Chapter 6
Lactobacillus plantarum WCFS1: 12 Years After the Genome

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Microbial Biotechnology
6.1. Abstract

*Lactobacillus plantarum* WCFS1 is one of the best studied Lactobacilli, notably as its genome was unravelled over ten years ago. *L. plantarum* WCFS1 can be grown to high densities, is amenable to genetic transformation and highly robust with a relatively high survival rate during the gastrointestinal passage. In this review we present and discuss the main insight provided by the functional genomics research on *L. plantarum* WCFS1 with specific attention for the molecular mechanisms related to its interaction with the human host and its potential to modify the immune system, and induce other health-related benefits. Whereas most insight has been gained in mouse and other model studies, only five human studies have been reported with *L. plantarum* WCFS1. Hence, we advocate the use of *L. plantarum* WCFS1 and other isolates of *L. plantarum* NCIMB8826 in human trials as to capitalize on the wealth of knowledge that is summarized here.
6.2. Introduction

There continues to be significant interest in lactic acid bacteria (LAB) that contribute to our quality of life by preserving and fortifying foods, producing flavours and texture, and providing health benefits (de Vos, 2011). Hence, recent years have seen the production of a panoply of publications that address these attributes in LAB with most of the applications relating to *Lactobacillus* spp. that are used in functional foods. While there are over 100 different *Lactobacillus* species, only few have been studied in detail and developed into paradigms, as is the case with many biotechnological systems. In 2003, the complete 3.3 Mb genome of *Lactobacillus plantarum* WCSF1, a single colony of the human saliva isolate *L. plantarum* NCIMB8826, was published as the first genome of a Lactobacillus species (Kleerebezem *et al*., 2003). This was well before the genomes of other well-studied *Lactobacillus* spp. were reported, such as those from *L. acidophilus* NCFM (Altermann *et al*., 2005) and *L. rhamnosus* GG (Morita *et al*., 2009) that are widely marketed as probiotics (Saxelin *et al*., 2005). Currently, there are 6 complete genomes of *L. plantarum* strains publicly available and draft genomes of another 20 strains have been submitted to public databases (NCBI genome database; January 2015). However, no comparative genomic studies have yet been reported though it has been noted that there is a high degree of gene content variation among *L. plantarum* strains (Molenaar *et al*., 2005). A recent review describes the functional comparison of the *L. plantarum* WCFS1 genome with that of a 36 other complete genomes of lactic acid bacteria (Douillard & de Vos, 2014). Due to the presence of its genome sequence, its excellent growth properties and high transformation efficiency with newly developed genetic tools, *L. plantarum* WCFS1 has been extensively studied. In retrospect, these were exactly the attributes why this particular strain was selected for genome sequencing a dozen years ago (Kleerebezem *et al*., 2003).

The subsequent scientific progress developed with *L. plantarum* WCSF1 has provided detailed molecular insight into its characteristics, notably those relating to its interaction with the human host. This is of considerable interest as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, are defined as probiotics by the World Health Organization (WHO) and recently reincorfoed by Hill *et al*. (2014). There is growing consensus that certain probiotic Lactobacillus strains that are known to survive human gastrointestinal (GI) passage could be effective for infectious childhood diarrhoea (Allen *et al*., 2010), the prevention of antibiotic associated diarrhoea (Hempel *et al*., 2012; Goldenberg *et al*., 2013) and necrotising enterocolitis (NEC) in premature infants (AlFaleh & Anabrees, 2014). For other clinical conditions, like atopic dermatitis (AD), inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS), there are good indications that certain probiotic lactobacilli could be effective (Floch *et al*., 2011; Sanders *et al*., 2013). It should be noted that although some mechanisms are widespread among probiotic species, other effects are solely strain-specific (Hill *et al*., 2014). In contrast to some other well-studied Lactobacilli, *L. plantarum* WCSF1 has reportedly only been used in a few clinical trials. The strain *L. plantarum* 299v, of which no public genome sequence is yet available, has been reported to have some...
probiotic effects, as it has beneficial effects in patients with irritable bowel syndrome and it has shown to reduce colonization of *Clostridium difficile* in critically ill patients treated with antibiotics (Ducrotte *et al*., 2012; Klarin *et al*., 2008). Moreover, other *L. plantarum* strains have been used in probiotic mixtures reportedly showing a health benefit, including *L. plantarum* in VSL#3 for inflammatory bowel diseases, and *L. plantarum* W62 in EcologicRelief and EcologicAAD for constipation and antibiotic associated diarrhea, respectively (Bekkali *et al*., 2007; Chapman *et al*., 2006; Koning *et al*., 2008). As *L. plantarum* WCFS1 has only be limited studied in clinical trial, it is not marketed in probiotic products but the extensive genetic and physiological work done on this strain provides an excellent basis for its further development as a probiotic strain. Hence, in this review we present an overview of the main insight that the molecular research on *L. plantarum* WCFS1 has provided in relation to the interaction with the host and potential health benefit for humans. Moreover, we indicate a variety of avenues that can be followed for future industrial applications of this strain and summarize suggestions further research that is needed for such applications.

### 6.3. From Genome to Function

The initial genome sequence of *L. plantarum* WCFS1 was based on Sanger dideoxy-sequencing (Kleerebezem *et al*., 2003) and has been revised by next generation sequence analysis on an Illumina platform, providing a genome sequence predicted to code for 3,042 proteins (18 pseudogenes) and 83 RNA-encoding genes (Siezen & van Hylchama Vlieg, 2011). The genome contains two large regions between 2.70 – 2.85 Mb and 3.10 – 3.29 Mb with a high flexibility, termed life style islands that include a total of 293 genes, mostly involved in sugar utilization (Molenaar *et al*., 2005). Furthermore, *L. plantarum* WCFS1 contains three plasmids, including two small ones, pWCFS101 and pWCFS102, that are rolling-circle replicating plasmids with an unclear function and a size of 1,917 and 2,365 bp, respectively. A third plasmid, pWCFS103 has a size of 36,069 bp, the capacity for conjugative transfer, and encodes genes involved in heavy-metal resistance (cadmium and arsenate) and NADH oxidase activity (van Kranenburg *et al*., 2005).

The genome of *L. plantarum* WCFS1 has been well annotated not only by automated methods but also by detailed manual curation. However, there is still a large fraction (approximately 30 %) of genes for which no function can be predicted. Moreover, as is the case with all genomes, in some cases the annotated genes are not correctly predicted and a combination of genetic and physiological experiments is needed to demonstrate the functionality of a gene. Due to the high transformation efficiency of *L. plantarum* WCFS1 (routinely 106 transformants/µg), a variety of useful inactivation systems (such as cre-lox; Lambert *et al*., 2007), and controlled expression platforms (such as NICE; Pavan *et al*., 2000), a great number of isogenic mutants have been generated in *L. plantarum* WCFS1. Many of these are relevant for its growth, cell shape or surface properties and its interactions with the environment – hence these are listed here and some of these are discussed further (see Table 6.1). Apart from the genetic systems, a useful set of high throughput
tools have been developed in recent years, varying from various microarray platforms, RNAseq approaches and advanced proteomics (Marco et al., 2010; De Vos, 2011; Fredriksen et al., 2013; Douillard & De Vos, 2014). This allowed detailed phenotypic analysis of mutants, functional studies of overexpressed genes, and the evaluation of genome-wide expression in response to environmental cues. Many of these approaches have been instrumental in not only confirming the predicted function of genes but also defining new and relevant properties, notably for GI tract survival and interactions with food, other bacteria and the host, as will be discussed below.

6.4. Gastro-Intestinal Tract Survival

*L. plantarum* NCIMB 8826, the parental strain of *L. plantarum* WCFS1, shows high survival capacity in the human GI tract, as after a single oral dose of $1.5 \times 10^{10}$ cfu/ml, it appeared possible to recover $1.0 \times 10^{8}$ cfu/ml (approximately 1% of the dose) from the ileum of healthy volunteers, which at least remains for five hours above $1.0 \times 10^{5}$ cfu/ml (Vesa et al., 2000). This contrasts to other Lactobacilli such as the *L. fermentum* strain KLD used in probiotic products that died off much more rapidly. The survival rate of *L. plantarum* NCIMB 8826 was 7%, and could be retrieved from faecal samples, one week after consumption had stopped, in contrast to 0.8% and 0.5% for *Lactococcus lactis* and *L. fermentum* respectively (Vesa et al., 2000). In addition, *L. plantarum* WCFS1 was readily obtained from the ileal effluents of ileostoma patients fed an oral dose (Marco et al., 2010). Another study demonstrated that *L. plantarum* WCFS1 in healthy human volunteers survived the *in vivo* gastrointestinal passage, as a 100-1000 fold increased level of *L. plantarum* could be recovered from faecal samples until 3–4 days after administration (Van Bokhorst-van de Veen et al., 2012b). The relative survival rate of *L. plantarum* WCFS1 in a GI-tract mimicking assay was high compared to other *L. plantarum* strains (e.g. a difference in survival of 7 log10 cfu/ml compared to *L. plantarum* CECT4646). In stationary and logarithmic growth phase, the *L. plantarum* strains LP80 and NCIMB12120 demonstrated an even higher survival rate compared to *L. plantarum* WCFS1.
Table 6.1. Overview of relevant *L. plantarum* WCFS1 mutants, the involved gene and their phenotypes, classified according to their gene function. Some mutants with mutations in homologous genes and similar phenotypes are combined. GI, Gastrointestinal; EPS, Extracellular polymeric substance; SPS, Surface Poly Saccharide; QS, Quorum Sensing. ND, not detected.

<table>
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<th>Gene(s)</th>
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<th>Gene Function</th>
<th>Affected Phenotype</th>
<th>Reference</th>
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<td><em>melA</em></td>
<td><em>Lp_3485</em></td>
<td>α-Galactosidase</td>
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<td><em>lacS</em></td>
<td><em>Lp_P48</em></td>
<td>Sugar Permease</td>
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<td><em>Lp_1125</em></td>
<td>Subunit Cytochrome (bd type)</td>
<td>Oxidative Respiration</td>
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<tr>
<td><em>narG</em></td>
<td><em>Lp_1497</em></td>
<td>Subunit Nitrate Reductase</td>
<td>Nitrate Respiration</td>
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<td><em>rpoN</em></td>
<td><em>Lp_0787</em></td>
<td>Sigma Factor 54</td>
<td>Global Expression</td>
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<td><em>Lp_0585</em></td>
<td>Mannose Operon Regulator</td>
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<td><em>manIIC</em></td>
<td><em>Lp_0230</em></td>
<td>Mannose Transport</td>
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<td>Glucose Repression</td>
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<td><em>Lp_0271</em></td>
<td>Gallate Decarboxylase</td>
<td>Tannine Utilization</td>
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<td>QS/EPS/Biofilm Production</td>
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<td>Response Regulator</td>
<td>QS/EPS/Biofilm Production</td>
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<td><em>plnGHSTUVWX</em></td>
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<td><em>bsh1</em></td>
<td><em>Lp_3538</em></td>
<td>Choloyl Glycin Hydrolase</td>
<td>Bile Resistance</td>
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<td><em>Lp_0067-Lp_3362-Lp_2572</em></td>
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<td>Acylase Activity</td>
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<td>Phenotype/Condition</td>
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<td>Lp_1177</td>
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<td>Reduced SPS and Rhamnose Level</td>
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<td>Lp_1197</td>
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<td>Lp_1215-Lp_2108</td>
<td>SPS Production</td>
<td>Reduced SPS Levels</td>
<td>Remus et al, 2012</td>
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<td>gfsA</td>
<td>Lp_1299</td>
<td>Glycosyl Transferase</td>
<td>Surface Protein Glycosylation</td>
<td>Lee et al., 2014</td>
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\textit{L. plantarum} WCFS1 is thus relatively highly robust in surviving the GI-transit, mainly in stationary phase. Nevertheless, there could be no \textit{L. plantarum} retrieved in the ileum after 8 hours (Vesa et al., 2000). These data suggest that \textit{L. plantarum} WCFS1 is a passenger in the GI-tract, and not an effective intestinal colonizer as found for various other Lactobacilli (Douillard & De Vos, 2014). It should be stressed that only the lumen was analysed, whereas the bacteria could have colonized the intestinal epithelium. Furthermore, during the GI passage the microorganism is able to exert its effect on the physiological and immunological systems of the host. For instance, the well-studied probiotic strain \textit{L. rhamnosus} GG, which is among the best mucus-adhering strains due its mucus-binding pilus protein SpaC, is also only able to temporarily colonize the gut (Kankainen et al., 2009; Goldin et al., 1992; Alander et al., 1999; Segers & Lebeer, 2014). It is assumed that the majority of lactobacilli are passengers in the GI-tract, rarely exceeding 1% of the total number of bacteria, and therefore have a profound health effect on the human host (Douillard & de Vos, 2014).

The first hurdle a consumed bacterium encounters when entering the GI-tract is the acidic stomach. An in vitro GI tract-survival model, in which bacteria were exposed to gastric juice containing pepsin and lipase at a pH of approximately 2.5, and subsequently subjected to pH-neutralizing pancreatic juice containing pancreatin and bile salts, \textit{L. plantarum} WCFS1 proved to be highly robust, with a relative small decrease in viable cells (Van Bokhorst-van de Veen et al., 2012a). The gastric juice exerted the highest impact on \textit{L. plantarum} WCFS1 survival, demonstrated by a million-fold decrease in living cells, whereas the condition resembling the small intestines hardly affected the survival (Van Bokhorst-van de Veen et al., 2012a). Another oro-gastric-intestinal tract model demonstrated that survival of \textit{L. plantarum} WCFS1 is unaffected by the initial oro-gastric stress, however, the viability decreased significantly when pH was downshifted to approximately 2.0 (Bove et al., 2013). There are several mechanism upregulated in response to low-pH conditions, for instance increased proton export by \( F_0 F_1 \)-ATPase to retain a proper intracellular pH. Furthermore, the gastric stress was associated with an increased expression of the chaperone genes \textit{dnaK}, \textit{groEL}, \textit{clpB} and \textit{clpE}, small heat shock proteins \textit{hsp1}, \textit{hsp2} and \textit{hsp3}. In addition, the expression of the adhesion factors \textit{mub} and \textit{msa}, and that of the operon \textit{plnEFI}, an ABC transporter involved in plantaricin production, was increased in response to gastric stress (Table 6.1; Bove et al., 2013). The expression levels of three genes (\textit{php2A, napA3} and \textit{lp_1669} – see Table 6.1) were negatively correlated with in vitro GI-survival and encode a penicillin-binding protein 2A, an Na\(^+\)/H\(^+\)-antiporter and an AraC family regulator that may control the expression of surface polysaccharide production, respectively (Van Bokhorst-van de Veen et al., 2013).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Function</th>
<th>Description</th>
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<td>Glycosyl Transferase</td>
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<td>oatA</td>
<td>Lp_0856</td>
<td>O-Acetyl Transferase</td>
<td>Cell Septation</td>
<td>Bernard et al., 2011</td>
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<td>oatB</td>
<td>Lp_0925</td>
<td>O-Acetyl Transferase</td>
<td>Cell Septation</td>
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2012a). Remarkably, all of these proteins are associated with the cell envelope and it is conceivable that by reducing non-essential cell wall proteins, the membrane integrity can be maintained and the cell’s resistance to low pH can be increased as is illustrated in Figure 6.1.

After survival of the stomach passage, an ingested bacterium reaches the duodenum, where it encounters a variety of stressful conditions, including the presence of conjugated bile salts. Not only do these bile salts disperse and absorb fat, these compounds also function as surfactants and disrupt the cells membrane integrity, generate free radicals, and when protonated, can lower the intracellular pH (Bron et al., 2004a; Van Bokhorst-van de Veen et al., 2012b). Bile salts are deconjugated by bacterial bile salt hydrolases (Bsh) and reabsorbed in the colon. *L. plantarum* WCFS1 contains four bile salt hydrolases (*bsh1, bsh2, bsh3* and *bsh4*; Table 6.1), compared to e.g. *L. acidophilus* NCFM which only possess two *bsh* genes (Begley et al., 2006) With Bsh1 being the major Bsh and Bsh3, 2 and 4 were able to hydrolyze penicillin V and penicillin G (Lambert et al., 2008a; 2008b). However, under selective conditions, Bsh2, Bsh3 and Bsh4 could be able to hydrolyze bile salts (Figure 6.1; Table 6.1). It is suggested that Bsh plays a role in bile detoxification,
An artificial GI-tract environment, consisting of 0.1% oxgall, a bovine bile salt, only marginally affected growth of *L. plantarum* WCFS1 but its morphology was severely changed (Bron *et al.*, 2004a). *L. plantarum* reacted on this physiological stress by upregulating the expression of proteins involved in bile export (*lp_0085, lp_2564* and *lp_3160*) and four oxidoreductases and a redox protein to restore the oxidative and redox imbalance (Bron *et al.*, 2004a). Additionally, the genes *lp_0237* and *lp_0775* were found to be bile-inducible. Overall, 62 and 28 open-reading frames are down- and upregulated. Among the upregulated genes are the oxidative stress-associated glutathione reductase and the metC-cysK operon (Bron *et al.*, 2006). By reducing the expression of non-essential membrane proteins, the cell might compensate for the bile-induced loss of membrane integrity (Bron *et al.*, 2006). Proteomic and transcriptomic analysis revealed that *L. rhamnosus* GG and *L. casei* BL23 also have a reduced expression of proteins involved in cell wall function in response to bile stress, suggesting this is a more common in LAB (Douillard & de Vos, 2014). *L. plantarum* WCFS1 seems to have a large array of response mechanisms to bile salts. Since apparently only the morphology is altered and not the viability, WCFS1 is able to efficiently cope with this stressful environment. The hydrolysis of bile salts is associated with lowering of serum cholesterol as well as mucin production (Lambert *et al.*, 2008a). Recently, bile salt hydrolases in the GI-tract have been implied in providing specific signals that reduce weight gain in mice after a high fat diet (Joyce *et al.*, 2014). With four copies of the *bsh* gene that are all expressed in *L. plantarum* WCFS1, this bacterium has the highest number of *bsh* genes in over 200 *Lactobacillus* species (Sun *et al.*, unpublished data). Hence, when extrapolated to the human system, it is tempting to assume that administration of *L. plantarum* WCFS1 could have the potential to lower serum cholesterol levels in individuals with high cholesterol or reduce weight gain (see Figure 6.1).

The colonic lumen, where the majority of the microbiota resides, is deprived of oxygen (anoxic), whereas the mucosal surface is more oxygen-rich. In addition, a high osmolarity predominates in the colon (Kleerebezem *et al.*, 2010). *L. plantarum* WCFS1 was found to respond to oxidative stress by using thioredoxin (TRX), the only active thiol-reducing system in this strain. Gram-positive bacteria cannot synthesize the antioxidant glutathione, therefore the TRX system is essential for this organism (Serrano *et al.*, 2007). The expression of the *trxA2* and *trxB1* genes was increased following oxidative stress and *trxB2* in combination with *trxA2* were involved in reductive stress and a temperature shift (Serrano *et al.*, 2007). The *trxB1* gene codes for a thioredoxin reductase (TR), which regenerates oxidized TXR (Arnér & Holmgren, 2000; see Figure 6.1).

Using a special in vivo expression technology (IVET) system, a set of 72 genes of *L. plantarum* WCFS1 was identified whose expression was induced during mouse GI tract passage (Bron *et al.*, 2004b). Many of these genes were predicted to be involved in cell wall anchoring, exporters and metabolism. By inactivating a selection of these (Table 6.1), it was observed that the GI-tract survival of the mutants Δlp_1164, Δlp_2940 and Δlp_3055 was decreased compared to the control strains, whereas the mutants Δlp_1403, Δlp_3281 and Δlp_3659 showed no differences (Bron *et al.*, 2007). The gene *lp_1164* is suggested to encode a...
component of the celllobiose transport and could be involved in the host-specific signaling, $lp_{2940}$ encodes and extracellular protein and $lp_{3055}$ is important in the copper homeostasis, as it is predicted to encode a copper-transporting ATPase. These genes thus play an important role in the GI-survival in mice and while the GI-tract of mouse and human differ in architecture, pH and microbial composition, it is very well possible that these genes also play a role in the survival in the human system. Other survival factors such as extracellular polysaccharides (EPS) also appear to be essential in the survival of the GIT (Gastrointestinal tract). Deletion of long galactose-rich EPS in $L.\ rhamnosus$ GG leads to significant reduced in vivo survival (Segers and Lebeer, 2014). The role of EPS in $L.\ planatrum$ WCFS1 in the interaction with the host remains to be determined.

Administration of $L.\ plantarum$ WCFS1 to a mouse model demonstrated that strains could be retrieved from faecal samples up to 7 days (Van Bokhorst-van de Veen et al., 2013). The GI-tract persistence could be increased to over 32 days when isolated faecal strains were re-administered to the mice (Van Bokhorst-van de Veen et al., 2013). This adaptation to the intestinal tract of mice could be ascribed to single nucleotide polymorphisms (SNPs) leading to structural variations in the cell envelope. The persistent strains all demonstrated a SNP in genes coding for membrane associated proteins. The cfus of $L.\ plantarum$ in the stomach and small intestine remained high for at least 4 hours after administration of $2\times10^{10}$ cfu $L.\ plantarum$ WCFS1 in mice but thereafter declined to background levels. This amount remains, however, at $1\times10^9$ cfu/g tissue for at least eight hours in the caecum and colon (Marco et al., 2007). A single intragastric gavage consisting of $1\times10^9$ cfu $L.\ plantarum$ WCFS1 in GF mice on a chow or “Western” diet, showed colonization over the intestinal epithelium (Marco et al., 2009); remarkably, a significantly higher colonization in the colon and caecum was achieved when mice were on a chow diet. The host diets were associated with dramatically different transcription profiles. For instance, the western diet, consisting of mainly simple sugars, is restricting growth, demonstrated by 3 to 5- times lower expression of genes involved in transcription, translation, and nucleotide biosynthesis (Marco et al., 2009).

### 6.5. Interaction with Food Components

Current dietary recommendations include the consumption of fruits and vegetables. In addition to the vitamins and dietary fibre content of these products, it is a source of the polyphenol tannin. Tannins can form indigestible protein complexes and bind heavy metals. Tannins have also been associated with hepatotoxicity and cancer (Jiménez et al., 2013). On the other hand, tannins have antimicrobial properties; thereby potentially alter the gut composition. $L.\ plantarum$ is so far the only tannin-degrading Lactobacillus species of human origin and contains tannase (tannin acyl hydrolase; Reverón et al., 2013). $L.\ plantarum$ WCFS1 is able to hydrolyze tannin into glucose and gallic acid, a harmful and anti-nutritional compound, which is decarboxylated by LpdB and LpdC ($lp_{0271}$ and $lp_{2945}$) that encode gallate decarboxylase.
activity (Jiménez et al., 2013). Other *L. plantarum* strains that are suggested to possess tannase-activity are *L. plantarum* CNRZ 1228, CNRZ 184, ATCC 8014 and ATCC 14917 (Osawa et al., 2000). Culturing of *L. plantarum* WCFS1 in the presence of tannic acid induces significantly higher expression of persistence and survival genes *copA*, *lp_2940*, *ram2* and *argG* that are highly induced in the GIT in response to high osmolarity and bile in mice and humans (Reverón et al., 2013). *L. plantarum* WCFS1 is thus able to respond to these toxic compounds as well as use these as an energy source, thereby selectively stimulating its growth. Although tannins are not a major constituent of the human diet, bacterial strains that have tannase-activity might be beneficial for human health. Further exploration of these properties is warranted (Osawa et al., 2000).

Plant cell walls contain many phenolic compounds which, when released, have shown to have several beneficial effects on the host (e.g. anti-inflammatory and anti-oxidants; Esteban-Torres et al., 2013). Ferruloyl esterase (FE; *lp_0796*), an enzyme involved in the release of these compounds would be able to release these beneficial compounds. Unfortunately, an efficient transport system for Lp_0769 lacks in *L. plantarum* WCFS1 as it was unable to hydrolyze any of the extracellular model substrates (Esteban-Torres et al., 2013); cell extracts could, however, partially hydrolyze methyl ferulate and methyl p-coumarate, demonstrating that the enzyme Lp_0769 is functional and is likely to be released upon lysis of *L. plantarum* WCFS1. Whether the activity of FE in *L. plantarum* WCFS1 is of significance remains to be determined, as other strains such as *L. fermentum* NCIMB 5221 and *L. fermentum* 11976 have superior FE-activity and already demonstrate potential health effects (Bhathena et al., 2009; Tomaro-Duchesneau et al., 2012).

*L. plantarum* WCFS1 encodes a p-nitrobenzoate reductase (PnbA; encoded by *lp_0050*). The PnbA enzyme catalyzes the reduction of nitroaromatics which are highly abundant food products due to several industrial processes (Guillen et al., 2009). These nitroaromatic compounds have been shown to be cytotoxic and mutagenic, therefore bacterial nitroreductases can have beneficial health effects on the host. PnbA is a highly selective reductase as it only reduces 4-nitrobenzoate and 2,4-dinitrobenzoate (Guillen et al., 2009).

Currently, many commercial products contain prebiotics, which are substances that selectively stimulate growth and/or activity of one or a limited number of bacteria and can thereby be beneficial for the host (Gibson et al., 2004). Short chain fructooligosaccharides (scFOS), a well-studied prebiotic, is converted by *L. plantarum* WCFS1 by a sucrose phosphoenolpyruvate transport system, a β-fructofuranosidase and a fructokinase (Saulnier et al., 2007). Although growth on scFOS was relatively slow, possibly since detailed analysis showed that preferentially the trisaccharide 1-ketose was used and its conversion was heterofermentative as the end products were mainly lactate and acetate.
6.6. Interaction with Other Microorganisms

For successful GI-transit, adaptation and response to environmental cues, *L. plantarum* needs a sensory system to react to other mutualistic and competing microorganisms. Gene expression depending on cell-density is referred to as quorum sensing, and can significantly aid in the survival of the bacteria (Kleerebezem et al., 1997; Sturme et al., 2007). The quorum sensing systems are regulated by signal molecules, autoinducing peptides (AIPs) that are sensed by a two component systems (TCS), that include a histidine protein kinase (HPK) and a response regulator (RR; Sturme et al., 2005). The quorum-sensing systems of *L. plantarum* WCFS1 have been well-studied, notably for the production of bacteriocins. *L. plantarum* WCFS1 possesses the *pln* locus that contains five operons; *plnABCD*, that encodes the AIP termed plantaricin A (*plnA*) which also is a bacteriocin, the HPK PlnB (*plnB*) and the two RRs PlnC and PlnD (Table 6.1; Sturme et al., 2007). *L. plantarum* WCFS1 shares this locus (to some extend) with *L. plantarum* C11, NC8 and J23 (Rojo-Bezares et al., 2008). At a certain bacterial cell density, the plantaricin A concentration reaches a threshold, thereby activating the HPK PlnB and this subsequently phosphorylates the RRs PlnC an PlnD. The RRs regulate the transcription of all the genes involved in bacteriocin synthesis. In this way there is a density-dependent expression of plantaricin A. The operons *plnEFI* and *plnJKLR* encode the plantaricins EF and JK with their respective immune proteins (Rojo-Bezares et al., 2008). The bacteriocins are subsequently transported and secreted by an ABC-transporter and accessory proteins (PlnGH) encoded by the operon *plnGHSTUVWXY*, the role of which remains to be determined (Sáenz et al., 2009).

Bacteriocins play an important role in the competition with other micro-organisms. Based on their characteristics, bacteriocins are distinguished into several classes. The antimicrobial peptides of *L. plantarum* WCFS1 can be classified into the non-lantibiotic family (class II) and include the well-studied plantaricin A, which is a class Iic bacteriocin, and the bacteriocins EF and JK belonging to the class IIb two-peptide bacteriocins (Table 6.1; Diep et al., 2009). The antimicrobial activity of plantaricin A has a relatively narrow spectrum and is significantly lower activity than that of the bacteriocins EF and JK (Diep et al., 2009). The latter bacteriocins PlnEF and PlnJK are mostly active against *Lactobacillus* species and closely related Gram-positive bacteria (e.g. *L. plantarum*, *L. casei*, *L. sakei*, *L. curvatus*, *Pediococcus pentosaceus* and *P. acidilactici*), whereas plantaricin A is effective against *Lactobacillus* species, such as *L. casei*, *L. sakei*, *L. plantarum* and *L. viridescens* (Diep et al., 2009). *L. plantarum* WCFS1 demonstrated bacteriocin production with a low minimum inhibitory concentration (MIC) against *Enterococcus faecalis* CNRZ135, *L. pentosus* CECT4023T, *L. plantarum* CECT748T, *Listeria innocua* BL86/26 and *Pediococcus pentosaceus* FBB63. The bacteriocin production however depends on the inoculation size and is dependent on quorum sensing as described above. Hence, at low cell densities, the bacteriocin production is too low to inhibit growth of competing microorganisms (Maldonado-Barragán et al., 2009). Of more interest would be to determine the antimicrobial effect against human pathogens such as *Salmonella enterica*, Shigella sonei and *Staphylococcus* strains, for which *L. rhamnosus* GG already demonstrated to reduce viability (Segers and Lebeer, 2014).
Quorum sensing is also essential in the formation of biofilms. Biofilms render the bacteria less sensitive to antimicrobials due to reduced penetration and resistance mechanisms. In addition, bacteria are less susceptible due to a lower growth rate (Van der Veen et al., 2011). For instance, cells of *L. plantarum* WCFS1 in a mixed biofilm with *L. monocytogenes* were more resistant to disinfection treatments by benzalkonium chloride and peracetic acid than the single species biofilms (Van der Veen et al., 2011). The formation of biofilms with other species might be beneficial for the host as it may involve co-aggregation with pathogens, thereby decreasing their colonization potential (Goh & Klaenhammer, 2010). Auto-aggregation entails the aggregation of genetically identical cells, and can enhance the resistance to stress in the intestines (Hevia et al., 2013). Aggregation promoting factors (APFs) are extracellular proteins, highly expressed in the stationary phase, that are directly linked to the ability to co-aggregate (Boris et al., 1997).

In *L. plantarum* NCIMB 8826 the serine/threonine domain of the APF, D1, binds to (porcine) mucin III and is involved in auto-aggregation as it has been found that *L. plantarum* loses its auto-aggregative abilities when gene D1 is knocked-out (Hevia et al., 2013). Moreover, gene D1 overproduction in *Lactococcus lactis* leads to aggregation. As *L. plantarum* WCFS1 has been derived from strain NCIMB 8826, it was not a surprise to find the gene D1 in the *L. plantarum* WCFS1 genome as Lp_0304, which has been annotated as an extracellular transglycosylase. However, gene D1 contained several SNPs as compared to the known sequence of Lp_0304 (Kleerebezem et al., 2003), either reflecting sequence errors or strain heterogeneity in NCIMB 8826, but it is likely that Lp_0304 also can bind mucus as it has the serine/threonine domain.

The accessory gene regulatory system (Agr system) performs a key role in biofilms formation and the lamBDCA operon of *L. plantarum* WCFS1 controls the expression of around 100 genes (Sturme et al., 2005). This system includes encodes a HPK (LamC) and the RR (LamA) that form a TCS, as well the AIP (LamD) and the export and modification protein (LamB). The expression of the lamBDCA operon seems to correlate with growth, as expression increased during the log-phase. The AIP was found to be a novel cyclic thiolactone autoinducing peptide that seems to control adherence, most likely via its effect on the expression of EPS operons (Sturme et al., 2005). The lamBDCA operon was found to be engaged in cross-talk with another TCS encoded by the lamKR operon, as a lamA/lamR mutant demonstrated a highly reduced adherence to glass compared to a single mutant or wild-type (Fujii et al., 2008). TCSs monitor and respond to environmental cues such as stress (Sturme et al., 2007). Quorum sensing is thus essential in the formation of biofilms, which not only renders *L. plantarum* WCFS1 less susceptible to external stressors but also may provide it with a means to trap pathogenic bacteria.

Peptidoglycan hydrolases (PGHs) are major actors in cell division, cell wall turnover, autolysis and biofilm formation. By cleavage of the bacteria peptidoglycan, they may even play a role in host interaction by the release of muramyl-peptides and PG fragments (Rolain et al., 2012). The genome of *L. plantarum* WCFS1 encodes for at least 12 PGHs, with N-acetylglucosaminidase (Acm2) and γ-D-Glu-mDAP muropeptidase (LytA) as the most pivotal proteins for physiology and morphogenesis (Rolain et al., 2012; Table 6.1). It has recently been observed that Acm2 is post-translationally modified by glycosylation (Fredriksen et al., 2012).
and this may further enhance the interaction with bacteria, contributing to biofilm formation.

### 6.7. Interactions with the Host – Epithelial Barrier

Extracellular proteins, that together constitute the secretome, are involved in variable processes such as host-adherence, recognition, degradation and uptake of luminal nutrients and transduction of signals (Buck et al., 2005). The genome of *L. plantarum* WCFS1 encodes 223 extracellular proteins of which 57 have predicted to be secreted or anchored to the surface (Boekhorst et al., 2006). Analysis of the secretome identified 12 adhesion factors; three contained a domain to adhere to collagen, one to chitin, one to fibronectin and seven to mucus. *L. plantarum* WCFS1 also contains a mucus-binding (MUB) domain, a domain that is unique for LAB and present in the MUB products of four genes (*lp*_1229, *lp*_3114, *lp*_3059 and *lp*_1643; Boekhorst et al., 2006; **Figure 6.2**). These include *lp*_1229, which encodes the mannose-specific adhesion (Msa; **Table 6.1**). Deletion of the msa gene resulted in loss of the ability of *L. plantarum* WCFS1 to agglutinate with yeast (Pretzer et al., 2005). When comparing to other *Lactobacillus* strains, 14 mucus-binding proteins were identified in *L. gasseri* ATCC 33323, and 18 proteins with potential adhesive properties in *L. acidophilus* L-92 (Douillard & de Vos, 2014); demonstrating the large repertoire of adhesive proteins within LAB.

*L. plantarum* encodes 32 proteins with a LPxTG-motif, which are proteins that are covalently bound to the cell wall as they are recognized and cleaved by sortase A, the product of the *srtA* gene. In Gram-positive pathogens these LPXTG motif-containing proteins are often virulence factors, and associated with functions as adhesion and receptors. In some cases these proteins are post-translationally glycosylated and are suggested to play a major role in cell to cell interaction (Fredriksen et al., 2013). O-linked glycosylated extracellular proteins are of interest due to their matrix interaction. For instance, the *L. plantarum* WCFS1 major autolysin Acm2 and the MUB protein *lp*_1643 (see above), are O-linked glycosylated (Fredriksen et al., 2013). Our current understanding of glycosylation in LAB is limited, but these glycoproteins likely play a role in the bacteria-host interaction (Fredriksen et al., 2012; Tytgat & LeBeer, 2014).

An increased or altered gastrointestinal permeability is associated with a variety of illnesses. It has been suggested that IBD and IBS are characterized by infiltration of antigens due to mucosal barrier dysfunction and subsequent on-going inflammation of the intestines (Bruewer et al., 2006; Barbara, 2006). The epithelial integrity is mainly controlled by tight junctions (TJs), which are multifunctional complexes of integral membrane proteins located at the apical parts of the epithelial cell (Schneeberger & Lynch, 2004). These structures interconnect the cells and include occludins, claudins and junction adhesion molecules. *L. plantarum* WCFS1 has the potential to enhance the intestinal integrity in cell lines through activation of TLR-2, which is expressed on intestinal epithelial cells (Karczewski et al., 2010).
Figure 6.2. Putative proteins involved in the host-microbe interaction of L. plantarum WCFS1. APF(D1), Aggregation promoting factor D1; WTA, Wall teichoic acid; LTA, Lipoteichoic acid; TLR, Toll-like receptor; Msa, Mannose specific adhesion; ZO-1, Zonulin-1; ZO-2, Zonulin-2; ZO-3, Zonulin-3; IL, Interleukin; TNF, Tumor necrosis factor; TH1, T-helper cell 1; Treg, Regulatory T-cell.
In vitro activation of TLR-2 transiently enhanced the epithelial resistance through zonula occludens 1 (ZO-1) translocation (Cario et al., 2004). A Caco-2 human epithelial model demonstrated a significant translocation of ZO-1 to the TJ-region due to L. plantarum WCFS1 (Figure 6.2). This was also observed in the duodenum of healthy individuals after short-term (6-hour period) administration of L. plantarum WCFS1, suggesting that this also occurs in humans (Karczewski et al., 2010). In addition, the drop in trans epithelial electrical resistance (TER) induced by phorbol 12,13-dibutyrate (PDBu), which dislocates occludin and ZO-1, was decreased in combination with L. plantarum WCFS1 (Karczewski et al., 2010). The enhanced barrier function is likely due to an altered TJ composition rather than an increase in TJ proteins, as transcription levels were not significantly altered by L. plantarum WCFS1 (Troost et al., 2008). Other L. plantarum strains were also able to prevent a reduction in TER in Caco-2 cells when co-cultured with pathogenic Escherichia coli strains (Ulluwishewa et al., 2011), indicating that L. plantarum can play a beneficial role in maintaining epithelial integrity.

Lipoteichoic acid, major constituents of the cell wall of gram-positive bacteria (and suggested as equivalent of the Gram-negative LPS), are important molecules for interaction with TLR-2. The inflammatory properties of LTA greatly depend on the decoration of this protein by D-Ala. A L. plantarum NCIMB 8826 Dlt- mutant that results in LTA with significantly less incorporated D-Ala units induces significantly less pro-inflammatory cytokines when incubated with PBMCs (Gangrette et al., 2005). In addition, deletion of lp_2991, a repressor of the LTA glycosylation enzyme Gtca3, led to significant higher IL-10, IL-12p70 and TNF-α levels (Meijerink et al., 2010). In agreement, a D-Ala mutant in L. rhamnosus GG or complete removal of LTA in L. acidophilus NCFM led to strongly reduced pro-inflammatory responses (Segers and Lebeer, 2014). Effects on the epithelial barrier have not yet been investigated for these mutants.

6.8. Interaction with the Host – Immune Systems

In vitro as well as in vivo research has shown immune modulatory capacities for L. plantarum WCFS1. Co-culture of peripheral blood mononuclear cells (PBMCs) with L. plantarum NCIMB 8826 showed significant increases in different markers of activated T-cells (Dong et al., 2012). L. plantarum WCFS1 induces expression of different pro-inflammatory cytokines as well as the anti-inflammatory cytokine IL-10 by PBMCs (Larché et al., 2003; van Hemert et al., 2010; Dong et al., 2012). Although the concentration of induced IL-10 and IL-12 were relatively low and moderate compared to other L. plantarum strains (van Hemert et al., 2010). Co-culture of immature monocyte derived dendritic cells (DCs) with L. plantarum WCFS1 activated the DCs and induced expression of the cytokines IL-10, TNF-α and the Tp1 inducing cytokine IL-12p70 (Larché et al., 2003; Smelt et al., 2012; Remus et al., 2013). A cytokine profile with an increased IL-10/IL-12 ratio would be beneficial in an allergic and autoimmune disorder. However, one should wonder whether these subtle changes will lead to significant effect in vivo. Genes of L. plantarum WCFS1
involved in immunomodulation include an N-acetyl-glucosamine/galactosamine phosphotransferase system, the LamBDCA quorum sensing system, components of the plantaricin (bacteriocin) biosynthesis and transport pathway, and transcription regulator \textit{lp\_2991} (Meijerink \textit{et al.}, 2010; van Hemert \textit{et al.}, 2010). However, the function of these genes is quite different and hence it is likely that different mechanisms underlie the observed phenotypes. Moreover, no human data are available as the mutants are generated by genetic modification (GMO), precluding human trials. Non-GMO approaches as recently described for \textit{L. rhamnosus} GG and coupled to next generation sequencing may be used to overcome this and provide avenues for human trials to address cause-effect relations (Rasingkangas \textit{et al.}, 2014).

In healthy wild-type mice, \textit{L. plantarum} WCFS1 leads to an increase in the number of regulatory DCs and regulatory T cells in the spleen (Smelt \textit{et al.}, 2012). In the small intestine a decrease in the T$_{\text{H}1}$/T$_{\text{H}2}$ ratio was seen, whereas in the large intestine a more regulatory phenotype was induced (Smelt \textit{et al.}, 2013a; \textbf{Figure 6.2}). Some of these effects were dependent on the D-alanylation of teichoic acids, as the \textit{L. plantarum} WCFS1 induced immune changes were not observed when the D-alanylation negative mutant \textit{dltX-D} was used (Smelt \textit{et al.}, 2013b). Also a human cross-over study with healthy volunteers indicated establishment of immune tolerance (van Baarlen \textit{et al.}, 2009). The volunteers consumed \textit{L. plantarum} WCFS1 every half an hour for 6 hours and thereafter gene expression responses in the duodenal cells were investigated. Among the regulated genes were numerous genes involved in immune regulation. Although this extensive administration does not reflect a “real life” setting, it provided insightful biological data in healthy human (van Baarlen \textit{et al.}, 2009). Induction of a regulatory phenotype can dampen inflammatory conditions, for instance such as observed in UC. Indeed, in a murine TNBS-induced colitis model, administration of NCIMB 8826 led to a dose-dependent protection level in weak to moderate colitis (Foligné \textit{et al.}, 2006).

The effect of \textit{L. plantarum} WCFS1 on the healthy intestinal mucosa transcriptional response was assessed after a short 1 and 6 hour exposure in human volunteers. In a randomized, placebo controlled, cross-over study 15 healthy individuals were exposed to 1x10$^{11}$ cfu \textit{L. plantarum} WCFS1 after which duodenal samples were taken (Troost \textit{et al.}, 2008). A one hour exposure demonstrated an upregulation of genes involved in the complement pathway (Troost \textit{et al.}, 2008). At the same time, genes involved with lipid and fatty acid metabolism, and the major transcriptional regulators were downregulated. Initial contact between \textit{L. plantarum} WCFS1 seems to down-regulate the proliferation and there is a primary immune response induced to the microbial presence (Troost \textit{et al.}, 2008). In agreement, in a comparable set-up using \textit{L. rhamnosus} GG the mucosal response was characterized by induction of T$_{\text{H}1}$ development (van Baarlen \textit{et al.}, 2011). A prolonged exposure of 6 hours is associated with upregulation of lipid/fatty acid metabolism and oxidative stress. In addition, genes involved in the antigen presentation are upregulated (Troost \textit{et al.}, 2008). These data suggest that the mucosa is initially alarmed, but after six hours return to their non-inflammatory proliferative state (Troost \textit{et al.}, 2008). No inflammatory signals were expressed both time-points.

A decrease in the T$_{\text{H}1}$/T$_{\text{H}2}$ ratio might have potential for T$_{\text{H}2}$-skewed allergic disease and \textit{L. plantarum} NCIMB 8826 dampened the response of DCs derived from house dust mite allergic individuals stimulated
with the dust mite allergen Der-p1 (Pochard et al., 2005). This is in contrast with a mice study using a well-established pathogen-free mouse peanut sensitization model (Meijerink et al., 2012). Administration of WCFS1 increased the peanut-extract (PE) specific IgG1, IgG2, IgE and mouse mast cell protease-1 (mMCP-1) levels in serum significantly. In addition, when splenocytes were re-stimulated with PE, there was an increase in the production of the unwanted T_{H2} associated cytokine IL-4 (Meijerink et al., 2012). As these allergy studies report contradiction results, it becomes evident that these afflictions are driven by many complex interactions.

### 6.9. Conclusions and future directions

According to the European Food Safety Authority (EFSA), not a single product has yet been studied well enough to allow a health claim on the package for the general (healthy) population. Nevertheless, there is an increasing scientific attention for probiotics, recently redefined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hill et al., 2014). *L. plantarum* WCFS1 is unique in the large amount of molecular work performed with this strain. However, no controlled trials have been reported that address its potential probiotic functions, except for a study addressing its effect on autistic spectrum disorders in children (Parracho et al., 2010). While this study showed an increased level of LAB following consumption and thus adds to the safe use of *L. plantarum* WCFS1, no specific impact could be determined and no follow up of this work have been reported. This is important as for EFSA trials should target the normal population and to be credible should consist of double blind placebo controlled randomized trials (Rijkers et al., 2011).

During the last ten years a large number of mutants of *L. plantarum* WCFS1 have been made by scientists to investigate effects of single or multiple genes (Table 1). Most of these mutants have been studied in only one or a few screening assays and it would be interesting to investigate these mutants in other assays with a focus on host-microbe interactions. As indicated above, non-GMO mutants can now be generated and characterized much faster than before using high throughput sequencing (Rasingankas et al., 2014). By using these and other non-GMO mutants in human studies, further insight into mechanisms of host-microbe interaction could be obtained.

Whether *L. plantarum* WCFS1 can be developed into a successful probiotic remains to be determined and clinical trials showing a health benefit will be necessary. Based on the previous studies, different areas seem to have potential, like treatment of people with elevated cholesterol levels, individuals with increased epithelial permeability, and diseases where stimulation of T_{H1} and/or regulatory T cells is beneficial. In addition the effect of *L. plantarum* WCFS1 on autism should be further explored. Although the study of Parracho et al. (2010) was significantly limited by the study design, it paves the way for future clinical trials.
in humans using *L. plantarum* WCFS1 as a probiotic. Not only to explore the effect of probiotics on the gut-brain axis, but also on a variety of other health parameters.

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6.11. References


Chapter 6


Chapter 6


Lactobacillus plantarum WCFS1: 12 Years After The Genome


