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In vitro gut modeling as a tool for adaptive evolutionary engineering of *Lactiplantibacillus plantarum*

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Abstract

Research and marketing of probiotics demand for holistic strain improvement considering both, the biotic and abiotic gut environment. Here we aim to establish the continuous *in vitro* colonic fermentation model PolyFermS as a tool for adaptive evolutionary engineering. Immobilized fecal microbiota from adult donors were steadily cultivated up to 72 days in PolyFermS reactors, providing a long-term compositional and functional stable ecosystem akin to the donors gut. Inoculation of the gut microbiota with immobilized or planktonic *Lactiplantibacillus plantarum* NZ3400, a derivative of the probiotic model strain WCFS1, led to successful colonization. Whole genome sequencing of 45 recovered strains revealed mutations in 16 genes involved in signaling, metabolism, transport and cell surface. Remarkably, mutations in LP_RS14990, LP_RS15205 and intergenic region LP_RS05100<LP_RS05095 were found in recovered strains from different adaptation experiments. Combined addition of the reference strain NZ3400 and each of those mutants to the gut microbiota resulted in increased abundance of the corresponding mutant in PolyFermS microbiota after 10 days, showing the beneficial nature of these mutations. Our data show that the PolyFermS system is a suitable technology to generate adapted mutants for colonization in colonic conditions. Analysis thereof will provide knowledge about factors involved in gut microbiota colonization and persistence.

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Importance

Improvement of bacterial strains to specific abiotic environmental factors is broadly used to enhance strain characteristics for processing and product quality. However, there is currently no multidimensional probiotic strain improvement approach for both, abiotic and biotic factors of a colon microbiota. The continuous PolyFermS fermentation model allows stable and reproducible continuous cultivation of colonic microbiota and provides conditions akin the host gut with high control and easy sampling. This study investigated the suitability of PolyFermS for adaptive evolutionary engineering of a probiotic model organism for lactobacilli, *Lactiplantibacillus plantarum*, to an adult human colonic microbiota. The application of PolyFermS controlled gut microbiota environment led to adaptive evolution of *L. plantarum* strains for enhanced gut colonization characteristics. This novel tool for strain improvement can be used to reveal relevant factors involved in gut microbiota colonization and develop adapted probiotic strains with enhanced functionality in the gut.

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Introduction

Microorganisms play a pivotal role in pharmaceutical, biotechnological and food industries. The latter depends heavily on microorganisms for starter cultures, biopreservation agents and flavor producers (1-3). Moreover, since the 1990s, there has been an increase in the production of probiotics, which are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (4). Strain improvement of probiotic bacteria is of major importance to meet consumer demands for functional foods and enhance competitiveness of probiotic strains. However, it demands for a multi-dimensional approach since biotic and abiotic factors are involved.

A promising solution for strain improvement is evolutionary engineering, which steers microbial evolution by exerting selective pressure (5-7). Desired mutants can be selected based on e.g. growth rate, increased survival, or retention time. This method is feasible with bacteria because short generation times and large population sizes facilitate rapid emergence and selective sweeps of mutants (8-11). It is a well established approach to improve targeted strain characteristics such as the acidification rate of *Lactococcus lactis* (12), growth of *Escherichia coli* (13) or enhanced succinate production in *Actinobacillus* and *Mannheimia* (10, 14, 15). Nonetheless, the potential of evolutionary engineering as multi-dimensional engineering within microbial consortia is not well established yet. Previously, the residence time of *Lactiplantibacillus plantarum* in the murine digestive tract was increased after repetitive administration of the longest persisting *L. plantarum* (16). However, *in vivo* models like mice have societal, ethical and monetary restrictions and might therefore be substituted by *in vitro* models. Furthermore, gastrointestinal physiology and gut species composition of mice are different from humans, possibly limiting the translation (17, 18).

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Continuous fermentation models are best suited for *in vitro* cultivation of gut microbiota in conditions akin to the gut (19, 20). Different PolyFermS models were successfully developed for cultivating colonic microbiota of humans of different age and conditions, swine, murine and chicken cecum microbiota (21-25). The continuous PolyFermS model allows testing several treatments in parallel in second-stage treatment reactors (TRs) seeded with the same gut microbiota produced in the inoculum reactor (IR) containing immobilized microbiota (21). Gut microbiota immobilization in polysaccharide gel beads leads to the maintenance of high cell density, long-term stability due to prevention of cell washout, and diversity of the simulated gut microbiota (23, 26, 27). It moreover creates a sessile bacterial fraction on the gel beads and a planktonic fraction resulting from the growth and release of sessile bacteria and further growth of planktonic cells in the bulk medium (27, 28). This mimics the gastrointestinal environment consisting of free and biofilm or mucus associated bacteria (29, 30). The PolyFermS colonic fermentation model enables operation up to several months in a highly controllable environment with multiple parameters to operate on, which is needed for evolutionary adaptation (26). We therefore hypnotized that the PolyFermS model can provide a long-term stable gut microbiota akin to the human adult colon that allows for adaptive evolution of an exogenous single strain.

In this study, we investigated the PolyFermS fermentation model as a novel tool for strain improvement via adaptive evolutionary engineering, using *L. plantarum* as model strain. *L. plantarum* originates from fermented foods (31, 32) and is detected at low levels in approximately half of healthy human gut microbiota (33). *L. plantarum* WCFS1 is a well-characterized model strain for transient probiotic lactobacilli (34, 35). A WCFS1 derivative harboring a chloramphenicol (CM) resistance gene for tracking was repeatedly exposed to and cultivated in PolyFermS reactors inoculated with

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112 immobilized adult fecal microbiota. Engineered strains were phenotypically and
113 genotypically characterized and tested for improved colonization in the PolyFermS
114 model.
115
116 **Material and Methods**
117 **Bacterial strains and growth conditions**
118 Bacterial strains used during this study are listed in Supplementary Table S1.
119 *L. plantarum* NZ3400 (36) was used as reference strain. NZ3400 is a derivative of
120 WCFS1, harboring a CM resistance cassette (P_{32}-*cat*) in a neutral locus on the
121 chromosome. *L .plantarum* was grown in De Man, Rogosa and Sharpe (MRS, Labo-
122 Life Sàrl, Pully, Switzerland) broth at 37°C, overnight. *L. plantarum* viable cells were
123 enumerated by plating on MRS agar supplemented with CM (10 µg/ml), aerobic
124 incubation at 37°C, overnight. *E. coli* MC1000 (37) was grown overnight in Brain Heart
125 Infusion Broth (BHI, Labolife Sàrl, Pully, Switzerland) at 37°C with agitation.
126
127 **Immobilization of adult fecal microbiota**
128 Fecal samples were obtained from four healthy individuals (27-31 years old) who did
129 not take antibiotics and probiotics for at least three months and did not show detectable
130 microbial growth on MRS + CM plates to avoid interference with *L. plantarum* NZ3400
131 recovery. The Ethics Committee of ETH Zürich exempted this study from review
132 because the sample collection procedure has not been performed under conditions of
133 intervention. Informed written consent was obtained from fecal donors. Fecal samples
134 were immediately transferred to an anaerobic chamber within 2 h after defecation and
135 suspended at 20% (w/v) in reduced peptone water (0.1%, pH 7; Thermo Fisher
136 Diagnostics AG, Pratteln, Switzerland). Immobilization in polymer gel beads (gellan
137 gum (2.5%, w/v), xanthan (0.25%, w/v) and sodium citrate (0.2%, w/v)) was performed

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as previously described (21, 38). The inoculum bioreactor (IR) (Sixfors, Infors, Bottmingen, Switzerland) was filled with 140 ml of vitamin supplemented MacFarlane medium, formulated to mimic the chyme entering the colon (20, 39), and inoculated with 60 ml of fecal gel beads. Beads were colonized in two fed-batch fermentations carried out at 37°C, pH 5.8 by controlled addition of NaOH (2.5 M), stirring at 180 rpm, and replacing 100 ml of spent medium with fresh medium after 16 h (21). Anaerobiosis was set by purging the bioreactor headspace with low CO_2 flow.

Proximal colon fermentation in the PolyFermS model using immobilized human gut microbiota

Continuous proximal colonic *in vitro* fermentations with immobilized human adult gut microbiota were performed in bioreactors as reported previously (26, 40). All fermentations were operated to mimic the adult proximal colon as described above for batch fermentation. Fresh medium, was continuously added to the IR (25 ml/h) and fermented medium was removed to maintain a working volume of 200 ml. The IR was run in continuous mode for at least 10 days to reach metabolic stability indicated by lower than 10% day-to-day variation in short chain fatty acids (SCFAs) concentration, before connecting second-stage TRs. TRs were inoculated by the IR at 1.25 ml/h and fed at 23.75 ml/h. TRs were operated continuously for four days prior to *L. plantarum* supplementation in order to establish a gut microbiota activity akin to the IR. Addition of planktonic or immobilized *L. plantarum* was tested in IR and TRs with different donor microbiota (Fig. 1). In case of two successive treatment periods in the same reactor, the reactor was disconnected after the first treatment period, sterilized, re-connected and stabilized for four days before starting a second treatment.

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Reactor effluent samples were taken daily to monitor the fermentation process, centrifuged for 10 min at 14 000 g, 4°C and stored at -20°C. Pellets were used for DNA extraction and supernatants for metabolite analysis.

Adaptive evolutionary engineering of *L. plantarum*

Adaptive evolution using immobilized L. plantarum NZ3400

Adaptive evolution of immobilized *L. plantarum* NZ3400 was tested in a single stage IR (IR1) inoculated with donor 1 fecal beads (Fig. 1, A). A *L. plantarum* overnight culture was harvested at 4°C, 4000 g for 10 min and washed twice in Phosphate-Buffered Saline (PBS), pH 6.2. Immobilization of *L. plantarum* was done as described for fecal samples under aerobic conditions. Beads were colonized during two pH-controlled batch cultures at 37°C for 16 h with stirring at 150 rpm. Colonized beads were washed in PBS, supplemented with cryoprotective buffer (41) and stored at - 80°C. Before use, *L. plantarum* beads were reactivated during two batch cultures as described for colonization, and washed twice in PBS. *L. plantarum* viable cell counts were determined by crushing 1 g of beads in PBS with a spatula and plating serial dilutions. Four g of beads ($5*10^9$ colony forming units (CFU) *L. plantarum*/g) were added to the single stage IR1 and cultivated for 67 days.

Adaptive evolution using planktonic L. plantarum NZ3400

Adaptation of planktonic *L. plantarum* NZ3400 was tested in TRs continuously inoculated by the effluent from IR2 containing beads with immobilized fecal microbiota of donor 2 (Fig. 1, B). *L. plantarum* strains were grown overnight, harvested, washed twice in PBS, re-suspended in MacFarlane medium and added to the TRs to a final level of 10^9 CFU *L. plantarum*/ml effluent. Long-term planktonic adaptation was tested

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187 in TR2 operated for 72 days, and repeated in TR1 (period1) and TR1 (period 2) for 44
188 and 23 days, respectively (Fig. 1, B).
189 Furthermore, the strain IA10, recovered from the immobilized adaptation, was added
190 in planktonic state to TR3 and TR5 containing donor 2 gut microbiota to investigate
191 effects of adaptations that occurred during the immobilized adaptation (Fig. 1, B).
192

193 **Metabolite analysis of the continuous colon fermentation**

194 Reactors were sampled daily for analysis of the SCFAs acetate, butyrate and
195 propionate, branched-chain fatty acids isobutyrate, isovalerate and valerate, and
196 intermediate metabolites lactate and formate (42). Concentrations were determined by
197 high-performance liquid chromatography (HPLC) as described previously (23).
198

199 **Microbial profiling by 16S rRNA gene amplicon sequencing**

200 Genomic DNA of fecal and effluent samples was extracted using the FastDNA® SPIN
201 Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's
202 instructions. The V4 region of the 16S rRNA gene was amplified with the primers 806R
203 (5′- GGACTACHVGGGTWTCTAAT- 3′) and 515F
204 (5′- GTGCCAGCMGCCGCGGTAA-3′). Library preparation and sequencing (Illumina,
205 CA, USA) using an Illumina MiSeq flow cell with V2 2 × 250-bp paired-end chemistry
206 supplemented with 10% of PhiX was performed in collaboration with the Genetic
207 Diversity Center (GDC, ETH Zürich, Switzerland).
208 Raw data obtained from 16S rRNA sequencing were processed using Cutadapt (43)
209 and DADA2 pipeline (44) to obtain amplicon sequence variants. Taxonomy was
210 assigned using SILVA database (v.132) (45) (full method in Supplementary Data S1).
211

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212 **Recovery of *L. plantarum* from the gut microbiota**

213 *L. plantarum* colonization was determined by plating on MRS + CM agar. Data were
214 compared to the theoretical washout curve determined for absence of growth, from the
215 following equation: $c_t=c_0*e^{(t/RT)}$ (40), where c_0 and c_t represent cell concentration at
216 time point 0 and t, respectively, and RT corresponds to the retention time. Colonies
217 were randomly picked, incubated in MRS + CM overnight, mixed 1:1 with 60% (v/v)
218 glycerol (Sigma-Aldrich Chemie GmbHm Buchs, Switzerland) and stored at -80°C.

219 Natural biofilm formed in TR2, used for long-term planktonic adaptation, and the
220 repetition experiment in TR1 (period 1). To recover *L. plantarum* from biofilms, the
221 vessels were emptied and washed twice with PBS. Remaining biofilm was removed
222 and homogenized with glass beads (5 mm, VWR International AG, Dietikon,
223 Switzerland) in dilution solution containing 0.85% NaCl and 0.1% peptone from casein
224 (w/v, VWR International AG, Dietikon, Switzerland). Dilutions were plated with
225 subsequent strain recovery and storage performed as described above.

226

227 **Phenotypic characterization of recovered *L. plantarum* strains**

228 Growth behavior of recovered *L. plantarum* strains was analyzed in MRS
229 supplemented with each of the main SCFAs of the human gut microbiota, acetate (50,
230 75, 100 mM), butyrate (15, 30, and 45 mM) and propionate (15, 30, and 45 mM) in
231 similar concentrations as measured during colonic fermentations. The abiotic gut
232 fermentation environment was simulated in Effluent-MacFarlane-Sugar (EMS) medium
233 consisting of sterile filtered PolyFermS effluent, MacFarlane medium in a ratio 9:1 and
234 0.75% (w/v) glucose (see Fig. S1 in the supplemental material). For comparison of the
235 effects on adaption in immobilized and long-term planktonic adaptation trials performed
236 in TR2 (Fig. 1, B), recovered strains were divided into four groups based on their origin

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of isolation: (1) 11 strains from the effluent at the late stage of immobilized adaptation after 53 days, (2) 14 strains from the effluent during day 7 and day 23 (early planktonic adaptation), (3) 19 strains from the effluent during day 60 and day 72 (late planktonic adaptation) and (4) 25 strains from the biofilm of planktonic adaptation after 72 days. Growth analysis was done in 96-well tissue culture test plates (Bioswisstec AG, Schaffhausen, Switzerland). Wells were filled with 200 µl of medium and inoculated with the potentially adapted *L. plantarum* at 37°C. Growth was monitored by optical density (OD) measurement at 600 nm in a plate reader after 24 h (PowerWaveTMXS, Bio-Tek Instrument Inc., Winooski, VT, US) in biological triplicates.

Phenotype stability was assessed by repeated daily culturing of strains in MRS for 28 days, approximately 190 generations, as presented above. Stability was measured after 1, 7, 14, 21 and 28 days.

Complete genome sequencing and data analysis

L. plantarum genomic DNA was isolated via lysozyme-based cell lysis (46) followed by purification using the Wizard® Genomic DNA Purification Kit according to the manufacturer's instructions (Promega, Dübendorf, Switzerland). The genome of the reference strain NZ3400 was sequenced at the Functional Genomics Center Zurich (Zürich, Switzerland) on PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) using one SMRT cell. Reads were assembled using HGAP assembly as described previously (47). All other *L. plantarum* strains were sequenced using Illumina Miniseq (Illumina, CA, US) with 250 bp paired-end reads at the Institute for Food Safety and Hygiene, University of Zurich (47). Reads were merged and mapped to the reference genome *L. plantarum* NZ3400 using CLC Genomic Workbench 11.0 (Qiagen CLC bio, Aarhus, Denmark) using default parameters. Single nucleotide polymorphisms (SNPs)

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were extracted using Parsnp (48). SNPs were confirmed by Sanger Sequencing (Eurofins GATC, Biotech GmbH, Konstanz, Germany). SNP related changes in amino acid sequence were determined in CLC Genomic Workbench.

Competition experiments in the human gut microbiota

Competition experiments between NZ3400 and the mutant strains *L. plantarum* IA01, PA1.2_01 and PA2_06 were performed to determine the effect of the mutations on gut microbiota colonization (Fig. 1, B). NZ3400 was paired with each of the three mutant strains in a 1:1 ratio to reach 10^9 CFU/ml reactor effluent to the gut microbiota and cultivated for ten days. Relative abundance of the strains in the complex community was determined by measuring allele frequency of the genes LP_RS14990 and LP_RS15205, respectively, via Pyrosequencing (full method in Supplementary Data S1).

Stability of mutations under standard cultivation conditions

Stability of the mutations in *L. plantarum* IA01 and PA2_06 was investigated during repeated daily cultures in MRS for 12 days, approximately 81 generations, performed in triplicates. Allele frequency was determined via Pyrosequencing as described above.

Plasmid construction and LP_RS14990 gene replacement of *L. plantarum* NZ3400

Six mutants carrying the mutation in LP_RS14990 were recovered from independent adaptation experiments. To investigate the involvement of this gene in gut microbiota colonization, a *L. plantarum* ΔLP_RS14990 knockout was constructed by double-crossover gene replacement in *L. plantarum* NZ3400* (full method in Supplementary

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Data S1). The knockout strain was subsequently added to the gut microbiota and colonization levels were compared to the reference strain NZ3400 (Fig. 1, B).

In silico analysis of LP_RS15205 in *L. plantarum*

LP_RS15205 was affected by identical mutation in four strains recovered from two independent adaptation experiments. Since this mutation was also found in the background gut microbiota, in silico analysis of this locus was performed. Complete genome sequences of Firmicutes (n = 557), Bacteroidetes (n = 218), Actinomycetes (n = 457) and γ-Proteobacteria (n = 638) from the NCBI genome database were downloaded in May 2020. The amino acid sequence of LP_RS15205 was blasted against these genomes using standard settings and significant hits were aligned using MUSCLE (49).

Data analysis

Statistics for growth experiments were calculated in R (version 3.6.2) using one-sample t-test for comparison to *L. plantarum* NZ3400 and paired samples t-test for comparison within recovered *L. plantarum* groups. Values represent mean values ± standard deviation. The heatmap was generated using the R pheatmap package and euclidean distance measure. Allele frequency determination by Pyrosequencing was calculated based on three extracted DNA samples of the same time point. Graphs were created using GraphPad Prism® version 8 (GraphPad Software Inc., San Diego, CA, USA).

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Results

PolyFermS model allows stable cultivation of adult gut microbiota

The PolyFermS model operated to mimic the adult proximal colon was used to provide a gut microbiota environment for evolutionary adaptation of *L. plantarum* NZ3400. Adaptation of immobilized *L. plantarum* was performed in IR1 and adaptation of planktonic *L. plantarum* was tested in TRs continuously inoculated with IR2 microbiota (Fig. 1). Metabolic stability of IR1 (see Fig. S2, A in the supplemental material) was achieved after one week with main SCFAs acetate, propionate and butyrate at 73 ± 7, 21 ± 4 and 19 ± 3 mM over two month fermentation, respectively. IR1 microbiota was dominated by Firmicutes and Bacteroidetes accounting for 44 ± 5 and 47 ± 2% of the total population during days 44-46, respectively, 48 ± 7 and 41 ± 2% during days 57-59, respectively, and 52 ± 4 and 36 ± 4% during days 61-65, respectively (see Fig. S3, A in the supplemental material). This stability was also observed on family level (see Fig. S3, B in the supplemental material). IR2 microbiota reached metabolic stability (see Fig. S2, B in the supplemental material) after one week with the main SCFAs acetate, propionate and butyrate at 84 ± 5, 32 ± 8 and 23 ± 4 mM, respectively, during two months of culture. IR2 had a different microbiota composition from IR1 (see Fig. S3, C&D in the supplemental material). Bacteroidetes dominated the gut microbiota compared to Firmicutes with 63 ± 1% and 36 ± 1% during days 24-26, respectively, 68 ± 4 and 30 ± 4% during days 41-43, respectively, and 66 ± 1 and 30 ± 1% during days 89-91, respectively (see Fig. S3, C in the supplemental material). Stability was maintained up to 90 days on family level (see Fig. S3, D in the supplemental material). Microbiota composition of the IR was successfully transferred to and maintained in the TRs (see Fig. S3, D in the supplemental material). Both, metabolic activity (see Fig. S2, C-E in the supplemental material) and composition were reproduced in the TRs.

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337 **Prolonged cultivation of *L. plantarum* in *in vitro* human gut microbiota**

338 To investigate the potential of the continuous *in vitro* gut fermentation model
339 PolyFermS for evolutionary engineering, immobilized *L. plantarum* NZ3400 was added
340 to the stabilized microbiota in IR1 at an initial concentration of 10^8 CFU/ml effluent.
341 NZ3400 decreased at the rate of the theoretical wash-out during the first four days
342 (Fig. 2, A), followed by colonization between 10^2 CFU/ml and 10^4 CFU/ml during the
343 50-days fermentation.

344 The PolyFermS model was further evaluated for long-term adaptation of planktonic
345 *L. plantarum* NZ3400 in TR2, fed by donor 2 gut microbiota. Cell counts decreased
346 from 10^9 to 10^5 CFU/ml during the first four days after spiking (Fig. 2, B), at the rate of
347 the wash-out. Thereafter, colonization steadily increased to 10^6 CFU/ml during
348 72 days. Repeated colonization by planktonic cells in the same gut microbiota added
349 10^6 and 10^9 CFU *L. plantarum*/ml in TR1 (period1) and TR1 (period2), yielded stable
350 cell counts of 10^6 and $3*10^4$ CFU/ml after 44 and 23 days, respectively. Taken
351 together, *L. plantarum* colonization was demonstrated for more than 50 days and
352 150 generations, settings that could allow evolution.

353

354 **Recovered *L. plantarum* strains are phenotypically adapted to the gut microbiota**
355 **environment**

356 To test for *L. plantarum* adaptation during gut microbiota cultivation, recovered strains
357 were grown in SCFA concentrations comparable to those in the gut fermenter. Average
358 growth of strains from immobilized adaptation measured by OD was impaired in MRS
359 and in MRS supplemented with 30 mM propionate or butyrate compared to the
360 reference strain NZ3400 (Table 1). Strains from early stage of long-term planktonic
361 adaptation behaved similarly to the reference NZ3400 in all tested media. However,
362 strains from late planktonic adaptation and biofilm grew better in MRS plus acetate

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(+0.11 and +0.07, respectively), less in standard MRS (-0.14 and -0.11, respectively) and similar in MRS plus propionate or butyrate (Table 1) compared to NZ3400. Moreover, strains from biofilm and late planktonic adaptation grew significantly better in the reactor-effluent-mimicking EMS medium compared to strains from early adaptation (Table 1).

When clustered according to growth performance in MRS and MRS supplemented with acetate (50 mM), butyrate (30 mM) and propionate (30 mM), strains from immobilized and planktonic adaptation were clearly separated (Fig. 3). Further, strains recovered from immobilized adaptation exhibited a higher growth variability than strains isolated from the long-term planktonic adaptation (see Fig. S4 in the supplemental material). The reference NZ3400 clustered with strains isolated from early planktonic adaptation and clearly separate from strains isolated from effluent and biofilm at the end of adaptation (Fig. 3). All together, these results strongly hint towards adaptation of *L. plantarum* during prolonged cultivation in the gut microbiota. Furthermore, strains did not cluster according to the reactor they were isolated from, suggesting that the adaptation pattern is not dependent on reactor but rather time point of isolation.

Phenotypes of these adaptations were stable for at least 190 generations (see Fig. S5 in the supplemental material). This strongly suggests that observed phenotypes are caused by mutations rather than physiological variations.

Mutations in adapted *L. plantarum* strains hint towards adaptive evolution

Stable altered phenotypes of recovered *L. plantarum* strains strongly suggest that these strains harbor mutations. Therefore, whole-genome sequencing of 45 strains randomly selected from adaptations experiments was performed. Comparison to the reference genome NZ3400 revealed 15 strains without any genotypic differences. Out of 18 SNPs confirmed by Sanger sequencing, two were detected in non-coding

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389 regions. The remaining 16 SNPs were found in genes involved in signaling,
390 metabolism, transport and cell surface (Table 2).
391 The 11 mutated of 12 sequenced strains from the immobilized adaptation belonged to
392 four different genotypes. Among these 11 strains, a mutation in the cell surface protein
393 gene encoded by LP_RS14255 was found seven times. Further, a mutation in
394 LP_RS14990, encoding a histidine kinase domain, was found thrice (Table 2). To
395 prevent carry-over of possible mutants present in the initial NZ3400 stock culture, a
396 new stock from a single NZ3400 colony isolate, designated NZ3400*, was produced.
397 NZ3400* was subjected to PacBio sequencing and used for all subsequent planktonic
398 supplementation trials. Eight of 15 *L. plantarum* strains that were recovered at late
399 stage of the long-term planktonic adaptation were mutated, resulting in five different
400 genotypes (Table 2). Strikingly, a SNP in LP_RS14990 occurred independently during
401 planktonic and immobilized adaptation. Moreover, the SNP in LP_RS15205 and in the
402 intergenic region between LP_RS05100 and LP_RS05095 were found in strains
403 isolated from two different reactors.
404 Immobilized adaptation resulted in a bigger fraction of mutated strains but consisted
405 predominantly of two isogenic lineages. Planktonic adaptation resulted in less frequent
406 mutagenesis, yet higher mutant diversity. This suggests a difference in adaptation
407 pressure, as already observed for the phenotypic screening. Recovery of some
408 identical mutants from different adaptation experiments suggests that some of the
409 observed mutations are involved in adaptation to the gut microbiota.
410
411 **Mutations in LP_RS14990 and LP_RS15205 are beneficial for *in vitro* gut**
412 **microbiota colonization**
413 The mutation in the histidine kinase protein gene LP_RS14990 and the ROK protein
414 gene LP_RS15205 occurred independently more than once in adaptation experiments.

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We therefore tested the fitness each of the mutants *L. plantarum* PA2_06 (C837A in LP_RS15205), IA01 (C979T in LP_RS14990) or PA1.2_01 (C979T in LP_RS14990 and C837A in LP_RS15205) in competition experiments with the reference strain NZ3400. Ten days of cultivation resulted in an increased abundance of all tested mutants in the gut microbiota when compared to NZ3400 (Table 3).

Pyrosequencing indicated that donor 2 gut microbiota had a *L. plantarum* background. Remarkably, the Pyrogram of this background at the position LP_RS15205 C837A was identical to the Pyrogram of a sample containing NZ3400 and PA2_06. This shows that the nucleotide variation of both, NZ3400 and PA2_06, also occurs naturally (see Fig. S6 in the supplemental material).

Mutation C979T in LP_RS14990 is stable under standard culturing conditions

After observing increased fitness of mutants compared to the reference strain, it was investigated whether the mutations of *L. plantarum* IA01 in LP_RS14990 and *L. plantarum* PA2_06 in LP_RS15205 are stable during daily repeated batch cultures without the adaptation pressure of the gut microbiota. Stability of the LP_RS14990 mutation in the IA01 strain was observed during 12 batch cultures. However, the mutation C837A in strain PA2_06 was not stable since the reference strain nucleotide reoccurred at 3.5 ± 0.15% after 12 days (see Table S3 in the supplemental material).

LP_RS14990 gene replacement in *L. plantarum* NZ3400 results in delay of gut microbiota colonization

To investigate the role of the LP_RS14990 gene in gut microbiota colonization, a ΔLP_RS14990 gene replacement strain was constructed and its colonization compared to NZ3400. NZ3400 started to colonize the gut microbiota of donor 3 on day one and donor 4 at day three, since levels were above the wash-out curve (Fig. 4).

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441 Levels of ΔLP_RS14990 in both gut microbiota decreased more rapidly than the wash-
442 out curve until day three, suggesting cell death (Fig. 4). Strain ΔLP_RS14990 started
443 to colonize the gut microbiota of both donors only after four days, later than the
444 reference strain. NZ3400 colonized donor 4 gut microbiota at $8*10^3$ CFU/ml, whereas
445 a lower colonization of $1*10^3$ CFU/ml was observed for ΔLP_RS14990 (Fig. 4, B).
446 However, this seems to be a donor microbiota dependent response since no difference
447 in colonization was observed in donor 3 gut microbiota (Fig. 4, A).
448
449 **In silico analysis of LP_RS15205 in *L. plantarum***
450 Occurrence of the SNP C837A (L279F) in LP_RS15205 in *L. plantarum* strains from
451 different reactors and in the background of the gut microbiota suggests an important
452 function of this SNP in survival of *L. plantarum* in the gut microbiota. In silico analysis
453 of LP_RS15205 revealed that C837A lies in a conserved domain with an ANLxGA
454 motive that is found in Firmicutes and γ-Proteobacteria (data not shown). The function
455 of this domain is unknown, but its conservation throughout two phyla strongly suggest
456 that it might be important for protein function and the mutation ANLxGA to ANFxGA
457 might have an impact on protein functionality.
458

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Discussion

Strain improvement is of major importance for food industries and probiotic cultures. Classical evolutionary adaptation approaches focus on specific strain characteristics in abiotic environments like improved performance of starter cultures or lactic acid bacteria (50, 51). However, for probiotics, improvement of gut colonization needs to consider abiotic and biotic factors. Here we examined the suitability of the *in vitro* gut fermentation model PolyFermS to provide a gut microbiota environment for evolutionary engineering of *L. plantarum* NZ3400 towards a human adult gut microbiota. Immobilization of the donor gut microbiota and cultivation in the PolyFermS allowed reproduction of distinct gut microbiota representative for human adults (52, 53). The achieved long-term metabolic and compositional stability allowed the generation of adapted mutants. *L. plantarum* supplementation method and donor microbiota seem to influence phenotypic and genotypic adaptations. *L. plantarum* strains recovered from the immobilized adaptation showed very limited adaptation in contrast to recovered strains from planktonic adaptation trials, although abiotic conditions were highly similar. It thus is assumed that differences in adaptation patterns are caused by the supplementation method. Immobilization provides protection against stress and entraps cells physically in the system, which leads to decreased adaptation pressure (54-56), which is in accordance with our phenotypic screening where strains from immobilized adaptation showed less adaptation. Polymer beads create mucosal-like attachment sites (57) and growth in beads results in a gradual release of sessile cells from the surface that then grow as planktonic cells in the bulk medium (38). The result is a mixed population consisting of cell lineages undergoing varying number of growth cycles in the planktonic state. This might explain the more diverse phenotypic adaptation pattern among strains from the immobilized adaptation. It was further shown that immobilized cells are genetically more stable than planktonic cells (58-61),

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485 which was observed in our study where recovered strains from immobilized adaptation
486 mainly consisted of two isogenic lineages.
487 Two observations suggest that the observed genotypic changes in isolated strains
488 were involved in adaptive evolution. The first is the known function of several of the
489 genes affected by SNPs. Activated genes of *L. plantarum* in the murine digestive tract
490 were involved in carbohydrate transport, metabolism and cell surface and in sugar-
491 related functions, molecule biosynthesis and stress response (62, 63). Further,
492 exposure of *L. plantarum* to the murine intestinal tract predominantly resulted in
493 mutations in genes encoding cell wall associated proteins (16). The SNPs identified in
494 our study belong to the categories mentioned above. Remarkably, the latter study
495 identified a SNP in a glycerophosphodiester phosphodiesterase encoding gene, a
496 gene also affected by a SNP in a recovered strain in our study. In summary, the
497 function of mutated genes are in agreement with previously observed responses of
498 *L. plantarum* to the *in vivo* intestinal environment. This shows similar selection
499 pressures in the PolyFermS system compared to *in vivo* settings.
500 The second observation indicating adaptive evolution in our experiment is that three
501 mutations were found independently in multiple experiments. Since mutations occur
502 continuously, -80°C stock cultures are rarely isogenic and the mutants found in our
503 study could have been already present as subculture in the NZ3400 stock (16). To
504 counteract this bias, two different stock cultures, NZ3400 and NZ3400*, were used for
505 the immobilized and planktonic adaptation, respectively. Occurrence of identical
506 mutants recovered from immobilized and planktonic adaptation with the SNP in
507 LP_RS14990 seems therefore unlikely to originate from a stock subculture.
508 Furthermore, the mutation was not detected by Pyrosequencing of NZ3400* prior to
509 inoculation (data not shown), minimizing a role for subcultures in our experiments.
510 Moreover, the increased fitness of mutants and delayed colonization of the

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511 ΔLP_RS14990 gene replacement strain show the beneficial effect of detected
512 mutations. This strongly supports that observed mutations are caused by adaptive
513 evolution and demonstrates the suitability of the PolyFermS model to select for relevant
514 mutations related to the human gut microbiota.

515 In conclusion, we demonstrated successful application of the continuous PolyFermS
516 gut fermentation model to provide a long-term stable gut microbiota to generate
517 adapted mutants to this environment. Immobilization of strains does not only allow
518 adaptive evolution of non-colonizers but also creates a culture consisting of sessile
519 and planktonic cells mimicking the human gastrointestinal tract. The conditions of the
520 model can be easily changed to other needs and selective pressures, including the
521 source of microbiota and abiotic conditions. This novel technology enables
522 identification of genes involved in gut microbiota colonization, persistence and
523 metabolism. The PolyFermS system could further be designed and applied for different
524 fermentations, to trace and identify evolutionary and ecological processes between an
525 exogenous single strain and a complex ecosystem.

526

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Tables

Table 1: Growth of recovered *L. plantarum* strains in different media.

***L. plantarum* origin**	**MRS**	**MRS+acetate (50 mM)**	**MRS+propionate (30 mM)**	**MRS+butyrate (30 mM)**	**EMS**
Immobilized adaptation	1.25±0.11^{c*}	1.02±0.15^{cb}	1.00±0.24^{b*}	0.84±0.24^{c*}	ND
Early planktonic adaptation	1.46±0.05^{b}	1.06±0.05^{b}	1.22±0.03^{a}	1.22±0.02^{a*}	0.69±0.05^{a*}
Late planktonic adaptation	1.34±0.05^{a*}	1.17±0.06^{a*}	1.25±0.07^{a}	1.21±0.07^{a}	0.74±0.04^{b*}
Biofilm of planktonic adaptation	1.37±0.07^{a*}	1.13±0.09^{a*}	1.22±0.07^{a}	1.15±0.06^{b*}	0.77±0.05^{c*}
NZ3400	1.48±0.04	1.06±0.04	1.23±0.03	1.20±0.03	0.63±0.07

The values reported are OD_{600nm} after 24 hours. Values obtained for NZ3400 represent mean±standard deviation of three biological triplicates. All other values represent mean±standard deviation of all strains recovered during the corresponding time period, whereas each recovered strain was measured in biological triplicates. Values labeled with the same letters within a column are not significantly different from each other ($p > 0.05$ in a paired samples t-test, adjusted for unequal variance or normal distribution when needed). Stars represent significant differences between NZ3400 and *L. plantarum* groups (one-sample t-test, $p \leq 0.05$). ND is not determined. EMS: effluent-MacFarlane-Sugar medium. Immobilized adaptation: n= 11 strains, early planktonic adaptation: n= 14 strains, late planktonic adaptation: n= 19 strains, biofilm of planktonic adaptation: n= 25 strains

Table 2: Identified genotypic changes of recovered *L. plantarum* strains compared to NZ3400

SNP position[a]	SNP location within gene	AA change[b]	AA charge[c]	AA polarity[d]	Immobilized adaptation cycle											Planktonic adaptation cycle											Recovered *L. plantarum* IA_10					Locus Tag[e]	Description
																TR1, period2			TR2								TR1, period1								
					IA01	IA02	IA03	IA04	IA05	IA06	IA07	IA08	IA09	IA10	IA11	PA1.2_01	PA1.2_02	PA1.2_03	PA2_01	PA2_02	PA2_03	PA2_04	PA2_05	PA2_06	PA2_07	PA2_08	PA1.1_01	PA1.1_02	PA1.1_03	IA_10.1	IA_10.2	IA_10.3	IA_10.4	IA_10.5		
					(i)	(i)		(ii)	(ii)	(ii)	(ii)	(ii)	(ii)	(ii)					(iii)	(iii)	(iv)	(iv)	(iv)												
3197325	C979T	Glu -> Lys	- = +	P = P	x	x	x									x	x	x																LP_RS14990	GHLK domain-containing protein
1441981	G645T	Met -> Ile	N = N	A = A																								x						LP_RS06730	Glycerophosphodiester phosphodiesterase
2635801	A487C	Thr -> Pro	N = N	P = A																									x					LP_RS12455	Lipase/esterase
2832369	C328T	Thr -> Ile	N = N	P = A																									x					LP_RS13325	class I SAM-dependent methyltransferase
3244391	C837A	Leu -> Phe	N = N	A = A																		x	x	x						x				LP_RS15205	ROK family protein
945932	C2340T	None																x																LP_RS04385	*rpoC* ; DNA-directed RNA polymerase subunit beta
488529	G1192A	Asp -> Asn	- = N	P = P																								x						LP_RS02260	aminoacetyl-tRNA hydrolase
1536407	G881A	None																								x								LP_RS07205	*rsgA* ; ribosome small subunit-dependent GTPase A
1737654	G173A	Ala -> Val	N = N	A = A																							x							LP_RS08140	HAD_IC family P-type ATPase
3261513	C749T	None																	x	x	x													LP_RS15260	extracellular solute-binding protein
1286590	A767G	Ile -> Thr	N = N	A = P																					x									LP_RS05980	MFS transporter
300072	C995T	Thr -> Met	N = N	P = A																						x								LP_RS01370	acetoin ABC transporter permease
332157	G382A	Ala -> Thr	N = N	A = P												x	x	x																LP_RS01530	hypothetical protein
2947006	G39T	Met -> Ile	N = N	A = A			x																											LP_RS13860	VanZ family protein
3025391	C569A	Gly->Val	N = N	A = A				x	x	x	x	x	x	x																			x	LP_RS14255	WxL domain-containing protein
2307096	G984A	Met -> Ile	N = N	A = A																										x				LP_RS10985	LPXTG cell wall anchor domain-containing protein
1116951		N.A	N.A	N.A													x			x														gen. DNA	intergenic; LP_RS05100 < LP_RS05095
67503		N.A	N.A	N.A											x																				gen. DNA	intergenic; LP_RS00275 < LP_RS00270

a) Indicates SNP position in relation to the *L. plantarum* WCFS1 genome
b) Indicates amino acid change in the mutant compared to NZ3400 caused by the SNP. None: no change in amino acid, N.A. = amino acid change not identifiable.
c) Indicates change of the amino acid charge compared to NZ3400 caused by the SNP. N: neutral, +: positive charge, -: negative charge.
d) Amino acid polarity, P: polar, a: apolar.
e) Location of the SNP. Intergenic SPS are labeled with genomic (gen.) DNA.
* X indicates a SNP, ⌀ indicates loss of a mutation. ** Roman letters represent isogenic strains.

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777 Table 3: Increase in relative abundance of mutants during 10 days of competition

778 against NZ3400.

	IA01, T (%)	PA2_06, G (%)	PA1.2_01, T (%)
Donor 2, rep.1	38±5	-	-
Donor 2, rep.2	25±13	-	-
Donor 3, rep.1	66±1	26±3	11±2
Donor 3, rep.2	51±1	31±3	39±2
Donor 4, rep.1	-	20±2	-
Donor 4, rep.2	-	6±2	-

779 Values represent mean±standard deviation of three DNA samples isolated at the same time point from the same
780 reactor, calculated as difference between time point of addition and 10 days of cultivation. Values depict. *Allele
781 frequency was measured for G since Pyrosequencing was done on the complementary DNA strand.

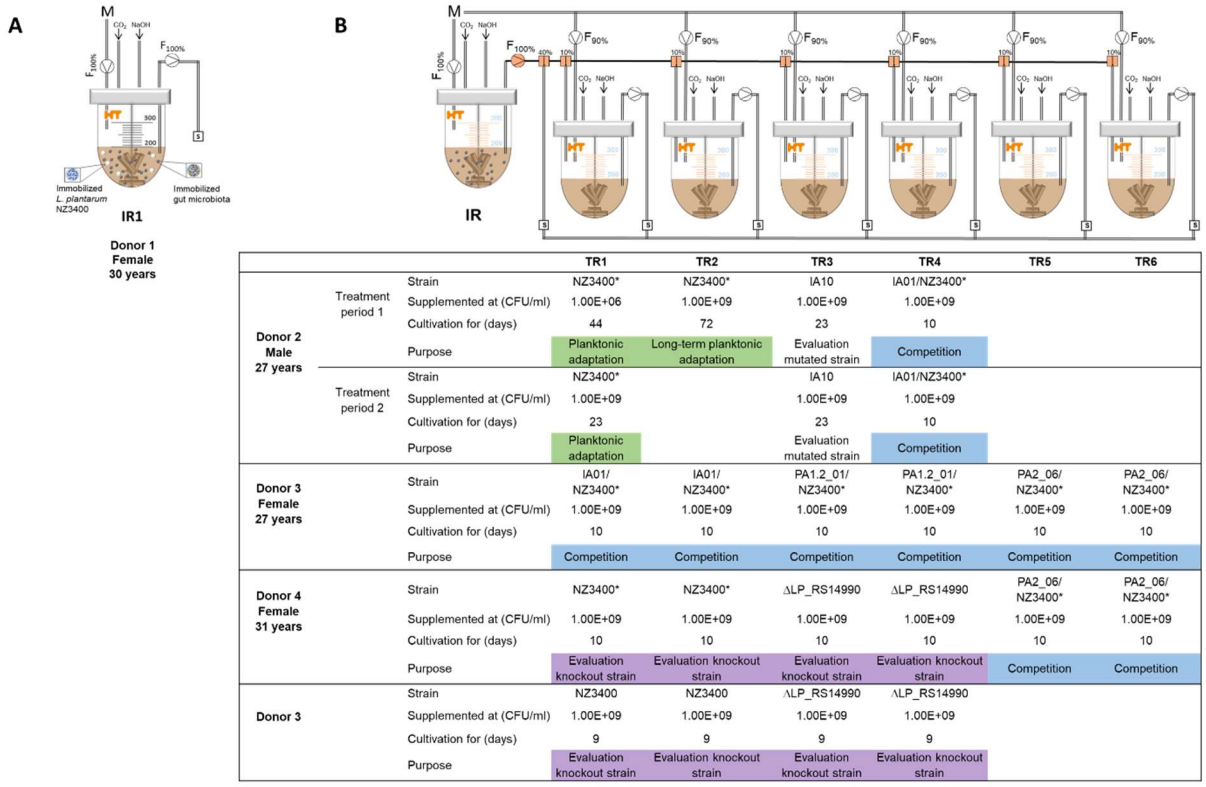
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Figures

Figure 1

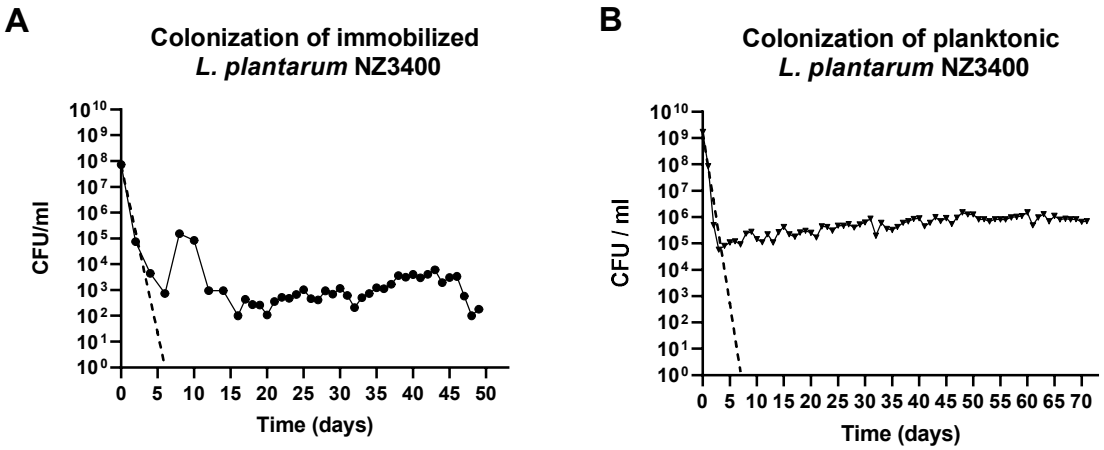


			TR1	TR2	TR3	TR4	TR5	TR6
Donor 2 Male 27 years	Treatment period 1	Strain	NZ3400*	NZ3400*	IA10	IA01/NZ3400*		
		Supplemented at (CFU/ml)	1.00E+06	1.00E+09	1.00E+09	1.00E+09		
		Cultivation for (days)	44	72	23	10		
		Purpose	Planktonic adaptation	Long-term planktonic adaptation	Evaluation mutated strain	Competition		
	Treatment period 2	Strain	NZ3400*		IA10	IA01/NZ3400*		
		Supplemented at (CFU/ml)	1.00E+09		1.00E+09	1.00E+09		
		Cultivation for (days)	23		23	10		
		Purpose	Planktonic adaptation		Evaluation mutated strain	Competition		
Donor 3 Female 27 years		Strain	IA01/ NZ3400*	IA01/ NZ3400*	PA1.2_01/ NZ3400*	PA1.2_01/ NZ3400*	PA2_06/ NZ3400*	PA2_06/ NZ3400*
		Supplemented at (CFU/ml)	1.00E+09	1.00E+09	1.00E+09	1.00E+09	1.00E+09	1.00E+09
		Cultivation for (days)	10	10	10	10	10	10
		Purpose	Competition	Competition	Competition	Competition	Competition	Competition
Donor 4 Female 31 years		Strain	NZ3400*	NZ3400*	ΔLP_RS14990	ΔLP_RS14990	PA2_06/ NZ3400*	PA2_06/ NZ3400*
		Supplemented at (CFU/ml)	1.00E+09	1.00E+09	1.00E+09	1.00E+09	1.00E+09	1.00E+09
		Cultivation for (days)	10	10	10	10	10	10
		Purpose	Evaluation knockout strain	Evaluation knockout strain	Evaluation knockout strain	Evaluation knockout strain	Competition	Competition
Donor 3		Strain	NZ3400	NZ3400	ΔLP_RS14990	ΔLP_RS14990		
		Supplemented at (CFU/ml)	1.00E+09	1.00E+09	1.00E+09	1.00E+09		
		Cultivation for (days)	9	9	9	9		
		Purpose	Evaluation knockout strain	Evaluation knockout strain	Evaluation knockout strain	Evaluation knockout strain		

Figure 1: PolyFermS setup of immobilized and planktonic adaptation and further experiments in *in vitro* human adult gut microbiota. A) Immobilized *L. plantarum* NZ3400 was added to immobilized fecal gut microbiota in IR1 from donor 1 during a single stage fermentation. B) Immobilized fecal microbiota of donors 2-4 was cultivated in the inoculum reactor (IR), which was used to inoculate second stage reactors (TRs) that were supplemented with *L. plantarum* after stabilization. NZ3400: reference strain; NZ3400*: new stock from single colony isolate of NZ3400; IA10, IA01, PA1.2_01, PA2_06: recovered *L. plantarum* mutants; *L. plantarum* ΔLP_RS14990: LP_RS14990 gene deletion strain. M: MacFarlane medium, S: sampling, F: feed.

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800 Figure 2



801

802 **Figure 2:** Colonization levels of *L. plantarum* during immobilized and planktonic
803 adaptation in adult gut microbiota. A) Immobilized *L. plantarum* NZ3400 (four g of
804 colonized beads containing $5*10^9$ CFU *L. plantarum*/g) was added to the gut
805 microbiota (●) and B) at 10^9 CFU/ml effluent in planktonic state (■). Dashed line
806 indicates the theoretical wash-out of the system. Cell count is depicted on the y-axis
807 and days of cultivation in the microbiota on the x-axis, whereas 0 corresponds to the
808 day of *L. plantarum* NZ3400 supplementation.

Figure 3

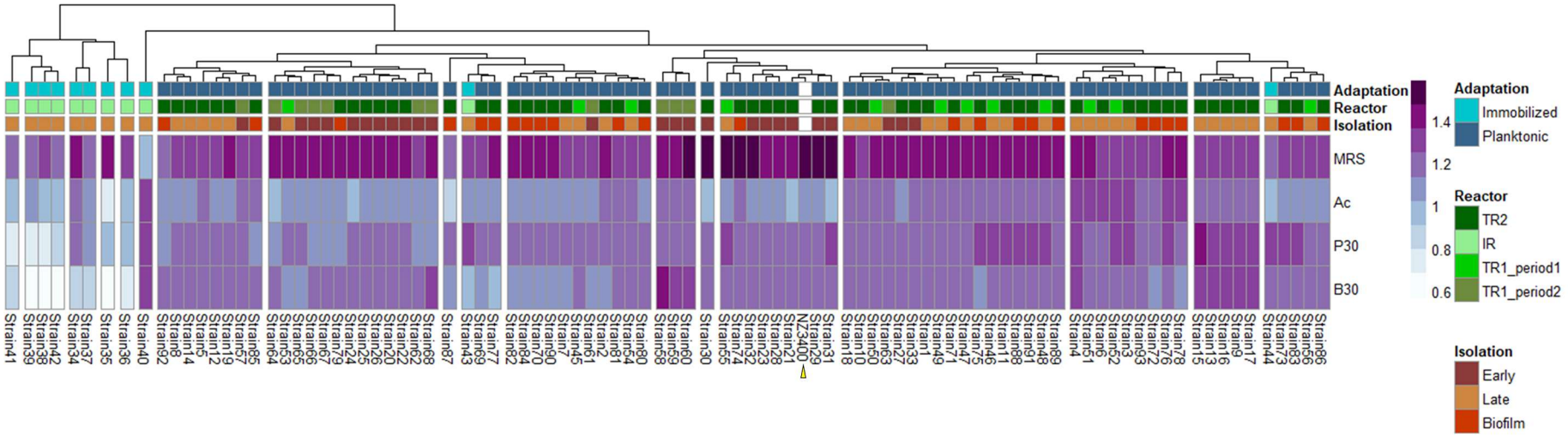
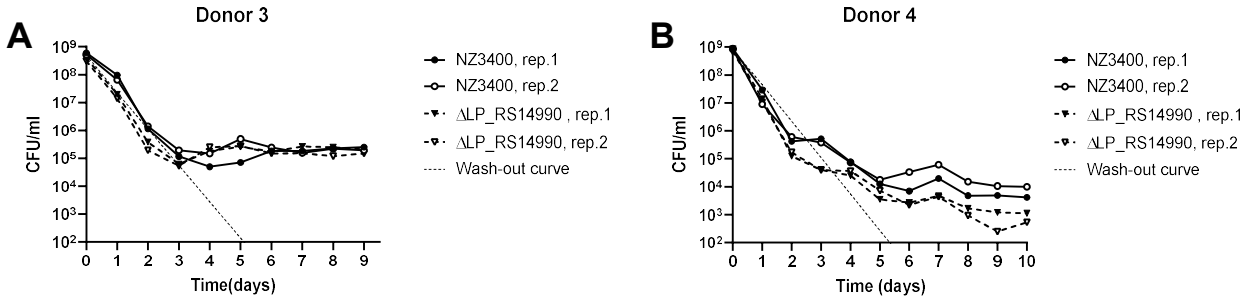


Figure 3: Heatmap visualization of growth behavior of potential mutant *L. plantarum* strains versus their reference strain in modified MRS medium (single combination of MRS with acetate (50 mM), butyrate (30 mM) or propionate (30 mM)). Image symbols: (i) Adaptation: indicates strain origin from immobilized or planktonic adaptation trials. (ii) Reactor: describes the general experiment type for long-term planktonic adaptation (TR2) and the repetition of planktonic adaptation in TR1 (period1) and TR1 (period2). (iii) Isolation: indicates biofilm / time point of isolation (early or late) of a strain during the adaptation cycles. Heatmap colors stand for measured ΔOD_{600nm} values over 24h. NZ3400 represents growth of *L. plantarum* NZ3400. Ac: acetate , P: propionate, B: butyrate.

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821 Figure 4



822

823 **Figure 4:** Colonization capacity of ΔLP_RS14990 compared to the reference strain
824 NZ3400. *L. plantarum* strains were added to a level of 10^9 CFU/ml reactor effluent.
825 A) *L. plantarum* ΔLP_RS14990 and NZ3400 were added into each of the two reactors
826 (rep.1, rep.2) in donor 3 gut microbiota. B) *L. plantarum* ΔLP_RS14990 and NZ3400
827 were added into each of the two reactors (rep.1, rep.2) in donor 4 gut microbiota.
828 Dotted lines indicate the theoretical wash-out curve of the system.