001
lp_3234	lp_3234	Transcription regulator	0.032
lp_3523	lp_3523	Glucokinase regulatory protein	0.049
Transport and binding proteins			
lp_0265	pts5ABC	PTS system, trehalose-specific IIBC component	0.026
lp_0286	pts6C	Cellobiose PTS, EIIC	0.004
lp_2780	pts20A	Cellobiose PTS, EIIA	0.001
lp_3635	msmK2	Multiple sugar ABC transporter, ATP-binding protein	0.001

B

ORF	name	Product	p-value
Hypothetical proteins			
lp_0111	lp_0111	Oxidoreductase	0.049
lp_0927	lp_0927	Unknown	0.022
Regulatory functions			
lp_2256	CcpA	Catabolite control protein A	0.001
lp_3579	spx5	Regulatory protein Spx	0.001

4.3.4 Comparison of transcriptome and proteome of the CcpA mutant

Comparative analysis of the proteomes of both strains showed in total 198 protein spots to be differentially regulated between the wild-type and the *ccpA* mutant. Application of the proteome reference map from a previous study (45), allowed the identification of 38 protein spots. Comparison of the wild-type and the CcpA mutant, showed that 81% (17 out of 21) and 80% (15 out of 18) of the identified proteins shared similar regulation during logarithmic growth (OD$_{600}$ 1.0) (Table 5A) and late logarithmic growth phase (OD$_{600}$ 3.0) (Table 5B), respectively.

Table 5: Proteomics results (P) and transcriptomics result (T) of the wild-type versus the *ccpA*-mutant of samples taken at mid log phase (A) and late log phase (B). P: fold-change in protein abundance detected by proteomics. T: fold-change in gene expression detected by transcriptomics Proteins showed at least 2 times differential production. ORF-numbers depicted in bold indicate the difference between the proteomic and transcriptomic data. Missing: not present in array. *: three different protein spots were identified as the same protein (45). [1]: Protein spot has been identified as a mixture of two proteins (45).

ORF	Name	Product	Main Class	P	T
lp_0233	mtlD	mannitol-1-phosphate 5-dehydrogenase	Energy metabolism	0.1	0.14
lp_0330	Fba	fructose-bisphosphate aldolase	Energy metabolism	0.3	1.18
lp_0369	gshR1	glutathione reductase	Biosynthesis of cofactors, prosthetic groups, and carriers	2	2.06
lp_0575	pts9AB	mannose PTS, EIIAB*	Transport and binding proteins	10	3.94
lp_0575	pts9AB	mannose PTS, EIIAB*2	Transport and binding proteins	10	3.94
lp_0575	pts9AB	mannose PTS, EIIAB*3	Transport and binding proteins	10	3.94
lp_0622	rplL	ribosomal protein L12/L7	Protein synthesis	2	missing
lp_0786	clpP	ATP-dependent Clp protease proteolytic subunit	Cellular processes	0.5	1.21
lp_0929	asp1	alkaline shock protein	Cellular processes	5	4.66
[1]lp_1321/ lp_2193	pepV /ftsZ	Dipeptidase/ cell division proteins FtsZ	Protein fate	0.2	missing
lp_1563	greA2	transcription elongation factor GreA	Transcription	3	1.3
lp_1872		hypothetical protein	Hypothetical proteins	0.1	0.6
lp_1874	dapB	dihydrodipicolinate reductase	Amino acid biosynthesis	10	0.27
lp_1874	dapB	dihydrodipicolinate reductase	Amino acid biosynthesis	4	0.63
lp_2057	ldhD	D-lactate dehydrogenase	Energy metabolism	0.25	0.63
[1]lp_2119 /lp_0790	Tuf /pgk	elongation factor Tu/ phosphoglycerate kinase	Protein synthesis	0.3	0.79
lp_2267	xtp1	xanthosine triphosphate pyrophosphatase	Purines, pyrimidines, nucleosides and nucleotides	2	missing
lp_2652		hypothetical protein	Cellular processes	2	0.86
lp_2697	pyre	orotate phosphoribosyltransferase	Purines, pyrimidines, nucleosides and	10	1.49

			nucleotides		
lp_2702	pyrC	Dihydroorotase	Purines, pyrimidines, nucleosides and nucleotides	10	missing

B

ORF	Name	Product	Main Class	P	T
lp_0233	mtlD	mannitol-1-phosphate 5-dehydrogenase	Energy metabolism	0.1	0.34
lp_0244	lp_0244	oxidoreductase (putative)	Hypothetical proteins	0.3	0.45
lp_0622	rplL	ribosomal protein L12/L7	Protein synthesis	5	missing
lp_0727	groES	GroES co-chaperonin	Cellular processes	10	1.80
lp_0792	enoA1	phosphopyruvate hydratase	Energy metabolism	0.5	1.62
lp_0929	asp1	alkaline shock protein	Cellular processes	4	2.62
lp_0930	asp2	alkaline shock protein	Cellular processes	10	1.26
lp_1163	lp_1163	nucleotide-binding protein	Hypothetical proteins	4	0.69
[1]lp_1321 /2193	pepV/ ftsZ	Dipeptidase/ cell division proteins FtsZ	Protein fate	0.3	0.78

4.4 Discussion

In this study, we compared the proteomics versus transcriptomics data in *L. plantarum* WCFS1 wild-type as well as its ΔCcpA derivative mutant. CcpA is activated by HPr-Ser46 whose formation is dependent on the energy status of the cell (221). *L. plantarum* cells produce high amounts of lactate in the early growth phase, indicating high glycolytic flux and high energy status under these conditions (231), which explains the high CcpA activity in the early phases of growth as demonstrated by the transcriptional analysis in this study. It was observed that CcpA acts as a global regulator in *L. plantarum* WCFS1, since transcriptome analyses revealed that the expression level of approximately 8% of all genes was affected by deletion of the *ccpA* gene. The most prominent CcpA-mediated transcription control was found in the control of carbon utilization, since the majority of the regulated genes was involved in carbohydrate-catabolic reactions. Furthermore, the full genome transcription analyses revealed that the main function of CcpA in *L. plantarum* is carbon catabolite repression and regulation of

sugar metabolism, which parallels the reported role of CcpA in other Gram-positive bacteria like *B. subtilis* and *L. lactis* (169, 261).

The up regulation of two *cre*-site containing bile salt hydrolases suggests a role for CcpA in the GI-tract, which is not surprising since relief of catabolite repression in the GI-tract has already been suggested in other studies that evaluated in situ gene expression of *L. plantarum* in the GI tract (29). Moreover, a role for CcpA in the adaptation of *L. plantarum* to specific niches is also illustrated by the *ccpA*-mediated regulation of putative oligo- and polysaccharide degrading protein complexes in the cell wall (208).

The potential correlation between transcriptomics and proteomics data was initially investigated in the wild-type strain (*L. plantarum* WCFS1). A previous proteome analysis by 2-DE of wild-type *L. plantarum* displayed differential regulation of proteins during growth of the wild-type (45). The present study supported these observations with transcriptomic analyses, revealing a good correlation (70 to 80%) between changes in protein abundance and mRNA levels. Nevertheless, some discrepancies between these two post-genomic analyses were observed, which mainly corresponded to genes involved in amino acid metabolism, cellular processes, and purine, pyrimidines, nucleosides and nucleotide biosynthesis. One protein that was identified on the 2-DE gels in three different isoforms was the enzyme dihydrodipicolinate reductase, which is involved in L-lysine biosynthesis and catalyzes a controlling step in L-lysine production in *Corynebacterium glutamicum* (65). Since this function could be controlled through secondary, post-translational regulatory mechanisms a lack of correlation between mRNA- and protein-levels might have been anticipated. In order to verify the similarity between both transcriptomics and proteomics approaches, the wild-type WCFS1 was compared to a *ccpA* gene deletion strain. The variation observed between both –omics approaches can be explained by several reasons. One of these is a putative difference in half-life between mRNA and proteins. The turn-over of bacterial mRNA is known to be very high and previous studies showed that in *Escherichia coli*, the mRNA half-life varies between 3 and 8 minutes (20), while the half-life of proteins can reach 60 min during growth and starvation (172). Due to the short life time of mRNA molecules, it is more likely that the activity of a microbe can be measured more accurately by proteome analysis than transcriptome analysis. In contrast, transcriptome analyses

provide a methodology that enables a more accurate evaluation of the dynamics and regulatory networks underlying microbial responses to environmental changes. The half lives of mRNA and proteins depends on various factors, that have impact on the differences of microbial response analyses when using either mRNA or proteins analysis. The presence of Clp-proteases on the 2-DE gels (Tables 2A, 3A, and 3C) might support this reasoning as these proteases are involved in cytolytic protein degradation (39) and thus might affect relative abundance and turn-over of particular proteins. Differential turnover of specific biomolecules could possibly explain the dissimilar observations related to the abundance of purine- and pyrimidine- biosynthesis genes (Table 3), since the corresponding mRNAs are known to be relatively unstable in *E. coli* (20) and probably also in *L. plantarum* (217). Analogously, the mRNA of a universal stress protein in *B. subtilis* (118) and genes encoding protection responses in *E. coli* (14) are highly stable, which might explain the contradictory results in gene expression and protein regulation found for stress related genes in the transcriptome and proteome analyses (Table 3). Fructose-bisphosphate aldolase (Table 5A), phosphopyruvate hydratase (Table 5B), and phopshoglycerate mutase (Table 3A) are glycolytic enzymes of the main energy generating pathway in glucose-grown lactic acid bacteria. Regulation of glycolytic enzymes at least partially depends on increased mRNA stability as has initially been described for the Gram negative bacterium *Zymomonos mobilis* (64) and was later confirmed in the Gram positive model organism *B. subtilis* (148). Further support for high mRNA stability of glycolytic transcripts has been described for *E. coli* where mRNAs corresponding to the energy metabolism functional category were found to be highly stable (14). The analogous results found for lactate dehydrogenase (Table 3A), would support relatively high mRNA stability for this gene transcript in *L. plantarum*. The high stability of these mRNAs might lead to the differential mRNA and protein turnover and to the different results for RNA and protein abundance.

The advantage of transcriptomics is the global gene expression analysis of the almost complete genome (97% coverage) compared to proteomics which shows approximately 600 protein spots per gel (45). However, post-translational modification (PTM) can be visualized by proteomics. Three protein spots were shown on 2-DE (Fig. 1F) and identified as mannose PTS, EIIAB protein. The spots differ only in molecular weight which can indicate that PTM has occurred. This

protein has two domains (EIIA and EIIB) and both domains can be phosphorylated at the histidyl residue and in addition, domain EIIB can also be phosphorylated at cysteinyl residue (59).

Growth conditions that provide excess glucose concentrations lead to an almost homolactic fermentation pattern in the wild-type cells of *L. plantarum* (73), generating two molar equivalents of ATP per glucose consumed. However, several pathways leading to acetate appear to be present in this microorganism: anaerobic conversion via pyruvate formate lyase (PFL) (144), aerobic conversion via pyruvate oxidase (POX) (82), and conversion via pyruvate dehydrogenase (PDH) (126). The genes encoding the latter enzyme appeared to be tightly controlled by CcpA as revealed by the transcriptome analysis. However, several studies suggested that PDH activity is lacking in *L. plantarum* (61, 102, 170), indicating that acetate production via this pathway is not unambiguously established. Pyruvate oxidase (*pox*) expression has previously been shown to be under control of CcpA (147), which is confirmed in our transcriptome analysis. Nevertheless, due to the anaerobic conditions applied in this study acetate production via the POX-dependent pathway is not the most likely explanation for the observed acetate production in the *ccpA* mutant strain. Therefore, acetate production is most probably formed through the pyruvate format lyase (PFL), phosphotransacetylase (PTA), and acetate kinase (ACK) pathway. Acetate production leads to additional ATP gain compared to lactate production and the *ccpA* mutant thereby produces more energy from the same amount of glucose consumed. The higher cell density that is ultimately reached by the *ccpA* mutant is most likely related to the reduced lactate and pH stress during growth as well as the increased energy gain through the production of acetate.

Overall, these transcriptomic and proteomic analyses confirmed a global role for CcpA in regulation of carbohydrate utilization by *Lactobacillus plantarum* WCFS1 with emphasis at the levels of transport, energy metabolism, and specific regulatory functions, which clearly parallels the ccpA-regulons reported for other species (169, 261). These results are in good agreement with the observation that *ccpA*-deletion in *L. plantarum* WCFS1 resulted in altered growth and fermentation characteristics. Strain NZ7304 reached higher cell densities and produced reduced amounts of lactate, while producing higher levels of acetate. Combined, the affected functional classes present a coherent response that includes transport

and degradation functions for the same sugars, the pentose phosphate pathway, the tricarboxylic acid cycle (TCA-cycle), glycolysis, and pyruvate metabolism. Moreover, post-genomic analyses showed a 70 to 80% confirmation of proteome data by transcriptome data, independent of the growth phase or strain that was analyzed in a particular comparison. Genes with inconsistent or deviating transcript and protein levels mainly classified among purine and pyrimidines biosynthesis, energy metabolism, and stress proteins.

In conclusion, our study has shown that parallel transcriptome and proteome analyses provide a powerful approach to determine abundance of molecules in the cell. Ultimately, combining these techniques could lead to new targets which are possibly regulated via mRNA and/or protein stability and could enable the elucidation of novel (post-translational) regulatory networks in the cell.

Chapter 5

Validation of a *Lactobacillus plantarum* Specific 16S Ribosomal RNA Probe for Application in Fluorescent *In Situ* Hybridization in the Human Intestinal Ecosystem

DAVID PA COHEN, WILLEM M DE VOS AND ELAINE E VAUGHAN
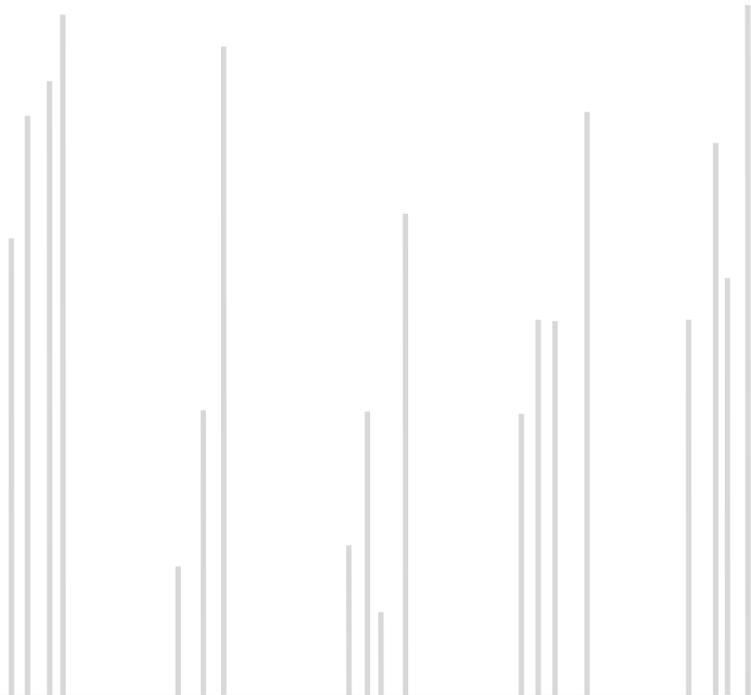
Abstract

The oligonucleotide probe Plant67 that was developed for targeting the 16S ribosomal RNA of *Lactobacillus plantarum* in dot-blot hybridizations, was validated for application in fluorescent *in situ* hybridization of whole cells from human intestinal samples. The probe was tested against 11 *Lactobacillus* species and 18 other gram-positive representatives from various genera. Moreover, to use this probe for enumeration and cell sorting from gastrointestinal samples, we validated it against 40 different bacterial species that are commonly found in the human intestine. No unspecific hybridization of the probe occurred. The probe was subsequently successfully applied to label *L. plantarum* cells from the complex intestinal ecosystems, namely ileal effluent and fecal samples.

5.1 Introduction

The human gastrointestinal tract (GI) harbors a complex and diverse microbial community. Cultivation studies have indicated that the number of bacterial cells increases gradually from the upper part to the lower part of the GI tract, varying from 10^4 cells/gram in the stomach to 10^{11} cells/g in the colon (209). While the small intestine is colonized by a majority of facultative anaerobes like lactobacilli, streptococci and enterobacteria, the colon is essentially a reservoir of strict anaerobic bacteria, including bacteroides, clostridia and bifidobacteria (103).

Lactobacilli are gram-positive and non-spore forming rods able to convert sugars into lactic acid (36). One of these, *Lactobacillus plantarum*, is commonly used in the production of fermented foods, including cheese products, meat and vegetables (55). Moreover, *L. plantarum* is a commensal inhabitant of the human intestinal tract where it can be found in numbers between 10^6 to 10^8 colony forming units/g of mucosa along the human GI tract (4, 196). Several *L. plantarum* strains are marketed as probiotics, defined as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (55, 70). There is considerable interest in determining the activity of probiotic bacteria inside the host as a means to providing a mechanistic basis for their activity (53). Recently, the complete genome of *Lactobacillus plantarum* WCFS1 has been sequenced (3.3 Mb) (126), and it has been shown that this human isolate is able to survive the passage through the stomach of man efficiently (241). Various genetic and transcriptomic approaches showed that *L. plantarum* WCFS1 induces specific survival mechanisms upon encountering bile salts and during passage of the GI tract (29, 31, 53, 159). To complement these nucleic acid-based approaches, we developed a proteomics platform (45) that could provide insight into the survival and activity of *L. plantarum* WCFS1 after passage through the GI tract.

The aim of this study is to specifically label *L. plantarum* using fluorescent in situ hybridization (FISH), allowing for its detection in the human intestinal tract ecosystem, and potentially for sorting and isolation by flow cytometry following previously established procedures (17). Subsequently, the proteome of the isolated *L. plantarum* cells could be characterized. In this study, we have validated an existing probe for *L. plantarum* that had been developed for dot-blot hybridization of *L. plantarum* in dairy niches by Hensiek and colleagues (100). Because the human microbiota in the gastrointestinal tract consists of

approximately 1,000 different bacterial species (260), an extensive validation of the probe's specificity against representative bacterial species of this ecosystem was required. Furthermore, during dot-blot hybridization. the ribosomal RNA (rRNA) molecule is denatured facilitating its hybridization properties, while in whole cells it maintains its native conformation that can obstruct the probe to hybridize with the target DNA (9, 243). Therefore, the previously designed *L. plantarum* probe (100) was validated against more than 40 representatives of the human GI tract inhabitants using FISH combined with epifluorescent microscopy. Finally, the *L. plantarum*-specific probe was tested in FISH of ileal effluent and fecal samples.

5.2 Material and methods

5.2.1 Oligonucleotide probes

A specific probe (5'-CCAATCAATACCAGAGTTCG-3') targeting the 16S rRNA gene sequence of *L. plantarum* was previously designed and validated for a dot blot hybridization assay specifically against lactic acid bacteria by Hensiek et al (100). In this study, this *L. plantarum* probe was aligned with other *Lactobacillus* strain sequences retrieved from GenBank (www.ncbi.nlm.nih.gov) using ClustalX software (112) and the ARB software package (149). The probe was validated *in silico* for its specificity based on comparative analysis of 16S rRNA sequences from other microbes in the Ribosomal Database Project (RDB, release v.9) (46). The probe was designated S-S-Plant-0067-a-A-20 (Plant67), according to the nomenclature of the Oligo Probe Database (OPD) as recommended by Alm *et al* (5). The oligonucleotide probes were purchased from MWG Biotech AG (Ebersberg, Germany) and were labeled at the 5'-end with indocarbocyanine (Cy3) or fluorescein isothiocyanate (FITC). The universal eubacterial probe Eub338 (5'-GCTGCCTCCCGTAGGAGT-3') (8) and the Lab158 (5'-GGT ATTAGCAYCTGTTTCCA-3') (92), that target the majority of bacteria and the lactic acid bacteria population, respectively, served as positive controls.

5.2.2 Bacterial growth conditions and fixation

L. plantarum WCFS1 used throughout this study was routinely cultured in MRS broth, if appropriate solidified with 1.5 % agar (155). Growth was carried out at 37°C without shaking. All the reference strains used in this study for the validation

of the Plant67 probe were obtained from the American Type Culture Collection (ATCC; Manassas, Va**.,** USA), Deutsche Sammlung von Mikroorganismen und Zellcultures (DSM; Braunschweig, Germany), and the VTT culture collection (VTT, Espoo, Finland): *Akkermansia muciniphila* (ATCC BAA-835), *Atopobium minutum* (DSM 20586), *Bacteroides distasonis* (DSM 20701), *Bacteroides fragilis* (DSM 2151), *Bacteroides ovatus* (ATCC 8483)*, Bacteroides pyogenes* (CCUG 15419), *Bacteroides uniformis* (DSM 6597), *Bacteroides tectus* (CCUG 25929), *Bifidobacterium lactis* (DSM 10140), *Bifidobacterium bifidum* (DSM 20082), *Bifidobacterium adolescentis* (DSM 20083), *Bifidobacterium breve* (DSM 20091), *Bifidobacterium longum* (DSM 20090), *Butyrivibrio fibrisolvens* (DSM 3071), *Clostridium aminophilum* (ATCC 49906), *Clostridium sticklandii* (ATCC 12662), *Colinsella aerofaciens* (DSM 3979), *Escherichia coli* (NCTC 12900)*, Eubacterium cylindroides* (DSM 3983), *Eubacterium rectale* (ATCC 33656), *Eubacterium ruminantium* (ATCC17233), *Enterococcus faecalis* (DSM 2146), *Fusobacterium prausnitzii* (ATCC 27766), *Lachnospira multipara* (DSM 3073), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus amylovorus* (DSM 200531), *Lactobacillus casei* (VTT E85225), *Lactobacillus crispatus* (DSM 20531), *Lactobacillus delbruekii* (DSM 20076), *Lactobacillus fermentum,* (DSM 20052, *Lactobacillus paracasei* (VTT E92467)*, Lactobacillus plantarum, Lactobacillus reuteri* (DSM 20016), *Lactobacillus rhamnosus* (VTT E78016)*, Lactobacillus sakei* (DSM 20017)*, Lactococcus lactis* MG1363, *Peptostreptococcus anaerobius* (ATCC 27337), *Prevotella albensis* (DSM 11370), *Prevotella bryantii* (DSM 11371), *Prevotella ruminocola* (ATCC 19189), *Roseburia intestinalis* (DSM 14610), *Ruminococcus albus* (DSM 20455), *Ruminococcus bromii* (ATCC 27255), *Ruminococcus flavefaciens* (ATCC 19208), *Ruminococcus hansenii* (DSM 20583), *Ruminococcus productus* (DSM 2950), *Streptococcus bovis* (ATCC 33317), *Veillonella parvula* (ATCC 10790), *Victivallis vadensis* (DSM 14823)*.* They were grown under conditions described by the suppliers. Exponentially grown cultures were harvested (10,000 x g, 10 min), washed with a 0.2 µm-pore-sized-filtered phosphate buffered saline (PBS; per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4; pH 7.2) and fixed in 4% paraformaldehyde (PFA) as previously described (259).

5.2.3 Preparation of ileum and fecal samples

Ileum effluent samples were obtained from healthy volunteers carrying an ileostoma as described before (53). After fasting overnight the volunteers consumed 300 ml of a flavored milk containing *L. plantarum* WCFS1 as previously described (53), and 4 h after administration a standardized lunch was consumed. Immediately after the ingestion of the milk, ileal effluent was collected from the stoma pouches of the volunteers at one-hour intervals for six hours in total. The ileum effluent samples were frozen on dry ice and stored at -80°C. Fecal sample was obtained freshly from a healthy volunteer who had not consumed any drinks containing *L. plantarum* WCFS1 and stored shortly at 4°C until fixation. The use of these samples was approved by the Ethics committee of the University Hospital Maastricht, Maastricht, The Netherlands.

For FISH analysis 4.5 ml of PBS was added to 0.5 g ileal or fecal sample followed by its homogenization by vortexing for 3 min in the presence of 5 sterile glass beads (diameter of 3 mm). The resulting suspension was subsequently centrifuged at 700 x g for 1 min at 4°C. The supernatant was fixed with 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) for 3 h. The cells were sedimented by centrifugation at 10,000 rpm for 5 min at 4°C. The pellet was washed twice with PBS and stored in a 50% ethanol-PBS solution until analysis.

For evaluation of the Plant67 probe, one ileal sample with the lowest amount of *L. plantarum* WCFS1 and a fecal sample were spiked with *L. plantarum* WCFS1 (2 x 10^7 CFU) to bring the numbers above the minimal detection threshold as the unspiked samples (< 10^5 *L. plantarum* cells) did not give any signal with the probe specific for *L. plantarum*. Subsequently, hybridizations were performed as described below.

5.2.4 Whole cell hybridization

In order to determine the most stringent conditions for the *L. plantarum*-specific probe Plant67, its dissociation behavior was determined. Pure cultures of *L. plantarum* WCFS1 and the non-target organism *Butyrivibrio fibrisolvens* were hybridized with increasing formamide concentration in the hybridization buffer (0-40%) and washed with washing buffer. Increasing the formamide percentage in the hybridization buffer reduces the nonspecific binding of the probe.

The specificity of the *L. plantarum*-specific probe was examined with species of the genus *Lactobacillus* and other reference strains that are commonly found in the human GI tract as listed above. In order to permeabilise *L. plantarum* cells, the fixed cells were incubated in a lysozyme (final concentration 100 mg/ml) solution for 1 h at 37°C as described before (56). The hybridization procedure was essentially performed as previously described (56) with the main difference that 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St Louis, USA) was added to the washing buffer to detect all present double-stranded DNA. Briefly, 10 µl of fixed bacterial cells of *L. plantarum* were spotted onto slides and dried for 10 min at 46°C. The cells were then dehydrated on the slide by incubating in increasing ethanol concentrations of 50, 80 and 96% (v/v) during 3 min. After dehydration, 10 µl of hybridization buffer without any formamide were spotted onto the wells with either 1 µl of a FITC-labeled probe solution containing Eub338 or Lab158 (50 ng/µl each), depending on the target strain, and 1 µl Cy3-labelled Plant67 probe (30 ng/µl). The hybridization was carried out in a humid hybridization chamber at 46°C for 1.5 h. This step was followed by washing for 10 min in 50 ml of preheated (48ºC) high salt washing buffer containing DAPI (final concentration 20 µg/ml), which stains the DNA of all intact cells. After washing, the slides were rinsed briefly with ice-cold milliQ water, air-dried with compressed air and embedded with Vectashield (Vector Laboratories, Burlingame, CA, USA). The microscopic analyses were performed with a Leica HC fluorescent microscope and photographs were made with a DC camera using Leica Qfluor software from the manufacturer (Leica Microsystems, Rijswijk, The Netherlands).

5.2.5 Statistical analysis

The digital images were analyzed using CMEIAS Ver. 1.27 (145) operating in UTHSCSA ImageTool Ver. 2.00 (252). At least one hundred hybridized cells per formamide concentration were counted and the intensity of the probe signal was measured as described by the manual with the software (CMEIAS). The average intensity of the hybridized cells was calculated and plotted against the formamide concentration, to determine the optimal hybridization and washing conditions for Plant67 probe.

5.3 Results

A specific probe, termed here Plant67, targeting the 16S rRNA gene sequence of *L. plantarum,* was previously designed and applied for dot blot hybridization in dairy niches (100). In order to use this probe for FISH experiments, it had to be validated against whole intact cells from different species**.** Moreover, to be of use in detecting or isolating FISH-labeled *L. plantarum* from human intestinal samples, its efficacy should be evaluated.

5.3.1 Validation of Plant67 probe specific for *L. plantarum* for FISH

The *L. plantarum*-targeting probe sequence was analyzed in the RDP databases for hybridization to species commonly found in human intestinal microbiota (data not shown). Besides *L. plantarum* species to which there were no mismatches, the probe was predicted to hybridize to the 16S rDNA *Butyrivibrio fibrisolvens* with one mismatch**.** As *B. fibrisolvens* is a common member of the human intestinal microbiota (190), it was used as a non-target species in order to validate the Plant67 probe for unspecific binding. No hybridization signal was detected with *B. fibrisolvens* after lysozyme treatment since this caused cell lysis of gram-negative bacteria. Hence no additional formamide was needed to avoid unspecific binding of the probe to non-target organisms (data not shown). Furthermore, previous studies showed that incubation in 50% ethanol-PBS solution resulted in lysis of gram-negative bacteria during FISH (49, 230) which was an advantage for this study.

Forty-seven reference strains were chosen to examine the specificity of the probe including *Lactobacillus* species other than *L. plantarum* and 38 common

human intestinal species**.** None of the reference strains showed hybridization with the probe (data not shown), confirming the specificity of the probe**.**

5.3.2 Application of Plant67 probe in ileal and fecal samples

To test the probe in environmental samples, bacterial cells obtained from the intestinal samples were fixed with PFA. Prior to fixing the cells, the ileum and fecal samples were spiked with 2 x 10^7 *L. plantarum* WCFS1 cells as pilot-study results showed no hybridization of the Plant67 probe (data not shown). Both intestinal samples were treated with lysozyme in order to allow the probes to enter the gram-positive bacteria. The samples showed significant hybridization of the Plant67 probe with *L. plantarum* WCFS1 in the samples to a level of approximately 10^7 as expected (Fig. 1). No unspecific binding of the probe occurred to other bacteria and matrix particles in the samples. This was confirmed by using the Eub338 probe and DAPI, which hybridizes with rRNA of most bacteria and stains double stranded DNA, respectively.

The application of the Plant67 probe was further confirmed using several ileum effluent samples (Fig. 2). 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 bacterial 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*. The FISH analysis revealed that the highest number of *L. plantarum* cells could be found in the ileostoma effluent samples collected between three to six hours after intake (data not shown), correlating to the 4.1 ± 2 h transit time that was determined previously for ileostomy patients (225). Furthermore, enumeration of the cells with FISH indicated that the samples with highest levels of *L. plantarum* contained 3-7 x 10^6 cells/ g ileal effluent, which was between 7 and 35% of the total ileal cells detected with DAPI.
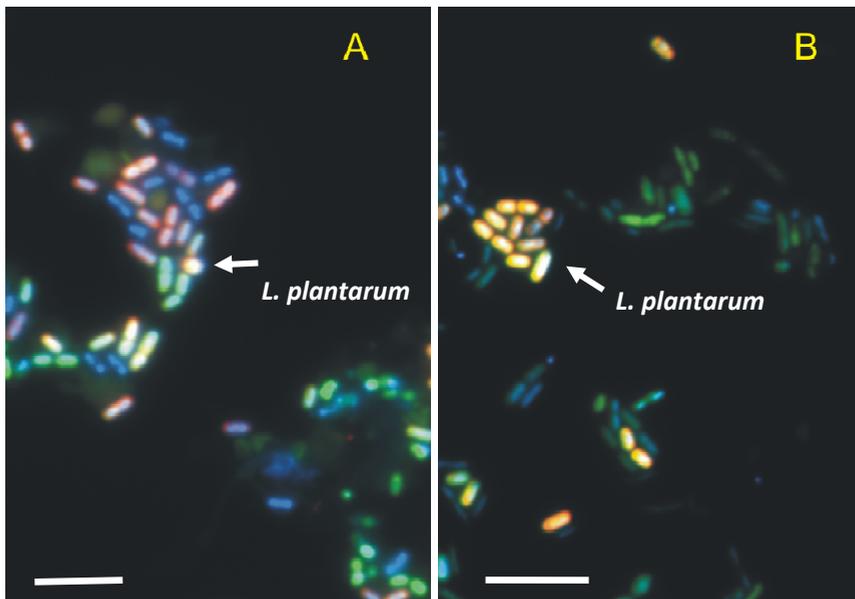
Figure 1. Micrograph of intestinal cells hybridized with the Cy3-labeled oligonucleotide probe Plant67, FITC-labeled Eub338, and stained with DAPI in ileal effluent (panel A) and in fecal samples (panel B). FITC-labeled Eub338 probe detects all bacteria and DAPI detects all cells. *L. plantarum* WCFS1 appear either red (Cy3), or red-blue (Cy3-FITC) or orange (Cy3-FITC, DAPI) depending on the stain. Non *L. plantarum* cells appear green (eubacteria) or blue (non eubacteria). Bar represents 10 μm.
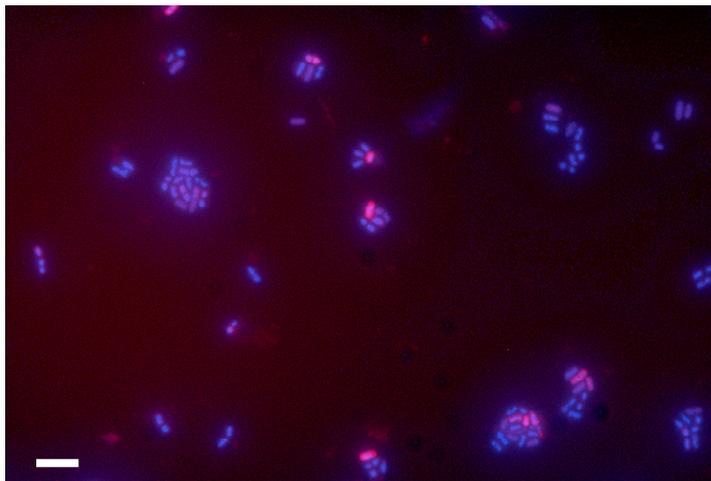
Figure 2: Micrograph of intestinal cells hybridized with the Cy3-labeled oligonucleotide probe Plant67 (red) and stained with DAPI (blue) in ileal effluent from patients who consumed flavor milk containing *L. plantarum* WCFS1. Bar represents 10 μm.

5.4 Discussion

In the present study the Plant67 probe was demonstrated to be specific for *L. plantarum* and has been applied successfully to visualize and enumerate *L. plantarum* WCFS1 in human ileostomy samples. In these experiments the transit time varied and this can depend on the food ingested as well as other factors such as gastric emptying rates, gut motility and the intestinal physiology of the person as previously described (225). These ileostomy samples were further used for RNA isolation and quantitative reverse-transcription PCR of a set of genes from the *L. plantarum* cells which demonstrated their metabolic activity (53). Furthermore, as indicated by the results (Fig. 2B) the probe can be applied successfully to label *L. plantarum* cells from fecal samples. It is interesting that not all cells in the ileal and fecal samples (Fig. 1) were hybridized with either the Plant67 or Eub338 probes. One explanation is that the permeabilization of the cells was insufficient to allow the probes access to the rRNA. The permeability obtained by lysozyme treatment was shown to be dependent on the growth phase of *L. plantarum* which becomes particularly resistant to hybridization as stationary phase is approached (56). This can also be the case for other bacteria. Another explanation for non-hybridized cells is that the Eub338 probe used in this study, namely EUB338-I, was shown not to cover all phyla of the Bacteria domain and to hybridize to only approximately 70% of all bacterial cells. For this reason, more recently, two additional bacterial probes, namely EUB338-II and EUB338-III, were designed in order to cover the Bacteria domain (49).

FISH labeling of microbial cells allows for their specific isolation from a complex ecosystem via sorting by a fluorescent activated cell sorter (FACS) of a flow cytometer that can separate the fluorescently labeled bacteria from unlabeled ones (17, 50). This technique has been successfully applied to sort and recover bifidobacterial populations that were stressed with bile salts and stained for viability using carboxyfluorescein diacetate, propidium iodide, and oxonol (17). More recently, this approach has been used to sort fecal microbial cells stained with various viability indicators, and molecular fingerprinting techniques could be applied to the sorted fractions to determine the genetic diversity (16). Therefore, it may also be feasible to label *L. plantarum* cells in an intestinal sample with a FISH probe, and use this to obtain an enriched fraction for *L. plantarum* cells for further molecular analysis. A proteomics application would be particularly

attractive over mRNA-based approaches, as proteins are considerably more stable than mRNA and show an average half-life of 60 min during growth and various kinds of starvation (172). Nevertheless there are some challenges depending on the samples whether intestinal or from food fermentations. The recovery of *L. plantarum* from ileal effluent using FISH varied between 0.1-1% of the total number (1.2-1.4 x 10^8) of ingested cells by the volunteers, based on the enumeration of the ileal effluent samples above (53). Factors influencing this include the efficiency of FISH for detection of *L. plantarum* which is growth phase-dependent (56), and thus not all cells may have been detected following transit. Moreover, the ingested *L. plantarum* cells were distributed over multiple samples as a consequence of dilution, and possible interaction or adherence to the host mucosal tissue. This could explain 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 in the stomach (241). Consequently, a much greater number of *L. plantarum* cells should be ingested by the volunteers, perhaps in a smaller volume, in order to perform direct sorting with the Plant67 probe, to ensure the numbers would be within the detection limit of the FACS (10^4 cells), as well as maintain a concentration not higher than 10^8 bacterial cells/ml in order to have single cells for the FACS machine. Another approach is to use a series of probes with increasing specificity to ultimately target the desired population. For example, first a more general probe specific for low G+C % content bacteria or possibly the Lab158 probe that targets all lactic acid bacteria could be applied to sort this community, and then in a final round the Plant67 is applied to the enriched community. This strategy to use multiple probes was successfully applied to sort bacteria belonging to the *Clostridium leptum* subgroup from human microbiota (139). Thus a careful design of experiments and tiered approaches should allow application of Plant67 to food and intestinal samples for sorting with a subsequent proteomics approach.

Acknowledgements

Chapter 6

Proteome Analysis of *Lactobacillus plantarum* Recovered from the Human Intestinal Tract via Immunomagnetic Separation

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AND WILLEM M DE VOS
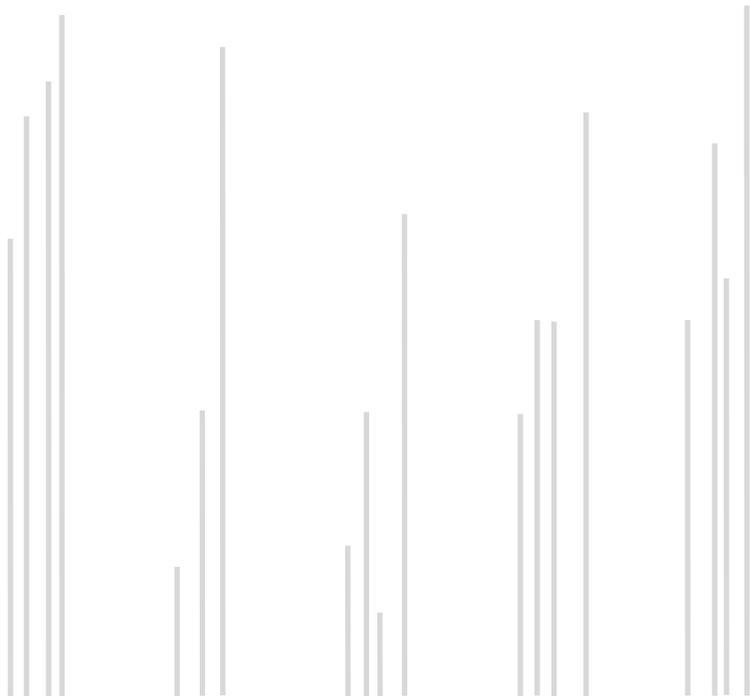
Abstract

In order to study the functionality of the human intestinal commensal *L. plantarum* WCFS1, an innovative approach involving an extraction method based on immunomagnetic beads with subsequent proteomic analysis has been developed. *L. plantarum* cells were sorted from other intestinal microbes and the proteome was analyzed using two-dimensional gel electrophoresis. A combination of a specific polyclonal antibody against *L. plantarum* together with magnetic beads was used to extract *L. plantarum* cells, and the strategy has been validated using both *in vitro* and *in vivo* samples containing *L. plantarum* WCFS1. In the enriched fraction from a human intestinal sample, *L. plantarum* accounted for 86% of the total bacterial cells based on quantitative PCR using specific primers. Furthermore, the proteome of *L. plantarum* cells isolated by the extraction method from the ileal effluent of a subject carrying an ileostoma that had been fed with *L. plantarum* WCFS1 was determined. The proteome was visualized on a two-dimensional electrophoresis gel which revealed a number of proteins, 13 of which could be identified based on the proteome reference map of *L. plantarum*. In conclusion, this study has shown that the use of the immunomagnetic separation method is successfully applicable to isolate *L. plantarum* WCFS1 cells from intestinal samples in order to perform further analysis to study the functionality of this bacterium in the human gastrointestinal tract.

6.1 Introduction

Lactic acid bacteria (LAB) are used to prepare fermented food products with improved preservation properties and with characteristic flavors and textures. Certain strains of LAB, in particular those belonging to the *Lactobacillus* genus, are also marketed as probiotics (146). LAB are gram-positive and non-spore forming bacteria that are characterized by their ability to ferment sugars to lactic acid and other by-products. One member of this group, *Lactobacillus plantarum*, is a natural inhabitant of the human gastro-intestinal (GI) tract and is predominantly found in the small intestine in numbers varying from 10^6 to 10^8 colony forming units per gram of ileal content (4, 196). Several *L. plantarum* strains are claimed to exert a beneficial effect on the host (55, 168). Moreover, there is evidence that *L. plantarum* may modulate the adaptive immune response (33, 178, 206) and inhibit the adherence of pathogenic bacteria in *in vitro* systems using intestinal epithelial cell lines (151). However, the molecular mechanisms underlying these potential beneficial effects have not yet been elucidated. One way to address these, is to study the expression of *L. plantarum* within the intestinal tract and various model studies have focused on the analysis of strain WCFS1, a human isolate that has been characterized at the genome level (54, 58). The first studies with *L. plantarum* WCFS1 were based on the recombination-based *in vivo* expression technology (R-IVET) that - together with the genome sequence and a Reverse-Transcription (RT) PCR approach - was instrumental in providing the first insight into global gene expression in the mouse intestine (29, 159). Since R-IVET can not be applied in human, alternative approaches are required to determine the global response of *L. plantarum* in man. One such approach was based on a transcriptomics analysis of surgically removed human colon and ileum biopsies, which showed that *L. plantarum* WCFS1 expressed numerous different genes in the human intestine compared to an *in vitro* culture, and some of these were also expressed in the mouse intestine (54). In this study, a proteomics-based technique has been selected based on the consideration that bacterial proteins are more stable than mRNA and have a lower turn-over rate (half-life of 60 min versus approximately 5 min during growth, respectively) (20, 172). Moreover, proteins are easier to obtain and to process as compared to the more sensitive mRNA. A previous study based on proteomics on infant fecal microbes has demonstrated

that proteomics of gut microbes isolated from human intestinal samples is a feasible approach (125).

In order to study the exclusive proteome of *L. plantarum* in a complex ecosystem, such as the GI tract, its cells must be separated from that of other microbes. In the present study, a strategy based on the use of a specific antibody for *L. plantarum* WCFS1 in combination with magnetic beads, has been used to obtain a bacterial fraction enriched for *L. plantarum* WCFS1 from intestinal samples. Quantitative PCR was applied to confirm the quality of the sorting of specifically *L. plantarum* WCFS1 prior to isolation and analysis of its proteome by two-dimensional electrophoresis (2DE). Overall, the results demonstrate the feasibility of this approach for mono-isolation of bacterial species from complex ecosystems such as intestinal samples prior to further analysis.

6.2 Material and Methods

6.2.1 Bacterial strains and growth

Throughout these studies, *L. plantarum* WCFS1, a single-colony isolate from *L. plantarum* NCIMB8826, from which the complete genome sequence has been determined (126); (GenBank, AL935263) was used. Other *Lactobacillus* species *e.g. Lactobacillus acidophilus* DSM 20079, *Lactobacillus brevis* DSM 20054 and *Lactobacillus fermentum* DSM 20052, obtained from the Deutsche Sammlung von Mikrooraganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and *L. plantarum* 299v, were also used in this study. All *Lactobacillus* species were cultured anaerobically in MRS broth (155) (Difco, Surrey, U.K.) at 37°C. *Escherichia coli* NCTC 12900 was kindly provided by Marion McRae (University of Aberdeen U.K.) and was grown aerobically on LB medium at 37°C under agitation (250 rpm). In case of interventions with *L. plantarum*, cells were harvested by centrifugation and washed three times with a physiological salt solution (Braun Melsungen AG, Melsungen, Germany) and further processed or fed to human volunteers as described previously (54). Shortly, bacterial cells were resuspended into 300 ml of a commercially available flavored milk product (Melk met Smaak, Campina, Woerden, The Netherlands) to a final concentration of 1.2-1.4 x 10^8 CFU/ml and consumed immediately by human volunteers. *L. plantarum* WCFS1 cells grown in MRS were used as control.

6.2.2 Isolation and processing of ileostoma and fecal samples

The ileal effluent sample was obtained as described before (53) from a volunteer who had been subject to colectomy at least three years ago and was considered healthy apart from having an ileostoma. The volunteer consumed 300 ml of the flavored milk containing *L. plantarum* WCFS1 as described above after fasting overnight. Immediately after the ingestion of the milk, ileal effluent was collected from the stoma pouch of the volunteer at a one-hour interval. The ileal effluent sample from the subject collected after 5 h post-consumption was used. The sample was frozen on dry ice and stored at -80°C until further use.

6.2.3 Production and purification of polyclonal serum

Polyclonal antibodies specific for *L. plantarum* WCFS1 were obtained by repeated subcutaneous injection of pathogen-free New Zealand Large rabbits and obtained from Eurogentec (Seraing, Belgium). The serum containing the polyclonal antibodies obtained after the third bleeding was purified on a Protein G agarose (Sigma, St. Louis, MO, USA) column. Before applying the serum (5 ml), the column (0.4 ml) was equilibrated with 20 mM sodium phosphate (Sigma) buffer pH 7.0. Prior to loading, the serum containing the polyclonal antibodies against *L. plantarum* WCFS1 was filter sterilized (0.2 μm) and loaded onto the column, followed by washing of the column with 20 mM sodium phosphate buffer. The antibodies were eluted from the column by adding 1.5 ml of 0.1 M glycine (Sigma) buffer into 0.3 ml 1 M Tris-HCl (Sigma), pH 8 buffer and stored at -20°C until further use.

6.2.4 Optimization of immunomagnetic isolation

To sort out specifically *L. plantarum* cells from other microbes within the ileum sample, a sandwich antibody method was applied in combination with magnetic beads. First, the polyclonal rabbit antibodies against *L. plantarum* WCFS1 were used to coat the desired bacterial cells. Secondly, a second antibody (goat anti-rabbit) covalently attached to a magnetic bead was used to capture *L. plantarum* cells coated with polyclonal antibodies. The magnetic beads attached to the second antibody were used to physically separate the bacterial cells from the samples. To determine the dilution factor of the purified antibody to be used for optimal extraction of the bacterial cells, different dilution factors were plotted against the DNA yield, which was used to quantify the amount of recovered

bacterial cells. A 4000 x dilution factor yielded the highest concentration of DNA and this dilution factor was applied in the further experiments. The magnetic beads were obtained from Invitrogen (Carlsbad, CA, USA), which have been coated with polyclonal goat anti-rabbit antibodies. To optimize the concentration of magnetic beads to be used for optimal extraction of *L. plantarum* WCFS1, the use of different amounts of the beads on the DNA yield was determined, which was used to quantify the amount of the recovered bacterial cells. The optimal amount of magnetic beads was reached using 80 µl of magnetic beads corresponding to an absolute number of 5.4×10^4 beads. Consequently, this was applied for the isolation of *L. plantarum* WCFS1 from ileal sample.

6.2.5 Immunomagnetic isolation of *L. plantarum* from ileal and fecal samples

Ten gram of ileostoma content from the subject - containing 5×10^6 *L. plantarum* cells/g, based on FISH enumeration (Chapter 5) - were homogenized for 3 min into 5 ml PBS containing chloramphenicol (Sigma) (PBS-CAM) with a final concentration of 100 µg/ml (93) using zirconium beads (diameter 3 mm). The homogenized solution was centrifuged at 700 x *g* for 1 min at 4° C and the supernatant was transferred to a new tube, and cells were pelleted at 8,000 x g for 15 min and washed three times with PBS-CAM, followed by centrifugation at 8,000 x *g* for 15 min at 4°C. The pellet was then resuspended in 1 ml PBS-CAM. The antibodies specific for *L. plantarum* WCFS1 were added to the solution with a final dilution factor of 4000 and incubated for 1 h at room temperature under gently shaking to avoid settling of the bacterial cells. After incubation, bacteria were washed once with PBS to remove the unbound antibodies. Subsequently, the pellet was resuspended in 1 ml PBS and 80 µl (final concentration 5.4×10^4 beads/ml) of magnetic beads were added and incubated for 1 h at room temperature using gentle shaking to avoid settling of the magnetic beads. Following incubation, the magnetic beads were concentrated to one side of the tube by exposing to a magnet for 5 min. The solution containing the unbound bacteria was discarded and the magnetic beads were washed 3 times with PBS. To elute the *L. plantarum* cells from the magnetic beads, the beads were resuspended in 0.1 M citric acid, pH 3 and incubated for 2 min under gently rotating. The magnetic beads were concentrated to one side of the tube using a magnet and the supernatant

containing *L. plantarum* were transferred to a clean tube. A second elution step with citric acid was performed and both supernatants were pooled together. The bacterial cells were washed once with PBS and stored at -80°C until further use.

6.2.6 DNA isolation and real time quantitative PCR

DNA was isolated from pure cultures and fractions enriched for *L. plantarum* derived from ileal effluent samples with the Fast DNA Spin kit soil (QBiogene, Carlsbad, Ca, USA). Extraction was used and performed according to the instructions of the manufacturer with the following modifications. After addition of the lysis buffer, cells were treated in a bead beater (Biospec Products inc., Bartlesville, USA) five times for 1 min at maximum speed and the protocol was continued according to the manufacturers instructions. Finally, the DNA was eluted from the matrix using 100 µl of Dnase-free water. The concentration of the purified DNA was determined spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, USA). In order to determine the number of *L. plantarum* cells isolated with the magnetic beads and the efficiency of the enrichment, quantitative PCR (qPCR) was performed on the isolated DNA. Two pairs of primers specific for the 16S ribosomal RNA (rRNA) of *L. plantarum* (30) and bacteria (171) were used as described before (159) and (211) with minor changes, respectively. In short, qPCR amplification was performed in 96-wells plates on an iQ5 Real-Time PCR Detection System (Bio-Rad). Samples were analyzed in a 25-µl reaction mix consisting of 12.5 µl Bio-Rad master mix SYBR Green (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 0.625 U i*Taq* DNA polymerase, 3 mM MgCl$_2$, 10 nM fluorescein), 0.2 µM of each primer and 5 µl of template DNA or water. The template DNA was obtained from DNA isolation as described before from extracted *L. plantarum* WCFS1 cells from ileal effluent samples. Standard curves of DNA from *L. plantarum* were created using serial 10-fold dilution of the genomic DNA corresponding to a range from 10^7 to 0 copies. The PCR amplification specific for *L. plantarum* was initiated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 60 s. PCR specificity and product detection were checked post amplification by examining the dissociation curves of the products. The melting curves profiles were generated by heating the samples to 95°C for 1 min and then cooling to 55°C for 1 min and slowly heating the samples to 95°C at 0.5°C/min. The PCR amplification specific for all bacterial 16S rRNA genes was

initiated at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The melting curve profiles were initiated by heating the samples to 95°C for 1 min and subsequently cooling to 60°C for 1 min and slowly heating to 95°C at 0.5°C/min. Control PCR samples without template DNA were included.

6.2.7 Protein isolation from *L. plantarum* WCFS1

The proteome of *L. plantarum* WCFS1 was extracted as described before (45) and the protein concentration was determined using 2-D Quant Kit (GE Healthcare, U.K.). In short, after extraction of *L. plantarum* WCFS1 cells from the ileostoma samples, the cells were washed with PBS and double distilled water (ddH$_2$O), respectively, and centrifuged at 5000 x *g* for 10 min at 4°C. The supernatant was discarded and the cells were resuspended in buffer containing 8 M urea (Bio-Rad, Hercules, CA, USA), 2% w/v CHAPS (Sigma) 65 mM DTT (Sigma), 0.5% v/v IPG buffer (pH 3-10 NL; Amersham Pharmacia Biotech, Uppsala, Sweden). The bacteria were lysed mechanically by beating with zirconium beads (diameter of 0.8 mm) using a mini bead beater (Biospec Products, Bartlesville, OK, USA) for 5 x 1 min with 1 min intervals on ice. The cell debris was removed and the supernatant was collected and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was collected and stored at -80°C until further use.

6.2.8 Two-dimensional electrophoresis and analysis

A 2-DE procedure optimized for *L. plantarum* was used (45). For isoelectric focusing (IEF), three µg of proteins were diluted into 185 µl rehydration buffer containing 8 M urea (Sigma), 2% w/v CHAPS (Sigma) 65 mM DTT (Sigma), 0.5% v/v Biolyte buffer (pH 3-10 NL; Bio-Rad). The protein solution was then applied to the IPG strip (pH 3-10 NL, 11 cm long; Bio-Rad). The strips were actively rehydrated at 50 V for 12 h on a PROTEAN IEF Cell (Bio-Rad). For the IEF, the following program was used: 250 V, 1 hr; 500 V, 1 hr; 1000 V, 1 hr; 8000 V for 2h; and 8000V for 40,000 VH at 20°C.

After the first dimension, the strips were equilibrated for 15 min in 50 mM Tris-HCl (Sigma), pH 6.8, 6 M urea (Sigma), 30% v/v glycerol , 2% w/v SDS, 1% DTT and for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% iodoacetamide (Sigma). The strips were placed on 12.5% SDS-PAGE gels and covered with 0.5% agarose containing a trace of bromophenol blue. The gels were

run at a constant voltage of 200 V in a Criterion gel system (Bio-Rad) until the marker dye had reached the base of the gel.

After the second dimension, the gels were silver stained according to (207). The gels were analyzed using PDQuest software version 7.2 (BIO-Rad). The visualized protein spots on the 2DE gels were compared with the proteome reference map of *L. plantarum* WCFS1 obtained from previous study (45). Furthermore ten protein spots were excised and subjected to MALDI-TOF analysis as described before (45).

6.3 Results

6.3.1 Specificity of the polyclonal antibody

Before optimizing the conditions for isolation of *L. plantarum*, the primary antibody preparation was tested for its specificity. Three *Lactobacillus* species, namely *L. acidophilus, L. brevis* and *L. fermentum,* and *E. coli* have been used in an ELISA assay that showed considerably lower affinity for the *L. plantarum*-specific antibodies than *L. plantarum* WCFS1 (data not shown). Moreover, other conditions for the specific recovery of *L. plantarum* were determined using specific PCR methods to quantify the number of *L. plantarum* and other bacterial cells. A 4000 x dilution of the primary antibody preparation was found to be optimal as was the use of a final concentration of 5.4 x 10^4 magnetic beads/ml and these conditions were used throughout this study to extract *L. plantarum* WCFS1 cells from the ileal effluent sample.

6.3.2 Quantification of *L plantarum* WCFS1 from ileal contents using quantitative PCR

The optimized immunomagnetic isolation procedure was applied on ileal effluent sample and the amounts of recovered *L. plantarum* cells were quantified using a quantitative PCR-based approach on DNA isolated from this sample. For the ileal sample, approximately 1.6 x 10^6 cells of *L. plantarum* per g of ileal effluent were recovered. qPCR was also performed on the same sample using universal primers for bacteria. Total bacteria accounted for 1.82 x 10^6 cells/g, indicating that the immunomagnetic separation was highly specific, as 86 % of the retrieved cells belonged to *L. plantarum*.

6.3.3 Two-dimensional electrophoresis and mass spectrometry

To analyze the functionality of *L. plantarum* during passage of the GI tract, 2-DE was applied on the proteins extracted from the cells recovered by immunomagnetic separation from the ileal sample. Numerous proteins could be detected (Fig. 1), and thirteen of these could be identified by using the *L. plantarum* proteome reference map (PRM) obtained from a previous study (45) (Table 1). To validate this PRM-based identification and to confirm that the visualized proteins were derived from *L. plantarum* cells, two of the protein spots (numbered 5 and 10) were excised and analyzed by MALDI-TOF mass spectrometry – this confirmed the PRM assessment as they were identified as fructose-bisphosphate aldolase and phosphoglycerate mutase as expected, respectively. The PRM-based identified proteins were classified into functional groups according to the KEGG database (see Table 1). The proteins were predicted to be involved in carbon metabolism, stress adaptation and translation.
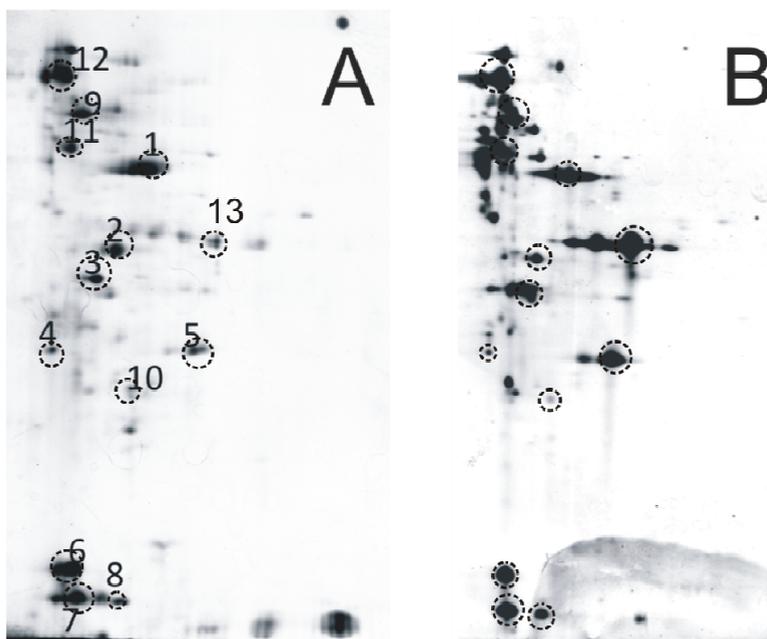
Figure 1. Two-dimensional gel electrophoresis of *Lactobacillus plantarum* WCFS1 recovered from ileal affluent. (A) *L. plantarum* WCFS1 cells isolated from the ileum content of a ileostoma patient with an ileostoma. Several protein spots were identified using the *L. plantarum* PRM established previously (45). The identified proteins are listed in Table 1. (B) Control proteome of *L. plantarum* WCFS1. The control cells were grown anaerobically at 37°C for 16 hr and subsequently, the proteome was isolated.

By comparing the intensities of the identified spots with the intensity of D-lactate dehydrogenase within the same gel, the glycolytic enzymes appeared to be down-regulated compared to the control, except for two proteins triosephosphate isomerase and phosphoglycerate mutase (Table 2). The stress proteins seemed to be present in a higher concentration than the proteins associated with carbohydrate metabolism in the proteome of the sorted *L. plantarum* WCFS1 cells.

Table 1. Proteins that have been identified using the PRM and by MALDI-TOF mass spectrometry (indicated in bold).

Spot no.	Reference Map	ORF	Functional class
1	Phosphoglycerate kinase	lp_0790	Carbohydrate metabolism
2	D-Lactate dehydrogenase	lp_2057	Carbohydrate metabolism
3	L-Lactate dehydrogenase	lp_0537	Carbohydrate metabolism
4	Triosephosphate isomerase	lp_0791	Carbohydrate metabolism
5	**Fructose-bisphosphate aldolase**	lp_0330	Carbohydrate metabolism
6	Alkaline shock protein	lp_0930	Stress Proteins
7	Alkaline shock protein	lp_0929	Stress Proteins
8	Small heat shock protein	lp_3352	Stress Proteins
9	Ribosomal protein S1	lp_1882	Translation
10	**Phosphoglycerate mutase**	lp_3170	Carbohydrate metabolism
11	Enolase	lp_0792	Carbohydrate metabolism
12	GroEL chaperonin	lp_0727	Stress Proteins
13	Glyceraldehyde 3-phosphate dehydrogenase	lp_0789	Carbohydrate metabolism

The spot number can be used to identify the spot in Fig. 1.

Table 2. Regulation of the identified proteins isolated from ileum effluent and control samples. The Ratio-Ileum and Ratio-control gives the fold-change of proteins compared to the intensity of the spot identified as D-lactate dehydrogenase of the ileum effluent sample and the control, respectively.

Identified proteins	Ratio-Ileum	Ratio-control
Phosphoglycerate kinase	1.64	2.07
D-Lactate dehydrogenase	1.00	1.00
L-Lactate dehydrogenase	0.46	0.85
Triosephosphate isomerase	0.63	0.14
Fructose-bisphosphate aldolase	0.47	4.69
Alkaline shock protein	1.66	3.74
Alkaline shock protein	1.40	0.72
Small heat shock protein	0.26	1.15
Ribosomal protein S1	0.42	1.27
Phosphoglycerate mutase	0.20	0.11
Enolase	0.49	0.72
GroEL chaperonin	0.95	1.21
Glyceraldehyde 3-phosphate dehydrogenase	0.47	4.76

6.4 Discussion

In this study, an innovative method based on immunomagnetic beads has been developed and evaluated for the isolation of the commensal *L. plantarum* WCFS1 in both *in vitro* and *in vivo* experiments. The combination of a polyclonal antibody specific for *L. plantarum* WCFS1 and a secondary anti-primary antibody, was found to capture specifically *L. plantarum* from ileal effluent sample from a subject who consumed a flavored milk containing *L. plantarum* WCFS1 (54). *L. plantarum* cells were quantified by qPCR after sorting from an ileal effluent, and accounted for the major part of the enriched fraction (86%). An explanation for the observation that the *L. plantarum* cell number in the fraction enriched from the ileal effluent sample was not higher might be due to the fact that *in vivo* bacteria are coated with IgA and IgG (237). This may affect the binding of the polyclonal antibody specific for *L. plantarum* by steric hindrance or recognition of the same ligand. The interference by human immunoglobulin binding, that is known to be variable (237), may also explain the observation that the recovery efficiency was found to vary between intestinal samples (data not shown).

Proteins were extracted from the *L. plantarum* cells that were isolated from the ileum sample and these were subsequently subjected to proteomic analysis. Approximately 20 spots could be visualized on 2-DE gels and 13 protein spots were identified using the *L. plantarum* PRM (45). This identification is expected to be reliable as two proteins that could be isolated in sufficient amounts for mass spectrometric analysis were identified correctly (Table 1). The proteins identified were mainly involved in carbon metabolism (9 of 13) or stress adaptation (4 of 13), and translation (1 of 13).

Concerning the carbohydrate metabolism, the proteome quantification suggested that the glycolysis in the sorted *L. plantarum* WCFS1 is down-regulated as compared to the control that consisted of an actively growing culture in a laboratory medium. This may be due to the decreased concentration of glycolytic sugars in the environment surrounding *L. plantarum* WCFS1 cell after a transition time of 5 h of the small intestine. The up-regulation of triosephosphate isomerase and phosphoglycerate mutase supports this, as these enzymes are involved in the gluconeogenesis. However, lactate production was still at a normal level as deduced from the presence of both D- and L-lactate dehydrogenase. It was shown previously that the gene encoding the L-lactate dehydrogenase was active in *L. plantarum* WCFS1 (53) during passage of the human GI tract. The other enzymes involved in the glycolysis have not previously shown to be induced during passage of *L. plantarum* WCFS1 through the intestine of human (53) or mice (29, 58). Nevertheless, these results were based on mRNA studies and, the proteomics technique presented here showed that these proteins were present in detectable amounts.

The identified stress proteins are alkaline shock proteins, which play a role in alkaline pH tolerance (134). It is known that in the small intestine the pH increases from the duodenum (pH 6) till the terminal part of the ileum (pH 7.4) (69). This is compatible with the presence of these alkaline shock proteins as is the presence of the GroEL chaperonin that is involved in protein refolding. Furthermore, *L. plantarum* WCFS1 cells in the ileum were translationally active, as suggested by the presence of a ribosomal protein.

The presence of enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as detected by 2-DE in the proteome of the sorted *L. plantarum* WCFS1 deserves specific attention. It was demonstrated that when GAPDH and enolase

are present in the extracellular proteome of *L. crispatus,* the cells can bind to plasminogen and mucin (79, 108).

Overall, the immunomagnetic sorting of *L. plantarum* WCFS1 from ileal effluent has revealed the presence of proteins involved in carbohydrate metabolism, stress proteins and translation. The presence of these proteins indicated that the bacterium is metabolically and translationally active and is able to survive the increasing pH in the small intestine likely facilitated by alkaline stress proteins. Furthermore the production of GAPDH and enolase suggests that the bacterium can bind to human matrix to aid persistence or colonization of the small intestine. This study shows for the first time the validation of an innovative approach based on immunomagnetic separation of the intestinal commensal bacterium *L. plantarum* WCFS1 in combination with a subsequent proteomic analysis. This method has been successfully applied to isolate the bacterium from intestinal samples and can offer a novel alternative to study functionality of intestinal microbes.

Acknowledgements

Chapter 7

General Discussion and Concluding Remarks
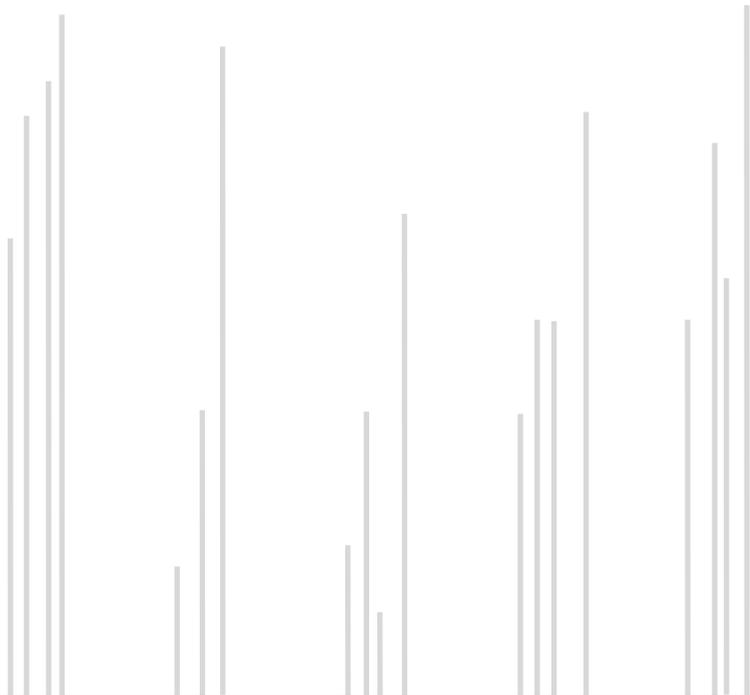
The aim of the thesis was to develop, validate and apply a proteomics approach to study the global response of *Lactobacillus plantarum* under laboratory conditions and during passage of the human gastrointestinal (GI) tract. Proteomics is the large-scale study of proteins and has several advantages over other global approaches such as transcriptomics, since it is addressing the functional molecules themselves and allows for the detection of protein modifications. Moreover, bacterial proteins have a much longer half-life than mRNA molecules. Hence, a proteomics approach was used to complement previous transcriptomics and genetic studies that revealed gene expression of *L. plantarum* during intestinal passage in men and mice (29, 53, 159).

7.1 Proteomics of *L. plantarum* WCFS1

L. plantarum WCFS1, a human isolate that has been extensively characterized (126) was selected as a model for lactic acid bacteria that colonize the human GI-tract as introduced in **Chapters 1** and **2**. A proteome reference map (PRM) of the intracellular soluble proteins of *L. plantarum* WCFS1 was constructed and found to be a valuable tool for the identification of protein spots on a 2-DE gel (**Chapter 3**). A variety of protein spots (75) were identified using MALDI-TOF mass spectrometry. It was shown in **Chapter 3** that during growth *L. plantarum* shows a dynamic proteome content reflecting the activity of specific metabolic pathways. In the early-log phase, proteins associated with energy generation were specifically present while in the late-log phase proteins catalyzing the synthesis of macromolecules were increased compared to the other growth phases. In the early-stationary phase, proteins predicted to be involved in strengthening of the cell-envelope were notably present and in the late-stationary phase, stress proteins were up-regulated probably to prepare for survival conditions, such as during nutrient starvation or the acidifying environment. It has been shown that the PRM is valuable tool and including additional identified spots into this map will strengthen this tool only more. Furthermore, our results showed that each growth phase has its own metabolic status: e.g. in the late-log phase the emphasis is on the synthesis of macromolecules. Keeping the cells in this growth phase, may yield more produced proteins than cells that are in the early-log phase (OD600 4.7). This can be of value for the production of recombinant proteins in L. plantarum as a vaccine (189).

Lately, a proteomics analysis was reported on two other *L. plantarum* strains, MLBPL1 and REB1, respectively (129). The data obtained showed similar proteome changes during growth as did *L. plantarum* WCFS1. However, the regulation of the glucose phosphoenolpyruvate:sugar transferase system (PTS)-EIIAB protein differed between the studies. In the WCFS1 strain, the glucose PTS-EIIAB protein was up-regulated during early- and late-stationary phase. This may be explained by the fact that this reaction is energetically more favorable than the use of permeases to transport glucose into the cell. In the case of strains MLBPL1 and REB1, the PTS-EIIAB was up-regulated in the lag phase. This might be due to the low energy state in these cells, as it was reported that in the lag phase also pyruvate oxidase and phosphoketolase that produce acetyl phosphate were up-regulated which in later growth phases can be converted into ATP (129). The availability of this pool of ATP-molecules might explain in general why the glucose PTS-EIIAB was down-regulated and use of permeases might be induced during log phase in *L. plantarum*.

The constructed PRM (**Chapter 3**) was used for the analysis of the potential similarity between the proteomic approach – as discussed in this thesis - and the transcriptomic approach based on microarray analysis of mRNA **(Chapter 4)**. The proteome and transcriptome of *L. plantarum* were investigated in the wild-type and in the mutant derivative lacking the *ccpA* gene (strain NZ7304). CcpA has been shown to play a key role in the carbon metabolism of the bacterium (231). This regulator is activated by the Ser-P form of HPr, a component of the PTS. To provide a global view of the molecular processes within the cells and generate insight into the global role of CcpA in these processes, a comparison of the proteomes and transcriptomes of the wild-type strain and the CcpA-mutant was provided **(Chapter 4).** Proteomics and transcriptomics data of the wild-type showed 50-80% similarity between the regulation of abundance of mRNA and proteins at different growth phases. The divergence between the results was observed for genes associated with the cellular response, energy metabolism, and purines, pyrimidines, nucleosides and nucleotides metabolism. Analysis of the proteome and transcriptome of the *ccpA* mutant showed 70-80% similarity (at OD_{600} 1.0 and OD_{600} 3,0, respectively) between both approaches. The difference in results between transcriptomics and proteomic approaches was observed for genes associated with energy and amino acid metabolism. The variation observed

between the approaches might be due to the difference in half-life of mRNA and protein, 3-8 min and up to 60 min, respectively (20, 172) but other factors including cytolytic enzymes (proteases) activity might have influence on the protein abundance (39). Although there was some discrepancy between both techniques, they both showed that the genes that are regulated by CcpA are dispersed in a similar manner amongst the different functional classes.

This study indicates that proteomic and transcriptomic approaches are more powerful when combined as they providea more complete information on the activity of the bacteria. Eventually, the combination of these techniques could lead to new targets that are possibly regulated via mRNA and/or protein stability and could enable the elucidation of novel (post-translational) regulatory networks in the cell.

7.2 Host-Microbe Interaction Studies

In the interaction between *L. plantarum* WCFS1 and the human host, the cell surface of the bacterium is of great interest as it is expected to contain the molecules that are involved in the first events. Previous studies have shown that binding of *L. plantarum* cells to eukaryotic cells is mannose-dependent and a large cell-surface protein with a membrane anchor was implied in this process (1, 187). Furthermore, it was shown that the composition of cell-envelope constituents of *L. plantarum* NCIMB8826, notably the alanylation state of the techoic acids can modulate pro- or anti-inflammatory immune responses (84). A protein fraction of the outer surface of *L. plantarum* WCFS1 and cell-envelope was prepared and analyzed by one-dimensional gel electrophoresis (1-DE) (Fig. 1) to investigate proteins potentially involved in the binding of this bacterium to the host cell. The fraction containing the outer surface proteins of *L. plantarum* WCFS1 showed 3 protein bands (Fig. 1A) and these proteins have been identified by MALDI-TOF mass spectrometry. Two unexpected glycolytic proteins were present in the enriched outer cell surface fraction but also a muramidase.
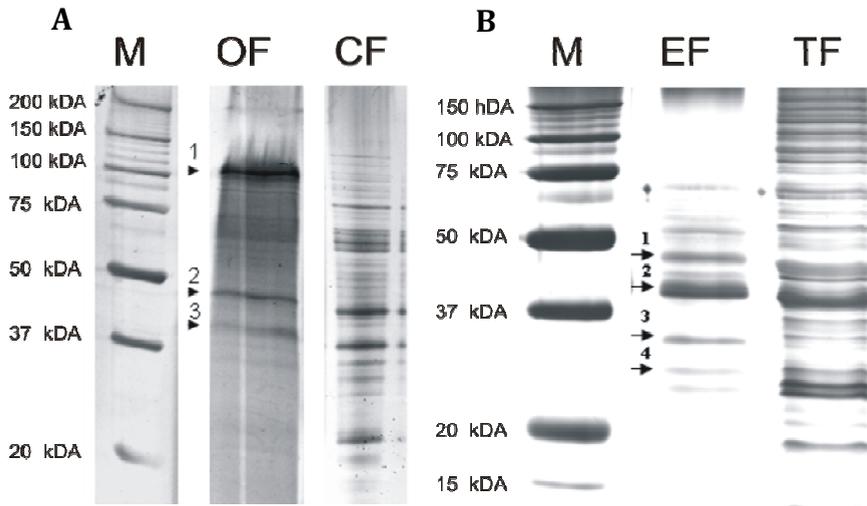
Figure 1. SDS-PAGE of protein fractions of *L. plantarum* WCFS1 grown in MRS and the identification of proteins bands by MALDI-TOF mass spectrometry. **A)** Outer cell surface protein fraction. M: protein marker OF: Outer cell surface protein fraction CF: cytosolic protein fraction as control 1: muramidase 2: glucose 6-phosphate isomerase 3: glyceraldehyde 3-phosphate dehydrogenase. **B)** Cell envelope protein fraction. EF: cell envelope protein fraction TF: total protein fraction as control 1: enolase 2: glyceraldehyde 3-phosphate dehydrogenase 3: mannose/glucose PTS, EIIAB 4: H^+-transporting two-sector ATPase.

The presence of two cytosolic proteins typically associated with glycolysis would suggest lysis of the cells during the preparation of this protein fraction. However, the presence of the third protein (muramidase) that has a secretion signal peptide in its sequence confirms that the method for enriching outer cell surface proteins is applicable. Furthermore, cell lysis would have given a more complex protein fraction than that detected on the 1-DE gel (Figure 1A). The fraction enriched for cell envelope proteins showed four protein bands (Fig. 1B) and these proteins also identified using MALDI-TOF again showed the presence of two glycolytic enzymes. An explanation for the fact that no other proteins are found that have a signal peptide, is that those proteins may contain LPxTG motif (see Table1) which is cleaved during assembly of the peptidoglycan cell wall by a sortase (164). Hence, such proteins are covalently attached to the cell envelop and likely washed away along with the cell debris during the sample preparation. Another factor that has a major influence is the hydrophobic property of those proteins and cell-envelope

proteins are not easily solubilized and therefore poorly detected by this gel method.

Table 1. The sec-dependent secretome (23) of *Lactobacillus plantarum* WCFS1 predicted from its complete genome sequence (126).

	Number of proteins	Domain
	27	LPxTG[1]
	10	LysM[2]
	9	C-terminal membrane anchor[3]
	72	N-terminal membrane anchor[4]
	48	Lipobox[5]
	56	Secreted/Unknown anchor[6]
Total	222	

1) The domain LPxTG is cleaved by sortase and covalently attached to peptidoglycan
2) The LysM motif binds non-covalently to peptidoglycan
3) The proteins have a hydrophobic tail close to the C-terminus
4) The proteins have a hydrophobic tail close to the N-terminus
5) Lipoproteins are normal membrane proteins but have a signal sequence (110)
6) Proteins that are secreted but have an unknown anchor

To overcome the difficulties with the hydrophobic properties of a protein, a non-gel based proteomics approach can improve the recovery of cell envelop proteins as the proteins do not have to be resuspended in aqueous buffer needed for 2-DE gels. The use of Liquid Chromatography-Mass Spectrometry (LC-MS) or Surface Enhanced Laser Desorption/Ioniziation-Time Of Flight (SELDI-TOF) can indeed improve the recovery of the hydrophobic proteins but the resolution of the LC-MS has to be improved if the desired proteins do not differ largely in molecular weight. To avoid the low resolution of separation by LC, SELDI-TOF can be applied by using antibodies for the separation of the protein mixture. By using antibodies, enrichment for outer cell surface or cell envelope proteins will be highly specific for the desired proteins and this approach can be fast and accurate.

Recently, a new Desorption Electrospray Ionization (DESI) technique has been developed in which samples could be ionized from several surfaces including living tissues (224). This technique should make it possible to analyze the proteins present on the cell surface without isolating these proteins prior to analysis and hence is a promising tool to study host-microbe interaction at the protein level.

Several recent studies have shown that canonical glycolytic enzymes are present on the surface of bacteria (24, 34, 133, 177). It was shown that the extracellular proteome of *Lactobacillus crispatus* containing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase was able to enhance activation of the human plasminogen (Plg) (108). Furthermore the genes coding for enolase and GAPDH were cloned into *E. coli* and expressed as His$_6$-fusion proteins which bound Plg and enhanced its activation by tissue-type Plg activator (108). This suggests that the identified enzymes belonging normally to classical metabolic pathways including glycolysis might also be involved in binding of *Lactobacillus* spp. to host cells. It is noteworthy that the *L. plantarum* WCFS1 proteome obtained following passage of the human GI tract (**Chapter 6**) showed the presence of enolase.

7.3 Proteome Analysis of *L. plantarum* WCFS1 Recovered from the Human Intestine

Proteomics has given insight in the dynamics of *L. plantarum* WCFS1 and its isogenic CcpA-mutant strain during growth (**Chapters 3 and 4**). These results, which were obtained during growth under laboratory conditions, were complemented with further analysis to assess the metabolic activity of this microbe during its passage through the GI tract. In order to isolate *L. plantarum* from intestinal samples, the feasibility of several approaches has been analyzed. The first strategy to obtain the desired bacteria from *in vivo* samples was to use the combination of fluorescent *in situ* hybridization (FISH) and fluorescent activated cell sorter (FACS) (**Chapter 5**). For this purpose, a oligonucleotide probe previously designed for *L. plantarum* in dairy niches (100) has been shown to be applicable and selective in FISH-labeling *L. plantarum* cells in ileal samples (**Chapter 5**). However, due to low numbers of *L plantarum* in the GI tract, the high viscosity of the ileal samples used and the possible presence of food particles, direct sorting of the bacterium was not feasible and therefore a high risk for proteome changes during sorting of the microbe was expected. Therefore, another approach to isolate *L. plantarum* WCFS1 from different sites of the GI tract was developed. The selected approach was based on a sandwich antibody assay; a polyclonal antibody specific for *L. plantarum* WCFS1 was raised by injecting whole bacterial cells in rabbits. To extract the desired bacterial cells - bound to the first antibody - magnetic beads coated with goat anti-rabbit antibodies were used

(Chapter 6). The quality of this method has been validated by quantitative PCR and 85% of the isolated bacterial cells were found to be exclusively *L. plantarum* WCFS1, suggesting that this method was suitable for its isolation. Subsequently, the *L. plantarum* WCFS1 proteome was isolated from ileal effluent and analyzed by 2-DE. Thirteen spots were identified using the PRM and the identification of two spots (fructose-bisphosphate aldolase and phosphoglycerate mutase) was confirmed by MALDI-TOF, proving that the PRM is a reliable method for fast identification of protein spots.

Further improvements to this method can be made by using a larger working volume to decrease the viscosity of the sample and also the total number of magnetic beads can then be increased (final concentration constant), and consequently, more antibody-labeled *L. plantarum* WCFS1 can be isolated. A second improvement is the use of a single antibody instead of a sandwich-based approach. When the magnetic beads are coated directly with antibodies against *L. plantarum,* less non-desired microbes are captured due to unspecific binding to the antibody. In conclusion: the immunomagnetic sorting is an innovative method for isolating specific microbes from complex ecosystems and is compatible with proteomics.

7.4 Concluding remarks and futures perspectives

Overall, this thesis has brought new insights into the functionality of the commensal *Lactobacillus plantarum* WCFS1. In this study, we have focused on the proteomic approach to advance the understanding of the proteome changes in both *in vitro* and *in vivo* conditions. The construction of the PRM for *L. plantarum* was shown to provide the basis for rapid protein identification, and consists of 200 proteins that can now enable a fast screening of proteins changes in *L. plantarum* under different conditions in both *in vitro* and *in vivo*.

Application of a proteomic approach provides a realistic insight, based on actual protein, into the activity of a commensal intestinal microbe. This work complements previous studies in which the impact of the host on *L. plantarum* was analyzed using a transcriptomic approach (53). The proteomic approach has been applied in this thesis to study the behavior of one single commensal intestinal strain present in a complex microbial community in human. Proteomics has several advantages over transcriptomics as mentioned in **Chapter 2.** In time a

metaproteomics approach involving the large-scale characterization of the entire protein complement of the gut microbiota at a given point should be possible. However due to the high complexity of the intestinal microbiota, which has been shown to harbor thousands of different species of which the vast majority has been assessed only by 16S ribosomal RNA gene sequencing (190), application of metaproteomics is rather limited at this stage. The first application using infants fecal samples showed the restrictions in our predictive capacity due to insufficient sequence information of intestinal microbes (125). However, there are great advances being made with increasing numbers of whole genome annotation of intestinal microbes due to genomics, and metagenomics, i.e. the study of the collective genomes in an environmental community. Therefore, at this stage, the proteomics should be used on model organisms, but its application can be expanded in the not too distant future to both commensal and pathogenic microorganisms (**Chapter 2**). Ultimately, this would lead to an overview of the functionality of diverse microbes in the human GI tract of healthy and compromised subjects.

It may be expected that further technical improvements of the proteomics approach will help in advancing the understanding of the host-microbe interactions. These improvements can include 1) advanced staining methods that have a broader dynamic range, 2) availability of larger gels, 3) resolving power of non-gel based method, and 4) sensitivity of the mass spectrometers. Moreover improving algorithms to predict/annotate genomes will result in more rapid progress for the metagenomics approach, and in turn, this will facilitate the identification of proteins in the metaproteomic approach.

All these developments will make the proteomic approach more reliable as a high-throughput technique. Despite the fact that the link between the concentration of mRNA and the concentration of proteins is still not fully understood and the activity of enzymes is not solely dependent on the concentration of the enzyme (214), the proteomic approaches have a great potential for studying the functionality of intestinal microbes and elucidating the interactions between the host, diet, and microbes.

Previously, approaches have addressed the activity of intestinal microbes using transcriptional analysis, and showed highly relevant interaction between the host and the intestinal microbes (58, 104, 213). Furthermore, the metabolomics

approach can give insight in the relationship between the concentration of an enzyme/protein and its activity. The future integration of all –omics technologies will contribute to the understanding of the functionality of host-microbe interactions, and developing a system biology approach for intestinal functionality.

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Samenvatting

Lactobacillus plantarum WCFS1 is een bacterie die behoort tot de groep van melkzuurbacteriën. Deze Gram-positieve bacteriën worden veelvuldig gebruikt tijdens preparatie en conserveren van voedselprodukten. Aan sommige melkzuurbacteriën worden gezondheidsbevorderende eigenschappen toegedicht en deze worden op de consumentenmarkt gebracht als probiotica.. Uit voorgaand onderzoek is gebleken dat *L. plantarum* na consumptie door de mens of een diermodel, specifieke effecten optreden, zoals een regulerend effect op de werking van het immuunsysteem, een remmend effect op de adhesie van de pathogene bacterie *Helicobacter pylori aan* humane maag-epitheel cellen, en een reductie van de darmpermeabiliteit en translocatie van bacteriën. In dit proefschrift zijn de veranderingen op eiwitniveau in *L. plantarum* WCFS1 onderzocht door gebruik te maken van een proteomics benadering waarbij getracht wordt alle eiwitten die tijdens een bepaalde conditie geproduceerd worden tegelijkertijd te onderzoeken. Op deze wijze wordt geprobeerd een zo compleet mogelijk te beeld te verkrijgen van de functionaliteit van *L. plantarum* WCFS1.

Hoofdstuk 1 beschrijft een algemene introductie over melkzuurbacteriën en over de meest gebruikte proteomics benaderingen. In **Hoofdstuk 2** wordt een samenvatting gegeven van de resultaten van proteomics analyses van melkzuurbacteriën uit eerdere studies. In **Hoofdstuk 3** wordt er een proteoom referentie map beschreven die gemaakt is van *L. plantarum* WCFS1 opgegroeid in laboratorium medium, en gebruikt kan worden voor toekomstige studies. Ongeveer 200 eiwitspots zijn geïdentificeerd door middel van MALDI-TOF massa spectrometrie. Tegelijkertijd zijn de proteoom veranderingen onderzocht tijdens de groei van de bacterie onder laboratorium omstandigheden. Hieruit is gebleken dat tijdens de groei voor elke groeifase een specifieke set van eiwitten differentieel gereguleerd is. In **Hoofdstuk** 4 wordt met proteomics en transcriptomics technieken onderzocht wat de respons is tijdens de groei van *L. plantarum* WCFS1 en een isogene mutant, waarvan het gen coderend voor een belangrijke catabole regulator eiwit CcpA geïnactiveerd was. De resultaten op eiwit en RNA niveau vertoonden een aanzienlijke correlatie (70-80%) voor alle verschillende groeifases. De belangrijkste genen en eiwitten waarvoor geen

correlatie gevonden werd waren betrokken bij pyrimidine biosynthese, energie metabolisme, en stress respons.

In de thesis worden voorts twee methoden beschreven om specifiek *L. plantarum* te labelen zodat isolatie van deze bacteriën mogelijk is uit humane darm monsters. Een methode is gebaseerd op het gebruik van een probe die specifiek bindt aan het DNA van *L. plantarum* (**Hoofdstuk 5**). Deze probe werd getest op zijn specificiteit tegen verscheidene melkzuurbacteriën en 40 andere bacteriën die gewoonlijk aanwezig zijn in de menselijke darm. Deze probe werd toegepast op monsters verkregen uit de effluent van de ileum en uit fecale monsters maar bleek onvoldoende selectief en snel toepasbaar te zijn. De tweede methode die beschreven wordt, is gebaseerd op scheiding met behulp van immunomagnetische bolletjes (**Hoofdstuk 6**). Een polyclonaal antilichaam opgewekt tegen *L. plantarum* werd gebruikt om deze bacterie te coaten met antilichaam. Een tweede antilichaam gebonden aan een magnetisch bolletje werd gebruikt om de *L. plantarum* bacteriën weg te vangen uit de humane darm monsters. Quantitatieve PCR met specifieke primers bevestigde de identiteit van de geïsoleerde bacteriën. Het bleek dat *L. plantarum* met een hoge efficientie (86 %) geisoleerd kon worden uit ileum effluent monsters van vrijwilligers die *L .plantarum* WCFS1 hadden geconsumeerd. Vervolgens werd van de geïsoleerde bacteriele fractie, het proteoom geïsoleerd en geanalyseerd met behulp van twee-dimensionale gel electrophorese (2-DE). De 2-DE gelen tonen eiwit spotjes die geïdentificeerd konden worden met behulp van de eerder ontwikkelde proteoom referentie map (**Hoofdstuk 3**). De identificatie van twee van deze eiwit spotjes werd bevestigd door MALDI-TOF massa spectrometrie. De geidentificeerde eiwitten zijn betrokken bij carbohydraat metabolisme, stress adaptatie en translatie, wat de metabolische activiteit van *L. plantarum* tijdens de passage van de humane ileum illustreert. Ook glycolytische eiwitten die betrokken kunnen zijn bij gastheer interacties of adhesie van andere Gram-positieve bacteriën waren aanwezig op de 2-DE gel.

In **Hoofdstuk 7** worden de belangrijkste experimentele resultaten samengevat en wordt aangegeven hoe dit proefschrift de basis legt voor het gebruik van proteomics om de functionaliteit van *L. plantarum* uit humane darmmonsters te bestuderen.

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About the author

David Cohen was born on the 13th December 1976 in Semarang, Indonesia. After finishing high school in Terneuzen, Zeeland in 1995, he moved to Utrecht where he studied Fundamental Biomedical Science at the University of Utrecht. The major subjects during this study were human physiology, immunology, and neural sciences. During this period he performed a training period at the Research Institute of Toxicology (RITOX) in Utrecht for 9 months where he studied ochratoxin A-mediated nephrotoxicity. During this training period, he set up new eukaryotic cell lines that were used to study the effect of Cytochrome P_{450} enzyme system during exposure to ochratoxin A. He performed his second training period at the Sylvius laboratory in Leiden. During this period (6 months) he assisted with the construction of a hybrid vector for application in gene therapy. Here, a viral gene delivery vector was constructed consisting of DNA elements of both Adeno virus and Adeno-associated virus to obtain a high DNA-uptake capacity with improved genome integration vector. After graduation, David spent some time traveling and climbing. In 2002 he started his PhD-study at the Laboratory of Microbiology under the supervision of prof. dr. Willem M. de Vos and dr. Elaine E. Vaughan. Currently David is working as a post-doctorate at the Laboratory of Virology in the University of Wageningen on a EU-project to use Baculoviruses for application in gene therapy.

List of publications

D.P.A. Cohen, J. Renes, F.G. Bouwman, E.G. Zoetendal, E. Mariman, W.M. de Vos and E.E. Vaughan. 2006. Proteomic analysis of log to stationary growth phase *Lactobacillus plantarum* cells and a 2-DE database. *Proteomics*: 6(24): 6485-

R. te Biesebeke, R. Boesten, E.S. Klaassens, C.C.G.M. Booijink, M.C. de Vries, M. Derrien, **D.P.A. Cohen**, F. Schuren, E.E. Vaughan, M. Kleerebezem, and W.M. de Vos. 2004. Microbial functionality in the human gastrointestinal tract. *Microbes and Environments*. 19: 276-280

D.P.A. Cohen, E.E. Vaughan, W.M de Vos and E.G. Zoetendal. A proteomic approach to study lactic acid bacteria. *In* Therapeutic Microbiology: Probiotics and Other Strategies, ed J. Versalovic, M. Wilson. *Submitted*

D.P.A. Cohen, E.G. Zoetendal, E.E. Vaughan and W.M. de Vos. Proteome analysis of *Lactobacillus plantarum* recovered from the human intestinal tract via immunomagnetic separation. *Manuscript in preparation*

Education

Discipline specific activities
Courses

Ecophysiology of the GI-tract, VLAG, 2003	1.4
Bioinformation Technology – 1 VLAG 2003	1.9
Mass Spectrometry, Universiteit Utrecht, 2003	1.4
Masterclass Proteomics, Universiteit Maastricht, 2004	0.6
Genetics and Physiology of Food Associated Microorganisms, VLAG, 2004	1.0
International FISH course, University of Vienna, Austria, 2004	1.4

Meetings

Dutch Proteomics platform, Utrecht, the Netherlands	0.3
Proteomic Forum, Munich, Germany, 2003	0.8
Seminar Proteomics, Bio-Rad, the Netherlands	0.3
Darmendag, the Netherlands, 2002-2005	1.1
Symposium on Lactic Acid Bacteria, Egmond aan Zee, the Netherlands	1.1
FEMS congress, Madrid, Spain, 2006	1.4

Poster/Oral presentations

FISH course, Vienna, Austria	0.5
Darmendag	0.5
Masterclass proteomics	0.5
Symposium on Lactic Acid Bacteria, Egmond aan Zee, the Netherlands	0.5
FEMS congress, Madrid, Spain	0.5

General courses

VLAG PhD week, 2003	1.1
Radiation expert 5B, Larenstein, Velp, 2003	1.4

Optionals

Preparation PhD research proposal	6.0
PhD/Postdoc meetings, Laboratory of Microbiology 2002-2006	3.0
Literature study microbiology, 2004-2005	3.0
PhD study tour, Japan, 2004	2.5
Training period Maastricht University (6 months) 2005	6.0

Total	***38.2***