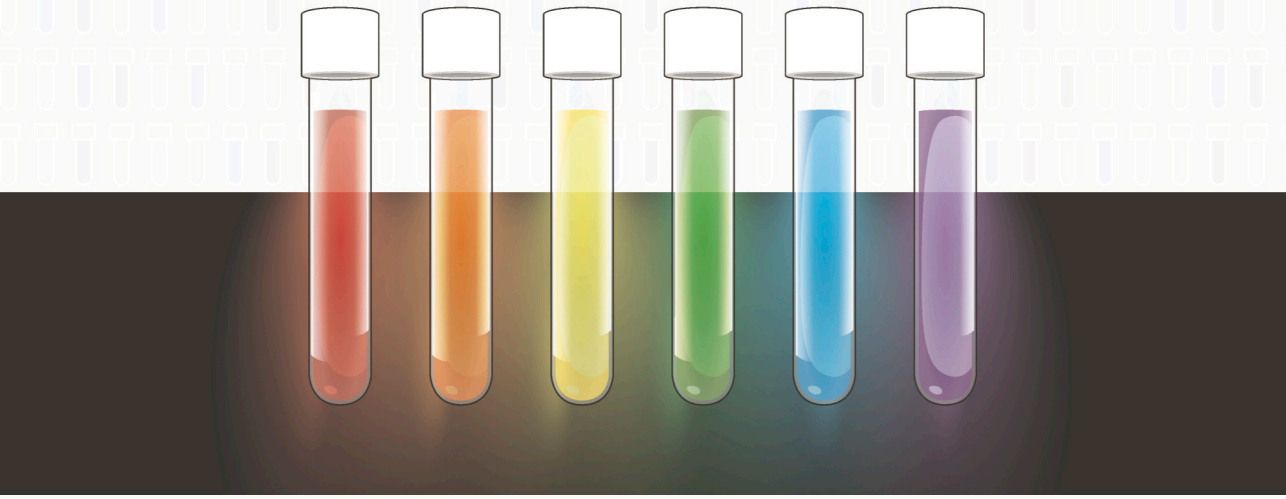
Stress responses and digestive tract robustness of *Lactobacillus plantarum*



Hermien van Bokhorst-van de Veen

Propositions

1. *In vitro* gastrointestinal tract assays are able to predict *in vivo* digestive tract persistence of lactic acid bacteria. (This thesis)

2. Relatively small variations in fermentation conditions of lactic acid bacteria lead to large differences in their capacity to survive gastrointestinal tract conditions. (This thesis)

3. To increase safety and reduce bacterial false positives after analysis of human blood samples, venipuncture in the elbow pit should be reconsidered since it contains a relatively high abundance of bacterial species, including common pathogens. (Grice *et al*, Science, 2009 324:1190-2, Conlan *et al*, PLoS ONE 7(10): e47075)

4. The modification of consumed long-chain polyunsaturated fatty acids from breast milk by babies contributes to the intelligence quotient, thereby demonstrating the importance of breast milk consumption by babies. (Caspi *et al*, PNAS, 2007 104:18860-5)

5. Although scientists believe that their work has nothing to do with faith, they can only have confidence that future experiments will give the same results as in the past when they are performed under identical conditions.

6. Street dance and solo dancing are good alternatives for ballroom dancers who do not have a partner.

Propositions belonging to the thesis entitled:

"Stress responses and digestive tract robustness of *Lactobacillus plantarum*"

Hermien van Bokhorst-van de Veen

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Stress responses and digestive tract robustness of *Lactobacillus plantarum*

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Stress responses and digestive tract robustness of *Lactobacillus plantarum*

Hermien van Bokhorst-van de Veen

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Voor mijn ouders

Summary

Lactobacillus plantarum is one of the most versatile lactic acid bacteria that can successfully inhabit a variety of environmental niches. It is a common inhabitant of the human and animal gastrointestinal (GI) tract and it is used as starter culture in various fermentation processes for different food raw-materials, including milk, fruits, vegetables, and meat. Moreover, *L. plantarum* is marketed as a health-promoting culture, i.e. a probiotic. In these different environments and processes the bacteria encounter stress conditions, such as heat, cold, acid, salt, and oxygen stress. Since starter cultures and probiotics require metabolic activity to contribute to the taste and texture of the fermented products, and/or viability to exert their *in situ* beneficial effect on the consumer, it is important to understand and improve the gene-regulatory adaptation that sustains their function and viability under these challenging conditions. Nowadays, genomic approaches are available that enable the global, genome-wide analysis of stress responses in lactic acid bacteria. The work presented in this thesis employs such tools and also developed some novel strategies to understand stress responses in *L. plantarum*.

During wine fermentation, *L. plantarum* is exposed to ethanol and global transcriptome profiling demonstrated the gene expression adaptation of this microorganism upon short- and long-term exposure to sublethal levels of this solvent. The results suggested that the ethanol induced activation of the CtsR-related stress regulon contributes to its adaptation to ethanol exposure which also provides cross-protection against heat stress. Transcriptome analyses under different growth conditions of gene deletion derivatives of the *L. plantarum* WCFS1 strain that lack the genes encoding the stress response regulators *ctsR* and/or *hrcA*, enabled the refinement of the gene regulation repertoire that is controlled by these central regulators of stress responses in this species. Notably, the deletion of both stress-regulators, elicited transcriptome changes that affected a large variety of additional gene-functions in a temperature-dependent manner, which prominently included genes related to cell-envelope remodelling.

Culturing of *L. plantarum* WCFS1 under different fermentation conditions led to large differences in GI-tract survival and robustness, which was addressed using a simple *in vitro* survival assay. Enhanced GI-tract survival and robustness could be associated with low salt and low pH conditions during the fermentations. The transcriptomes obtained for each of the fermentation conditions employed, were correlated with the observed GI-tract survival rates, enabling the identification of candidate genes involved in the robustness phenotype. They included a transcription regulator involved in capsular polysaccharide remodelling (Lp_1669), a penicillin-binding protein (Pbp2A) involved in peptidoglycan biosynthesis, and a Na+/H+ antiporter (NapA3). A role of these candidate genes in actual survival in the GI-tract assay could be confirmed by mutation analysis, further confirming their contribution to GI-tract stress robustness in *L. plantarum*.

This thesis also describes the use of a novel, next-generation sequencing-based method, for the assessment of the *in vivo* GI-tract persistence of different *L. plantarum* strains that were administered to healthy human volunteers in specifically designed strain-mixtures. A remarkable consistency of

the strain-specific *in vivo* persistence curves was observed when comparing data obtained from different volunteers. Moreover, a striking congruency was observed between the strain-specific *in vivo* persistence curves and the predicted GI-tract survival based on the simple *in vitro* assay. Finally, evolutionary adaptation of *L. plantarum* WCFS1 to the murine GI-tract was studied by extended exposure of the strain to the mice digestive tract through consecutive rounds of (re)feeding of the longest persisting bacterial colonies. Re-sequencing of the genomes of more persistent derivatives of the original strain, and the evaluation of the genomic modifications identified, implied that genes encoding cell envelope-associated functions and energy metabolism play an important role in the determination of GI-tract persistence in *L. plantarum*.

The results described in this thesis strive to obtain an improved understanding of the gene-regulatory adaptations of *L. plantarum* that allow its survival under stress conditions, including those associated with residence in the gastrointestinal tract of animals and humans, with the intention to exploit such understanding to rationally improve the robustness of these bacteria.

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General Introduction

Post-genomics tools for the identification of stress responses in lactic acid bacteria

Hermien van Bokhorst-van de Veen

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Introduction

The natural habitat of lactic acid bacteria (LAB) varies from plants, to animals and humans, including the oral, genital, and gastrointestinal tract (GI-tract). LABs have long been thought to be strictly fermentative and convert sugar to lactic acid as one of the main end-point metabolites. However, more recently it was shown that the addition of heme to growth media enables aerobic respiration in lactococcal cultures, supporting increased biomass yields without acidification and enhanced stationary phase survival [1]. Analogously, a recent survey confirmed that heme and/or menaquinone could also stimulate respiration in a subset of *Lactobacillus* species [2]. Nevertheless, LAB-containing fermented food and beverages, including fruits, vegetables, cereal grains, meat, and milk [3,4], have been used for centuries, as the lactic acid produced acts as a preservative due to the pH lowering effect. Moreover, these bacteria greatly contribute to the flavor and texture of the fermentation end-products [5]. More recently, specific strains of *Lactobacillus* have been associated with health-promoting effects in the consumers, including a suppressive effect of *L. johnsonii* [6] and *L. acidophilus* [7] on *Helicobacter pylori* infection, as well as alleviation of lactose intolerance [8] and inflammatory bowel disease [9]. Although exact numbers depend on the strain and type of application, it is recommended that probiotic products contain at least 10^7 microorganisms per g or ml [10]. Moreover, by definition, appropriate amounts of probiotics are required to be alive during consumption to confer a health benefit on the host [11]. In addition, it is desired that they reach their target site (usually the intestine) alive. Hence, an important prerequisite for the industrial application of these starter and probiotic cultures is their persistence towards the stresses encountered in the industrial pipeline, ranging from temperature, osmotic, oxidative, and/or solvent stress during industrial fermentation to industrial processing stresses such as freeze-drying (Fig. 1). For instance, during wine fermentation, lactic acid bacteria are responsible for the de-acidification of the product via malolactic fermentation. In addition, malolactic fermentation enhances microbial stability and improves the aroma and flavour attributes of the wine. In this fermentative product, the stresses encountered by the lactic acid bacteria are ethanol, low pH, sulfur dioxide, low temperature, fatty acids, and decreased nutrient content [12,13]. For probiotics the plethora of stresses encountered is even greater, as they require survival during shelf-life of the fermentation end-product. Subsequently, more stresses are met during residence in and travel through the different parts of the host's GI-tract, such as the gastric acidity in the stomach, bile salt and digestive enzyme challenges in the duodenum, a relatively high osmolarity in the colon, as well as stress conditions associated with oxygen gradients that are steep at the mucosal surface, while the colonic lumen is virtually anoxic [14].

During the last decade, genome sequencing of LAB and the application of functional genomics has drastically enhanced our insight in this group of industrially important bacteria, their overall molecular make-up, metabolic capacities, evolutionary relatedness, and molecular adaptation to environmental conditions including those associated with industrial applications and/or their residence in the mammalian GI-tract. The fact that starter cultures and probiotics require either metabolic activity to contribute to the taste and texture of the fermentation end-products or vitality to exert their *in situ* beneficial effect on the consumer, respectively, justifies the increasing interest in the molecular mechanisms behind the observed stress responses in these bacteria (Fig. 1). This

chapter describes the state-of-the-art tools available in the post-genomic era to identify specific LAB stress response.

LAB genomics

Following the first publication of the genome sequence of an autonomously growing microbe, *Heamophilus influenzae*, in 1995 [15] the field of genomics has initially concentrated strongly on the determination of genome sequences of pathogenic bacteria and several model organisms that were traditionally used for molecular research. Genome sequencing of microbes of biotechnological importance, including the LAB, lagged behind, but has caught-up in the last 10 years. A landmark study in the field of LAB genomics is the release of the complete genome sequence of *Lactococcus lactis* spp. *lactis* strain IL1403 [16]. This 2.4 Mbp genome was annotated to encode 2310 proteins and its comparison to the microbial genomes available at that time confirmed the relatedness of *Lactococcus* to the streptococcal genus, and revealed genes predicted to be involved in fermentative and respiratory pathways. Shortly after this first LAB genome, the genome sequences of two lactobacilli, *L. plantarum* [17] and *L. johnsonii* [18], were determined. Their initial comparison already highlighted the relatively high diversity encountered within the genus *Lactobacillus* [19], while the determination of the genome sequence of *L. acidophilus* [20] underlined the higher degree of similarity within subgroups of the *Lactobacillus* genus, e.g. the "acidophilus complex" or "acidophilus group". Since these initial LAB genome sequence releases more than 100 genome

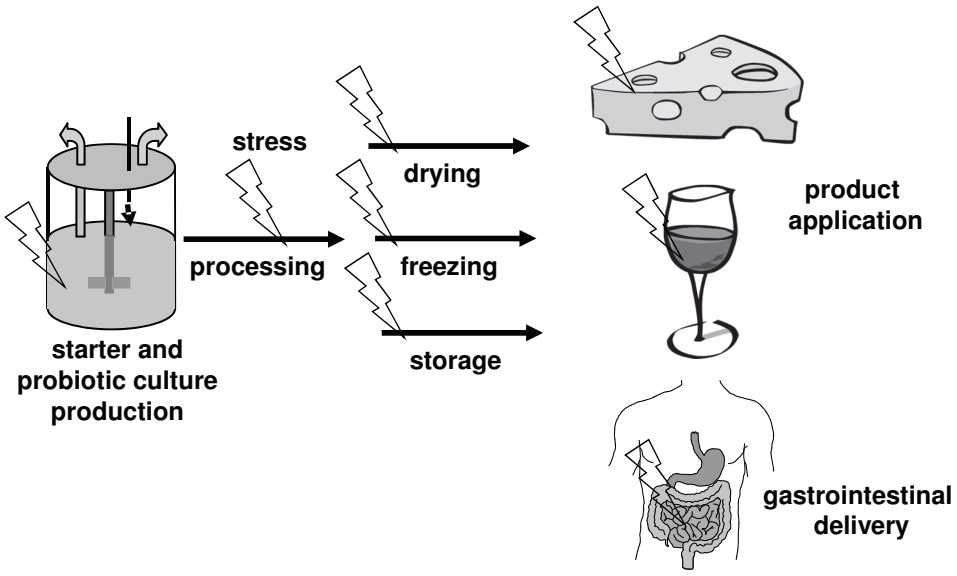

Fig. 1. Schematic representation of the various stresses encountered by starter and probiotic cultures in the industrial pipeline.

sequences of industrially important LAB have appeared in the public domain [21], whereas many genome-sequencing projects are currently ongoing. Thereby genomic research is contributing enormously to our knowledge of the genetics of this group of organisms. Nevertheless, we should not ignore that many industrially relevant traits of LAB are encoded on mobile genetic elements, e.g., plasmids and/or transposons, rather than on the chromosome [22-24], underlining that sequence determination of these genetic elements should be included to complement the function-blueprint prediction of an organism.

The wealth of genomic sequence information of LAB has stimulated a variety of *in silico* analyses to compare the available LAB genomes. A landmark study in this field is associated with the release of 9 novel LAB genome sequences of various species, and their comprehensive comparison that included the definition of so-called LaCOGs that represent a refinement of the existing categories of orthologous genes (COGs) dedicated to the LAB genomes. LaCOG distribution analyses were subsequently used to reconstruct an initial view of the evolutionary relationship between LAB [25]. The same LaCOG and COG analyses were also employed to identify a set of LAB genes that are associated to various stress responses in these bacteria (Table 1). This overview shows that in most LAB HrcA is involved in control of heat shock protein expression (all except *Oenococcus oeni*; Table 1). Notably, for *O. oeni* it has been proposed that heat-shock and general stress response may be controlled via a complex regulatory network encompassing various regulatory proteins [26]. In agreement with HrcA conservation, the canonical heat shock proteins that are commonly under HrcA control and perform chaperonin like functions (GroELS, DnaKJ, GrpE), are universally conserved, while the majority of LAB species also encode the additional chaperones HtpX, and HSP20 (IbpA). The involvement of CtsR in regulation of class III stress proteins, including the Clp proteases and related functions, is predicted for all LAB genomes except *Leuconostoc mesenteroides*, and the lactobacilli belonging to the "acidophilus complex". Nevertheless, the corresponding Clp proteases (COG associated gene names: ClpA, X, Q, and P) appear to be universally present in these LAB genomes, although ClpYQ (also designated HslUV) presence varies. The conservation of the oxidative stress components involved in thioredoxin metabolism (TrxA and B) supports an important role for this module in protection against oxidative stress, which has been recently been experimentally confirmed in *L. plantarum* [27]. All LAB genomes appear to encode a virtually complete machinery associated with DNA damage stress responses, including the pathways for homologous recombination and double-strand break repair (RecABDFJNOR, RuvAB, and Ssb) and its homology independent facilitator complex (GyrAB and TopA), as well as the global genome repair pathway involved in base and nucleotide excision repair (Mfd, UvrABCD, and Xth), although the endonuclease IV (Nfo) that plays a role in base excision repair appears to be absent in many species. Notably, the canonical DNA mismatch repair function encoded by *mutS* and *mutL* appears absent from *O. oeni*, while this species as well as *Streptococcus thermophilus* also lack a *recQ*-like gene. The lack of *recQ* in *S. thermophilus* has been proposed to partially explain the genome decay observed in this species, which is characterized by a high frequency of pseudo-genes and function loss and is likely due to its extensive adaptation to the benign and nutrient-rich environment encountered during growth in milk [28,29]. These examples illustrate how genome sequencing and comparative genomics may accelerate our understanding of conserved and differential mechanisms

underlying LAB stress tolerance and its control. In the section below, this strategy will be further refined, to illustrate how genome diversity among strains of a species might be exploited to identify chromosomally encoded genes that are involved in functional properties of interest, including stress-tolerance phenotypes.

Species diversity mining to elucidate genotype-phenotype correlations

Although many LAB species are currently represented by a genome sequence of an exemplary isolate, it is clear that many phenotypic differences exist among strains of a certain species. This phenotypic variation among strains has a major impact on their performance in fermentation applications, and has been an important source of product diversification and innovation in the past decades. As an example, the application of different strains of *Lactococcus lactis* in cheese production can impact dramatically on the flavor and texture characteristics of the end-product (for a review see: [5]), which has stimulated the development of high-throughput, miniaturized cheese manufacturing procedures that enable product-related functionality screening of individual strains to accelerate product diversification [30]. This phenotypic variation among strains is at least partially due to their diversity in gene-content. Several approaches are available to determine the genomic diversity among strains. The comparative genome hybridization (CGH) approach employs one-directional comparison of gene-content profiles per strain using genome-wide microarrays that are designed on basis of the genome of a single strain. This approach enables the construction of high-resolution genome-wide presence-absence patterns for each of the strains that is analyzed. Many array platforms that are currently used for transcriptome analyses contain several probes per annotated gene and are generally suitable for CGH. However, even higher resolution can be achieved by using so-called tiling-arrays that contain probes that cover the entire genome sequence through minimal tiling probe-design. CGH has been applied to determine the genomic diversity of several LAB, including *L. plantarum* [31,32], *L. sakei* [33], and *O. oeni* [34]. The gene-specific diversity database obtained in this way can readily be applied to identify the gene(s) responsible for specific phenotypic traits that are variable among the strains analyzed by gene-trait matching (GTM). This approach is exemplified by the diversity-based identification of the mannose specific adhesin (Msa) of *L. plantarum*, which is proposed to be involved in its probiotic functionality related to reducing the severity of infection of enterotoxic *Escherichia coli* in humans by competitive exclusion. Subsequently, the role of Msa in mannose specific adherence proposed by GTM could be confirmed by *msa* mutation analysis [35]. Intriguingly, transcriptome analyses of pig intestinal mucosa revealed that mucosal interaction with the *msa* mutant of *L. plantarum* fails to elicit the expression of the host bacteriocidal pancreatitis-associated protein, in amounts comparable to those observed for the wild-type strain, suggesting *msa* dependent interaction with the host innate immune system [36]. Moreover, the sequence of the *msa* gene in different *L. plantarum* strains appeared to encode a protein with strain-specific domain composition, which can be associated with strain-specific quantitative mannose adherence capacities [37]. This work underlines the discovery power of the GTM approach for the elucidation of genetic determinants underlying specific phenotypes.

Table 1. Overview of stress associated genes and cluster orthologous genes (COG; sorted by their degree of conservation among LAB species) predicted in 12 LAB genomes, focusing on variable COG categories.

COG annotation	Cold shock protein	Rad3-related DNA helicase	ATPase of helicase subunit of the Holliday junction resolvase	Superfamily II DNA/RNA helicase required for DNA uptake	Nucleotidyltransferase/DNA polymerase involved in DNA repair	DNA gyrase	DNA structure-specific ATPase, suppression of recombination	Homologous recombination, DNA repair[1]	Holliday junction resolvasome	ATPase involved in DNA repair	Single-stranded DNA-binding protein	Topoisomerase IA	Excinuclease ATPase subunit	excision repair complex	Integrase	Exonuclease VII	Exonuclease III	Transcription-repair coupling factor (superfamily II helicase)	NUDIX family hydrolase
corresponding COG	COG1278	COG1199	COG2256	COG4098	COG0389	#	COG1193	#	#	#	COG0629	COG0550	COG0178	#	COG0582	#	COG0708	COG1197	COG0494
COG gene	*cspC*	*dinG*	-	*comEA*	*dinP*	*gyrA, B*	*mutS2*	*recA, B, D, F, J, N, O, R*	*ruvA, B*	*sbcC, D*	*ssb*	*top A*	*uvrA*	*uvrB, C, D*	*xerC*	*xseA, B*	*xthA*	*mfd*	*mutT*
COG Functional group	K	KL	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	LK	LR
Number of proteins	24	16	20	15	28	24	16	12	12	14	23	16	20	12	29	12	14	12	66
Number of organisms	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
***L. casei* ATCC 334**	3	2	2	1	2	2	1	1	1	1	2	1	3	1	2	1	1	1	6
***L. bulgaricus* ATCC BAA-365**	2	1	1	2	2	2	2	1	1	1	1	2	1	1	2	1	1	1	8
***L. gasseri* ATCC 33323**	1	1	3	1	2	2	2	1	1	1	1	1	1	1	3	1	2	1	5
***L. johnsonii* NC533**	1	1	2	1	1	2	2	1	1	1	1	1	1	1	3	1	2	1	4
***O. oeni* PSU-1**	1	1	2	2	1	2	1	1	1	1	1	3	2	1	2	1	1	1	3
Le. mesenteroides	1	1	1	1	4	2	1	1	1	1	3	2	2	1	2	1	1	1	3
***P. pentosaceus* ATCC 25745**	2	2	1	1	2	2	1	1	1	1	3	1	1	1	4	1	1	1	2
***L. plantarum* WCFS1**	3	2	3	1	2	2	1	1	1	2	1	1	3	1	2	1	1	1	8
***L. brevis* ATCC 367**	1	2	2	1	3	2	1	1	1	2	1	1	3	1	2	1	1	1	4
***La. lactis* ssp. *cremoris* SK-11**	5	1	1	2	3	2	1	1	1	1	3	1	1	1	4	1	1	1	9
***La. lactis ssp. lactis* IL1403**	2	1	1	1	2	2	2	1	1	1	4	1	1	1	2	1	1	1	8
***S. thermophilus* LMD-9w**	2	1	1	1	4	2	1	1	1	1	2	1	1	1	1	1	1	1	6

3	1	1	1	2	4	1	1	1	1		1	1	2		1								
3	1	1	1	2	3	2	1	1	1	1		1	3		1								
3	1	1	1	2	3	1	1	1	1	1		1	2		1	1							
3	1	2	1	2	3	1	1	1	1	2	1	1	1	2	1	1	1						
4	1	2	1	2	4	1	1	1	1	2	3	1	1		1	1	1						
4	1	1	1	2	3	2	1	1	2	2	1		1		1	1	1	2	1				
5	1	2	1	3	5	2	1	1	1	1	1			1	1			1					
3	1	2	1	2	3	1					1		1					2					
5	1	1	1	2	2	1	1	1	1	1	1	1	2		1								
4	1	1	1	2	2	2	1	1	1	1	1	1	2		1	3							
5	2	2	1	2	4	1	1	1	1	2	2	1	1	1		1	1	3	1				
12	12	12	12	12	12	12	11	11	11	10	10	9	8	8	7	7	6	6	4	4	3	2	
47	13	17	12	25	38	16	11	11	12	14	13	9	8	15	11	7	11	10	12	4	5	3	3
O	O	O	O	O	OC	OU	K	L	L	L	O	L	K	L	O	L	O	KL	KL	L	L	L	L
dpA	*dpX*	*degQ*	*groEL,S, dnaJ, K, grpE*	*trxB*	*trxA*	*dpP*	*hrcA*	*mutS, L*	*radC*	*recQ*	*ibpA*	*nth*	*ctsR*	*xerD*	*htpX*	*mutY*	*hslU, V*	*hepA*	*SSL2*	*pinR*	*nfo*	*rdlB*	*recT*	*rus*	
COG0542	COG1219	COG0265	#	COG0492	COG0526	COG0740	COG1420	#	COG2003	COG0514	COG0071	COG0177	COG4463	COG4974	COG0501	COG1194	#	COG0553	COG1061	COG1961	COG0648	COG3077	COG3723	COG4570	
ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	ATP-dependent protease Clp, ATPase subunit	Serine protease (heat-shock protein)	Chaperonin functions	Thioredoxin reductase	Thiol-disulfide isomerase or thioredoxin	Protease subunit of ATP-dependent Clp protease	Transcriptional regulator of heat shock gene	DNA mismatch repair enzyme, ATPase	DNA repair proteins	Superfamily II DNA helicase	Molecular chaperone (HSP20 family)	Predicted EndoIII-related endonuclease	Transcriptional repressor of class III stress genes	Site-specific recombinase XerD	Zn-dependent protease with chaperone function	A/G-specific DNA glycosylase	ATP-dependent protease HslVU (ClpYQ)	Superfamily II DNA/RNA helicase, SNF2 family	DNA or RNA helicase of superfamily II	Site-specific recombinase, DNA invertase Pin homolog	Endonuclease IV	DNA-damage-inducible protein J	Recombinational DNA repair protein, RecT	Holliday junction resolvase	

[1] two copies of *recA* and *recD* are predicted for *Lactobacillus casei*
Variation in presence vs absence per genome as well as variation in predicted number of gene copies per genome are provided. Adapted from [25].

In addition, strain diversity can nowadays also be addressed by the determination of multiple genome sequences of individual isolates of a particular species. Especially the emergence of the highly effective next-generation sequencing technologies [38,39] facilitates this approach, which is illustrated by the appearance of multiple genome sequences of specific LAB species in the public domain, including the *Lactobacillus* species *L. plantarum*, *L. casei*, *L. delbrueckii*, *L. reuteri*, and *L. rhamnosus*. This trend is bound to accelerate gene-function assignment, including the identification of genes involved in relevant phenotypes. A clear example of such novel gene-function assignment potential is provided by the recent completion of the genome sequence of the best-documented probiotic strain, *L. rhamnosus* GG [40], and its comparison to the closely related strain LC705. The two *L. rhamnosus* genomes (both approximately 3.0 Mbp) are very similar and syntenous, but also contain strain specific genomic islands. One of the GG-specific genome islands encodes a pilin-like surface structure that is important in adherence to intestinal mucus and is proposed to aid persistence of *L. rhamnosus* GG *in vivo* in the intestine [40]. Analogously, comparative genome sequence analysis of two or more LAB strains of the same species that display a high difference in survival capacity under specific stress conditions might enable the identification of the genetic determinants underlying this phenotypic difference.

However, despite the successes of GTM approaches described above, it is also clear that many phenotypes do not depend on the presence or absence of specific genes, but are predominantly determined by the difference in expression levels of conserved genes. A clear illustration of this is provided by the very high diversity in gene expression-regulation phenotypes observed in individual strains of *Lactococcus lactis*, which was based on the comparative analysis of the activity levels of 5 enzymes in two different growth media. The enzymes analyzed are considered relevant for their flavor forming capacities during cheese-making, illustrating the potential impact of this regulatory diversity on eventual product properties [41]. Moreover, it is likely that the majority of stress-tolerance genes are conserved among strains of a particular species and that strain specific survival capacities depend on their relative levels of expression rather than their presence or absence. Therefore, to unravel the contribution of conserved genes in stress-tolerance phenotypes, comparative genomics should be performed at the functional (e.g., transcriptome, proteome) level.

Functional-genomics approaches to unravel LAB stress responses; *in vitro* approaches to identify robustness genes in LAB

The intrinsic underrepresentation of conserved stress factors identified utilizing different LAB strains and GTM approaches described above can be complemented by comparing transcriptome profiles derived from an individual strain grown under normal and (a) stress condition(s). To this end, DNA microarray technology has been exploited widely to identify several of the (conserved) genetic factors regulated during stress imposed on LAB during industrial fermentation (e.g. lactate production), processing (e.g. hydrostatic pressure) and storage (e.g. high osmolarity / low water activity) or after consumption by the human host (low pH encountered in the stomach and pancreatic enzyme and bile associated stress in the duodenum) (Fig. 1). For example, a transcriptome profiling approach revealed the effect of lactic acid stress in *L. plantarum* strain WCFS1 [42]. Strikingly, 3

cell surface complex (*csc*) operons [43] were found to be among the highly induced gene clusters in response to lactic acid stress, suggesting the corresponding proteins are abundantly present on the cell surface. Indeed, cells pre-exposed to lactic acid displayed striking morphological changes, including a rough morphology, as compared to the smooth appearance of unstressed control cells. The observed morphology changes might be associated with the observed lactic acid tolerance [42]. Unfortunately, a subsequent dedicated mutagenesis approach in which these *csc* gene clusters were deleted could not confirm the involvement of these cell surface proteins in lactate stress tolerance, as the mutants displayed tolerance levels comparable to the wild-type [44], which may be due to genetic compensation as is suggested by the high degree of redundancy of the *csc* clusters in the *L. plantarum* genome [43]. Similarly, transcriptome analysis in *L. sanfranciscensis* revealed the high pressure-regulated gene expression of genes of several (conserved) functional classes, including protein and fatty acid biosynthesis, energy metabolism, as well as transport and cell envelope proteins [45].

Transcriptome profiling of *L. reuteri* ATCC55730 after exposure to acid revealed the induction of several genes with potential functions in membrane fluidity regulation or peptidoglycan biosynthesis and organization, including a putative phosphatidyl glycerophosphatase and a putative esterase gene, belonging to the family of penicillin-binding proteins [46]. A mutant lacking the latter gene displayed a gastric juice and bile sensitivity phenotype [46], establishing a definite role for the penicillin-binding protein of this LAB in its robustness under GI conditions. Similarly, DNA microarray experiments using bile exposed *L. acidophilus* NCFM [47] or *L. plantarum* WCFS1 [48] revealed induction of several genes potentially involved in cell envelop and surface protein biosynthesis. These data corroborate earlier observations made when the bile response in *L. plantarum* WCFS1 was investigated utilizing a genetic screen [49]. Furthermore, *L. acidophilus* gene disruption mutants in a cell-division protein (*cdpA*) and surface layer protein A (*slpA*) displayed an increased bile resistant, while their osmotolerance was negatively affected [50,51], further highlighting the importance of subtle modifications in cell envelop composition on the robustness of LAB to persist in different stress conditions relevant for industrial processing and GI-tract survival. The DNA microarray studies in *L. plantarum* WCFS1 also revealed the induction of the *dlt* operon during bile stress, suggesting the importance of D-Ala decoration of wall-teichoic acid and/or lipo-teichoic acid for cell envelope integrity and robustness of this LAB [48]. Notably, an *L. rhamnosus dltD* mutant displayed a reduced survival capacity in simulated gastric juice [52], whereas a similar approach in *L. reuteri* revealed a pronounced effect on *in vitro* growth at low pH [53].

Although the DNA microarray analyses described above performed for *L. acidophilus* [47] and *L. reuteri* [54] also indicated that genes involved in their respective exopolysaccharide (EPS) production are regulated upon bile exposure, no phenotypic analysis of dedicated mutants have been reported to date [55]. To this end, detailed physiological characterization of recently construction *L. rhamnosus* GG showed that EPS molecules need to be downregulated for optimal adherence to intestinal epithelial cells [56] and they seem to be required for protection of *L. rhamnosus* GG against the antimicrobial factors of the lower regions of the GI-tract [57]. In addition to *L.*

rhamnosus GG, *L. plantarum* WCFS1 mutants with reduced production of (specific forms of) capsular polysaccharides showed increased TLR-2 activation [58].

Overall, these studies investigating alterations in transcriptome profiles under industrially relevant stress conditions have led to substantial insight into the candidate factors important for bacterial robustness when encountering these stresses. Importantly, several of these studies have been followed by dedicated mutagenesis approaches and subsequent reassessment of stress robustness, establishing a definite role for the robustness factors in the industrial and GI performance of LAB. However, most data has been obtained in simplified laboratory systems that fail to accurately assess the physiochemical complexity encountered during industrial fermentation and processing [3,59] or the multitude of stresses and bacterial competition of the intestinal environment [60]. These issues have been addressed by *in vivo* approaches that are discussed below.

(R-)IVET

In vivo expression technology (IVET) and its resolvase-based variant (R-IVET) are powerful methodologies that allow the genome-wide identification of *in vivo* induced (*ivi*) promoters and their corresponding genes utilizing a promoter trapping system [61,62]. By applying IVET to *L. sakei* 23K, 15 genetic loci could be identified which display increased expression levels during raw-sausage fermentation. These *in carne* induced genes included several genes which are likely to contribute to known stress-related functions, as well as a gene involved in acquisition of ammonia from amino acids and several genes encoding unknown functions. Subsequently, mutants in the *ivi* genes encoding an L-asparaginase, a hypothetical metallo-β-lactamase, and a hypothetical membrane protein displayed a hampered *in carne* performance, establishing a definite role for these proteins during raw-meat fermentation [3]. Similarly, Bachmann *et al*. developed an optimized R-IVET system that enables double-positive selection of responding clones by the implementation of a MelA and a luciferase-based promoter probe system into the R-IVET vector [4]. Following the initial validation of this system by the identification of genes specifically induced in minimal media as compared to rich laboratory media [4], this system was applied to identify genes that are induced specifically during cheese manufacturing [63]. Subsequent luciferase activity profile analysis of individual R-IVET clones in a micro-cheese model system [30] enabled the real-time *in situ* assessment of promoter strength, generating temporal expression patterns for the associated genes during cheese ripening [63].

Besides the utilization of (R)-IVET to study fermentation processes *in situ*, this technique was also exploited for the identification of 72 *L. plantarum ivi* genes in the mouse GI-tract [64]. Nine *ivi* genes encode sugar-related functions, including several sugar PTS transport systems. Another nine genes appear to be involved in acquisition and synthesis of amino acids, nucleotides, cofactors and vitamins, indicating their limited availability in the GI-tract. Furthermore, surface adaptations were suggested by the *in vivo* induction of four predicted extracellular proteins, while the *in situ*

induction of several stress-related genes reflects the harsh conditions *L. plantarum* encounters in the GI-tract. [64]. Importantly, a dedicated mutagenesis approach underlined the critical contribution of *lp_2940*, encoding a protein of unknown function predicted to be covalently attached to the cell wall, and *copA*, encoding a copper transporting ATPase, to murine gut persistence of *L. plantarum* WCFS1 [65]. Notably, a similar strategy to identify *ivi* genes in *L. reuteri* revealed 3 genes [59], including a gene encoding a conserved protein sharing homology to the *L. plantarum lp_2718* gene-product [59,64].

In situ transcriptome profiling

In situ transcriptome profiling during fermentation, application, or intestinal residence is a complimentary approach to reveal gene expression patterns elicited by the physico-chemical stress conditions encountered during these complex processes. Subsequently, this information can be harnessed for the production and/or selection of more robust strains or cultures on basis of stress-response mimicking expression patterns of these candidate tolerance or robustness factors. Recently, this strategy was used to unravel the adaptive behavior of *S. thermophilus* during the late stages of milk fermentation, revealing strong regulation in sugar metabolism pathways. Moreover, the induction of nitrogen metabolism was eminent, particularly in the transport and biosynthetic pathways for sulfur-containing amino acids [66]. Although insightful, this study ignores the fact that *S. thermophilus* is naturally associated with *L. delbrueckii* subsp. *bulgaricus* during industrial yoghurt production. Hence, a follow-up study from the same research group investigated *S. thermophilus* gene and protein expression profiles in milk whilst being co-cultured with *L. delbrueckii*. Specific co-culture regulation could be established for 77 genes, including several genes encoding functions in nitrogen metabolism, reiterating the importance of a prompt response towards the limited availability of (specific) amino acids during milk fermentation. Moreover, the expression of nearly all genes predicted to be involved in iron transport were downregulated, whereas that of iron-chelating *dpr* and that of the *fur* regulator were induced, suggesting a reduction in the intracellular iron concentration, likely in response to H_2O_2 production by *L. delbrueckii* [67].

Due to recent technical advances, the isolation of high-quality bacterial RNA derived from intestinal samples nowadays is a routine laboratory procedure [68], allowing *in situ* transcriptome approaches to monitor changes in bacterial gene expression in the GI-tract. For example, transcriptomes of *L. plantarum* were obtained from samples derived from the caeca of mono-associated mice that were fed differential diets (either western-style [high fat, low fiber] or standard chow [low fat, high fiber]) [69], and in intestinal biopsies removed from patients diagnosed to have colon cancer who volunteered to participate in a probiotic trial prior to surgery [70]. Comparative analyses of these human-derived transcriptomes and both the mouse caecum-derived and more than 100 *in vitro* transcriptomes revealed significant convergence of the *L. plantarum* response to human and mouse intestinal conditions. Altered carbohydrate acquisition and cell surface composition were among the most pronounced altered functional classes. For example, the capsular polysaccharide biosynthesis

operon *cps3* and the cell-surface protein clusters *cscI* and *cscVIII* were consistently induced in all *in vivo* samples [71]. These overlapping responses for *L. plantarum* in different GI compartments and using different mammalian model systems support a diet-, host-, and microbiota-independent core response in *L. plantarum*. Hence, the cognate extracellular molecules of this LAB are key-performance factors involved in (probiotic) functionalities, likely to include robustness, in the GI-tract [14]. In another study, *in situ* transcriptomes of *L. johnsonii* residing in different compartments of the mouse GI-tract were obtained [72]. Colon-specific gene expression was not detected, whereas the induction of specific sugar PTS transport systems was demonstrated in the jejunum, the stomach, and the caecum. Moreover, the stomach-specific genes include several multidrug transport systems, a cation-efflux protein, as well as a copper transporting ATPase, closely resembling the alterations in gene expression found in the *L. plantarum* R-IVET approach described above [64].

Assessment of multiple stress responses and regulatory network reconstruction

Although these *in situ* studies have shed light on the molecules involved in the stress responses in LAB, these analysis have generally focused on one particular stress during one aspect of the LAB application pipeline. However, a few studies have reported the LAB stress response towards multiple industrially relevant stresses, e.g. alterations in *L. lactis* gene expression after exposure to heat, acid, and osmotic stress were assessed utilizing DNA macroarrays, focusing on 375 metabolic genes. Although the majority of stress-regulated genes was specific for an individual stress condition, a number of stress responses were common for the different stresses, including repression of several transporters and induction of two nucleotide kinases [73]. Another elegant study described the elucidation of the response of *Bifidobacterium breve* towards heat, osmotic, and solvent stress [74]. Data obtained from transcriptome analysis, DNA-protein interaction data, and GusA reporter fusion studies were combined with an *in silico* analysis, allowing the construction of a model for an interacting regulatory network for stress responses in this probiotic bacterium. This model revealed HspR controls the SOS response and the ClgR operon, which in turn regulates and is regulated by HrcA. As exemplified by the bifidobacterial study described above, such a multiple variable "stressomics" approach is highly valuable for comprehensive stress response analyses, as it does not only identify the genes directly involved in robustness and/or stress survival, but can also reveal the regulatory networks and complete regulons involved.

Understanding of stress responses to improve robustness

Improving our understanding of stress responses in biotechnologically important bacteria like the lactic acid bacteria will enable the rational design of robustness enhancing strategies. The application of (functional) genomics approaches allows a holistic view of stress responses and their intertwined

regulation, which will accelerate the development of such improvement strategies. Identification of marker genes for robustness as well as the genomics based development of comprehensive cross-protection strategies that can be applied during starter or probiotic strain production, hold promise for the improvements in the production of robust biotechnological workhorses.

Outline of the thesis

Overall, this introductory **Chapter 1** underlines the value of genomics approaches to increase our understanding of the molecular biology of LAB in general, and illustrates how a variety of post-genomic approaches can accelerate the identification of genes involved in stress response and tolerance, in particular. In this thesis, several of the technologies described above, but also newly developed technologies, were employed to unravel the genes and molecular mechanisms involved in the GI robustness of the probiotic model *Lactobacillus plantarum* WCFS1.

Chapter 2 depicts the transcriptional and phenotypic responses of *L. plantarum* WCFS1 towards the solvent ethanol which predominantly influences the cell envelope. In addition, the cross-protective effect of ethanol for the survival of *L. plantarum* after exposure to several other stresses was assessed. It appeared that proteins of the class I and class III stress response like chaperones and Clp-proteases are important for the bacterial adaptation towards ethanol stress. To further investigate the importance of these proteins in stress adaptation, their regulators HrcA and CtsR were deleted from *L. plantarum* WCFS1. The impact of deregulation of the HrcA and CtsR regulons was determined by employing transcriptomics to compare the genome-wide expression patterns in these deletion derivatives with those of the wild-type strain (**Chapter 3**). Furthermore, the impact of fermentative conditions on the *in vitro* digestive tract survival of *L. plantarum* WCFS1 was determined by using a fermentation-genomics platform. The mild-stresses applied in this platform together with the GI-tract characteristics of the strain are described in **Chapter 4**. It appeared that low salt concentrations and a relatively low pH during fermentation enhance GI-tract survival. Moreover, bacterial robustness marker-genes were discovered through transcriptome-trait matching, and validated by the construction and phenotypic characterization of gene-specific deletion strains.

In **Chapter 5** the strain-specific intestinal persistence characteristics of members of the *L. plantarum* species were determined. The digestive tract robustness of 40 *L. plantarum* strains was assessed using the GI-tract mimicking *in vitro* assay. Moreover, by using genotypic diversity of the strains, barcoding, and pyrosequencing, the intestinal persistence curve of these strains could also be analyzed *in vivo* in human volunteers. To this end, the strains were consumed by the volunteers in a mixture of ten different strains, and the strain-specific intestinal persistence curves were quantitatively determined. Notably, the strain-specific *in vivo* GI-tract persistence curves appeared to significantly correlate with their survival curves determined in the *in vitro* digestive tract assay, supporting the predictive value of this 'simple' *in vitro* assay. In **Chapter 6** *L. plantarum* WCFS1

was exposed to several rounds of murine GI-tract passage and re-isolation from feces. This approach aimed to recover adapted strains that had acquired mutations that allowed them to persist longer in the murine intestine. Resequencing of the adapted isolates revealed specific genomic adaptations in the adapted strains, among which cell-envelope associated functions appeared to be enriched. **Chapter 7** discusses the findings presented in this thesis, in the light of probiotic applications and requirements. Moreover, it provides several clues for the future application of the molecular knowledge generated in this thesis, aiming to improve robustness of industrially applied strains.

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Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*

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Abstract

This paper describes the molecular responses of *Lactobacillus plantarum* WCFS1 towards ethanol exposure. Global transcriptome profiling using DNA microarrays demonstrated adaptation of the microorganism to the presence of 8% ethanol over short (10 min and 30 min) and long (24 h) time intervals. A total of 57 genes were differentially expressed at all time points. Expression levels of an additional 859 and 873 genes were modulated after 30 min and 24 h exposure to the solvent, respectively. Ethanol exposure led to induced expression of genes involved in citrate metabolism, cell envelope architecture, as well as canonical stress response pathways controlled by the central stress-regulators HrcA and CtsR. Correspondingly, cells grown for 24 h in medium containing 8% ethanol exhibited higher levels of citrate consumption, modified cell membrane fatty acid composition and showed invaginating septa compared with cells grown in liquid medium without ethanol. In addition, these physiological changes resulted in cross-protection against high-temperatures, but not against several other stresses tested. To evaluate the role of HrcA and CtsR in ethanol tolerance, *ctsR* and *hrcA* gene deletion mutants were constructed. The growth rate of the *L. plantarum* Δ*ctsR::cat* was impaired in MRS containing 8% ethanol, whereas growth of the *L. plantarum* Δ*hrcA::cat* and Δ*ctsR*Δ*hrcA::cat* mutants was indistinguishable from wild-type cells. Overall, these results suggest that the induction of CtsR class III stress responses provides cross-protection against heat stress.

Introduction

Lactic acid bacteria (LAB) are essential for the fermentation of numerous foods and beverages, including yoghurt, sausages, olives, and wine [1-4]. During the application of LAB in food and beverage fermentations, these bacteria are typically required to survive and remain metabolically active under diverse environmental conditions, including specific stresses. For example, wine LAB are exposed to several stresses, such as an acidic pH, a high alcoholic content, suboptimal growth at room temperature, and growth-inhibitory compounds originating from both yeast and bacterial metabolism [4].

In order to understand the mechanisms of stress tolerance of lactobacilli, numerous studies have examined the physiological and genetic adaptations of these organisms during growth and survival in diverse environmental stresses [4-6]. Recently, the availability of complete genome sequences (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) and post-genomic-approaches have accelerated our understanding of the global (genome-wide) stress responses in lactobacilli to acid, lactate, oxidative, bile, and heat stress [7-12]. These studies have shown that lactobacilli respond rapidly to their environment by modulating expression levels of genes involved in different cellular processes including stress response pathways, cell division, transport, and cell envelope composition. Adaptation to the harsh environmental conditions is at least partially under the control of HrcA and CtsR, canonical class I and III stress response regulators present in many Gram-positive bacteria [6].

The stress-responses of the model LAB *Lactobacillus plantarum* WCFS1 have also been the subject of numerous reports employing transcription profiling and targeted mutation analysis of individual genes encoding either stress response genes or their regulators [7,8,13]. Interpretation of the results obtained in these studies have been accelerated by the availability of the *L. plantarum* WCFS1 genome sequence [14], its advanced gene-function annotation [15], a stochiometry-based genome scale metabolic model [16], as well as effective mutagenesis tools [17]. Thus far, the detrimental effects of ethanol on *L. plantarum* are poorly understood, and ethanol toxicity is generally attributed to the interaction of ethanol with the cell membrane resulting in a loss of membrane integrity and secondary effects on metabolism and stress-response pathways [18]. Ethanol stress is encountered by *Lactobacillus plantarum* in a variety of beverage fermentations, most notably beer and wine, and strains of this species have been reported to display high levels of tolerance to this solvent [19,20].

This study aimed to identify the global adaptive and cross-protective responses of *L. plantarum* WCFS1 during growth in the presence of ethanol. The molecular responses of *L. plantarum* WCFS1 to short- and long-term exposure to 8% ethanol were investigated by whole genome transcription profiling. Determination of specific metabolic and morphological adaptations in *L. plantarum* and the cross-protective effects of ethanol exposure towards other environmental stresses complemented the transcriptome-based results. In addition, mutagenesis approaches revealed that the molecular adaptations are at least partly controlled by CtsR as previous studies revealed the direct interaction between CtsR and the promoter regions of the *ctsR-clpC* operon and *hsp1* gene [21].

Material and Methods

Strains and growth conditions

Strains used in this study are described in Supplementary Table S1 of the supplemental materials *Lactobacillus plantarum* WCFS1 [14] was grown at 20°C in MRS (de Man-Rogosa-Sharpe) broth (Difco, West Molesey, United Kingdom) with either 8% (v/v) additional water or 8% (v/v) ethanol. Growth and cell density were determined by measurement of the OD_{600} of the culture using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK). Citrate, lactate, formate, pyruvate, 2,3-butadiol, acetoin, succinate, acetate, propionate, and ethanol concentrations were measured in culture supernatants by high-performance liquid chromatography (HPLC) as described previously [22]. Cells were harvested at OD_{600} = 1.0 for transcript profiling, cross-protection experiments, microscopy, and lipid extraction.

RNA isolation and transcriptome analysis

Transcriptome analysis was performed in duplicate immediately prior (t =0) and subsequent to exposure to 8% (v/v) ethanol in MRS for 10 min, 30 min, and 24 h. RNA extraction, reverse transcription, labeling, hybridization, and data analysis were performed as described previously [23]. In short, following quenching, RNA was phenol-chloroform extracted and purified using the High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). Quality of the RNA obtained was measured with the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA), and samples with a 23S/16S RNA ratio equal or higher than 1.6 were taken for cDNA synthesis. cDNA was synthesized using the Superscript TMIII RT enzyme (Invitrogen, Carlsbad, CA, USA), purified with the CyScribe GFX purification kit (GE Healthcare, Buckinghamshire, UK) and labeled differentially using Cyanine 3 or Cyanine 5 labels (Amersham^{TM}, Cy^{TM}Dye Post-labeling Reactive Dye Pack, GE Healthcare, Buckinghamshire, UK). After a second purification with the CyScribe GFX purification kit (GE Healthcare, Buckinghamshire, UK), *L. plantarum* WCFS1 cDNA was hybridized to oligonucleotide DNA microarrays for this strain (Agilent Technologies, Santa Clara, CA, USA). The DNA microarray design and gene expression data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GPL4318 and GSE17847, respectively. *L. plantarum* WCFS1 DNA microarrays were hybridized according to a modified loop design which included comparisons of all conditions within three steps (Fig. S1). The transcript data was normalized by local fitting of an M-A plot applying the LOESS algorithm [24], using the Limma package [25] in R (http://www.R-project.org) as previously described [23], and genes with FDR-adjusted p-values less than 0.05 were considered to be significantly differently expressed. To analyze the results, heat maps of gene expression levels were constructed for the transcript profiles using the Genesis platform [26]. Blastn was performed using http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Lipid and fatty acid extraction

Approximately $1x10^{11}$ *L. plantarum* cells grown in MRS [with or without 8% (v/v) ethanol] until OD_{600} = 1.0 at 20°C were collected by centrifugation (15,300 x *g* for 10 min at 23°C) and washed with phosphate-buffered saline (PBS), pH 7.4. Cell walls were degraded using 0.05 $g{\cdot}ml^{-1}$ lysozyme (Merck, Damstadt, Germany) and 250 $units{\cdot}ml^{-1}$ mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) in 100mM $K_2HPO_4^-$ buffer (pH 6.2) under agitation at 10 rpm for 3 h at 44°C (Hybridization oven/shaker RPN2511E, Amersham pharmacia biotech, Little Chalfont, UK). The cells were collected by centrifugation at 4000 x *g* for 10 min at 23°C and the cell membranes were harvested by dissolving the pellets thoroughly in 3 ml diethylether:heptane (1:1) acidified with 2.5M sulphuric acid. Following centrifugation at 500 x *g* for 5 min at 23°C, the upper organic phase was collected for total fatty acid methyl esthers (FAMEs) analysis. FAMEs were generated and analyzed according to Badings and de Jong [27]. A gas chromatograph (GC) (Carlo Erba, Mega 8060, Milan, Italy) with flame ionization detection (FID) and on-column injector was used to separate the FAMEs. The GC column (Varian, WCOT Fused Silica with stationary phase CP-Wax 52 CB, The Netherlands) contained hydrogen as a carrier gas and was 15 m in length, with an inside diameter of 0.32 mm and a film thickness of 0.50 µm. Data were analyzed with EZChrom Elite, version 3.1.4 (Agilent Technologies, Santa Clara, CA, USA).

Microscopy

For scanning electron microscopy (SEM), round (8 mm diameter) cover slips were coated with Poly-L-lysine [0.01% (w/v) in water] and incubated for 30 min in *L. plantarum* cultures (OD_{600} = 1.0). Cells adhering to the cover slips were then fixed with 4% (v/v) glutaraldehyde for 30 minutes, rinsed with water and subsequently dehydrated by serial incubation in an acetone solution, starting from 10% acetone and going up to 30%, 50%, 70% and 100% acetone. After critical point drying with carbon dioxide (CPD 030, BalTec, Balzers, Liechtenstein), the cover slips were affixed to a sample holder by carbon adhesive tabs (EMS, Washington, USA) and sputter coated with 5 nm platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Cambridge, UK). The bacteria were analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at room temperature at a working distance between 8 and 15 mm, with SE detection at 3.5 kV. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl., Brussels, Belgium) and contrast and brightness were optimized using Adobe Photoshop CS (Adobe, San Jose, California, USA).

For phase-contrast microscopy, *L. plantarum* cultures were examined directly by phase contrast at a magnification of 1250-fold with a Dialux 20 microscope (Leitz, Wetzlar, Germany). Fluorescence microscopy was performed as described previously [28] with several modifications. In short, control, 30 min ethanol-exposed, and 24 h ethanol-exposed cultures (OD_{600} = 1.0) were 10 times diluted, incubated for 20 min on low melting point agarose-coated microscope slides containing 20 $µg{\cdot}ml^{-1}$ FM4-64 (Molecular Probes, Eugene, USA) and 0.5 $µl{\cdot}ml^{-1}$ Syto9 (Molecular Probes, Eugene, USA), and imaged by oil immersion fluorescence microscopy (BX51TRF Fluorescence Microscope, Olympus Corporation, Tokyo, Japan) at a 500-fold magnification.

Mutant construction

Gene deletion mutants were constructed by using the mutagenesis vector pNZ5319 according to Lambert et al. [17]. The *L. plantarum* WCFS1 *ctsR* and *hrcA* genes were replaced with a *lox66*-P_{32}-*cat*-*lox71* cassette resulting in strains NZ3410^{CM} (Δ*ctsR::cat*) and NZ3425^{CM} (Δ*hrcA::cat*) respectively. Primers used to construct the *L. plantarum* WCFS1 mutants are described in Table S2. In short, upstream and downstream flanking regions of *hrcA* and *ctsR* were amplified with primers A, B, C, and D for *hrcA* and E, F, G, and H for *ctsR*. Primers B, F and C, G contained an overhang region homologous to the ultimate 5' and 3' regions of the *lox66*-P_{32}-*cat*-*lox71* cassette (amplified with primers I and J), respectively, to enable the joining of the three PCR products in a Splicing by Overlap Extension (SOEing) PCR [29] with primers E and H for *ctsR* and A and D for *hrcA* (Table S2). The obtained amplicons were blunt-ligated into *Ecl*136II-*Swa*I digested pNZ5319 [17] and resulted in plasmids pNZ3410, pNZ3423, and pNZ3425. After introduction of the mutagenesis plasmids into competent *L. plantarum* WCFS1, cells were plated on MRS containing 10 μg·ml^{-1} chloramphenicol. After 48 h, double cross-over deletion mutants were initially selected by colony-PCR using primer pairs M plus O and N plus P (named 87 (30)) for *ctsR* and K plus O and L plus P for *hrcA* (Table S2). For each mutant, a colony that generated both flanking-PCR products was selected and plated on MRS with and without 30 μg·ml^{-1} erythromycin. A single colony for each mutant displaying the anticipated erythromycin sensitive phenotype was selected and designated NZ3410^{CM} (Δ*ctsR::cat*) and NZ3425^{CM} (Δ*hrcA::cat*), the latter resulting from the use of plasmid pNZ3425. The *L. plantarum* WCFS1 *ctsR-hrcA* mutant was constructed in the NZ3410^{CM} (Δ*ctsR::cat*) background in two steps. Firstly, strain NZ3410 (Δ*ctsR*) was constructed by excision of the *lox66*-P_{32}-*cat*-*lox71* cassette by expression of the Cre resolvase enzyme from pNZ5348 according to methods described by Lambert et al. [17]. Introduction of pNZ3423 and colony confirmation by PCR resulted in strain NZ3423^{CM} (Δ*ctsR*, Δ*hrcA::cat*) (Table S1).

To evaluate relative growth efficiency, the wild type (WCFS1) and mutant strains NZ3410^{CM} (Δ*ctsR::cat*), NZ3425^{CM} (Δ*hrcA::cat*) and NZ3423^{CM} (Δ*ctsR,* Δ*hrcA::cat*) were inoculated at OD_{600} = 0.1 in 96-wells plates and incubated in MRS with or without 8% (v/v) ethanol at 20°C. OD_{600} of the cultures was monitored spectophotometrically (Safire2, Tecan Austria GmbH, Grödig, Austria) in a robotic set-up (Genesis Workstation 150/8, Tecan Austria GmbH, Grödig, Austria). Significance of differences in growth rates of wild-type and mutants were evaluated by analysis of variance (ANOVA) using R (http://www.R-project.org). Differences were considered significant if the p value was <0.05.

Cross-protection studies

Wild-type *L. plantarum* WCFS1 was grown in MRS in the absence or presence of ethanol 8% (v/v) until OD_{600} = 1.0 at 20°C. Cells were washed in PBS before exposure to various stresses. For all stress tolerance assays, serial dilutions of the samples were prepared immediately after stress exposure and these serial dilutions were immediately plated on MRS agar. Plates were incubated at 30°C for 2 days for colony forming unit (CFU) enumeration according to the technique described by Sieuwerts

et al [30]. Oxidative stress tolerance was determined upon suspending the *L. plantarum* cells in PBS containing 40 mM hydrogen peroxide, a concentration which is lethal to *L. plantarum* WCFS1 [13]. Cells were collected every 5 min for 60 min for CFU enumeration. To quantify *L. plantarum* survival at low pH, cells grown in MRS or MRS with 8% (v/v) ethanol were suspended in PBS with an adjusted pH of 2.4 (acidified by 5 M HCl) and subsequently sampled at 5 min intervals, followed by assessment of the amounts of viable cells as described above. Heat resistance of wild-type and mutant *L. plantarum* cultures grown in MRS in the presence or absence of 8% (v/v) ethanol MRS until OD_{600} = 1.0 was assessed after suspending the cells in PBS or PBS containing 8% (v/v) ethanol followed by incubation in a thermocycler (Biometra Thermocycler, Westburg, the Netherlands) at the following temperatures: 37.0, 37.5, 39.1, 41.7, 44.4, 47.1, 49.9, and 52.6 °C. Cell survival was determined every 10 min for 60 min by CFU enumeration. To analyze heat tolerance levels of *L. plantarum*, the $\log_{10}$ of the time and temperature when 1% of the original population was able to form a colony were plotted. To determine the impact of ethanol stress adaptation on salt tolerance, *L. plantarum* WCFS1 was cultured in MRS with or without the addition of 8% (v/v) ethanol until OD_{600} = 1.0. These cultures were inoculated into MRS broth containing 0.6, 0.7, or 0.85 M NaCl, and culture density was monitored at 20°C for 72 hours with a spectrophotometer (SPECTRAmax PLUS384, Molecular Devices, UK). To determine UV radiation tolerance, serial dilutions of wild-type *L. plantarum* broth cultures were plated on MRS agar and exposed for 0 to 180 sec to UV radiation at 254 nm (E-series hand-held UV lamp, Spectroline, Westbury, NY, USA), with a lamp height of 9 cm. After exposure, the MRS agar plates were incubated at 30°C for 2 days prior to CFU determination.

Results

L. plantarum WCFS1 growth and metabolism in the presence of 8% ethanol

Cell growth and fermentation profiles of *L. plantarum* WCFS1 in MRS containing either 8% additional water or 8% ethanol and were monitored over 24 h at 20°C (Fig. S2 and Fig. S3). The growth temperature and alcohol concentration were selected because these conditions mimic wine fermentations and *L. plantarum* WCFS1 was able to reach a final OD_{600} close to the control condition within a few days of growth. *L. plantarum* WCFS1 was able to grow in the presence of 8% ethanol, albeit with an approximately 5-fold lower growth rate (0.06 h^{-1} +/- 0.003) compared with MRS cultures (0.32 h^{-1} +/- 0.03). The final optical density also was approximately 1.4-fold reduced in MRS containing ethanol, and this amount coincided with a more than 2.3-fold lower cell yield (Fig. S2). Culture media pH values when *L. plantarum* reached an OD_{600} = 1.0 were slightly lower for the MRS cultures (pH 5.09 +/- 0.02) than for ethanol-containing MRS (pH 5.15 +/- 0.02). This result might have been due to the 10-fold higher amounts of citrate consumed per 100 μmol lactate produced during *L. plantarum* growth in the presence of ethanol (0.59 μmol citrate consumed) compared with control cultures (0.06 μmol citrate consumed). Conversely, lactate was the primary fermentation end-product of the actively dividing cultures, but also low amounts of formate and acetate were detected (Fig. S3).

Global transcript profiles of *L. plantarum* WCFS1 during growth in ethanol

The transcriptomes of *L. plantarum* WCFS1 after short (10 min and 30 min) and extended (24 h) incubation in ethanol-containing MRS medium were identified using DNA microarrays specific for this strain. The 24 h time point was selected because at that time *L. plantarum* was in mid-exponential phase of growth (OD_{600} = 1.0), enabling comparisons to transcript profiles of reference MRS cultures (t = 0) harvested at the same cell density and growth-phase. Genes differentially expressed by *L. plantarum* during exposure to ethanol were identified by comparisons to transcriptomes of *L. plantarum* WCFS1 cells grown on MRS. After 10 min exposure to 8% ethanol in MRS, 57 genes were significantly differentially expressed compared with MRS cultures (t = 0) (Fig. 1A). These genes constitute a core transcriptional response by *L. plantarum* to ethanol since their expression levels remained similarly up-regulated and down-regulated after 30 min and 24 h exposure to this compound (Fig. 1A). The core ethanol-response included 1.3- to 5.4-fold activation of established stress-associated genes including *groEL*, *groES*, *hsp3*, *grpE*, *lp_0752* (putative stress-responsive transcription regulator), *lp_0726* (membrane-bound protease of the CAAX family), and *lp_3128* (stress-induced DNA binding protein) (Fig. 1B). In *L. plantarum* WCFS1, *lp_3128* was up-regulated after exposure to hydrogen peroxide stress [7]. The gene *lp_3128* shares 98% identity with the DNA starvation/stationary phase protection protein Dps of *L. delbrueckii* subsp. *bulgaricus* ND02. The Dps protein of *E. coli* was previously shown to protect against DNA damage [31-33]. Genes required for citrate metabolism, specifically *citCDEF* and *fum*, were also induced 1.7- to 7.0-fold at all time points (Fig. 1B). In contrast, genes coding for fatty acid biosynthesis including *fabZ1, fabH2, acpA2,* and *fabD* were down-regulated between 1.5- and 4.0-fold (Fig. 1B). Approximately 30% of the protein-encoding genes annotated in the *L. plantarum* genome were differentially expressed at 30 min (916 genes) and 24 h (930 genes) after inoculation into ethanol-containing MRS (Fig. 1A). At both time points, stress-response pathways were induced, cell division as well as lipid and amino acid metabolism were down regulated. In the sections below, these and additional modifications in *L. plantarum* gene expression patterns and their associated phenotypes in response to ethanol are described.

Effects of ethanol on cell envelope composition, cell division, and morphology.

According to transcriptome analysis, *L. plantarum* cell membrane and cell wall components were influenced by ethanol. Expression of the *dlt* operon required for D-alanylation of teichoic acids was induced 1.3- to 1.7-fold by the presence of ethanol. Two *tagE* genes, *tagE5* and *tagE6*, possibly involved in wall teichoic acid biosynthesis were induced 1.4-fold after ethanol exposure for 24 h (Fig. 2A). Several lipoprotein precursor-encoding genes were induced 1.2- to 3.5-fold in the presence of ethanol after 30 min or 24 h or at both time points. In addition, three out of four *L. plantarum* capsular polysaccharide biosynthesis loci (*cps1*, *cps3,* and *cps4*) were down-regulated 1.4- to 3.1-fold in the ethanol-containing MRS for 30 min, 24 h, or at both time points (Fig. 2A).

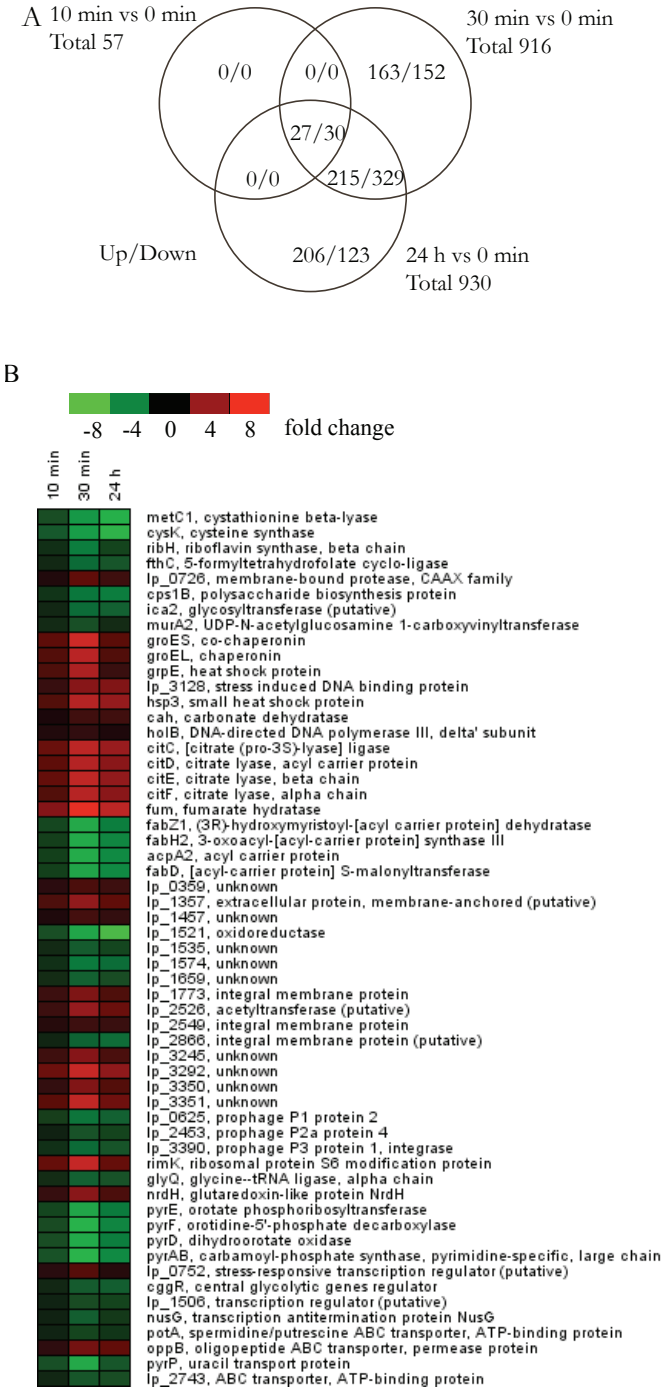

Fig. 1. Venn diagram of the number of *L. plantarum* WCFS1 genes differentially expressed during 10 min, 30 min and 24h incubation in MRS in the presence of 8% ethanol compared with MRS incubation (0 min) (A). Numbers before and after the slash represent up- and down-regulated genes, respectively, compared with cells incubated in MRS. The heat map shows expression levels of the 57 core-response genes differentially expressed at all time points (10 min, 30 min, and 24 h) in MRS containing ethanol compared with MRS cultures (B). The Lp_number indicates gene number on *L. plantarum* WCFS1 chromosome [14]. Genes with FDR-adjusted p-values less than 0.05 were considered to be significantly differentially expressed.

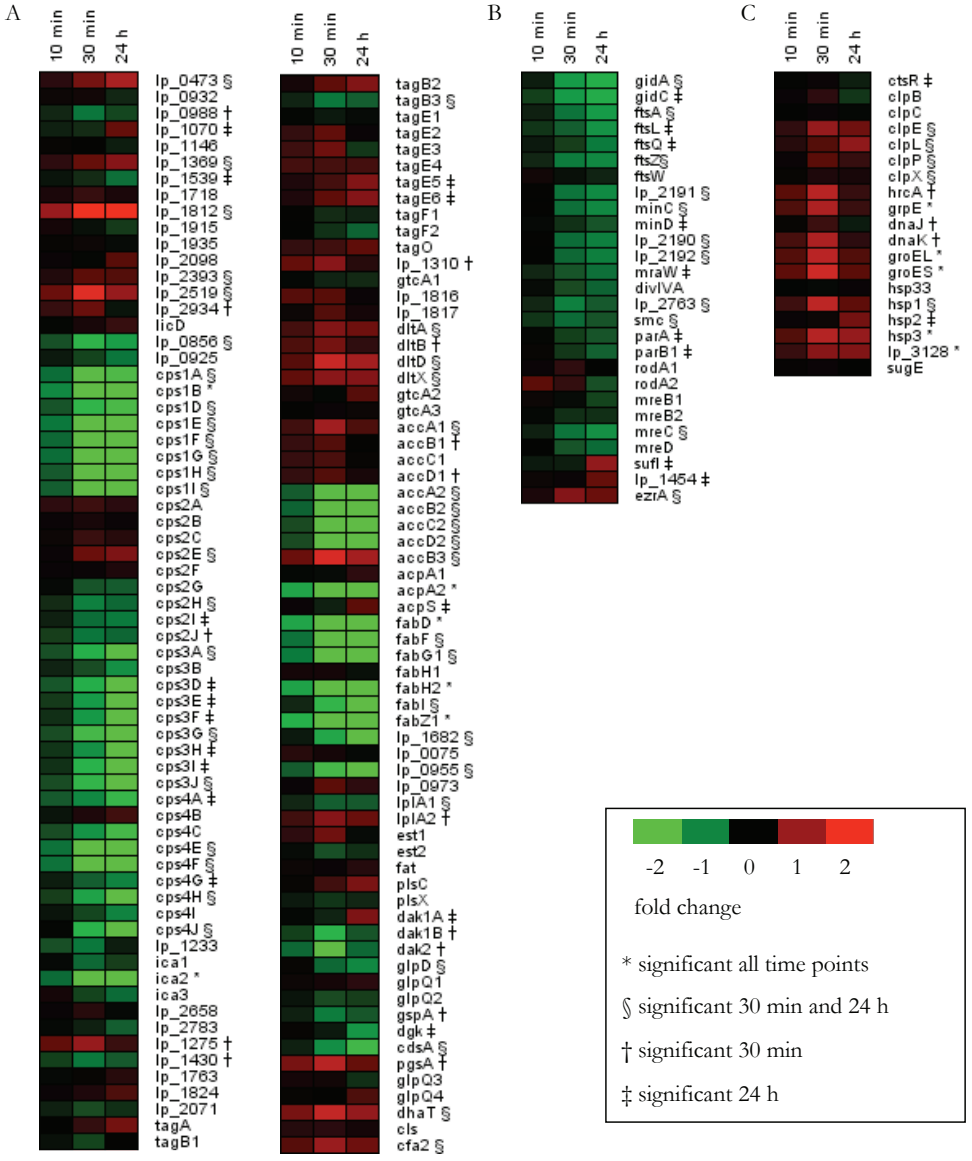

Fig. 2. Heat map of *L. plantarum* WCFS1 genes differentially expressed in the presence of 8% ethanol for 10 min, 30 min, and 24 h. Genes are grouped based on duration of the response, gene annotation and functional category. Gene expression levels of the cultures grown in MRS containing 8% ethanol compared with control MRS cultures are shown according to annotation for cell envelope associated functions (A), cell division (B), and genes involved in stress response pathways (C). The Lp_number indicates gene number on *L. plantarum* WCFS1 chromosome [14]. Genes with FDR-adjusted p-values less than 0.05 were considered to be significantly differentially expressed.

Ethanol stress also significantly affected the expression of *L. plantarum* genes associated with the fatty acid biosynthesis pathways. In general, the majority of genes required for membrane lipid biosynthesis were down-regulated including genes coding for fatty acid elongation proteins (*fab*) and an acyl carrier protein (ACP). The *fab* locus constitutes 12 genes which were repressed at least 1.5-fold starting 10 min after exposure to ethanol in MRS and remained down-regulated after 30 min and 24 h in that culture medium (Fig. 2A). In contrast, expression of the two *L. plantarum* WCFS1 acetyl-CoA carboxylase (ACC) operons involved in the initiation phase of fatty acid (FA) biosynthesis differed such that *acc1* was induced and *acc2* was repressed. Finally, increased expression levels were observed for the gene encoding an acyl carrier protein synthase which maintains the ACP pool in its active form (*acpS*; 1.2-fold at 24 h) [34], and cyclopropane-fatty-acyl-phospholipid synthase (*cfa2*; 1.5- and 1.3-fold at 30 min and 24 h, respectively) (Fig. 2A).

Fatty acid methyl esters (FAMEs) analyses showed increases in the amounts of saturated fatty acids (SFA) palmitric acid (C16:0, 1.9-fold) and, stearic acid (C18:0, 3.8-fold) in cells after 24 h growth in ethanol-containing MRS. A 1.6-fold decrease of the amounts of the mono-unsaturated fatty acid C18:1 was detected, whereas polyunsaturated acid C18:3 increased 1.5-fold (Fig. 3). Collectively, the *L. plantarum* membranes from cultures grown in MRS with 8% ethanol contained an approximately 2.7-fold lower ratio of unsaturated fatty acids (USFA) relative to saturated fatty acids (USFA/SFA = 2.85 +/- 0.29) compared with control MRS cultures (USFA/SFA = 7.80 +/- 1.20) (Fig. 3).

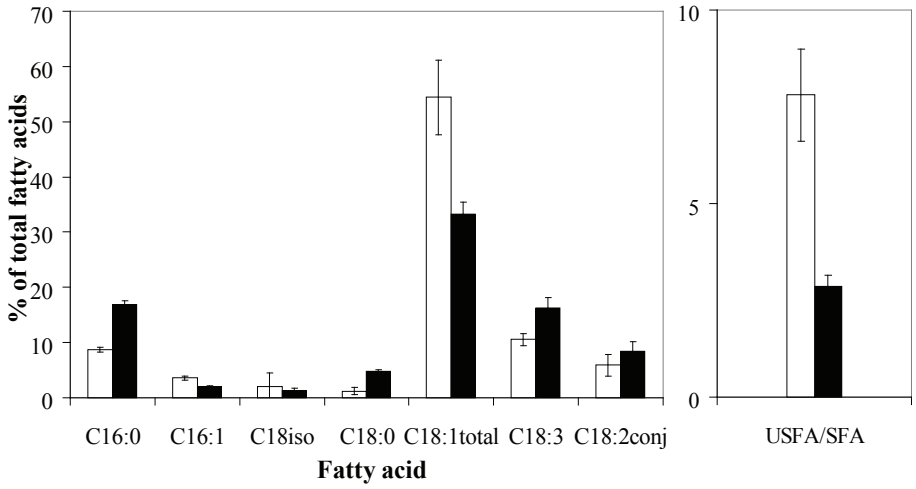

Fig. 3. Fatty acid composition of *L. plantarum* WCFS1 grown in presence or absence of 8% ethanol. Proportions of total membrane fatty acids were determined in mid-logarithmic cultures (OD_{600} = 1.0) grown at 20°C in MRS (white bars) or MRS containing 8% ethanol (black bars). All fatty acids detected for the cells are shown. Iso = isomer, conj = conjugated, USFA = unsaturated fatty acids, SFA = saturated fatty acids. The average (+/- standard deviation) out of four independent cultures is shown.

Because the transcript profiles indicated significant changes to the cell surface of *L. plantarum* in the presence of ethanol (Fig. 2A), global cell morphology and appearance were also determined for the *L. plantarum* cells using SEM. Mid-exponential phase cells grown for 24 h in the presence of ethanol exhibited a rougher appearance and counterclockwise, spiral-shaped invaginating septa which were absent in *L. plantarum* cells harvested from MRS (compare Fig. 4A and C with Fig. 4B and D). The unusual chain angles conferred by the spiral-shaped cells were also observed by phase-contrast microscopy (data not shown). Control and ethanol-exposed *L. plantarum* cells stained with the lipophilic cationic styryl FM4-64 dye did not show membrane lipid spirals, as was detected in *B. subtilis* [35], nor was a difference observed between the two cultures in membrane lipid distribution (Fig. S4). Although the physiological changes which resulted in these aberrantly shaped cells are unclear, it is likely that cell division is disturbed during ethanol exposure. This is supported by the finding that *L. plantarum* genes coding for septum site-determination proteins MinC and MinD and the tubulin-like FtsZ protein required for establishing the site of cell division were down-regulated (1.4-, 1.2-, and 1.4-fold respectively) during growth in the presence of ethanol (Fig. 2B). Simultaneously, the gene cording for EzrA, a protein which inhibits Z-ring formation

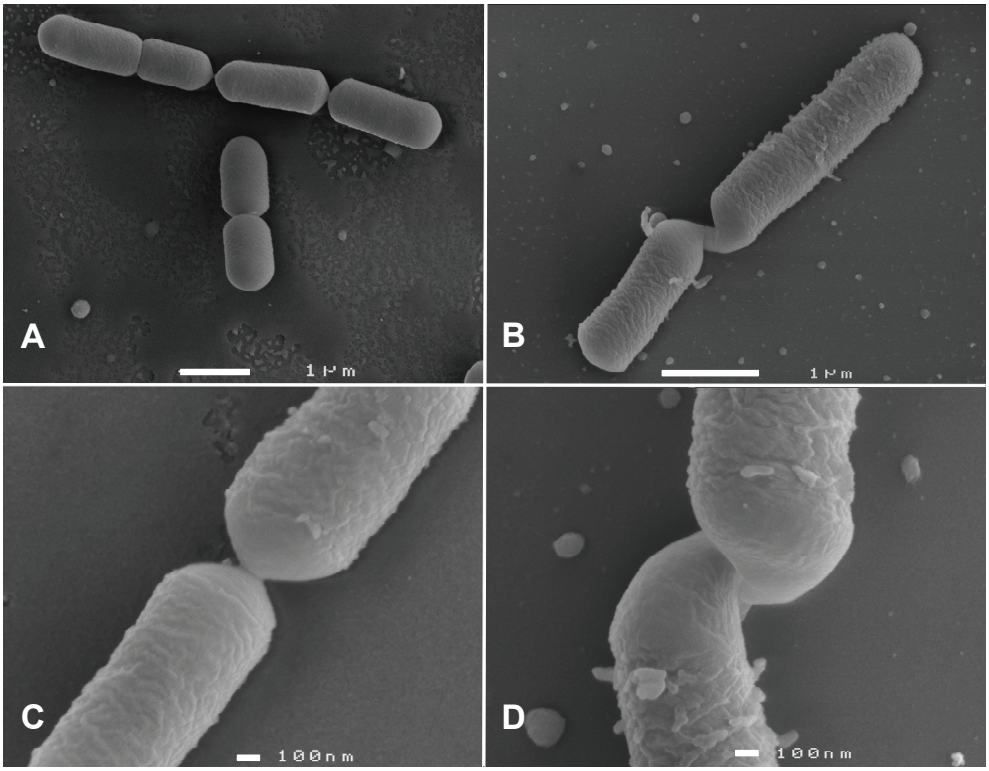

Fig. 4. SEM analysis of *L. plantarum* WCFS1 cultures grown in the presence or absence of ethanol. *L. plantarum* WCFS1 was grown at 20°C and harvested during exponential phase (OD_{600} = 1.0) from MRS (A and C) or 8% ethanol-containing MRS (B and D). Scale bars: 1 µm for A and B and 100 nm for C and D.

[36] was expressed at higher levels (Fig. 2B). Gene expression levels of other cell division and shape determination proteins were reduced (*mreC*, 1.3- and 1.5-fold for 30 min and 24 h, respectively), while several cell division associated genes including *mreB, mreD,* and *rodA* were not differentially expressed in *L. plantarum* exposed to 8% ethanol in MRS (Fig. 2B).

Induction of stress response pathways in *L. plantarum* during growth in ethanol

Genes coding for the class I and class III stress-response transcriptional regulators HrcA and CtsR, as well as the genes under their control, were differentially expressed in the presence of 8% ethanol. The regulons of both regulators are shown in Table S3 in the supplemental material. Transcription of *hrcA* and two genes which are predicted to be regulated by HrcA [37], *dnaK* (encoding a heat shock protein) and *dnaJ* (a chaperone protein), were significantly up-regulated in ethanol-containing MRS at 30 min (Fig. 2C). Other genes at least partially controlled by HrcA coding for chaperones GroES, GroEL, GrpE, and the putative membrane-bound protease *lp_0726* were up-regulated at all time points (Fig. 1B and 2C). Transcription of *ctsR* was significantly reduced in cells exposed to ethanol for 24 h. Genes shown to be repressed by CtsR, including *clpP*, *clpE* (encoding proteases), and *hsp1* (small heat shock protein) [21,38] were up-regulated after 30 min and 24 h of ethanol exposure (Fig. 2C).

Other genes associated with tolerance to one or more environmental stresses were also differentially regulated during growth in ethanol. Stress-response genes primarily known for roles in heat resistance were up-regulated at all time points and include a small heat shock protein (*hsp*3, HSP 19.3) and transcriptional regulator (*lp_3128*; stress induced DNA binding protein) (Fig. 2C). Other genes associated with heat tolerance were intermittently up-regulated in *L. plantarum* and include *hsp2* (HSP 18.55), *clpL* and *clpX*, (proteases), and *tig* (trigger factor) (Fig. 2C). Finally, a cell-surface localized protease encoded by *htrA* was also expressed at an elevated level (1.4-fold) after ethanol incubation for 30 min and 24 h. This gene was induced in *Lactococcus lactis* and *Lactobacillus helveticus* upon exposure to ethanol, NaCl, and heat [39,40].

Genes coding for adaptation to oxidative stresses, including a glutathione peroxidase (*gpo*), thioredoxin (*trxA1*), stress-induced DNA binding protein (lp_3128), catalase (*kat*), and a ferric uptake regulator (*fur*) [41], were induced in ethanol-exposed cultures after 24 h growth. In comparison, genes in the SOS regulon important for survival under conditions which induce DNA damage were either down-regulated or not differentially expressed during extended ethanol exposure (24 h). Similarly the expression of three cell surface complexes (*lp_2173-lp_2175, lp_2975-lp_2978* and *lp_3676-lp_3679*) that were previously shown to be strongly induced during lactate stress were unaffected by ethanol stress [9].

Cross-protection of ethanol-exposed *L. plantarum* cells against high temperatures

Because known stress-response pathways were activated in *L. plantarum* WCFS1 during growth in the presence of ethanol, we examined whether this strain could withstand higher levels of other chemical or environmental stresses after exposure to ethanol as compared to normally grown cells. The cross-protective stress tolerance levels of *L. plantarum* cultures grown for 24 h in the presence of ethanol were determined by exposing the cells to lethal levels of hydrogen peroxide (40 mM), UV radiation (254 nm, ranging from 0 to 180 sec), acid pH (pH 2.4), and elevated temperatures (37°C to 53°C), as well as growth in high NaCl concentrations (0.6, 0.7, and 0.85 M).

Among the stress conditions tested, the only difference between the ethanol-exposed and control *L. plantarum* cultures was the increased capacity of the ethanol-exposed cells to survive at elevated temperatures. Although all cultures exhibited an exponential decay in viability in the presence of heat, *L. plantarum* cells grown until exponential phase in MRS containing 8% ethanol for 24 h (OD_{600} = 1.0) survived longer and at higher temperatures between 37°C and 53°C over a range of 0 to 60 min exposure times compared with cells harvested at the same optical density in normal MRS. This was observed by plotting the $\log_{10}$ values of temperature and time when 1% of the starting population was still able to form a colony after heat exposure (Fig 5). *L. plantarum* grown in MRS with 8% ethanol was able to survive at temperatures approximately 4°C higher than control MRS cultures. This cross-protective effect was observed when ethanol-exposed *L. plantarum* cultures were subjected to heat both in presence and absence of 8% ethanol, although heat resistance was higher when ethanol was absent (Fig 5). Viability of cells suspended in 8% ethanol at the time of heat exposure declined at higher rates (between -18 $\log_{10}$ min/°C and -19 $\log_{10}$ min/°C) compared with cells exposed to heat alone (-13 $\log_{10}$ min/°C), independently of whether the cultures were grown in the presence of ethanol.

ctsR and not *hrcA* influences growth of *L. plantarum* in ethanol

To identify the roles of *L. plantarum* CtsR and HrcA stress response pathways during ethanol-stress conditions, *ctsR* and *hrcA* deletion mutants were constructed (Δ*ctsR::cat*, Δ*hrcA::cat,* and Δ*ctsR*Δ*hrcA::cat*). Growth of the Δ*hrcA::cat* mutant was similar to wild-type *L plantarum* at 20°C. The growth rates of *L. plantarum* Δ*ctsR::cat* and Δ*ctsR*Δ*hrcA::cat* grown in MRS at 20°C were slightly, but significantly lower compared with the wild-type strain (1.1- and 1.2-fold respectively) (Fig. 6A).

When grown at 20°C in MRS containing 8% ethanol, the Δ*ctsR::cat* strain exhibited a 1.2-fold ($p = 0.01$) faster growth rate relative to the parental strain, whereas the Δ*hrcA::cat* and Δ*ctsR*Δ*hrcA::cat* mutants grew similarly as wild-type cells (Fig. 6B). This indicates that CtsR negatively influences the growth rate in MRS containing ethanol at 20°C and that the growth advantage of the CtsR-

deficient strain in ethanol is abolished when HrcA is absent. This result indicates an overlap in the CtsR and HrcA regulatory networks as was previously predicted [4].

Discussion

Lactobacillus species are able to grow and survive under sub-optimal conditions during food and beverage fermentations. Here, we unraveled the adaptations expressed by *L. plantarum* WCFS1 which enabled growth in media containing 8% ethanol, a level found in some alcoholic beverages. *L. plantarum* WCFS1 was shown to adapt by modulating basic metabolic pathways, cell envelope

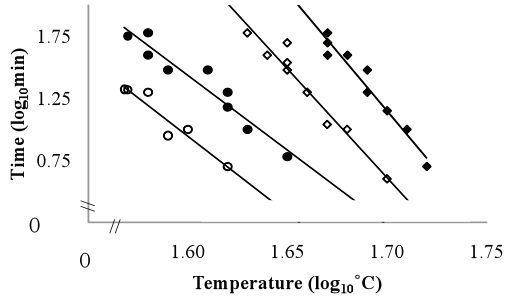

Fig. 5. Heat resistance of *L. plantarum* WCFS1 grown in presence or absence of 8% ethanol subjected to heat stress for 60 min. Shown are the time and temperature when 1% of the original population was able to form a colony. Cultures grown in MRS (open symbols) and in MRS containing 8% ethanol (filled symbols) were subjected to heat stress with (circles) and without (diamonds) the presence of 8% ethanol. Representative values of three independent cultures are shown. ○ R2 of linear trendline = 0.96, ● R2 = 0.95, ◇ R2 = 0.96, ◆ R2 = 0.98.

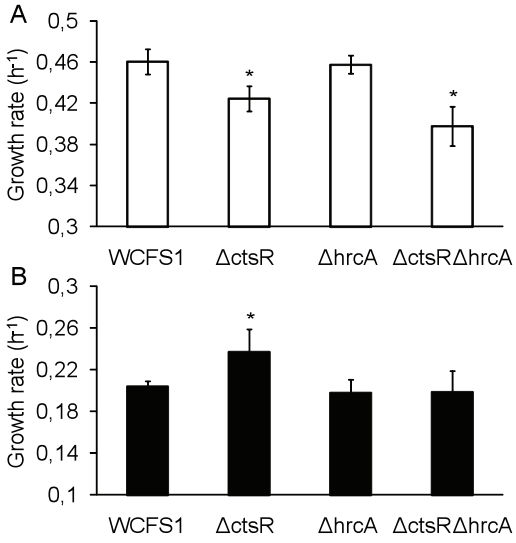

Fig. 6. Growth rates of wild-type and mutant *L. plantarum* WCFS1 in MRS and MRS containing 8% (v/v) ethanol. *L. plantarum* WCFS1 and Δ*cts*R::*cat*, Δ*hrcA*::*cat* and Δ*cts*RΔ*hrcA*::*cat* deletion mutants were grown in MRS (A) or MRS containing 8% ethanol (B) at 20°C. Significant differences in the observed growth rates of the mutants in comparison to the parental (wild-type) strain are marked by asterisks ($p < 0.05$). The growth rates are given as the average (+/- 95% confidence interval) out of three independent experiments.

composition, and by inducing stress-response pathways. Transcriptional responses were elicited within 10 min of exposure to 8% ethanol and expanded during extended incubation (30 min and 24 h). These adaptations resulted in cross-protection against thermal stress, but not other stresses.

Ethanol is known to interfere with bacterial cell membrane integrity by interacting at the lipid-water interface. Ethanol influences membrane lipid-ordering and bilayer stability and affects membrane-characteristics like, permeability, fluidity, and the functioning of membrane-embedded enzymes [18]. Genome-wide analyses of *L. plantarum* gene expression in the presence of 8% ethanol revealed that this organism responds immediately upon exposure to this solvent. This response is sustained under continuous ethanol stress and can be seen as a core-response to ethanol. In addition, extended incubation in ethanol resulted in the expansion of the *L. plantarum* transcriptional changes beyond this core response.

The core-response to ethanol stress included activation of citrate metabolism (*citCDEF* operon) which was accompanied by increased utilization of citrate from the medium. In *L. plantarum,* citrate is converted to acetate and oxalacetate by citrate lyase and oxaloacetate is subsequently decarboxylated to form pyruvate [42]. Activation of citrate metabolism in response to ethanol stress was also observed in *Oenococcus oeni* [43], and is probably explained by its membrane potential and pH-gradient generating effects which can support cellular energy supplies [44,45].

Modification of cellular FA metabolism was another core-response of *L. plantarum* to ethanol. Overall, the transcript profiles suggest that a reduction in FA biosynthesis led to changes in the composition of the cell membrane. Exponential phase *L. plantarum* cells collected after growth in ethanol-containing MRS harbored reduced levels of C18:1, increased levels of palmitic acid, stearic acid and C18:3, and an overall decrease in USFA/SFA ratios compared to cells grown in MRS. These membrane modifications resemble those observed in *O. oeni* ATCC BAA-1163 grown in similar conditions [46]. The observed alterations in *L. plantarum* FA composition probably resulted from changes in *de novo* FA biosynthesis. Although it is possible that desaturases could modify existing phospholipid acyl chains in the membrane bilayer [47,48], evidence that this occurred in *L. plantarum* is lacking. Phospholipid acyl desaturase, phospholipid *cis-trans* isomerase and CFA synthase [47] are the known bacterial enzymes which catalyze FA desaturation, however, *L. plantarum* appears to encode only a CFA synthase. CFA appears to be absent from *L. plantarum* membranes and hence CFA synthase likely does not confer a major role in the observed changes in FA composition under ethanol stress.

Transcriptome analyses also identified differential expression of several genes involved in cell wall-associated functions under ethanol stress. These adaptations included induction of the *dlt* operon, a locus which is involved in D-alanylation of teichoic acids. Induction of these genes was observed previously when *L. plantarum* was exposed to bile, another surface-active component [8]. In addition, changes in expression of *tagE* (a gene possibly involved in wall teichoic acid biosynthesis) and certain genes coding for cell-surface lipoproteins and capsular polysaccharides suggest that

there were significant modifications to the cell envelope structure of *L. plantarum* upon ethanol exposure. The cell wall acts as a binding scaffold for enzymes, and thereby has an important role in control of cell-division and morphology [49]. Remarkably, growth of *L. plantarum* in the presence of 8% ethanol resulted in invaginating spirals at the septum site of dividing cells. This phenotype resembles that of a conditional *ftsZ* mutant of *Escherichia coli* when it was grown at non-permissive growth temperatures. The division defect of the *E. coli ftsZ* mutant was explained by a failure in FtsZ-ring assembly and closure [50,51]. The morphology of ethanol-exposed *L. plantarum* cells might have resulted from changes in FtsZ-ring assembly or other cell-division associated functions due to alterations in cell envelope composition, as was shown previously for *E. coli* [52].

Although there was some overlap between gene expression of *L. plantarum* during ethanol stress and the transcriptional responses of this organism to other environmental insults, growth of *L. plantarum* in the presence of ethanol cross-protected this organism exclusively against thermal stress. Similarly, exposure of *Bacillus cereus* to sub-lethal concentrations of ethanol induced cross-protection against thermal, but not oxidative or high-salt stress [53]. In *L. plantarum*, transcriptional modifications in response to ethanol included the induction of known heat shock response genes [11,54], including *hsp2* (Hsp 18.55) and *hsp3* (Hsp 19.3), two genes which were previously shown to support growth of *L. plantarum* at elevated temperatures and in 12% ethanol [55]. Heat shock responses of LAB are classified into six classes depending on their mode of transcriptional regulation in *B. subtilis* [56]. HrcA is commonly regarded as a class I transcriptional repressor and its regulon was predicted in *L. plantarum* on the basis of a cognate *cis*-acting element, designated CIRCE, in the promoter regions of *groEL-groES*, *hrcA-grpE-dnaK-dnaJ,* and *lp_0726* [57]. Transcriptional regulation by HrcA is dependent on availability of the GroELS complex such that HrcA is inactive when GroELS is unavailable during periods of cellular stress [56]. Transcription of *groELS*, *grpE,* and *lp_0726* was elevated in *L. plantarum* after 10 min, 30 min and 24 h incubation in MRS containing ethanol, indicating a rapid and continuous unfolding of proteins due to the presence of the alcohol. In contrast, induction of the heat-shock genes *dnaK-dnaJ* was only observed after 30 min exposure to ethanol. This result might be due to the differential processing of the polycistronic *hrcA-grpE-dnaK-dnaJ* transcript as has been proposed as the mechanism of differential transcription of this operon in *B. subtilis* and *L. sakei* [58,59].

The class III heat-shock regulon is controlled by CtsR, a transcriptional repressor which binds to a heptanucleotide direct repeat referred as the CtsR-box [60]. CtsR negatively auto-regulates its own synthesis by the same mechanism [61]. The CtsR regulon was previously shown to be involved in ethanol and heat-stress responses in *B. subtilis* [62] and *L. plantarum* [63]. Analogously, our results show that the CtsR regulon was partially induced after 30 min and 24 h of ethanol exposure and included elevated expression of ClpP, ClpE, and Hsp1 encoding genes. This finding suggests that the chaperonin function of GroELS was not sufficient to sustain the correct folding of proteins during ethanol-stress, and the accumulation of denatured and aggregated proteins resulted in the activation of Clp-mediated proteolysis [64]. The temporal activation of class I and III stress regulon members refines our knowledge of the sequential involvement of these stress regulons to the maintenance of appropriate protein functioning under ethanol stress conditions.

To further investigate the role of *ctsR* and *hrcA* in ethanol adaptation, mutants of *L. plantarum* WCFS1 were constructed that lack one or both of these genes. The role of the transcriptional repressor CtsR in adaptation of *L. plantarum* to ethanol and heat-stress was observed previously [65]. The slightly higher growth rate of *L. plantarum* Δ*ctsR::cat* compared with wild-type cells in the presence of 8% ethanol confirms the contribution of the *ctsR* regulon members to counteracting ethanol-induced stress. The growth rate of this mutant under normal growth conditions in MRS was slightly reduced relative to wild-type cells. While inactivation of *hrcA* did not affect the growth rate of *L. plantarum* in MRS culture medium with or without ethanol present. Notably, in the presence of 8% ethanol the *L. plantarum hrcA-ctsR* mutant grew with a rate equal to the wild type, suggesting an interaction between the *ctsR* and *hrcA* stress response regulons in *L. plantarum*[4,21,65].

This study advances knowledge on the stress-tolerance mechanisms of *L. plantarum*, which are important to control this organism in industrial processes that may include exposure to ethanol or similar stress conditions. Improved understanding of adaptive behavior of bacteria under stress conditions could pave the way towards rational design of methods to maximize cell survival and targeted improvement of stress-robustness in LAB.

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Supplemental material

Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*

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Table S1. Strains and plasmids used in this study.

Strain or plasmid	Relevant feature(s)[a]	Reference
Strains		
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	[1]
NZ3410^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *ctsR* (Δ*ctsR::cat*)	This work
NZ3410	Derivative of WCFS1 containing a *lox72* replacement of *ctsR*	This work
NZ3425^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *hrcA* (Δ*hrcA::cat*)	This work
NZ3423^{CM}	Derivative of NZ3410 containing a *lox66*-P32-cat-*lox71* replacement of *hrcA* (Δ*ctsR*Δ*hrcA::cat*)	This work
E. coli		
TOP-10	Cloning host; F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^r) *endA1* λ$^-$	Invitrogen
Plasmids		
pNZ5319	Cm^r Em^r; for multiple gene replacements in gram-positive bacteria	[2]
pNZ3410	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *ctsR*	This work
pNZ3425[1]	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *hrcA*	This work
pNZ3423[1]	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *hrcA*	This work
pNZ5348	Em^r; containing *cre* under the control of the *pcrA* (lp_1144) promoter	[2]

[a] Str^r, streptomycin resistant; Cm^r chloramphenicol resistant; Em^r, erythromycin resistant.

[1] Plasmids contain a piece of 42 random nucleotides after the *cat* stop codon. This can be used to discriminate between the *hrcA* and *ctsR-hrcA* mutant in for instance competitive experiments.

Table S2. Primers used in this study.

ID	Name	Sequence (5' to 3')	Reference
A	KOhrcA-2028F	GTTCATGACTATCGTTTGACCAACG	This work
B	KOhrcA-2028R2	CATTAGTCTCGGACATTCTGCTCCCG CGTGATCATCACCTCTTTTTAGCAC	This work
C	KOhrcA-2030F2	CCGATCGCTACGAGAAGACGCACTA GACGAGTGACGGACAGGGAGATG	This work
D	KOhrcA-2030R	GCCACAACTGAAGGAACCGTCCGGC	This work
E	KOctsR-1017F	CCTGCGGTTAGTGATAACCGTACCGG	This work
F	KOctsR-1017R2	CATTAGTCTCGGACATTCTGCTCCCG TTGACTTTGCATGTGCTTCACCC	This work
G	KOctsR-1019F3	CCGATCGCTACGAGAAGACGCACTA AGCTAAAGAAAGCGAGGAATCGCAATG	This work
H	KOctsR-1019R	GAGCATCATCAAGCGCTTATCTGCC	This work
I	TAG-lox66-F2	CGGGAGCAGAATGTCCGAGACTAATG	This work
J	TAG-lox71-catR2	TAGTGCGTCTTCTCGTAGCGATCGG	This work
K	hrcA-outI	GCGCAATTAGCTGCAATCACACAAACTG	This work
L	hrcA-outII	TTGCTTGCCGCTTGGCAACTTCACC	This work
M	ctsR-outI	GCGGAATTGGCAGACGCACAGGAC	This work
N	ctsR-outII	TCGAATTCACCACGATACTTTGTCCC	This work
O	86	AACGGTAGATTTAAATTGTTTAAACG	This work
P	87	GCCGACTGTACTTTCGGATCCT	[2]

Table S3. CtsR and HrcA regulon members.

ID[1]	Name	Remarks	Reference
CtsR			
lp_0786	*clpP*		[3]
lp_1269	*clpE*		[3]
lp_1903	*clpB*		[3]
lp_0129	*hsp1*		[3]
lp_1019	*clpC*	In operon with *ctsR*	[3]
lp_0547	*fisH*		[4]
lp_0836	*nrpR1* (*spsx1*)		[3]
lp_2942	*lp_2942*		[3]
lp_1995	*lp_1995*		[3]
lp_2090	*elaC*		[3]
HrcA			
lp_0727	*groEL*	In predicted[2] operon with *groES*	[5]
lp_0728	*groES*	In predicted[2] operon with *groEL*	[5]
lp_2029	*hrcA*	In predicted[2] operon with *grpE, dnaK,* and *dnaJ*	[5]
lp_2028	*grpE*	In predicted[2] operon with *hrcA, dnaK,* and *dnaJ*	[5]
lp_2027	*dnaK*	In predicted[2] operon with *hrcA, grpE,* and *dnaJ*	[5]
lp_0726	*lp_0726*		[5]
lp_0129	*hsp1*		[6]

[1] The lp_number indicates gene number on *L. plantarum* WCFS1 chromosome [7].
[2] [8]

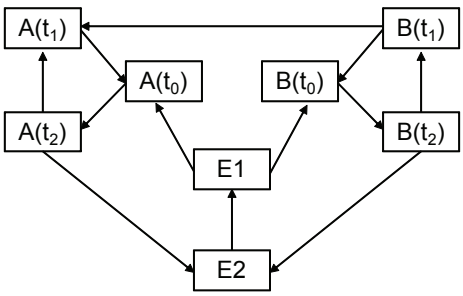

Fig. S1. Experimental design and hybridization scheme. A0, A1, and A2 represent control (t = 0, OD_{600} = 1.0), 10 min, and 30 min incubation in MRS containing 8% ethanol, respectively. B0, B1, and B2 are technical duplicates of A0, A1, and A2, respectively. E1 and E2 are technical duplicates of each other and represent 24 h incubation in MRS containing 8% ethanol (OD_{600} = 1.0). Arrow represents Cyanine 3 (tail) and Cyanine 5 (head) label.

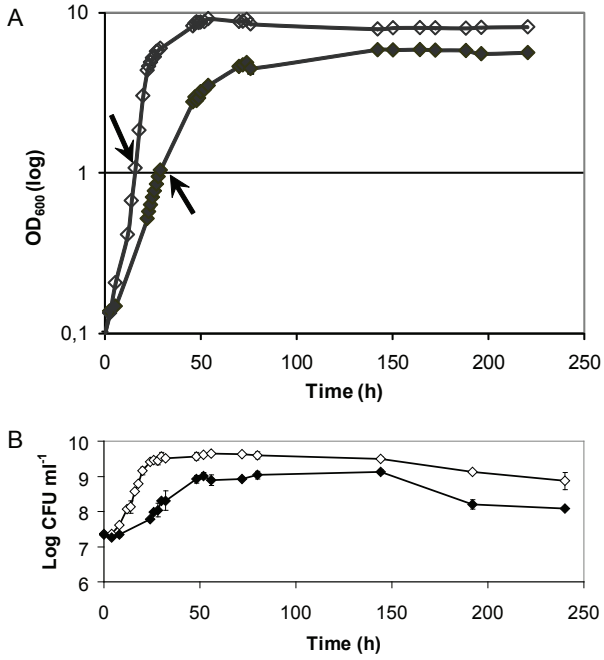

Fig. S2. Growth curves of *L. plantarum* WCFS1 in MRS and in MRS containing 8% (v/v) ethanol. Culture optical density (OD_{600}) (A) and colony forming units (CFU)·ml^{-1} (B) were determined for *L. plantarum* during growth in MRS (open symbols) and MRS containing 8% ethanol (closed symbols) at 20°C. Arrow indicates sampling point for microarray and phenotypic experiments. For (A) and (B) the average (+/- standard deviation) out of two independent experiments is shown.

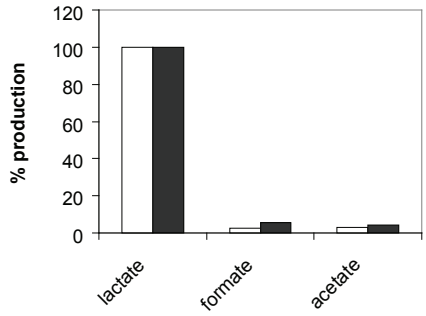

Fig. S3. Primary metabolites of *L. plantarum* WCFS1 in MRS and in MRS containing 8% ethanol. Detected primary metabolites in the supernatants of *L. plantarum* WCFS1 cultures grown in MRS (white bars) and in MRS containing 8% (black bars) until OD_{600} = 1.0. Lactate levels were set at 100%.

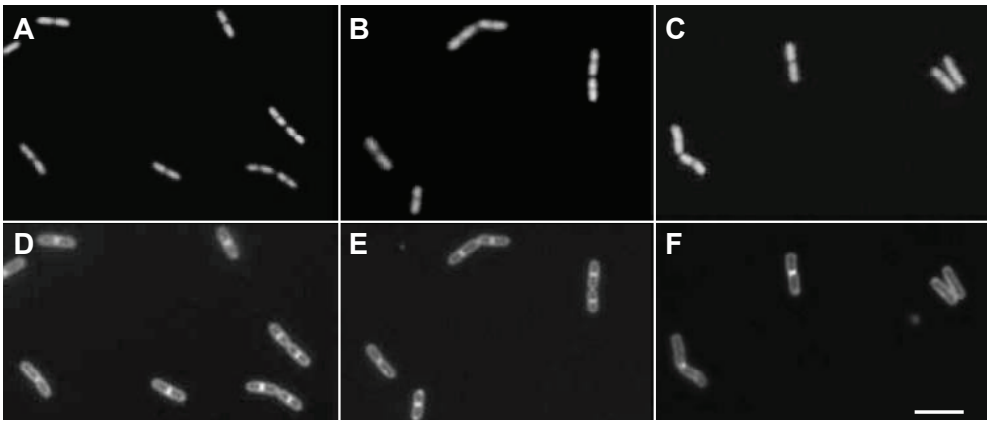

Fig. S4. Lipid distribution in *L. plantarum* WCFS1 grown in presence or absence of 8% (v/v) ethanol. Visualization of DNA by Syto9 (A, B, C) and membranes by FM4-64 (D, E, F) of 0 min (control A, D), 30 min (B, E) and 24 h (C, F) cultures grown in MRS containing 8% until OD_{600} = 1.0 at 20°C. Scale bar (panel F) 5 µm for all photos.

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3

Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation

Van Bokhorst-van de Veen H, Bongers RS, Wels M, Bron PA, Kleerebezem M. Submitted for publication.

Abstract

To cope with environmental challenges bacteria possess sophisticated defense mechanisms that involve stress-induced adaptive responses. The canonical stress regulators CtsR and HrcA play a central role in the adaptations to a pleithora of stresses in a variety of organisms. Here, we determined the CtsR and HrcA regulons of *Lactobacillus plantarum* WCFS1 grown under reference (28°C) and elevated (40°C) temperatures, using *ctsR*, *hrcA*, and *ctsR-hrcA* deletion mutants. While the maximal growth rates of the mutants and the parental strain were similar at both temperatures, DNA microarray analyses revealed that the CtsR or HrcA deficient strains displayed altered transcription patterns of genes encoding functions involved in transport and binding of sugars and other compounds, primary metabolism, transcription regulation, capsular polysaccharide biosynthesis, as well as fatty acid metabolism. These transcriptional signatures enabled the refinement of the gene repertoire that is directly or indirectly controlled by CtsR and HrcA of *L. plantarum*. Deletion of both regulators, elicited expression changes of a large variety of additional genes in a temperature-dependent manner, including genes encoding functions involved in cell-envelope remodeling. Moreover, phenotypic assays revealed that both transcription regulators contribute to regulation of resistance to hydrogen peroxide stress. The integration of these results allowed the reconstruction of CtsR and HrcA regulatory networks in *L. plantarum*, highlighting the significant intertwinement of class I and III stress regulons and illustrating the complex nature of adaptive responses to stress conditions in these bacteria.

Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that occupy a variety of habitats. LAB are acid tolerant and produce lactate as a major metabolic end-product, thereby generating preservative characteristics to fermented foods and beverages. Due to their long history of use in food products, LAB are generally regarded as safe (GRAS) [1]. Next to their prominent role in food fermentation, LAB can be found on (decaying) plant materials and are among the natural inhabitants of the gastrointestinal (GI) tract of animals and humans [2-4]. Specific *Lactobacillus* strains are marketed as probiotics which are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [5]. The gastrointestinal tract is the site of action where probiotics are predominantly considered to confer these health benefits, where they may inhibit colonization and infection by pathogens, they may strengthen the intestinal epithelial barrier, or modulate immune responses [6]. Probiotics encounter a variety of stresses during industrial production and storage, e.g. temperature shifts and low water availability during freeze- or spray-drying, or acid stress during storage. Moreover, during GI passage probiotic bacteria are exposed to acid stress in the stomach, as well as exposure to bile salts and digestive enzymes, while they also have to cope with severe nutrient-competition with the endogenous gut microbiota [7].

To persist under stress conditions, probiotics have an arsenal of molecular defense mechanisms [8-11]. Many stress conditions induce protein denaturation and aggregation, and bacteria, including lactobacilli, possess conserved chaperones and proteases to restore or remove misfolded or denatured proteins. This process has extensively been studied in the paradigm Gram-positive bacterium *Bacillus subtilis* using abruptly or constantly elevated temperatures as the inducing stress condition. The repertoire of heat shock responses in *Bacillus subtilis* was stratified in six classes depending on their mode of transcriptional regulation [12-14]. Several of these stress response classes observed in *Bacillus subtilis* are conserved among the LAB, including the highly conserved Class I regulon. Expression of the Class I stress regulon members is controlled by the repressor HrcA, which specifically binds to the inverted repeat element, CIRCE (controlling inverted repeat for chaperon expression), under non-stressed conditions. The highly conserved CIRCE element (TTAGCACTC-N9-GAGTGCTAA) is typically found in the promoter regions of the *groE* and *dnaK* operons, which encode the two chaperon complexes GroES-GroEL and HrcA-DnaK-GrpE-DnaJ, respectively [15]. The *hrcA* gene is commonly part of the *dnaK* operon, placing this gene under autorepression control. HrcA-repression is dependent on the availability of the GroELS complex and is relieved when the GroELS chaperon complex is not available, i.e. during stress conditions where non-native proteins arise [12]. The HrcA regulon is not only induced during heat shock, but is also activated by a variety of other stress conditions, including acid, bile, and salt stress [8-10,16]. The class III regulon appears to be less conserved among LAB. Although the class III stress regulon repressor CtsR (class three stress gene repressor) appears to be consistently present in LAB, the members of the regulon member genes are more variable [17]. CtsR specifically binds to a heptanucleotide repeat (A/GGTCAAA/T), referred to as the CtsR box [18]. This *cis*-acting regulatory element is commonly encountered in the promoter regions of *clpP* and several other, but not all, *clp* genes, which encode Clp-proteases that are involved in protein quality control during

both stress and non-stress conditions [19]. ClpP mediated proteolysis removes misfolded proteins from the cell, but Clp proteases can also function in cellular differentiation processes [19]. In some organisms other transcription regulators, including HrcA, are involved in co-regulation of the CtsR target genes [19,20]. In conclusion, HrcA and CtsR are key components in stress response regulation, which may include cross-regulation between their respective regulons.

Lactobacillus plantarum is encountered in several environmental niches, including fermented foods and the human GI tract, and specific strains are marketed as probiotics [21]. *L. plantarum* WCFS1, a single colony isolate of strain NCIMB 8826, has been shown to actively survive passage through the human digestive tract [22,23], and it was the first *Lactobacillus* species of which the complete genome sequence was determined [24]. Besides the genome sequence, advanced functional annotations, as well as sophisticated bioinformatics and mutagenesis tools have been developed, enabling the investigation of gene-regulatory mechanisms at the molecular level [25-27]. For example, the *hrcA* and *ctsR* regulon members could be predicted on basis of the conserved *cis*-acting elements involved, which has in part been confirmed experimentally [10,28-31]. Some of the HrcA and CtsR regulon members in *L. plantarum* WCFS1 have been detected through phylogenetic footprinting [30], large scale analysis of co-regulation of expression [31], or via DNA binding assays [28,29]. Moreover, gene-expression responses in *L. plantarum* have been unraveled for various stress conditions, including lactate [32], low pH [32], oxidative [33,34], solvent [35,36], bile [37], cold [35], and heat stress [35]. Analysis of available transcriptome data indicates that some but not all of the predicted HrcA and CtsR regulon members of *L. plantarum* WCFS1 are differentially expressed during these different stress challenges [31]. Despite the characterization of these stress responses, the exact regulons of HrcA and CtsR in *L. plantarum* remain undetermined, to date.

This manuscript describes the regulons of CtsR and HrcA at reference and elevated growth temperatures by determination of the whole-genome transcriptome patterns of *ctsR*, *hrcA*, and *ctsR-hrcA* deletion mutants [36]. The data revealed that the CtsR or HrcA deficient strains displayed altered transcription patterns of genes encoding functions involved in transport and binding of sugars and other compounds, primary metabolism, as well as cell envelope remodeling. Moreover, deficiency of both transcription factors elicited temperature-dependent and pleiotropic transcriptional adaptation of the cell. Stress-phenotyping of the mutants revealed a role of both regulators in the regulation of oxidative stress tolerance. Taken together, our results enabled the refinement of the CtsR and HrcA regulatory networks in *L. plantarum*.

Materials and methods

Strains and growth conditions

L. plantarum WCFS1 [24], Δ*ctsR* (NZ3410) [36], Δ*hrcA::cat* (NZ3425^{CM}) [36], and Δ*ctsR*Δ*hrcA::cat* (NZ3423^{CM}) [36] were grown in MRS (de Man-Rogosa-Sharpe) broth (Difco, West Molesey, United Kingdom) in pH-controlled batch fermentations at 0.5 L scale in a Multifors mini-in parallel fermentor system (Infors-HT Benelux, Doetinchem, the Netherlands) [38]. A single colony isolate of *L. plantarum* WCFS1 or its derivatives was used to inoculate 5 mL of MRS followed by overnight growth at 37°C. The full-grown culture was used to prepare a dilution range from 10^{-1} to 10^{-6} in fresh medium and these dilutions were grown overnight. Subsequently, the culture density was assessed by determination of the optical density at 600 nm (OD_{600}) and the culture that had an OD_{600} closest to 1.5 (representing logarithmically growing cells) was used to inoculate the fermentors at an initial OD_{600} of 0.1. During fermentation the cultures were stirred at 125 rpm, the pH of the culture was maintained at 5.8 by titration of 2.5M NaOH, and temperature was set at 28°C or 40°C. A biological duplicate, derived from independent colonies and performed on different days, was included for all strains and temperatures. Cells were harvested at an OD_{600} of 1.0 for RNA isolation.

RNA isolation and microarray analysis

RNA extraction, labeling and hybridization, as well as data analysis were performed as described previously [39,40]. Briefly, following quenching and cell disruption by bead beating, RNA was isolated using the High Pure kit including 1 h treatment with DNaseI (Roche Diagnostics, Mannheim, Germany). The resulting RNA was reverse transcribed to obtain cDNAs which were labeled using Cyanine 3 or Cyanine 5 labels (AmershamTM, CyTMDye Post-labelling Reactive Dye Pack, GE Healthcare, UK). The cDNAs were hybridized (Fig. S1) on WCFS1-specific, custom-made Agilent arrays (GEO accession number GPL13984; http://www.ncbi.nlm.nih.gov/geo/). Each array contained 15k probes. All probes were present on the array in duplicate and all genes had at least two, but most often three different probes represented on the microarray. Subsequently, the slides were washed and scanned using routine procedures [39,40] and the obtained transcriptome profiles were normalized using Lowess normalization [41]. The data were corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [42]. The median intensity of the different probes per gene was selected as the gene expression intensity. This analysis resulted in genome-wide, gene expression levels for *L. plantarum* WCFS1, NZ3410, NZ3423^{CM}, and NZ3425^{CM}. CyberT was used to compare the different transcriptomes [43]. This analysis resulted in a gene expression ratio and false discovery rate (FDR) for each gene. Genes were considered significantly differentially expressed when FDR-adjusted p-values was < 0.05. The DNA microarray data is available under GEO accession number GSE31253.

Data analysis tools

Visualization of the genes displaying differential expression in the mutants as compared to the wild-type was performed by loading Excel files into the Cytoscape software suite [44]. Data were first ordered using the spring embedded sorting algorithm in the Cytoscape tool. Coloring of the edges (up- or downregulation of the mutants over wild type) and nodes (annotated main class) and structuring of the network were performed manually. The SimPheny^{TM} software package (Genomatica InC., San Diego, USA) loaded with the *L. plantarum* WCFS1 genome-scale model [26] was used to visualize differentially expressed genes that encode enzymes in metabolic pathways. Over-represented main classes and subclasses in the transcriptome data were identified using the Biological Networks Gene Ontology (BiNGO) [45] Cytoscape plugin. MEME software [46] was used with default settings to predict conserved *cis*-acting motifs from 300 nt upstream regions preceding the predicted translation start of the first genes of the operons of all genes. Subsequently, MAST [47] was used to perform genome-wide searches for the MEME-predicted *cis*-acting elements of HrcA and CtsR [30,31].

Phenotypic assays

To determine growth efficiency of the different mutant strains, *L. plantarum* WCFS1 or its derivatives were grown in MRS at 28°C, 37°C, 40°C, or 42°C, and growth was monitored by OD_{600} measurement during 72 hours (SPECTRAmax PLUS384, Molecular Devices, UK). To quantify the colony forming capacity at elevated temperature, the wild type and gene deletion derivatives were grown at 30°C, serially diluted on MRS agar plates, and incubated for 1 week at 30°C or 42°C. Hydrogen peroxide stress tolerance was measured as described before [36]. In short, PBS washed cultures (OD_{600} = 1.0) were resuspended in PBS containing 40 mM hydrogen peroxide at RT and samples were taken from this suspension, every 5 min for 60 min, and colony forming units were enumerated by plating of serial dilutions. Bile resistance was monitored as described before [48]. Briefly, cultures were inoculated in MRS containing 0.1% (w/v) porcine bile (Sigma, Zwijndrecht, The Netherlands) at 28°C and growth was monitored by OD_{600} determination (SPECTRAmax PLUS384, Molecular Devices, UK). Two-sided Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered significant.

Results

HrcA and CtsR are involved in the heat stress response of *L. plantarum*

HrcA and CtsR are regulators of class I and class III stress responses, respectively, including heat induced stress [12]. The role of these repressors at reference and elevated temperature was investigated in *L. plantarum* and its previously constructed derivatives that are deficient in either CtsR or HrcA alone, or both [36]. The maximum growth rate of the Δ*ctsR*, Δ*hrcA::cat*, and Δ*ctsR*Δ*hrcA::cat*

strains at 28, 37, and 40°C did not differ from the *L. plantarum* WCFS1 wild-type strain (Fig. 1). These findings expand earlier observations demonstrating unaltered growth characteristics of another *L. plantarum ctsR* mutant relative to its parental strain at 28°C [29]. However, although the maximum growth rate of Δ*hrcA::cat* was comparable to the wild-type at 42°C, the Δ*ctsR* and Δ*ctsR*Δ*hrcA::cat* mutants displayed 2.0- and 4.1-fold ($p < 0.001$; Fig. 1) decreased growth rates, respectively. This result indicates that CtsR is required to sustain normal growth rates at 42°C. When serial dilutions of stationary phase cultures grown at 30°C were spotted on MRS plates, followed by continued incubation at 30°C, the wild-type and mutant strains gave approximately equal numbers of colonies, which were in all cases within the range anticipated for full-grown cultures. This observation indicates that HrcA and CtsR do not influence the colony forming unit (CFU) numbers of *L. plantarum* WCFS1 at 30°C. Notably, when the plates were incubated at 42°C, the wild type strain generated approximately 100-fold lower CFU as compared to incubation at 30°C ($p < 0.001$). Importantly, the CFU numbers obtained with the Δ*ctsR* mutant were even stronger reduced at 42°C ($p < 0.001$), and this effect

was even more pronounced for the Δ*ctsR*Δ*hrcA::cat* mutant (Fig. 2). Conversely, CFU numbers for the mutant lacking a functional *hrcA* were approximately equal at 30°C, and 42°C, indicating that this mutation contributes to increased robustness as compared to the wild-type at this elevated temperature (Fig. 2).

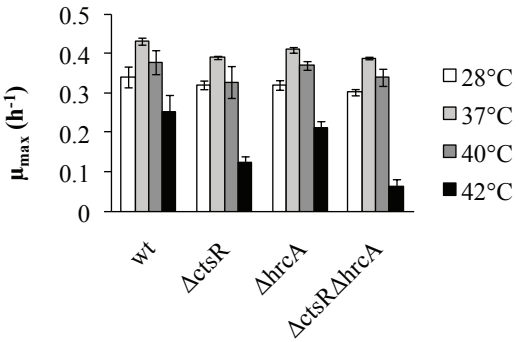

Fig. 1. Maximum growth rates of *L. plantarum* WCFS1 (wt), NZ3410 (Δ*cts*R), NZ3425CM (Δ*hrcA*), and NZ3423^{CM} (Δ*cts*RΔ*hrcA*). Growth rates are shown for reference (28°C) and elevated (37°C, 40°C, and 42°C) temperatures as indicated in the figure legend. Asterisks indicate P-value < 0.001. Data shown are mean ± standard deviation of 3 independent experiments.

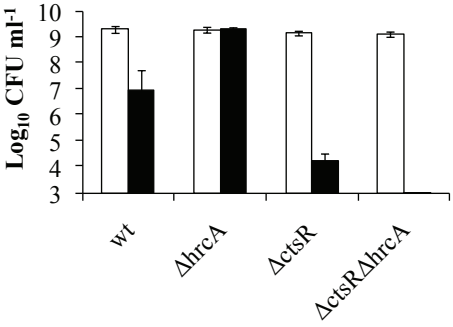

Fig. 2. Involvement of CtsR and HrcA in the ability to form colonies at elevated temperature. *L. plantarum* WCFS1 (wt), NZ3410 (Δ*cts*R), NZ3425^{CM} (Δ*hrcA*), and NZ3423^{CM} (Δ*cts*RΔ*hrcA*) cultures were serial diluted on MRS plates and incubated at control (30°C; white bars) or elevated temperature (42°C; black bars). Asterisks indicate P-value < 0.001. Data shown are mean ± standard deviation of 3 independent experiments.

Transcriptional response of *L. plantarum* during heat stress

To investigate the transcriptional response of *L. plantarum* to elevated temperature and the role of CtsR and HrcA herein, transcriptome profiles of *L. plantarum* WCFS1 at control and elevated temperatures were determined. The control temperature of 28°C and elevated temperature of 40°C were selected since *L. plantarum* wild type displays similar growth rates at these temperatures as compared to the CtsR and HrcA deficient derivatives (see above). This prevents blurring of the results by genes responding to differential growth rates. When comparing the transcriptomes obtained for the wild-type strain at the two temperatures, more than 1000 genes were significantly differentially expressed. At 40°C *hrcA* expression was reduced, while that of *groEL* and *groES* were induced. In addition, *clpP*, *clpB*, and *clpE*, expression were induced at the elevated temperature. Of the other (predicted) HrcA or CtsR regulon members (see Table 1) only *hsp1* (small heat shock protein 1, which has been shown to be regulated by CtsR [29] and is also predicted to be regulated by HrcA [10]) was induced. In addition, at 40°C many genes coding for proteins with regulatory functions were transcribed at an elevated level, suggesting that their regulons contribute to maintenance of normal growth rates at this elevated growth temperature, while genes coding for proteins involved in degradation of proteins, peptides, and glycopeptides were repressed. Other transcriptional changes observed at elevated temperature were the downregulation of the capsular polysaccharide (*cps*)-clusters *1*, *3*, and *4*, while many cell surface proteins, including *cscII*, encoding one of 9 cell surface complexes (*lp_2173-lp_2175* (50)) were upregulated. Moreover, the majority of genes required for membrane lipid biosynthesis were down-regulated, including genes encoding fatty acid elongation proteins (*fab*), acyl carrier proteins (ACP), and acetyl-CoA carboxylases (ACC). The *fab*-locus encompasses 12 genes, which were all repressed at least 3.3-fold. In addition, expression levels of *dak1A*, involved in glycerolipid metabolism, and cyclopropane-fatty-acyl-phospholipid synthase (*cfa-1*) were increased, while its paralogue *cfa-2* was repressed. These results strongly suggest that *L. plantarum* adapts its cell envelope in response to growth at elevated temperature.

Impact of CtsR and HrcA deficiency on expression of their predicted regulons members

To unravel the role of HrcA and CtsR regulation in adaptation to growth at elevated temperatures, we evaluated the transcriptome profiles of the Δ*ctsR*, Δ*hrcA::cat*, and Δ*ctsR*Δ*hrcA::cat* mutants grown at 28°C and 40°C (Fig. 3). Relative to the wild-type strain, the expression of the *ctsR* gene was dramatically decreased in the mutants that lack a functional *ctsR* gene copy (161- to 984-fold), irrespective of the temperature of growth, confirming the integrity of the *ctsR* mutation in these strains (Table 1). Similarly, *hrcA* was decreased in the Δ*hrcA::cat*, and Δ*ctsR*Δ*hrcA::cat* mutants as compared to the wild type (145- to 241-fold; Table 1). The predicted HrcA and CtsR promoter binding motifs (*cis*-elements) [30,31] were used for MAST [47] analyses to predict the members of the HrcA and/or CtsR regulons, revealing several genes that appear to harbor the *cis*-acting motif of at least one of the transcription regulators (Table 1). Several of the CtsR regulon members that

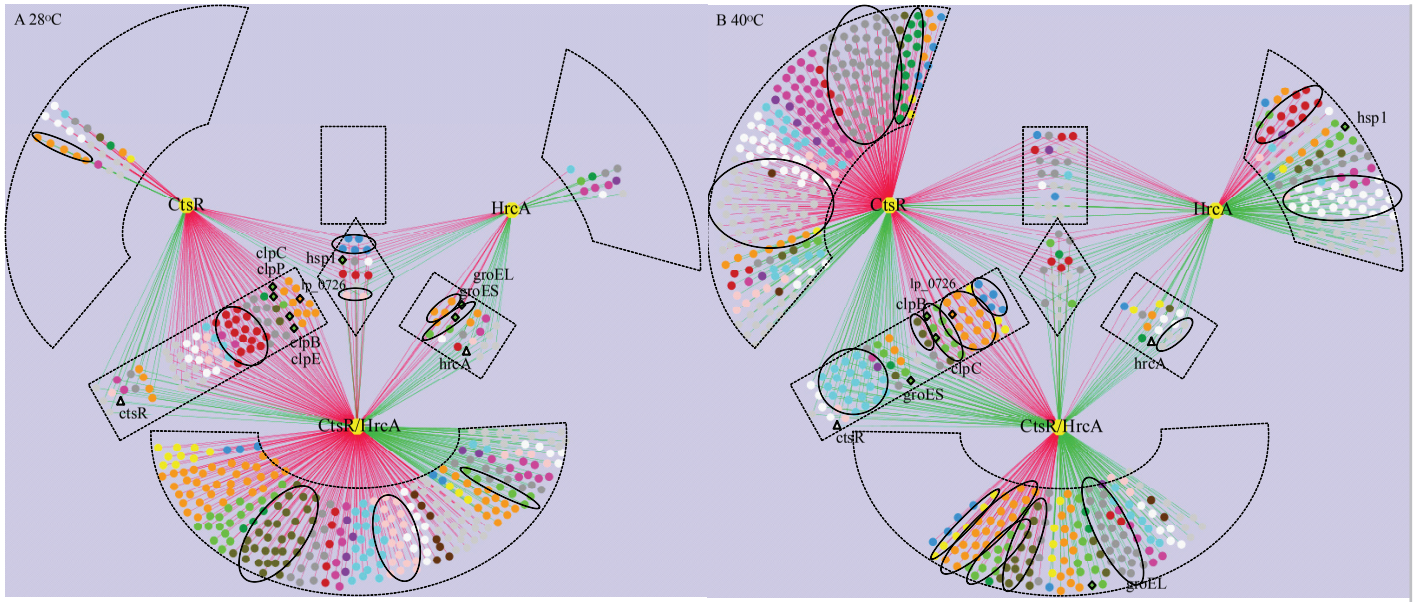

Fig. 3. Significantly differentially transcribed genes in NZ3410 (Δ*ctsR*), NZ3425^{CM} (Δ*hrcA*), and NZ3423^{CM} (Δ*ctsR*Δ*hrcA*) as compared to the wild-type grown at 28°C (A) or 40°C (B). Yellow colored octangular nodes represent the mutants and other colored nodes indicate main classes. The red and green lines indicate up- or downregulation, respectively. Triangle nodes indicate the CtsR or HrcA transcription regulator, diamond nodes indicate genes that are predicted to be part of the CtsR and/or HrcA regulon, whereas black ovals indicate over-represented main classes or subclasses in that particular main class. The main class "hypothetical proteins" was excluded.

Table 1. Fold-changes of predicted and verified CtsR and HrcA regulon members[a] in the NZ3410 (Δ*ctsR*), NZ3425^{CM} (Δ*hrcA::cat*), and NZ3423^{CM} (Δ*ctsR*Δ*hrcA::cat*) strains compared with the wild type.

ID[b]	Name	Function	E-value[c]	28°C			40°C		
				Δ*ctsR*	Δ*hrcA*	Δ*ctsR* Δ*hrcA*	Δ*ctsR*	Δ*hrcA*	Δ*ctsR* Δ*hrcA*
CtsR									
lp_0786	*clpP*	endopeptidase Clp, proteolytic subunit		**2.42**[d]	-1.09	**2.33**	1.27	-1.29	1.12
lp_1269	*clpE*	ATP-dependent Clp protease, ATP-binding subunit ClpE	$1.9{\cdot}10^{-4}$	**2.27**	-1.02	**2.12**	-1.01	-1.20	-1.12
lp_1903	*clpB*	ATP-dependent Clp protease, ATP-binding subunit ClpB	$4.9{\cdot}10^{-4}$	**7.01**	1.00	**6.92**	**4.24**	-1.33	**3.71**
lp_1018	*ctsR*	transcription repressor of class III stress genes		**-696**	-1.15	**-984**	**-526**	-1.31	**-161**
lp_1019	*clpC*	ATP-dependent Clp protease, ATP-binding subunit ClpC		**1.92**	-1.09	**1.84**	**1.76**	-1.30	**1.58**
lp_0129	*hsp1*	small heat shock protein	$3.8{\cdot}10^{-5}$	**5.57**	**3.16**	**12.70**	1.12	**-1.38**	1.21
lp_2945	*lp_2945*	aromatic acid carboxylyase, subunit C (putative)	$3.4{\cdot}10^{-4}$	1.27	-1.08	1.57	1.21	1.20	1.46
lp_2451	*lp_2451*	prophage P2a protein 6; endonuclease	0.48	1.05	1.11	1.12	1.03	**1.40**	1.32
lp_2926	*lp_2926*	unknown	2.7	1.08	-1.08	-1.10	1.30	-1.19	1.05
lp_2426[e]	*lp_2426*	prophage P2a protein 31; phage transcriptional regulator, ArpU family	2.7	-1.18	-1.56	-2.07	**8.85**	-1.87	1.31
lp_2540	*lp_2540*	unknown	3.9	1.09	-1.31	4.11	-1.27	1.27	-1.14
lp_2541	*lp_2541*	ABC transporter, substrate binding protein	3.9	-1.15	-1.03	1.01	1.07	1.31	**1.44**
lp_2542	*lp_2542*	ABC transporter, permease protein (putative)	3.9	-1.03	-1.12	-1.06	-1.02	1.09	1.15
lp_2543	*lp_2543*	ABC transporter, ATP-binding protein	3.9	-1.18	1.02	**1.27**	-1.14	1.15	1.01
lp_3530	*treP*	trehalose phosphorylase	3.9	-1.20	-1.25	-1.05	2.30	-1.32	-1.13
lp_2061	*lp_2061*	unknown	3.9	**1.38**	**1.53**	**1.47**	-1.21	1.10	1.07
lp_2029	*hrcA*	heat-inducible transcription repressor HrcA	5.7	-1.32	**-241**	**-147**	1.15	**-176**	**-145**
lp_2028	*grpE*	heat shock protein GrpE	5.7	-1.04	1.48	1.23	-1.21	1.26	-1.27
lp_2027	*dnaK*	chaperone, heat shock protein DnaK	5.7	-1.23	1.30	1.16	-1.28	1.09	**-1.43**
lp_2842	*lp_2842*	transcription regulator, LysR family	6.5	1.08	1.14	-1.04	-1.17	-1.34	1.03
lp_1843	*lp_1843*	aldose 1-epimerase family protein	9.6	-1.06	-1.14	1.06	**1.50**	1.19	1.20
lp_1845	*hslU*	ATP-dependent Hsl protease, ATP-binding subunit HslU	9.6	1.10	-1.02	1.23	**1.65**	1.08	**1.44**
lp_1846	*hslV*	ATP-dependent protease HslV	9.6	1.16	1.14	**1.31**	**1.78**	1.11	**1.50**
lp_1847	*lp_1847*	integrase/recombinase, XerC/CodV family	9.6	1.22	1.22	**1.36**	**1.73**	1.11	**1.36**
lp_0547	*ftsH*[f]	cell division protein FtsH, ATP-dependent zinc metallopeptidase		1.12	-1.04	1.08	-1.07	1.00	-1.25
lp_0836	*nrpR1* (*spx1*)[g]	regulatory protein Spx		**2.24**	-1.04	**2.15**	**2.13**	-1.11	**2.06**
lp_2942	*lp_2942*[g]	transcription regulator, LysR family		1.10	1.11	1.08	1.37	1.02	-1.06
lp_1995	*lp_1995*[g]	lipoprotein precursor (putative)		1.09	1.11	1.02	1.28	-1.09	1.18
lp_2090	*elaC*[g]	ribonuclease Z		1.12	-1.04	1.20	1.10	1.05	1.25

HrcA									
lp_0727	*groEL*	GroEL chaperonin	$5.6 \cdot 10^{-3}$	-1.19	**2.00**	**1.59**	-1.46	1.06	-1.50
lp_0728	*groES*	GroES co-chaperonin	$5.6 \cdot 10^{-3}$	-1.21	**2.13**	**1.62**	-1.55	1.14	-1.50
lp_2029	*hrcA*	heat-inducible transcription repressor HrcA	$4.7 \cdot 10^{-8}$	-1.32	**-241**	**-147**	1.15	**-176**	**-145**
lp_2028	*grpE*	heat shock protein GrpE	$4.7 \cdot 10^{-8}$	-1.04	1.48	1.23	-1.21	1.26	-1.27
lp_2027	*dnaK*	chaperone, heat shock protein DnaK	$4.7 \cdot 10^{-8}$	-1.23	1.30	1.16	-1.28	1.09	**-1.43**
lp_2026	*dnaJ*	chaperone protein DnaJ		-1.13	1.05	1.17	-1.07	1.08	1.14
lp_0726	*lp_0726*	membrane-bound protease, CAAX family	0.1	**1.90**	-1.07	**1.56**	**2.26**	-1.22	**2.44**
lp_0129	*hsp1*	small heat shock protein		**5.57**	**3.16**	**12.70**	1.12	**-1.38**	1.21
lp_0413	*plnQ*	plantaricin biosynthesis protein PlnQ	1.1	-1.03	1.23	1.51	-2.14	-1.16	1.16
lp_3578	*kat*	catalase	1.5	1.02	1.02	1.03	1.28	-1.20	-1.13
lp_3617	*tal3*	transaldolase	2.7	-1.19	-1.04	1.22	1.26	1.14	-1.28
lp_3618	*pts37A*	sorbitol PTS, EIIA	2.7	1.03	1.02	1.33	**4.51**	1.33	1.26
lp_3619	*pts37BC*	sorbitol PTS, EIIBC	2.7	2.15	1.31	2.50	2.68	-1.40	-1.23
lp_3620	*pts37C*	sorbitol PTS, EIIC	2.7	1.00	-1.33	-1.10	1.88	1.19	1.46
lp_3621	*srlM1*	sorbitol operon activator	2.7	1.39	1.17	2.13	2.22	1.08	1.40
lp_3622	*srlR1*	sorbitol operon transcription antiterminator, BglG family	2.7	-1.36	-1.13	-1.05	2.17	1.01	1.30
lp_3623	*srlD1*	sorbitol-6-phosphate 2-dehydrogenase (EC 1.1.1.140)	2.7	-1.10	-1.35	-1.43	1.37	-1.16	1.88
lp_1268	*lp_1268*	integrase/recombinase	3.4	**-2.21**	-1.10	**-1.56**	**-3.07**	1.49	**-3.38**
lp_0387	*lp_0387*	unknown	3.8	1.18	1.04	1.25	1.06	-1.00	1.35
lp_1879	*hbsU*	DNA-binding protein	4.2	-1.14	1.04	-1.14	-1.23	1.04	**-1.27**
lp_1880	*lp_1880*	unknown	4.2	-1.13	1.11	-1.14	**-1.59**	1.20	**-1.71**
lp_1723	*lp_1723*	hydrolase, HAD superfamily, Cof family	5.1	-1.05	-1.10	-1.06	1.51	-1.11	1.06
lp_2851	*lp_2851*	short-chain dehydrogenase/oxidoreductase	5.6	3.42	1.12	2.21	1.83	-1.89	1.40
lp_2852	*cmd*	4-carboxymuconolactone decarboxylase (putative)	5.6	-1.71	-1.70	-1.73	1.01	**-3.03**	-1.53
lp_2853	*lp_2853*	unknown	5.6	1.16	-2.12	1.11	1.34	-1.16	-1.17
lp_2732	*lp_2732*	oxidoreductase	6.1	-1.04	1.01	-1.11	**3.26**	-1.46	-1.26
lp_2733	*lp_2733*	NADPH-dependent FMN reductase family protein	6.1	1.30	-1.01	1.19	**3.31**	-1.40	-1.04
lp_0800	*lp_0800*	cell surface protein precursor	6.2	1.49	1.09	1.32	**12.66**	-1.41	1.13
lp_1932	*gph2*	phosphohydrolase, possibly inorganic pyrophosphatase (3.6.1.1)	7.5	1.11	-1.23	1.22	-2.00	-1.47	-1.19
lp_1933	*thgA2*	galactoside O-acetyltransferase	7.5	1.29	-1.25	1.51	-1.39	-1.32	-1.18

[a] Adapted from [36].
[b] The lp_number indicates gene number on *L. plantarum* WCFS1 chromosome [24].
[c] E-value is a combination of all matches on the upstream sequence after comparing the canonical regulatory factor binding site. Values until 10 were included.
[d] Fold-changes in bold are significant (FDR adjusted *p*-value < 0.05).
[e] The *cis*-element is predicted to be in front of this operon that contains *lp_2426* until *lp_2431*, which all encode proteins of prophage P2a. Fold-changes are only given for *lp_2426*.
[f] [28]
[g] [29]

have previously been experimentally verified [29], were transcribed at higher levels in the Δ*ctsR* and Δ*ctsR*Δ*hrcA::cat* mutants grown at 28°C as compared to the wild-type, including *clpP, clpE, clpB, clpC, hsp1,* and *spx1* (Fig. 3 and Table 1). In addition, a gene with unknown function (*lp_2061*) and an operon including 2 proteases (*hslU* and *hslV*) were expressed at elevated levels in the Δ*ctsR* strain. Of the predicted *hrcA* regulon members (Table 1), no altered expression pattern was detected for the *grpE*, *dnaK,* and *dnaJ* genes, which are located in the same operon as *hrcA*, while *groEL* and *groES* expression patterns were increased in the Δ*hrcA::cat* mutant, at 28°C. The list does include a gene with unknown function (*lp_1880*) and an integrase/recombinase (*lp_1268*) that were differentially expressed in the Δ*hrcA::cat* and Δ*ctsR*Δ*hrcA::cat* strains. Remarkably, the *hrcA* operon seems to have 2 CIRCE elements and a CtsR-targeted *cis*-element in its promoter region, which may suggest dual control of this regulon by both regulators. However, *hrcA* was not differentially expressed in the Δ*ctsR* mutant at control or elevated temperature. When identifying possible dually regulated genes, only *hsp1* had CtsR and HrcA *cis*-acting elements in the promoter region of this gene (Table 1), as was described previously [10]. This was supported by the upregulation of this gene in all three mutants compared to wild type at 28°C (Fig. 3A and Table 1). Together this indicates that the deregulation of class I and/or class III stress responses by mutation of their regulators induces a partial alteration of expression of their (predicted) regulon members under the conditions tested. Besides the predicted regulon members, the transcription of genes classified to various functional categories appeared to be affected by *ctsR* and/or *hrcA* mutation, which will be discussed below.

HrcA and CtsR mutation affect expression of genes encoding proteins with diverse functions

Additional genes coding for proteins from several functional categories were displaying altered transcription levels in the Δ*hrcA::cat* and Δ*ctsR* mutants as compared to the wild type. The *hrcA* mutation led to induced transcription of 29 transcription regulator encoding genes, including transcription regulators belonging to the AraC, LysR, MarR and TetR/AcrR family regulators. Several genes involved in primary metabolism were induced in the Δ*ctsR* strain compared to the wild type. These genes were involved in a variety of central metabolism reactions, centering around pyruvate dissipation and fermentation related reactions, including *pox*, *pfl*, *pdh*, *pps*, *mae*, *als*, and *cit* (Fig. 4). In addition, genes involved in pentose-5-phosphate pathway, producing D-xylulose-5-phosphate, which can be used for nucleotide synthesis or energy production, (including *xpkA*, *tkt1*, *deoM*, *rpiA1*, *gntK*, and *xfp*) were induced in the Δ*ctsR* strain compared to the wild type (Fig. 4). Moreover, genes involved in sugar metabolism, such as *scrB* (sucrose), *pbg* (glucose), *lac* (galactose), *ara* (ribulose), and *iol* (inositol), were induced in this strain, as were genes involved in transport of other unspecified carbohydrate substrates and organic acids. These genes included sucrose (*pts26BCA*), glucose (*pts32*), maltodextrin (*mdx*, *msmX*), mannitol (*pts2A*), mannose (*lp_3643*, *pts9*), arabinose (*araP*), trehalose (*pts4ABC*) and sorbitol (*pts37A*, *pts38BC*) transporters. These results illustrate the impact of CtsR deregulation on the expression of metabolic genes, mainly affecting functions of primary carbohydrate import and central metabolic pathways, which was

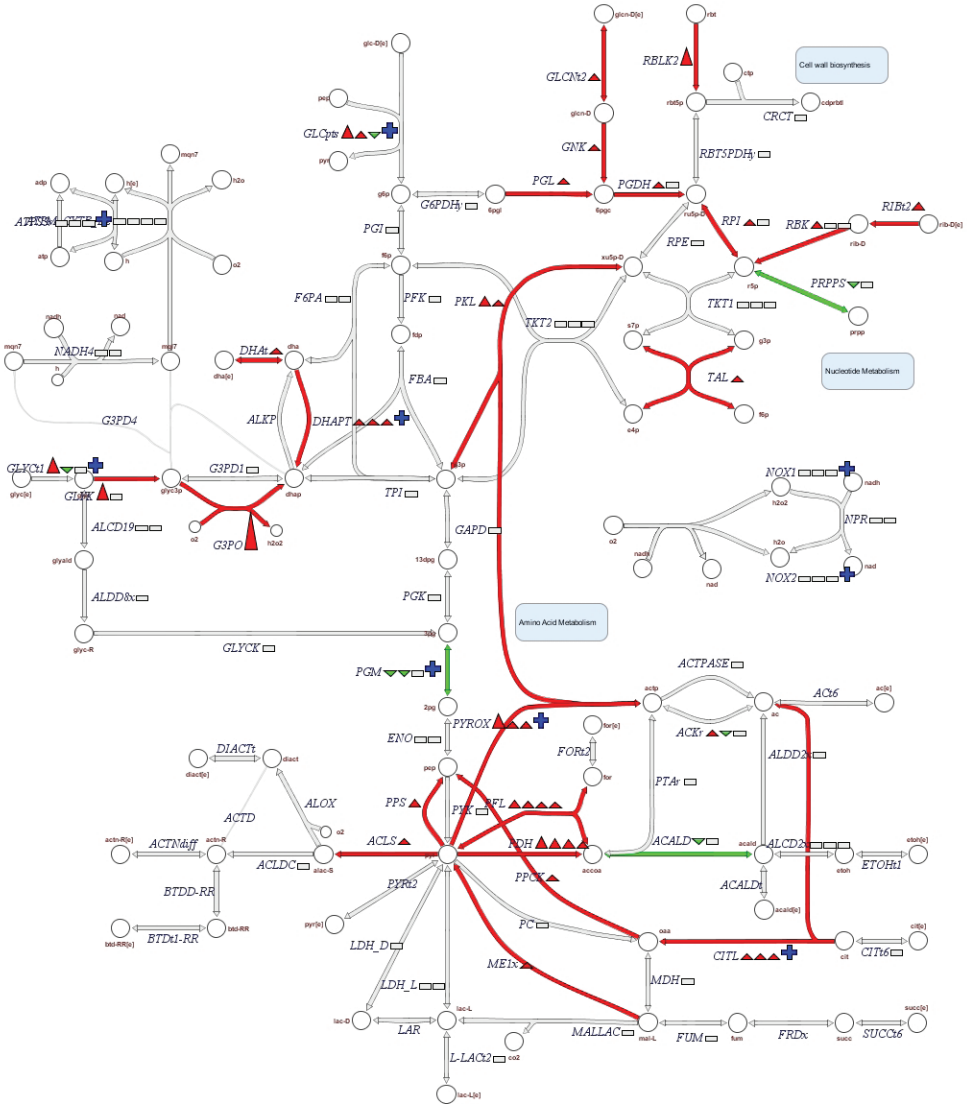

Fig. 4. Primary metabolic pathway of *L. plantarum* NZ3410 (Δ*ctsR*) compared to *L. plantarum* WCFS1 grown at 40°C. Green lines and triangles indicate downregulation, whereas red lines and triangles indicate upregulation, open rectangles indicate no change, and plus symbols indicate that expression of more than 3 genes is acquired for enzyme production. Abbreviations are addressed in the supplementary information, according to Teusink *et al*. [26].

not observed in the *hrcA*-deficient strain. Nevertheless, the *hrcA*-mutation led to repression of genes involved in transport and binding functions, like those involved in transport of phosphate (*pst*), amino acids (*cho*, *sdA*, *lp_1722*, and *lp_3324*), and unknown substrates. Taken together these observations illustrate that deregulation of CtsR or HrcA elicits different response-profiles of transport and metabolism functions.

In addition, the mutations of *hrcA* and/or *ctsR* appeared to play a role in the control of expression of some of the genes and functions that were affected by the temperature of growth in the wild-type strain (see above). Temperature-mediated regulation appeared to be (partially) lost in the Δ*ctsR* mutant (*cps1*), in the Δ*hrcA::cat* mutant (*fab* operon, *dak1A*, and *cfa2*), or in the Δ*ctsR*Δ*hrcA::cat* mutant [*lp_0988* (lipoprotein precursor), *cps1*, and *cfa2*] compared to that seen in the wild-type strain (Fig. 5). This indicates that inactivation of both class I and III transcription regulation leads to deregulation of different combinations of cell envelope biosynthesis processes compared to deregulation of one of the regulators in a temperature-dependent way. Taken together, these findings indicate that some of the more prominent adaptations that the wild-type strain employs to combat elevated growth temperatures, appear to be deregulated in the HrcA and CtsR mutant strains.

Combined HrcA and CtsR deficiency elicits pleiotropic deregulation of the stress control network

To characterize the gene-regulation consequences of the *hrcA* and *ctsR* single mutation relative to the double mutation, the significant regulatory profiles were reconstructed in gene-regulation networks for these strains relative to the wild-type strain at both 28°C (Fig. 3A) and 40°C (Fig. 3B). A relatively large number of genes displayed significant differential expression when comparing the Δ*ctsR*Δ*hrcA::cat* and wild type strains grown at either 28°C (513 genes) or 40°C (603 genes). At 28°C, these genes included almost all differentially expressed genes of the Δ*ctsR* and Δ*hrcA::cat* strains (Fig. 3A). Conversely, less than one quarter and less than one third of the genes differentially expressed in the double mutant at 28°C were affected in the *ctsR* and *hrcA* single mutation at 40°C, respectively. Genes that are not differentially expressed in the other mutants than the Δ*ctsR* strain comprised for instance induction of energy metabolism (genes associated with TCA cycle, sugars, and glycolysis) and transport and binding proteins (e.g. the PTS system) and comprised 24 genes associated with regulatory functions for the Δ*hrcA::cat* strain. Overlapping genes of the *ctsR* or *hrcA* single mutation grown at 40°C with the double mutant grown at both temperatures included genes associated with the pentose phosphate pathway (*tkt1A* and *tkt1B*) and cell division (*ftsQ*, *parB1*, *parA*, and *parB2*), for the *ctsR* mutation and included genes associated with transport and binding proteins (e.g. ABC transporters and multidrug transporter proteins) for the *hrcA* mutation. In addition, genes associated with the cell envelope (such as genes encoding cell surface proteins and genes involved in fatty acid

biosynthesis) were differentially expressed in all three mutants at 40°C. All three mutants affect temperature-independently the *dak1B* operon that is involved in glycerolipid metabolism. Moreover, approximately one third of the genes appeared to be consistently affected by the Δ*ctsR*Δ*hrcA::cat* mutation at both growth temperatures. The genes consistently affected by the Δ*ctsR*Δ*hrcA::cat* mutation included induction of genes associated with the cellular processes (such as cell division protein-encoding genes *ftsZ*, *ftsA*, and *ftsQ*), DNA metabolism (DNA ligase *ligA*, DNA helicase *pcrA*, and DNA-directed DNA polymerase I *polA*), transport and binding proteins (Na^+/H^+ antiporter *napA2*, mannose PTS *pts9D*, and 10 ABC transporters), and cell envelope remodeling (*cps*-cluster *1*, *fab*-locus, lipoprotein precursors *lp_1146* and *lp_1539*).

To further analyze the transcriptome profile of the Δ*ctsR*Δ*hrcA::cat* mutant grown at 28°C and 40°C, over-representative functional classes were identified (Fig. 3). The BiNGO analysis tool was used to compare the Δ*ctsR*Δ*hrcA::cat* strain to the wild type, indicating that functional classes associated

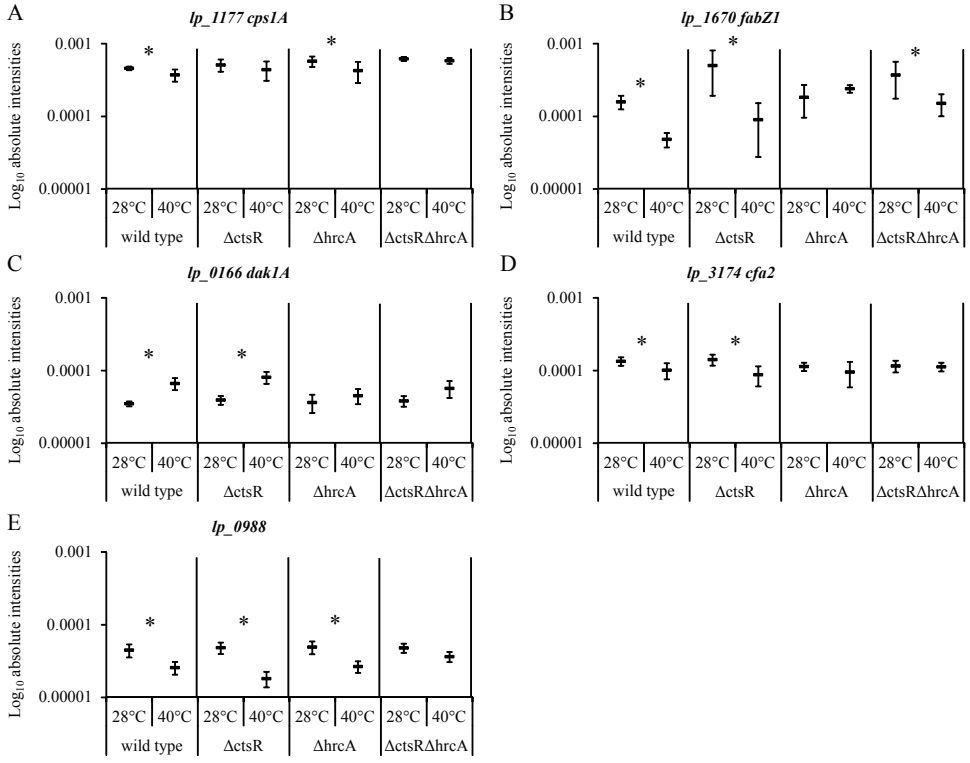

Fig. 5. Box plots displaying the absolute intensity of the first gene of the *cps* cluster *1* (*lp_1177*; A), the *fab*-operon (*lp_1670*; B), *dak1A* (*lp_0166*; C), *cfa2* (*lp_3174*; D), and *lp_0988* (E) of *L. plantarum* WCFS1 (wild type), NZ3410 (Δ*ctsR*), NZ3425^{CM} (Δ*hrcA*), and NZ3423^{CM} (Δ*ctsR*Δ*hrcA*) grown at 28°C or 40°C. Asterisk indicates that (part) of the loci are significant differentially expressed when compared to the strains growth at the other temperature.

with cell envelope remodeling were induced, including the main class "cell envelope" with the sub-class "surface polysaccharides, lipopolysaccharides and antigens", which were induced at both temperatures of growth. In addition, the main classes "cellular processes" and "DNA metabolism" were temperature-independently induced. Temperature specific cell envelope remodeling was also apparent from over-representation of the main class "fatty acid and phospholipid metabolism" when grown at 28°C, while several subclasses of cell surface proteins ("LPxTG anchored", "membrane bound", and "other") were over-represented at 40°C. The main class "protein synthesis" was reduced in the *ctsR* and *hrcA* deficient strain only when grown at 40°C (Fig. 3). Taken together, these data indicate that the cell employs highly adaptable, temperature-dependent systems involving many cell envelope associated functional classes to compensate for the absence of CtsR and HrcA regulation and that the expression of a large variety of additional genes appeared to be modulated compared to deregulation of one of the transcription factors.

HrcA and/or CtsR are required for hydrogen peroxide resistance regulation in *L. plantarum*

Besides involvement of CtsR and HrcA to combat temperature stress, it is known that the transcription factors are associated with other stresses. To evaluate whether *ctsR* and/or *hrcA* may be involved in gastrointestinal (GI)-tract survival, the overlap between the differentially expressed genes in the constructed mutant and the genes identified as being induced in the murine intestine [49] were compared, revealing a substantial overlap (26%) with the genes that were induced in the *ctsR* deletion mutant compared to the wild type grown at 40°C. In addition, *L. plantarum* WCFS1 genes differentially expressed in response to porcine bile exposure [50], were also affected by the *ctsR* gene deletion when grown at 40°C (27%), albeit in the opposite direction. The possible role(s) of CtsR and/or HrcA in bile-stress response and tolerance was investigated by determination of the relative bile-tolerance of the three mutants relative to the wild type, revealing no significant role of either *ctsR* or *hrcA* in growth in the presence of bile (MRS containing 0.1% porcine bile; data not shown), suggesting that the *ctsR* and *hrcA* regulators do not play a role in bile tolerance. Although we cannot rule out the occurrence of polar effects that may have altered the expression of some genes. In addition, the 3 mutant strains also displayed similar survival characteristics as the wild type in an *in vitro* assay that aims to mimick conditions encountered in the GI-tract [38]. Overall, these data suggest that although deregulation of CtsR and HrcA affects the expression of genes that were also differentially expressed under conditions relevant for the GI-tract, no experimental support could be found for a role of the *ctsR* and/or *hrcA* responses in survival under these conditions.

Another comparison between gene expression profiles of the Δ*ctsR*Δ*hrcA::cat* strain grown at 28°C and the response of *L. plantarum* to hydrogen peroxide [34], also revealed overlapping responses (21 %). Analogous to what was observed for the bile responses (see above), the direction of gene expression changes were opposite for a number of genes affected both by H_2O_2 exposure, i.e., H_2O_2 induced expression of *lp_1163*, *dak1B*, *dak2*, *dak3*, *lp_1539*, the *cps1*-cluster and the

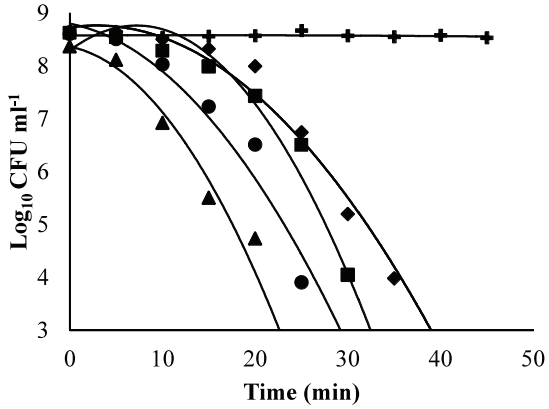

Fig. 6. Involvement of CtsR and HrcA in hydrogen peroxide resistance. Colony forming units of *L. plantarum* WCFS1 (wt, squares), NZ3410 (Δ*cts*R, diamonds), NZ3425^{CM} (Δ*hrcA*, circles), and NZ3423^{CM} (Δ*cts*RΔ*hrcA*, triangles) cultures when subjected to 40 mM H_2O_2 exposure. As a control, the Δ*cts*RΔ*hrcA* strain was taken for incubation in PBS without H_2O_2. Data shown are representative for 3 independent experiments.

Δ*ctsR*Δ*hrcA::cat* mutation elicited their repression. To evaluate the potential involvement of *ctsR* and *hrcA* in the oxidative-stress response and cognate tolerance towards H_2O_2 exposure, the wild type and mutant strains were grown to the exponential phase of growth (OD_{600} of 1) and their rate of loss of survival upon lethal H_2O_2 exposure (40mM H_2O_2, [51]) was followed over time by enumeration of colony forming units (Fig. 6). Compared to the wild-type strain, the Δ*ctsR* strain displayed similar rates of loss of survival, while the Δ*hrcA::cat* and especially the Δ*ctsR*Δ*hrcA::cat* strain were substantially reduced in their capability to tolerate H_2O_2 compared to the wild-type strain. This was already apparent after relatively short exposure to lethal peroxide stress levels, as is illustrated by the 10-fold reduced viability of the Δ*ctsR*Δ*hrcA::cat* strain after 10 min exposure to peroxide relative to the wild-type. These data establish that deregulation of the HrcA and CtsR regulons might influences H_2O_2 tolerance.

Discussion

In this paper, transcriptome profiles of *L. plantarum* WCFS1 were determined at reference and elevated temperatures. In the wild type strain, elevated temperature already induced relatively major alterations in gene expression patterns. Many of these alterations suggest that adaptation of the cell envelope architecture is among the most important adaptive responses to elevated temperature. Relative to growth at 28°C, growth at 40°C induced the expression of several of the predicted CtsR and/or HrcA regulon members, e.g., *groES*, *groEL*, *clpP*, *clpB*, *clpE,* and *hsp1* [30,31]. This is in accordance with the study of Russo *et al.* that performed a global proteomic analysis of *L. plantarum* WCFS1 and Δ*ctsR* mutant strains under optimal and heat stressed conditions [52]. Growth characteristics of the HrcA and CtsR deficient strains were considerably different from those of the wild-type, which was especially apparent from the mutants' phenotype at 42°C. At this temperature, CtsR appeared to be required for maximum growth rates, while HrcA deletion increased colony forming capacity. While in several other organisms, *ctsR* mutation has been

shown to enhance survival under stress conditions [53-56] this seemed not to be the case for *L. plantarum*, which is in agreement with previous studies in this organism [29]. Conversely, the unimpaired colony forming capacity of the *hrcA* mutant at 42°C can be related to the deregulation of the class I stress response network, which is in agreement with the observation that similar mutations in other species enhanced their robustness under stress conditions [56,57]. However, in *Listeria monocytogenes*, *hrcA* deletion is suggested to be associated with increased heat sensitivity [58]. Overall, the impact of deregulation of the class I and class III stress responses on bacterial robustness is not very consistent and seems to vary considerably between species, which implies that extrapolation of the results obtained in specific species or strains to other organisms should be performed with great care.

To understand the HrcA and CtsR mediated stress adaptation, transcriptome analyses were performed comparing the transcriptional profiles of the HrcA- and CtsR-deficient strains at 28°C and 40°C. In addition, to unravel the intertwinement of the class I and class III stress response networks, a strain that lacked both repressors was included in this study. Transcriptome analyses of similar single mutants of either *hrcA* or *ctsR* have been reported for other species [3,59-63], and mutants lacking both repressors have been constructed in *Listeria monocytogenes* [59] and in *Staphylococcus aureus* [63]. Nevertheless, to the best of our knowledge, this study presents the first transcriptome analysis of a strain that is deficient for both regulators. Of the predicted *hrcA* regulon members, no altered expression pattern was detected for the *grpE, dnaK* and *dnaJ* genes. Other transcriptional regulators might be involved in their regulation, e.g. it has been demonstrated that CcpA affects the expression of the *groELS* and *dnaK* operons in *L. plantarum* [64]. Although *lp_0726* is a predicted *hrcA* regulon member, its transcription level was increased in the Δ*ctsR* and Δ*ctsR*Δ*hrcA::cat* mutants. Previous studies indicated that *ftsH, lp_2942, lp_1995,* and *elaC* belonged to the CtsR regulon [29], but in our experiments these genes did not display altered expression relative to the wild type in any of the mutants and conditions tested. Besides transcriptional changes in the predicted regulons, *hrcA* and *ctsR* mutation led to a differential expression of genes involved in many functional classes during control and elevated temperature.

One of the deteriorating consequences encountered by cells growing at temperatures that can be considered as stress temperatures is denaturation and aggregation of proteins [65]. Lack of appropriate control of both the protein folding support (chaperones) and protein quality (Clp proteolysis) may elicit affecting gene expression responses involving genes belonging to different functional classes and affecting numerous cellular processes. These responses may include altered levels of regulator proteins in the cell, which may elicit changes in expression of a variety of regulons. Moreover, the levels of regulator protein may be differentially affected by the temperature of growth, leading to temperature-specific response of various regulatory networks, as was observed in this study. The drastic transcriptome changes elicited in the strain that lacks both CtsR and HrcA at control temperature is illustrative for the magnitude and complexity of the response required for the compensation for the deregulation of both class I and III stress responses. In addition, the results pinpoint that cell envelope remodeling plays an important role in the temperature adaptation in the wild-type strain, but is also prominently affected by the disruption of class I and III stress

response networks. Intriguingly, it has been proposed that in prokaryotes heat shock responses are predominantly controlled by the membrane physical state [66-68], which is in agreement with the finding that adaptive responses include many membrane and envelope modulating functions. Moreover, HrcA has been proposed to be a membrane-associated protein in *Helicobacter pylori*, and even an integral membrane protein in *Streptococcus pneumoniae*. In addition, the *hrcA*-regulon member GroELS of *Escherichia coli* is involved in folding of both soluble and membrane-associated proteins, while concomitantly stabilizing lipid membranes [47,69,70].

To understand the role of HrcA and CtsR in other stress conditions besides elevated temperature, the deregulation responses in the *hrcA* and *ctsR* mutant strains were compared with responses in the wild-type *L. plantarum* strain upon its exposure to specific stress conditions. The mutant lacking both *ctsR* and *hrcA* displayed decreased H_2O_2 tolerance levels compared with the wild type, suggesting that appropriate classI and III stress-regulation are required for optimal peroxide stress adaptation in *L. plantarum*. Similarly, class I and class III stress responses were previously reported to be involved in oxidative stress tolerance in *Fusobacterium nucleatum*, which was associated to induction of ClpB and DnaK in response to H_2O_2 stress [71]. A potentially more indirect link may exist between the Clp protease and H_2O_2 stress responses in *B. subtilis*, where Clp protease activity is involved in regulation of Spx [19], which in its turn was shown to be induced upon H_2O_2 exposure [72].

Overall, deregulation of the CtsR and HrcA regulons in *L. plantarum* elicits compensatory responses that can be characterized by differential transcriptome analyses. These analyses reveal the modulation of several major functional classes, which appears to be temperature-dependent. Therefore, proper control of the CtsR and HrcA regulons are essential for maintaining optimal cell function in changing environments. Moreover, gene regulatory network reconstructions are essential to survey the full regulatory response of an organism. In these networks, the role of the canonical class I and III stress response regulators will be of great importance, because of their pleiotropic character.

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3

Supplemental material

Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation

Van Bokhorst-van de Veen H, Bongers RS, Wels M, Bron PA, Kleerebezem M. Submitted for publication.

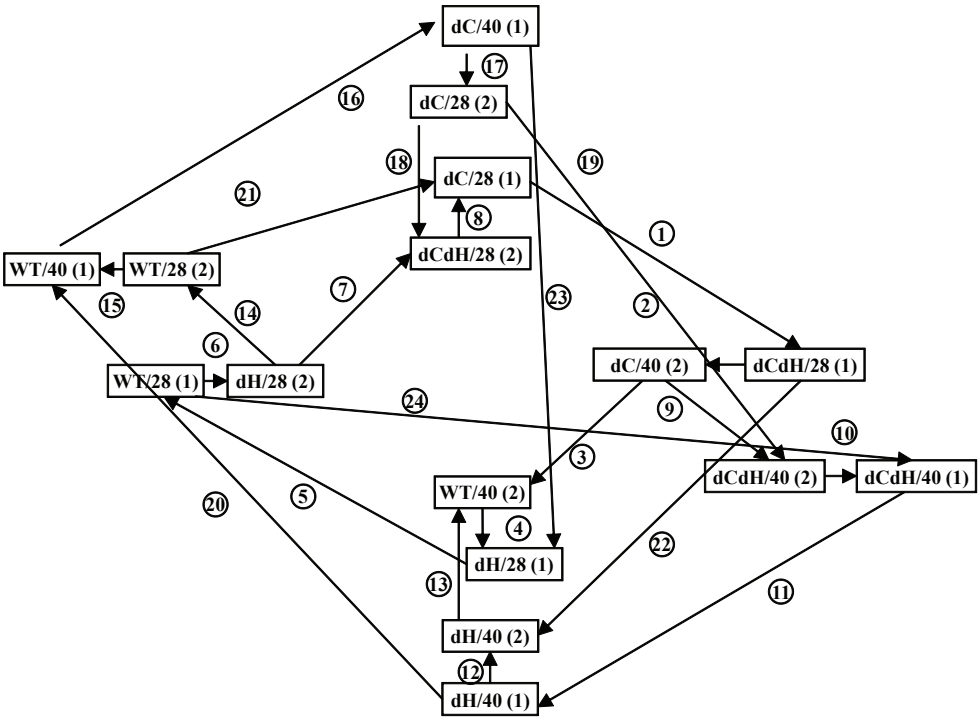

Fig. S1. Hybridization scheme for DNA microarrays using cDNA derived from *L. plantarum* WCFS1 (WT), NZ3410 (Δ*ctsR*; dC), NZ3425^{CM} (Δ*hrcA::cat*; dH), and NZ3423^{CM} (Δ*ctsR*Δ*hrcA::cat*; dCdH). Temperature in °C is indicated after the slash. Duplicates were included (between brackets) and circled number indicates hybridization number. Tail and head of the arrow represent Cy3 and Cy5 labeling, respectively.

Supplemental information for Fig. 4.

Abbreviation	Component
ACALD	acetaldehyde dehydrogenase (acetylating)
ACALDt	acetaldehyde reversible transport
ACKr	acetate kinase
ACLDC	acetolactate decarboxylase
ACLS	acetolactate synthase (Also catalyzes ACHBS)
ACt6	acetate transport in/out via proton symport
ACTNdiff	(R)-acetoin diffusion
ACTPASE	acylphosphatase

ALCD19	alcohol dehydrogenase (glycerol)
ALCD2x	alcohol dehydrogenase (ethanol: NAD)
ALDD2x	aldehyde dehydrogenase (acetaldehyde, NAD)
ALDD8x	aldehyde dehydrogenase (D-glyceraldehyde, NAD)
ALOX	oxidative decarboxylation of acetolacate (chemical)
ATPM	ATP maintenance requirement
BTDD-RR	(R,R)-butanediol dehydrogenase
BTDt1-RR	(R,R)-butanediol transport in/out via diffusion reversible
CITL	citrate lyase
CITt6	citrate transport in/out via proton symport
CRCT	CTP:D-ribitol-5-phosphate cytidylyltransferase
DHAPT	dihydroxyacetone phosphotransferase
DHAt	dihydroxyacetone transport via facilitated diffusion
DIACTt	diacetyl diffusion
ENO	enolase
ETOHt1	ethanol transport in/out via diffusion
F6PA	fructose-6-phosphate aldolase
FBA	fructose-bisphosphate aldolase
FORt2	formate transport in via proton symport
FRDx	fumarate reductase (NADH)
FUM	fumarase
G3PD1	glycerol-3-phosphate dehydrogenase (NAD)
G3PD4	glycerol-3-phosphate dehydrogenase (NAD)
G3PO	glycerol 3-phosphate oxidase
G6PDHy	glucose 6-phosphate dehydrogenase
GAPD	glyceraldehyde-3-phosphate dehydrogenase (NAD)
GLCNt2	D-gluconate transport via proton symport
GLCpts	D-glucose transport via PEP:Pyr PTS
GLYCK	glycerate kinase
GLYCt1	glycerol transport via uniport (facilitated diffusion)
GLYK	glycerol kinase
GNK	gluconokinase
LAR	lactate racemase
LDH_D	D-lactate dehydrogenase
LDH_L	L-lactate dehydrogenase
L-LACt2	L-lactate reversible transport via proton symport
MALLAC	malolactic enzyme
MDH	malate dehydrogenase
ME1x	malic enzyme (NAD)
NADH4	NADH dehydrogenase (Menaquinone 7 & no proton)
NOX1	NADH oxidase (H2O2 forming)
NOX2	NADH oxidase (H2O forming)

NPR	NADH peroxidase
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PFK	phosphofructokinase
PFL	formate C-acetyltransferase
PGDH	phosphogluconate dehydrogenase
PGI	glucose-6-phosphate isomerase
PGK	phosphoglycerate kinase
PGL	6-phosphogluconolactonase
PGM	phosphoglycerate mutase
PKL	phosphoketolase
PPCK	phosphoenolpyruvate carboxykinase
PPS	phosphoenolpyruvate synthase
PRPPS	phosphoribosylpyrophosphate synthetase
PTAr	phosphotransacetylase
PYK	pyruvate kinase
PYROX	pyruvate oxidase
PYRt2	pyruvate reversible transport via proton symport
RBK	ribokinase
RBLK2	L-ribulokinase (ribitol)
RBT5PDHy	ribitol-5-phosphate 2-dehydrogenase (NADP)
RIBt2	ribose transport in via proton symporter
RPE	ribulose 5-phosphate 3-epimerase
RPI	ribose-5-phosphate isomerase
SUCCt6	succinate transporter in/out via proton symport
TAL	transaldolase
TKT1	transketolase
TKT2	transketolase
TPI	triose-phosphate isomerase

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Modulation of *Lactobacillus plantarum* gastrointestinal robustness by fermentation conditions enables identification of bacterial robustness markers

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Abstract

Lactic acid bacteria (LAB) are applied worldwide in the production of a variety of fermented food products. Additionally, specific *Lactobacillus* species are nowadays recognized for their health promoting effects on the consumer. To optimally exert such beneficial effects, it is considered of great importance that these so-called probiotic bacteria reach their target sites in the gut alive. The probiotic model organism *Lactobacillus plantarum* WCFS1 was cultured under different fermentation conditions, which was complemented by the determination of the corresponding molecular responses by full-genome transcriptome analyses. In addition, the gastrointestinal (GI) survival of the cultures produced was assessed in an *in vitro* assay. Variations in fermentation conditions led to dramatic differences in GI-tract survival (up to 7-log) and high robustness could be associated with low salt and low pH during the fermentations. Moreover, random forest correlation analyses allowed the identification of specific transcripts associated with robustness. Subsequently, the corresponding genes were targeted by genetic engineering, aiming to enhance robustness, which could be achieved for 3 of the genes that negatively correlated with robustness and where deletion derivatives displayed enhanced survival compared to the parental strain. Specifically, a role in GI-tract survival could be confirmed for the *lp_1669*-encoded AraC-family transcription regulator, involved in capsular polysaccharide remodeling, the penicillin-binding protein Pbp2A involved in peptidoglycan biosynthesis, and the Na^+/H^+ antiporter NapA3. Moreover, additional physiological analysis established a role for Pbp2A and NapA3 in bile salt and salt tolerance, respectively. In conclusion, transcriptome trait matching enabled the identification of biomarkers for bacterial (gut-)robustness, which is important for our molecular understanding of GI-tract survival and could facilitate the design of culture conditions aimed to enhance probiotic culture robustness. Moreover, the molecular robustness markers can also facilitate the targeted selection of novel, more robust strains from culture collections.

Introduction

According to the world health organization (WHO) probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [1]. The most widely applied probiotics belong to the genera *Lactobacillus* and *Bifidobacterium* [2,3]. Their beneficial effects are considered to encompass several mechanisms, including the modulation of the intestinal microbiota, the production of antibacterial substances, improvement of epithelial barrier function, and reduction of intestinal inflammation [4-6]. Probiotics are most commonly provided through ingestion of freshly fermented food products or as dried bacterial preparations. The viability of bacteria is considered an important trait for probiotic functionality, justifying the interest to unravel the mechanism(s) involved in gastrointestinal (GI)-tract survival at the molecular level [7-10].

During passage through the GI-tract, probiotics encounter several stresses, including acidity in the stomach, exposure to bile and digestive enzymes in the intestine. Perhaps the greatest determinant of probiotic survival in the gut is tolerance to gastric acid present in the stomach which may reach a pH as low as 1 during fasting [9]. A low extracellular pH affects the proton motive force, thereby disrupting the energy supply required for processes such as membrane transport [11]. In addition, lower intracellular pH values caused by acidic conditions may inhibit specific pathways by damaging acid-sensitive associated enzyme functions [11]. In the small intestine, bile acids act primarily as a surfactant that can disrupt bacterial membranes [12] and damage macromolecules such as RNA and DNA through the generation of free oxygen radicals [13]. Moreover, protonated bile acids can freely pass cell membranes and release protons intracellularly which might lead to lowering of the intracellular pH, analogous to acid stress [9].

Among the lactobacilli, *Lactobacillus plantarum* is encountered in a plethora of fermentations, ranging from vegetables to dairy and meat [14]. Next to this dietary abundance, *L. plantarum* is frequently encountered as a natural inhabitant of the GI-tract of several mammals, including humans [15]. Specific strains of this species are marketed as probiotics. In addition, *L. plantarum* NCIMB8826 was demonstrated to effectively survive passage of the human stomach, reached the ileum in high numbers, and was detected in the colon [16]. A single colony isolate of this strain (designated *L. plantarum* strain WCFS1) was the first *Lactobacillus* strain of which the full genome sequence was published [17]. Subsequently, sophisticated bioinformatics tools were developed for this LAB, including an advanced genome annotation [18], and genome-based metabolic models [19], as well as effective mutagenesis tools [20]. This enables the molecular investigation of gene-regulatory mechanisms underlying the observed GI-tract persistence of *L. plantarum* WCFS1.

Another post-genomic approach employs the exploration of genomic diversity among *L. plantarum* strains, using comparative genome hybridization databases to identify candidate bacterial effector molecules responsible for phenotypic differences between the strains by genotype-phenotype correlations [14,21]. Results obtained utilizing such approaches include the *in vitro* identification of the gene encoding the mannose-specific adhesin in *L. plantarum* WCFS1 that was subsequently

shown to elicit specific innate immune responses in pig mucosal tissues *in vivo* [22,23]. Other examples are the association of specific genetic loci with the immunomodulatory capacity of *L. plantarum* WCFS1 on both dendritic and peripheral blood mononuclear cells [24,25]. Despite the success of genotype-phenotype matching strategies, this approach is intrinsically limited to the identification of factors of which the gene absence/presence varies in different *L. plantarum* strains [10]. However, differences in phenotypes like stress tolerance are likely to be predominantly determined by differential gene expression levels of genes that are conserved among all strains, or even all lactobacilli (e.g. the HrcA regulator and its regulon [26]). This notion is supported by a recent study that concluded that closely related *Lactococcus lactis* strains express very different levels of conserved enzyme functions as a consequence of highly differential and strain specific regulation of gene expression under different environmental conditions [27]. Analogously, the survival capacities of probiotic bacterial strains can be strongly influenced by the way they are produced or at which stage of growth they are harvested [28,29].

To enable the identification of genes of which the expression level is correlated to the phenotype of interest, we recently developed a transcriptome-phenotype matching fermentation platform that has been shown to allow detection of transcripts involved in growth and stress response of *L. plantarum* [30]. Here we employed this fermentation genomics platform to correlate transcriptome data to GI-tract survival using the random forest algorithm [31]. These correlations led to the identification of 13 candidate effector molecules for GI-tract persistence. A subsequent gene deletion strategy established a definite role in GI-tract persistence for the AraC-family transcription regulator encoded by *lp_1669*, the penicillin-binding protein Pbp2A involved in peptidoglycan biosynthesis, and the Na^+/H^+ antiporter NapA3.

Materials and Methods

Strains and growth conditions

Strains used in this study and their relevant characteristics are listed in Table 1. To induce differential transcriptome in *L. plantarum* WCFS1, a fermentation scheme was designed with five variable parameters, namely temperature (28 or 37°C), pH (5.2, 5.6 or 6.2), and/or amino-acid (1.1× or 2.0× standard amounts, see below), oxygen (N_2 or air), and NaCl (0 or 0.3M) availability (Table 2).These variable parameters were combined into a combinatorial fermentation scheme on the basis of a balanced fractional factorial design [32].

The fermentation scheme designed above was applied to pH-controlled batch fermentations at 0.5 L scale in a Multifors mini-in parallel fermentor system (Infors-HT Benelux, Doetinchem, The Netherlands). For inoculation of the fermentors, a single colony isolate of *L. plantarum* WCFS1 [17] was used to inoculate 5 mL of chemically defined medium (2× CDM) [18] and grown overnight at 37°C. This full-grown culture was used to prepare a dilution range ranging from

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant feature(s)[a]	Reference
Strains		
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	[17]
NZ3412^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *pbp2A* (Δ*pbp2A::cat*)	This work
NZ3412	Derivative of WCFS1 containing a *lox72* replacement of *pbp2A* (Δ*pbp2A*)	This work
NZ3417^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *lp_1669* (Δ*lp_1669::cat*)	This work
NZ3414^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *lp_1817* (Δ *lp_1817::cat*)	This work
NZ3415^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *pacL3* (Δ *pacL3::cat*)	This work
NZ3416^{CM}	Derivative of WCFS1 containing a *lox66*-P32-cat-*lox71* replacement of *napA3* (Δ*napA3::cat*)	This work
NZ3416	Derivative of WCFS1 containing a *lox72* replacement of *napA3* (Δ*napA3*)	This work
NZ3419^{CM}	Derivative of NZ3412 containing a *lox66*-P32-cat-*lox71* replacement of *napA3* (Δ*pbp2A-*Δ*napA3::cat*)	This work
NZ3418^{CM}	Derivative of NZ3416 containing a *lox66*-P32-cat-*lox71* replacement of *napA3* (Δ*napA3-*Δ*lp_1669::cat*)	This work
SIP411	Derivative of WCFS1 harboring the pSIP411 plasmid	This work
SIP411B	Derivative of WCFS1 harboring the pSIP411B plasmid (empty vector)	This work
NZ3430	Derivative of WCFS1 harboring the pNZ3430 plasmid (over-*lp_1357*)	This work
NZ3431	Derivative of WCFS1 harboring the pNZ3431 plasmid (over-*hicD3*)	This work
NZ3432	Derivative of WCFS1 harboring the pNZ3432 plasmid (over-*thrC* and *lp_2759*)	This work
NZ3433	Derivative of WCFS1 harboring the pNZ3433 plasmid (over-*lp_0148~0150*)	This work
NZ7021	Derivative of WCFS1 harboring the pNZ2021 plasmid (empty vector)	[49]
NZ7026	Derivative of WCFS1 harboring the pNZ2026 plasmid (over-*folB, folP, folk, folE, xtp2,* and *folC2*)	[49]
E. coli		
TOP-10	Cloning host; F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^r) *endA1* λ$^-$	Invitrogen
MC1061	Cloning host; *araD139* Δ(*araA-leu*)7697 Δ*lacX74 galK16 galE15*(*GalS*) λ$^-$ *e14*$^-$ *mcrA0 relA1 rpsL150*(str^r) *spoT1 mcrB1 hsdR2*	[72]
Plasmids		
pNZ5319	Cm^r Em^r; for multiple gene replacements in Gram-positive bacteria	[20]
pNZ3412	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *pbp2A*	This work
pNZ3417	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *lp_1669*	This work
pNZ3414	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *lp_1817*	This work
pNZ3415	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *pacL3*	This work
pNZ3416	Cm^r Em^r; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *napA3*	This work
pSIP411	Em^r; cloning vector	[46]
pSIP411B	Em^r; pSIP11 derivative without the *gusA* gene (empty vector)	This work
pNZ3430	Em^r; pSIP411 derivative containing the *lp_1357* gene of WCFS1	This work
pNZ3431	Em^r; pSIP411 derivative containing the *hicD3* gene of WCFS1	This work
pNZ3432	Em^r; pSIP411 derivative containing the *thrC* and *lp_2759* operon of WCFS1	This work
pNZ3433	Em^r; pSIP411 derivative containing the *lp_0148~0150* operon of WCFS1	This work
pNZ7021	Cm^r; (empty vector)	[49]
pNZ7026	Cm^r; pNZ7021 derivative containing the *folB, folP, folk, folE, xtp2,* and *folC2* gene cluster of WCFS1	[49]
pNZ5348	Em^r; containing *cre* under the control of the *pcrA* (*lp_1144*) promoter	[20]

[a] Str^r, streptomycin resistant; Cm^r chloramphenicol resistant; Em^r, erythromycin resistant.

10^{-1} to 10^{-6} in fresh medium and the dilutions were grown overnight. Subsequently, the cultures were photospectrometrically assessed and the culture with an OD_{600} nearest to 1.5, representing logarithmic growing cells, was used to inoculate the fermentors at an initial OD_{600} of 0.1. Prior to inoculation the media in the fermentors were adjusted to the appropriate pH and temperature according to the design. During fermentation the cultures were stirred at 125 rpm, the initial pH was maintained by automated titration with 2.5M NaOH, (Infors-HT Benelux, Doetinchem, The Netherlands), and the cultures were sparged with N_2 or Air at a rate of 150mL/min. Moreover, CO_2 was mixed into these gas-phases at a final concentration of 2.5%, to prevent previously established growth stagnation of *L. plantarum* WCFS1 under aerobic conditions [33]. Cells were harvested at OD_{600} = 1.0 for full-genome transcriptome profiling, while the GI-tract survival was determined in the same cells, as well as in cells that were harvested 25 h after inoculation.

GI-tract assay

For GI-tract survival analysis, cultures were washed with prewarmed (37°C) PBS and resuspended in prewarmed (37°C) filter sterilized gastric juice [53mM NaCl, 15mM KCl, 5mM Na_2CO_3, 1mM $CaCl_2$, 0.1 mg ml^{-1} lipase (Fluka 62301-1G-F; derived from *Aspergillus niger*), and 1.2 mg ml^{-1}

Table 2. Fermentation conditions used in this study.

Fermentation	NaCl (mM)	Amino acid concentration[a]	Temperature (°C)	pH	O_2
A	0	2.0	37	5.2	-
B	300	2.0	37	5.2	-
C	0	1.1	37	5.2	+
D	300	1.1	37	5.2	+
E	0	2.0	28	5.2	+
F	300	2.0	28	5.8	+
G	300	2.0	28	5.2	+
H	0	1.1	28	5.2	-
I	300	1.1	28	5.2	-
J0	0	2.0	37	6.4	+
K	300	2.0	37	6.4	+
L	0	1.1	28	5.8	-
M	0	1.1	37	6.4	-
N	300	1.1	37	6.4	-
O	0	2.0	28	6.4	-
P	300	2.0	28	6.4	-
Q	0	1.1	28	6.4	+
R	0	2.0	37	5.8	-
S	0	2.0	28	5.8	+
T	300	2.0	28	5.8	+
U	0	1.1	28	5.8	-
V	300	1.1	28	5.8	-
W	0	2.0	28	5.8	+
X[b]	0	2.0	37	5.8	-
Y	300	1.1	28	6.4	+
Z	0	2.0	37	5.8	-
AA	300	2.0	37	5.8	-
AB	0	1.1	37	5.8	+
AC	300	1.1	37	5.8	+
AD	0	1.1	28	5.2	-

[a] Fold change based on the original CDM [18].

[b] From this fermentation no samples were taken at logarithmic phase.

pepsin (Sigma P-7125 from porcine) that had a pH adjusted to 2.4 with HCl (logarithmic cells) or 2.3 (stationary cells)]. The gastric juice enzymes were added immediately prior to the treatment. After 60 min incubation while rotating at 10 rpm in a Hybridization oven/shaker (RPN2511E, Amersham pharmacia biotech, Little Chalfont, UK) at 37°C, the cultures were neutralized to pH 6.5 with 0.5M $NaHCO_3$, and prewarmed (37°C) pancreatic juice [85mM NaCl, 5mM KH_2PO_4, 2mM Na_2HPO_4, 10mM $NaHCO_3$, 30 mg ml^{-1} pancreatin (Sigma P7545; derived from porcine stomach) and bile acid mixture (latter two were added immediately prior to pancreatic juice prior to the treatment)] was added, followed by continued incubation for another 60 min (rotating 10 rpm, 37°C). The bile acid mixture consisted of 3.0 mM (final concentration in assay) sodium glycocholate hydrate, 1.3 mM sodium glycodeoxycholate, 2.4 mM sodium glycochenodeoxycholate, 1.0 mM taurocholic acid sodium salt hydrate, 0.4 mM sodium taurodeoxycholate hydrate and 1.0 mM sodium taurochenodeoxycholate to mimic human bile components and concentrations [34]. Preceding and during GI-tract assay incubation (t = 0, 20, 40, 60, 90, and 120), samples were taken for colony forming unit (CFU) enumeration by spot-plating [35]. In total a reduction of 8 logs could be detected. Relative GI-tract survival of the different cultures was expressed as the fraction of the corresponding input numbers of viable cells (t=0 was set at 1.00).

RNA isolation and DNA microarrays

RNA isolation from *L. plantarum*, subsequent cDNA synthesis and indirect labeling, as well as DNA microarray hybridizations were performed using routine procedures [24,36]. Briefly, 10 mL samples derived from the fermentors at an OD_{600} of 1.0 were quenched [37] prior to RNA isolation, and 5 μg of isolated RNA was used for cDNA synthesis and indirect labeling with cyanine 5 (Cy5) or cyanine 3 (Cy3) [24,36]. The DNA microarray hybridization scheme consisted of a loop design that consisted of smaller sub-loops containing all samples gathered from a single fermentation run (Fig. 1). A two-color microarray-based gene expression analysis was performed on a custom-made 60-mer oligonucleotide array [Agilent Biotechnologies, submitted in GEO under platform (GPL13984)] to determine genome-wide, absolute gene transcription levels. Co-hybridization of

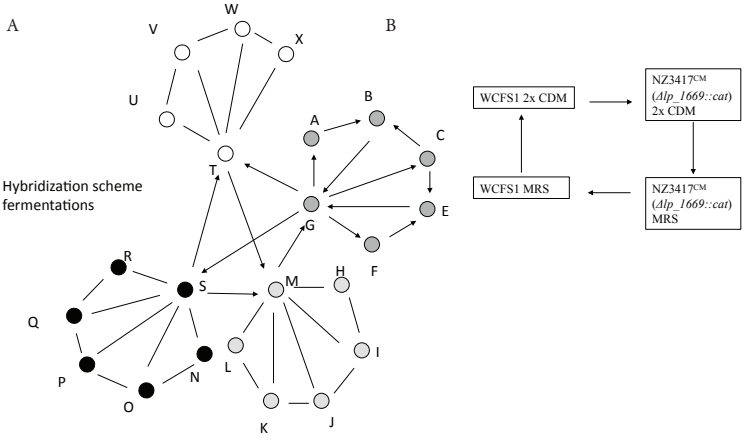


Fig. 1. Hybridization schemes. Tail and head of the arrow represent Cy3 and Cy5 labeling, respectively. Panel A: Fermentations hybridization schemes. Letters represent fermentations. All loops are labelled as the A-G loop. Panel B: Lp_1669 regulon hybridization scheme.

Cy5- and Cy3-labeled cDNA probes was performed on these oligonucleotide arrays at 42°C for 16 h in Slidehyb#1 (Ambion, Austin, USA). Subsequently, the slides were washed twice in 1× SSC containing 0.1% sodium dodecyl sulfate and twice in 1× SSC before they were scanned. Slides were scanned with a ScanArray Express 4000 scanner (Perkin Elmer, Wellesley, USA), and image analysis and processing were performed using the ImaGene Version 7.5 software (BioDiscovery Inc., Marina Del Rey, USA). The microarrays were scanned at different intensities and for each of the microarrays the best scan was selected on the basis of signal distribution (low number of saturated spots and a low number of low signal spots). The data were normalized using Lowess normalization [38] as available in MicroPrep [39]. The data were corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [39]. The median intensity of the different probes per gene was selected as the gene expression intensity. This analysis resulted in genome-wide, absolute gene expression levels for *L. plantarum* WCFS1 derived from the fermentations. In addition, the transcriptome of *L. plantarum* WCFS1 and NZ3417^{CM} (Δ*lp_1669::cat*, see below) grown in 2× CDM and MRS (de Man-Rogosa-Sharpe) broth (Difco, West Molesey, United Kingdom) at 37°C was analyzed as described above. The hybridization scheme is shown in Figure 1. In addition, CyberT [40] was used for calculation of gene expression ratios and false discovery rate (FDR) p-values. Genes of the Lp_1669 regulon with FDR-adjusted p-values less than 0.05 together with a fold-change than 2.0 or lower than 0.5 were considered to be significantly differently expressed. All microarray data is MIAME compliant and is available in the GEO database (GSE31076 and GSE31254 for the fermentations and Lp_1669 regulon, respectively).

Data storage, visualization tools, and correlation statistics

A MySQL-based storage system for data produced from the fermentation, transcriptomics and phenotypical experiments (e.g. the gastrointestinal survival presented here but also other functional characteristics such as metabolite profiles [30]) was developed. Statistical methods, ANOVA [41] and Random Forest [31], were implemented to enable data significance and correlation analysis, respectively. Both the storage system and the statistical methods have been integrated into a freely accessible, web-based platform designated FermDB (www.cmbi.ru.nl/fermdb). One set of fermentations was excluded from the data analysis as the GI-tract survival data appeared unreliable, likely caused by minor deviations in the pH of the batch of GJ applied which is known to heavily influence GI survival (Van Bokhorst-van de Veen *et al.* unpublished data). The biomolecular interaction network of the Lp_1669 regulon in 2× CDM and MRS was visualised using the Cytoscape software (version 2.8.1) [42], and the Biological Networks Gene Ontology (BiNGO) tool [43] was employed to detect significantly overrepresented categories in the regulon of Lp_1669.

Deletion mutant construction

Gene deletion mutants were constructed using the mutagenesis vector pNZ5319 according to Lambert et al. [20]. The *L. plantarum* WCFS1 *pbp2A, lp_1669, lp_1818, pacL3,* and *napA3* genes were replaced with a *lox66*-P_{32}-*cat*-*lox71* cassette resulting in strains NZ3412^{CM} (Δ*pbp2A::cat*),

NZ3417^{CM} (Δ*lp_1669::cat*), NZ3414^{CM} (Δ*lp_1817::cat*), NZ3415^{CM} (Δ*pacL::cat*), and NZ3416^{CM} (Δ*napA3::cat*), respectively. Primers sequences used to construct the gene-targeted knock-out vectors for *L. plantarum* WCFS1 are provided in Table S1. In short, upstream and downstream flanking regions (left flank, LF; right flank, RF, respectively) of the target genes (i.e., *pbp2A, lp_1669, lp_1817, pacL3,* and *napA3*) were amplified with primer pair combinations as listed in Table S2. Primers at the 3'-end of the upstream and 5'-end of the downstream flanking regions (A3, A4, B3, B4, C3, C4, D3, D4, E3, and E4) were extended with an overlap-sequence complementary to the 5' and 3' end of the *lox66*-P_{32}-*cat-lox71* cassette (amplified with primers I and J [44]), to enable knock-out construction by a Splicing by overlap extension (SOE) PCR [45] with primer pairs as listed in Table S2. The obtained (SOE-ing) amplicons were blunt-ligated into *Ecl*136II-*Swa*I digested pNZ5319 [20] resulting in plasmids pNZ3412, pNZ3417, pNZ3414, pNZ3415, and pNZ3416 (see Table 1). *Escherichia coli* was used as an intermediate cloning host and after introduction of the mutagenesis plasmids into competent *L. plantarum* WCFS1, cells were plated on MRS containing 10 μg·ml^{-1} chloramphenicol. After 48 h, grown colonies were plated on MRS with and without 30 μg·ml^{-1} erythromycin. Colonies from each mutant displaying the anticipated erythromycin sensitive phenotype were selected for colony-PCR using primer pairs as listed in Table S3. Mutant colonies with the expected genetic organization were selected for each of the knock-out target loci; NZ3412^{CM} (Δ*pbp2A::cat*), NZ3417^{CM} (Δ*lp_1669::cat*), NZ3414^{CM} (Δ*lp_1817::cat*), NZ3415^{CM} (Δ*pacL::cat*) and NZ3416^{CM} (Δ*napA3::cat*). The *L. plantarum* WCFS1 *pbp2A* plus *napA3* and *napA3* plus *lp_1669* double-mutants were constructed in the NZ3412^{CM} (Δ*pbp2A::cat*) and NZ3416^{CM} (Δ*napA3::cat*) background, respectively, in a two-step procedure. Firstly, strains NZ3412 (Δ*pbp2A*) and NZ3416 (Δ*napA3*) were constructed by excision of the *lox66*-P_{32}-*cat-lox71* cassette by transient expression of the Cre resolvase enzyme from pNZ5348 according to methods described by Lambert et al. [20]. In these deletion mutant strains, pNZ3416 and pNZ3417 were introduced and double mutant strains were selected using the approach described above, resulting in the isolation of strains NZ3419^{CM} (Δ*pbp2A*-Δ*napA3::cat*) and NZ3418^{CM} (Δ*napA3*-Δ*lp_1669::cat*), respectively (Table 1).

Overexpression mutant construction

Gene overexpression mutants were constructed using the expression vector pSIP411 [46]. Primers were designed (Table S1) to introduce a restriction enzyme site for cloning the target gene(s) into the expression vector pSIP411 at the *Nco*I site. The *lp_1357* and *thrC+lp_2759* overexpression mutants were designed with *BspH*I site, which has compatible ends with *Nco*I site. The target gene(s) were amplified by PCR using corresponding primers for each mutant (F1/F2, G1/G2, H1/H2 and I1/I2 for *lp_1357, lp_2349, thrC+lp_2759*, and *lp_0148~0150* mutants, respectively). The reactions were carried out with KOD polymerase (Novagen, Darmstadt, Germany) according to the instructions of the manufacturer. The purified PCR products were digested by restriction enzymes (Invitrogen, Molecular probes, Inc, USA) for which sites were introduced in the primers (see Table S1) and cloned in NcoI-SmaI digested pSIP411. Ligation mixtures were transformed to *Escherichia coli*, and re-isolated from primary transformants. Correctly assembled overexpression plasmids were identified by PCR, restriction and sequence analysis. Re-isolated plasmids were propagated into *L.*

plantarum WCFS1 and transformants were selected on MRS containing 30 μg·ml^{-1} erythromycin.

For protein analysis of the overexpression mutants, the induction and sample preparation procedures were modified from the description by Sørvig et al. [46]. The 19-amino-acid inducing peptide (of Met-Ala-Gly-Asn-Ser-Ser-Asn-Phe-Ile-His-Lys-Ile-Lys-Gln-Ile-Phe-Thr-His-Arg [47]) was custom-synthesized by BACHEM (Budendorf, Switzerland). The inducing peptide was dissolved in degassed water, as recommended by BACHEM to avoid oxidation of the peptides. The overnight cultures of the overexpression strains were diluted 50-fold and then incubated at 37°C. After OD_{600} had reached 0.3, the inducing peptide was added to the cultures at varying concentrations of 0, 0.1, 1, 10, and 50 ng/ml. Incubation was continued at 37°C for another 4 hours until the OD_{600} had reached approximately 1.8. Bacterial cells were collected by the centrifugation at 5,200× *g* for 10 min, followed by resuspension of the pellet in 50 mM Sodium-phosphate buffer pH 7. The cells were disrupted with 1 g zirconium beads by using a FastPrep™ (Qbiogene Inc, Cedex, France). After the disruption, the samples were centrifuged 5 min at 20,800× *g* to obtain cell-free extracts for analysis by SDS-PAGE.

Phenotypic assays of mutant strains

Gene deletion mutants were analyzed for their gastrointestinal survival characteristics in a procedure identical to that described for the wild-type (see above). To evaluate the relative GI-tract survival of the overexpression mutants, the mutant strain SIP411B (empty vector) and the overexpression mutants were sakacin-induced (50ng/ml) (see above). Additionally, to measure the relative GI-tract survival of the folate overexpression strain, strains NZ7021 (empty vector) and NZ7026 (folate overproducing strain) [48] were inoculated at OD_{600} = 0.1 in MRS containing 80 mg/ml chloramphenicol and 0 or 10mg/ml *p*-aminobenzoic acid (*p*ABA) according to Wegkamp et al. [49], grown at 37°C until OD_{600} was 1.0, and subjected to the GI-tract survival assay. To evaluate relative growth efficiency of the deletion mutants, the parental strain (WCFS1) and mutant strains NZ3412^{CM} (Δ*pbp2A::cat*), NZ3417^{CM} (Δ*lp_1669::cat*), and NZ3416^{CM} (Δ*napA3::cat*) were inoculated at OD_{600} = 0.1 in 96-wells plates and incubated in MRS broth at 28°C. OD_{600} of the cultures was monitored spectophotometrically (GENios, Tecan Austria GmbH, Grödig, Austria).

Capsular polysaccharide isolation and determination

Capsular polysaccharide (CPS) was purified and chain lengths and sugar composition were determined essentially as described before [50]. Briefly, 500 ml cultures of *L. plantarum* WCFS1 and NZ3417^{CM} (Δ*lp_1669::cat*) were grown in 2× CDM at 37°C until stationary phase (25 h). After 1 h incubation at 55°C, the cells were separated from the CPS containing growth medium by centrifugation for 15 min (6000× *g*) and to prevent overgrowth during dialysis, erythromicine was added to the supernatant to a final concentration of 10μg/ml. A dialyzing tube 12-1400 Da (Fisher Scientific) was prepared by boiling twice 2% $NaHCO_3$ / 2 mM EDTA, and once in reverse osmosis water. After overnight dialysis against running tap water followed by 4 h dialysis using reverse osmosis water, the samples were freeze-dried and stored at -20°C until further analysis.

The samples were dissolved in eluent (100 mM $NaNO_3$ + 0.02% NaN_3), filtered over 0.2 µm, and placed in a thermally controlled sample holder at 10°C and 200 µl was injected (model 231 Bio, Gilson) on the columns connected in series and remained at 35°C with a temperature control module (Waters, Milford, USA) to perform size exclusion chromatography (SEC) [TSK gel PWXL guard column, 6.0 mm × 4.0 cm, TSK gel G6000 PWXL analytical column, 7.8 mm × 30 cm, 13.0 µm and TSK gel G5000 PWXL analytical column, 7.8 mm x 30 cm, 10 µm (TosoHaas, King of Prussio, USA)]. Light scattering was measured at 632.8 nm at 15 angles between 32° and 144° (DAWN DSP-F, Wyatt Technologies, Santa Barbara, USA). UV absorption was measured at 280 nm (CD-1595, Jasco, de Meern, The Netherlands) to detect proteins. The specific viscosity was measured with a viscosity detector (ViscoStar, Wyatt Technologies, Santa Barbara, USA) at 35°C and sample concentration was measured by refractive index detection, held at a fixed temperature of 35°C (ERC-7510, Erma Optical Works, Tokyo, Japan).

During the analysis with SEC the polysaccharide peak was collected (2 min x 0.5 ml/min = 1 ml). The acid hydrolyses of the collected polysaccharide was carried out for 75 min at 120°C with 2 M trifluoro acetic acid under nitrogen. Following hydrolyses, the solutions were dried overnight under vacuum and dissolved in water. High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on a gold electrode was used for the quantitative analyses of the monosaccharides rhamnose, galactosamine, arabinose, glucosamine, galactose, glucose, mannose, xylose, galacturonic acid, and glucuronic acid. The analyses were performed with a 600E System controller pump (Waters, Milford, USA) with a helium degassing unit and a model 400 EC detector (EG&G, Albuquerque, USA). With a 717 autosampler (Waters, Milford, USA), 20 µl of the sample was injected on a Dionex Carbopac PA-1, 250 × 4 mm (10-32), column thermostated at 30°C. The monosaccharides were eluted at a flow rate of 1.0 ml/min. The monosaccharides were eluted isocratic with 16 mM sodium hydroxide, followed by the elution of the acid monosaccharides starting at 20 min with a linear gradient to 200 mM sodium hydroxide + 500 mM sodium acetate in 20 minutes. Data analysis was done with Dionex Chromeleon software version 6.80. Quantitative analyses were carried out using standard solutions of the monosaccharides (Sigma-Aldrich, St. Louis, USA).

Results

Gastric acidity is a critical determinant of *L. plantarum* survival

An *in vitro* assay was developed that allows a high-throughput assessment of bacterial GI-tract survival (Fig. 2A). Two independent reference *L. plantarum* WCFS1 cultures that were harvested during logarithmic phase of growth (OD_{600}=1) displayed a 6-log decrease in CFU ml^{-1} in the GI-tract assay (Fig. 2B). The survival curves of these reference cultures demonstrated the major impact on survival exerted by gastric juice on *L. plantarum* viability and the relatively minor effect of the conditions which resembled the small intestine (Fig. 2B). This differential effect on survival during the two stages within the GI-tract assay was consistently observed for all cultures tested, irrespective of the fermentation conditions applied or the growth phases from which bacterial cells were harvested.

The strongest determinant in the loss of survival during the gastric juice treatment appeared to be the pH. For screening log-phase cells of *L. plantarum,* a pH of 2.4 was used for cells, because lowering or increasing of the gastric juice pH by 0.1 pH unit resulted in death or survival of almost all cells, respectively (data not shown). *L. plantarum* cells harvested at the stationary phase of growth consistently displayed a higher tolerance to the gastric juice treatment, which is exemplified by their higher survival rate in the GI-tract assay when a reduced pH of 2.3 was used (Fig. 2B), a at which the cells harvested from the logarithmic phase of growth were nearly all killed within 60 minutes of incubation.

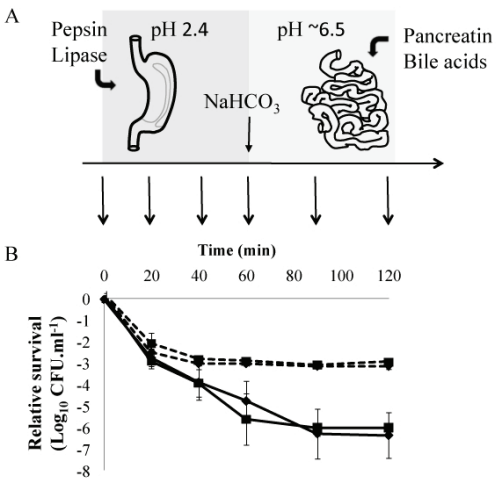


Fig. 2. Relative survival of *L. plantarum* cells, subjected to an upper gastrointestinal-tract mimicking assay. *L. plantarum* WCFS1 cultures were grown aerobically at 28°C in 2× CDM containing normal acid concentration, at a pH of 5.8 and without NaCl. The cultures were harvested at mid-exponential phase (OD_{600}=1.0) and subjected to an upper GI-tract mimicking assay (A): After 60 min incubation in gastric juice containing pepsin and lipase at a pH of 2.4 (logarithmic cells) or 2.3 (stationary cells), cultures were neutralized with $NaHCO_3$ and pancreatic juice containing pancreatin and bile acids was added and incubation continued for 60 min (see materials and methods for details). Preceding and during incubation, samples were taken for CFU determination (aligned arrows). Panel B shows the relative survival of two independent cultures in logarithmic phase (solid lines) and stationary phase (dashed lines) during the GI-tract mimicking assay. Input is set at 0 Log_{10} CFU ml^{-1}, data presented are averages of technical sextuplicates (+/- standard deviation).

Fermentation-enhanced digestive tract survival

We then examined the effects of different growth conditions on *L. plantarum* WCFS1 GI-tract survival. *L. plantarum* WCFS1 was harvested from the logarithmic and stationary phase (25h of growth) of growth in fermentors in which mild stresses were applied. Notably, these fermentations employed a fractional factorial experimental design to assess the combined effect of mild stresses using a relatively small number of fermentations that varied in pH, temperature, NaCl concentration, oxygen, and amino acid availability (Fig. 3.). The results demonstrate that fermentation conditions used to culture *L. plantarum* WCFS1 conferred a profound influence on the GI-tract survival of that microorganism. Fermentation conditions resulted major differences (a reduction of 7 logs for

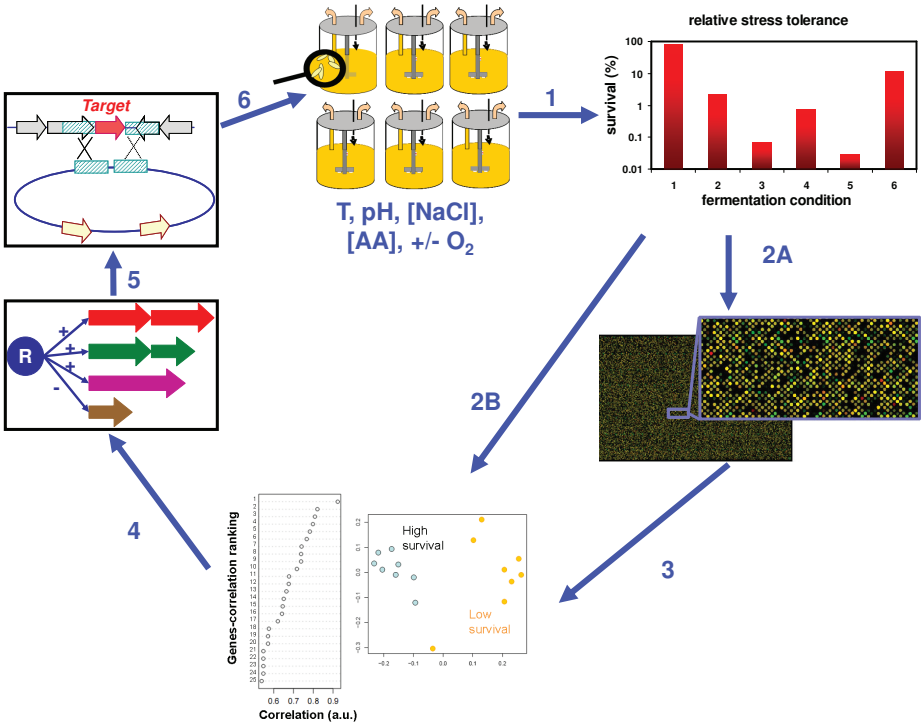


Fig. 3. Workflow for the fermentation genomics platform. Variations in fermentation conditions were coupled to a GI-tract mimicking assay aiming to determine the survival characteristics (arrow 1). Transcriptome profiles of the bacteria obtained from the different fermentations (arrow 2A) were determined and were via gene expression pattern comparison and regulatory network reconstructions (arrow 3) correlated to stress tolerance characteristics using the correlation algorithms random forest (arrow 2B). This approach lead to the identification of candidate genes, that are potentially of importance for GI-tract survival generated by the varying growth conditions (arrow 4). Mutagenesis (arrow 5) of these candidate stress factors (either gene deletion or overexpression strategies) and their subsequent evaluation using the same fermentation-coupled stress tolerance set-up (arrow 6) enabled validation of the postulated correlation. Figure adapted from [10].

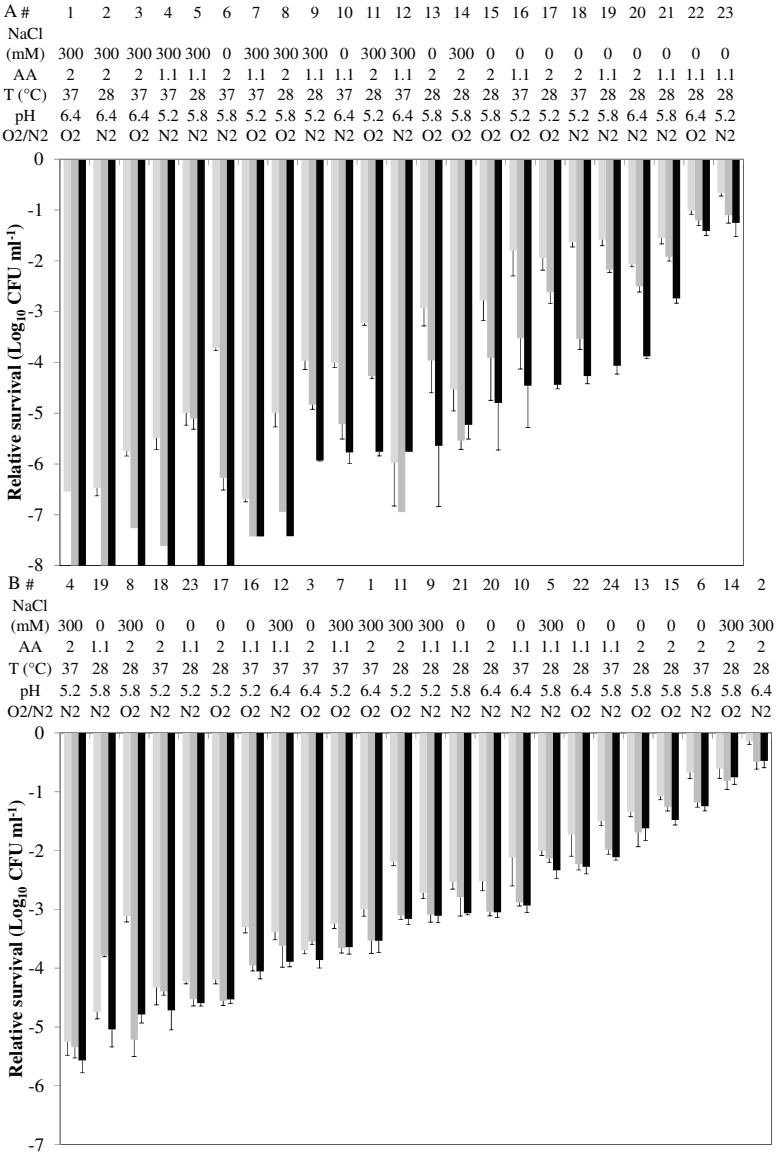


Fig. 4. Relative GI-tract survival of differently grown *L. plantarum* WCFS1. Log_{10} CFU ml^{-1} determination of *L. plantarum* WCFS1 in logarithmic phase (A) and stationary phase (B) after 20 (light grey), 40 (dark grey), and 60 min (black) gastric juice incubation. Input is set at 0 Log_{10} CFU ml^{-1}, # = fermentation number, cultures were grown in 2× CDM with (300 mM) or without (0) NaCl; with normal amino acid concentration (2) or reduced (1.1); at 28°C or 37°C; medium buffered at a pH of 5.2, 5.8, or 6.4; and aerobically (O_2) or anaerobically (N_2). Data presented are averages of technical sextuplicates (– standard deviation).

the logarithmic population and 5 logs for stationary cells) in *L. plantarum* WCFS1 survival after incubation in gastric juice (Fig. 4). Notably, survival of cultures grown in different fermentation conditions strongly exceeded the levels of variation in survival observed in independent GI-tract assays (Fig. 2B).

To identify the fermentation conditions that significantly affected the survival rate in the simulated GI-tract conditions, a Mann-Whitney U test was performed on all time points measured. The presence of 300 mM additional NaCl in the growth medium resulted in a significant ($P < 0.05$) negative influence on *L. plantarum* GI-tract survival irrespective whether cells were analyzed after collection from either logarithmic or stationary phase of growth (shown for 60 min incubation in Fig. 5A and B). *L. plantarum* grown in more acidic conditions (pH 5.2 instead of pH 6.4) and harvested in stationary phase showed a significantly ($P < 0.05$) enhanced the gastric juice survival rate (shown for 60 min Fig. 5C).

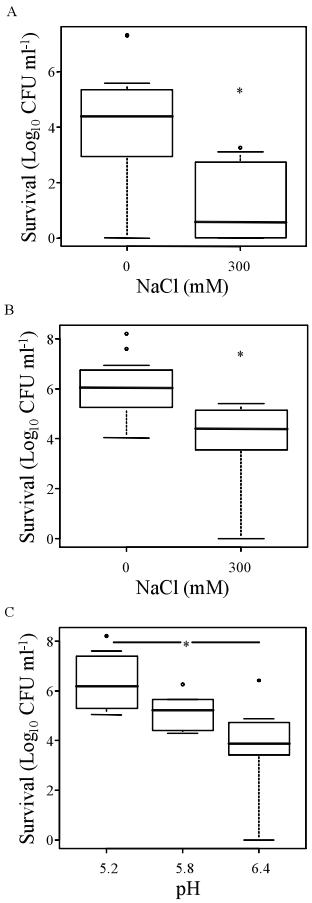
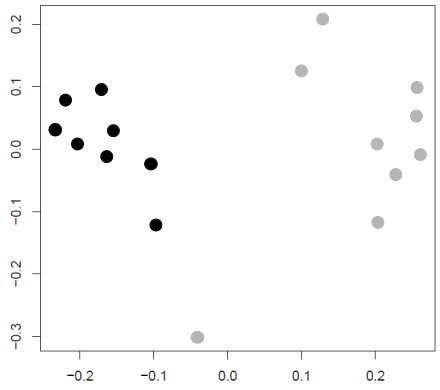


Fig. 5 (left). Effect of medium components on GI-tract survival of *L. plantarum* WCFS1. Box plots of NaCl and 60 min GI-tract survival of logarithmically (A) and stationary (B) grown cultures and of pH and 60 min GI-tract survival of stationary cells (C). Results are based on data from all fermentations used in this study (see Fig. 4A). * P-value < 0.05 compared with 0 mM NaCl (A and B) or pH 6.4 (C).

Fig. 6 (below). MDS plot of the eight best and the eight poorest surviving *L. plantarum* WCFS1 cultures grown under different growth conditions after GI-tract passage. Sample distances of good (black circles) and poor (grey circles) surviving cultures (see Fig. 4A). Classification is based on the transcriptomes of these cultures just before subjection to the GI-tract survival assay.



Transcriptome to phenotype association identifies candidate effector molecules for GI-tract survival

In parallel with the GI-tract survival patterns, transcriptome profiles were identified for logarithmic cells harvested from all fermentation conditions employed in this study (Fig. 3). To investigate whether high- and low-rate surviving cultures in the GI-tract assay could be distinguished based on the expression of specific genes, the cultures were first ranked on their GI-tract survival after gastric juice incubation (t=60 min). For cultures that had retained undetectable survival rates after 60 min of gastric incubation, the relative survival rates after 20 min and 40 min of gastric incubation, were employed to refine their relative survival ranking (Fig. 4A).

The transcriptomes of the eight cultures with highest survival rates and the eight cultures with the lowest survival in the GI-tract assay were clearly distinguishable according to principal component analysis (PCA) (Fig. 6). This result indicated that the transcriptomes contained information (genes) within the first two components of the PCA which might allow the discrimination between high- and low-survival rates in the GI-tract. To identify specific transcripts that discriminate between low and high GI-tract survival, and thus can be regarded as candidate robustness markers, the random forest algorithm was applied [31]. This allowed the identification of transcripts that have a high contribution to accurately predict the low- and high-survival outcomes (Table S4).

The initial list of genes predicted to be associated with GI-tract survival was further refined by application of several selection criteria that are based on transcript ranking. Firstly, only transcripts with an importance factor higher than 1 according to the random forest algorithm were selected for further analysis. Secondly, the quantitative correlation of individual transcripts with the survival rate observed in individual cultures was evaluated, selecting those transcripts (genes) that had the highest quantitative correlation with survival (expressed in R^2 in Table S4, see Figure 7 for two examples). Lastly, genes encoding prophage associated functions that are typically hypervariable among *L. plantarum* strains were discarded [14,21]. The remaining transcripts and their associated genes (Table 3) were considered to have the strongest correlation with the measured gastric juice tolerance and were therefore selected for validation.

Table 3. Candidate genes linked with GI-tract survival of L. plantarum selected for genetic engineering.

ORF[a]	Name	function	Subcellular localization prediction[b]	Correlation with high survival[c]	R^{2d}	Importance[e]	KO / over[f]	Strain[g]
lp_1413	*pbp2A*	transpeptidase-transglycosylase (penicillin binding protein 2A)	N-terminally anchored (No CS)	-	0.702	1.832	KO	NZ3412^{CM}
lp_2827	*napA3*	Na(+)/H(+) antiporter	Multi-transmembrane	-	0.686	1.503	KO	NZ3416^{CM}
lp_1669	*lp_1669*	transcription regulator, AraC family	Intracellular	-	0.601	1.156	KO	NZ3417^{CM}
lp_3398	*pacL3*	cation transporting P-type ATPase	Multi-transmembrane	-	0.474	1.790	KO	NZ3415^{CM}
lp_1817	*lp_1817*	ribitol-5-phosphate 2-dehydrogenase (putative)	Intracellular	-	0.378	1.156	KO	NZ3414^{CM}
lp_2758	*thrC*	threonine synthase	Intracellular	+	0.714	1.227	over	pNZ3432[h]
lp_3299	*folB*	dihydroneopterin aldolase	Intracellular	+	0.638	1.772	over	pNZ7026[i]
lp_0149	*lp_0149*	ABC transporter, ATP-binding protein, Cobalt (or cobalamine)	Intracellular	+	0.634	1.977	over	pNZ3433[j]
lp_3297	*folE*	GTP cyclohydrolase I	Intracellular	+	0.554	1.356	over	pNZ7026[i]
lp_0148	*lp_0148*	ABC transporter, permease protein, Cobalt (or cobalamine)	Multi-transmembrane	+	0.523	1.156	over	pNZ3433[j]
lp_2349	*hicD3*	L-2-hydroxyisocaproate dehydrogenase	Intracellular	+	0.441	1.001	over	pNZ3431
lp_3296	*folC2*	folylpolyglutamate synthase / dihydrofolate synthase	Intracellular	+	0.432	1.081	over	pNZ7026[i]
lp_1357	*lp_1357*	extracellular protein, membrane-anchored (putative)	N-terminally anchored (No CS)	+	0.233	1.001	over	pNZ3430

a ORF, open reading frame.
b Subcellular localization prediction according to LocateP [71].
c +, positive correlation; -, negative correlation.
d R^2 based on linear regression of transcript intensity and GI-tract survival of the eight best and eight worst surviving cultures (see Fig. 4).
e Importance according to random forest [31].
f KO, knock out; over, overexpression.
g *L. plantarum* KO strains with NZ number or *L. plantarum* strains harboring plasmids (pNZ number).
h pNZ3432 contains *thrC* and *lp_2759*.
i pNZ7026 contains *folB-folK-folE-folC2-xtp2-folP*.
j pNZ3433 contains *lp_0148*, *lp_0149*, and *lp_0150*.

Validation of target GI-tract survival effector molecules by mutagenesis

To validate the association of the expression level of specific genes in *L. plantarum* with GI-tract survival, the 13 genes with the highest ranking based on the criteria described above were targeted by genetic engineering aiming to improve GI-tract survival beyond the observed levels with the wild-type strain (Table 3). Therefore, the direction of the correlation between transcript intensity and survival in the GI-tract assay determined whether a gene would be targeted for overexpression (positive correlation, see Fig. 7A for an example) or gene-deletion (negative correlation, Fig. 7B).

Genes targeted for overexpression were *folB, thrC*, *lp_0149*, *hicD3*, and *lp_1357* (Table 3). For *folB* overexpression, we used a previously constructed mutant that overexpresses the entire *folB-folK-folE-folC2-xtp2-folP* cluster [48,49]. To achieve overexpression of *thrC*, *lp_0149*, *hicD3*, and *lp_1357* (Table 3), the genes were cloned under control of the sakacin P inducible *orfX* promoter [46]. For the candidate genes selected for overexpression that were part of a predicted operon [51], the whole operon was cloned in the sakacin induction vector (Table 3). Sakacin P induced overexpression of the cytoplasmic *hicD3* and *thrC* and the downstream *lp_2759* gene products could readily be confirmed by SDS-PAGE analysis of cell-free extracts of induced cultures (Fig. S1). In contrast, overproduction of the membrane-anchored (*lp_1357*) and transmembrane proteins

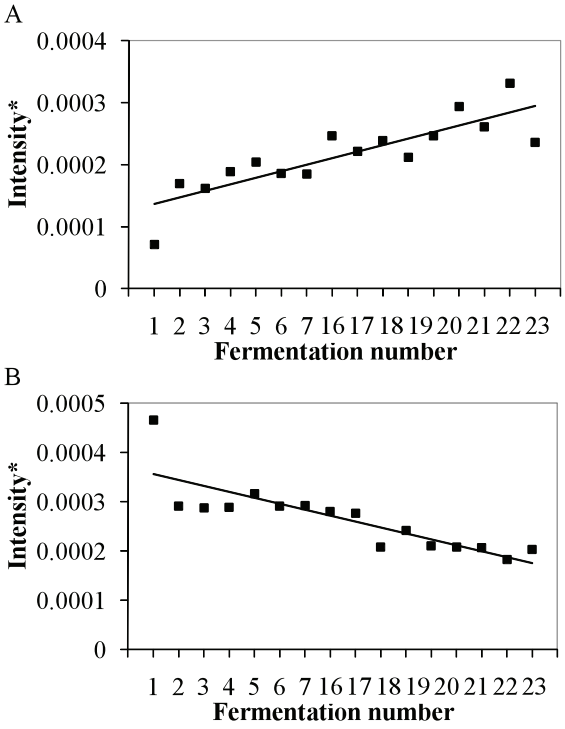


Fig. 7. Correlation of *L. plantarum* WCFS1 GI-tract survival and transcript intensity of *thr*C (A) and *pbp2A* (B). The eight best and eight worst fermentations (see Fig. 4A) are ranked with increasing GI-tract survival. *Data was normalized to correct for between slide variation [24]. R^2 *thr*C = 0.71, R^2 *pbp2A* = 0.70.

(*lp_0148~0150*) were not distinguishable by SDS-PAGE (data not shown). Nevertheless, because of the successful overexpression of the two other proteins, it can be assumed that at least the transcripts of these genes were present at increased levels in these cells upon sakacin induction, suggesting that also protein production is likely to be elevated.

The constructed mutants were grown until the logarithmic growth phase and subjected to the GI-tract assay. The survival of the Sakacin P induced overexpression mutants was improved when compared to a control strain harboring the empty induction plasmid (Fig. 8). Although not significant, the contrary seemed to be the case, since the slight effects that were observed in some of the experiments suggested that the expression of the cloned genes reduced the survival capacity of these cells rather than improve.

In contrast, survival *L. plantarum* Δ*pbp2A::cat*, Δ*lp_1669::cat*, and Δ*napA3::cat* mutants showed significantly improved survival in the GI-tract assay, as compared to their parental strain (Fig. 9). These strains harbored disruptions in genes associated with poor survival in gastric stress. Notably, we have combined the individual mutants described here to construct Δ*pbp2A-*Δ*napA3::cat* and Δ*napA3-*Δ*lp_1669::cat*. However, these double gene deletion derivatives displayed robustness phenotypes comparable to the single Δ*napA3::cat* gene deletion derivative, indicating that the

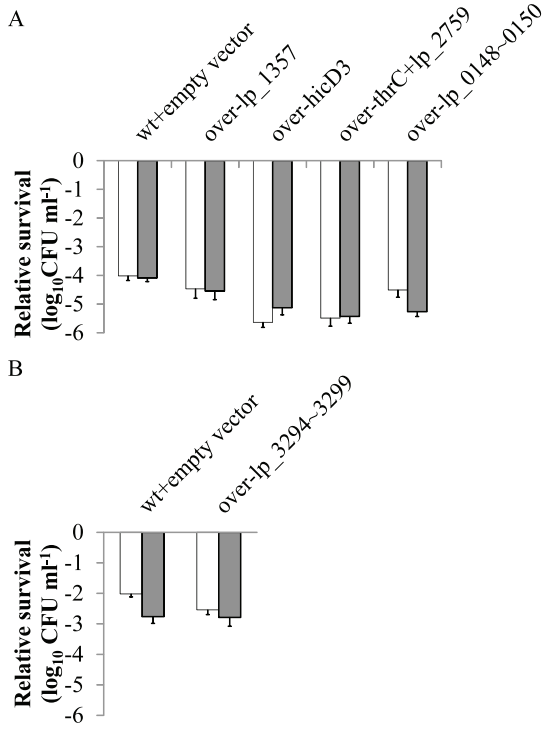


Fig. 8. Relative GI-tract survival of *L. plantarum* mutants overexpressing genes potentially involved in GI-tract survival. Log10 CFU ml^{-1} determination of mid-exponentially grown in batch *L. plantarum* mutants after 60 min gastric juice incubation (white bars) and subsequent 60 min pancreatic juice incubation (grey bars). Input is set at 0 Log_{10} CFU ml^{-1}. Empty vectors are pSIP411 (A) and pNZ7021 (B). *L. plantarum* harboring pNZ3430 (over-*lp*_1357), pNZ3431 (over-*hic*D3), pNZ3432 (over-*thr*C+*lp*_2759), pNZ3433 (over-*lp*_0148~0150), and pNZ7026 (over-*lp*_3294~3299). Data presented is the average of technical sextuplicates (- standard deviation).

positive effect on GI robustness of these mutations appears not cumulative (data not shown). Nevertheless, these results establish the involvement of certain fermentation-condition dependent gene products in GI survival.

Pbp2A is annotated as a penicillin-binding protein involved in peptidoglycan biosynthesis, Lp_1669 is predicted to be a transcription regulator, and NapA3 is homologous to Na^+/H^+ antiporters. To gain more insight in the mechanisms by which these proteins influence robustness, growth of the parental strain and the Δ*pbp2A::cat*, Δ*lp_1669::cat*, and Δ*napA3::cat* derivatives was monitored under standard- and stress-conditions. At 28°C in laboratorial culture medium (MRS), the growth rates of the mutants did not differ from the wild-type, nor did the addition of H_2O_2 (1 to 5 mM), lysozyme (0.025 to 3.2 g/ml), or SDS (0.9 to 30 g/l) induce differences in growth rate of the mutants compared with the wild type strain (data not shown). However, the presence of bile salts (10 to 50 mM) in the culture medium reduced the maximum growth rate of Δ*pbp2A::cat* to 20% as compared to the parental strain. This result indicates that Pbp2A contributes to the survival capacity of *L. plantarum* in low-pH, stomach like conditions, but also improves bile tolerance, but not to tolerance to detergents in general.

The addition of NaCl to the growth medium reduced the growth rate of Δ*napA3::cat* to 20% (400 mM) and 80% (1 M) of the wild type (data not shown). Because NapA3 is a Na^+/H^+ antiporter which might be affected by extracellular pH?, the growth of the Δ*napA3::cat* mutant was monitored under different starting pH conditions (pH 4.6 to 6.4) in the presence and absence of NaCl (300 mM). The growth rate of the mutant appeared unaltered during growth in the absence of salt. Only the presence of NaCl reduced the growth rate of Δ*napA3::cat* under all measured conditions (data not shown). These results support a role of this function in salt tolerance, which in our experiments, appeared to be independent of the pH.

Contrary to Δ*napA3::cat* and Δ*pbp2A::cat*, a specific phenotype was not established for the transcription regulator Lp_1669. To elucidate the regulon associated with this regulator, the transcriptome profile of the NZ3417^{CM} (Δ*lp_1669*::*cat*) strain was compared to that of the wild-type strain grown in 2× CDM [18] or MRS. Differential transcriptome datasets were mined for

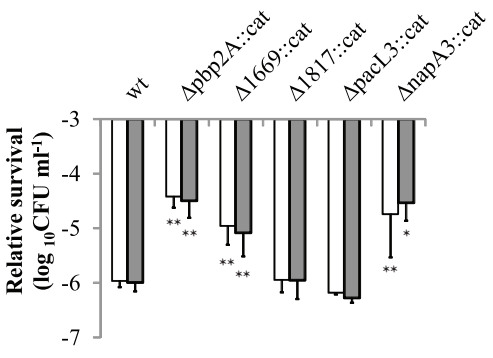


Fig. 9. Relative GI-tract survival of *L. plantarum* mutants with *cat* replacements of candidate genes involved in GI-tract survival. Log_{10} CFU ml^{-1} determination of logarithmic (OD_{600} = 1.0) batch *L. plantarum* mutants after 60 min gastric juice incubation (white bars) and subsequent 60 min pancreatic juice incubation (grey bars). Input is set at 0 Log_{10} CFU ml^{-1}. * *P-value* < 0.05, ** *P-value* < 0.01 compared with wild type (wt). Representative of two independent experiments, data presented are averages of technical sextuplicates (- standard deviation).

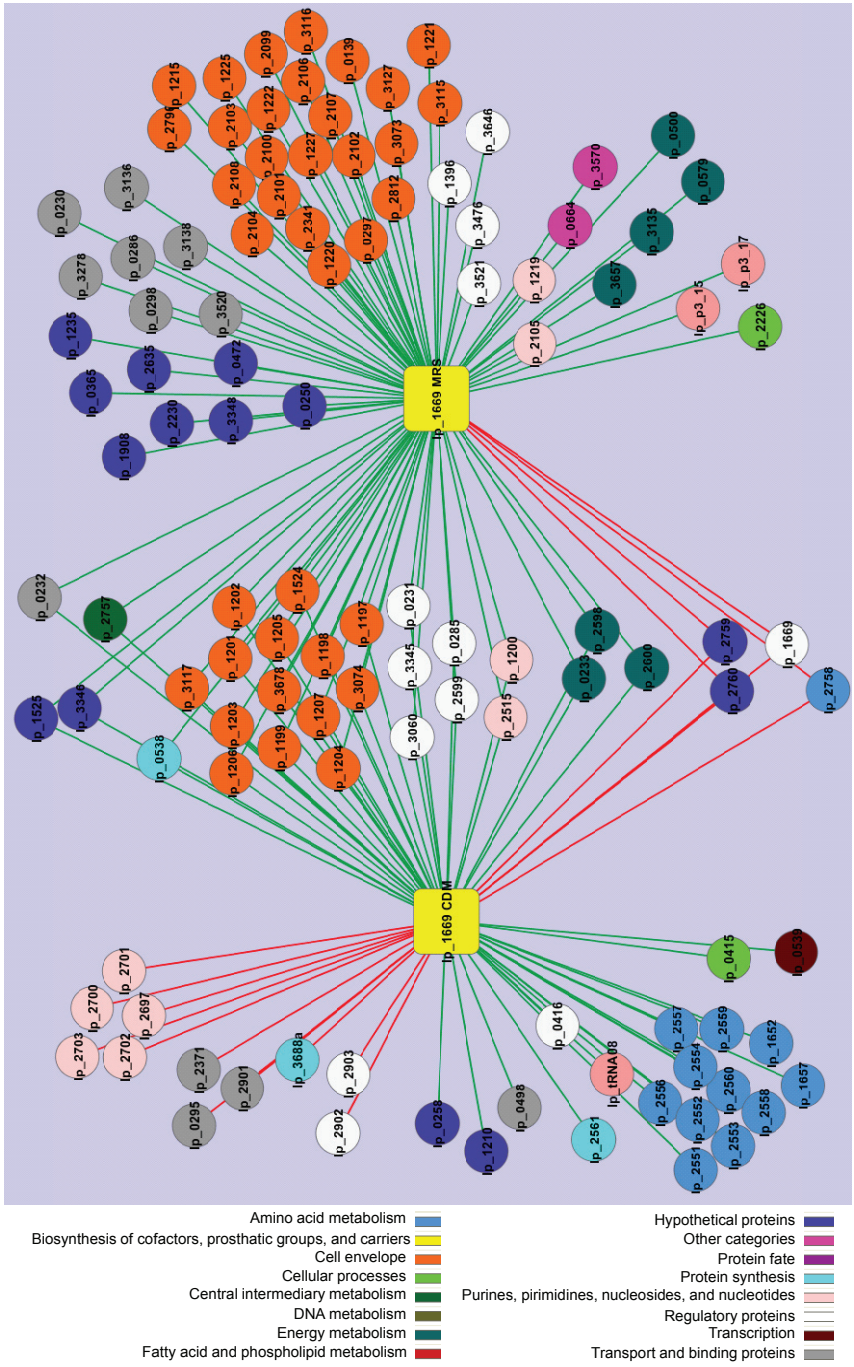


Fig. 10. Regulatory network of Lp_1669 grown in 2× CDM or MRS. Yellow rectangle nodes represent growth in 2× CDM (left node) or in MRS (right node). Round nodes represent single genes with their corresponding lp_number, which is the number as annotated in the sequenced parental *L. plantarum* WCFS1 strain [17], green lines represent down-regulation, and red lines represent up-regulation of the gene compared to the parental strain. The colors of the round nodes represent the annotated main class.

overrepresented (main and sub-) functional classes using the Biological Networks Gene Ontology (BiNGO) tool [43]. The results showed that the Lp_1669-deficient strain displayed enhanced expression of genes belonging to the main functional class of cell envelope associated functions, and more specifically to its subclass of surface polysaccharides, lipopolysaccharides, and antigens. This effect of the mutation was observed independent of the medium used (Fig. 10). Analysis at the individual transcript level revealed that the capsular polysaccharide (CPS) clusters *cps2*, *cps3*, and *cps4* are induced in the MRS-grown Lp_1669-deficient strain as compared to the wildtype, suggesting that the regulatory function encoded by *lp_1669* is involved, either directly or indirectly, in the regulation of CPS biosynthesis. Notably, especially the expression of the *cps2* cluster was induced in 2× CDM grown Lp1669 deficient cells. To confirm the involvement of Lp_1669 in CPS modification, CPS of the NZ3417^{CM} (Δ*lp_1669::cat*) strain and the wild-type was isolated and molar mass and sugar composition were determined by using a HPLC-based method developed previously by Looijesteijn and Hugenholtz [50]. Minor changes in CPS sugar composition of the Lp_1669-deficient strain were found in comparison to the wild type strain (Table 4). Galactosamine was only detected in the mutant strain, whereas arabinose was found only in the wild-type strain. Rhamnose and glucosamine also tended to be slightly more abundant in the wild type *L. plantarum* WCFS1. Moreover, the average molar mass of Δ*lp_1669::cat* strain-derived CPS was 1.5-fold higher compared to the wild type (Table 4). This indicates that Lp_1669 seems to be involved in subtle CPS modification, specifically in chain length determination. These observations might also (partially) explain the observed increased gastrointestinal survival of the *L. plantarum* Lp_l669-deficient strain.

Table 4. Molar mass and sugar composition of CPS isolated from *L. plantarum* WCFS1 and NZ3417^{CM} (Δ*lp_1669::cat*).

Strain	**WCFS1**	**Δ*lp_1669::cat***
Total molar mass (kg/mol)	20 (±1.4)	30 (±1.5)
Sugar (% of total sugars)[a]		
Rhamnose	3.2	2.6
Galactosamine	ND	1.3
Arabinose	0.5	ND
Glucosamine	3.7	2.8
Galactose	12.6	12.8
Glucose	27.8	26.4
Galacturonic acid	52.3	54.1

[a] ND is not detected.

Discussion

This study demonstrates that the production method, medium composition, and stage of growth strongly influenced the GI-survival efficacy of this model-probiotic organism. Combining the fermentative and survival data pinpointed to specific fermentation conditions that may enhance robustness (low salt and low pH), whereas genome association analysis of the transcriptome and survival data revealed 13 genes potentially involved in GI-survival.

Cells harvested from stationary phase generally were more robust than logarithmically growing cells, and in particular, those cells displayed enhanced survival in gastric juice which overall had a dramatically larger impact on survival compared to pancreatic juice. The influence of acidity on GI-tract survival was also emphasized by the observation that lowering the gastric juice pH by as little as 0.1 unit had a pronounced impact on survival. Differences among bacterial species in their sensitivity to gastric and intestinal secretions have been observed before [52-54] and a higher sensitivity for acid than bile stress was also noted for *L. rhamnosus*, as well as for other *L. plantarum* strains [55,56].

The finding that exposure to low pH during growth enhances GI-survival is in agreement with earlier observations that pre-adaptation to sublethal stress conditions enhances the subsequent robustness of bacteria to lethal stress conditions [11], supporting the suitability of the fermentation genomics platform and bioinformatics tools employed in this study. For salt it is known that it can protect against, but also increase susceptibility to, other stresses [57,58]. Moreover, these results clearly establish that fermentation conditions have a major impact on the GI-tract associated stress tolerance of bacterial cultures, and that specifically mild salt stress and lower pH adaptation may elicit adaptive responses that reduce and support such stress tolerance, respectively.

Genotype-phenotype matching strategies have been applied successfully to increase our understanding of probiotic functionality [22,24,25,59,60]. However, this approach intrinsically disallows the identification of conserved mechanisms, since it is solely based on strain-specific gene content [10]. The fact that approximately 90% of all genes are conserved within the species *L. plantarum* [14] further exemplifies the limited identification-power of gene-trait matching (GTM). Indeed, 9 out of the 13 genes identified here are conserved among all 42 *L. plantarum* strains used in this study [14] and could thus not have been identified with GTM, establishing the complementarity of our transcriptome-trait matching (TTM) approach. Moreover, GTM identified robustness markers might not be present in industrially applied strains, disallowing improvement of GI-tract survival of these strains. Industrial strains are generally selected on basis of a combination of traits, e.g. flavor-formation, probiotic functionality, or robustness. Therefore, the TTM results seem more applicable than GTM efforts, since TTM pinpoints the possibilities for fermentation-enhanced improvement of a specific trait whilst applying the same strain, whereas industrial implementation of gene-trait matching results might require tedious selection of alternative strains on basis of identified robustness markers that also express other desired functionalities. Moreover, when applying this TTM strategy, trait specific biomarkers can be identified rather than universal biological markers

as were found for adaptation-stress induced microbial robustness towards challenge-stresses in *Bacillus cereus* [61]. The non-involvement of certain ubiquitous markers of *L. plantarum* in GI-tract survival could be confirmed, as the GI-tract persistence of strains lacking the canonical stress response regulators CtsR and HrcA did not differ from the wild type *L. plantarum* WCFS1 (Van Bokhorst-van de Veen *et al.*, unpublished results). In addition, a TTM strategy can be used for a wide range of functional industrial applications and the improved trait-correlated transcripts can be used in further research as biomarkers to fine-tune the quality control of the product.

The transcription levels of the 13 genes potentially involved in robustness were either positively or negatively correlated with survival in the GI-tract assay. To confirm the role of these genes in this phenotype, mutagenesis of the identified genes was performed aiming in all cases to improve GI-tract survival characteristics. To this end, overexpression and gene deletion derivatives of the parental strain were constructed, depending on the direction of the predicted correlation. Three of the five constructed gene deletion derivatives displayed enhanced GI-tract survival, confirming the predicted role of the targeted gene. By contrast, none of the overexpression derivatives displayed improved robustness behavior in the GI-tract assay, and all had survival characteristics that were virtually identical to those of the parental strain. A possible explanation for these observations may be found in the potential disruption of a gene-regulatory network by the deletion of a single gene in that network, while overexpression of a single element from a complementary gene-function network may not provide the same effect as the enhanced expression of all elements in the network. From the five constructed gene deletion derivatives, three showed improved survival, which is a relatively good success rate. This is in line with earlier observations [22,24,25] and can be explained by the fact that the random forest algorithm also leads to the identification of non-causal relationships, reiterating the importance of follow-up mutagenesis approaches to establish a definite role for candidate biomarkers identified with this algorithm.

The 3 genes for which the importance in GI-tract survival could be confirmed by gene deletion encode a AraC family regulator (Lp_1669), a Na^+/H^+ antiporter (NapA3), and a penicillin binding protein (Pbp2A). Notably, all three proteins are associated with cell wall modification and transport, and their mutation may lead to cell envelope modulation. This finding per se, may not be qualified as surprising, because the cell envelope is the first line of defense against stresses [62]. Moreover, the resistance to acid and adaptation to bile stress in *L. plantarum* WCFS1 has been associated with membrane integrity and cell envelope modifications, respectively [63,64]. The AraC family of regulators to which Lp_1669 belongs [17] is characterized by transcriptional regulators that act mostly as activators. However, in some cases these regulators serve as repressors of transcription or as both activators and repressors [65]. The observed effect of Lp_1669 on GI-tract survival is likely to be indirect, possibly via CPS remodeling, because the Lp_1669-deficient strain had CPS with a higher molar mass that might result in a thicker CPS layer around the cells. It has been demonstrated that the presence of EPS/CPS improved the *in vivo* GI survival of *L. rhamnosus* GG [66]. The Na^+/H^+ antiporter NapA3 might affect GI survival via a role in pH homeostasis. Because disruption of *napA3* improved GI-tract survival, it seems likely that NapA3 exports sodium ions associated with the influx of protons, thereby decreasing its internal pH and proton motive

force due to the acid stomach conditions. This is also in line with our observation that the gene deletion derivative is only reduced during growth in the presence of sodium salts. Finally, *pbp2A* encodes the penicillin binding protein 2A which is annotated to be involved in peptidoglycan biosynthesis [17]. Disruption of *pbp2A* improved the acid stomach condition survival, while it decreased the growth rate in the presence of bile. Noteworthy in this respect is the finding that the compositions of peptidoglycan directly affects the integrity of the cells and can influence the acid- and bile-tolerance [11,67-69]. Moreover, transcriptome analysis of *L. acidophilus* NCFM and *L. plantarum* WCFS1 demonstrated that many genes related to cell membrane and peptidoglycan biosynthesis displayed altered expression profiles during exposure to bile [67,70]. An increased acid sensitivity by the inactivation of penicillin binding proteins is found in *Lactococcus lactis* and *L. reuteri* [11,68]. However, we found the deletion of *pbp2A* improves the GI-tract survival, which suggests that disruptions in peptidoglycan biosynthesis genes could either improve or decrease the survival of probiotics, reiterating the general concept of subtle inter-strain and species differences in survival mechanisms.

In conclusion, this study demonstrated that fermentation conditions have a large influence on the GI-tract survival of *L. plantarum*. We showed that TTM enables the identification of genetic loci involved in gastrointestinal robustness and this approach can also be employed to rationally design fermentation and process conditions that aim for the production of probiotics with improved GI survival and consequently have a higher potential to achieve their desired health-beneficial effects on the consumer.

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Supplemental material

Modulation of *Lactobacillus plantarum* gastrointestinal robustness by fermentation conditions enables identification of bacterial robustness markers

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Table S1. Primers used in this study.

ID	Name	Sequence (5' to 3')[a,b]	Reference
A1	pbp2A-outI	AGTTCTGTGCGTAGTTTGCC	This work
A2	pbp2A-1412F	TTTGCTATAATGTATTCATTAC	This work
A3	ppbp2A-1412R	gcatacattatacgaacggtagatttTTTTTGCATAATCTTCCCCTTGTTCAGC	This work
A4	pbp2A-1414F	cggttacagcccgggcatgagTAGTAAAGCTAGCTTCTGAACG	This work
A5	pbp2A-1414R	GACCGTGCAAGGTACCAATC	This work
A6	pbp2A-outII	TAGTGGTCACCCGCCACACC	This work
B1	lp-1669-outI	ATCATGGCTTAATCAACAGCG	This work
B2	lp-1669-1668F	CGCCAGGCGTAATGAGTGTG	This work
B3	lp-1669-1668R-inverted cat	gcatacattatacgaacggtagatttAATCTTCACACTAATCACTCCTAC	This work
B4	lp-1669-1670F-inverted cat	cggttacagcccgggcatgagTAACAAGCGTTGCCGTTTAGG	This work
B5	lp-1669-1670R	CGAAAAATTAGTTGTCATGG	This work
B6	lp-1669-outII	AAATTAGTTGTCATGGTTGG	This work
C1	lp-1817-outI	CGCGACAGAGAAGTCCAACC	This work
C2	lp-1817-1816F	TTTCGTAGACGAGTCAAAG	This work
C3	lp-1817-1816R	gcatacattatacgaacggtagatttATTTAACATCTTATGACCTCTTTTTC	This work
C4	lp-1817-1818F	cggttacagcccgggcatgagTAAAGACGGTAAAGCTCGTGTTAC	This work
C5	lp-1817-1818R	ATATGATCAACTTCCTGATT	This work
C6	lp-1817-outII	CATGTACATAAGATAGATCC	This work
D1	pacL3-outI	GGTAATCATAGCAACATTAG	This work
D2	pacL3-3397F	CATACCAGGTTGTGTCACGG	This work
D3	pacL3-3397R	gcatacattatacgaacggtagatttATTCTGCATCGTTTATTCCGTAATTCG	This work
D4	pacL3-3399F	cggttacagcccgggcatgagTAAGGATGATCAATTCAAGTTAGTTAAAATG	This work
D5	pacL3-3399R	GTTGATTAACAAAATTACTG	This work
D6	pacL3-outII	TCAATATCATTTTCAGTTTG	This work
E1	napA3-outI	AGTCTGGGCATGCATGAAGC	This work
E2	napA3-2826F	AACGAGCAGGCCGACGAGC	This work
E3	napA3-2826R	gcatacattatacgaacggtagatttGTAATCCATTAAAAACCTC-CTAAAAAAGG	This work
E4	napA3-2828F	cggttacagcccgggcatgagTAAAGCAATTGAAAATCCCAACTTG	This work
E5	napA3-2828R	TCCTGGGAAGTTTACGAACC	This work
E6	napA3-outII	CCGATAACTGAAGTTCTTGG	This work
F1	lp-1357-overexpression F	CCCCCTCATGAAGCAGTTCTGGTCACTAATC	This work
F2	lp-1357-overexpression R	CTAACTCTTTGTCCCGGTTGG	This work
G1	hicD3-overexpression F	CCCCCCCATGGCTCGTAAATATGGTGTGATCGGG	This work
G2	hicD3-overexpression R	TTATGCTTGCGGTAAAACGTCC	This work

H1	thrC+lp-2759 overexpression F	CCCCCTCATGAAAACACTTTATCGCAGTACC	This work
H2	thrC+lp-2759 overexpression R	TCAGTTGAAGTAATTTTCTAGGAAAA	This work
I1	lp-0148~0150 overexpression F	CCCCCACATGTCTCAAAACAAGCAATCCAATTCAATTCG	This work
I2	lp-0148~0150 overexpression R	TTATGCCTTAAACGGATTCCAG	This work
I	TAG-lox66-F2	CGGGAGCAGAATGTCCGAGACTAATG	[1]
J	TAG-lox71-catR2	TAGTGCGTCTTCTCGTAGCGATCGG	[1]
R87	87	GCCGACTGTACTTTCGGATCC	[2]
Is169	169	TTATCATATCCCGAGGACCG	This work
S1-2	Sequencing primer R of pSIP411	GTAATTGCTTTATCAACTGCTGC	This work
S2-3	Sequencing primer 3 of thrC+lp-2759	ACCATACTTACAACAACTTGAACTCAACC	This work
S3-4	Sequencing primer 4 of lp-0148~0150	GATCTCTACAACGATGATTTTTGATGAAG	This work

[a] The lower-case letters indicates the overhang sequences that homolgous to the ultimate regions of the *cat* (chloramphenicol acetyltransferase) amplicon.
[b] Underlined are the restriction sites.

Table S2. Primer pair combinations used for LF and RF amplification and for the SOE step of the deletion mutants.

Label	Target gene	Left flank primer pair	Right flank primer pair	SOE primer pair
A	*pbp2A*	A2 / A3	A4 / A5	A2 / A5
B	*lp-1669*	B2 / B3	B4 / B5	B2 / B5
C	*lp-1817*	C2 / C3	C4 / C5	C2 / C5
D	*pacL3*	D2 / D3	D4 / D5	D2 / D5
E	*napA3*	E2 / E3	E4 / E5	E2 / E5

Table S3. Primer pair combinations used for each deletion mutant to confirm the correct integration in the genome.

Label	Target gene	Left side	Right side
A	*pbp2A*	A1 / Is169	R87 / A6
B	*lp-1669*	B1 / R87	Is169 / B6
C	*lp-1817*	C1 / Is169	R87 / C6
D	*pacL3*	D1 / Is169	R87 / D6
E	*napA3*	E1 / Is169	R87 / E6

Table S4. Candidate genes associated with GI-tract survival of *L. plantarum* WCFS1.

ORF[a]	name	function	Subcellular localization prediction[b]	Correlation with high survival[c]	R^{2d}	Importance[e]	KO / over[f]	Strain[g]
lp_0148	*lp_0148*	ABC transporter, permease protein, Cobalt (or cobalamine)	Multi-transmembrane	+	0.523	1.156	over	pNZ3433[h]
lp_0149	*lp_0149*	ABC transporter, ATP-binding protein, Cobalt (or cobalamine)	Intracellular	+	0.634	1.977	over	pNZ3433[h]
lp_0217	*lp_0217*	ABC transporter, permease protein	Multi-transmembrane	-	0.626	2.268		
lp_0315	*potD*	spermidine/putrescine ABC transporter, substrate binding protein	N-terminally anchored (No CS)	+	0.348	1.001		
lp_0332	*lp_0332*	extracellular protein (putative)	N-terminally anchored (No CS)	+	0.555	1.001		
lp_0404	*plnL*	immunity protein PlnL	Multi-transmembrane	+	0.415	1.001		
lp_0415	*plnA*	plantaricin A precursor peptide, induction factor	Secreted via minor pathways (no CS)	+	0.147	1.001		
lp_0490	*lp_0490*	unknown	Intracellular	+	0.155	1.001		
lp_0625	*lp_0625*	prophage P1 protein 2, mitogenic factor, cell surface lipoprotein	Lipid anchored	+	0.499	1.443		
lp_0630	*lp_0630*	prophage P1 protein 7	Intracellular	+	0.498	1.001		
lp_0820	*glmM*	phosphoglucosamine mutase	Intracellular	+	0.345	1.001		
lp_0869	*lp_0869*	extracellular protein	Secretory (released) (with CS)	-	0.257	1.001		
lp_1188	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	Intracellular	+	0.290	1.001		
lp_1189	*rfbB*	dTDP-glucose 4,6-dehydratase	Intracellular	+	0.302	1.001		
lp_1357	*lp_1357*	extracellular protein, membrane-anchored (putative)	N-terminally anchored (No CS)	+	0.233	1.001	over	pNZ3430
lp_1413	*pbp2A*	transpeptidase-transglycosylase (penicillin binding protein 2A)	N-terminally anchored (No CS)	-	0.702	1.832	KO	NZ3412[CM]
lp_1515	*infC*	translation initiation factor IF-3	Intracellular	-	0.043	1.119		
lp_1562	*udk*	uridine kinase	Intracellular	+	0.346	1.156		
lp_1669	*lp_1669*	transcription regulator, AraC family	Intracellular	-	0.601	1.156	KO	NZ3417[CM]
lp_1817	*lp_1817*	ribitol-5-phosphate 2-dehydrogenase (putative)	Intracellular	-	0.378	1.156	KO	NZ3414[CM]

lp_1838	*lp_1838*	transcription regulator, LysR family	Intracellular	-	0.009	1.092		
lp_1958	*lp_1958*	acetoin ABC transporter, ATP-binding protein	Intracellular	-	0.550	1.092		
lp_2349	*hicD3*	L-2-hydroxyisocaproate dehydrogenase	Intracellular	+	0.441	1.001	over	pNZ3431
lp_2451	*lp_2451*	prophage P2a protein 6; endonuclease	Intracellular	+	0.727	1.156		
lp_2643	*lplA1*	lipoate-protein ligase	Intracellular	+	0.411	1.001		
lp_2651	*lp_2651*	transcription regulator, GntR family	Intracellular	-	0.334	1.001		
lp_2758	*thrC*	threonine synthase	Intracellular	+	0.714	1.227	over	pNZ3432[i]
lp_2761	*lp_2761*	O-acetyltransferase	Intracellular	+	0.432	1.688		
lp_2827	*napA3*	Na(+)/H(+) antiporter	Multi-transmembrane	-	0.686	1.503	KO	NZ3416^{CM}
lp_2960	*lp_2960*	acyltransferase (putative)		+	0.308	1.001		
lp_3019	*lp_3019*	extracellular protein (putative)	N-terminally anchored (No CS)	-	0.170	1.001		
lp_3296	*folC2*	folylpolyglutamate synthase / dihydrofolate synthase	Intracellular	+	0.432	1.081	over	pNZ7026[j]
lp_3297	*folE*	GTP cyclohydrolase I	Intracellular	+	0.554	1.356	over	pNZ7026[j]
lp_3299	*folB*	dihydroneopterin aldolase	Intracellular	+	0.638	1.772	over	pNZ7026[j]
lp_3398	*pacL3*	cation transporting P-type ATPase	Multi-transmembrane	-	0.474	1.790	KO	NZ3415^{CM}
lp_3493	*aroC2*	3-dehydroquinate dehydratase	Intracellular	+	0.025	1.260		
lp_3661	*rbsR*	transcription regulator, LacI family, ribose	Intracellular	+	0.197	1.417		

a ORF, open reading frame.
b Subcellular localization prediction according to LocateP [3].
c +, positive correlation; -, negative correlation.
d R^2 based on linear regression of transcript intensity and GI-tract survival of the eight best and eight worst surviving cultures (see fig 4).
e Importance according to random forest [4].
f KO, knock out; over, overexpression.
g *L. plantarum* KO strains with NZ number or *L. plantarum* strains harboring plasmids (pNZ number).
h pNZ3433 contains *lp_0148*, *lp_0149*, and *lp_0150*.
i pNZ3432 contains *thrC* and *lp_2759*.
j pNZ7026 contains *folB-folK-folE-folC2-xtp2-folP*.

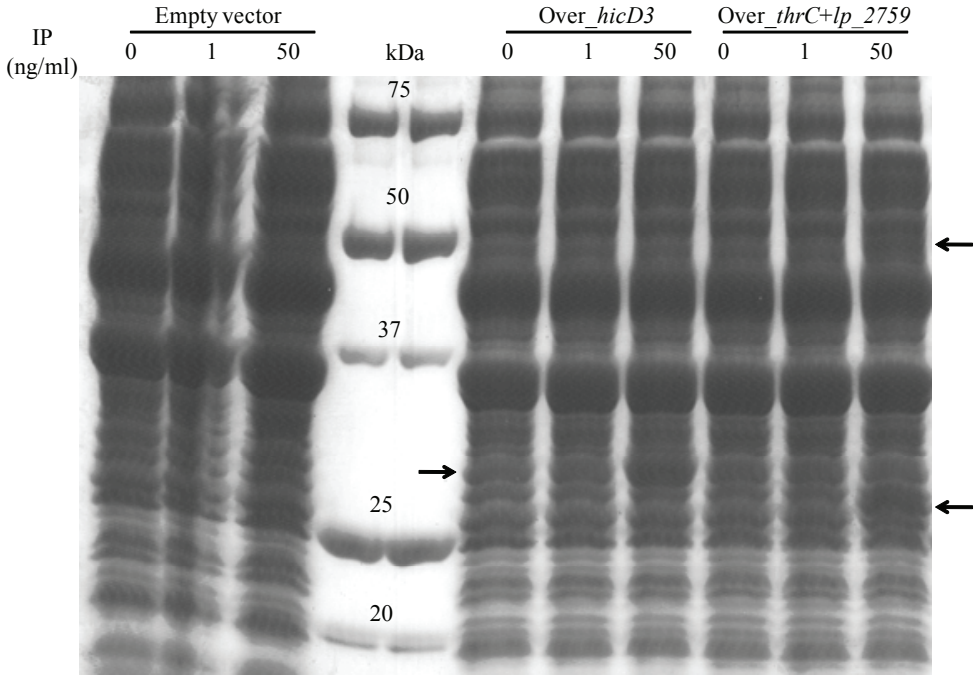

Fig. S1. SDS-PAGE of cell-free extracts logarithmic *L. plantarum* strains overexpressing *hicD3* (*lp_2349*) and overexpressing *thrC* (*lp_2758*) and *lp_2759*. The arrows indicate protein bands increasing with increasing amounts of Sakacin P (inducing peptide, IP). Empty vector = pSIP411B. *L. plantarum* harboring pNZ3431 (over-*hicD3*), and pNZ3432 (over-*thrC+lp_2759*). Marker sizes are indicated in kDalton (kDa).

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5

Congruent strain specific intestinal persistence of *Lactobacillus plantarum* in an intestine-mimicking *in vitro* system and in human volunteers

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Abstract

An important trait of probiotics is their capability to reach their intestinal target sites alive to optimally exert their beneficial effects. Assessment of this trait in intestine-mimicking *in vitro* model systems has revealed differential survival of individual strains of a species. However, data on the *in situ* persistence characteristics of individual or mixtures of strains of the same species in the gastrointestinal tract of healthy human volunteers have not been reported to date. The GI-tract survival of individual *L. plantarum* strains was determined using an intestine mimicking model system, revealing substantial inter-strain differences. The obtained data were correlated to genomic diversity of the strains using comparative genome hybridization (CGH) datasets, but this approach failed to discover specific genetic loci that explain the observed differences between the strains. Moreover, we developed a next-generation sequencing-based method that targets a variable intergenic region, and employed this method to assess the *in vivo* GI-tract persistence of different *L. plantarum* strains when administered in mixtures to healthy human volunteers. Remarkable consistency of the strain-specific persistence curves were observed between individual volunteers, which also correlated significantly with the GI-tract survival predicted on basis of the *in vitro* assay. In conclusion, the survival of individual *L. plantarum* strains in the GI-tract could not be correlated to the absence or presence of specific genes compared to the reference strain *L. plantarum* WCFS1. Nevertheless, *in vivo* persistence analysis in the human GI-tract confirmed the strain-specific persistence, which appeared to be remarkably similar in different healthy volunteers. Moreover, the relative strain-specific persistence *in vivo* appeared to be accurately and significantly predicted by their relative survival in the intestine-mimicking *in vitro* assay, supporting the use of this assay for screening of strain-specific GI persistence.

Introduction

Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' [1]. The most widely applied probiotics belong to the genera *Lactobacillus* and *Bifidobacterium* [2,3]. To be able to exert their beneficial effects in the intestine, it is a prerequisite for probiotic cultures to counteract the stressful conditions encountered during production, shelf life, and exposure to the harsh conditions of the (upper) digestive tract [4,5].

A straightforward strategy that is typically applied for the selection of robust probiotic strains is to subject these bacteria to a series of conditions that mimic the gastrointestinal (GI)-tract *in vitro*, including survival at low pH (resembling the stomach) and/or upon exposure to bile salts and digestive enzymes (resembling the duodenum) [6-8]. A diverse range of lactobacilli and bifidobacteria have also been tested in more sophisticated GI-tract simulators, e.g. the TNO Intestinal Models (TIM-1 and TIM-2) [9,10], the Simulator of Human Intestinal Microbial Ecosystem (SHIME) [11], and the Dynamic Gastric Model (DGM) [12]. Although physicochemical properties and/or microbial interactions of the strains of interest can be investigated in these models, they lack the interactions of the bacteria with host cells such as epithelial and immune cells.

Besides the *in vitro* work discussed above, a limited number of *in vivo* studies have assessed the GI survival and persistence of candidate probiotic strains. For example, 7% of the single administered *L. plantarum* NCIMB8826 reached the ileum alive, while of *L. fermentum* KLD and *Lactococcus lactis* MG1363 only 0.5 and 1.0% of the consumed bacteria could be recovered, respectively [13]. In addition, distinct persistence and survival characteristics of *L. gasseri* [14], *L. reuteri* [15], and *L. plantarum* [15] mixed with other species were reported. Moreover, several studies using three strains of *L. reuteri* illustrated the wide-range of GI persistence characteristics of these strains, which ranged from detection on 14 to 49 days following consumption by volunteers [16-18]. These studies indicate that strains of the same species may display considerable variation in GI-tract persistence. However, this information is only available for very few species, and is restricted to only few strains of these species.

L. plantarum is encountered in a variety of artisanal and industrial fermentations, ranging from vegetables to milk and meat [19]. Next to this dietary abundance, *L. plantarum* is frequently encountered as a natural inhabitant of the GI-tract of several mammals, including humans [20], and specific strains are commercially exploited as probiotics [21]. A single colony isolate of *L. plantarum* NCIMB8826, designated *L. plantarum* WCFS1, was the first *Lactobacillus* strain of which the full genome sequence was reported [22]. An *in vitro* GI-tract assay combined with transcriptome-trait matching, followed by mutagenesis approaches [23], established a role of an AraC-family transcription regulator (Lp_1669), a penicillin-binding protein (Pbp2A), and a Na^+/H^+ antiporter (NapA3) in survival under intestinal conditions [7]. Furthermore, specific stress responses in *L. plantarum* have been deciphered [24-27], including GI-tract relevant conditions like bile exposure [27,28]. Finally, studies also have addressed the transcriptional response to specific GI conditions in mice [29,30] and humans [31].

Here we present the different survival capacities of a set of *L. plantarum* strains in an *in vitro* assay that mimicks the physicochemical conditions encountered during the initial stages of passage through the human GI tract. To validate these findings to the real-life situation, a next-generation sequencing-based method was developed that is able to discriminate individual strains based on a variable intergenic region. This method was employed to quantitatively follow mixtures of *L. plantarum* strains during digestive tract transit in healthy human volunteers, allowing the determination of the competitive population dynamics persistence of 21 *L. plantarum* strains *in vivo*. This approach revealed that strain-specific GI persistence profiles appeared highly stable across volunteers. Moreover, quantitative ranking of *in vivo* human GI-tract persistence levels of the individual strains was significantly correlated to the ranking obtained for the *in vitro* GI-tract survival assay, providing qualitative predictive value to the *in vitro* method used.

Materials and methods

In vitro GI-tract assay

All strains used in this study are listed in Table S1. Strains were grown in 2× chemically defined medium [32] at 37°C. Prior to exposure to the GI-tract assay, the strains were washed in prewarmed PBS at 37°C. The GI-tract assays were performed as described previously for *L. plantarum* WCFS1 [7]. Briefly, gastric juice (GJ) containing freshly added pepsin and lipase was added to the cultures and the samples were incubated at 37°C while rotating at 10 rpm. GJ at a pH of 2.5 was used for cells harvested from logarithmic phase [optical density at 600 nm (OD_{600}) = 1.0 as measured photospectroscopically (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK)] and pH 2.4 for stationary phase *L. plantarum* cells (harvested 25 h after inoculation). After 60 min incubation in GJ, the samples were pH-neutralized and pancreatic juice (PJ) containing pancreatin and bile salts was added, followed by incubation for another 60 min. Samples were taken prior to incubation, and after GJ- and PJ-incubation to determine relative survival rates on basis of colony forming units (CFUs) by spot plating of serial dilutions followed by incubation at 30°C for 2 days.

Human trial

The study protocol was approved by the Medical Ethical Committee of Wageningen University, registered under number NL29812.081.09, and the study was conducted according to the principles of the Declaration of Helsinki. Volunteers were aged between 18 and 65 years, had no known health problems, consumed no commercially available probiotic products during the month prior to first fecal sample donation, and had a routine defecation frequency of approximately once per day. Participants were asked to maintain their normal diet, whilst not consuming any commercial probiotic products. Exclusion criteria were defined as digestive tract or organ complaints, any symptoms that are likely to be related to a digestive tract disease, intake of antibiotics during the 3 months prior to the experiment, intake of antacids, and pregnancy. Ten healthy volunteers

Table 1. Combinations of 10 *L.plantarum* strains consumed as mixtures by the 10 volunteers.

Subject	1 to 5	6	7	8	9	10
Strain[a]	**WCFS1**	**WCFS1**	**WCFS1**	**WCFS1**	**WCFS1**	**WCFS1**
	ATCC14917	Lp95	LD3	LD3	ATCC14917	Lp95
	NCTH19-2	**NCTH19-2**	**NCTH19-2**	**NCTH19-2**	**NCTH19-2**	**NCTH19-2**
	CIP104450	CIP104450	CIP104450	Q2	Q2	Q2
	CIP104440	H14	CIP104441	CIP104440	H14	CIP104441
	KOG18	LP80	KOG18	LP80	KOG18	LP80
	ATCC8014	KOG24	KOG24	CIP104448	CIP1044448	ATCC8014
	LP85-2	NCIMB12120	DKO22	NCIMB12120	LP85-2	DKO22
	299v	299v	299	299	299v	299
	NC8	**NC8**	**NC8**	**NC8**	**NC8**	**NC8**

[a] Strains indicated in bold are consumed by all volunteers.

participated in the study, which all signed a written informed consent form and were informed that they could withdraw from the study at any time without providing a reason.

*L. plantarum s*trains were isolated from highly variable habitats (Table S1). Bacterial preparations containing 10 *L. plantarum* strains (Table 1) mixed in equal amounts based on culture optical density at 600 nm (OD_{600}) were prepared essentially as described previously [33]. Briefly, *L. plantarum* strains were cultured at 37°C in MRS (Difco, West Molesey, United Kingdom), washed with peptone-physiologic salt [0.1% (w/v) peptone and 0.85% (w/v) sodium chloride], and mixed in equal amounts [according to their OD_{600} as measured photospectroscopically (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK)]. Cells were collected by centrifugation at 4000 x *g* for 10 min at room temperature and pellets were dissolved in 20% (w/v) maltodextrin, 2% (w/v) glucose solution prior to consumption. Each portion contained approximately 10^{11} CFU. Four mixtures were prepared in which a 10-fold dilution range of strain WCFS1 was included in a standard mixture of 9 other strains (ATCC14197, NCTH19-2, CIP104450, CIP104440, KOG18, ATCC8014, LP85-2, 299v, and NC8). Fecal samples were collected on two different days prior to the intake of the bacterial preparation, and subsequently on the day the volunteers received the bacterial preparation (day 0) and daily during the 10 subsequent days, as well as after 14 and 21 days. Fecal samples obtained were stored at -20°C until DNA isolation (see below). Moreover, to detect *L. plantarum* viability, the fecal samples collected from volunteers 1, 4, and 5 on day 1, 2, 3, 5 and 7 were mixed with glycerol [final concentration of approximately 20% (v/v)] and stored at -80°C prior to plating of serial dilutions. To this end, approximately 2 g feces in glycerol were mixed with 1 ml reduced physiological salt [0.1% (w/v) peptone, 0.05% (w/v) cysteine hydrochloride

and 0.8% (w/v) sodium chloride; RPS], serial diluted, plated on MRS agar plates containing 50 μg/ml streptomycin and 10 μg/ml tetracycline, to which (most, if not all) *L. plantarum* strains are naturally resistant, and incubated at 37°C. From subject 2, the plates appeared to contain no or hardly any colonies with the typical *L. plantarum* colony-phenotype and these samples were therefore excluded in the analysis. Colonies of the other 2 subjects were collectively recovered from the plates containing a high density of single colonies by the addition of 2 ml RPS followed by gentle scraping using a spatula. After washing with RPS, these suspensions were stored at -20°C prior to DNA isolation (see below).

DNA isolation, pyrosequencing, and data analysis of the mixed strains

DNA from *in vitro* bacterial cultures was extracted using InstaGene™ Matrix (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. For variable locus selection and intergenic region sequence determination, the DNA was amplified with primers A to V according to Table S2 and the resulting amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, USA), followed by sequencing (BaseClear, Leiden, The Netherlands). To visualize strain-specific variation in the intergenic region between *lp_0339* and *lp_0340*, the Clone Manager program (version 9.03, Scientific & Educational Software, Cary, USA) was used to align the sequences.

DNA isolation from feces was performed as previously described [34,35]. Briefly, after bead-beating, DNA was purified by 2 to 3 phenol-chloroform extractions, followed by overnight precipitation of the DNA using 1 volume of isopropanol and 1/10 volume of sodium acetate. The resulting pellets were washed with 70% (v/v) ethanol, and dissolved in 100 μl TE buffer by overnight incubation at 4°C. All PCR reactions were performed using KOD Hot Start DNA polymerase (EMD Bioscience, Gibbstown, USA) according to the manufacturer's instructions with primer combinations as listed in Table S2 and S3. The reverse primers used to generate amplicons for high-throughput sequencing of amplicons derived from DNA isolated from the fecal material harbored a unique 6 nt barcode, allowing discrimination of all the samples derived from different time-points and volunteers in a pooled amplicon mixture (Table S2). After amplification of the variable intergenic region from fecal DNA, the resulting amplicons were purified using the Invitek MSB® HTS PCRapace kit (STRATEC Molecular, Birkenfeld, Germany) and their concentrations were measured by NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, USA). Subsequently, the amplicons were pooled in equimolar amounts and ran on, and isolated from a 1.5% agarose gel using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, USA), and analyzed by massive parallel sequencing on a GS FLX (titanium chemistry, GATC Biotech AG, Konstanz, Germany). Sequence data were binned per sampling time point on basis of the unique 6 nt barcodes using the Qiime pipeline [36]. Subsequently, for each of the sequences within a sample, the best hit was determined among the sequences of the 10 variable regions using BLAST [37] in combination with *ad hoc* Python scripts to quantify the relative amount of each strain, using the strictest sequence identity criteria possible (cutoff of 100 % sequence identity across the barcode and the relevant region of the intergenic sequence). In total 89% of the sequences could be linked with a sample.

Quantitative PCR to determine *L. plantarum* amounts

Quantitative PCR using SYBR Green was applied to determine total *L. plantarum* amounts or amounts of the 10 consumed *L. plantarum* strains with the *L. plantarum* 16S-specific primer pair Lp-16Sfo(2) plus Lp-16Sre(2) [28] (Table S2) or the intergenic locus-specific primers Q-PCR_10LP_strains_F plus Q-PCR_10LP_strains_R, respectively (Table S2). 1× Power SyberGreen (Molecular Probes, Eugene, USA), 10 pmol forward primer, 10 pmol reverse primer, and 1000- or 10,000-fold diluted DNA were used as starting material. Reactions were initiated at 95°C for 3 min, followed by 40 amplification cycles consisting of a denaturation step at 95°C for 15 sec, primer annealing at 50°C for 30 sec, and extension at 72°C for 30 sec. Similarly, for the determination of the 10 consumed *L. plantarum* strains, reactions were initiated at 50°C for 2 min and 95°C for 10 min, and followed by 40 amplification cycles consisting of a denaturation step at 95°C for 15 sec and primer annealing and extension at 60°C for 1 min. All runs were completed with amplicon-integrity verification by melting curve analysis. All reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Cycle threshold values were obtained upon manual setting of the baseline at a threshold value at which fluorescence was appreciably above background and within the exponential phase of amplification for all reactions.

Statistical analyses and strain clustering

A Spearman's Ranktest was used to determine the correlation of the *L. plantarum* strains' survival in the *in vitro* GI-tract assay using cells harvested from logarithmic phase compared to stationary phase-harvested cells. Furthermore, this test was used to determine the correlation of the *in vitro* GI-tract survival (stationary phase harvested) compared to the *in vivo* GI-tract persistence of the strains consumed by the first 5 subjects (Table 1). Strains were ranked for robustness according to their $\log_{10}$CFU/ml survival rate after 60 min of gastric juice incubation or according to the averaged difference in relative numbers of sequences after intake of all 5 subjects divided by the relative numbers of sequences of the input sample, respectively. The strains from the latter ranking only got a distinctive ranking if their average value of the different measurements was outside the standard deviation of the nearest strain, while if this was not the case, both strains received the same ranking. The statistical significance of differences between Spearman correlations was determined by Fisher's Z transformation, and P-values < 0.05 were considered significant.

Hierarchical clustering of the individual *L. plantarum* strains based on their absence/presence of genes [19,38] was performed using average linkage agglomeration and Pearson correlation in Genesis [39].

Results

A GI-tract mimicking assay reveals extensive diversity in survival of 42 *L. plantarum* strains

To determine the dynamic range of survival, 42 *L. plantarum* strains, including the reference strain WCFS1, were subjected to a GI-tract mimicking assay. This experiment revealed that the relative GI survival of the strains exceeded a 7 $\log_{10}$ CFU/ml difference for cells harvested either from the logarithmic or stationary phase of growth (Fig. 1). Cells harvested from the stationary phase commonly displayed higher survival compared to cells harvested from the logarithmic phase (Fig. 1A and B). Irrespective of the growth phase from which the cells where harvested, the best surviving strain was *L. plantarum* NCIMB12120, while strains ATCC8014 and CECT4645 displayed the lowest GI survival (Fig. 1). A positive and significant ($p<0.01$) correlation was observed between the strain-specific relative survival when sampled from the logarithmic phase or from the stationary phase, indicating that the differences in survival were independent of the growth phase. Notably, the reference strain WCFS1 was one of the better surviving strains as it was ranked as 6th (logarithmic phase) and 4th (stationary phase) most robust strain, displaying survival rates that were within 1-$\log_{10}$ difference relative to the most robust strain NCIMB12120 (Fig. 1).

To identify candidate genes of *L. plantarum* that affect GI-tract robustness, the survival data of each strain were correlated to genomic diversity data obtained by comparative genome hybridization (CGH) using *L. plantarum* WCFS1 as the reference genome [19]. The colony enumeration of the 42 *L. plantarum* strains (both for logarithmic and stationary phase cells) after exposure to the GI-tract assay conditions were correlated with the CGH derived diversity data using the random forest algorithm [40]. Unfortunately, these analyses did not reveal significant correlations between gene presence and absence patterns in individual strains in relation to their relative GI robustness. The genes that were identified by this correlation with the highest relative significance were consistently belonging to the *L. plantarum* prophages, which are known to be highly variable between strains [19,38], and were considered not plausible as candidate effector-genes in relation to GI-tract survival.

Discrimination of mixed *L. plantarum* strains on basis of a variable intergenic region

To enable assessment of the *in vivo* GI-tract persistence and survival of mixtures of *L. plantarum* strains, and to compare the obtained data to the *in vitro* results, we aimed to identify and exploit a variable region in the genomes of 40 *L. plantarum* strains. Notably, the 2 strains excluded in this analysis as compared to the *in vitro* assay presented above were isolated from spinal fluid or tooth abscess and were therefore considered unsuitable for the human volunteer study. As a source of anticipated variable DNA sequences, non-coding intergenic regions were explored based on the

genome sequence of *L. plantarum* WCFS1 [22]. Candidate intergenic loci were selected on basis of (i) convergent orientation of the flanking genes, (ii) universal conservation of the flanking genes among the strains according to comparative-genome hybridization [38], (iii) length of intergenic region (150-200 bp) and (iv) absence of expression correlation of the flanking regions [41,42]. Moreover, the candidate genetic loci were not allowed to be conserved in other species to prevent the targeting of conserved multi-gene loci. Eleven regions fulfilling these criteria were selected for design of degenerated primers based on the amino acids sequences of the proteins encoded by the flanking genes present in *L. plantarum* WCFS1 (Table S3). These degenerated primers (Table S2, Fig. 2A) were used for amplification of the intergenic regions by PCR using chromosomal DNA from at least 8 *L. plantarum* strains as a template. The target loci that yielded a single amplicon of a length comparable to that obtained with WCFS1 in at least 5 strains were subjected to amplicon sequencing. Some of the amplicons evaluated contained little variation between the strains and thereby were considered unsuitable for the purpose of sequence-based strain tracking, while other amplicons were excluded because their sequencing generated ambiguous results (Table S3). The

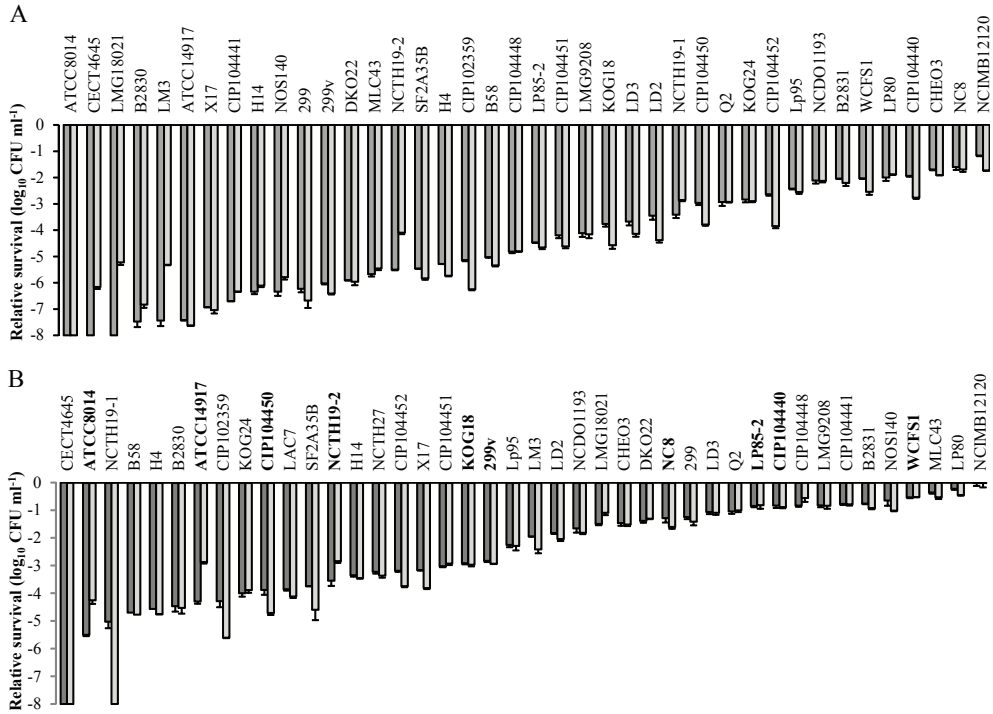

Fig. 1. Relative survival of *L. plantarum* strains subjected to an *in vitro* GI-tract assay. Relative viability loss of *L. plantarum* strains harvested from logarithmic phase (panel A) or stationary phase (panel B) of growth after 60 min (dark grey) gastric juice incubation and subsequent 60 min (light grey) pancreatic juice incubation. The starting population size is set at 0 Log_{10} CFU ml^{-1}, the data presented are averages of technical triplicates (- standard deviation). Strains depicted in bold in panel B were present in the bacterial preparation consumed by subjects 1 to 5.

intergenic region between *lp_0339* and *lp_0340* (designated 339-IR-340) satisfied all criteria mentioned above. To enhance amplification reliability, novel, non-degenerated primers were designed on basis of conserved nucleotide sequences within the amplicon sequences corresponding to the flanking genes of 339-IR-340 (Table S2, Fig. 2A). The isolated genomic DNA of the 40 *L. plantarum* strains was used as template in PCR reactions, resulting in 0.5 kb amplicons using template DNA derived from 34 strains. Subsequent sequencing of these amplicons revealed 10 distinct intergenic sequences in these 34 strains (Fig. 2B and Table S1).

To investigate the distribution of the different variable regions among these 34 strains, the 339-IR-340 regions were projected on the dendogram that was created on basis of the CGH data available for these strains [19,38]. Only 4 of the different sequence variations of the 339-IR-340 region did not co-cluster with the subgroups of strains as they clustered together in the CGH-based dendogram (Fig. 3). This observation indicates that the strain-specific gene absence / presence distributions (based on CGH) are largely, but not universally, correlated with the sequence variation in the 339-IR-340 intergenic region selected. This variable sequence-tag present in the genomes of these strains of *L. plantarum* was employed for sequence based strain-specific quantification in strain-mixtures as described below.

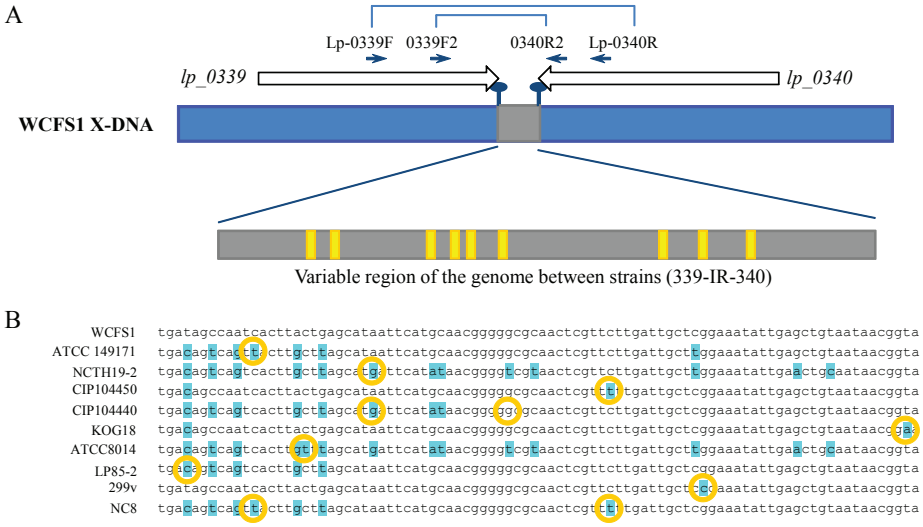

Fig. 2. Schematic representation of the 339-IR-340 region of *L. plantarum* strains. Panel A: Schematic representation of the variable region (grey area) between the *lp_0339* and *lp_0340* genes (white open arrows) of *L. plantarum* WCFS1 with the single nucleotide polymorphism positions (yellow areas) detected in the other strains. Primers used to generate amplicons for sequencing are displayed. Panel B: Sequence comparison of the 10 sequence variations in the 339-IR-340 intergenic region. Yellow circles indicate the nucleotide(s) that distinguish the 339-IR-340 sequence types.

Four mixtures were designed that each contained 10 *L. plantarum* strains with 10 distinctive 339-IR-340 sequences. Using the DNA isolated from these mixtures of 9 strains with a variable amount of the tenth strain (reference WCFS1), revealed that reproducibility of the relative contribution of the 9 strains to the overall bacterial preparation was very high (maximal 11% variation, Table 2). Moreover, the titration of different amounts of the reference strain WCFS1 in this mixture (10-fold dilution range) revealed that within a range of 100-fold dilution the relative

abundance of this strain could still be assessed with high accuracy, while higher dilutions of the WCFS1 population appeared to lead to overestimation of the WCFS1 relative abundance as compared to its actual size (Fig. S1). These experiments establish that the amplicon sequence distribution data allow the accurate detection of strain-specific relative-abundance decreases within a community up to 100-fold, which was clearly sufficient for the reliable determination of strain-specific relative abundances in fecal samples (see below).

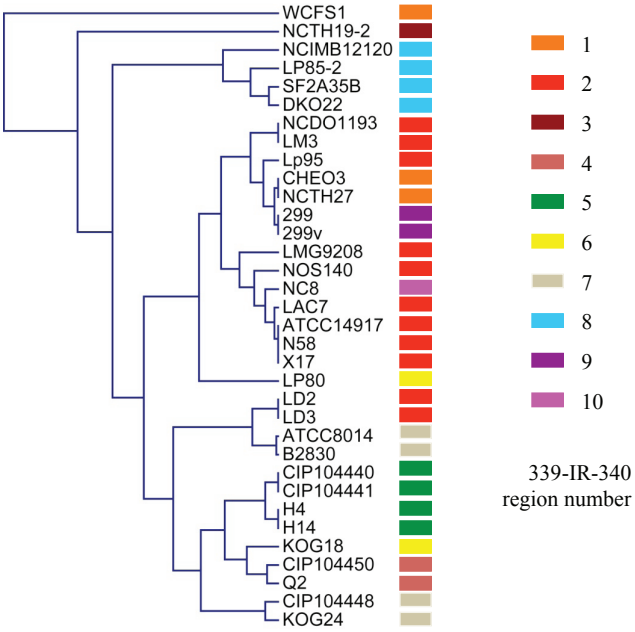

Fig. 3. Co-clustering of 34 *L. plantarum* strains based on the presence/absence gene profiles and the 339-IR-340 region distribution. The previously published comparative genome hybridization datasets [19] were used to construct the genomic relatedness tree presented, which was complemented with the distribution of the 10 distinct 339-IR-340 sequence types, indicated by the colored bars.

Human trial setup

The size of the endogenous *L. plantarum* populations were determined in 2 fecal samples collected from each volunteer prior to initiation of the trial, using Q-PCR with total fecal-DNA as template with primers specific for the *L. plantarum* 16S rRNA gene [28]. The endogenous population of all subjects was on average 3.4 (± 0.41) $\log_{10}$ ng/µg DNA. To assess the population dynamics of a single dosage of 10^{11} bacteria of a mixed population of *L. plantarum* strains in the GI-tract of healthy volunteers, mixtures were designed to contain 10 *L. plantarum* strains with 10 unique variable regions (Table 1). Subsequently, the abundance of individual *L. plantarum* strains was quantitatively monitored in fecal samples collected at different time-points after administration.

Five subjects received a preparation with an identical mixture of *L. plantarum* strains, to assess the variation in population dynamics in individual volunteers using a fixed input community. Next to this group of 5 subjects, the amount of strains that could be assessed in this human trial was enlarged by providing alternative mixtures of 10 *L. plantarum* strains that can be distinguished on basis of their 339-IR-340 sequence to the other 5 volunteers. Overall, this enabled the evaluation of competitive persistence of a total of 21 strains using a universal DNA amplification and sequence analysis regime. Notably, both the reference strain WCFS1 as well as the two strains (NCTH19-2 and NC8) that harbor unique 339-IR-340 sequences (Table S1) were included in all strain mixtures provided to the volunteers. These common strains functioned as reference strains to allow persistence evaluation of the 21 strains relative to these references (Table 1). Following administration, fecal sample collection was performed on a daily basis for a period of 10 days, as well as on days 14 and 21 after consumption. In addition, to determine whether all strains survived the digestive tract, DNA was isolated and amplified from plated fecal samples of 2 subjects (see materials and methods section for more details). These samples indicated that indeed all 10 strains survived GI passage (data not shown).

Table 2. Relative *L. plantarum* strain abundance of 4 independent replicates[a]

Stain / Nr[b]	ATCC14917	NCTH19-2	CIP104450	CIP104440	KOG18	ATCC8014	Lp85-2	299v	NC8	Total
1	0.143	0.188	0.059	0.079	0.120	0.194	0.029	0.099	0.088	1
2	0.140	0.206	0.046	0.083	0.118	0.200	0.028	0.096	0.084	1
3	0.142	0.183	0.050	0.091	0.125	0.195	0.028	0.097	0.088	1
4	0.144	0.187	0.054	0.080	0.121	0.182	0.034	0.100	0.098	1
Average	0.142	0.191	0.052	0.083	0.121	0.193	0.030	0.098	0.090	
St dev[c]	0.002	0.010	0.006	0.006	0.003	0.008	0.003	0.002	0.006	

[a] Four mixtures were designed that each contained 10 *L. plantarum* strains with 10 distinctive 339-IR-340 sequences. The variable amount of the tenth strain (reference WCFS1) was a dilution series and is subtracted from the other strains.

[b] Nr indicates sample number.

[c] St dev indicates standard deviation of the 4 replicates.

Q-PRC was used to determine the total *L. plantarum* community size, using primers designed on the universal part flanking the 339-IR-340 region of the 21 strains included in this study. The first fecal samples collected (usually obtained within 1.5 days after the bacterial mixture intake by the subjects) contained an approximately 2-3 log increased *L. plantarum* population. However, after 3 to 4 days, the *L. plantarum* population sizes returned to the levels prior to intake (data not shown). Fecal DNA samples from which amplicons could be generated were included in the amplicon pyrosequencing analysis. After barcode-based assignment of the sequence data to specific samples, the total numbers of sequences recovered per sample varied between 4805 to 16,905 sequence reads.

Conserved GI-tract persistence patterns of *L. plantarum* strains among human subjects

Initially focusing on the 5 volunteers who consumed the same mixture of strains, it appeared that in all volunteers a consistent group of 5 strains in this mixture were recovered in an approximately equal relative abundance as compared to the input mixture (Fig.4). In contrast, the strains CIP104450 and Lp85-2 were recovered in substantially higher relative amounts as compared to their relative abundance in the input mixture. Conversely, strains ATCC14917, NCTH19-2, and ATCC8014 appeared to be underrepresented in the fecal output compared to their abundance in the input mixture (Fig. 4). Remarkably, the *L. plantarum* community composition remained virtually identical over time in all 5 subjects (Fig. 4). Moreover, evaluation of the relative abundance of the 3 strains that were consumed by all 10 volunteers revealed that, although the variation was larger compared to the 5 subjects who consumed the fixed strain mixture, the same trend was observed for these strains, i.e., WCFS1 and NC8 were stable over time, whereas the relative abundance of NCTH19-2 decreased consistently compared to the input mixture (Fig. 5).

Evaluation of the strain-specific abundance profiles obtained from the other 5 subjects (6-10) that consumed variable *L. plantarum* mixtures, revealed that, despite the small sample numbers, consistent observations were made with respect to the relative abundance of particular strains in the fecal preparations in comparison to their abundance in the corresponding input mixture (Fig. S2). For example, strains LD3, NCIMB12120, and DKO22 seemed to be consistently present in increased amounts compared to their relative population size in the input mixture. In contrast, KOG24, CIP10448, and Lp80 were consistently recovered in smaller relative amounts in comparison to their relative abundance in the input mixture (Fig. S2). Strain Lp95 was administered in mixtures provided to subject 10 and 6, and was recovered in relative high amounts in fecal populations analyzed for subject 10, but was only recovered with relatively low abundance from fecal material of subject 6 (Fig. S2A and E). Notably, strain DKO22 that belongs to the ssp. *argentoratensis* [43] and was consumed by subjects 7 and 10 was detected as the strain with the highest relative abundance increase among all strains tested in this study (Fig. S2B and E), which exceeded the increasing relative abundance described for strains CIP104450 and Lp85-2 (see above).

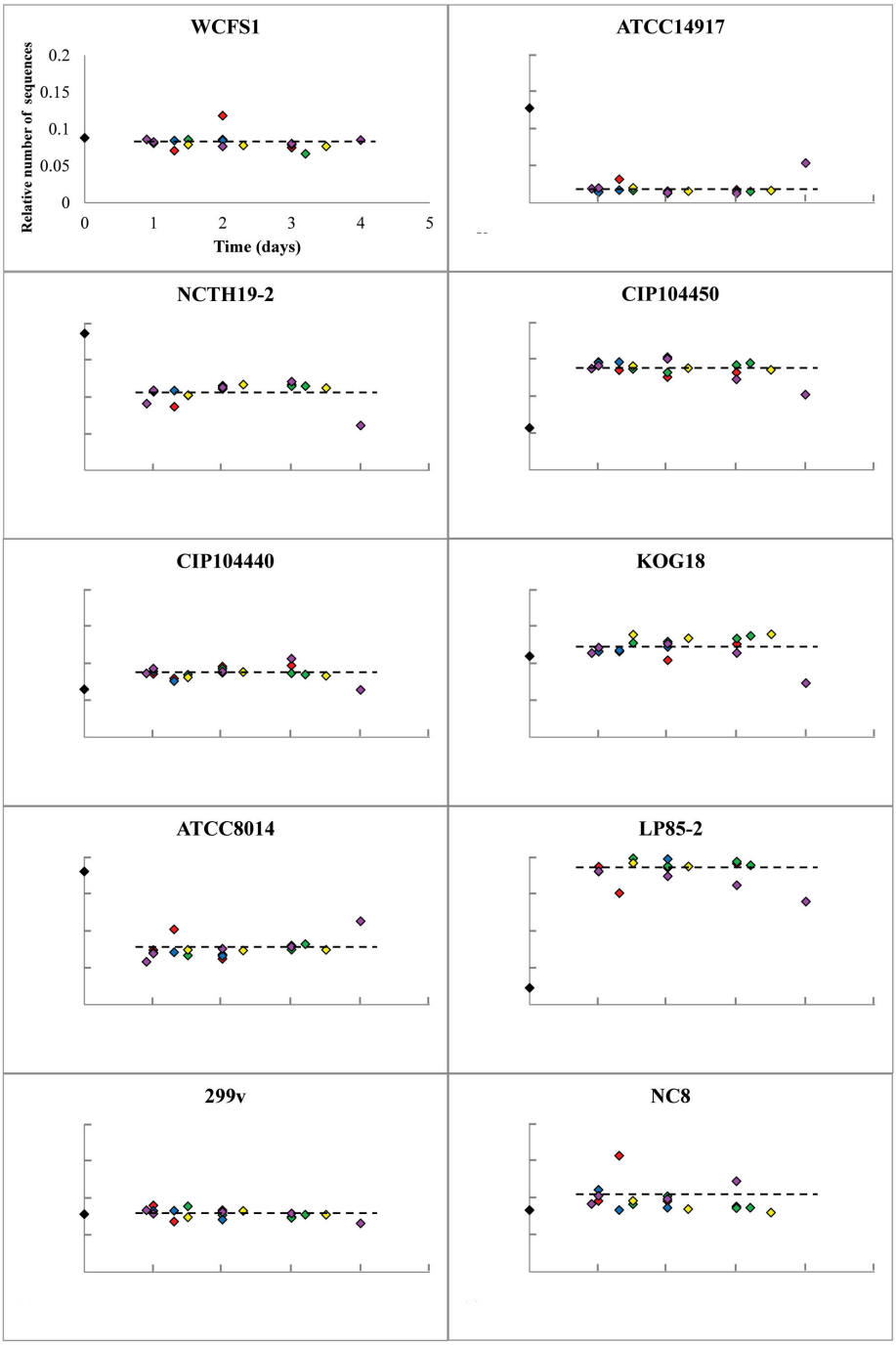
WCFS1
ATCC14917
NCTH19-2
CIP104450
CIP104440
KOG18
ATCC8014
LP85-2
299v
NC8
Relative number of sequences
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
Time (days)

Fig. 4. (left page) Strain-specific *L. plantarum* relative abundance after human consumption as detected by pyrosequencing. Relative strain abundances of the bacterial preparations consumed by the volunteers are depicted in black diamonds and those determined in time-specified post-consumption fecal material from the subjects 1 to 5 in red, green, blue, purple, and yellow diamonds, respectively. The graphs represent the number of strain specific sequences in the amplicons generated from DNA derived from fecal samples, divided by the number of strain-specific sequences identified in the input mixture amplicon. The total number of sequences per sample was set at 1 for normalization purposes. Axis-scaling in all the graphs is the same as depicted for strain WCFS1.

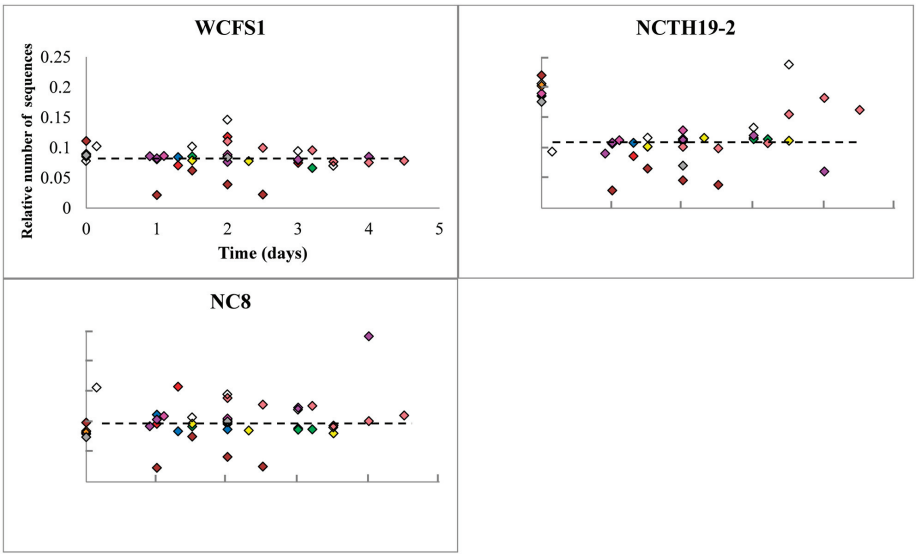

Fig. 5. *L. plantarum* strain WCFS1, NCTH19-2, and NC8 relative abundance after human consumption as assessed by pyrosequencing. Relative strain abundances from subjects 1 to 10 are depicted in red, green, blue, purple, yellow, pink, brown, orange, white and grey diamonds, respectively. The graphs represent the number of strain specific sequences in the fecal amplicons, divided by the number of strain-specific sequences identified in the input mixture amplicon. The total number of sequences per sample was set at 1 for normalization purposes. Axis-scaling in all the graphs is the same as depicted for strain WCFS1.

Correlation of *in vivo* and *in vitro* GI-tract persistence profiles

As the magnitude of the effect on strain specific survival/persistence is considerably different between the *in vitro* and *in vivo* analyses, the ranking of the persistence of individual strains was compared using a Spearman's rank test. This statistical analysis revealed that the *in vivo* strain persistence of the strains from the fixed strain mixture and their *in vitro* GI-tract survival (harvested from stationary phase, Fig. 1B) were positively and significantly (p=0.001) correlated, demonstrating the predictive value of the *in vitro* assay for the pre-selection of strains that are anticipated to display relatively high persistence in the human GI tract. Overall, these data indicate that there are conserved persistence patterns in human individuals that are strain specific, and that the relative persistence may be qualitatively predicted using the simplified *in vitro* screening model presented here.

Discussion

Our *in vitro* GI-tract assay revealed that individual *L. plantarum* strains displayed dramatic differences in GI-tract survival. These data expand earlier *in vitro* observations of variation of GI-robustness among small numbers of *L. plantarum* strains [14,15], towards an extensive cohort of strains of this species that were isolated from various geographical locations and diverse habitats [19]. Considerable variations between *L. plantarum* strains have been reported for other phenotypes as well, such as degradation of carbohydrates, growth at 45°C, and tolerance to NaCl or nisin in the growth medium [19]. Despite the reported success of CGH approaches for the identification of the genetic basis for phenotypes such as mannose specific adhesion and the immunomodulatory capacities of *L. plantarum* [44-46], no significant and plausible correlations between gene presence and absence patterns in individual strains was revealed in relation to their relative GI robustness. This finding suggests that the differences in GI-tract survival are unlikely to be caused by the absence or presence of specific genes compared to the reference strain *L. plantarum* WCFS1. Consequently, it seems likely that the survival differences in the GI-tract assay are predominantly determined by differential gene expression levels of genes that are conserved among the strains included in this collection [19]. This notion is also supported by a recent study performed in our laboratory that demonstrated that the *L. plantarum* WCFS1 GI-tract robustness can be correlated to the transcription level of specific genes [7].

To determine competitive *in vivo L. plantarum* persistence, the variable intergenic region 339-IR-340 was used to develop a novel, high-throughput method to study the population dynamics of mixtures of strains in (complex) matrices like feces. Methods that were already available to discriminate *in vivo* digestive tract survival of specific strains in a mixture include selective plating of fecal samples followed by confirmation of strain/species identity, e.g. by methods based on physiological characteristics like sugar utilization capacity [15]. Alternative discriminatory methods rely on molecular typing techniques like plasmid or genomic DNA profiling using restriction enzyme analysis (REA) [15], pulsed-field gel electrophoresis (PFGE) [47,48], or PCR based fingerprinting

techniques like random amplification of polymorphic DNA (RAPD) [49], arbitrarily primed PCR (AP-PCR) [14], PCR-denaturing gradient gel electrophoresis (PCR-DGGE) [14], internal transcribed spacer PCR (ITS-PCR) [47], or Real-Time PCR [14,50]. Generally, these techniques are labor-intensive and cannot be applied in a high-throughput manner. Alternative methods that can quantitatively discriminate individual strains in a large set of closely related mixed strains (e.g. from the same species) depend on introduction of different antibiotic resistance markers in the genome [51] or on discriminative insertions in the DNA (for example tags [24] or transposons [52]) in closely related strains. The method described here is analogous to the traditional multi-locus sequence typing (MLST), which relies on the natural genetic variance between strains. However, the method employed here targets an intergenic region with a high degree of sequence variability among strains rather than the commonly applied targets of housekeeping protein encoding genes in MLST. The intergenic region used here displayed 10-different sequence types among the strains analyzed but its sequence diversity may be expanded by sequencing this region in a larger panel of strains. Importantly, the method described here is compatible with barcoded next-generation sequencing for the quantitative determination of strain specific abundance levels in a complex mixture enabling low labor intensity, high-throughput analysis of community dynamics.

The detection of the 10 strains in the feces after consumption by healthy human volunteers via plating and pyrosequencing showed that all these strains are able to survive GI passage. Several studies have used inert radiopaque markers to establish that the upper limit of total GI transit time in normal individuals is 96 hour [53,54]. The GI persistence of *L. plantarum* WCFS1 in human volunteers appeared similar to what has been detected before, i.e., detectable up to 3, but not up to 7 days after the last intake [13]. The shape of the persistence curve obtained for all *L. plantarum* strains also reflects the passage of *Bacillus stearothermophilus* spores that are considered to pass the intestine inertly [13]. Despite the typical transient behavior of *L. plantarum* in the human intestine, it is still very possible that *L. plantarum* influences the host, for instance by stimulating the immune system as has been demonstrated for different lactobacilli *in vivo*, including *Lactobacillus plantarum* [33,55].

Remarkably, the persistence of individual strains appeared to be strongly conserved between human individuals. This suggests that intestinal passage is not drastically influenced by the subject-specific characteristics, such as gender, dietary intake, or endogenous microbiota composition. Moreover, the equal distribution of the 3 strains that were consumed by all volunteers indicates that the persistence is independent of the combination of *L. plantarum* strains used in the bacterial preparations. Although only measured in two volunteers, the strain with the most distinguishable enhanced persistence compared to the rest of the strains was DKO22. Intriguingly, the strains that cluster together on basis of their gene content with DKO22, namely NCIMB12120 and Lp85-2, also displayed a higher persistence as compared to the majority of the strains. These 3 strains all belong to the ssp. *argentoratensis* [43], suggesting that this subspecies may display enhanced GI persistence relative to the *L. plantarum* strains. A larger group of spp. *argentoratensis* strains should be tested to get a more accurate impression of the strain-specific GI-tract persistence of representatives of this subspecies.

The most discriminative factor involved in the determination of gut-persistence of *L. plantarum* consistently appears to be their capacity to survive the acid conditions encountered in the stomach. Following the loss of viability of the individual strains in the stomach mimicking conditions of the *in vitro* GI-tract assay, the subsequent small intestine-like conditions did not appear to drastically influence viability. This characteristic is also reflected by the recovery curve obtained in the *in vivo* persistence analysis in humans, where the strains all displayed identical recovery/persistence curves, suggesting that once they have passed the stomach, the rest of the intestinal tract does not provide any strain-discriminative selection conditions. Apparently the combination of strains in the mixture did not influence the survival capacity of its individual components, which is remarkable since competition is commonly expected to especially affect closely related strains. This observation may be related to the fact that *L. plantarum* is apparently not an effective colonizer of the intestinal tract of humans, and displays persistence curves that resemble that of a mere passant of the GI-tract, for which the gastric pH is the main hurdle for survival of intestinal passage.

The work presented here demonstrates that there is considerable variation in strain-specific GI-tract survival among *L. plantarum* strains, which is especially apparent from the *in vitro* assay results. These differences were substantially smaller in the *in vivo* persistence analysis, but the two approaches generated a congruent relative ranking of strains with respect to their GI-tract survival and/or persistence. Remarkably, the data presented imply that the *in vivo* persistence of *L. plantarum* strains is not strongly affected by the undoubtedly substantially different host-specific factors, like gender, genetic background, life-style and/or dietary habits.

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5

Supplemental material

Congruent strain specific intestinal persistence of *Lactobacillus plantarum* in an intestine-mimicking in vitro system and in human volunteers

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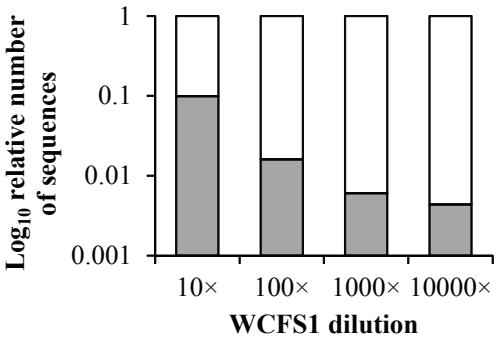
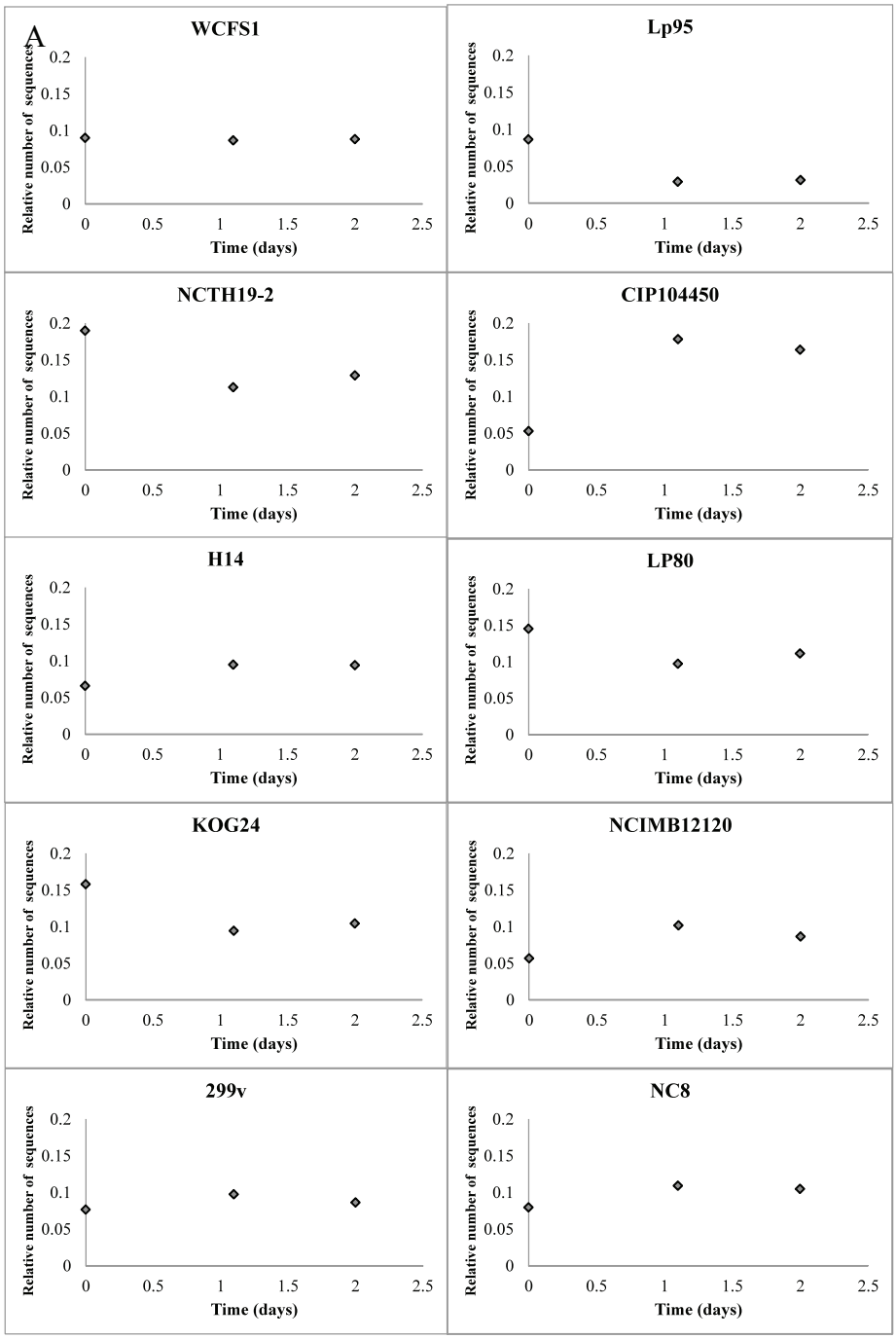
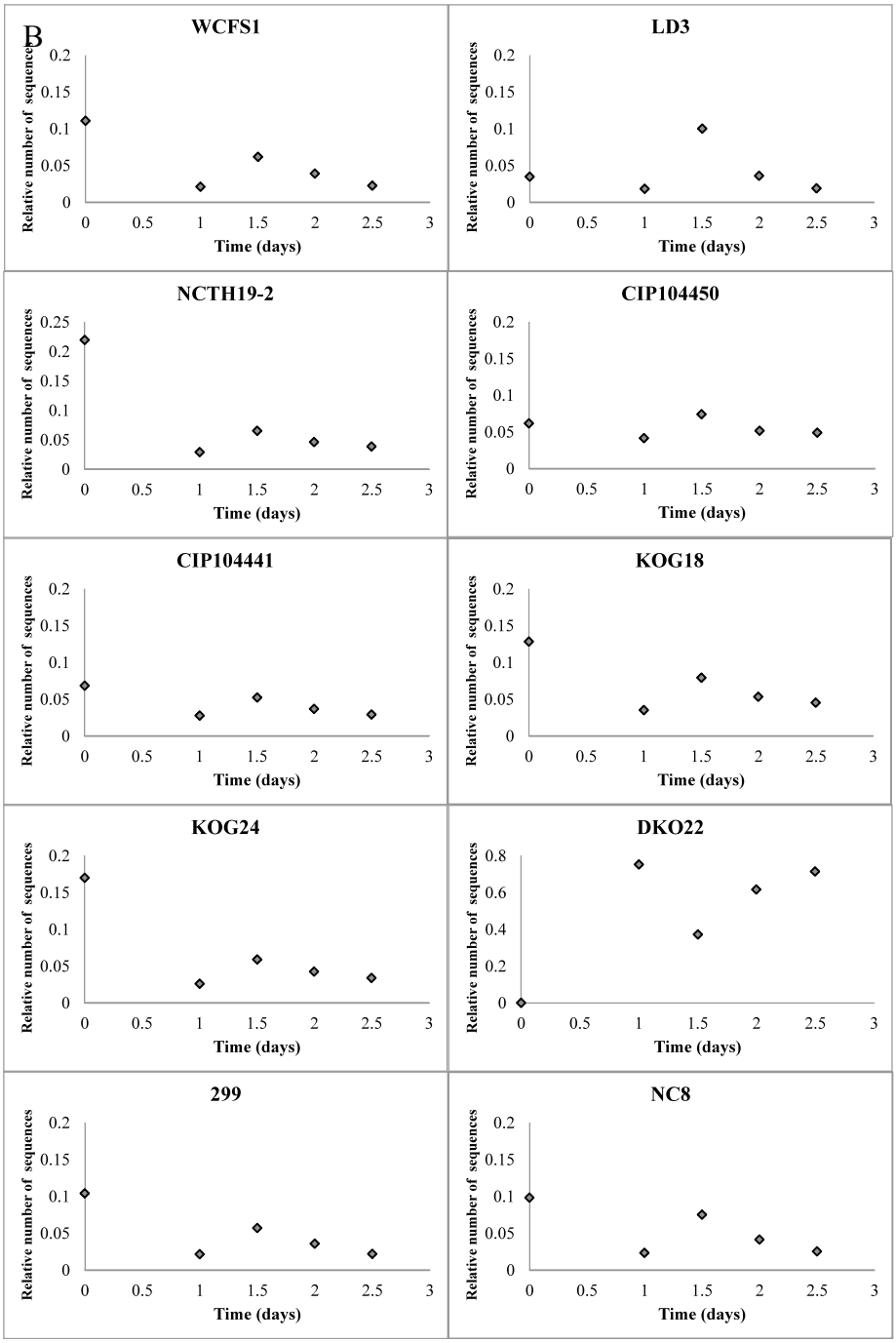
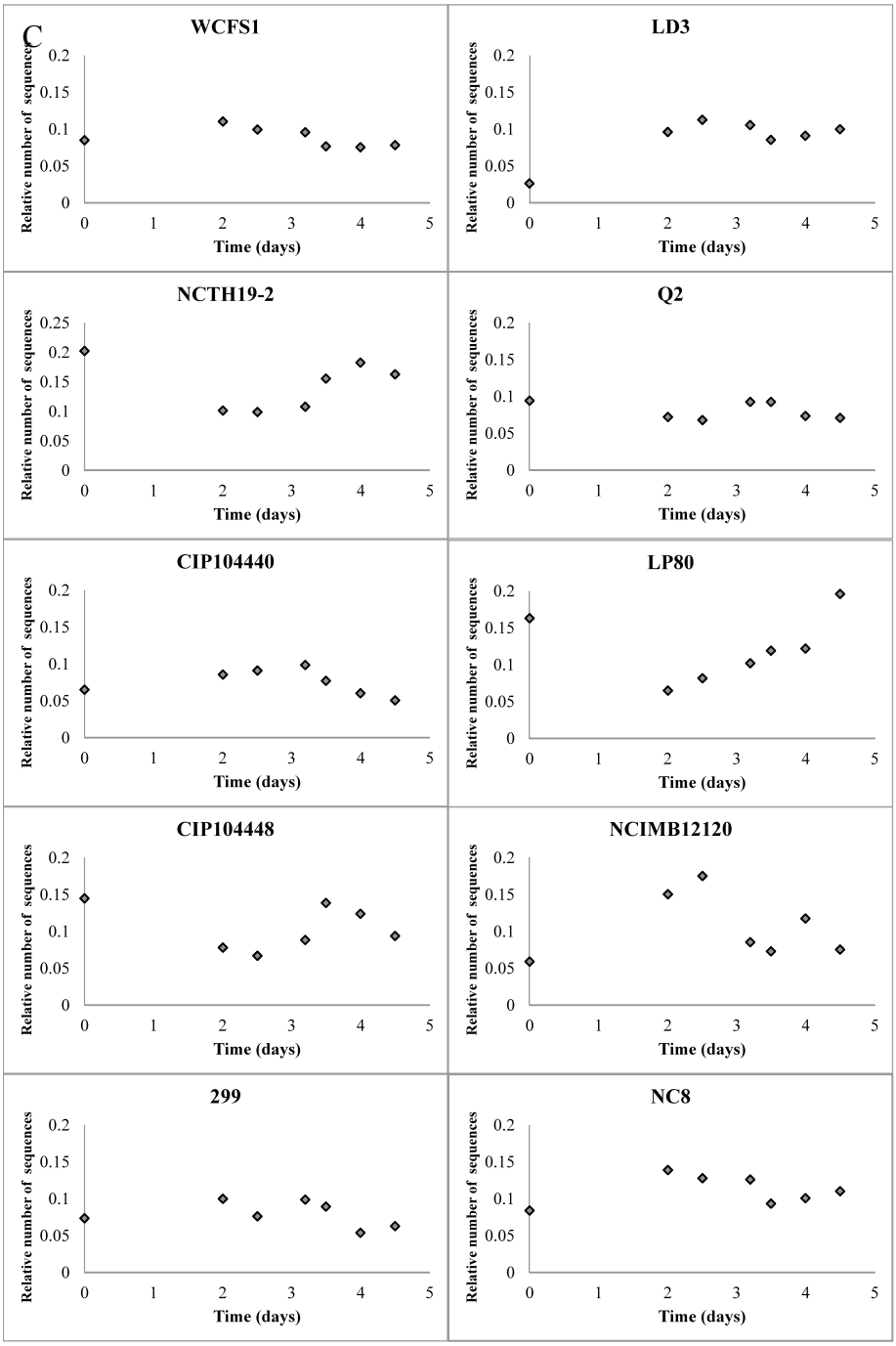


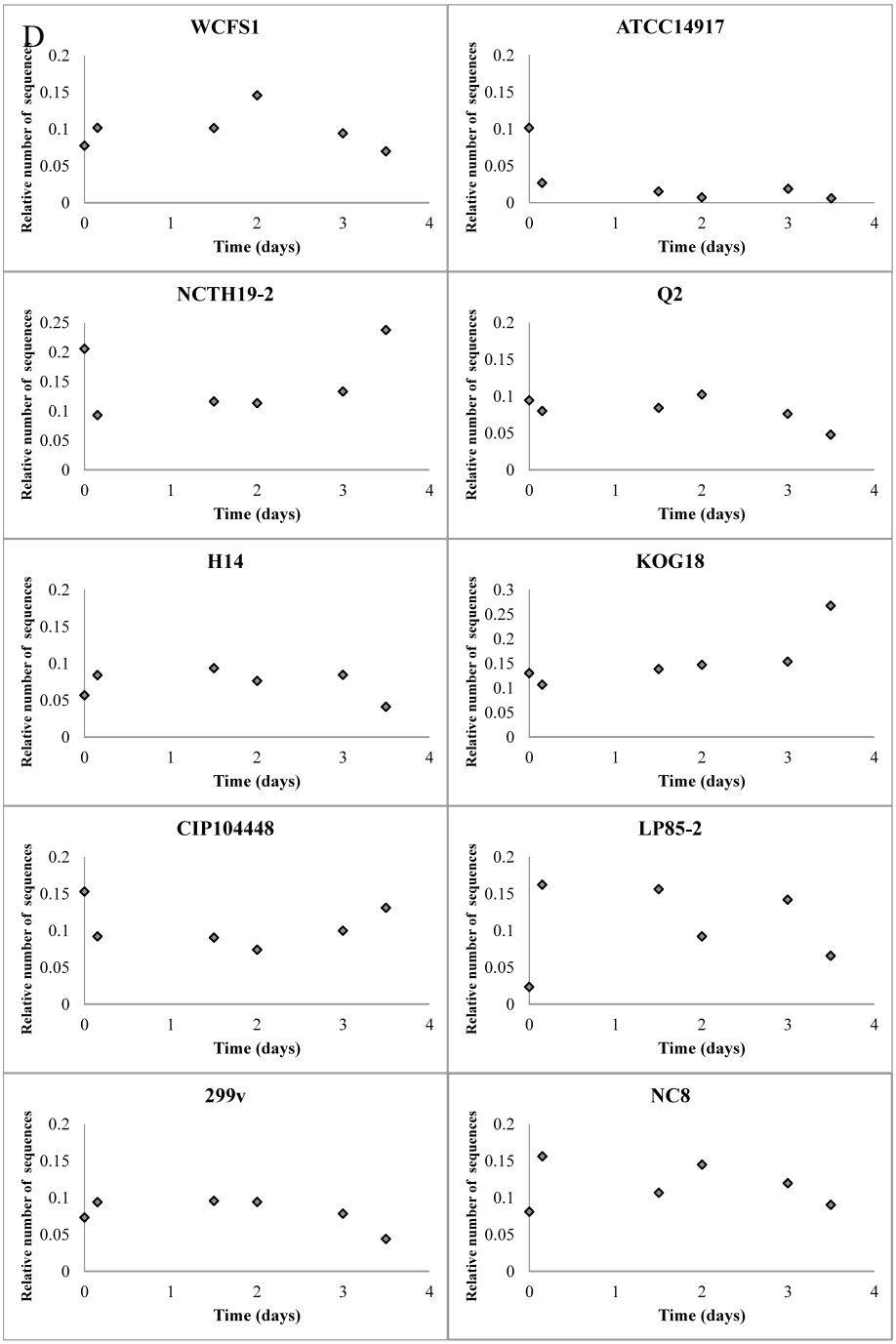
Figure S1. *L. plantarum* mixture of 10 strains with 10-fold dilution range of *L. plantarum* WCFS1 relative abundance. The relative number of sequences of 4 10-fold dilution steps is depicted for WCFS1 (grey bars) and 9 undiluted other strains together (white bars). Total number of sequences per sample is set at 1.

Figure S2 (see below). Strain specific *L. plantarum* relative abundance in human fecal samples detected by pyrosequencing. Individual strain abundance is shown for subject 6 to 10. Graphs represent number of strain-specific sequences divided by the number of sequences identified for the same strain in the input mixture. Total number of sequences per sample is set at 1, for normalization purposes. Panel A to E represent subjects 6 to 10, respectively.


A
WCFS1
Lp95
NCTH19-2
CIP104450
H14
LP80
KOG24
NCIMB12120
299v
NC8
Relative number of sequences
Time (days)
0
0.05
0.1
0.15
0.2
0.5
1
1.5
2
2.5


B
WCFS1
LD3
NCTH19-2
CIP104450
CIP104441
KOG18
KOG24
DKO22
299
NC8
Relative number of sequences
Time (days)


C
WCFS1
LD3
NCTH19-2
Q2
CIP104440
LP80
CIP104448
NCIMB12120
299
NC8
Relative number of sequences
Time (days)


D
WCFS1
ATCC14917
NCTH19-2
Q2
H14
KOG18
CIP104448
LP85-2
299v
NC8
Relative number of sequences
Time (days)

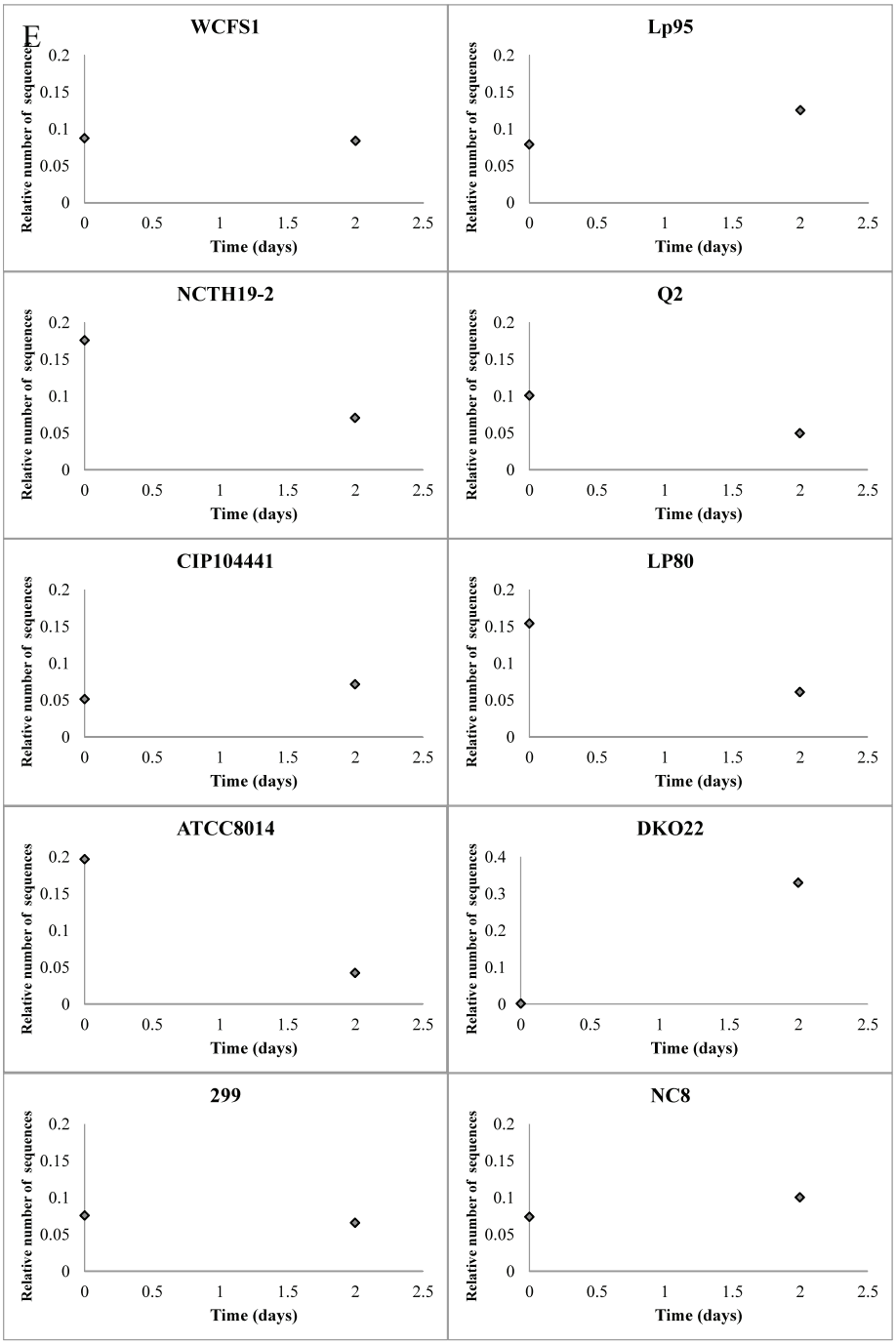

E
WCFS1
Lp95
NCTH19-2
Q2
CIP104441
LP80
ATCC8014
DKO22
299
NC8
Relative number of sequences
Time (days)

Table S1. Strains used in this study[a].

Strain	Alternative designation	Origin	339-IR-340 region #[b]	Source or reference[c]
WCFS1d	NCIMB 8826	Human saliva, UK	1	[1]
ATCC14917	LMG 6907	Pickled cabbage, Denmark	2	ATCC
MLC43		Raw cheese with rennet, Italy		WUR
CHEO3		Pickled sour sausage, Vietnam	1	NIZO
NCTH19-1		Pickled sour sausage, Vietnam		NIZO
NCTH19-2		Pickled sour sausage, Vietnam	3	NIZO
NCTH27		Pickled sour sausage, Vietnam	1	NIZO
LD2		Fermented orange, Vietnam	2	NIZO
NOS140		Cabbage kimchi, Japan	2	NIZO
Q2		Fermented sourdough, Italy	4	DSDA
H4		Fermented sourdough, Italy	5	DSDA
H14		Fermented sourdough, Italy	5	DSDA
CECT4645		Cheese		NIZO
KOG18		Turnip pickled with rice bran, Japan	6	NIZO
KOG24		Cheese, Japan	7	NIZO
LMG9208		Sauerkraut, UK	2	NIZO
Lp95		Wine red grapes, Italy	2	NIZO
B2830		Cassava sour	7	NIZO
B2831		Cassava sour		NIZO
N58		Pickled sour sausage, Vietnam	2	FIRI
X17		Hotdogs, Vietnam	2	NIZO
LAC7		Banana fermented, Vietnam	2	NIZO
LD3		Radish pickled, Vietnam	2	NIZO
DKO22e		Cassava sour, Nigeria	8	NIZO
299	DSM 6595	Human colon, UK	9	[2]
CIP104440	61A	Human stool, France	5	CIP
SF2A35B[d]		Sour cassava, South America	8	[3]
NCIMB12120e		Ogi, Nigeria	8	NCIMB
CIP104441	61P	Human stool, France	5	CIP
CIP104450	61BR	Human stool, France	4	CIP
CIP104451	61CA	Human urine, France		CIP
CIP104452		Human tooth abcess, France		CIP
299v	DSM 9843	Human intestine, UK	9	[4]
NC8		Grass silage, Sweden	10	[5]
LM3		Silage	2	[6]
LP80	DSM 4229	Silage	6	C. Platteeuw
LP85-2e		Silage, France	8	[7]
ATCC8014	LMG 1284	Maize ensilage	7	ATCC
NCDO1193	LMG 9209	Vegetables	2	NCIMB
CIP102359		Human spinal fluid, France		CIP
CIP104448	61BB	Human stool, France	7	CIP
LMG18021		Milk, Senegal		BCCM

[a] Adapted from Molenaar *et al.* [8] and Siezen *et al.* [9].
[b] Number of the variable intergenic region between *lp_0339* and *lp_0340*. The number is the same as in Figure 3.
[c] NCIMB, National Collections of Industrial, Marine and Food Bacteria, United Kingdom; ATCC, American Type Culture Collection, USA; WUR, Wageningen University and Research Center, the Netherlands; NIZO, NIZO food research collection, the Netherlands; DSDA, Dipartimento di Scienza degli Alimenti, Universitá degli Studi di Napoli Federico, Italy; FIRI, Food Industries Research Institute, Vietnam; CIP, Collection of Institute Pasteur, France; and BCCM, Belgian Co-ordinated Collections of Micro-organisms, Belgium.
[d] Strains in bold are consumed by the subjects, as they could be discriminated on basis of their 339-IR-340 region.
[e] Putative subspecies *argentoratensis* [10].

Table S2. Primers used in this study.

ID	Name[a]	Sequence (5' to 3')[b]	Subject or sample description	Time after intake (days)[c]
A	Lp-0166R	CCCCARTGDGCNGGWTCRTGWCC	166-IR-168	
B	Lp-0168R	DGCRTGDGHNGGYTCRTGWCC	166-IR-168	
C	Lp-0339F	CNTWYAAYATGGCDGGNTGGCG	339-IR-340	
D	Lp-0340R	GCCNGCNATGACNGGNTAYCCNGG	339-IR-340	
E	Lp-0396F	ATNCCYTGRTGCCARTGNGGNGC	396-IR-397	
F	Lp-0397R	HNCVCCAGCNADNGGNCGNCC	396-IR-397	
G	Lp-0415F	GTATTCTTTGCAGATGGGGGC	415-IR-416	
H	Lp-0416R	TAGTGTCATCCAAGATAGCTCC	415-IR-416	
I	Lp-0587F	GGTGTTTGCGCAGAAAGTCCC	587-IR-588	
J	Lp-0588R	YTGAATCCAYTCRTCRYTRGTRTCC	587-IR-588	
K	Lp-0631F	TTCTTCNGTAAGATCTTCACCYCC	631-IR-632	
L	Lp-0632R	CCAACACTWGGTGTTCTATGHCC	631-IR-632	
M	Lp-2464R	TCRCTMGCDATAATGTTAATYGCHGC	2464-IR-2466	
N	Lp-2466F	GTDAAAGCDATCGCTTWTGACCC	2464-IR-2466	
O	Lp-2602R	ACRTAHTKTTGHTGATTDAWVACRCG	2602-IR-2603	
P	Lp-2603R	AAATCACGAAACCCATGAAACCC	2602-IR-2603	
Q	Lp-3124R	CAATATCCTGAGCAGTGCCC	3124-IR-3125	
R	Lp-3125R	CGGCTTCTAGGGCTGCCGC	3124-IR-3125	
S	Lp-3233R	AAATCAAACGAAATGAGCGCCC	3233-IR3234	
T	Lp-3234R	CTACGGTAATGGGCGAGAGC	3233-IR3234	
U	HlociF1	TTAGTTGTTCAGATTCCAGGC	Hloci-IR-Hloci	
V	HlociR1	CCCTGGTACAATGGGACC	Hloci-IR-Hloci	
W	0339F2	CGCCGTAATCAGTTCTTTACG	339-IR-340	
X	0340R2	CCTTTGGGTACATGGACGCG	339-IR-340	
PS00	PS.001 B lp_0339f HvB[d]	CCTATCCCCTGTGTGCCTTGGCAGTCT-CAGTATACCAGTGAAGCATTTGCCG	All subjects	All
PS01	PS.001 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccaataGCGTACCTGTTAGAGAAGCGG	1	1
PS02	PS.002 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccacaaGCGTACCTGTTAGAGAAGCGG	1	2
PS03	PS.003 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccacgcGCGTACCTGTTAGAGAAGCGG	1	3
PS04	PS.004 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccactgGCGTACCTGTTAGAGAAGCGG	Input mix 1-5	
PS05	PS.005 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccagacGCGTACCTGTTAGAGAAGCGG	Input mix 6	
PS06	PS.006 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccagcaGCGTACCTGTTAGAGAAGCGG	2	1.3
PS07	PS.007 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccagttGCGTACCTGTTAGAGAAGCGG	2	1.5
PS08	PS.008 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccatctGCGTACCTGTTAGAGAAGCGG	2	2
PS09	PS.009 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccatggGCGTACCTGTTAGAGAAGCGG	2	3
PS10	PS.010 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccattcGCGTACCTGTTAGAGAAGCGG	2	3.2
PS11	PS.011 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgacaGCGTACCTGTTAGAGAAGCGG	Input mix 7	
PS12	PS.012 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgatcGCGTACCTGTTAGAGAAGCGG	3	1
PS13	PS.013 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgcacGCGTACCTGTTAGAGAAGCGG	3	1.3
PS14	PS.014 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgcctGCGTACCTGTTAGAGAAGCGG	3	2
PS15	PS.015 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgcggGCGTACCTGTTAGAGAAGCGG	Input mix 8	

PS16	PS.016 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgctaGCGTACCTGTTAGAGAAGCGG	Input mix 9	
PS17	PS.017 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccggaaGCGTACCTGTTAGAGAAGCGG	4	0.9
PS18	PS.018 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccggccGCGTACCTGTTAGAGAAGCGG	4	1
PS19	PS.019 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgtcgGCGTACCTGTTAGAGAAGCGG	4	2
PS20	PS.020 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccgtgtGCGTACCTGTTAGAGAAGCGG	4	3
PS21	PS.021 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctaagGCGTACCTGTTAGAGAAGCGG	4	4
PS22	PS.022 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctaccGCGTACCTGTTAGAGAAGCGG	Input mix 10	
PS23	PS.023 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctagaGCGTACCTGTTAGAGAAGCGG	5	1.5
PS24	PS.024 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctcgtGCGTACCTGTTAGAGAAGCGG	5	2.3
PS25	PS.025 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctgcgGCGTACCTGTTAGAGAAGCGG	5	3.5
PS26	PS.026 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcctgtaGCGTACCTGTTAGAGAAGCGG	Input mix 1-5 with strain WCFS1 100× diluted	
PS27	PS.027 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccttatGCGTACCTGTTAGAGAAGCGG	Input mix 1-5 with strain WCFS1 1000× diluted	
PS28	PS.028 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccttcaGCGTACCTGTTAGAGAAGCGG	6	1.1
PS29	PS.029 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGccttgcGCGTACCTGTTAGAGAAGCGG	6	2
PS30	PS.030 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgaacaGCGTACCTGTTAGAGAAGCGG	1 (scraping from plate)	1
PS31	PS.031 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgaaggGCGTACCTGTTAGAGAAGCGG	7	1
PS32	PS.032 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgacagGCGTACCTGTTAGAGAAGCGG	7	1.5
PS33	PS.033 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgacgtGCGTACCTGTTAGAGAAGCGG	7	2
PS34	PS.034 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgagcgGCGTACCTGTTAGAGAAGCGG	7	2.5
PS35	PS.035 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgagtaGCGTACCTGTTAGAGAAGCGG	5 (scraping from plate)	1.5
PS36	PS.036 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgatccGCGTACCTGTTAGAGAAGCGG	1 (scraping from plate)	3
PS37	PS.037 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgatgaGCGTACCTGTTAGAGAAGCGG	8	2
PS38	PS.038 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgattgGCGTACCTGTTAGAGAAGCGG	8	2.5
PS39	PS.039 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcactGCGTACCTGTTAGAGAAGCGG	8	3.2
PS40	PS.040 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcatgGCGTACCTGTTAGAGAAGCGG	8	3.5
PS41	PS.041 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgccacGCGTACCTGTTAGAGAAGCGG	8	4
PS42	PS.042 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcgatGCGTACCTGTTAGAGAAGCGG	8	4.5
PS43	PS.043 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcggaGCGTACCTGTTAGAGAAGCGG	9	0.15

PS44	PS.044 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcgtcGCGTACCTGTTAGAGAAGCGG	9	1.5
PS45	PS.045 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgctagGCGTACCTGTTAGAGAAGCGG	9	2
PS46	PS.046 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcgcttaGCGTACCTGTTAGAGAAGCGG	9	3
PS47	PS.047 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcggaagGCGTACCTGTTAGAGAAGCGG	9	3.5
PS48	PS.048 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcggaccGCGTACCTGTTAGAGAAGCGG	1 (scraping from plate)	2
PS49	PS.049 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcggagtGCGTACCTGTTAGAGAAGCGG	10	2
PS50	PS.050 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcggataGCGTACCTGTTAGAGAAGCGG	Input mix 1-5 with strain WCFS1 10000× diluted	
PS51	PS.051 A lp_0340r HvB	CCATCTCATCCCTGCGTGTCTCCGACT-CAGcggcaaGCGTACCTGTTAGAGAAGCGG	5 (scraping from plate)	2.3
Q1	Lp-16Sfo(2)[c]	TGATCCTGGCTCAGGACGAA	Total *L. plantarum* population	
Q2	Lp-16Sre(2)[c]	TGCAAGCACCAATCAATACCA	Total *L. plantarum* population	
Q3	Q-PCR_10LP_strains_F	GCGGGTGGCGAAGGCTATGTGCGC	339-IR-340	
Q4	Q-PCR_10LP_strains_R	CGAATAAGTGCAGTTTTGCAATTCGC	339-IR-340	

[a] Primers starting with PS in the name are the primers used for pyrosequencing.
[b] Nucleotides in non-capitals are the barcode and underlined nucleotides are complementary to the *L. plantarum* strain DNA.
[c] If applicable.
[d] All primers starting with PS in the name are combined with this forward primer.
[e] Reference for primers Q1 and Q2 is [11]. The other primers were designed in this work.

Table S3. Primer pair combinations used for intergenic variable region amplification and summary PCR and sequencing results.

Primer combinations	Expected product length (bp)	PCR and sequencing results including observed products lengths (bp)	Further use
A + B	530	Seven out of 8 strains yielded a weak product at 530 + non-specific products	No
C + D	800	All 8 tested strains yielded a product at 800 + non-specific products	Yes
E + F	420	No products	No
G + H	400	Six out of 8 strains yielded a product at 400 + non-reliable sequence results	No
I + J	360	All 8 tested strains yielded a product at 360, but little variation was observed	No
K + L	600	Four out of 8 strains yielded a product at 600, possible prophage	No
M + N	690	Thirteen out of 19 strains yielded a product at 690 + variation observed, possible prophage	No
O + P	450	Four out of 12 stains yielded a product at 450	No
Q + R	460	Six out of 8 strains yielded a product at 460 + 2 strains yielded non-specific products	No
S + T	360	One out of 8 strains yielded a product at 360	No
U + V	500	All 19 strains yielded a product at 500, but little sequence variation was observed	No

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6

Genotypic adaptations associated with prolonged persistence of *Lactobacillus plantarum* in the murine digestive tract

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Abstract

Probiotic bacteria harbor effector molecules that confer health benefits, but also adaptation factors that enable them to persist in the gastrointestinal tract of the consumer. To study these adaptation factors, an antibiotic resistant derivative of the probiotic model organism *Lactobacillus plantarum* WCFS1 was repeatedly exposed to the mice digestive tract by three consecutive rounds of (re)feeding of the longest persisting colonies. This exposure to the murine intestine allowed the isolation of intestine-adapted derivatives of the original strain that displayed prolonged digestive tract residence time. Re-sequencing of the genomes of these adapted derivatives revealed single nucleotide polymorphisms as well as a single nucleotide insertion in comparison with the genome of the original WCFS1 strain. Detailed *in silico* analysis of the identified genomic modifications pinpointed that alterations in the coding regions of genes encoding cell envelope-associated functions and energy metabolism, appear to be beneficial for gastrointestinal tract survival of *L. plantarum* WCFS1.

Introduction

The human gastrointestinal (GI)-tract is colonized by trillions of microbial cells termed the microbiota, which outnumbers the amount of human somatic cells by approximately 10-fold [1,2]. Intestinal colonization is initiated immediately after birth, followed by a period of high community composition dynamics. Finally, after infancy, the microbiota reaches a more stable but personal community [3,4] that plays a pivotal role in maintaining gut homeostasis [5,6]. GI diseases such as inflammatory bowel disease and irritable bowel syndrome are associated with altered microbiota compositions that deviate from healthy controls [7]. Moreover, disease symptoms can be counteracted by the dietary consumption of probiotics [8,9], which are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [10]. One possible mechanism of action for probiotics may lie in the fact that they can modulate the immune system of the host [6,11]. This mechanism seems especially feasible in the small intestine, as this region of the GI-tract contains a relatively large amount of the immunomodulatory capacity of the body, while the population size of the endogenous microbiota is relatively small, allowing transient dominance of dietary microorganisms, including probiotics [12,13]. Other mechanisms by which probiotic bacteria are postulated to influence host health include competitive exclusion of pathogens and gut barrier improvement [5,11].

It is recommended that probiotic products contain at least 10^7 live microorganisms per gram or milliliter [14]. Therefore, an important prerequisite for the industrial application of probiotic cultures is their persistence under conditions that include the stresses encountered during the residence in and the travel through the different parts of the host's GI-tract, such as the low pH in the stomach, bile salt and digestive enzymes in the duodenum, a relatively high osmolarity in the colon, as well as stress conditions associated with oxygen gradients that are steep at the mucosal surface, whereas the colonic lumen is virtually anoxic [15]. Hence, to understand and improve probiotic performance, it is important to identify the adaptation factors that promote survival and persistence of probiotics in the GI-tract. Stimulated by this industrial interest, GI stress has been relatively well studied in probiotic species, notably in the lactic acid bacterial genus *Lactobacillus*. For example, GI survival of dedicated gene deletion mutants has been assessed [16-19], and *in situ* induction of gene expression was studied using *in vivo* expression technology [18,20] and transcriptome analysis [16,21] in mice and humans. Adaptation factors of probiotic lactobacilli include adhesins, molecules conferring stress tolerance and nutritional versatility, antimicrobial compounds targeting competing microbes, and factors promoting tolerance to the immune system´s antimicrobial activities [22]. Another interesting technology to study GI-tract adaptation factors is experimental evolution. This strategy was successfully applied to study GI colonization of *Escherichia coli*, demonstrating the importance of mutations in the flagellar *flhDC* operon and in *malT*, the transcriptional activator of the maltose regulon [23]. Although, to our knowledge, adaptive evolution has not been applied to study GI persistence of lactic acid bacteria, this technology was successfully implemented in several species of this group of bacteria. For example *Lactococcus lactis* strain KF147 was adapted from its original plant environment to a dairy environment within 1000 generations [24], and *Lactobacillus plantarum* strain WCFS1 could be adapted to growth on glycerol [25].

In this study, we applied experimental evolution by repeated isolation and feeding of mice GI-tract-adapted *L. plantarum* WCFS1, a model organism for probiotic lactobacilli. We employed an antibiotic-resistant derivative of the sequenced and re-annotated *L. plantarum* WCFS1 strain [26,27]. Derivative strains with extended GI persistence were identified after two rounds of re-isolation. Subsequent re-sequencing and comparison of adaptively selected strains with the original strain revealed the independent enrichment of specific mutations, several of which were located in and upstream of genes related to cell envelope and energy metabolism functions, implying that these functions contribute to the GI-tract adapted phenotype.

Table 1. Bacterial strains used in this study.

Strain	Relevant feature(s)	Reference
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	[27]
NZ3400^{CM}	Derivative of WCFS1 containing a *lox66*-P32-*cat*-*lox71* insertion in the neutral H-locus (*H-locus::cat*)	[28]
NZ3400$^{CM\text{-}RIF}$	Rifampicin resistant derivative of NZ3400^{CM}	This work
NZ3439A$^{CM\text{-}RIF}$	Single colony isolate of NZ3400$^{CM\text{-}RIF}$	This work
NZ3439B $^{CM\text{-}RIF}$	Single colony isolate of NZ3400$^{CM\text{-}RIF}$	This work
NZ3440$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 7 from round 1	This work
NZ3441$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 5 from round 1	This work
NZ3442$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 0 from round 2	This work
NZ3443$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 0 from round 2	This work
NZ3444$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 14 from round 2	This work
NZ3445$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 10 from round 2	This work
NZ3446$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 19 from round 3	This work
NZ3447$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 17 from round 3	This work
NZ3448$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 32 from round 3	This work
NZ3449$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 32 from round 3	This work
NZ3450$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 5 from round 1	This work
NZ3451$^{CM\text{-}RIF}$	Mouse 1 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 23 from round 3	This work
NZ3452$^{CM\text{-}RIF}$	Mouse 2 GI-tract adapted NZ3400$^{CM\text{-}RIF}$, isolated at day 7 from round 2	This work

Materials and methods

Strains and growth conditions

All strains (Table 1) were cultured in de Man-Rogosa-Sharpe (MRS, Merck, Darmstadt, Germany) medium at 37°C. When appropriate, 10 μg/ml chloramphenicol, and/or 50 μg/ml rifampicin were added to the medium. To allow selective plating of the adapted strains from fecal samples (see below) *L. plantarum* NZ3400^{CM} [28] was adapted to 50μg/ml rifampicin by culturing in the presence of increasing concentration of this antibiotic, resulting in strain NZ3400$^{CM\text{-}RIF}$ (Table 1).

Mice and experimental setup

Two wild-type male Balb/c mice were purchased from Harlan (Harlan, Horst, The Netherlands). At the start of the experiments the mice were 10 weeks old. The animals were fed standard chow and water *ad libitum* and were housed in separate cages during the course of the experiment. All animal experiments were performed after receiving approval of the institutional Animal Care Committee of the Groningen University (The Netherlands) and all animals received animal care in compliance with the Dutch law on Experimental Animal Care. NZ3400$^{CM\text{-}RIF}$ was grown overnight, washed twice in peptone-physiologic salt (0.1% (w/v) peptone and 0.85% (w/v) sodium chloride), and concentrated 30-fold in peptone-physiologic salt containing 20% glycerol prior to storage at -20°C. Immediately prior to gavage, the cultures were thawed and washed twice

Table 2. Input for gavage (strain mixtures).

Round number	Colonies					
	Mouse 1			Mouse 2		
	Name or number	Day	Round	Name or number	Day	Round
1	NZ3400$^{CM\text{-}RIF}$			NZ3400$^{CM\text{-}RIF}$		
2	1, NZ3440$^{CM\text{-}RIF}$ [a]	7	1	40, NZ3441$^{CM\text{-}RIF}$ [a]	5	1
	9, NZ3450$^{CM\text{-}RIF}$ [a]	5	1	41	5	1
	10	5	1	42	5	1
				44	5	1
3	46	8	2	16, NZ3443$^{CM\text{-}RIF}$ [a]	0	2
	47	10	2	17	0	2
	49, NZ3444$^{CM\text{-}RIF}$ [a]	14	2	19	0	2
	50	14	2	24	0	2
				25	0	2
				26	0	2
				27	0	2
				28	0	2
				29	0	2
				30	0	2

[a] These strains were selected for genome re-sequencing.

with peptone-physiological salt. Two mice were used for this study. Each animal was subjected to ingestion of one dose containing 1×10^9 colony forming units in 200 µl MRS via gavage. Fecal samples were collected daily until no bacterial cells could be recovered, or for a maximum of 32 days per round. Fecal samples were stored in MRS containing 20% glycerol at -80°C until further use. Fecal samples were serially diluted, plated on MRS agar plates containing 10 µg/ml chloramphenicol plus 50 µg/ml rifampicin, and incubated at 37°C. To confirm that the colonies were derived from the original NZ3400$^{CM\text{-}RIF}$ strain, a PCR with the TaqMan® Universal PCR Master mix (Invitrogen, Molecular probes, Inc, USA) was performed with primers for the *cat* gene (5'-GTTTGTGATGGTTATCATGCAGG-3' and 5'-TGTAACGGTAAGTGCACCG-3') and for an *L. plantarum* WCFS1 specific gene (*nspA* [29]; 5'-ATGCTCAATACTATTATTACACG-3' and 5'-TGTCGATAGTTTAACTTTTTCTGACC-3') according to the manufacturer's instructions. Template material was part of a colony that was lysed by 2 min incubation at 800 W in a microwave (Intellowave, LG, Amstelveen, The Netherlands) and amplicons were visualized on a 2% agarose gel. To obtain pure cultures, single colonies with the correct genotype were streaked on MRS agar plates and incubated at 37°C. This procedure was repeated twice. Subsequently, single colonies were grown overnight in 10 ml of MRS (Merck, Darmstadt, Germany) at 37°C and stored in MRS containing 20% glycerol at -80°C. The second and third round of gavage were performed with the same mice and bacterial cell preparation procedures as the first round with the notion that each mouse received only cultures that were isolated from its own feces and consisted of equally mixed liquid cultures derived from the colonies as listed in Table 2.

DNA isolation, re-sequencing, and data analysis

Genomic DNA isolation of cultures selected for re-sequencing (Table 1) was performed using a cell lyses method followed by proteinase K-treatment and phenol-chloroform extraction as described previously [30]. Full genome re-sequencing using Illumina technology (paired end, 100 nt) was performed by GATC-Biotech (Konstanz, Germany), resulting in a genome coverage per sample between 500 and 1100× the *L. plantarum* WCFS1 genome. Structural variations (SVs; single nucleotide polymorphisms (SNPs) and small insertions and deletions) in the Illumina reads of the *L. plantarum* WCFS1 derivatives compared to the *L. plantarum* WCFS1 genome sequence were identified using an in-house developed tool RoVar (SAFT van Hijum, VCL de Jager, B Renckens, and RJ Siezen, unpublished data; http://trac.nbic.nl/rovar). To prevent that reads were aligned to ambiguous regions in the reference sequence, repeat masking of the reference sequence was done by (i) creating 30-bp fragments, (ii) aligning these fragments to the reference sequence by using BLAT [31] with a tile size of 8, and (iii) masking regions (replace the original sequence by N nucleotides) to which fragments align perfectly in multiple positions in the reference sequence. To detect SVs, read alignment to the reference was performed by BLAT (tile size of 8). To reduce read alignment artifacts, alignments were allowed, provided that SVs were at least 4 bp from either the 3' or 5' end of a given read. SVs were used for further analysis provided that they were supported by at least 20 reads of which at most 5% of the reads were allowed to suggest an alternative allele. SVs that were detected in only one of the original strains but not in the genomically adapted strains were excluded. In addition, if all strains contained the alternative allele at a frequency higher than 50%, the SV was

also excluded. Protein structure analysis was performed using the webserver Project HOPE [32] by submitting the original and mutated proteins. Area under the curve was calculated according to the trapezoidal rule.

Results and Discussion

The persistence of *L. plantarum* to the murine GI-tract environment can be extended by repetitive exposure

To assess whether it is possible to adapt *L. plantarum* WCFS1 to the murine GI environment, a single dose of a chloramphenicol- and rifampicin-resistant derivative strain of *L. plantarum* WCFS1 ($NZ3400^{CM\text{-}RIF}$) was administered to two individually housed mice by gavage. Notably, when fecal samples of these mice were plated prior to gavage, no chloramphenicol- and rifampicin-resistant colonies were detected (data not shown), demonstrating our antibiotic-based plating method is fully selective. Moreover, the identity of the obtained colonies after GI passage was determined by employing PCR on individual colonies. This analysis confirmed that for all colonies distinct amplicons of the anticipated size were obtained using both an $NZ3400^{CM\text{-}RIF}$ specific- primer pair that amplifies the chloramphenicol resistant gene (*cat*), as well as an *L. plantarum* WCFS1 specific primer pair (targeting *nspA*) (data not shown) [29]. *L. plantarum* $NZ3400^{CM\text{-}RIF}$ could be isolated from the fecal samples by selective plating for up to five and seven days following gavage-based feeding of mouse 1 and 2, respectively (Fig. 1). It appeared that *L. plantarum* $NZ3400^{CM\text{-}RIF}$ passes quickly through the digestive tract, since at day one the vast majority of colonies of the strain could be isolated from the feces and this number decreased relatively rapidly at the subsequent time points. For the second round of gavage the colonies obtained from the later time-points (the mixture for mouse 1 contained colonies isolated from day five and seven, whereas mouse 2 received a mixture of colonies isolated from day five, Table 2) were purified, cultured in broth, mixed, and administered again as a single dose to the same mouse from which they were originally isolated (Fig. 1). During selective plating of mice fecal samples after the second round of gavage, it appeared that *L. plantarum* $NZ3400^{CM\text{-}RIF}$ was still present in the mice GI-tracts, as colonies of this strain were also detectable on day zero in both mice (prior to gavage) (Fig. 1). Furthermore, the relative number of *L. plantarum* colonies at day one of both mice was lower when compared to the first round ($3.6{\cdot}10^4$ vs $1.5{\cdot}10^7$ CFU/ml), although the highest numbers of colonies was still detected at day 1. However, the persistence curves revealed that colonies could be detected up to 14 and 10 days after the second gavage of mice 1 and 2, respectively. This indicates that the last day at which $NZ3400^{CM\text{-}RIF}$ could be detected had approximately doubled as compared to the first round experiment. Moreover, the area under the curve was slightly increased (1.7 and 1.1 × for mouse 1 and 2, respectively) as compared to the first round (Fig. 1), which suggests a slightly increased proliferation *in situ* in the murine GI-tract. To assess whether the prolonged residence time could be further increased, a second round of re-isolation was initiated. The mixture for this round of gavage for mouse 1 contained mixed cultures based on colonies isolated on day nine, 10, and 14

of round two, while mouse 2 received a mixture of 10 colonies isolated from day zero of round two (Table 2). During this third round, no chloramphenicol- and rifampicin-resistant bacteria were detectable prior to intake. Again, a prolonged persistence curve was observed as compared to the former two rounds, e.g. colonies were still detectable after 32 days (Fig. 1). The area under the curve appeared at least doubled during round three as compared to round two (Fig. 1), indicating a further prolongation of transit time and/or *in situ* proliferation of the strain. Taken together, this experiment demonstrates that extended persistence of *L. plantarum* can be achieved by repetitive exposure to the murine digestive tract.

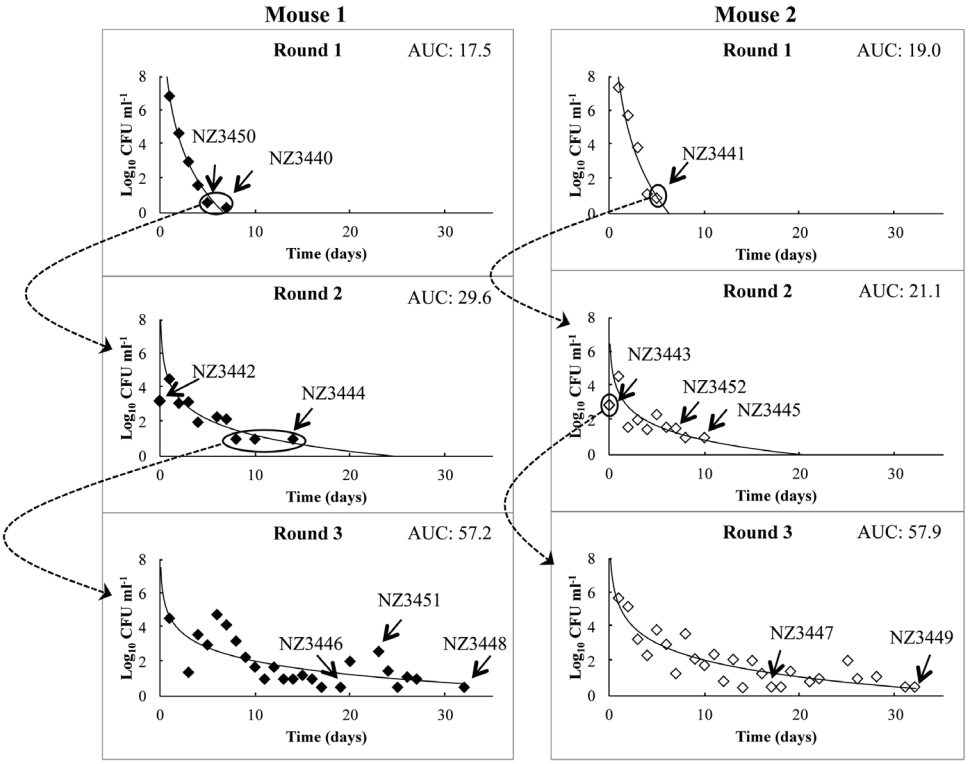

Fig. 1. Experimental setup for the repetitive murine GI-tract passage experiment and colony forming units (CFUs) obtained during this experiment. Dashed arrows indicate the ingested mixed culture for the next round of gavage from the isolated colonies (circled). Small arrows and NZ numbers indicate re-sequenced strains. AUC = area under the curve (in Log_{10} CFU ml^{-1} × days).

Strains adapted to the GI tract harbor genomic adaptations

To evaluate whether genomic adaptations can be identified that may explain the enhanced persistence and/or survival of the identified isolates, 13 isolates were subjected to full genome-resequencing. Besides the 13 adapted isolates, two randomly picked colonies of the original strain were included in the re-sequencing strain collection. The re-sequencing datasets obtained were analysed for structural variations (SVs) using the published and re-annotated genome as a reference [26] (Fig. 1 and Table 1), revealing 26 SVs within the collection of the 13 adapted strains, encompassing 25 single nucleotide polymorphisms (SNPs) and one single nucleotide insertion (Table 3). Of these mutations, 21 SNPs were located within the coding region of annotated genes, whereas the remaining mutations (SNPs and insertion) were localized outside coding regions and their genetic location is referred to as the most proximal gene (either up or downstream of the mutation). Remarkably, several SNPs and the insertion were encountered in more than one isolate of the adapted strain collection, even among isolates that were identified in the two independent experimental set-ups (i.e., mouse 1 and mouse 2; Table 3). This result might be due to the creation of new mutations in the genome that will be accumulated over time or by increasing existing *L. plantarum* cell numbers of a subpopulation that already contain the mutation. Since the experimental procedure allowed for independent adaptive selection starting with more than a single strain-lineage during the three passages, these enriched mutations do not appear in all adapted strains and could thus be selectively adapted in more than one ancestral lineage. Therefore, it is more likely that that the corresponding mutations were selectively enriched in the identified subpopulation of adapted strains of *L. plantarum*. This is supported by the finding that some of the more frequently encountered mutations were also encountered in the genomes of the two randomly picked isolates from the starting (wild-type) population.

Functional distribution of selective genomic adaptations

To evaluate whether genes that belong to a certain functional category (categories as defined in [27]) were more frequently affected by the SVs that were encountered in the more persistent isolates, we analysed the functional category distribution of the mutation patterns found in the isolates, to identify overrepresented functional classes. Remarkably, within the entire list of 26 SVs that were identified, 10 were associated with genes predicted to encode proteins localized in the cell envelope [33], which is highly significant (Fisher exact p-value of 3.8×10^{-5}). Moreover, all resequenced GI-tract persistent strains except one contained at least one SNP associated with a gene that is predicted to encode a protein that is lipid-anchored, membrane embedded by multi-transmembrane domains, N-terminally anchored, involved in glycerolipid metabolism, or glycosyltransfer involved in cell envelope metabolism (Table 3). These findings support the importance of cell envelope-associated functions in the molecular adaptation during GI-tract passage. The glycosyltransferase protein (Lp_1276) in the wild-type strain contains a negatively charged aspartate residue at position 319 that was modified to a glycine residue in four of the higher-persistence derivatives isolated, which derived from both independent mouse experiments. Submission of the alternative amino acid

Table 3. Identified SVs in the strains.

SNP position on genome	Base change	NZ3439A[a]	NZ3439B	Mouse 1							Mouse 2						Gene number[b]	Description	Protein length (AA) and AA change[c]	AA charge change[d]
				NZ3450 (1-5)	NZ3440 (1-7)	NZ3442 (2-0)	NZ3444 (2-14)	NZ3446 (3-19)	NZ3451 (3-23)	NZ3448 (3-32)	NZ3441 (1-5)	NZ3443 (2-0)	NZ3452 (2-7)	NZ3445 (2-10)	NZ3447 (3-17)	NZ3449 (3-32)				
55634	g=t								x								i	*lp_0056*	**cation transport protein**[g]	(471) Glu4Stop[e]	- = N
263132	g=t							x									u	*lp_0291*	FAD/FMN-containing dehydrogenase		
339201	g=t														x		i	*lp_0370*	*glpK*, glycerol kinase	(505) Arg106Leu	+ = N
496786	g=t					x											i	*lp_0547*	***ftsH*, cell division protein FtsH, ATP-dependent zinc metallopeptidase**	(745) Thr135	N
897574	c=a								x								i	*lp_0966*	**integral membrane protein**	(260) Ala23Asp	N = -
1008906[f]	-=c		x	x			•	x	•	x	•	•		•	•	•	u	*lp_1112*	*fum*, fumarate hydratase		
1030257	c=a											x					d	*lp_1132*	**unknown**		
1112413	t=g	x	x	·		•	•	x			•	•		x			i	*lp_1222*	***cps3F*, polysaccharide polymerase**	(296) Gly294	N
1161890	a=g	·	•		x	x	x				x	·	·	·			i	*lp_1276*	glucosyldiacylglycerol 6-beta-glucosyltransferase	(340) Asp319Gly	- = N
1223290	g=a									x							i	*lp_1328*	*glpQ1*, glycerophosphodiester phosphodiesterase	(228) Asp39Asn	- = N
1239032	g=c														x		i	*lp_1348*	unknown	(132) Gly111Arg	N = +
1373534	g=t							x									i	*lp_1499*	*narJ*, nitrate reductase, delta chain	(190) Ala58Ser	N
1630848	g=a				x								x				u	*lp_1801*	integral membrane protein		
1796781	g=a												x				i	*lp_1983*	fructosamine kinase family protein	(280) Met163Ile	N
1848765	c=t					x											i	*lp_2045*	*polC*, DNA-directed DNA polymerase III, alpha chain PolC-type	(1437) Pro526	N
1882497	c=t					x											i	*lp_2087*	*recJ*, single-strand DNA-specific exonuclease RecJ	(778) Trp36Stop[e]	N

2376669	c=t														x	i	*lp_2675*	**multidrug transport protein, major facilitator superfamily**	(387) Arg380Gln	+ = N
2771436	c=a				x								x				i	*lp_3112*	short-chain dehydrogenase/ oxidoreductase	(278) Ala50Glu	N = -
2776w469	g=a														x		i	*lp_3114*	**mucus-binding protein (putative)**	(2032) Thr748Met	N
2797196	g=a					x											i	*lp_3128*	stress induced DNA binding protein	(155) Glu155Lys	- = +
2839736	c=t													x			i	*lp_3185*	**branched-chain amino acid transport protein**	(261) Val195	N
2846257	g=a			x			x	x	x	x							i	*lp_3193*	***prtM2*, peptidylprolyl isomerase**	(309) Thr147Met	N
2925643	c=g								x								u	*lp_3285*	***qacH*, quaternary ammonium compound-resistance protein**		
3034205	c=t			x			x	x	x	x							i	*lp_3418*	*pck*, phosphoenolpyruvate carboxykinase (ATP)	(553) Arg229Cys	+ = N
3169301	c=a														x		i	*lp_3551*	*xfp*, xylulose-5-phosphate phosphoketolase	(803) Ala79Asp	N = -
3298983	c=t					x					x	x					i	*lp_3681*	*gidA*, cell division protein GidA	(636) Glu172Lys	- = +

[a] Indicates in what strain the SV was detected compared with the reference genome of *L. plantarum* WCFS1[26]. NZ3439A and NZ3439B are single colonies isolated from the culture ingested by both mice. Mouse 1: NZ3450 and NZ3440 were ingested in round 2, NZ3444 was ingested in round 3. Mouse 2: NZ3441 was ingested in round 2, NZ3443 was ingested in round 3. A cross indicates ≥ 20 reads and ≤ 5% alternative alleles, a large dot indicates ≥ 20 reads and between 5 and 10% alternative alleles, a small dot indicates < 20 reads, and blank spaces indicate no reads containing mutations were identified at all.

[b] SNP was located in the gene (i), in the upstream region of the nearest gene (u), or in the downstream region of the nearest gene (d).

[c] Indicates the amino acid change caused by the SNP as compared with the wild type. If no other amino acid is stated after the first one, the SNP caused no amino acid change.

[d] Indicates the charge change of the amino acid caused by the SNP as compared with the original amino acid charge. If no charge is stated after the first, the amino acid change caused no charge change. N = neutral charge, - = negative charge, + = positive charge.

[e] Due to the introduction of a stop codon, the gene possibly becomes a pseudogene.

[f] Indicates that this position is located in a *Lactobacillus plantarum* supermotif (LPSM) [35].

[g] Descriptions in bold indicate that the encoding proteins are predicted to be localized in the cell envelope (i.e. the proteins are multi-transmembrane, N-terminally anchored, LPxTG cell wall anchored, or lipid anchored) according to LocateP [33].

sequence to Project HOPE, a toolbox to predict the consequences of specific mutations on protein structure [32], revealed that the loss of the charged residue (Asp) is likely to cause loss of interactions within the protein structure, whereas also the peptide chain flexibility introduced by the alternative glycine residue is predicted to disturb the required rigidity of the native protein at this position. Therefore, it seems conceivable that this Asp-319-Gly substitution leads to loss of function for the encoded glycosyltransferase. Another mutation among the identified cell-envelope associated genes is the SNP detected in the gene encoding a putative mucus binding protein (*lp_3114*), although this mutation was identified in only a single adapted isolate (NZ3449; isolated in the last passage round, and therefore with the most prolonged persistence), it may contribute to the extended persistence observed for this strain. Mucus binding capacity of lactobacilli has been associated with extended intestinal tract persistence, which was clearly evidenced by the comparative genomic analysis of two *L. rhamnosus* strains [34].

Another functional category of genes that were frequently associated with adaptively selected SNPs and insertion was the category of metabolic functions. SNPs were encountered in the coding regions of genes encoding a glycerol kinase (*lp_0370*), glycerophosphodiester phosphodiesterase (*lp_1328*), δ-chain nitrate reductase (*lp_1499*), fructosamine kinase (*lp_1983*), and a xylulose-5-phosphate phosphoketolase (*lp_3551*) in single adapted isolates. However, some (combinations of) mutations were also encountered in several of the adapted isolates, including the accumulation of an insertion in the upstream region of the fumarate hydratase (*lp_1112*) in at least three of the isolates recovered from mouse 1. It also appears to accumulate in the strains isolated from the other mouse, but with lower certainty. Although the consequences of the mutation upstream of the *fum* gene remains unclear, it is especially intriguing that it resides within a previously identified *L. plantarum* supermotif (LPSM) of which the biological function remains unknown, but which may play a role in regulation of expression of up- or downstream located genes [35], possibly under specific conditions like those encountered *in situ* in the intestine. Importantly, adjustment of metabolic functions has previously been associated with the *in situ* adaptation of *L. plantarum* WCFS1 to the murine and human intestinal tract conditions [20,21].

Intriguingly, two independent (derived from different mice) but identical SNPs were encountered within the coding region of a short-chain dehydrogenase oxidoreductase (*lp_3112*), which leads to the replacement of the neutral alanine residue (Ala-50) by a negatively charged glutamate residue. Moreover, identical and independent mutations were also detected in the upstream region of an integral membrane protein (*lp_1801)*. These findings imply that the evolutionary pressure exerted by intestinal tract conditions can elicit the adaptive selection of highly specific genetic variations that provide improved adjustment to these conditions. Analogously, the selective pressure exerted by the intestinal tract conditions also appeared to have led to enrichment of particular mutations, as evidenced by the five-fold identification of the SNPs in both a peptidylprolyl isomerase (*prtM2*; *lp_3193*) as well as a phosphoenol carboxylase (*pck*; *lp_3418*) in the seven isolates derived from mouse 1 in our experiment.

An intriguing and unique combination of SNPs is encountered in the adapted isolate NZ3442,

in which a SNP in the gene encoding a single-strand DNA-specific exonuclease (*recJ*; *lp_2087*) introduces a stop codon in this gene, presumably leading to loss of the RecJ function. Notably, this adapted strain also contains amino acid-altering SNPs in the genes encoding a stress induced DNA binding protein (*lp_3128*) and the α-chain of a DNA-directed DNA polymerase III, of the PolC-type (*polC*; *lp_2045*). Taken together, these findings imply that the impaired RecJ-mediated processing of blocked replication forks may affect the fidelity of the replication-recovery process [36], which may in part be compensated by the additional *lp_3128* and *lp_2045* SNPs identified in this strain. Impaired or reduced efficacy of replication fidelity may result in a mutator phenotype that has previously been implicated in adaptation rates in the (experimental) evolution in bacteria [37]. Moreover, the proposed impact on replication fidelity may affect cell division processes, which in its turn be reflected in the additionally unique SNP in the cell division ATP-dependent zinc metallopeptidase protein FtsH (*ftsH*; *lp_0547*) as well as the cell division protein GidA (*gidA*; *lp_3681*) that were also identified in this strain, although the latter mutation only induced a synonymous amino acid change in the *gidA* encoded protein.

Conclusion

The work presented here demonstrates the feasibility of experimental evolution for the extension of the GI residence time of *L. plantarum* WCFS1. This is relevant considering that the initial persistence-curves that were determined revealed that this strain is rapidly passing the murine GI-tract and does not appear to colonize effectively, which is in agreement with earlier experiments performed with this strain [38]. Moreover, this persistence curve is comparable to that observed for other lactobacilli exposed to the murine digestive tract, including *L. casei* [39], *L. acidophilus*, *L. sakei* [40,41], and the vast majority of *L. fermentum* strains tested [42]. Similarly, when lactobacilli were administered to humans, bacterial fecal counts rapidly decreased when the oral administration of the strain was stopped, as was observed for *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. acidophilus*, *L. paracasei*, *L. gasseri*, and *L. fermentum* in trials with at least nine subjects [43-45]. This also appeared to be the case for *L. plantarum* WCFS1 in human feeding trials with the single strain [46], and several *L. plantarum* strains that were ingested as a mixture [47], which could be largely attributed to the detrimental effects of the low pH in the stomach [48]. Taken together, all these studies generally suggest relatively poor colonization characteristics of lactobacilli in both the murine and human GI-tract, and improvement of this trait, as showcased here for *L. plantarum*, may be feasible for other lactobacilli as well. This approach is likely to result in enhancement of the efficacy of delivery of viable probiotics *in situ* in the GI-tract for several, if not all, of the strains that are currently marketed. Despite the fact that colonization profiles in mice and humans appear very similar, it remains to be determined whether the improved phenotype for the murine GI-tract observed here using an antibiotic resistant derivative of *L. plantarum* WCFS1, can also be achieved in humans using non-GMO approaches. Several strategies seem feasible here, e.g. the chloramphenicol acetyl transferase gene (*cat*) used here is flanked by *loxP* sites, allowing its removal from the murine GI adapted strains by temporal expression of the Cre recombinase [49]. The genetic modification of

the resulting 'resolved' strain would then be restricted to the residual *loxP72* oligonucleotide in the chromosome of the strain, but would lack the antibiotic resistance marker used to facilitate its selection in the mouse experiment. Subsequently, for such a resolved strain it could be tested whether it also displays enhanced robustness and/or colonization in the human GI-tract. Alternatively, we have previously demonstrated the feasibility of antibiotic-based selective plating using naturally occurring antibiotic resistances [47,50], offering the possibility to repeat the experimental approach presented here directly in human volunteers. In conclusion, besides demonstrating the feasibility of achieving enhanced GI-tract robustness, our resequencing efforts of the adapted derivatives advance our knowledge on the GI-tract-persistence mechanisms of *L. plantarum*, which are important to predict and control this organism's *in situ* delivery. Improved understanding of adaptive behavior of bacteria under stress conditions could pave the way towards rational design of methods to maximize cell survival and targeted improvement of digestive tract robustness in *L. plantarum*, but also in many other lactobacilli currently marketed as probiotics.

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7

General Discussion

Hermien van Bokhorst-van de Veen

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General Discussion

This thesis describes the study of stress responses of *Lactobacillus plantarum*, a model organism for probiotic lactobacilli, in relation to its digestive tract robustness. The species was subjected to generally sub-lethal and industrially relevant stresses during its growth and functional readouts included transcriptional and phenotypic adaptation of the bacteria. These strategies were employed to better understand and exploit the gene-regulatory adaptations involved in digestive tract survival of this species, but also evaluated adaptive evolution approaches, to improve the gastrointestinal (GI) persistence of this bacterium. GI-persistence monitoring employed an *in vitro* model, or an *in vivo* mouse or human model. The majority of these studies were performed with the model strain *L. plantarum* WCFS1. However, also the diversity of GI-tract persistence in different strains of this species was addressed *in vitro* and *in vivo*, using a novel strategy to monitor strain-specific intestinal tract persistence in human volunteers.

Below a short summary is given of the novel tools developed in the work presented in this thesis. Subsequently, some future directions for development and GI-tract research in the light of probiotic performance are discussed.

Tool development

An important trait of probiotics is their capability to reach their intestinal target sites alive to optimally exert their beneficial effects. A straightforward strategy to determine GI persistence is to subject probiotic bacteria to *in vitro* GI-tract mimicking assays. Several of these assays have already been developed previously, and many of these models characteristically employ relatively simple assays that subject bacteria to a low-pH solution to resemble stomach conditions, and/or neutral pH solutions containing bile salts to resembling duodenal or small intestinal conditions [1-3]. Alternatively, also more sophisticated GI-tract simulators such as the TNO Intestinal Models and the Simulator of Human Intestinal Microbial Ecosystem can be employed for the same purpose [4-6]. For the research performed in this thesis, a human GI-tract simulating assay was developed that allows assessment of survival of bacterial (e.g. *L. plantarum*) cultures in a standardized and relative high-throughput manner, encompassing exposure to human intestine mimicking conditions and compounds in relevant concentrations (e.g. bile acids, [7]). The assay enabled the detection of both improved or diminished survival rates of differently grown bacterial cultures compared to control growth conditions, but also allowed the comparative evaluation of survival rates of wild-type and mutant strains or a panel of different strains of a species (e.g. *L. plantarum*). Using this assay, the survival of *L. plantarum* WCFS1 cultures grown in media that contained sublethal concentrations of ethanol (Chapter 2) or were exposed to other mild stresses (Chapter 4), was assayed to evaluate cross protective responses that were elicited by these mild stress conditions. In addition, the assay was employed to evaluate the impact of additives in the delivery matrix to explore their impact on bacterial survival (Fig. 1 and see below). Moreover, the assay was employed to compare the survival

rates of the wild-type strain, *L. plantarum* WCFS1 strain with, (i) several mutant derivatives of this strain (Chapters 3 and 4), (ii) other strains of the *L. plantarum* species (Chapter 5), (iii) or strains that belong to other *Lactobacillus* or *Bifidobacterium* species (Table 1). The work described in this thesis revealed that small changes in growth conditions introduced large differences in GI survival (up to 7-log; Chapter 3). Large differences in GI-tract survival were also detected for other *L. plantarum* strains (Chapter 5) and also for strains of other *Lactobacillus* species and bifidobacteria (Table 1). Besides the phenotypic description of survival rates of cultures and strains, the *L. plantarum* WCFS1 survival data were also employed as a 'trait' to identify fermentation condition or bacterial transcripts that correlate with digestive tract robustness. These transcripts can be used as digestive tract robustness markers (see also below). In addition for further study, it would be of great value to subject different mixtures of strains to the same conditions and challenges simultaneously, which enhances efficacy of research but allows strict and direct comparison of strain-specific results. Employing strain-specific detection methods to differentially quantify survival of individual strains that are mixed is also very attractive in animal or human intervention studies, requiring fewer animals or volunteers as compared to parallel evaluation of individual strains. To this end, a novel method to enable such strain-specific detection in mixed bacterial population was developed in this thesis (Chapter 5) and is discussed below.

Several methods are available that discriminate mixed strains and include molecular typing techniques using DNA and restriction enzymes, PCR based fingerprinting techniques, or Real-Time PCR. Less laborious and more high-throughput are techniques that make use of genomically inserted markers like antibiotic resistance genes, transposons, or sequence-tags in closely related strains. Advantages of these markers are that competition experiments can be performed by following the growth of individual strains in a standardized mixture of strains to evaluate how

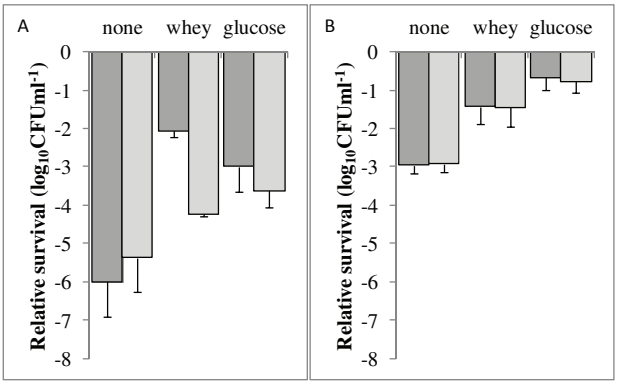

Fig. 1. Relative survival of *L. plantarum* WCFS1, subjected to an upper GI-tract mimicking assay in the absence or presence of additives. *L. plantarum* cultures were grown in MRS at 37°C. The cultures were harvested at mid-exponential phase (OD_{600}=1.0, A) or stationary phase (25 h after inoculation, B) and subjected to an upper GI-tract mimicking assay containing no additive, whey protein isolate (1 mg/ml), or glucose (1.5%). Dark grey bars represent the colony forming units (CFUs) after 60 min gastric juice incubation and light grey bars represent CFUs after subsequent 60 min small intestine incubation. Input is set at 0 Log_{10} CFU ml^{-1}, data presented are averages of technical sextuplicates (- standard deviation).

Table 1. Strain-dependent characteristics of the GI-tract survival assay.

Species	Strain	St resistance*	SI tolerance*
L. rhamnosus	GG#	↑	–
	NCIMB 8824#	↑	↓↓↓
	LMG 10772	↓↓↓	↓↓↓
	LMG 6400#	↑	↓↓
L. acidophilus	NCFM	↑↑	↑
	LAFTI-10	–	–
	I233	↓↓↓	↓↓↓
	LMG 9433	↑↑	↑
L. reuteri	DSM20016	↑↑	↑↑
	100-23	↑↑	↑↑
	DSM 17938	↑	↑
	LMG 9213	↑↑	↑↑
L. casei	P2	↓↓	↓↓
	ATCC334	↓↓↓	↓↓↓
	BL23	↓↓	↓↓↓
	LMG 6904	↓↓↓	↓↓↓
L. helveticus	DPC 4571	↓↓	↓↓↓
	CNRZ32	↓↓	↓↓
L. delbrueckii ssp bulgaricus	LMG6901	↓↓↓	↓↓↓
	ATCC BAA365	↓↓↓	↓↓↓
B. animalis ssp lactis	Bb12	↓	↓
	HN019	–	–
B. longum	LMG 13196	↓↓↓	↓↓↓
	LMG 18899	↓↓↓	↓↓

St Stomach, SI small intestine, *L. Lactobacillus*, *B. Bifidobacteria*. *Compared to *L. plantarum* WCFS1, - no change. #These strains were more sensitive to SI when they were first challenged with St compared to no preceding St incubation.

the strains in the mixture influence and/or compete-with each other. The genetically modified *L. plantarum* WCFS1 derivatives constructed in this thesis all contain a unique sequence-tag at the genetic site of mutation, which enables high-throughput, parallel-, sequence-based analysis of population dynamics in mixtures of these mutants. For example, experiments were performed to evaluate the relative persistence of a mixture of *L. plantarum* WCFS1 mutant derivatives in the mouse gastrointestinal (GI)-tract in comparison to the same mixture grown under laboratorial conditions (unpublished results, P.A. Bron, I. van Swam, M.J. Smelt, M. Wels, P. de Vos, and M. Kleerebezem). Moreover, similar unique sequence tags can relatively easily be inserted in the genome of *L. plantarum* WCFS1 mutant derivatives that were constructed prior to the standard sequence tagging approach or in different strains of the species *L. plantarum*, provided that the insertion side

used for tag-insertion is conserved and other strains can be transformed. The latter post-mutation tagging approach could facilitate the further study of the adapted derivatives with extended persistence in the mouse GI-tract that are presented in this thesis (Chapter 6) in comparison with the parental WCFS1 strain. Ideally, the mouse-intestine adapted strains should also be evaluated in a human volunteer persistence trial, but in that case the genetic engineering to introduce the unique sequence tags would lead to legislator constraints on basis of the use of genetically modified organisms (GMOs) in humans. To enable strain-specific tracking of bacteria in complex ecosystems, the thesis also describes the development of a next-generation sequencing-based method that targets a *L. plantarum* variable intergenic region for sequence-based strain discrimination. Notably, this approach does not require genetic-engineering and employs wild-type strains (Chapter 5). This method was employed to evaluate the *in vivo* GI-tract persistence of strains when administered in mixtures to healthy human volunteers. Up to 10 strains could be combined and this number may be further increased when more sequence varieties of this intergenic region of *L. plantarum* strains are discovered. Alternatively, other hypervariable sequence regions in the genomes of *L. plantarum* strains may be discovered that enable a larger group of strains to be differentially quantified on basis of massive parallel sequence analysis. Notably, the principal approach employed here can be extrapolated and employed to other probiotic species. Especially in the current era where strain specific genome sequencing is becoming feasible through next-generation sequencing approaches and the constantly reducing financial investment required for such efforts.

Another approach that makes use of genetic variations among a series of *L. plantarum* strains is the gene-trait matching method as described in Chapters 1 and 5. This approach has been proven successful in identifying specific genes that are involved in eliciting immunomodulatory responses in dendritic or peripheral blood mononuclear cells, or are required for mannose adhesion that is associated with specific innate immune responses in pig mucosal tissues *in vivo* [8-11]. However, when employing phenotypic variations between strains of *L. plantarum* that were obtained in the *in vitro* assay for GI-tract persistence, the observed strain-specific phenotypic variations could not be credibly linked to a specific gene or a set of genes. This may imply that complex phenotypes (like GI-tract survival and persistence) may not be determined by 'simple' gene presence and absence variations, but are related to fine-tuned gene-regulatory responses that involve conserved gene repertoires. Therefore a functional genomics approach was chosen that employs transcriptome-trait matching rather than gene-trait matching. Chapter 4 presents the variation of *in vitro* determined GI-tract survival rates of different cultures of *L. plantarum* WCFS1 and correlates these to specific fermentation conditions as well as the corresponding *L. plantarum* transcriptomes. The fermentation conditions used encompassed a variety of mild-stress conditions and by combinations of stress conditions, the number of fermentations could be reduced while still allowing the evaluation of the effects of single or multiple stress conditions in combination [12]. Using these fermentation conditions, parallel analysis of genome-wide transcriptomics and physiological characteristics (e.g. maximum growth rate, yield, and organic acid profiles) of *L. plantarum* WCFS1, correlations between fermentation conditions and industrially relevant physiological characteristics could be identified [12]. It appeared that the presence of sodium chloride (NaCl) decreased GI-tract survival, while growth at a lower pH positively affected survival. Moreover, transcriptome-trait

matching enabled the identification of *L. plantarum* WCFS1 transcripts that play a role in survival in the *in vitro* GI-tract persistence assay, encoding a sodium-proton pump, a penicillin binding protein, and a transcription factor. The latter gene could subsequently be shown to influence the expression of a capsular polysaccharide gene cluster (Chapter 4). Notably, all these three functions appeared to be associated with cell envelope functions but could not *a priori* be predicted to play a role in GI-tract survival. Not only transcriptome trait matching allows the identification of genes involved in complex phenotypes like GI-tract persistence, also other 'omics' based technologies (e.g. proteomics) can be employed to unravel stress tolerance related functions [13]. These approaches can expand our understanding of the stress-specific and general stress response networks operating in bacteria in correlation to their contribution to tolerance to detrimental conditions. In addition, the transcriptome-trait matching approach can also be employed to decipher the involvement of genes in other complex phenotypes. This notion was exemplified by the considerable differences observed for the differently grown *L. plantarum* WCFS1 cultures when their immunomodulatory potential was evaluated in a dendritic cell assay *in vitro* (M. Meijerink *et al.* unpublished observations).

Thereby this functional genomics approach to identify the role of specific genes or proteins in complex phenotypes of a bacterium holds good promises and compared to gene-trait matching has the advantage that it allows the detection of the functional contribution to such phenotypes of conserved genes or proteins.

Stress responses and improving robustness

It is clear that gene-regulatory responses are important for robustness phenotypes. Pre-adaptation occurs when sub-lethal stress conditions elicit the activation of stress-response networks that can protect against detrimental conditions, including stress conditions of progressive intensity or stress conditions other than the initial sublethal stress, this phenomenon is called cross-protection. Pre-exposure to a lower pH growth condition was shown to improve the survival under more severe acid-stress conditions (such as the developed GI-tract assay) for *L. plantarum* WCFS1 (Chapter 4). The fact that relatively mild stress conditions can induce an adaptive response, suggests that pre-exposition of industrially relevant strains to sub-lethal stress conditions during their production could improve their robustness during stress-exposure exerted by subsequent application [14]. The observation that pre-exposure to sublethal acid stress increased survival under lethal acid-stress conditions has also been reported for several other lactic acid bacteria, including *L. sanfranciscensis* [15], *Lactococcus lactis* [16], and *L. acidophilus* [17]. Analogously, pre-exposure to low concentrations of bile elicited responses in *L. acidophilus* that protected this bacterium against subsequent exposure to relatively high bile concentration [18]. On the other hand, pre-exposure to a certain stress condition to subsequently protect against another (apparently unrelated) stress condition (i.e., cross-protection), could allow improvement of robustness without the application or addition of undesired conditions, or compounds (e.g. bile acids), during industrial production. There are many examples where cross-protection has been reported for a variety of bacteria, including industrially

relevant (lactic acid) bacteria. For example, pre-exposure of *Propionibacterium freudenreichii* to heat enhanced bile tolerance [19], *L. plantarum* pre-exposed to sublethal heat-treatment enhanced growth in media containing 6% NaCl or a low pH (pH 5.0) [20]. Moreover, acid pre-treatment in *Lactococcus lactis* results in improved heat, ethanol, H_2O_2, acid, and NaCl tolerance [21] and *L. paracasei* survival during spray-drying could be enhance either mild osmotic or sublethal heat-stress conditions, while similar pretreatments of *L. rhamnosus* improved its survival during 'storage' at 30°C for extended times [22]. In conclusion, these approaches can lead to significant improvement of generic stress robustness of several important starter and probiotic strains. This thesis unraveled the gene repertoires elicited by solvent stress (ethanol) in *L. plantarum* as well as the cross protective impact of sublethal ethanol exposure to subsequent 'unrelated' stresses (Chapter 2). To this end, *L. plantarum* was grown in media that contained sub-lethal ethanol concentrations and were subsequently exposed to other stresses like heat, oxidative, and GI-track mimicking stress conditions (Chapter 2). Sub-lethal ethanol stress exposure responses in *L. plantarum* were able to induce cross-protection against heat-stress, but not against stresses encountered in the GI-tract assay. Solvents are predicted to predominantly interact with lipid bilayers [23], and can be anticipated to destabilize bacterial membranes. Small heat-shock proteins function as chaperones that assist the protein-folding process by stabilizing unfolded or partially folded proteins [24] and have been reported to interact and stabilize the phospholipid bilayer [25]. Notably, small heat shock protein Lo18 from *Oenococcus oeni* is membrane-associated and its expression is induced by addition of benzyl alcohol [26]. In addition, Hsp2, another small heat shock protein, can affect membrane fluidity in *L. plantarum* [27] and was induced upon extended ethanol exposure in this species (Chapter 2). In *B. subtilis*, the class I stress chaperones DnaK and GroEL were associated with the cell membrane upon short-term ethanol exposure, and displayed enhanced kinase activity under these conditions, suggesting their contribution to membrane-function maintenance upon solvent exposure [28]. In *L. plantarum*, GroEL and DnaK are predicted to be regulated by the HrcA repressor [29] and thereby belong to the Class I heat shock regulon, which also encompasses the other proteins encoded within the *groE* and *dnaK* operons, GroES, GrpE, and DnaJ [29]. Next to the Class I regulon, *L. plantarum* also encodes members of the typical Class III stress regulon that is controlled by the dedicated repressor CtsR. The members of the CtsR regulon in *L. plantarum* were experimentally determined and include the predicted members of this class III stress regulon, ClpP, ClpB, ClpE, ClpC, Hsp1, and FtsH [30-32]. Although strain specific differences in the regulons associated with stress responses in LAB have been described [31], the core stress-responses in LAB appear to be conserved, including the predicted regulations of the CtsR and HrcA regulators [14,33-35]. Many reports describe the induction of expression of CtsR and/or HrcA regulon-members upon bacterial exposure to different stresses. Examples include increased expression of GroE and ClpP in *Lactococcus lactis* after exposure to heat, acid, or UV-irradiation [16,36], induction of *L. mesenteroides* DnaK and GroEL upon cold-shock [37], and GroES, GroEL, and DnaK induction upon osmotic upshift in *Lactococcus lactis* [38]. To unravel the role of the Class I and Class III stress regulators (HrcA and CtsR, respectively) in *L. plantarum* WCFS1, mutant-derivative strains of *ctsR*, *hrcA,* or both, were constructed. Growth of the *ctsR* mutant at elevated temperature (42°C) was impaired in *L. plantarum* (Chapter 3). It appeared that the general impact of CtsR and HrcA transcription factor deficiencies were temperature-dependent and encompassed an impressive network of genes,

encompassing many functional categories (Chapter 3). The single mutations of either *hrcA* or *ctsR* led to altered transcription patterns of genes encoding functions involved in transport and binding of sugars and other compounds, primary metabolism, transcription regulation, capsular polysaccharide biosynthesis, as well as fatty acid metabolism. Moreover, mutation of both regulators, elicited expression changes of a large variety of additional genes in a temperature-dependent manner, including many genes that encode functions associated with cell-envelope architecture, suggesting substantial cell envelope remodeling in this mutant. The mutant studies highlighted the interaction of class I and III stress regulons and illustrated the complex gene-regulatory networks involved in adaptive responses to stress conditions in *L. plantarum*.

Nowadays, a vast genetic toolbox for the manipulation of LAB gene expression levels is available, allowing complementary approaches to induce or repress expression levels of genes encoding stress regulon members or regulators, prior to stress exposure. Examples of genetic engineering approaches to increase stress tolerance include the overproduction of GroESL in *L. paracasei* and *Lactococcus lactis*, which resulted in an improved salt tolerance [39] Overexpression of a manganese-dependent catalase in *L. casei* resulted in a strain that displayed better survival characteristics upon H_2O_2 exposure [40]. Similarly, heterologous expression of superoxide dismutase in *L. acidophilus* [41,42], as well as heme catalase in *L. plantarum* [43], or *Lactococcus lactis* [44] resulted in elevated tolerance to oxidative stress. Furthermore, *L. plantarum* variants engineered to overexpress the heat shock proteins HSP 18.55 or HSP 19.3 displayed improved heat resistance and enhanced survival in the presence of ethanol [45]. Notably, an analogous approach, termed "pathobiotechnology" described by Sleator *et al*., exploits heterologous expression of the sophisticated compatible solute accumulation system derived from the food-borne pathogen *Listeria monocytogenes* in industrially relevant strains [46]. This concept is exemplified by the introduction of the *betL* gene, encoding the betaine uptake system of *Listeria monocytogenes*, into *L. salivarius*, which resulted in significantly increased betaine accumulation in this species and the corresponding elevated osmotic stress tolerance [47], as well as improved tolerance to high pressure exposure [48].

Besides the approaches discussed above which engineer strains towards the overexpression of one genetic locus important for stress tolerance, several studies describe manipulation of complete stress regulons by targeting CtsR. Following this strategy, a *ctsR* deletion mutant in *Streptococcus thermophilus* [49] and *L. sakei* [50] exhibited improved heat stress tolerance during exponentially growth and more efficient growth during sausage fermentation, respectively. However, the *S. thermophilus ctsR* mutant displayed increased osmotic- and oxidative-stress sensitivity [49], illustrating that elevation of tolerance to a particular stress condition can at the same time diminish tolerance to other stress conditions. The *ctsR* mutant in *L. plantarum* was more ethanol- and heat-sensitive as compared to the wild-type, despite the fact that several of the genes in the CtsR regulon (*hsp1*, *clpB*, *clpC*, *clpE*, and *clpP*) were demonstrated to be transcribed at a higher level [51]. The observed highly variable consequences of *ctsR* deletion with respect to different stress condition tolerance within one species suggest subtle stress-dependent differences in the induced regulon. Moreover, the highly variable phenotypic effects observed for *ctsR* mutants in different LAB species, underlines the interspecies differences in the *ctsR* regulon responsiveness under analogous stress

conditions, which severely complicates generic application of such mutants as more 'robust' strains. Moreover, the debate on the application of GMOs in the food industry is momentarily undecided, and legal issues and general public opinion are hampering industrial application of many of the more robust strains described here.

In summary, several strategies can be exploited to achieve more robust bacterial strains, ranging from pre-genomic approaches like modifying the growth medium and exploiting cross-protection strategies or genetic engineering to elevate or repress the expression of stress factors or their regulators, to post-genomic strategies that could exploit approaches analogous to the fermentation genomics platform described here for the prediction and improvement of robustness effector molecules.

Future directions

Although the concept of probiotics dates back more than a century [52] they have only been extensively researched during the past few decades. To move the probiotic field forward, several issues need to be addressed. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host [53]. The European Food Safety Authority (EFSA) is an organization of the European Union that provides independent scientific advice and communication on existing and emerging risks associated with food and feed [54]. The majority of the currently marketed probiotics targets prophylactic health benefits and thus claims to provide a reduction of disease-risk to healthy subjects. However, some products also claim to treat health-compromises or diseases and thus provide a therapeutic benefit to specific sub-populations. Until now, the EFSA rejected all health claims associated with probiotic products [55].

To date it remains largely unknown what molecular mechanism of interaction in the host gastrointestinal tract underlies the proposed health benefit elicited by probiotic strains. Moreover, to measure health beneficial effects in healthy human subjects has proven to be very difficult, since there are no reliable markers for health improvement in healthy populations [56,57]. Probiotic modes of action have been proposed to include (i) strengthening of epithelial integrity, (ii) reduction of infection risk through competitive exclusion of pathogens or via the production of antimicrobial activity, (iii) modulation of host immune responses, or (iv) contribution to the *in situ* metabolic conversions [56,58,59]. Recent studies, have also illustrated that probiotics may influence the functioning of the gut-brain axis, a bidirectional neurohumoral communication system that can be affected by the gut microbiota [60]. However, the latter domain of probiotic applications is still in its infancy, it is largely based on studies in (germfree) animal models and requires translational studies to evidence effects in humans as well as knowledge of the underlying mechanisms. Nevertheless, this can be a novel area of probiotic application and research and may be fruitful in the treatment of specific behavioral diseases.

Mechanistic insight in probiotic functioning and identification of the bacterial effector molecules involved in the observed health-stimulatory effects is required to improve the predictability of probiotic effects, and to further substantiate the strain specific dogma that is proposed for probiotic effects. The discovery of probiotic effector molecules and approaches to enhance their expression *in situ* at the site of probiotic action, could strengthen the position of these health-promoting cultures in the functional food market. In addition, methods to accurately monitor the improved health state of the consumer can be deduced from the molecular host responses underlying the proposed health promotion, which could strengthen the efficacy read-out of probiotic clinical trials. A clear prerequisite of probiotic products that can be deduced from the probiotic definition, is their viable state, which implies that the bacteria should survive the stresses that they encounter during for instance product preparation and storage [14]. However, expression of probiotic effector molecules may not correlate with maximum survival of the bacteria, but may depend on specific environmental conditions that are encountered *in situ* in the GI-tract. Nevertheless, such *in situ* responses will also depend on the bacterial viability and robustness.

At the moment several strategies have been applied to influence probiotic survival during gastrointestinal tract passage, including adjustment of growth conditions and media during production, exposure to mild-stress conditions during production to elicit cross-protective stress responses, or inclusion in the delivery matrix of specific additives, or even encapsulation. A relatively straightforward way to improve survival is the addition of specific compounds to the growth and production medium. For example, addition of Tween80 to the growth medium of several *L. rhamnosus*, *L. paracasei,* and *L. salivarius* strains resulted in up to a 3-log increased survival during exposure to gastric juice *in vitro*. Tween80 could be shown to alter the fatty acid composition of the *L. rhamnosus* GG membrane, which was apparent as a 55-fold higher oleic acid content, and a higher overall unsaturated/saturated fatty acid ratio. The authors suggest that these changes in the membrane composition and the consequences for membrane fluidity are most likely the explanation for the observed enhanced survival [1]. Another study revealed that addition of glucose resulted in up to 6-log enhanced survival of *L. rhamnosus* GG after 90 min exposure to gastric juice *in vitro* (pH 2.0) [61]. Notably, only *L. rhamnosus* cells pre-exposed to metabolizable sugars, such as glucose or fructose, displayed improved survival characteristics, suggesting that an energized state is essential for robustness of these bacterial cells [61]. Similar observations were made in our studies, where the survival of *L. plantarum* cells during the GI-tract assay could be increased at least 10-fold by the addition of 1.5% glucose to the bacterial suspension that was subjected to the assay (Fig. 1). Moreover, subsequent experiments revealed that the addition of only 0.05% glucose was sufficient to improve GI-tract assay survival. Importantly, metabolization of the available carbohydrate is essential for the observed enhanced survival effect, since the stimulation of *L. plantarum* survival in this assay was only achieved by addition of the fermentable D-glucose, and not by the addition of the non-fermentable L-glucose (Fig. 2). The fermentable nature of the carbohydrate added implies that ATP generation is most likely the survival enhancing consequence, which is in agreement with the acid tolerance dependency on the F_0F_1-ATPase as suggested by Corcoran and coworkers [61]. Other additives that were evaluated in our studies included whey proteins. This additive also appeared to improve survival rates during the GI-tract assay *in vitro* (Fig. 1), which was especially

apparent for *L. plantarum* WCFS1 when it was taken from the logarithmic phase of growth for which the survival could be enhanced 1000-fold by the addition of whey proteins. The molecular mechanism by which the whey proteins can improve survival during the GI-tract assay remains unknown.

To evaluate whether higher survival in the presence of glucose or whey protein is strain specific, several strains of different lactobacilli and bifidobacteria were subjected to the digestive tract with or without these additives (Table 2). All strains tested, except *L. acidophilus* NCFM displayed an improved survival rate upon the addition of glucose during the GI-tract assay. This indicates that the capacity for energy generation more or less universally protects bacteria against severe acid-stress conditions, which most likely involves the conserved F_0F_1-ATPase [62]. Analogously, whey proteins improved GI-tract survival in more than half of the tested strains and species.

Comparison of the digestive tract survival pattern in the *in vitro* assay of the other species in comparison to that obtained for *L. plantarum* WCFS1 revealed considerable species-specific variations (Table 1). For *L. plantarum* the predominant factor that influenced GI-persistence *in vitro* was the severe acid stress encountered in the stomach, while the exposure to small intestinal conditions that included bile acid exposure hardly affected the strains of this species (Chapters 4 and 5). The *in vivo* persistence curves obtained from the human volunteer study appear to support this notion of predominant stomach-associated killing of *L. plantarum* (Chapter 5). However, Table 1 shows that some of the tested species or strains were more sensitive to small-intestinal conditions, e.g. pancreatic juice and bile acid exposure as compared to stomach-like conditions, e.g. gastric juice. This group included most of the *L. rhamnosus* strains that were tested. Notably, the level of

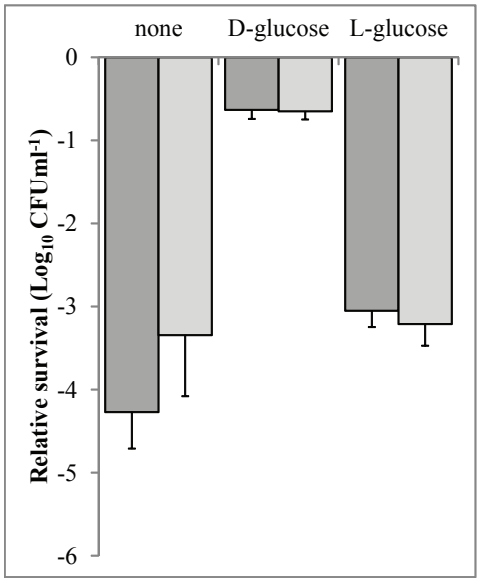

Fig. 2. Relative survival of *L. plantarum* WCFS1, subjected to an upper GI-tract mimicking assay in the absence of L-glucose or D-glucose. *L. plantarum* cultures were grown in 2× CDM at 37°C. The cultures were harvested at stationary phase (25 h after inoculation and subjected to an upper GI-tract mimicking assay containing no additive, D-glucose (1.5%) or L-glucose (1.5%). Dark grey bars represent the colony forming units (CFUs) after 60 min gastric juice incubation and light grey bars represent CFUs after subsequent 60 min small intestine incubation. Input is set at 0 Log_{10} CFU ml^{-1}, data presented are averages of technical sextuplicates (- standard deviation).

sensitivity of these strains for pancreatic juice and bile acid exposure was increased by pre-exposure to the stomach-mimicking acid conditions (Table 1). In conclusion, quantitative comparison of relative survival of different bacterial species and strains, revealed substantial differences between these bacteria, which is in agreement with several other studies that reported on species and strain variations in acid and bile tolerance [63,64].

In an ideal world, after measuring several parameters, consumption of the right probiotic strain that is grown, stored, and delivered under the right conditions, will give the desired beneficial effect on consumer health. As mentioned above, clinical trials are essential to corroborate probiotic effects and are a prerequisite in probiotic research. Mechanistic insight and analysis of *in situ* expression of probiotic effector molecules is bound to require live bacteria, although depending on the mechanism of action also specific cultivation conditions that maximize effector molecule expression during production may contribute to health benefit effects of the corresponding products. Live delivery to the intestinal tract is a prerequisite when *in situ* expression of effector molecules is the basis of the probiotic effect, and thus may benefit from GI-tract adapted strains with improved survival and persistence characteristics. These features can be enhanced in existing cultures through adaptive evolution employing relatively simple regimes of multiple, subsequent passages of the intestinal tract and consistently isolating the more persistent derivatives, as was exemplified in Chapter 6. To

Table 2. Strain-dependent effect of glucose (1.5%) or whey protein isolate (1 mg/ml) addition during GI-tract survival.

Species	Strain	Affected by
L. plantarum	WCFS1	Glucose Whey
L. acidophilus	NCFM	
	LAFTI-10	Glucose
L. rhamnosus	GG	Glucose Whey
	NCIMB8824	Glucose
	LMG10772	Glucose
	LMG6400	Glucose Whey
L. casei	P2	Glucose Whey
	ATCC334	Glucose
	BL23	Glucose Whey
	LMG6904	Glucose Whey
L. helveticus	DPC4571	Glucose Whey
	CNRZ32	Glucose Whey
L. delbrueckii ssp bulgaricus	LMG6901	Glucose Whey
	ATCC BAA365	Glucose Whey
B. animalis ssp lactis	Bb12	Glucose Whey
	HN019	Glucose Whey
B. longum	LMG18899	Glucose Whey

L. Lactobacillus, *B. Bifidobacteria*.

facilitate selective culturing of the administered Lactobacilli from fecal material, one could employ intrinsic antibiotic resistances of these microbes (e.g. tetracyclin, streptomycin for *L. plantarum*), or introduce specific resistances that do not require genetic modification like rifampin resistance. The latter resistance marker was employed to selectively recover a panel of lactic acid bacteria, including *L. plantarum*, from saliva to determine their relative persistence in the human oral cavity [65] or the human GI-tract (Chapter 5).

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Appendices

Nederlandse samenvating
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Nederlandse samenvatting

Introductie: *Lactobacillus plantarum* (afgekort *L. plantarum*) is een veelzijdige melkzuurbacterie die succesvol op veel plaatsen kan wonen. Het is een algemene inwoner van het menselijke maagdarmkanaal en wordt gebruikt als startcultuur in verschillende fermentatieprocessen, inclusief gras (dat wordt omgezet in kuilgras), melk (yoghurt, kaas, etc.), fruit (olijven), groenten (zuurkool), en vlees (saucijzen). Bovendien wordt *L. plantarum* verkocht als een gezondheidsbevorderende bacterie, ook wel een probioticum genoemd. In deze verschillende omgevingen en processen ondervinden bacteriën stress, zoals hitte, kou, zuur, zout en zuurstof stress. Aangezien startculturen en probiotica actief bijdragen aan de smaak en textuur van de gefermenteerde producten en/of levend moeten zijn om hun gunstig effect ter plekke in de darmen te geven, is het belangrijk om te begrijpen hoe bacteriën zich aanpassen in deze uitdagende omstandigheden en om ze vervolgens te verbeteren. Hierbij wordt gebruik gemaakt van het genoom; het erfelijke materieel van een organisme. Dit genetisch materiaal bestaat uit DNA; de bouwstenen van een organisme. Het DNA bevat genen welke omgezet kunnen worden in RNA (transcriptie) en vervolgens in eiwitten (translatie). Deze eiwitten zijn de 'werkers' van elke cel, dus ook van bacteriële cellen. Zij zorgen ervoor dat de cel functioneert en kan reageren op zijn omgeving. Toepassingsgeoriënteerde benaderingen zijn tegenwoordig beschikbaar waarmee de globale stress reacties (op DNA, RNA en eiwit niveau) van melkzuurbacteriën te analyseren zijn. Het werk dat in dit proefschrift gepresenteerd wordt, maakt gebruik van dergelijke bestaande methodes, maar ook van nieuw ontwikkelde strategieën om de stress reacties in *L. plantarum* te onderzoeken.

Doel: Het werk dat beschreven wordt in dit proefschrift streeft naar het verkrijgen van een beter begrip van de aanpassingen van *L. plantarum* onder stressvolle omstandigheden, inclusief het verblijf in het spijsverteringskanaal van dieren en mensen, om zo de robuustheid van deze bacteriën te verbeteren.

Resultaten en conclusies: Tijdens het maken van wijn wordt *L. plantarum* blootgesteld aan ethanol. Na korte en langere blootstelling aan een niet-dodelijke dosis ethanol toonden transcriptoomprofielen (=verzameling van RNAs) de aanpassing op RNA niveau van dit micro-organisme aan. Deze resultaten suggereerden dat de door ethanol geïnduceerde activering van de stressreactie op het CtsR regulon bijdraagt aan de kruis-bescherming tegen hitte stress. Na verwijdering van het *ctsR* gen (=stukje DNA dat voor een eiwit codeert) en het *hrcA* gen werden de transcriptomen van deze *L. plantarum* mutanten geanalyseerd. Verwijdering van deze toezichthouders van stressreacties (*ctsR* en/of *hrcA* dus) leidde tot het verfijnen van het repertoire van genen waarop ze invloed hebben. Voornamelijk het verwijderen van beide stress-regulatoren tegelijk veroorzaakte veel veranderingen in het transcriptoom op een temperatuur afhankelijke manier. Kweken van *L. plantarum* WCFS1 onder verschillende groeicondities leidde tot grote verschillen in maagdarmkanaaloverleving en robuustheid. Deze maagdarmkanaaloverleving werd bepaald met behulp van een eenvoudige test in het laboratorium. Verbeterde maagdarmkanaaloverleving en robuustheid kunnen worden geassocieerd met laag zout en lagere zuur gehalten tijdens het kweken. De transcriptomen van deze bacteriekweken werden gelinkt met de waargenomen overleving van het maagdarmkanaal.

Hieruit konden kandidaat-genen die betrokken zijn bij robuustheid worden geïdentificeerd. Na validatie bleken een transcriptieregelaar die betrokken is bij de samenstelling van de bacteriewand (Lp_1669), een penicilline-bindend-proteïne (Pbp2A) en een natrium/proton wisselaar (NapA3), een bijdrage te leveren aan de stress robuustheid van *L. plantarum* in het maagdarmkanaal. Dit proefschrift beschrijft ook het gebruik van een nieuwe methode om verschillende *L. plantarum* stammen te identificeren die werden toegediend aan gezonde menselijke vrijwilligers in speciaal ontworpen mengsels van *L. plantarum* stammen. Een opmerkelijke overeenkomst van de stam-specifieke persistentie werd waargenomen wanneer de gegevens van de verschillende vrijwilligers met elkaar werden vergeleken. Bovendien was er een overeenstemming gevonden tussen de stam-specifieke persistentie in de vrijwilligers en de voorspelde overleving van het spijsverteringskanaal op basis van de eenvoudige laboratoriumtest. Tot slot werd de evolutionaire aanpassing van *L. plantarum* WCFS1 aan het muizen maagdarmkanaal bestudeerd door de stam langdurig bloot te stellen aan het spijsverteringskanaal van deze dieren. Dit werd gedaan door opeenvolgende rondes van (her)voeden van de langst verblijvende bacteriële kolonies. De genomen van de oorspronkelijke en de aangepaste kolonies werden met elkaar vergeleken en het bleek dat genen coderend voor eiwitten met functies die te maken hebben met de vorming van de buitenkant van de bacterie en energiemetabolisme een belangrijke rol spelen bij de bepaling van maagdarmkanaalpersistentie van *L. plantarum*.

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About the author

Hermien van de Veen was born on November 18th 1982 in Amersfoort and grew up on an agricultural firm in Nijkerk. After finishing primary education at "basisschool met de Bijbel Diermen" in Putten, she continued her education at high school "Christelijk College Groevenbeek" in Ermelo. In 2004, she obtained her propaedeutic and Bachelor Biology at Utrecht University and subsequently the excellent Master Biomolecular Sciences. During this study, she compared young, stress induced premature senescence, and replicative senescence in endothelial cells under supervision of Dr. Elza Regan-Klapisz and Dr. Liesbeth Hekking at the faculty of Cellular Architecture and Dynamics from Utrecht University. Her second internship was a genetic research at DSM in Delft under supervision of Dr. Lucie Pařenicová.

In 2006, Hermien began as a Wageningen UR researcher on a TI Food & Nutrition project. Afterwards she started her PhD research under supervision of Prof. Dr. Michiel Kleerebezem. The work was part of the TIFN project entitled 'fermentation enhanced probiotic function', and was conducted at NIZO food research in Ede. The research performed during this period is presented in this thesis.

At the moment, Hermien van Bokhorst-van de Veen works as microbiologist at Food and Biobased Research, part of Wageningen UR, where she performs research on mild preservation techniques.

List of publications

Van Bokhorst-van de Veen H*, Bron PA*, Wels M, and Kleerebezem M. Engineering Robust Lactic Acid Bacteria. 2011. In: Stress Responses of Lactic Acid Bacteria. Edited by Tsakalidou E, Papadimitriou K: Springer US; 369-394

Van Bokhorst-van de Veen H, Abee T, Tempelaars M, Bron PA, Kleerebezem M, and Marco ML. Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*. 2011. Appl Environ Microbiol 77: 5247-5256

Bron PA*, Wels M*, Bongers RS, van Bokhorst-van de Veen H, Wiersma A, Overmars L, Marco ML, and Kleerebezem M. Transcriptomes reveal genetic signatures underlying physiological variations imposed by different fermentation conditions in *Lactobacillus plantarum*. 2012. PLoS One 7:e38720

Van Bokhorst-van de Veen H, Lee I, Marco M, Wels M, Bron PA, and Kleerebezem M. Modulation of *Lactobacillus plantarum* gastrointestinal robustness by fermentation conditions enables identification of bacterial robustness markers. 2012. PLoS One 7: e39053

Van Bokhorst-van de Veen H, van Swam I, Wels M, Bron PA, and Kleerebezem M. Congruent strain specific intestinal persistence of *Lactobacillus plantarum* in an intestine-mimicking *in vitro* system and in human volunteers. 2012. PLoS ONE 7: e44588

Van Bokhorst-van de Veen H, Smelt JM, Wels M, Van Hijum SAFT, De Vos P, Kleerebezem M, and Bron PA. Genotypic adaptations associated with prolonged persistence of *Lactobacillus plantarum* in the murine digestive tract. 2013. Biotechnology journal, DOI: 10.1002/biot.201200259

Van Bokhorst-van de Veen H, Bongers RS, Wels M, Bron PA, Kleerebezem M. Transcriptome signatures of class I and III stress response deregulation in *Lactobacillus plantarum* reveal pleiotropic adaptation. Submitted for publication

Patent

Van Bokhorst-van de Veen H, Lee I, Wels M, De Vos P, Bron PA, Bongers RS, Wiersma A, Kleerebezem M. Probiotics with enhanced survival properties. Patent filing no. 11164469.6

* Equal contribution

Overview of completed training activities

Discipline specific activities
Ecophysiology of the gastrointestinal tract, VLAG/WIAS, Wageningen (2007)
Probiotics: from start to finish, NIZO, Ede (2007)
Genetics and physiology of food-associated microorganisms, VLAG, Wageningen (2007)
Food fermentation, VLAG, Wageningen (2008)
Darmendag/Gut day (2006-2008, poster presentations and 2010, oral presentation)
Gut microbiota in health and disease, Amsterdam, The Netherlands (2007)
ALW Platform molecular genetics annual meeting, Lunteren, The Netherlands (2007)
9th and 10th Symposium on lactic acid bacteria, Egmond aan Zee, The Netherlands (2008 and 2011, poster presentation)
The 3rd congress of European microbiologists (FEMS), Gothenburg, Sweden (2009, poster presentation)
Prebiotics, probiotics and new foods, Rome, Italy (2009, oral presentation)
Microbial stress response (Gordon research conference), South Hadley, USA (2010, poster presentation)

General courses
PhD week, VLAG, Bilthoven (2007)
Scientific writing, WGS, Wageningen (2008)
Systems biology: Statistical analysis of ~omics data, VLAG/EPS, Wageningen (2008)
Basic statistics, PE&RC, Wageningen, (2010)

Optionals
Preparing PhD research proposal, VLAG, Wageningen (2007)
Work discussion meetings, NIZO food research (2007-2011)
Expert and work discussion meetings, TIFN (2006-2011)
Program 3 WE-days, TIFN (2006-2011)
PhD/Post-Doc meetings, Laboratory of Microbiology (2006-2011)
PhD study trip Laboratory of Microbiology, USA (2010)

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