Research Article

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

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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 mouse 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 appeared to be beneficial for the gastrointestinal tract survival of *L. plantarum* WCFS1. This work demonstrates the feasibility of experimental evolution for the enhancement of the gastrointestinal residence time of probiotic strains, while full-genome re-sequencing of the adapted isolates provided clues towards the bacterial functions involved. Enhanced gastrointestinal residence is industrially relevant because it enhances the efficacy of the delivery of viable probiotics in situ.

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Abbreviations: AUC, area under the curve; **CFU**, colony-forming unit; **CM**, chloramphenicol; **GI**, gastrointestinal; **MRS**, de Man-Rogosa-Sharpe; **RIF**, rifampicin; **SNP**, single nucleotide polymorphism; **SV**, structural variation

1 Introduction

The human gastrointestinal (GI) tract is colonized by trillions of microbial cells, termed the microbiota, which outnumber 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 that, when administered in adequate amounts, confer a health benefit on the host (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf). One possible mechanism of action for probiotics may lie in the fact that they can modulate the immune system of the host [6, 10]. This mechanism seems especially feasible in the small intestine, because 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 [11, 12]. Other mechanisms by which probiotic bacteria are postulated to influence host health include competitive exclusion of pathogens and gut barrier improvement [5, 10].

It is recommended that probiotic products contain at least 10^7 live microorganisms per gram or milliliter [13]. 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 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 [14]. 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 was relatively well studied in probiotic species, notably in the lactic acid bacterial genus *Lactobacillus*. For example, GI survival of dedicated gene-deletion mutants was assessed [15–18], and in situ induction of gene expression was studied using in vivo expression technology [17, 19] and transcriptome analysis [15, 20] 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 [21]. 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*, which is the transcriptional activator of the maltose regulon [22]. Although, to the best of our knowledge, adaptive evolution was not applied to study the 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 [23], and *Lactobacillus plantarum* strain WCFS1 could be adapted to grow on glycerol [24].

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 [25, 26]. 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 contributed to the GI-tract-adapted phenotype.

2 Materials and methods

2.1 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 (CM) and/or 50 μg/mL rifampicin (RIF) were added to the medium. To allow selective plating of the adapted strains from fecal samples (see below), *L. plantarum* NZ3400^{CM} [27] was adapted to 50μg/mL RIF by culturing in the presence of increasing concentrations of this antibiotic, resulting in strain NZ3400$^{CM\text{-}RIF}$ (Table 1).

2.2 Mice and experimental setup

Two wild-type male Balb/c mice were purchased (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 from the institutional Animal Care Committee of 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 physiological salt (0.1% (w/v) peptone and 0.85% (w/v) sodium chloride), and concentrated 30-fold in peptone physiological salt containing 20% glycerol prior to storage at –20°C. Immediately prior to gavage, the cultures were thawed and washed twice with peptone physiological salt. Each animal was subjected to ingestion of one dose containing 1×10^9 colony-forming units (CFUs) in 200 μL MRS by gavage. Fecal samples were collected daily until no bacterial cells could be recovered, or for a maximum of 32 days per round.

Table 1. Bacterial strains used in this study

Strain	Relevant feature(s)	Reference
L. plantarum		
WCFS1	Single-colony isolate of *L. plantarum* NCIMB8826	[26]
NZ3400^{CM}	Derivative of WCFS1 containing a *lox66*-P32-*cat*-*lox71* insertion in the neutral H-locus (*H-locus::cat*)	[27]
NZ3400$^{CM\text{-}RIF}$	RIF-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

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 CM plus 50 µg/mL RIF, and incubated at 37°C. To confirm that the colonies were derived from the original NZ3400$^{CM\text{-}RIF}$ strain, 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* [28]; 5'-ATGCTCAATACTATTATTACACG-3' and 5'-TGTCGATAGTTTAACTTTTTCTGACC-3'), according to the manufacturer's instructions. Template material was part of a colony that was lysed by two min incubation at 800 W in a microwave (Intellowave, LG, Amstelveen, The Netherlands) and amplicons were visualized on 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 at 37°C and stored in MRS containing 20% glycerol at –80°C. The second and third rounds of gavage were performed with the same mice and bacterial cell preparation procedures as those used in the

Table 2. Input for gavage (strain mixtures)

Round number	Mouse 1 Colony name or number	Day	Round	Mouse 2 Colony 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.

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 listed in Table 2.

2.3 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 [29]. Full genome re-sequencing using Illumina technology (paired end, 100 nt) was performed by GATC-Biotech (Konstanz, Germany), resulting in genome coverage per sample of 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 with the *L. plantarum* WCFS1 genome sequence were identified using a tool developed in-house, RoVar (SAFT van Hijum, VCL de Jager, B Renckens, and RJ Siezen, unpublished data; http://trac.nbic.nl/rovar). To prevent the alignment of reads to ambiguous regions in the reference sequence, repeat masking of the reference sequence was done (i) by creating 30 base pair (bp) fragments; (ii) by aligning these fragments to the reference sequence, using BLAT [30] with a tile size of 8; and (iii) by 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, providing that SVs were at least 4 bp from either the 3′ or 5′ end of a given read. SVs were used for further analysis, providing 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 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 web server Project HOPE [31] by submitting original and mutated proteins. The area under the curve (AUC) was calculated according to the trapezoidal rule.

3 Results and discussion

3.1 Extending the persistence of *L. plantarum* to the murine GI-tract environment by repeated exposure

To assess whether it was possible to adapt *L. plantarum* WCFS1 to the murine GI environment, a single dose of a CM- and RIF-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 CM- and RIF-resistant colonies were detected (data not shown); thus demonstrating our antibiotic-based plating method was 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 amplified the CM-resistant gene (*cat*), as well as an *L. plantarum* WCFS1-specific primer pair (targeting *nspA*; data not shown) [28]. *L. plantarum* NZ3400$^{CM\text{-}RIF}$ could be isolated from the fecal samples by selective plating for up to 5 and seven days following gavage-based feeding of mice 1 and 2, respectively (Fig. 1). It appeared that *L. plantarum* NZ3400$^{CM\text{-}RIF}$ passed 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 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 days 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 because 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 with the first round (3.6×10^4 vs. 1.5×10^7 CFU/mL), although the highest numbers of colonies were still detected on day one. 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 compared with the first-round experiment. Moreover, the AUC slightly increased (1.7 and 1.1× for mice 1 and 2, respectively) compared with the first round (Fig. 1), which suggested 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 days 9, 10, and 14 of round 2, whereas mouse 2 received a mixture of 10 colonies isolated from day 0 of round 2 (Table 2). During this third round, no CM- and RIF-resistant bacteria were detectable prior to intake. Again, a prolonged persistence curve was observed compared with the former two rounds, for example, colonies were still detectable after 32 days (Fig. 1). The AUC appeared to be

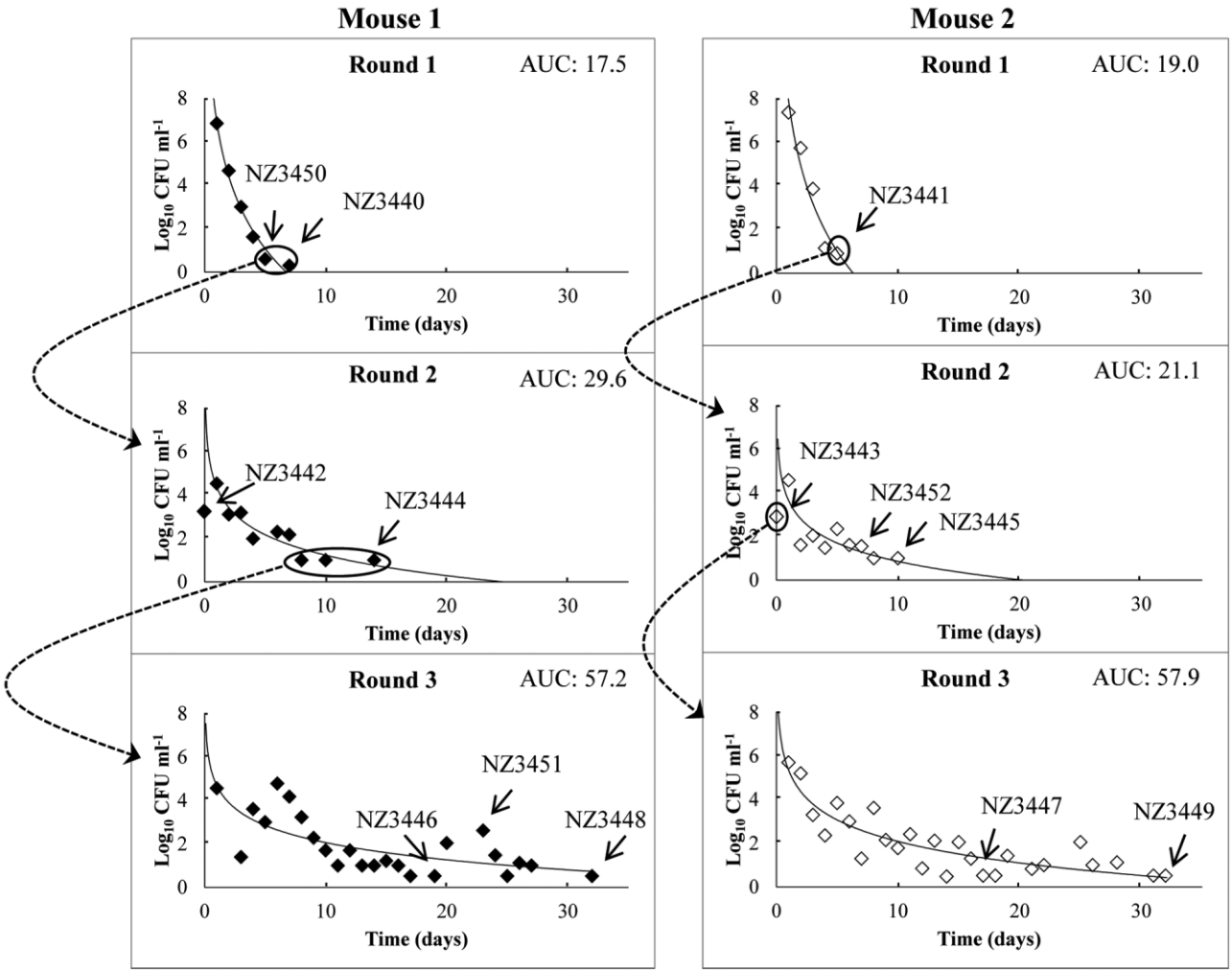

Figure 1. Experimental setup for the repetitive murine GI-tract-passage experiment and CFUs obtained during this experiment with two mice. Dashed arrows indicate the ingested mixed culture for the next round of gavage from the isolated colonies (circled). Small arrows and NZ numbers indicate re-sequenced strains. AUC = area under the curve (in $\log_{10}$ CFU mL^{-1} × day).

at least doubled during round three relative to round two (Fig. 1), indicating further prolonging of the transit time and/or in situ proliferation of the strain. This experiment demonstrates that extended persistence of *L. plantarum* can be achieved by repetitive exposure to the murine digestive tract.

3.2 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 re-sequencing. Besides the 13 adapted isolates, 2 randomly picked colonies of the original strain were included in re-sequencing strain collection. The re-sequencing datasets obtained were analyzed for SVs using the published and re-annotated genome as a reference [25] (Fig. 1 and Table 1), revealing 26 SVs within the collection of the 13 adapted strains, encompassing 25 SNPs and 1 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 was 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 identified in the two independent experimental setups (i.e. mouse 1 and mouse 2; Table 3). This result might be due to the introduction of new mutations in the genome that accumulate over time or by increasing existing *L. plantarum* cell numbers of a subpopulation that already contain the mutation. Since the experimental procedure allowed independent adaptive selection, starting with more than a single strain

Table 3. Identified SVs in the adapted strains compared with the published genome sequence and the input strain administered to two mice

SNP position on genome	Base change	NZ3439A[a]	NZ3439B	Mouse 1 NZ3450 (1–5)	Mouse 1 NZ3440 (1–7)	Mouse 1 NZ3442 (2–0)	Mouse 1 NZ3444 (2–14)	Mouse 1 NZ3446 (3–19)	Mouse 1 NZ3451 (3–23)	Mouse 1 NZ3448 (3–32)	Mouse 2 NZ3441 (1–5)	Mouse 2 NZ3443 (2–0)	Mouse 2 NZ3452 (2–7)	Mouse 2 NZ3445 (2–10)	Mouse 2 NZ3447 (3–17)	Mouse 2 NZ3449 (3–32)	Gene number[b]	Description and AA change[c]	Protein length (AA)	AA charge change[d]
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 = –
2776469	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) Indication of the strain in which the SV was detected compared with the reference genome of *L. plantarum* WCFS1 [25]. 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 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 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) This position is located in a *Lactobacillus plantarum* supermotif (LPSM) [34].
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 [32].

lineage during the three passages, these enriched mutations did not appear in all adapted strains and could thus be selectively adapted in more than one ancestral lineage. Therefore, it is more likely that the corresponding mutations were selectively enriched in the identified subpopulation of adapted strains of *L. plantarum*. This was 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.

3.3 Functional distribution of selective genomic adaptations

To evaluate whether genes that belonged to a certain functional category (categories as defined in [26]) were more frequently affected by the SVs encountered in the more persistent isolates, we analyzed the functional category distribution of the mutation patterns found in the isolates to identify over-represented functional classes. Remarkably, within the entire list of 26 SVs identified, 10 were associated with genes predicted to encode proteins localized in the cell envelope [32]; this is highly significant (Fisher exact p value of 3.8×10^{-5}). Moreover, all re-sequenced GI-tract-persistent strains, except one, contained at least one SNP associated with a gene predicted to encode a protein that is lipid anchored, membrane embedded by multi-transmembrane domains, N-terminally anchored, involved in glycerolipid metabolism, or involved in glycosyl transfer for 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, which was modified to a glycine residue in four of the higher-persistence derivatives isolated that derived from both independent mouse experiments. Submission of the alternative amino acid sequence to Project HOPE, a toolbox to predict the consequences of specific mutations on protein structure [31], revealed that the loss of the charged residue (Asp) was likely to cause loss of interactions within the protein structure, whereas peptide chain flexibility introduced by the alternative glycine residue was 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. The mucus-binding capacity of lactobacilli was associated with extended

intestinal tract persistence, which was clearly evidenced by the comparative genomic analysis of two *L. rhamnosus* strains [33].

Another functional category of genes frequently associated with adaptively selected SNPs and insertion was that of metabolic functions. SNPs were encountered in the coding regions of genes encoding a glycerol kinase (*lp_0370*), glycerophosphodiester phosphodiesterase (*lp_1328*), d-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 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 genes [34], possibly under specific conditions like those encountered in situ in the intestine. Importantly, adjustment of metabolic functions was previously associated with the in situ adaptation of *L. plantarum* WCFS1 to the murine and human intestinal tract conditions [19, 20].

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 led 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 fivefold identification of the SNPs in both a peptidylprolyl isomerase (*prtM2*; *lp_3193*) and 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 a-chain of a DNA-directed DNA polymerase III, of the PolC type (*polC*; *lp_2045*). These findings imply that the impaired RecJ-mediated processing of blocked replication forks may affect the fidelity of the replication-recovery process [35], which may in part be compensated for 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 previously implicated in adaptation rates in the (experimental) evolution in bacteria [36]. Moreover, the proposed impact on replication fidelity may affect cell division processes, which in turn may be reflected in the additionally unique SNP in the cell division ATP-dependent zinc metallopeptidase protein FtsH (*ftsH*; *lp_0547*) and the cell division protein GidA (*gidA*; *lp_3681*) also identified in this strain, although the latter mutation only induced a synonymous amino acid change in the *gidA* encoded protein.

4 Concluding remarks

The feasibility of experimental evolution for the extension of the GI residence time of *L. plantarum* WCFS1 was demonstrated. This was relevant when considering that the initial persistence curves determined revealed that this strain rapidly passed through the murine GI tract and did not appear to colonize effectively, which was in agreement with earlier experiments performed with this strain [37]. Moreover, this persistence curve was comparable to that observed for other lactobacilli exposed to the murine digestive tract, including *L. casei* [38], *L. acidophilus*, *L. sakei* [39, 40], and the vast majority of *L. fermentum* strains tested [41]. Similarly, when lactobacilli were administered to humans, bacterial fecal counts rapidly decreased when the oral administration of the strain stopped, as observed for *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. acidophilus*, *L. paracasei*, *L. gasseri*, and *L. fermentum* in trials with at least nine subjects [42–44]. This also appeared to be the case for *L. plantarum* WCFS1 in human feeding trials with a single strain [45] and several *L. plantarum* strains ingested as a mixture [46]; this could be largely attributed to the detrimental effects of low pH in the stomach [47]. All of these studies generally suggested relatively poor colonization characteristics of lactobacilli in both the murine and human GI tract, and improvement of this trait, as showcased herein for *L. plantarum*, may be feasible for other lactobacilli. This approach is likely to result in enhanced efficacy of the delivery of viable probiotics in situ in the GI tract for several, if not all, of the strains currently marketed. Despite the fact that colonization profiles in mice and humans appear to be very similar, it remains to be determined whether the improved phenotype for the murine GI tract observed herein using an antibiotic-resistant derivative of *L. plantarum* WCFS1 can also be achieved in humans using non-GMO approaches. Several strategies seem feasible, for example, the CM 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 [48]. 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 previously demonstrated the feasibility of antibiotic-based selective plating using naturally occurring antibiotic resistances [46, 49], offering the possibility to repeat the experimental approach presented herein directly in human volunteers. In conclusion, besides demonstrating the feasibility of achieving enhanced GI-tract robustness, our re-sequencing efforts of the adapted derivatives advance our knowledge on the GI-tract-persistence mechanisms of *L. plantarum*, which are important to predict and control the delivery of this organism in situ. Improved understanding of adaptive behavior of bacteria under stress conditions could pave the way towards the 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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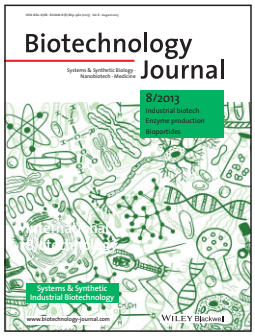

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