Bacterial adaptation to host diet is a key evolutionary force shaping host-microbe symbiosis

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Life on Earth was dominated by bacteria for billions of years[1]. About 600 million years ago, animal life emerged and micro-organisms played a crucial role in shaping animal development, physiology and evolution[2–4]. The two partners committed to a symbiotic relationship that persists in nearly all animals today. Such beneficial interactions are pervasive throughout nature and have been extensively characterized[5,6]. However, the ecological and evolutionary forces that drive the emergence and evolution of the symbiont benefits to their animal hosts remain largely elusive. Here we show that the host nutritional environment, instead of the host, is a predominant driving force in this evolutionary process and we identify a mechanism resulting from the bacterial adaptation to the diet, which confers improved functional benefit to the host. By applying experimental evolution to a model of host-bacteria beneficial symbiosis: *Drosophila melanogaster* associated with *Lactobacillus plantarum*, one of its growth promoting symbiotic bacteria[7,8], we found that the *de novo* mutations in the same acetate kinase (*ackA*) locus invariably emerge first, rapidly become fixed, and such evolution occurs with or without the host. Furthermore, we demonstrate that *ackA* mutations trigger the increased production of N-acetyl-glutamine, which is sufficient to confer improved host growth capabilities to the

evolved bacterial strains. Our study therefore identifies a specific mechanism by which a symbiotic bacterium increases its benefit to its animal host and reveals that adaptation to the host diet is a foremost step in the determination of the evolutionary course of symbiosis between an animal and its gut microbes.

Text

To study the ecological and evolutionary forces shaping animal-microbe symbioses, we experimentally tested microbial evolution using *Drosophila melanogaster* associated with one of its most abundant symbionts, *Lactobacillus plantarum*, a well-established model of host-bacteria symbiosis[9,10]. As growth promotion during undernutrition is one of the major advantages conferred by *L. plantarum* to its animal host[9,11], we asked if and how a bacterium can increase its potential to support animal growth while evolving with its host. To this end, we experimentally evolved NIZO2877 ($Lp^{NIZO2877}$), a strain of *L. plantarum* isolated from processed human food[12], which was previously shown to moderately promote growth both in *Drosophila* and mice[9,11]. We mono-associated germ-free (GF) *Drosophila* eggs with a fully sequenced clonal population of $Lp^{NIZO2877}$ on a low-nutritional diet and experimentally evolved the partners for 20 *Drosophila* generations (i.e 313 days, which correspond to a maximum of 2146 bacterial generations; see Methods and Extended Data Fig. 1,2). At each generation, we selected the first emerging pupae carrying a subpopulation of *L. plantarum* strains, and transferred them to a new sterile diet (Methods; Extended Data Fig. 1). The adults rapidly emerged from the pupae and deposited the new embryos and their associated *L. plantarum* strains that subsequently colonized and propagated in the new environment. We then isolated the $Lp^{NIZO2877}$-evolved strains associated with the adult flies eclosed from the transferred pupae, selected a representative set of isolates and measured individually their growth promoting capacity on an independent set of GF fly larvae. After only two fly generations (i.e. after a maximum of 124 bacterial generations, Fig. 1a,b), we identified a few evolved $Lp^{NIZO2877}$ strains that significantly improved larval growth and accelerated pupariation compared to the ancestor strain.

Specifically, the evolved strains exhibited the same effect as Lp^{WJL}, a potent *L. plantarum* growth promoting strain[13] (Fig. 1a,b). These results show that the evolution of $Lp^{NIZO2877}$ with its *Drosophila* host leads to the rapid improvement of *L. plantarum* animal growth promotion (Extended Data Fig. 3).

To identify the genetic changes underlying the rapid microbial adaptation responsible for the improved growth of the host, we sequenced the genomes of 11 evolved $Lp^{NIZO2877}$ strains (Extended Data Table 1, replicate 1) with increased host growth promoting potential across the 20 *Drosophila* generations. We identified a total of 11 mutations, including nine single-nucleotide polymorphisms (SNPs) and two small deletions (Fig. 1c; Extended Data Table 2). In particular, in the strain isolated from the second fly generation (FlyG2.1.8), we found a single change in the genome within one of the three acetate kinase genes (*ackA*). Remarkably, this first mutation was subsequently fixed and strictly correlated with the improved animal growth phenotype (Fig. 1c).

To confirm this finding, we conducted an independent replicate of *L. plantarum* experimental evolution with *Drosophila*. Both the phenotypic and genomic evolution of *L. plantarum* were again obtained: $Lp^{NIZO2877}$ improved its animal growth promoting potential by rapidly acquiring and fixing a mutation in the *ackA* gene (Extended Data Fig. 4, Extended Data Table 2). In the first experiment, the evolved $Lp^{NIZO2877}$ strains with improved animal growth potential all carried a three-nucleotide deletion in the *ackA* gene that removed one proline residue. From the second replicate, the evolved strains carried a SNP that resulted in a premature stop-codon leading to protein truncation (Extended Data Fig. 5). These independently isolated mutations likely generate an inactive *ackA* protein. Importantly, these two evolved strains each bearing only one mutation in *ackA* (FlyG2.1.8 and FlyG3.1.8) already showed a more statistically significant effect on *Drosophila* growth compared to their ancestor (Fig. 1a,b). Based on these observations, we propose that the novel appearance of the *ackA* mutation is the first fundamental step in shaping the evolutionary trajectory in the *L. plantarum*/*Drosophila* symbiosis model.

To fully establish that *ackA* mutation is responsible for the evolution of *L. plantarum*/*Drosophila* symbiosis, we employed CRISPR-Cas9 to re-insert the deleted CCT triplet in the FlyG2.1.8 *ackA* locus (Methods; Extended Data Fig. S6), so that we genetically revert the *ackA* allele in the FlyG2.1.8 isolate back to its ancestral form[14]. The reverted strain (FlyG2.1.8^{Rev}) bearing the ancestral *ackA* allele lost its increased capacity to promote animal growth when compared to the ancestor strain (Fig. 1d,e). These results therefore demonstrate that the *ackA* mutation in *L. plantarum* directly results in faster and increased *Drosophila* growth.

To investigate the complete *L. plantarum* population dynamics during *Drosophila* symbiosis evolution, we sequenced the metagenome of whole bacterial population samples across the 20 *Drosophila* generations of the first replicate experiment. We identified both segregating and fixed mutations and tracked their frequencies through time (Methods). We found that the *ackA* mutation was the first variant to appear in the population. Remarkably, it showed a rapid selective sweep and became fixed as early as after three *Drosophila* generations (Fig. 2a). This observation suggests a competitive advantage of the evolved *Lp*^{NIZO2877} strains bearing this variant. To test this hypothesis, we performed a competition assay between the ancestral *Lp*^{NIZO2877} strain and the derived FlyG2.1.8 isolate in symbiosis with *Drosophila* (Methods, Fig.2b, Extended Data Fig. 7). We find that the evolved strain bearing only the *ackA* mutation starts outcompeting the ancestor strain as early as after one day, demonstrating that the *ackA* mutation confers a strong competitive advantage in symbiosis with *Drosophila*. To test whether such advantage requires the host's presence, we performed the same competition assay by inoculating only the bacterial strains on the *Drosophila* nutritional environment (i.e. the diet). Surprisingly, we observed that FlyG2.1.8 outcompeted the ancestral strain even when the *Drosophila* host is absent (Fig. 2c). Therefore, the competitive advantage of *L. plantarum* isolates bearing the *ackA* variant is likely independent of the animal host.

Intrigued by this result, we questioned whether the animal host has any influence on the evolution of its symbiotic bacteria. To test this, we experimentally evolved *Lp*^{NIZO2877} in the same low-protein fly diet, but without *Drosophila* (Methods; Extended Data Fig. 8) and tested their

capacity to promote fly growth throughout the course of the experimental evolution. Strikingly, in this context, the *Lp*^{NIZO2877} strains evolved in total absence of the host isolated from two parallel experiments also showed an increase in their *Drosophila* growth promoting ability (Fig. 3a,b). Furthermore, genome sequencing of single evolved isolates from both experiments again revealed the acquisition of novel mutations in the *ackA* gene (Fig. 3c; Extended Data Fig. 9). Taken together, these findings show that the genomic evolution of *L. plantarum* is driven by the adaptation to host nutritional environment, rather than to its host *per se*; the acquisition of the *ackA* variant is sufficient to drive the adaptive process to the nutrition, which ultimately results in the improvement of *L. plantarum* symbiotic effect on *Drosophila*.

We next investigated how *L. plantarum* adaptation to the nutritional environment enhances *Drosophila* growth. We postulated that *L. plantarum* adaptation to the specific nutritional environment of *Drosophila* would lead to the production of metabolites that are beneficial for *Drosophila* growth. To test this hypothesis, we analyzed the metabolome of *Drosophila* diets colonized with either *Lp*^{NIZO2877} or the evolved FlyG2.1.8 strain that bears only the *ackA* variant. Among all of the metabolites differentially detected in the substrate (Extended Data Table 6), we observed a significant and robust increase in the levels of N-acetyl-amino-acids in the diet processed by the evolved strain (Fig. 4a). Specifically, N-acetyl-glutamine is one of the most differentially represented compounds between the two conditions. We therefore tested whether N-acetyl-glutamine is sufficient to improve the animal growth promoting capacity of *Lp*^{NIZO2877}. Remarkably, we find that, when N-acetyl-glutamine is added in a dose dependent manner in the diet, the ancestor strain *Lp*^{NIZO2877} is able to recapitulate the beneficial effect conferred by FlyG2.1.8 on *Drosophila* growth (Fig. 4b). We then asked whether N-acetyl-glutamine enhances fly growth by improving *Lp*^{NIZO2877} fitness. To test this, we performed a competition assay between *Lp*^{NIZO2877} and FlyG2.1.8 strains in the host diet supplemented with 0.1g/L of N-acetyl-glutamine. We find that FlyG2.1.8 outcompetes the ancestor strain even in presence of N-acetyl-glutamine (Extended Data Fig. 10). This result indicates that N-acetyl-glutamine does not confer a competitive advantage to *Lp*^{NIZO2877} over

FlyG2.1.8, nevertheless it benefits the host physiology. Taken together, these findings establish N-acetyl-amino-acids, and in particular N-acetyl-glutamine, as molecules produced by the evolved *L. plantarum* strains during growth on the *Drosophila* diet, which concur to enhance *Drosophila* growth.

Our results uncover the nature of the adaptive processes of *L. plantarum* during evolution with its fly host. To our knowledge, this is the first direct experimental evidence showing that the host nutritional environment, and not the host *per se*, drives microbial adaptation and metabolic changes that alter the functional outputs of microbe-animal symbiosis. In other words, the host diet represents the foremost selective pressure dictating evolutionary change of symbiotic bacteria and their consequent benefits to host physiology. Rapid adaptation of *L. plantarum* to the host nutritional environment occurred in multiple independent experimental lineages through the parallel fixations of different variants of a single gene, the acetate kinase *ackA*. This is an exceptional case of evolutionary convergence, indicating that the *ackA* mutation is the preferred or even the unique means for *L. plantarum*$^{\text{NIZO2877}}$ to adapt to its host nutritional environment. These harsh nutritional conditions of our experimental setting affect *L. plantarum* physiology by delaying its growth (Extended Data Fig. 2). It was shown that the expression of *L. plantarum ackA* (named *ack2* in the *L. plantarum* reference strain WCFS1) is down-regulated at low growth rates suggesting that silencing *ackA* would be required to cope with poor growth condition[15]. This observation may explain the observed strong selective pressure on *ackA* in our experimental settings, which led to the rapid *de novo* emergence of different variants in the population (Fig. 2a). As a consequence, the strong competitive advantage given by these mutations led to their fixation (Fig. 2). Indeed, the *ackA* mutations found in the independent lineages of adaptive evolution improve the growth rate of *L. plantarum* cells on the fly diet (Extended Data Fig. 11). It remains elusive how *ackA* variants confers competitive advantage to *L. plantarum* cells on the fly diet; however, our results indicate that these mutations cause a shift in the metabolism of *L. plantarum* by modifying the usage of cellular acetyl groups which would confer benefits to *Drosophila* larvae growth. *ackA* participates in the reversible conversion of acetate to acetyl-phosphate; *ackA* variants impede this reaction, and might therefore shunt the pools of cellular

acetyl groups into different metabolic routes leading to the accumulation of other acetylated compounds, such as N-acetyl-amino-acids, which, once secreted, are consumed and beneficial to the host.

Understanding how evolutionary forces shape host-microbe symbiosis is essential to comprehend the mechanisms of their reciprocal functional influence. Our results demonstrate that the primary selection pressure acting on a particular symbiotic bacterium originates from the nutritional substrate alone, which is strong enough to drive the rapid fixation of a *de novo* mutation. The resulting adaptations that confer a fitness advantage to the bacteria are quickly capitalized by the host as a physiological growth advantage in the same harsh nutritional environment, and symbiosis can henceforth be perpetuated. Our study demonstrates that nutritional substrate, rather than the host, is a major driving force of microbial genomic change, and this may be the first step in animal-microbe symbiotic evolution.

Figures

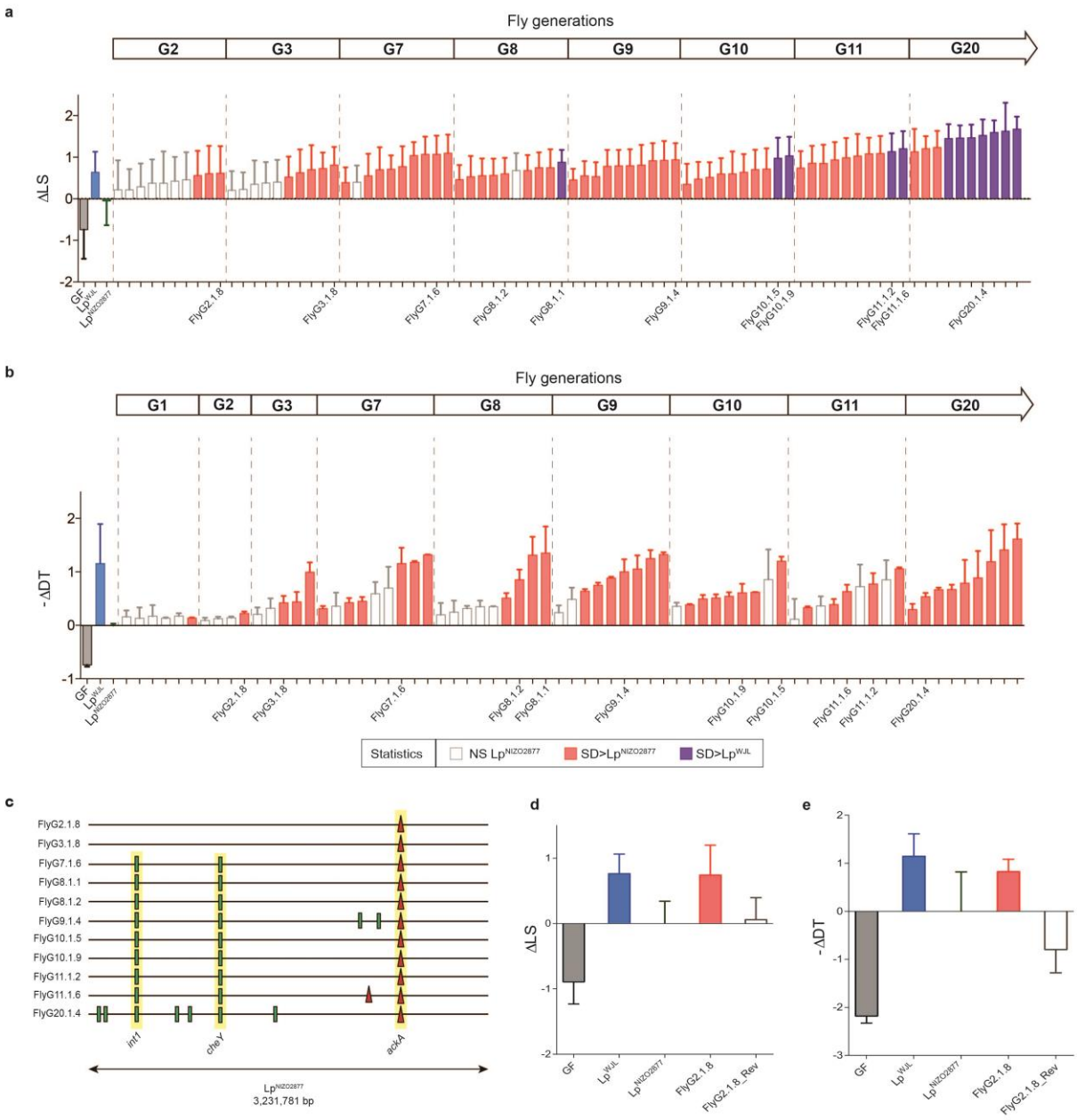

Figure 1. Experimental evolution of *L. plantarum* with *Drosophila melanogaster* improves its growth promoting effect. a, Longitudinal size of larvae (LS) measured 7 days after egg deposition (AED) on poor nutrient diet. Larvae were kept germ-free (GF) or associated with $Lp^{NIZO2877}$ (ancestor), Lp^{WJL} (growth-promoting *L. plantarum* strain) or $Lp^{NIZO2877}$-evolved strains. The delta (ΔLS) between the size of larvae associated with the respective condition and the size of larvae associated with $Lp^{NIZO2877}$ is shown from *Drosophila* generation 2 (G2) to generation 20 (G20). $Lp^{NIZO2877}$-evolved strains that exhibited a significant difference at promoting larval growth compared to their ancestor (Student's t test: $p<0.05$) are shown in red. $Lp^{NIZO2877}$-evolved strains that exhibited a significant difference at promoting larval growth compared to the beneficial *L. plantarum* Lp^{WJL}

strain are shown in purple. **b,** Developmental timing (DT) of individuals that were kept GF or associated with *Lp*^{NIZO2877}, *Lp*^{WJL} or *Lp*^{NIZO2877}-evolved strains isolated from *Drosophila* G1 to G20. The minus delta (-ΔDT) between the mean time of emergence of 50% of the pupae associated with the respective condition and the mean time of emergence of 50% of the pupae associated with *Lp*^{NIZO2877} is shown in the graph. *Lp*^{NIZO2877}-evolved strains that exhibited a significant difference at accelerating developmental timing compared to the ancestor (Student's t test: $p<0.05$) are shown in red. The evolved strains that have been selected for further analyses are labelled on the x axis. **c,** Mutations identified in *Lp*^{NIZO2877}-evolved strains from *Drosophila* generation 2 (G2) to generation 20 (G20) represented along *Lp*^{NIZO2877} genome. The genome of each evolved strain is represented as a horizontal line. Red triangles indicate deletions and small green bars show single nucleotide polymorphisms. Mutations occurring in the same gene of different strains and fixed along the experimental evolution are highlighted in yellow (*int1*, *cheY*, *ackA*). **d,** Longitudinal size of larvae measured 7 days AED on poor nutrient diet. Larvae were kept germ-free (GF) or associated with *Lp*^{NIZO2877}, *Lp*^{WJL}, FlyG2.1.8 or with FlyG2.1.8-reverted strain (FlyG2.1.8^{Rev}). The delta (ΔLS) between the size of larvae associated with the respective *L. plantarum* strain and the size of larvae associated with *Lp*^{NIZO2877} is shown. **e,** Developmental timing (DT) of individuals that were kept GF or associated with *Lp*^{NIZO2877}, *Lp*^{WJL}, FlyG2.1.8 or with FlyG2.1.8^{Rev} strain. The minus delta (-ΔDT) between the mean time of emergence of 50% of the pupae associated with the respective condition and the mean time of emergence of 50% of the pupae associated with *Lp*^{NIZO2877} is shown in the graph.

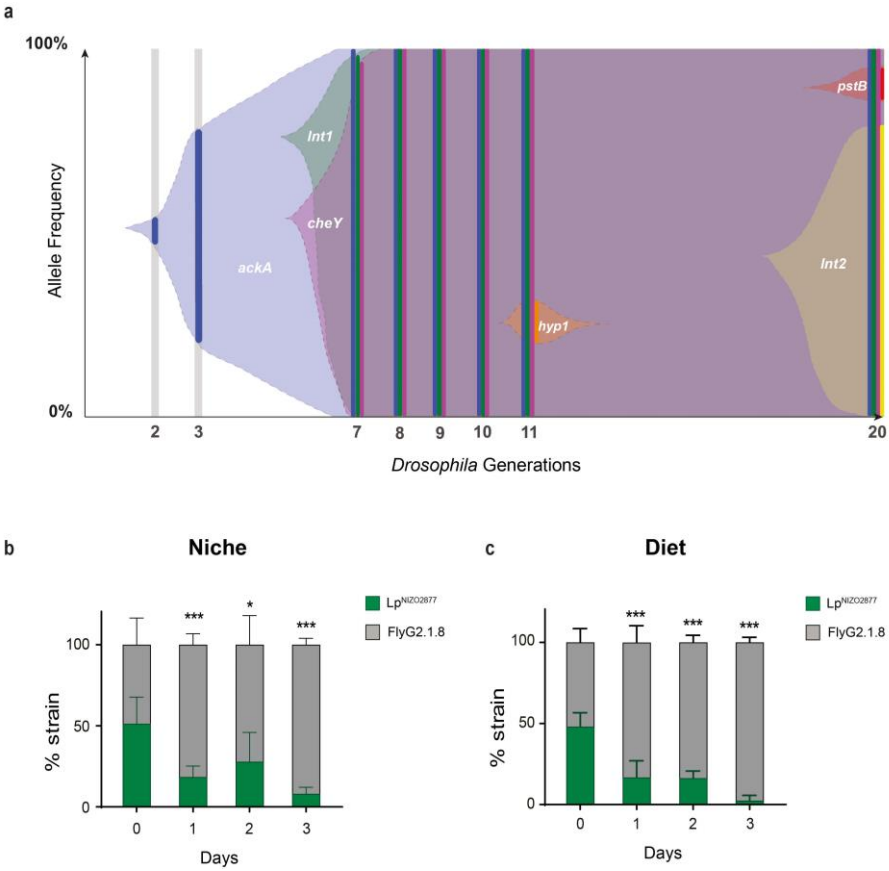

Figure 2. *Lp*$^{\text{NIZO2877}}$-evolved strain shows higher fitness compared to the ancestor. a, Muller diagram showing the genome evolutionary dynamics of *Lp*$^{\text{NIZO2877}}$ population (I replicate) along 20 *Drosophila* generations. The y-axis shows the percentage of the detected frequencies of each mutation (plain colours). Shaded areas represent the inferred mutation frequencies. Lower axis shows the fly generation where the sampling took place. **b,c,** 1:1 competitive assay between *Lp*$^{\text{NIZO2877}}$ and *Lp*$^{\text{NIZO2877}}$-evolved strain (FlyG2.1.8) in poor nutrient diet with *Drosophila* larvae (**b**) and without *Drosophila* larvae (**c**). Bars represent the percentage of each strain detected in each sample (Niche or Diet) by qPCR. *$P < 0.05$, ***$P < 0.01$, obtained by Student's t-test.

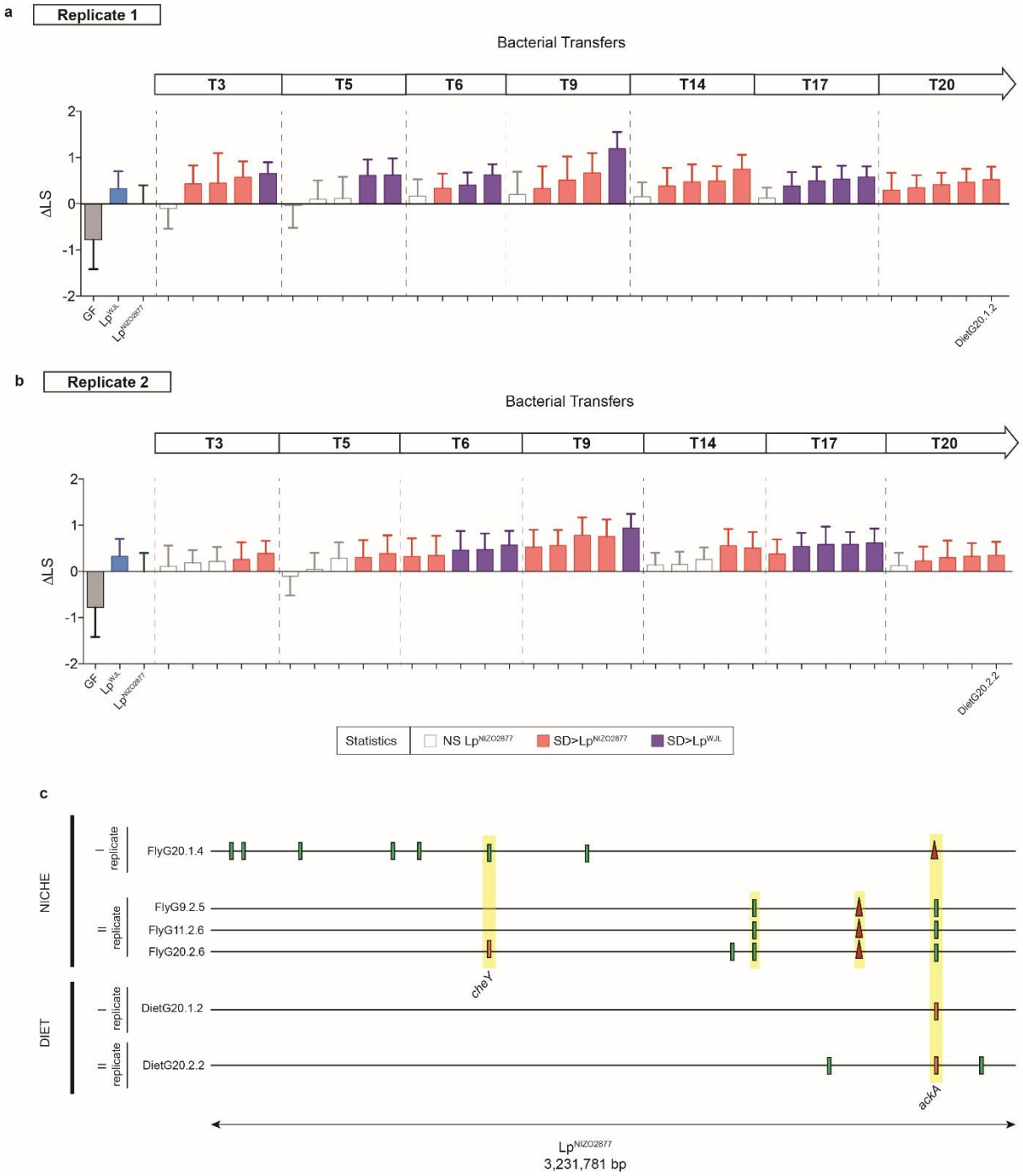

Figure 3. *Lp*$^{\text{NIZO2877}}$ adaptation to the diet increases its host's growth. a,b, Longitudinal size of larvae (LS) measured 7 days after egg deposition (AED) on poor nutrient diet. Larvae were kept germ-free (GF) or associated with *Lp*$^{\text{NIZO2877}}$, *Lp*^{WJL} and with *Lp*$^{\text{NIZO2877}}$-evolved strains evolved in poor nutrient diet in the absence of *Drosophila*. The delta (ΔLS) between the size of larvae associated with *Lp*$^{\text{NIZO2877}}$-evolved strains and the size of larvae associated with *Lp*$^{\text{NIZO2877}}$ is shown from transfer 3 (T3) to transfer 20 (T20) for the first replicate (**a**) and the second replicate (**b**) of evolution. *Lp*$^{\text{NIZO2877}}$-evolved strains that exhibited a significant difference at promoting larval growth compared

to their ancestor (Student's t test: $p<0.05$) are shown in red. *Lp*^{NIZO2877}-evolved strains that exhibited a significant difference at promoting larval growth compared to the beneficial *L. plantarum Lp*^{WJL} strain are shown in purple. The evolved strains that have been selected for further analyses are labelled on the x axis. **c,** Mutations identified in *Lp*^{NIZO2877}-derived strains of all replicates evolved in poor nutrient diet with *Drosophila* larvae (Niche) and in poor nutrient diet without *Drosophila* larvae (Diet). Each evolved strain genome is represented as a horizontal line. Red triangles indicate deletions and small bars shows single nucleotide polymorphisms. Different colours indicate different variants. Mutations occurring in the same gene and fixed along the experimental evolution are highlighted in yellow. The genes mutated in independent replicates of experimental evolution are labelled (*cheY*, *ackA*).

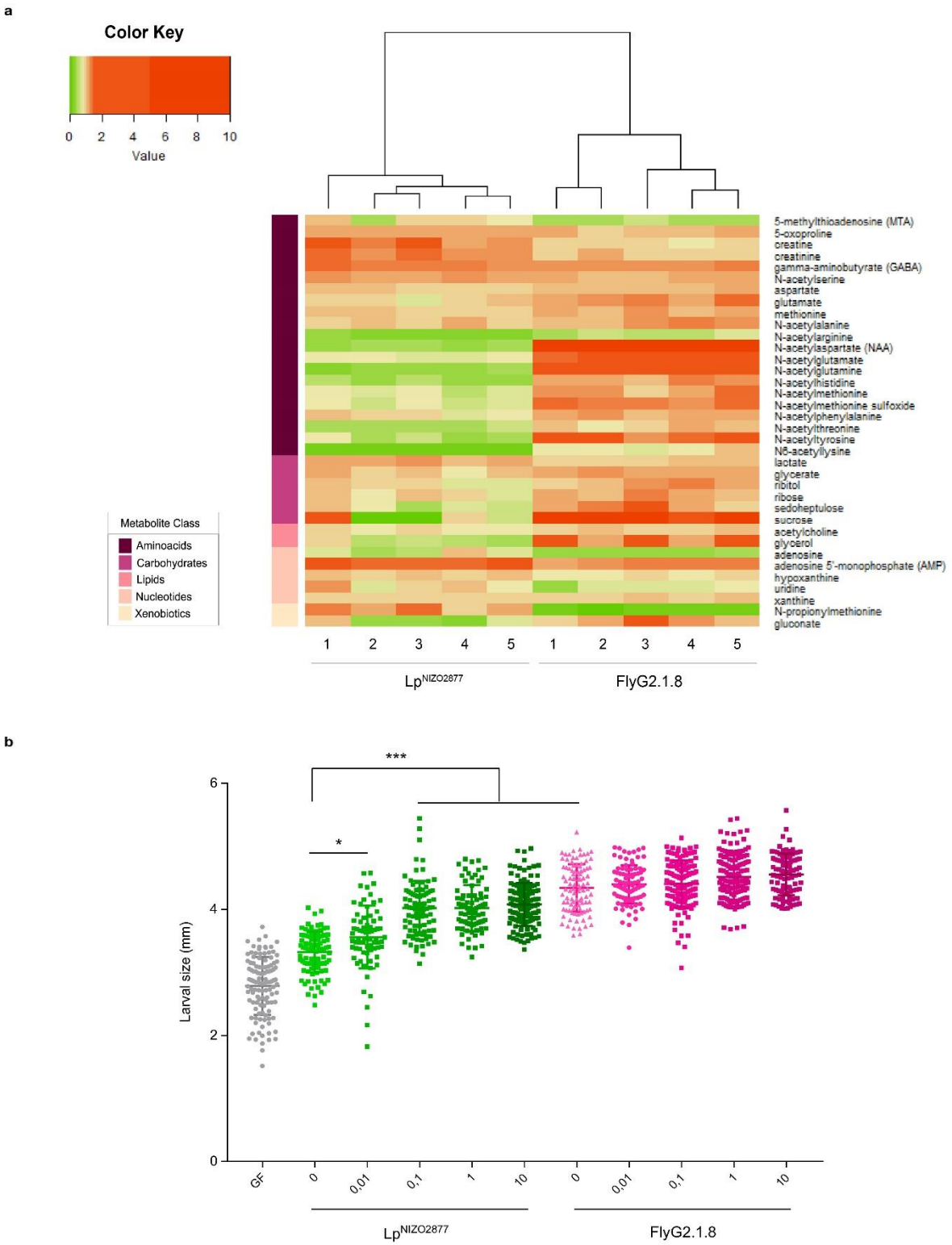

Figure 4. N-acetyl-glutamine recapitulates the beneficial effect of FlyG2.1.8 on *Lp*^{NIZO2877}-associated larvae. a, Heat map showing the metabolites that differ significantly between experimental groups (*Lp*^{NIZO2877} and FlyG2.1.8) (two-sided *t*-tests p<0.05). The heat map was generated with *heatmap.2* function in R. The compounds are ordered by the metabolite class given

by the left scale. **b,** Longitudinal size of larvae (n > 60 larvae/group) measured 7 days after egg deposition on poor nutrient diet supplemented with different concentrations (g/L) of N-acetyl-glutamine (x axis). Larvae were kept germ-free (no supplementation of N-acetyl-glutamine) or associated with *Lp*^{NIZO2877} (ancestor) and with Fly.G2.1.8 (evolved strain). Larval size is shown as mean ± s.e.m. * P < 0.05, *** P < 0.01.

Methods

Bacterial strains and culture conditions. The strains used in the present study are listed in Table S1. All experimentally evolved strains were derived from *L. plantarum*^{NIZO2877} (kind gift from Prof. Michiel Kleerebezem, NIZO Food Research BV, Netherlands) that was originally isolated from a sausage in Vietnam[12]. All strains were routinely grown overnight at 37°C in Man, Rogosa and Sharpe (MRS) medium (BD Bioscience) without agitation. Strains were stored at -80°C in MRS broth containing 20% glycerol.

***Drosophila* stocks and breeding.** *Drosophila yw* flies were used as the reference strain in this work. *Drosophila* stocks were cultured at 25°C with 12/12 hour dark/light cycles on a yeast/cornmeal medium containing 50g/l inactivated yeast (rich diet) as described by Storelli et al. (2011). Poor-nutrient diet was obtained by reducing the amount of yeast extract to 8 g/l. Germ-free (GF) stocks were established and maintained as described in Storelli et al.[9].

Experimental Evolution design. The experimental "Adaptive Evolution" (AE) model of *L. plantarum* in *Drosophila* Niche (*Drosophila* + Diet) was designed as follows: GF female flies laid GF embryos on poor nutrient GF medium. Forty GF eggs were transferred on 10 ml of new poor nutrient GF medium. *L. plantarum*^{NIZO2877} (ancestor) was cultured to stationary phase (18h). The culture was washed in sterile PBS and 300 µl (10^8 CFU) were added directly on the embryos and the fly food (bacterial load = 10^7 CFU/ml) (Extended Data Fig.1). Emerging larvae were allowed to develop on the contaminated medium at 25°C. The first 15 pupae were transferred to a new poor nutrient GF medium, and allowed to complete metamorphosis. Once the pupae were transferred, the

bacterial community associated with them (on average 10^6 CFU/ml) was also indirectly transferred to the new medium by contamination of the substratum from the surface of the transferred pupae and predominantly by defecation of the adults emerging from the transferred pupae. This allowed the propagation of an evolving bacterial subpopulation derived from the ancestor on the new medium. Once the emerging adults had laid eggs (a minimum of 40 and no more than 80 per tube), which were the founders for the next fly generation, they were collected and homogenized using the Precellys 24 tissue homogenizer (Bertin Technologies, France) and 0.75/1 mm glass beads in 800 µl of MRS broth and stored at -80°C by adding 20% glycerol. Single bacterial isolates were isolated at the end of each fly generation by plating the crushed adult flies on MRS agar plates at 37°C for 48h. Ten colonies were randomly selected from the plates and tested individually for *Drosophila* growth promotion (larval size and developmental timing assays) on new GF *yw Drosophila* embryos (see below). The AE model has been propagated for 20 fly generations.

The experimental evolution of *L. plantarum* in *Drosophila*'s nutritional medium (Diet) was designed as follows: *L. plantarum*^{NIZO2877} (ancestor) was cultured to stationary phase (18h). The culture was washed in sterile PBS and 3 µl (10^6 CFU) were added directly on 100 µl of poor nutrient GF diet (bacterial load = 10^7 CFU/ml) (Fig. S7) and kept at 25°C. After four days (time necessary for the microbial load to reach the same value found on the 15 pupae used for propagating the bacterial population in the Niche adaptive evolution setup), the medium was crushed using the Precellys 24 tissue homogenizer (Bertin Technologies, France) and 0.75/1 mm glass beads in 500 µl of PBS using glass beads and 10 µl of the crushed medium (10^5 CFU) were used to inoculate 100 µl of new poor nutrient GF medium (10^6 CFU/ml). This protocol has been repeated 20 times.

Strain-specific PCR tests were performed to confirm the unique presence of *L. plantarum*^{NIZO2877} throughout both experimental evolution models as reported in Schwarzer et al. [11].

Generation time of *L. plantarum* in experimental evolution model. To determine the generation time of *L. plantarum* strains in the *Drosophila* Niche (*Drosophila* + Diet) and Diet, we used a modified version of a method that reported the correlation between bacterial growth rate and

16SrRNA content[16]. *L. plantarum* strains were cultured to stationary phase (18h) and washed in sterile PBS. Serial dilutions have been prepared and 5 µl containing a total of 10^3 colony-forming units (CFUs) were added to 100 µl of GF poor nutrient diet with and without *Drosophila* larvae (Diet and Niche setup respectively) and kept at 25°C. Samples were snap-frozen in liquid nitrogen at different time points across five days of growth. Bacterial RNA was extracted using NucleoSpin RNA Isolation kit (Macherey-Nagel, Germany) following manufacturer's instructions. Reverse transcription of total extracted RNA into cDNA has been performed using Superscript II (Invitrogen, USA) according to manufacturer's instructions. Quantitative PCR was performed in a total of 20 µl on a Biorad CFX96 apparatus (Biorad) using SYBR GreenER qPCR Supermix (Invitrogen, USA). The reaction mixture consisted of 0.5 µl of each primer (10 µM each), 12,5 µl of SYBR GreenER mix, 10 µl of water and 1,5 µl of template cDNA. The PCR conditions included 1 cycle of initial denaturation at 95°C for 2 min, followed by 45 cycles of 95°C for 10 sec and 60°C for 40 sec. Absolute quantification of 16rRNA was conducted as follows: five 1:10 serial dilutions of the standard sample (100 ng/µl of cDNA extracted from *L. plantarum*^{NIZO2877} culture) were quantified by Real-time PCR using universal 16S primers (forward primer, UniF 5'-GTGSTGCAYGGYTGTCGTCA-3' and reverse primer, UniR 5'-ACGTCRTCCMCACCTTCCTC-3', Extended Data Table 4)[17]. Each dilution has been tested in triplicate. Melting curves of the detected amplicons were analysed to ensure specific and unique amplification. Standard curves were generated plotting threshold cycle (Ct) values against the log of the standard sample amount. Based on the data obtained from the standard curve, the Ct values of the Niche and Diet samples have been used to obtain the log of their 16SrRNA concentration at each time point. The 16S rRNA values during exponential phase have been used to infer the bacterial generation time following the equation reported by Widdel[18].

Colonization and infection of larvae for Larval Size and Developmental time measurements. 40 embryos collected from GF females were transferred to a fresh poor nutrient GF medium. Bacterial strains were cultured to stationary phase (18h) in MRS broth at 37°C. The embryos and the fly

medium were mono-associated with 300 µl (7 x10^7 CFU) of the respective bacteria. Emerging larvae were allowed to develop on the contaminated medium at 25°C.

Larval Size Measurements. Larvae (n ≥ 120) were collected 7 days after inoculation, washed in distilled water, transferred on a microscopy slide, killed with a short heat treatment (5s at 90°C) and mounted in 80% glycerol/PBS. The larvae were imaged under a Leica steromicroscope M205FA and larval longitudinal size (length) was measured using ImageJ software[19].

Developmental Timing. Developmental timing of *Drosophila* associated with different bacteria was quantified by counting the number of pupae emerging over time. These results are represented as the day at which 50% of the whole population pupariated (D50). Each graph represents the mean of 3 biological replicates, including at least 30 individuals each.

Genome sequencing. Genomic DNA of single bacterial strains was extracted from cultures grown to stationary phase in MRS broth using the UltraClean Microbial DNA isolation kit (Mo Bio, Qiagen, USA). For all single strain sequencing genomic libraries were prepared following Ion Xpress Plus gDNA Fragment Library construction protocol for 400bp reads. The strains were sequenced using the Ion Torrent PGM platform. The DNA library construction and sequencing was performed on the IGFL sequencing platform (Lyon, France). For community sequencing, the lysate obtained from the crushed adult flies was plated out on MRS agar and cultured at 37°C for 48h. A mixture of >1000 clones was used to extract the genomic DNA using the UltraClean Microbial DNA isolation kit (Mo Bio, Qiagen, USA). The DNA library construction and sequencing was carried out by the EMBL Genomics Core Facilities (Heidelberg, Germany). Each sample was pair-end sequenced on an Illumina MiSeq Benchtop Sequencer. Standard procedures produced data sets of Illumina paired-end 250 bp read pairs. The mean coverage per sample was 99x. Processed reads were aligned and analysed against their respective reference strain (ancestor) genome to identify mutations, using default settings in breseq[20] for single isolate genomes and using the '-polymorphism' setting for libraries constructed for bacterial communities. In order to discard false positive mutations, we generated an R script which used the breseq file as input to derive the real percentage of reads affected by mutation. Candidate

mutations were verified by targeted PCR amplification and Sanger sequencing. These data were used to build a decision tree in order to correlate the frequency of reads affected by a given mutation (%) and its real presence in the genome (obtained by Sanger sequencing). This allowed us to establish that the real mutation calls were those predicted by frequency values higher than 83,5%. All candidate mutations were subsequently confirmed by targeted PCR amplification and Sanger sequencing by using specific primers (Table S2). Non-synonymous mutations in genes belonging to pathway of interest were analysed with PROVEAN (Protein Variation Effect Analyzer)[21] to predict the functional impact of the genetic variant. The score threshold used was set to -2.5.

***L. plantarum* genomic editing with CRISPR-Cas9.**

Plasmid generation. The Cas9 targeting plasmid was assembled by first amplifying the pMSP3545 plasmid backbone (Extended Data Table 5) and the Cas9+tracrRNA from pCas9 with oligos oRL1-oRL4 (Extended Data Table 6). These PCR fragments were stitched together using Gibson assembly[22] (NEB CN#32611S) and transformed into *E. coli* NovaBlue cells. This synthesized plasmid was named p3545Cas9 and served as the non-targeting control vector used for subsequent transformation assays (Extended Data Figure 6a). The targeting sgRNA was synthesized as a repeat-spacer-repeat array under a constitutive *Lactobacillus* promoter (P_{pgm})[23]. This array (gBlock 1) was amplified with oligos oRL5-oRL6. The p3545Cas9 plasmid was digested with XbaI and PstI and the two fragments were inserted by Gibson assembly. This step created the pCas9+RSR plasmid. To insert a spacer to specifically targeting the mutated *ackA* gene, oligos oRL13-14 were phosphorylated with T4 PNK (NEB CN#M0201S), annealed, and ligated into the pCas9+RSR plasmid after digestion with PvuI and NotI. The spacer was designed so the CCT triplet (absent in Fly.G2.1.8) was inserted within the seed portion of the target sequence (Extended Data Figure 6b).

The repair template plasmid was assembled by amplifying the $Lp^{NIZO2877}$ *ackA* gene with oRL7-oRL8, digesting this PCR product and the pJP005 plasmid with SpeI and SacI, and ligating them together with T4 DNA ligase (NEB CN#M0202S). 300ng of backbone was ligated with 422 ng of insert, ethanol precipitated, and entirely transformed into *L. plantarum* WCFS1. The center of the amplified

region contained the CCT triplet absent in Fly.G2.1.8 *ackA* gene (Extended Data Table 5). Colony PCR was used to screen for successful clones using oligos oRL09-10. Colony PCR was performed by picking a single colony into 20 µL of 20 mM NaOH and incubating at 98ºC for 20 minutes. These tubes were then microwaved for 1 minute with the cap open, and 5 µL of this mixture was added to the PCR mix and amplified with PFU polymerase isolated from *Pyrococcus furiosus* (gift from R.M. Kelly).

Growth conditions and electroporation. All *L. plantarum* strains were grown on MRS liquid broth (BD CN# 288130) and MRS agar (BD CN# 288210) and incubated at 37ºC. Antibiotic concentrations for *L. plantarum* were as follows: rifampicin (25µg/mL), chloramphenicol (10µg/mL), and erythromycin (10µg/mL). All *E. coli* strains were cultured in LB medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) while being shaken at 250 RPM at 37ºC. Plasmids were maintained at the following antibiotic concentrations: erythromycin (50µg/mL), chloramphenicol (34µg/mL), and ampicillin (50µg/mL). Electroporation of *L. plantarum* was adapted from numerous protocols[24–26]. To prepare cells for electroporation, 1 mL of overnight culture of *L. plantarum* was back-diluted into 25 mL of MRS liquid broth containing 0.41 M glycine and any necessary antibiotics. This was performed in sealed 50-mL falcon tubes (VWR CN#21008-212) to prevent aeration of the bacteria. These tubes were cultured at 37ºC and 250 RPM for ~3 hours, or until the OD_{600} was approximately 0.85. Cells were centrifuged at 5,000 RPM for 10 minutes at 4ºC. The pelleted cells were kept on ice, then washed twice with ice-cold 10 mM $MgCl_2$ followed by a wash in SacGly (10% glycerol with 0.5 M sucrose). The washed cell pellet was then re-suspended in 1mL of SacGly and centrifuged at 20,000 RPM for 1 minute and the final pellet was re-suspended in 500 µL of SacGly. For all transformations, 60µL of this suspension was added to a 1-mm gap cuvette and transformed at 1.8kV, 200Ω resistance, and 25 µF capacitance. Following electroporation, cells were resuspended in 1 mL of MRS broth and transferred to a sterile tube and incubated at 37ºC without shaking for 3 hours. 250 µL of the recovered cells was then plated on MRS agar with appropriate antibiotics. Any dilutions prior to plating was done in MRS media.

CRISPR-Cas9 repair-template editing. To perform the genomic edits, the *ackA*_pJP005 plasmid was transferred from WCFS1 into Fly.G2.1.8 and prepared for electroporation using the previously described conditions, selecting for the ackA_pJP005 plasmid with chloramphenicol. Electrocompetent cells were then transformed with 5µg of p3545_Cas9 or pCas+RSR isolated from a methylation-free *E. coli* strain (EC135)[11]. Transformation efficiencies of the Cas9 plasmid into Fly.G2.1.8 were vastly improved in the presence of the homologous-recombination template (Extended Data Figure 6c). After transformation, cells were plated on solid MRS media supplemented with erythromycin to only select for the CRISPR-Cas9 plasmid. The *ackA* gene from the resultant colonies was amplified with oRL11-12 and subjected to Sanger sequencing (Extended Data Figure 6d). Seven colonies harbored the inserted CCT triplet (FlyG2.1.8^{Rev}, Extended Data Figure 6d), while two colonies were unedited and one colony did not yield an amplification product. The lack of editing in the two colonies may be due to mutation of Cas9 or the spacer as reported previously[27]. To clear the plasmids from the edited strains, colonies were subjected to multiple rounds of non-selective outgrowths in liquid medium (Extended Data Figure 6e). After each round of non-selective outgrowths, edited *Lactobacillus* cultures were struck out on non-selective plates and colonies were then struck out on selective chloramphenicol or erythromycin plates to determine if plasmids were successfully cleared. Once colonies were no longer able to grow on selective medium, antibiotic susceptibility was tested in liquid culture and strains were analyzed *in vivo*. Two isolates from separate colonies were subjected to whole-genome sequencing (using an Ion torrent PGM platform) to confirm the insertion of the CCT triplet in *ackA* and the absence of any mutations elsewhere in the genome (Extended Table 1).

Competitions tests. Competition assays between *Lp*^{NIZO2877} (ancestor) and FlyG2.1.8 (evolved beneficial strain) have been tested in *Drosophila* Niche and Diet. The competitions were performed at a ratio of 1:1, over 3 days of co-colonization, following the same inoculation procedure described for the Diet evolution experiment. A total of 10^4 CFUs has been used as inoculum. During the three days of co-colonization, the samples have been crushed using the Precellys 24 tissue homogenizer

(Bertin Technologies, France) and 0.75/1 mm glass beads in 500 µl of PBS. The lysate has been plated out on MRS agar and cultured at 37°C for 48h. 10000 colonies have been collected and bacterial DNA was extracted using the UltraClean Microbial DNA isolation kit (Mo Bio, Qiagen, USA). A specific Real-time PCR assay has been developed to distinguish and quantify the presence of *Lp*^{NIZO2877} (ancestor) and *FlyG2.1.8* (evolved beneficial strain). Specific primer pairs have been designed on the acetate kinase gene sequence using Geneious 9[28]. Quantitative PCR was performed in a total of 20 µl on a Biorad CFX96 apparatus (Biorad) using SYBR GreenER qPCR Supermix (Invitrogen, USA), bacterial DNA and the gene specific primer sets (forward primer *Lp*^{NIZO2877}-specific: ackA_NIZO2877_F_RT; forward primer FlyG2.1.8-specific: ackA_FlyG2_F_RT; and common reverse primer: ackA_R_RT; Extended Data Table 4). The reaction mixture consisted of 0.5 µl of each primer (10 µM each), 12,5 µl of SYBR GreenER mix, 10 µl of water and 1,5 µl of template cDNA. Each sample has been tested in triplicate. The PCR conditions included 1 cycle of initial denaturation at 95°C for 2 min, followed by 45 cycles of 95°C for 10 sec and 60°C for 40 sec. Melting curves of the detected amplicons were analysed to ensure specific and unique amplification. PCR efficiency was calculated for each primer set using six serial dilutions of DNA starting from 2 ng/µl. Relative quantification of each bacterial strain has been performed using 16SrRNA as reference gene (UniF-UniR primers, Extended Data Table 4).

Metabolome profiling. Microtubes containing axenic poor nutrient diet were inoculated with bacterial suspension (10^3 CFU/ml) or with PBS and incubated for 3 days at 25°C. Microtubes were then snap-frozen in liquid nitrogen and stored at -80°C before sending to Metabolon Inc. (www.metabolon.com). Five biological replicates per condition were generated. Samples were then extracted and prepared for analysis using Metabolon's standard solvent extraction method. Each resulting extract was divided into five fractions: two for analysis by two separate reverse phase/Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (RP/UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with

negative ion mode ESI, and one sample was reserved for backup. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities.

Data treatment and statistical analyses. Data representation and statistical analysis were performed using GraphPad Prism version 6.00 (La Jolla California USA, www.graphpad.com) using ANOVA test for multiple group comparison and two-tailed t-test for comparison between two groups. Groups with values of $P<0.05$ were considered statistically different.

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Author Contributions

M.E.M. and F.L. designed the project; M.E.M. and H.G. conducted the experiments; M.E.M. and P.J. conducted the bioinformatics analyses; R.L., M.S. and C.L.B. designed and performed the CRISPR-Cas9 engineering experiments; S.H. and B.G. generated the sequencing data; M.E.M. and F.L. analyzed the data and wrote the paper.

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