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 donor's 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.

42 **Importance**

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

56
57

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 like 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).

82 Continuous fermentation models are best suited for *in vitro* cultivation of gut microbiota in
83 conditions akin to the gut (19, 20). Different PolyFermS models were successfully
84 developed for cultivating colonic microbiota of humans of different ages and conditions,
85 swine, murine, and chicken cecum microbiota (21-25). The continuous PolyFermS model
86 allows testing several treatments in parallel in second-stage treatment reactors (TRs)
87 seeded with the same gut microbiota produced in the inoculum reactor (IR) containing
88 immobilized microbiota (21). Gut microbiota immobilization in polysaccharide gel beads
89 leads to the maintenance of high cell density, long-term stability due to prevention of cell
90 washout, and diversity of the simulated gut microbiota (23, 26, 27). It moreover creates a
91 sessile bacterial fraction on the gel beads and a planktonic fraction resulting from the
92 growth and release of sessile bacteria and further growth of planktonic cells in the bulk
93 medium (27, 28). This mimics the gastrointestinal environment consisting of free and
94 biofilm or mucus associated bacteria (29, 30). The PolyFermS colonic fermentation model
95 enables operation up to several months in a highly controllable environment with multiple
96 parameters to operate on, which is needed for evolutionary adaptation (26). We therefore
97 hypothesized that the PolyFermS model can provide a long-term stable gut microbiota
98 akin to the human adult colon that allows for adaptive evolution of an exogenous single
99 strain.
100 In this study, we investigated the PolyFermS fermentation model as a novel tool for strain
101 improvement via adaptive evolutionary engineering, using *L. plantarum* as model strain.
102 *L. plantarum* originates from fermented foods (31, 32) and is detected at low levels in
103 approximately half of healthy human gut microbiota (33). *L. plantarum* WCFS1 is a well-
104 characterized model strain for transient probiotic lactobacilli (34, 35). A WCFS1 derivative
105 harboring a chloramphenicol (CM) resistance gene for tracking, was cultivated in

106 PolyFermS reactors inoculated with immobilized adult fecal microbiota for at least
107 100 generations. Engineered strains were phenotypically and genotypically characterized
108 and tested for improved colonization in the PolyFermS model.
109

110 **Material and Methods**

111 **Bacterial strains and growth conditions**

112 Bacterial strains used during this study are listed in Supplementary Table S1.
113 *L. plantarum* NZ3400 (36) was used as reference strain. NZ3400 is a derivative of
114 WCFS1, harboring a CM resistance cassette (P_{32}-*cat*) in a neutral locus on the
115 chromosome. *L .plantarum* was grown in De Man, Rogosa and Sharpe (MRS, Labo-Life
116 Sàrl, Pully, Switzerland) broth at 37°C, overnight. *L. plantarum* viable cells were
117 enumerated by plating on MRS agar supplemented with CM (10 µg/ml), aerobic
118 incubation at 37°C, overnight.
119

120 **Immobilization of adult fecal microbiota**

121 Fecal samples were obtained from four healthy adult individuals (27-31 years old) who did
122 not take antibiotics and probiotics for at least three months and did not show detectable
123 microbial growth on MRS + CM plates to avoid interference with *L. plantarum* NZ3400
124 recovery. Out of 15 tested fecal samples, four samples that did not show microbial growth
125 on MRS+CM and were chosen for fermentation (donor 1-4). The Ethics Committee of ETH
126 Zürich exempted this study from review because the sample collection procedure has not
127 been performed under conditions of intervention. Informed written consent was obtained
128 from fecal donors. Fecal samples were immediately transferred to an anaerobic chamber
129 within 2 h after defecation and suspended at 20% (w/v) in reduced peptone water (0.1%,

130 pH 7; Thermo Fisher Diagnostics AG, Pratteln, Switzerland). Immobilization of 10 ml fecal
131 slurry in polymer gel beads (gellan gum (2.5%, w/v), xanthan (0.25%, w/v) and sodium
132 citrate (0.2%, w/v)) was performed as previously described (21, 37). The inoculum
133 bioreactor (IR) (Sixfors, Infors, Bottmingen, Switzerland) was filled with 140 ml of vitamin
134 supplemented MacFarlane medium (Supplementary Data S1), formulated to mimic the
135 chyme entering the colon (20, 38), and inoculated with 60 ml of fecal gel beads. Beads
136 were colonized in two fed-batch fermentations carried out at 37°C, pH 5.8 by controlled
137 addition of NaOH (2.5 M), stirring at 180 rpm, and replacing 100 ml of spent medium with
138 fresh medium after 16 h (21). Anaerobiosis was set by purging the bioreactor headspace
139 with CO_2 and monitored by redox potential probes.
140

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

143 Continuous proximal colonic *in vitro* fermentations with immobilized human adult gut
144 microbiota were performed in bioreactors as reported previously (26, 39). All
145 fermentations were operated to mimic the adult proximal colon as described above for
146 batch fermentation. Fresh medium, was continuously added to the IR (25 ml/h) and
147 fermented medium was removed to maintain a working volume of 200 ml. Because short-
148 chain fatty acids (SCFAs) are the main fermentation end products of the gut microbiota,
149 their stable production is a convenient, easily measurable marker for stability of
150 continuous intestinal fermentation models (21, 40). The IR was run in continuous mode
151 for at least 10 days to reach metabolic stability indicated by lower than 10% day-to-day
152 variation (25, 40) in SCFAs concentration, before connecting second-stage TRs. TRs
153 were inoculated by the IR at 1.25 ml/h and fed at 23.75 ml/h. TRs were operated

154 continuously for four days prior to *L. plantarum* supplementation in order to establish a gut
155 microbiota activity akin to the IR. Addition of planktonic or immobilized *L. plantarum* was
156 tested in IR and TRs with different donor microbiota (Fig. 1). In case of two successive
157 treatment periods in the same reactor, the reactor was disconnected after the first
158 treatment period, sterilized, re-connected and stabilized for four days before starting a
159 second treatment.
160 Reactor effluent samples were taken daily to monitor the fermentation process,
161 centrifuged for 10 min at 14 000 g, 4°C and stored at -20°C. Pellets were used for DNA
162 extraction and supernatants for metabolite analysis.
163
164 **Adaptive evolutionary engineering of *L. plantarum***
165 *Adaptive evolution using immobilized L. plantarum NZ3400*
166 Adaptive evolution of immobilized *L. plantarum* NZ3400 was tested in a continuously run
167 single-stage IR (IR1) inoculated with donor 1 fecal beads (Fig. 1, A). A *L. plantarum*
168 overnight culture was harvested at 4°C, 4000 g for 10 min and washed twice in
169 Phosphate-Buffered Saline (PBS), pH 6.2. Immobilization of *L. plantarum* was done as
170 described for fecal samples under aerobic conditions. Beads were colonized during two
171 pH-controlled batch cultures at 37°C for 16 h with stirring at 150 rpm. Colonized beads
172 were washed in PBS, supplemented with cryoprotective buffer (41) and stored at - 80°C.
173 Before use, *L. plantarum* beads were reactivated during two batch cultures as described
174 for colonization and washed twice in PBS. *L. plantarum* viable cell counts were determined
175 by crushing 1 g of beads in PBS with a spatula and plating serial dilutions. Four g of beads
176 ($5*10^9$ colony forming units (CFU) *L. plantarum*/g) were added to the single-stage IR1 and
177 cultivated for 53 days.

178 *Adaptive evolution using planktonic L. plantarum NZ3400**

179 Due to microbial growth on MRS+CM plates of donor 1 fecal sample at this time, a new
180 donor was chosen for the planktonic adaptation. To prevent carry-over of possible mutants
181 present in the initial NZ3400 stock culture, a new stock from a single NZ3400 colony
182 isolate, designated NZ3400*, was produced. NZ3400* was subjected to PacBio
183 sequencing and used for all subsequent planktonic supplementation trials. NZ3400 and
184 NZ3400* differed in eight SNPs but none thereof in SNP affected genes of recovered
185 *L. plantarum*. Adaptation of planktonic *L. plantarum* NZ3400* was tested in TRs
186 continuously inoculated by the effluent from IR2 containing beads with immobilized fecal
187 microbiota of donor 2 (Fig. 1, B). Because the PolyFermS model was built with six TRs,
188 two consecutive treatment periods were performed to test all treatments. *L. plantarum*
189 strains were grown overnight, harvested, washed twice in PBS, re-suspended in
190 MacFarlane medium and added to the TRs to a final level of 10^9 CFU *L. plantarum*/ml
191 effluent. Long-term planktonic adaptation was tested in TR2 operated for 72 days and
192 repeated in TR1 (period 1) and TR1 (period 2) for 44 and 23 days, respectively (Fig. 1, B).
193 Furthermore, the strain IA10, recovered from the immobilized adaptation, was added in
194 planktonic state to TR3 (period 1) and TR3 (period 2) containing donor 2 gut microbiota
195 to investigate effects of adaptations that occurred during the immobilized adaptation
196 (Fig. 1, B).

197

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

199 Reactors were sampled daily for analysis of the SCFAs acetate, butyrate and propionate,
200 branched-chain fatty acids isobutyrate, isovalerate and valerate, and intermediate

201 metabolites lactate and formate (42). Concentrations were determined by high-
202 performance liquid chromatography (HPLC) as described previously (23).
203

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

205 Genomic DNA of fecal and effluent samples was extracted using the FastDNA® SPIN Kit
206 for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions.
207 The V4 region of the 16S rRNA gene was amplified with the primers 806R
208 (5′- GGACTACHVGGGTWTCTAAT- 3′) and 515F (5′- GTGCCAGCMGCCGCGGTAA-
209 3′). Amplicons were barcoded PCR-based. Library preparation and sequencing (Illumina,
210 CA, USA) using an Illumina MiSeq flow cell with V2 reagent kit for 2 × 250-bp paired-end
211 Nextera chemistry supplemented with 10% of PhiX was performed in collaboration with
212 the Genetic Diversity Center (GDC, ETH Zürich, Switzerland).
213 Raw data obtained from 16S rRNA sequencing were processed using Cutadapt (43) and
214 DADA2 pipeline (44) to obtain amplicon sequence variants. Taxonomy was assigned
215 using SILVA database (v.132) (45) (full method in Supplementary Data S1).
216

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

218 *L. plantarum* colonization was determined by plating on MRS + CM agar. Combination of
219 the MRS selectivity for lactobacilli and enterococci together with aerobe incubation and
220 presence of antibiotics allowed growth repression of all other bacteria. Data were
221 compared to the theoretical washout curve determined for absence of growth, from the
222 following equation: $c_t=c_0*e^{(t/RT)}$ (39), where c_0 and c_t represent cell concentration at time
223 point 0 and t, respectively, and RT corresponds to the retention time. Colonies were

224 randomly picked, incubated in MRS + CM overnight, mixed 1:1 with 60% (v/v) glycerol
225 (Sigma-Aldrich Chemie GmbHm Buchs, Switzerland) and stored at -80°C.
226 Natural biofilm formed in TR2, used for long-term planktonic adaptation, and the repetition
227 experiment in TR1 (period 1). To recover *L. plantarum* from biofilms, the vessels were
228 emptied and washed twice with PBS. Remaining biofilm was removed and homogenized
229 with glass beads (5 mm, VWR International AG, Dietikon, Switzerland) in dilution solution
230 containing 0.85% NaCl and 0.1% peptone from casein (w/v, VWR International AG,
231 Dietikon, Switzerland). Dilutions were plated with subsequent strain recovery and storage
232 performed as described above.
233
234 **Phenotypic characterization of recovered *L. plantarum* strains**
235 Growth behavior of recovered *L. plantarum* strains was analyzed in MRS supplemented
236 with each of the main SCFAs of the human gut microbiota, acetate (50, 75, 100 mM),
237 butyrate (15, 30, and 45 mM) and propionate (15, 30, and 45 mM) in similar
238 concentrations as measured during colonic fermentations (see Fig. S2 in the
239 supplemental material). The abiotic gut fermentation environment was simulated in
240 Effluent-MacFarlane-Sugar (EMS) medium consisting of filter-sterilized PolyFermS
241 effluent, MacFarlane medium in a ratio 9:1 and 0.75% (w/v) glucose (see Fig. S1 in the
242 supplemental material). Glucose was added since *L. plantarum* was unable to grow in
243 MacFarlane medium. For comparison of the effect on adaption in immobilized and long-
244 term planktonic adaptation trials performed in TR2 (Fig. 1, B), recovered strains were
245 divided into four groups based on their origin of isolation: (1) 11 strains from the effluent
246 at the late stage of immobilized adaptation after 53 days, (2) 14 strains from the effluent
247 during day 7 and day 23 (early planktonic adaptation), (3) 19 strains from the effluent

248 during day 60 and day 72 (late planktonic adaptation) and (4) 25 strains from the biofilm
249 of planktonic adaptation after 72 days. Strains were isolated at an early stage after seven
250 (stable *L. plantarum* colonization) and 23 days and late stage of 60 and 72 days to
251 increase the chance to observe adaptation. Biofilm was sampled on the last day of
252 fermentation because the reactor had to be emptied for biofilm sampling. Growth analysis
253 was done in 96-well tissue culture test plates (Bioswisstec AG, Schaffhausen,
254 Switzerland). Wells were filled with 200 μl of medium and inoculated with the potentially
255 adapted *L. plantarum* at 37°C. Growth was monitored by optical density (OD)
256 measurement at 600 nm in a plate reader after 24 h (PowerWaveTMXS, Bio-Tek
257 Instrument Inc., Winooski, VT, US) in biological triplicates.
258 Phenotype stability was assessed by repeated daily culturing of strains in MRS for
259 28 days, approximately 190 generations, as presented above. Stability was measured
260 after 1, 7, 14, 21 and 28 days, which corresponds to the time of transcriptome
261 homogenization among *L. plantarum* strains isolated from different habitats (46).
262

263 **Complete genome sequencing and data analysis**

264 *L. plantarum* genomic DNA was isolated via lysozyme-based cell lysis (47) followed by
265 purification using the Wizard® Genomic DNA Purification Kit according to the
266 manufacturer's instructions (Promega, Dübendorf, Switzerland). The genome of the
267 reference strain NZ3400* was sequenced at the Functional Genomics Center Zurich
268 (Zürich, Switzerland) on PacBio RS II (Pacific Biosciences, Menlo Park, CA, USA) using
269 one SMRT cell. Reads were assembled using HGAP assembly as described previously
270 (48). All other *L. plantarum* strains were sequenced using Illumina Miniseq (Illumina, CA,
271 US) with 250 bp paired-end reads at the Institute for Food Safety and Hygiene, University

272 of Zurich (48). Reads were merged and mapped to the reference genome *L. plantarum*
273 NZ3400* using CLC Genomic Workbench 11.0 (Qiagen CLC bio, Aarhus, Denmark) using
274 default parameters. Single nucleotide polymorphisms (SNPs) were extracted using
275 Parsnp (49). SNPs were confirmed by Sanger Sequencing (Eurofins GATC, Biotech
276 GmbH, Konstanz, Germany). SNP related changes in amino acid sequence were
277 determined in CLC Genomic Workbench.
278
279 **Competition experiments in the human gut microbiota**
280 Competition experiments between NZ3400* and the mutant strains *L. plantarum* IA01,
281 PA1.2_01 and PA2_06 were performed to determine the effect of the mutations on gut
282 microbiota colonization (Fig. 1, B). NZ3400* was paired with each of the three mutant
283 strains in a 1:1 ratio to reach 10^9 CFU/ml reactor effluent to the gut microbiota and
284 cultivated for ten days. Ten days was sufficient to obtain stable *L. plantarum* colonization
285 for at least four days but also as short as possible to minimize the chance of proliferation
286 of new mutants. Relative abundance of the strains in the complex community was
287 determined by measuring allele frequency of the genes LP_RS14990 and LP_RS15205,
288 respectively, via Pyrosequencing (full method in Supplementary Data S1).
289
290 **Stability of mutations under standard cultivation conditions**
291 Stability of the mutations in *L. plantarum* IA01 and PA2_06 was investigated during
292 repeated daily cultures in MRS for 12 days, approximately 81 generations, performed in
293 triplicates. Cultivation of NZ3400* served as control. Allele frequency was determined via
294 Pyrosequencing as described above.
295

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

297 Six mutants carrying the mutation in LP_RS14990 were recovered from independent
298 adaptation experiments. To investigate the involvement of this gene in gut microbiota
299 colonization, a *L. plantarum* ∆LP_RS14990 knockout was constructed by double-
300 crossover gene replacement in *L. plantarum* NZ3400* (full method in Supplementary
301 Data S1). The knockout strain was subsequently added to the gut microbiota and
302 colonization levels were compared to the reference strain NZ3400* (Fig. 1, B).

303

304 **In silico analysis of LP_RS15205 in *L. plantarum***

305 LP_RS15205 was affected by identical mutation in four strains recovered from two
306 independent adaptation experiments. Since this mutation was also found in the
307 background gut microbiota, in silico analysis of this locus was performed. Complete
308 genome sequences of Firmicutes (n = 557), Bacteroidetes (n = 218), Actinomycetes
309 (n = 457) and γ-Proteobacteria (n = 638) from the NCBI genome database were
310 downloaded in May 2020. The amino acid sequence of LP_RS15205 was blasted against
311 these genomes using standard settings and significant hits were aligned using MUSCLE
312 (50).

313

314 **Data analysis**

315 Statistics for growth experiments were calculated in R (version 3.6.2) using one-sample t-
316 test for comparison to *L. plantarum* NZ3400* and paired sample t-test for comparison
317 within recovered *L. plantarum* groups. Values represent mean values ± standard
318 deviation. The heatmap was generated using the R pheatmap package and Euclidean
319 distance measure. Allele frequency determination by Pyrosequencing was calculated

320 based on three extracted DNA samples of the same time point. Graphs were created
321 using GraphPad Prism® version 8 (GraphPad Software Inc., San Diego, CA, USA).
322

323 **Results**

324 **PolyFermS model allows stable cultivation of adult gut microbiota**

325 The PolyFermS model operated to mimic the adult proximal colon was used to provide a
326 gut microbiota environment for evolutionary adaptation of *L. plantarum* NZ3400.
327 Adaptation of immobilized *L. plantarum* was performed in IR1 and adaptation of planktonic
328 *L. plantarum* was tested in TRs continuously inoculated with IR2 microbiota (Fig. 1).
329 Metabolic stability of IR1 (see Fig. S2, A in the supplemental material) was achieved after
330 one week with main SCFAs acetate, propionate and butyrate at 73 ± 7, 21 ± 4 and
331 19 ± 3 mM over two month fermentation, respectively. IR1 microbiota was dominated by
332 Firmicutes and Bacteroidetes accounting for 44 ± 5 and 47 ± 2% of the total population
333 during days 44-46, respectively, 48 ± 7 and 41 ± 2% during days 57-59, respectively, and
334 52 ± 4 and 36 ± 4% during days 61-65, respectively (see Fig. S3, A in the supplemental
335 material). This stability was also observed on family level (see Fig. S3, B in the
336 supplemental material). IR2 microbiota reached metabolic stability (see Fig. S2, B in the
337 supplemental material) after one week with the main SCFAs acetate, propionate and
338 butyrate at 84 ± 5, 32 ± 8 and 23 ± 4 mM, respectively, during two months of culture. IR2
339 had a different microbiota composition from IR1 (see Fig. S3, C&D in the supplemental
340 material). Bacteroidetes dominated the gut microbiota compared to Firmicutes with
341 63 ± 1% and 36 ± 1% during days 24-26, respectively, 68 ± 4 and 30 ± 4% during days
342 41-43, respectively, and 66 ± 1 and 30 ± 1% during days 89-91, respectively (see
343 Fig. S3, C in the supplemental material). Stability was maintained up to 90 days on family

344 level (see Fig. S3, D in the supplemental material). Microbiota composition of the IR was
345 successfully transferred to and maintained in the TRs (see Fig. S3, D in the supplemental
346 material). Both, metabolic activity (see Fig. S2, C-E in the supplemental material) and
347 composition were reproduced in the TRs.
348

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

350 To investigate the potential of the continuous *in vitro* gut fermentation model PolyFermS
351 for evolutionary engineering, immobilized *L. plantarum* NZ3400 was added to the
352 stabilized microbiota in IR1 at an initial concentration of 10^8 CFU/ml effluent. NZ3400
353 decreased at the rate of the theoretical wash-out during the first four days (Fig. 2, A),
354 followed by colonization between 10^2 CFU/ml and 10^4 CFU/ml during the 50-days
355 fermentation. *L. plantarum* was able to maintain a self-sustaining population, an
356 observation which will be referred to as colonization.
357 The PolyFermS model was further evaluated for long-term adaptation of planktonic
358 *L. plantarum* NZ3400* in TR2, fed by donor 2 gut microbiota. Cell counts decreased from
359 10^9 to 10^5 CFU/ml during the first four days after spiking (Fig. 2, B), at the rate of the wash-
360 out. Thereafter, colonization steadily increased to 10^6 CFU/ml during 72 days. Repeated
361 supplementation of *L. plantarum* with 10^6 and 10^9 CFU/ml in TR1 (period1) and TR1
362 (period2) resulted in stable colonization at different levels of 10^6 and $3*10^4$ CFU/ml,
363 respectively. Therefore, *L. plantarum* colonization at a donor specific level was
364 demonstrated for more than 50 days and 150 generations.
365

366 **Recovered *L. plantarum* strains are phenotypically adapted to the gut microbiota**
367 **environment**

368 To test for *L. plantarum* adaptation during gut microbiota cultivation, recovered strains
369 were grown in SCFA concentrations comparable to those in the gut fermenter. Average
370 growth of strains from immobilized adaptation measured by OD was impaired in MRS and
371 MRS supplemented with 30 mM propionate or butyrate compared to the reference strain
372 NZ3400* (Table 1). Strains from early stage of long-term planktonic adaptation behaved
373 similarly to the reference NZ3400* in all tested media. However, strains from late
374 planktonic adaptation and biofilm grew better in MRS plus acetate (+0.11 and +0.07,
375 respectively), less in standard MRS (-0.14 and -0.11, respectively), and similar in MRS
376 plus propionate or butyrate (Table 1) compared to NZ3400*. Moreover, strains from
377 biofilm and late planktonic adaptation grew significantly better in the reactor-effluent-
378 mimicking EMS medium compared to strains from early adaptation (Table 1).

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

390 Phenotypes of these adaptations were stable for at least 190 generations (see Fig. S5 in
391 the supplemental material). This strongly suggests that observed phenotypes are caused
392 by mutations rather than physiological variations.
393

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

395 Stable altered phenotypes of recovered *L. plantarum* strains strongly suggest that these
396 strains harbor mutations. Therefore, whole-genome sequencing of 45 strains randomly
397 selected from adaptations experiments was performed. Comparison to the reference
398 genome NZ3400* revealed 15 strains without any genotypic differences. Out of 18 SNPs
399 confirmed by Sanger sequencing, two were detected in non-coding regions. The
400 remaining 16 SNPs were found in genes involved in signaling, metabolism, transport, and
401 cell surface (Table 2).
402 The 11 mutated of 12 sequenced strains from the immobilized adaptation belonged to four
403 different genotypes. Among these 11 strains, a mutation in the cell surface protein gene
404 encoded by LP_RS14255 was found seven times. Further, a mutation in LP_RS14990,
405 encoding a histidine kinase domain, was found thrice (Table 2). Eight of 15 *L. plantarum*
406 strains that were recovered at late stage of the long-term planktonic adaptation were
407 mutated, resulting in five different genotypes (Table 2). Strikingly, a SNP in LP_RS14990
408 occurred independently during planktonic and immobilized adaptation. Moreover, the SNP
409 in LP_RS15205 and in the intergenic region between LP_RS05100 and LP_RS05095
410 were found in strains isolated from two different reactors.
411 Immobilized adaptation resulted in a bigger fraction of mutated strains but consisted
412 predominantly of two isogenic lineages. Planktonic adaptation resulted in less frequent
413 mutagenesis, yet higher mutant diversity. This suggests a difference in adaptation

414 pressure, as already observed for the phenotypic screening. Recovery of some identical
415 mutants from different adaptation experiments suggests that some of the observed
416 mutations are involved in adaptation to the gut microbiota.
417

418 **Mutations in LP_RS14990 and LP_RS15205 are beneficial for *in vitro* gut microbiota**
419 **colonization**

420 The mutation in the histidine kinase protein gene LP_RS14990 and the ROK protein gene
421 LP_RS15205 occurred independently more than once in adaptation experiments. We
422 therefore tested the fitness of each of the mutants *L. plantarum* PA2_06 (C837A in
423 LP_RS15205), IA01 (C979T in LP_RS14990) or PA1.2_01 (C979T in LP_RS14990 and
424 C837A in LP_RS15205) in competition experiments with the reference strain NZ3400*.
425 Ten days of cultivation resulted in an increased abundance of all tested mutants in the gut
426 microbiota when compared to NZ3400* (Table 3).
427 Pyrosequencing indicated that donor 2 gut microbiota had a *L. plantarum* background.
428 Remarkably, the Pyrogram of this background at the position LP_RS15205 C837A was
429 identical to the Pyrogram of a sample containing NZ3400* and PA2_06. This shows that
430 the nucleotide variation of both, NZ3400* and PA2_06, also occurs naturally (see Fig. S6
431 in the supplemental material).
432

433 **Mutation C979T in LP_RS14990 is stable under standard culturing conditions**

434 After observing increased fitness of mutants compared to the reference strain, it was
435 investigated whether the mutations of *L. plantarum* IA01 in LP_RS14990 and *L. plantarum*
436 PA2_06 in LP_RS15205 are stable during daily repeated batch cultures without the
437 adaptation pressure of the gut microbiota. Stability of the LP_RS14990 mutation in the

438 IA01 strain was observed during 12 batch cultures. However, the mutation C837A in strain
439 PA2_06 was not stable since the reference strain nucleotide reoccurred at 3.5 ± 0.15%
440 after 12 days (see Table S3 in the supplemental material). Investigation of NZ3400* in
441 repeated MRS batch cultures revealed no occurrence of the SNPs of the mutants.
442
443 **LP_RS14990 gene replacement in *L. plantarum* NZ3400* results in delay of gut**
444 **microbiota colonization**
445 To investigate the role of the LP_RS14990 gene in gut microbiota colonization, a
446 ∆LP_RS14990 gene replacement strain was constructed and its colonization compared
447 to NZ3400*. NZ3400* started to colonize the gut microbiota of donor 3 on day one and
448 donor 4 at day three since levels were above the wash-out curve (Fig. 4). Levels of
449 ∆LP_RS14990 in both gut microbiota decreased more rapidly than the wash-out curve
450 until day three, suggesting cell death (Fig. 4). Strain ∆LP_RS14990 started to colonize the
451 gut microbiota of both donors only after four days, later than the reference strain.
452
453 **In silico analysis of LP_RS15205 in *L. plantarum***
454 Occurrence of the SNP C837A (L279F) in LP_RS15205 in *L. plantarum* strains from
455 different reactors and in the background of the gut microbiota suggests an important
456 function of this SNP in survival of *L. plantarum* in the gut microbiota. In silico analysis of
457 LP_RS15205 revealed that C837A lies in a conserved domain with an ANLxGA motive
458 that is found in Firmicutes and γ-Proteobacteria (data not shown). The function of this
459 domain is unknown, but its conservation throughout two phyla strongly suggests that it
460 might be important for protein function and the mutation ANLxGA to ANFxGA might have
461 an impact on protein functionality.

462 **Discussion**

463 Strain improvement is of major importance for food industries and probiotic cultures.
464 Classical evolutionary adaptation approaches focus on specific strain characteristics in
465 abiotic environments like improved performance of starter cultures or lactic acid bacteria
466 (51, 52). However, for probiotics, improvement of gut colonization needs to consider
467 abiotic and biotic factors. Here we examined the suitability of the *in vitro* gut fermentation
468 model PolyFermS to provide a gut microbiota environment for evolutionary engineering of
469 *L. plantarum* NZ3400 towards a human adult gut microbiota. Immobilization of the donor
470 gut microbiota and cultivation in the PolyFermS allowed reproduction of distinct gut
471 microbiota representative for human adults (53, 54). The achieved long-term metabolic
472 and compositional stability allowed the generation of adapted mutants and the use of
473 several donors increased the external validity of results on observed adaptations.
474 *L. plantarum* supplementation method and donor microbiota seem to influence phenotypic
475 and genotypic adaptations.

476 Even though observed phenotypic differences of recovered *L. plantarum* were small,
477 *L. plantarum* strains recovered from the immobilized adaptation showed very limited
478 adaptation in contrast to recovered strains from planktonic adaptation trials, although
479 abiotic conditions were highly similar. It thus is assumed that differences in adaptation
480 patterns are caused by the supplementation method. Immobilization protects against
481 stress and entraps cells physically in the system, leading to decreased adaptation
482 pressure (55-57), which is in accordance with our phenotypic screening where strains
483 from immobilized adaptation showed less adaptation. Polymer beads create mucosal-like
484 attachment sites (58) and growth in beads results in a gradual release of sessile cells from
485 the surface that then grow as planktonic cells in the bulk medium (37). The result is a

486 mixed population consisting of cell lineages undergoing varying number of growth cycles
487 in the planktonic state. This might explain the more diverse phenotypic adaptation pattern
488 among strains from the immobilized adaptation. It was further shown that immobilized
489 cells are genetically more stable than planktonic cells (59-62), which was observed in our
490 study where recovered strains from immobilized adaptation mainly consisted of two
491 isogenic lineages.

492 Two observations suggest that the observed genotypic changes in isolated strains were
493 involved in adaptive evolution. The first is the known function of several of the genes
494 affected by SNPs. Activated genes of *L. plantarum* in the murine digestive tract were
495 involved in carbohydrate transport, metabolism and cell surface, and sugar-related
496 functions, molecule biosynthesis and stress response (63, 64). Further, exposure of
497 *L. plantarum* to the murine intestinal tract predominantly resulted in mutations in genes
498 encoding cell wall-associated proteins (16). The SNPs identified in our study belong to the
499 categories mentioned above. Remarkably, the latter study identified a SNP in a
500 glycerophosphodiester phosphodiesterase encoding gene, a gene also affected by a SNP
501 in a recovered strain in our study. In summary, the function of mutated genes are in
502 agreement with previously observed responses of *L. plantarum* to the *in vivo* intestinal
503 environment. This shows similar selection pressures in the PolyFermS system compared
504 to *in vivo* settings.

505 The second observation indicating adaptive evolution in our experiment is that three
506 mutations were found independently in multiple experiments. Since mutations occur
507 continuously, -80°C stock cultures are rarely isogenic and the mutants found in our study
508 could have been already present as subculture in the NZ3400 stock (16). To counteract
509 this bias, two different stock cultures, NZ3400 and NZ3400*, were used for the

510 immobilized and planktonic adaptation, respectively. Occurrence of identical mutants
511 recovered from immobilized and planktonic adaptation with the SNP in LP_RS14990
512 seems therefore unlikely to originate from a stock subculture, as confirmed by Sanger
513 sequencing (data not shown). Furthermore, the mutation was not detected by
514 Pyrosequencing of NZ3400* before inoculation (data not shown), minimizing a role for
515 subcultures in our experiments. Moreover, the increased fitness of mutants shows the
516 beneficial effect of detected mutations. This strongly supports that observed mutations are
517 caused by adaptive evolution and demonstrates the suitability of the PolyFermS model to
518 select relevant mutations related to the human gut microbiota. The exact nature of the
519 evolutionary pressure in our system is not known and remains to be elucidated. It is likely
520 the result of a combination of factors such as the competition for nutrients, metabolic
521 cross-feeding, physiochemical factors, or presence of antimicrobial metabolites.
522 In conclusion, we demonstrated successful application of the continuous PolyFermS gut
523 fermentation model to provide a long-term stable gut microbiota to generate adapted
524 mutants to this environment. Immobilization of strains does not only allow adaptive
525 evolution of non-colonizers but also creates a culture consisting of sessile and planktonic
526 cells mimicking the human gastrointestinal tract. The conditions of the model can be easily
527 changed to other needs and selective pressures, including the source of microbiota and
528 abiotic conditions. This novel technology enables identification of genes involved in gut
529 microbiota colonization, persistence, and metabolism. The PolyFermS system could
530 further be designed and applied for different fermentations, to trace and identify
531 evolutionary and ecological processes between an exogenous single strain and a complex
532 ecosystem.
533

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540

541

542

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773

774 **Tables**

775 **Table 1: Growth of recovered *L. plantarum* strains in different media.** The values
776 reported are OD_{600nm} after 24 hours. Values obtained for NZ3400* represent
777 mean ± standard deviation of biological triplicates. All other values represent
778 mean ± standard deviation of all strains recovered from one adaption period, whereas
779 each recovered strain was measured in biological triplicates. Immobilized adaptation:
780 n= 11 strains, early planktonic adaptation: n= 14 strains, late planktonic adaptation:
781 n= 19 strains, biofilm of planktonic adaptation: n= 25 strains.

	MRS	MRS+acetate (50 mM)	MRS+propionate (30 mM)	MRS+butyrate (30 mM)	EMS
***L. plantarum* origin**					
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

782 [a,b,c]: significantly significantly different from each other ($p > 0.05$ in a paired sample t-test,
783 adjusted for unequal variance or normal distribution when needed).

784 [#]: significant differences between NZ3400* and *L. plantarum* groups (one-sample t-test, p
785 ≤ 0.05).

786 ND: not determined

787 EMS: Effluent-MacFarlane-Sugar medium

788

789 **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, period 2			TR2								TR1, period 1							
					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	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

790 a) Indicates SNP position in relation to the *L. plantarum* WCFS1 genome

791 b) Indicates amino acid change in the mutant compared to NZ3400* caused by the SNP. None: no change in amino acid, N.A. = amino

792 acid change not identifiable.

793 c) Indicates change of the amino acid charge compared to NZ3400* caused by the SNP. N: neutral, +: positive charge, -: negative

794 charge.

795 d) Amino acid polarity, P: polar, A: apolar.

796 e) Location of the SNP. Intergenic SPS are labeled with genomic (gen.) DNA.

797 * X indicates a SNP, ⦸ indicates loss of a mutation. ** Roman letters represent isogenic strains.

798

799

800

801

802 **Table 3: Increase in relative abundance (in %) of *L. plantarum* mutants during**
803 **10 days of competition against NZ3400* in *in vitro* human gut microbiota.**
804 Presented values show the increase of the ratio of *L. plantarum* mutant to the reference
805 strain NZ3400* after 10 days of cultivation compared to the time point of inoculation.
806 Values represent mean ± standard deviation of three DNA samples isolated at the
807 same time point from the same reactor.

		***L. plantarum* mutant strains**		
		IA01	PA2_06	PA1.2_01
Donor 2	Replicate 1	38±5		
	Replicate 2	25±1		
Donor 3	Replicate 1	66±1	26±3	11±2
	Replicate 2	51±1	31±3	39±2
Donor 4	Replicate 1		20±2	
	Replicate 2		6±2	

808

809

810 **Caption Figures**

811 **Fig. 1**: PolyFermS setup of immobilized and planktonic adaptation and further
812 experiments in *in vitro* human adult gut microbiota. A) Immobilized *L. plantarum*
813 NZ3400 was added to immobilized fecal gut microbiota in IR1 from donor 1 during a
814 single-stage fermentation. B) Immobilized fecal microbiota of donors 2-4 were
815 cultivated in the inoculum reactor (IR), which was used to inoculate second stage
816 reactors (TRs) that were supplemented with *L. plantarum* after stabilization. NZ3400:
817 reference strain; NZ3400*: new stock from single colony isolate of NZ3400; IA10, IA01,
818 PA1.2_01, PA2_06: recovered *L. plantarum* mutants; *L. plantarum* ∆LP_RS14990:
819 LP_RS14990 gene deletion strain. F_M: Inflow MacFarlane medium, F_O: Reactor outflow
820 S/W: Sampling/Waste.

821

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

829

830 **Fig. 3**: Heatmap visualization of growth behavior of potential mutant *L. plantarum*
831 strains versus their reference strain NZ3400* in modified MRS medium (single
832 combination of MRS with acetate (50 mM), butyrate (30 mM) or propionate (30 mM)).
833 Image symbols: (i) Adaptation: indicates strain origin from immobilized or planktonic
834 adaptation trials. (ii) Reactor: describes the general experiment type for long-term
835 planktonic adaptation (TR2) and the repetition of planktonic adaptation in TR1

836 (period 1) and TR1 (period 2). (iii) Isolation: indicates biofilm/time point of isolation
837 (early or late) of a strain during the adaptation cycles. Heatmap colors stand for
838 measured ΔOD_{600nm} values after 24h. The black triangle represents growth of
839 *L. plantarum* NZ3400*. Ac: acetate, P: propionate, B: butyrate.
840
841 **Fig. 4**: Colonization of ΔLP_RS14990 compared to the reference strain NZ3400*.
842 *L. plantarum* strains were added to a level of 10^9 CFU/ml reactor effluent.
843 A) *L. plantarum* ΔLP_RS14990 and NZ3400* were added into each of the two reactors
844 (rep.1, rep.2) in donor 3 gut microbiota. B) *L. plantarum* ΔLP_RS14990 and NZ3400*
845 were added into each of the two reactors (rep.1, rep.2) in donor 4 gut microbiota.
846 Dotted lines indicate the theoretical wash-out curve of the system. Cell count is
847 depicted on the y-axis and days of cultivation in the microbiota on the x-axis, whereas
848 0 corresponds to the day of *L. plantarum* supplementation. *: significantly different
849 colonization levels between NZ3400* and ΔLP_RS14990 ($p \leq 0.05$, paired sample t-
850 test).
851

852 **Caption Supplemental Figures**

853 **Fig. S1**: Comparison of metabolite concentrations in EMS medium and the reactor
854 effluent. Barplot representing metabolite concentrations (mM) of the EMS medium
855 consisting of reactor effluent, MacFarlane medium and glucose and the reactor effluent
856 without *L. plantarum* supplementation. Samples were analyzed in biological triplicates.

857 **Fig S2**: Concentrations (mM) of main SCFAs of PolyFermS effluent samples
858 inoculated with immobilized and planktonic *L. plantarum*: A) IR1 inoculated with
859 donor 1 immobilized fecal microbiota and immobilized *L. plantarum*; B) IR2 inoculated
860 with donor 2 immobilized fecal microbiota and connected treatment reactors (TRs)
861 C) TR1 (period 1), D) TR1 (period 2)and E) TR2 spiked with planktonic *L. plantarum*.

862 **Fig. S3**: Microbial composition in relative abundance obtained by 16S rRNA amplicon
863 sequencing. Composition at phylum (A,C) and family (B,D) level of *in vitro* proximal
864 colon microbiota of A,B) IR1 inoculated with donor 1 immobilized fecal microbiota and
865 immobilized *L. plantarum* and of C,D) IR2 inoculated with donor 2 immobilized fecal
866 microbiota and connected treatment reactors (TRs) TR1 (period 1), TR1 (period 2) and
867 TR2 spiked with planktonic *L. plantarum*. Values at family level of < 1% are
868 summarized in the "Others" group. X-axis labeling indicates the day during
869 fermentation relative to the start of the IR.

870 **Fig. S4**: Principal component analysis (PCA) of growth pattern of recovered
871 *L. plantarum* strains. *L. plantarum* strains were recovered from the immobilized and
872 planktonic adaptation cycle and grown in MRS and MRS supplemented with acetate
873 (50 mM), propionate (30 mM) and butyrate (30 mM). Reference indicates the reference
874 strain NZ3400*. Plot is based on the R function fviz_pca_ind.

875 **Fig. S5**: Growth of *L. plantarum* in MRS during four weeks daily serial cultivation.
876 *L. plantarum* was serially cultivated in triplicates in MRS medium for four weeks. Final

877 OD was measured in the first overnight culture (t0) and after one, two, three and four
878 weeks (t1-t4). ΔOD600 values were calculated by subtracting values obtained after
879 16 h of growth at 37°C by values obtained at the time point of inoculation. Values
880 represent mean ± standard deviation of triplicates. The reference strain (RT) and
881 selected recovered *L. plantarum* strains from immobilized and planktonic adaptation
882 are depicted on the x-axis.

883 **Fig. S6**: Pyrogram obtained by Pyrosequencing of the C837A SNP in LP_RS15205.
884 A) Pyrogram obtained from donor 2 gut microbiota prior to *L. plantarum*
885 supplementation. B) Pyrogram obtained from donor 3 gut microbiota supplemented
886 with *L. plantarum* PA2_06 and NZ3400* in a ratio 1:1. X-axis depicts the added
887 nucleotide over time and y-axis represents the light signal induced by incorporated
888 nucleotides. Allele frequency was measured for G and T since Pyrosequencing was
889 done on the complementary DNA strand.

890

891 **Caption Supplemental Tables**

892 **Table S1**: Strains and plasmids used in this study.

893 **Table S2**: Primers used in this study.

894 **Table S3**: SNP stability in *L. plantarum* IA10 and PA2_06 during 12 days of continuous
895 cultivation in MRS medium.

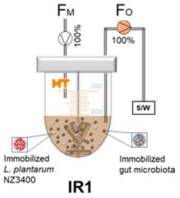
896

897 **Supplemental Material and Methods**

898 Supplementary Data S1

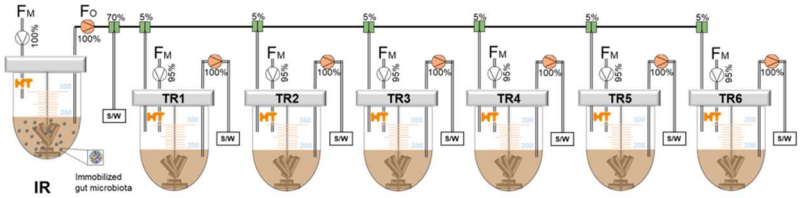
Figure 1

A



Donor 1
Female
30 years

B



			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 2

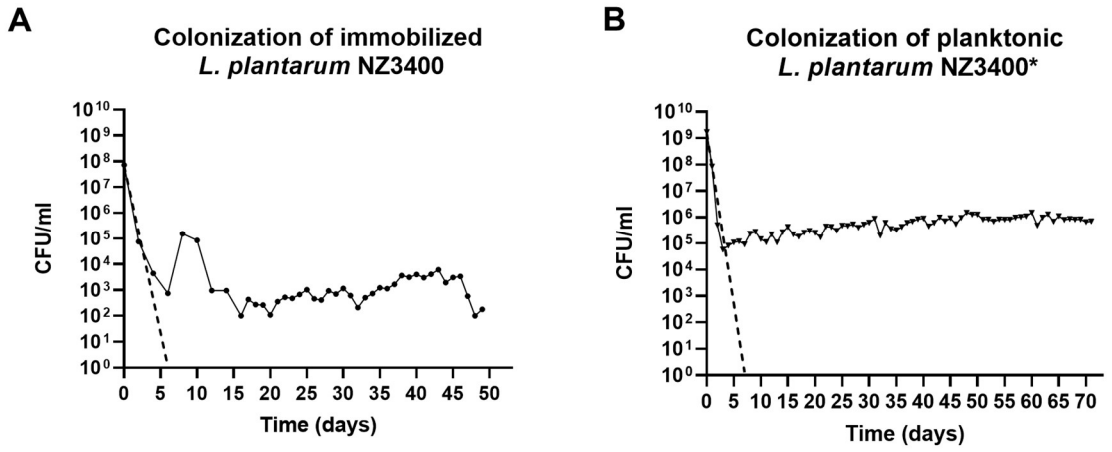


Figure 3

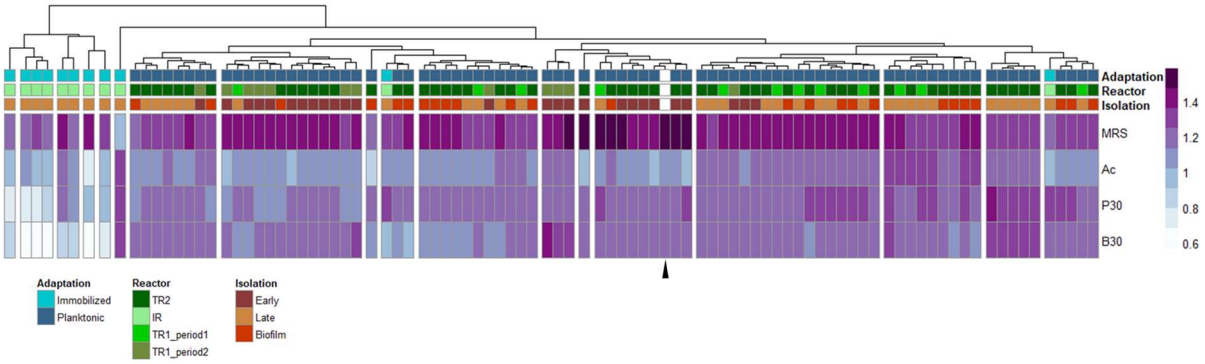
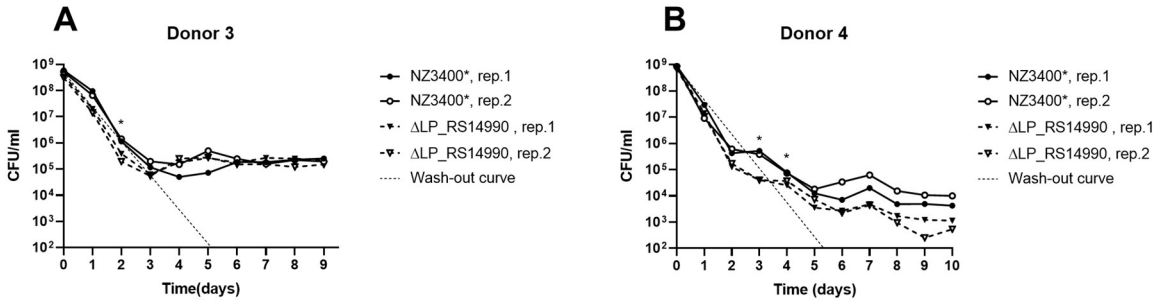



Figure 4