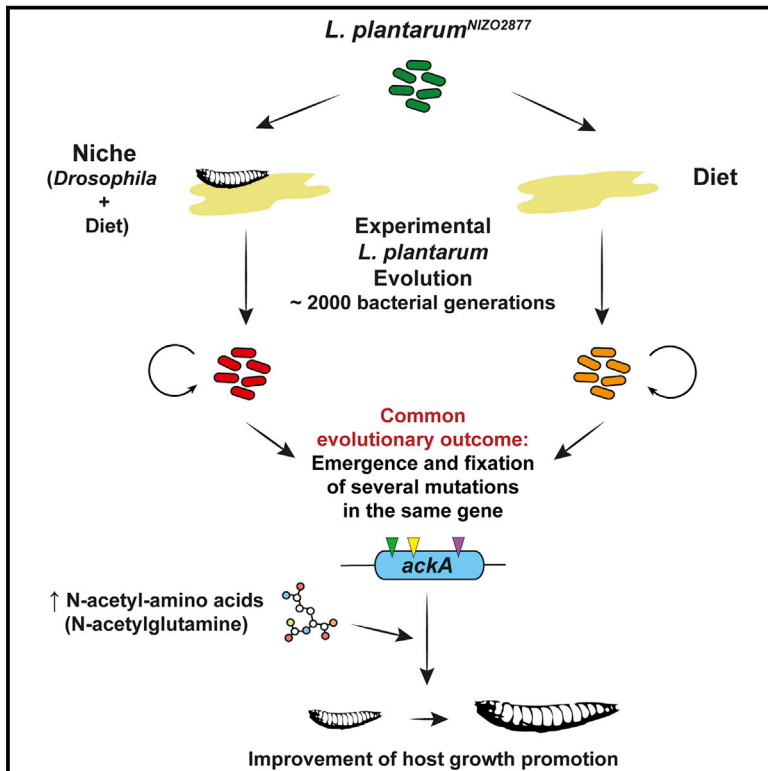


Cell Host & Microbe

Bacterial Adaptation to the Host's Diet Is a Key Evolutionary Force Shaping *Drosophila-Lactobacillus* Symbiosis

Graphical Abstract



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In Brief

Martino et al. demonstrate that, in the symbiosis between *Drosophila* and *Lactobacillus plantarum*, the host diet represents the driving force in the evolution of *L. plantarum* symbiotic effect. This is a clear example of by-product mutualism, where the host capitalizes on the by-products of the self-serving traits of their symbionts.

Highlights

- *L. plantarum* experimental evolution leads to the improvement of its symbiotic benefit
- *L. plantarum* increases its growth-promotion ability by adapting to *Drosophila* diet
- Mutation of *ackA* gene enhances both *L. plantarum* fitness and benefit to the host
- N-acetyl-glutamine production is sufficient to improve *L. plantarum* growth promotion



Bacterial Adaptation to the Host's Diet Is a Key Evolutionary Force Shaping *Drosophila-Lactobacillus* Symbiosis

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SUMMARY

Animal-microbe facultative symbioses play a fundamental role in ecosystem and organismal health. Yet, due to the flexible nature of their association, the selection pressures that act on animals and their facultative symbionts remain elusive. Here we apply experimental evolution to *Drosophila melanogaster* associated with its growth-promoting symbiont *Lactobacillus plantarum*, representing a well-established model of facultative symbiosis. We find that the diet of the host, rather than the host itself, is a predominant driving force in the evolution of this symbiosis. Furthermore, we identify a mechanism resulting from the bacterium's adaptation to the diet, which confers growth benefits to the colonized host. Our study reveals that bacterial adaptation to the host's diet may be the foremost step in determining the evolutionary course of a facultative animal-microbe symbiosis.

INTRODUCTION

Animal-microbe symbioses are ubiquitous and their nature can be extremely diverse. Mutualistic relationships are those symbioses whereby both partners benefit from each other (Bronstein, 1994). They can differ in the number of species involved, the duration of the symbiotic relationship, and how dependent the partners are on the interaction for their development, survival, and reproduction (Douglas, 2011). In obligate mutualism, the organisms depend on each other for their survival. In many cases, this co-dependency has occurred over time as each organism adapts to the reciprocal benefits (Holland and Bronstein, 2008). In facultative symbioses, microbes and their hosts are not fully dependent on each other: the host can survive without its bacterial symbionts, which, in turn, can live in different ecosystems regardless of the host presence (Gilbert and Neufeld, 2014). Nevertheless, facultative microbial symbionts confer

crucial benefits to their animal partners (Feldhaar, 2011; Ferrari and Vavre, 2011). The flexible nature of facultative mutualism suggests that there are both costs and benefits associated with maintaining such symbiosis (Bronstein, 1994; Douglas, 2011; Engel and Moran, 2013; Fisher et al., 2017). However, the ecological and evolutionary forces that drive the emergence and evolution of the benefits conferred by facultative symbionts to their animal hosts remain largely elusive.

To address this question, we experimentally tested microbial evolution using *Drosophila melanogaster* associated with one of its most abundant facultative symbionts, *Lactobacillus plantarum*, with whom it establishes nutritional mutualism (Douglas, 2011; Erkosar et al., 2015; Ma et al., 2015; Matos et al., 2017; Storelli et al., 2011, 2018). *L. plantarum* positively affects juvenile growth rate and maturation when *Drosophila* faces chronic undernutrition (Storelli et al., 2011). Such benefit results, at least in part, from the capacity of *L. plantarum* to promote the expression of larval intestinal peptidases and the consequent increase of dietary protein digestion and amino acid intake by the host (Erkosar et al., 2015; Matos et al., 2017). Conversely, *L. plantarum* benefits from its animal partner. Although *L. plantarum* encounters a strong cost during transit through larval gut, larvae secrete bacterial maintenance factors that counteract this cost and improve microbial fitness, thus perpetuating symbiosis (Storelli et al., 2018). *Drosophila/L. plantarum* association also represents a prototypical case of facultative symbiosis. Indeed, *L. plantarum*, as well as most *Drosophila* facultative symbionts, does not colonize the host intestine, but remains associated with *Drosophila* during its entire life cycle by constant reassociation through cycles of ingestion and excretion (Blum et al., 2013; Broderick et al., 2014; Storelli et al., 2011, 2018). In addition, it is vertically transmitted to progenies via the deposition of contaminated mother's feces on the surface of the embryo during egg laying and on the surrounding substratum (Matos and Leulier, 2014).

Here we show that the host nutritional environment, instead of the host, is a predominant driving force in the emergence and evolution of symbiotic benefits that *L. plantarum* confers to its animal partner. By applying experimental evolution to a moderate *Drosophila* growth-promoting strain *L. plantarum*^{NIZO2877} (Schwarzer et al., 2016), we found that the *de novo* mutations



in the same acetate kinase (*ackA*) locus invariably emerge first and rapidly become fixed, and such evolution occurs with or without the host. Furthermore, we demonstrate that *ackA* mutations trigger the increased production of N-acetylated amino acids by the evolved strain, including N-acetyl-glutamine, a compound that is sufficient to confer improved *Drosophila* growth capabilities when provided together with the ancestral bacterial strain. Our study therefore identifies a specific mechanism by which a symbiotic bacterial strain increases its benefit to its animal host, and reveals that adaptation to the host diet is a foremost step in the emergence and perpetuation of facultative animal-microbe symbioses.

RESULTS

Experimental Evolution of *L. plantarum* with *D. melanogaster* Improves Its Growth-Promoting Effect

As growth promotion during chronic undernutrition is one of the major advantages conferred by *L. plantarum* to its animal host (Schwarzer et al., 2016; Storelli et al., 2011), we asked if and how this bacterium can increase its potential to support animal growth while both partners face chronic undernutrition.

To this end, we performed experimental evolution of NIZO2877 (*Lp*^{NIZO2877}), a strain of *L. plantarum* isolated from processed human food (Martino et al., 2015a), which was previously shown to moderately promote growth both in *Drosophila* and mice (Schwarzer et al., 2016). We mono-associated germ-free (GF) *Drosophila* eggs with a fully sequenced clonal population of *Lp*^{NIZO2877} on a low-nutritional diet and studied the partners for 20 *Drosophila* generations (i.e., 313 days, corresponding to about 2,000 bacterial generations; see STAR Methods and Figure S1). At each generation, we selected the first emerging pupae carrying a subpopulation of *L. plantarum* strains and transferred them to a new sterile diet (STAR Methods and Figure S1A). 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 that emerged from the transferred pupae, selected a representative set of isolates, and measured individually their growth-promoting capacity on an independent set of naive GF fly larvae. After only two fly generations (i.e., after about 124 bacterial generations, Figures 1A and 1B), we identified a few evolved *Lp*^{NIZO2877} strains that significantly improved larval growth and accelerated pupariation timing compared with the ancestor strain. Specifically, the evolved strains exhibited the same effect as *Lp*^{WJL}, a potent *L. plantarum* growth-promoting strain (Martino et al., 2015b; Storelli et al., 2011) (Figures 1A and 1B). These results show that the evolution of *Lp*^{NIZO2877} in the context of its symbiosis with *Drosophila* leads to the rapid improvement of *L. plantarum* animal growth promotion (Figures 1C and 1D).

Genome Sequencing Reveals the Appearance and Fixation of a Single Mutation in *L. plantarum ackA* Gene

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 (Table S1, replicate 1) with increased host growth-promoting

potential sampled across the 20 *Drosophila* generations. We identified a total of 11 mutations, including nine SNPs and two small deletions (Figure 1E and Table S2). 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 (Figure 1E). Following *ackA* mutation, additional variants appeared along *L. plantarum* experimental evolution, which seem to correlate with further improvement of symbiotic benefit (Figure 1A).

Independent Replicate of Experimental Evolution Confirms that *L. plantarum*^{NIZO2877} Improves Its Growth-Promoting Effect through Mutation of the *ackA* Gene

To test the repeatability of our findings, we conducted an independent replicate of *L. plantarum* experimental evolution while in symbiosis 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 mutations, including variants in the *ackA* gene (Figure 2 and Table S2). 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 (Figure S2). These independently isolated mutations likely generate an inactive *ackA* protein. Although both replicates of *L. plantarum* experimental evolution show additional mutations besides the *ackA* variant (Figures 1E and 2E), the two evolved strains each bearing only one mutation in *ackA* (FlyG2.1.8 and FlyG3.1.8) already showed a statistically significant *Drosophila* growth improvement compared with their ancestor (Figures 1A and 1B). This suggests that acquiring an *ackA* mutation is sufficient to confer an increased *Drosophila* growth-promotion potential. Therefore, we propose that the *de novo* appearance of the *ackA* mutation is the first fundamental step in shaping the evolutionary trajectory in the *Lp*^{NIZO2877}/*Drosophila* symbiosis model.

ackA Mutation Is Necessary to Improve *L. plantarum*^{NIZO2877} Growth-Promoting Effect

To fully establish that *ackA* mutation is responsible for the evolution of *Lp*^{NIZO2877}/*Drosophila* symbiosis, we employed CRISPR/Cas9-based bacterial genetic engineering (Jiang et al., 2013) to reinsert the deleted CCT triplet in the FlyG2.1.8 *ackA* locus (Figure S3), so that we genetically reverted the *ackA* allele in the FlyG2.1.8 isolate back to its ancestral form. The reverted strain (FlyG2.1.8^{Rev}) bearing the ancestral *ackA* allele lost its increased capacity to promote animal growth when compared with the ancestor strain (Figure 3). These results therefore demonstrate that the *ackA* mutation in *Lp*^{NIZO2877} is a causative change resulting in faster and increased *Drosophila* growth.

ackA Confers Competitive Advantage to *L. plantarum* Evolved Strains in Both Presence and Absence of the Host

To investigate the complete *L. plantarum* population dynamics while in symbiosis with *Drosophila*, we sequenced the

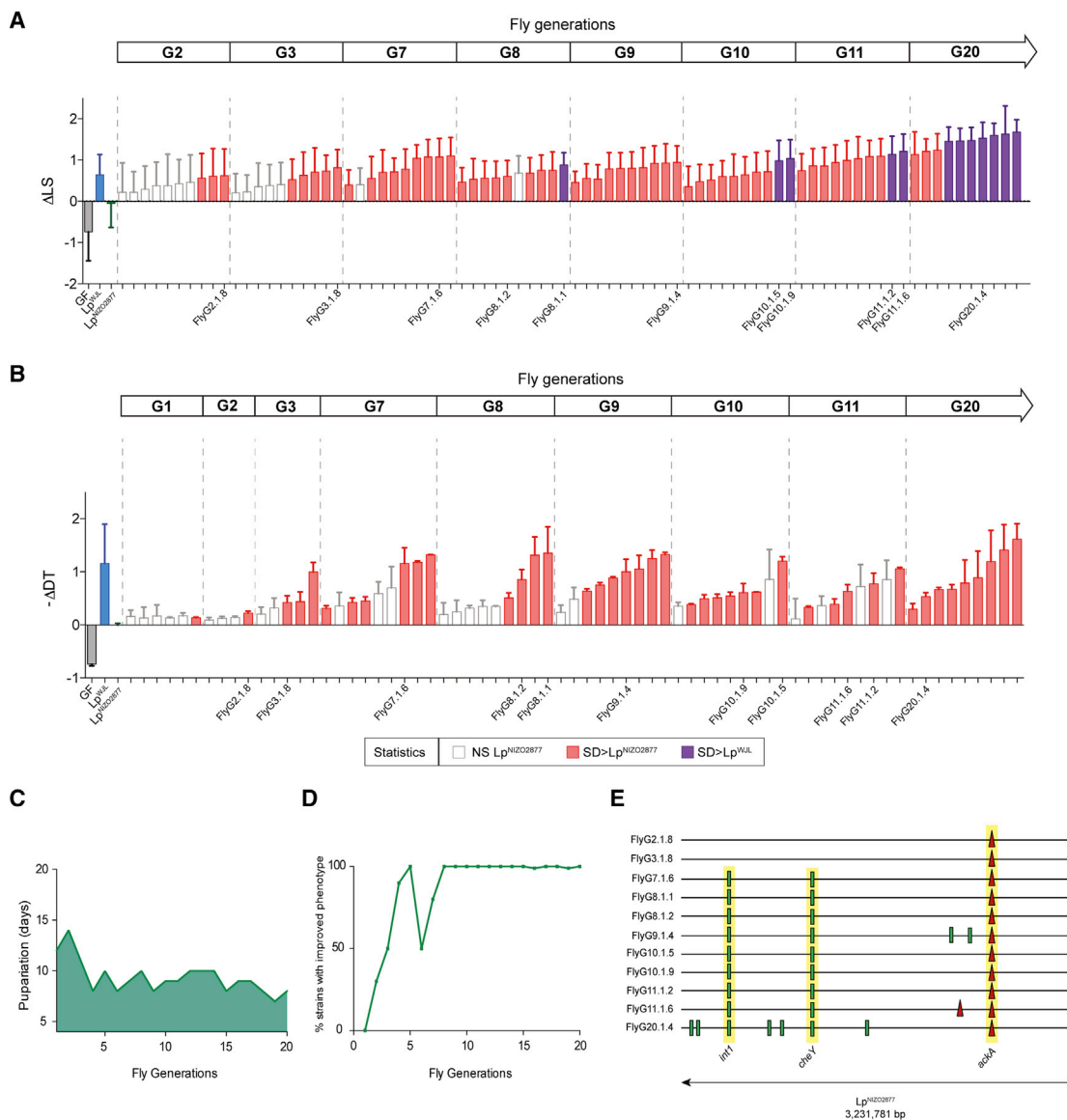


Figure 1. Experimental Evolution of *L. plantarum* with *D. melanogaster* Improves Its Growth-Promoting Effect

(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 in larval size (Δ LS) shows the difference between the size of larvae associated with the respective condition and the size of larvae associated with *Lp*^{NIZO2877} from *Drosophila* generation 2 (G2) to generation 20 (G20). Each bar refers to an *Lp*^{NIZO2877}-evolved strain isolated from the first replicate of experimental evolution from G2 to G20. *Lp*^{NIZO2877}-evolved strains that exhibited a significant difference (improved) at promoting larval growth compared with the ancestor strain (Student's t test: $p < 0.05$) are shown in red. *Lp*^{NIZO2877}-evolved strains that exhibited a significant difference (improved) at promoting larval growth compared with 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 in developmental timing ($-\Delta$ DT) is calculated from 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}, and shown in the graph. *Lp*^{NIZO2877}-evolved strains that exhibited a significant difference at accelerating DT compared with the ancestor strain (Student's t test: $p < 0.05$) are shown in red. The evolved strains that have been selected for further analyses are labeled on the x axis.

(C) Difference in maturation time of individuals associated with *Lp*^{NIZO2877}-evolved strains along the first replicate of *Lp*^{NIZO2877} adaptive evolution. The mean pupariation time of the first 15 individuals at each fly generation is shown on the y axis.

(D) Percentage of *Lp*^{NIZO2877}-evolved strains isolated at each fly generation that were found to be significantly better than the *Lp*^{NIZO2877} at increasing larval size. Ten bacterial isolates were randomly isolated at the end of each fly generation from newly emerged adult *Drosophila* (see Figure S1A) and reassociated with new GF *Drosophila* embryos to quantify their ability to promote larval growth (see A).

(E) Mutations identified in *Lp*^{NIZO2877}-evolved strains from *Drosophila* generation 2 (G2) to generation 20 (G20) represented along the *Lp*^{NIZO2877} genome. The genome of each evolved strain is represented as a horizontal line. Red triangles indicate deletions and small green bars show SNPs occurring in the same gene of different strains and fixed along the experimental evolution are highlighted in yellow (*int1*, *cheY*, *ackA*).

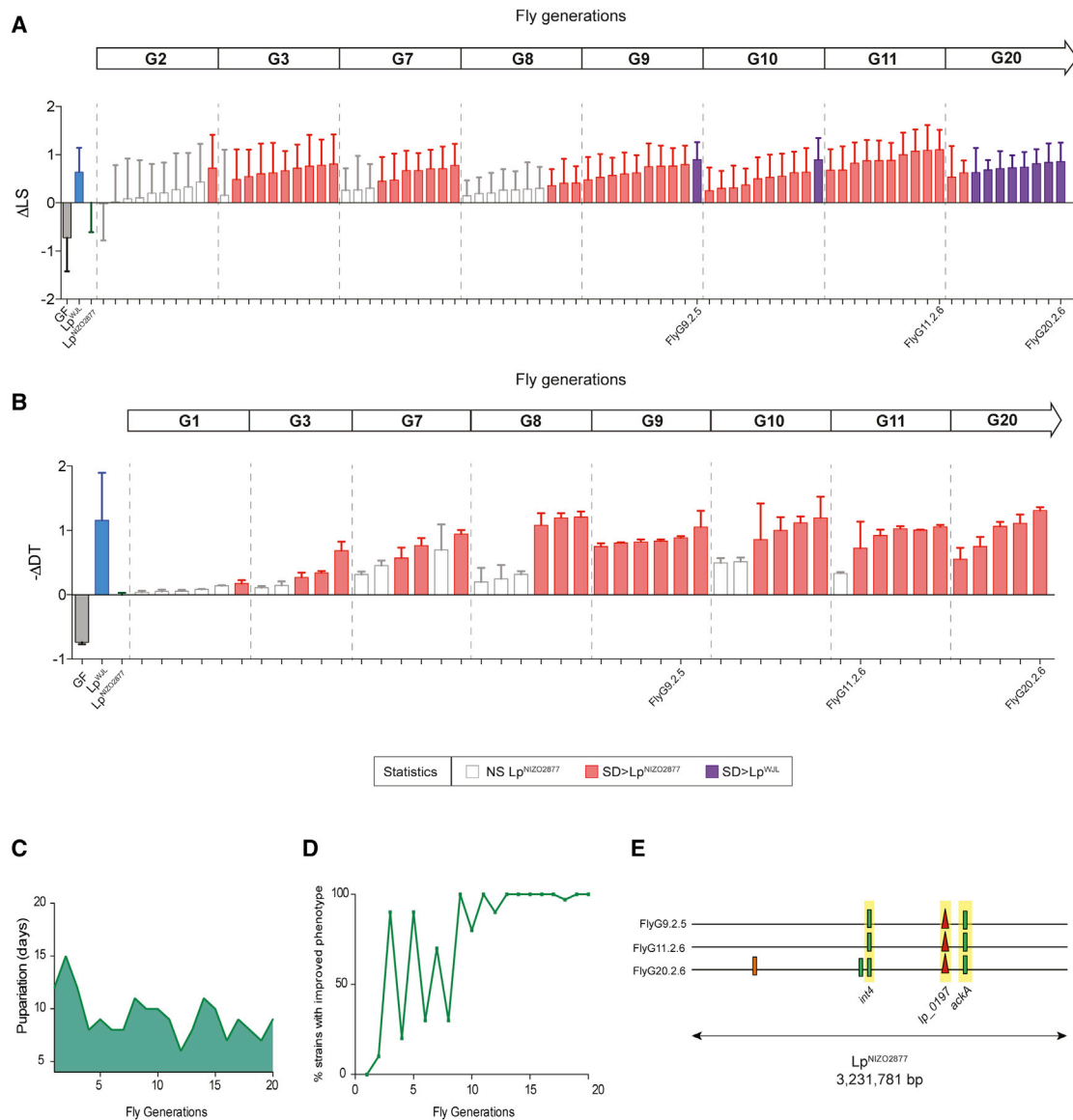


Figure 2. Second Replicate of $Lp^{NIZO2877}$ Adaptive Evolution Confirms the Ability of *L. plantarum* Evolved Strains to Improve Fly Growth

(A) Difference in longitudinal size of larvae (LS) measured 7 days AED on poor-nutrient diet. Larvae were kept GF or associated with $Lp^{NIZO2877}$, Lp^{WJL} , or $Lp^{NIZO2877}$ -evolved strains. The Delta in larval size (ΔLS) shows the difference between the size of larvae associated with the respective condition and the size of larvae associated with the ancestor strain (Student's t test: $p < 0.05$) are shown in red. $Lp^{NIZO2877}$ -evolved strains that exhibited a significant difference (improved) at promoting larval growth compared with 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 in developmental timing ($-\Delta DT$) is calculated from the mean time of emergence of 50% of the whole adult population associated with the respective condition and the mean time of emergence of 50% of the whole adult population associated with $Lp^{NIZO2877}$, and shown in the graph. $Lp^{NIZO2877}$ -evolved strains that exhibited a significant difference at accelerating developmental timing compared with the ancestor strain (Student's t test: $p < 0.05$) are shown in red. The evolved strains that have been selected for further analyses are labeled on the x axis.

(C) Difference in maturation time of individuals associated with $Lp^{NIZO2877}$ -evolved strains along the second replicate of $Lp^{NIZO2877}$ adaptive evolution. The pupariation time of the first 15 individuals at each fly generation is shown on the y axis.

(D) Percentage of $Lp^{NIZO2877}$ -evolved strains isolated at each fly generation that were found to be significantly better than the ancestor at increasing larval size during the second replicate of *L. plantarum* adaptive evolution. Ten bacterial isolates were randomly isolated at the end of each fly generation from newly emerged adult *Drosophila* (see Figure S1) and reassociated with new GF *Drosophila* embryos to quantify their ability to promote larval growth.

(E) Mutations identified in $Lp^{NIZO2877}$ -evolved strains isolated from the second replicate of experimental evolution from *Drosophila* generation 9 (G9) 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 SNPs. Mutations occurring in the same gene of different strains and fixed along the experimental evolution are highlighted in yellow (*int4*, *lp_0197*, *ackA*).

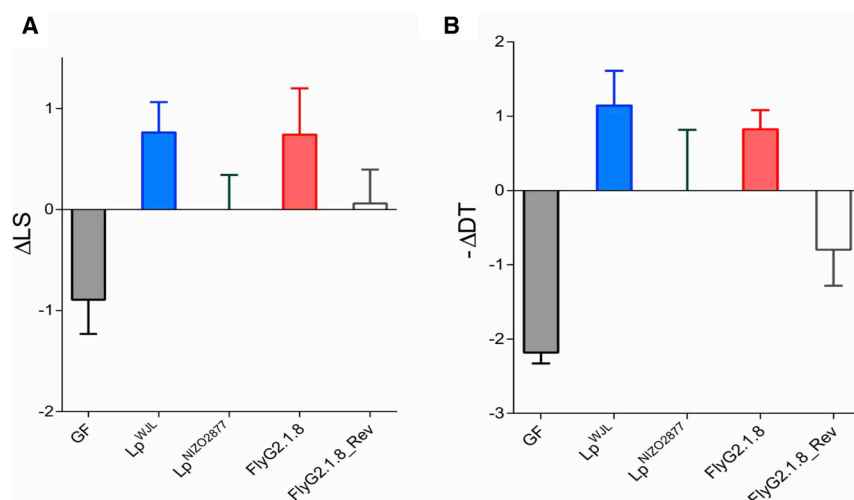


Figure 3. *ackA* Mutation Is Sufficient to Improve *L. plantarum*^{NIZO2877} Growth-Promoting Effect

(A) 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 in larval size (Δ LS), the difference between the size of larvae associated with the respective *L. plantarum* strain and the size of larvae associated with *Lp*^{NIZO2877}, is shown. FlyG8.2.1 strain, exhibiting a significant difference (improved) at promoting larval growth compared with *Lp*^{NIZO2877} (Student's t test: $p < 0.05$), is shown in red. Strains that did not exhibit a significant difference (improved) at promoting larval growth compared with *Lp*^{NIZO2877} are shown in white.

(B) 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 in developmental timing ($-\Delta$ DT) 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. FlyG8.2.1 strain, exhibiting a significant difference (improved) at promoting larval growth compared with *Lp*^{NIZO2877} (Student's t test: $p < 0.05$), is shown in red. Strains that did not exhibit a significant difference (improved) at promoting larval growth compared with *Lp*^{NIZO2877} are shown in white.

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 (STAR Methods). We found that the *ackA* mutation was the first variant to appear in the population. Remarkably, the *ackA* variant showed a rapid selective sweep and became fixed as early as after three *Drosophila* generations (Figure 4A). 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* (STAR Methods; Figures 4B and S4). We found that the evolved strain bearing only the *ackA* mutation started out-competing the ancestor strain as early as after 1 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 was absent (Figure 4C). To characterize the nature of such competitive advantage, we measured the growth rate of both strains on the *Drosophila* nutritional environment. We find that the evolved strain FlyG2.1.8 was able to replicate much faster than its ancestral strain *Lp*^{NIZO2877} in the *Drosophila* diet (Figure 4D), which contributes to establishing its competitive advantage. Taken together, these results show that *L. plantarum* evolved strains bearing the *ackA* variant shows higher fitness compared with their ancestor in our experimental settings, and that their competitive advantage is likely independent of the animal host.

***L. plantarum* Evolution and Improvement in Symbiotic Benefit Is Driven by the Adaptation to the Host Nutritional Environment Rather Than to Its Host**

To determine whether the animal host has an influence on the evolution of its symbiotic bacteria, we experimentally evolved

Lp^{NIZO2877} in the same low-yeast fly diet, but without *Drosophila* (STAR Methods and Figure S5) and tested the capacity of isolates sampled throughout the course of the experimental evolution to promote fly growth on a set of naive GF fly larvae. Strikingly, in two parallel experiments, the *Lp*^{NIZO2877} strains evolved in the absence of the host also show an increased ability to promote *Drosophila* growth when mono-associated with naive GF fly larvae (Figures 5A and 5B). Furthermore, genome sequencing of single evolved isolates from both experiments again revealed the acquisition of novel mutations in the *ackA* gene (Figures 5C and S5). Taken together, these findings show that the genomic evolution of *L. plantarum* is driven by the adaptation to the 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 fly diet, which ultimately results in the improvement of the *L. plantarum* symbiotic effect on *Drosophila*.

***L. plantarum* Improves *Drosophila* Growth through Secretion of N-Acetyl-Glutamine**

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 (Table S6), we observed a significant and robust increase in the levels of N-acetyl-amino acids in the diet processed by the evolved strain compared with the diet processed by the ancestral strain (Figure 6A). 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

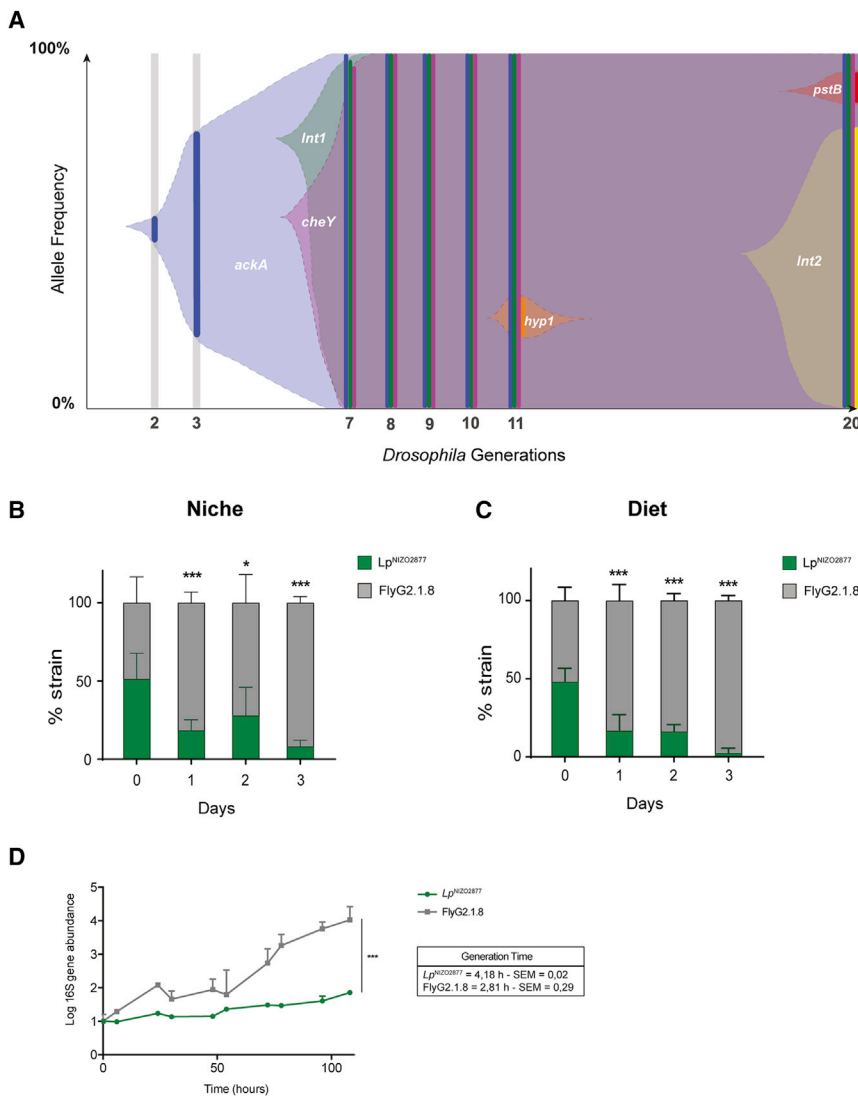


Figure 4. *Lp*^{NIZO2877}-Evolved Strain Shows Higher Fitness Compared with the Ancestor

(A) Muller diagram showing the genome evolutionary dynamics of *Lp*^{NIZO2877} population (replicate I) along 20 *Drosophila* generations. The y axis shows the percentage of the detected frequencies of each mutation (plain colors). Shaded areas represent the inferred allele frequencies. Lower axis shows the fly generation where the sampling took place.

(B and C) 1:1 competitive assay between *Lp*^{NIZO2877} and *Lp*^{NIZO2877}-evolved strain (FlyG2.1.8) in poor-nutrient diet with *Drosophila* larvae (B) and without *Drosophila* larvae (C). Error 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. (D) 16S rRNA kinetics of *Lp*^{NIZO2877} and FlyG2.1.8 in the *Drosophila* nutritional environment. Absolute quantification of the 16rRNA gene (ng/ μ L) has been conducted for each time point. 16S rRNA gene quantification is shown in logarithmic scale. The values have been normalized by the mean of t0 (time 0). The mean generation time (h) of each strain \pm SEM is reported on the graph (see STAR Methods). The result of the non-parametric analysis of covariance (*sm.ancova* function in R) between the curves is reported (****p* < 0.005).

DISCUSSION

Our results uncover the nature of the adaptive process of *L. plantarum* while in symbiosis with its fly host. We report 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 a facultative nutritional symbiosis. In our experimental context, the dietary substrate asserts the predomi-

nant selective pressure dictating the evolutionary change of facultative symbiotic bacteria and their consequent benefits to host physiology. Rapid adaptation of the *L. plantarum*^{NIZO2877} strain 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 a spectacular case of parallel evolution, indicating that the *ackA* mutation is the preferred or possibly the unique means for *L. plantarum*^{NIZO2877} to adapt to its host nutritional environment. Our experimental settings represent a harsh nutritional condition, which only allows *L. plantarum* slow growth (calculated generation time: 3.2 hr; Figure S1B). It was shown that the expression of *L. plantarum* *ackA* (*ack2* in the *L. plantarum* reference strain WCFS1) is downregulated at low growth rates, suggesting that silencing *ackA* would be required to cope with poor growth condition (Goffin et al., 2010). This observation may explain the observed strong selection pressure on *ackA* in our experimental settings, which led to the rapid *de novo* emergence of variants in the population (Figures 1 and 2). *ackA* mutation significantly improved *L. plantarum* fitness on

manner in the diet, the ancestor strain *Lp*^{NIZO2877} is now able to recapitulate the beneficial effect conferred by FlyG2.1.8 on *Drosophila* growth (Figure 6B). However, the molecule alone is not sufficient to improve the growth of GF flies at low concentrations, but benefits the larvae when added at the highest concentration (10g/L) (Figure 6B). 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 0.1 g/L of N-acetyl-glutamine (a concentration sufficient to confer improved symbiotic benefit to the ancestral strain). We find that FlyG2.1.8 still outcompetes the ancestor strain even in presence of N-acetyl-glutamine (Figure 6C). This result indicates that N-acetyl-glutamine does not confer a competitive advantage to *Lp*^{NIZO2877} over FlyG2.1.8 while growing on the diet; 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 enhance *Drosophila* growth but not *Lp*^{NIZO2877} fitness.

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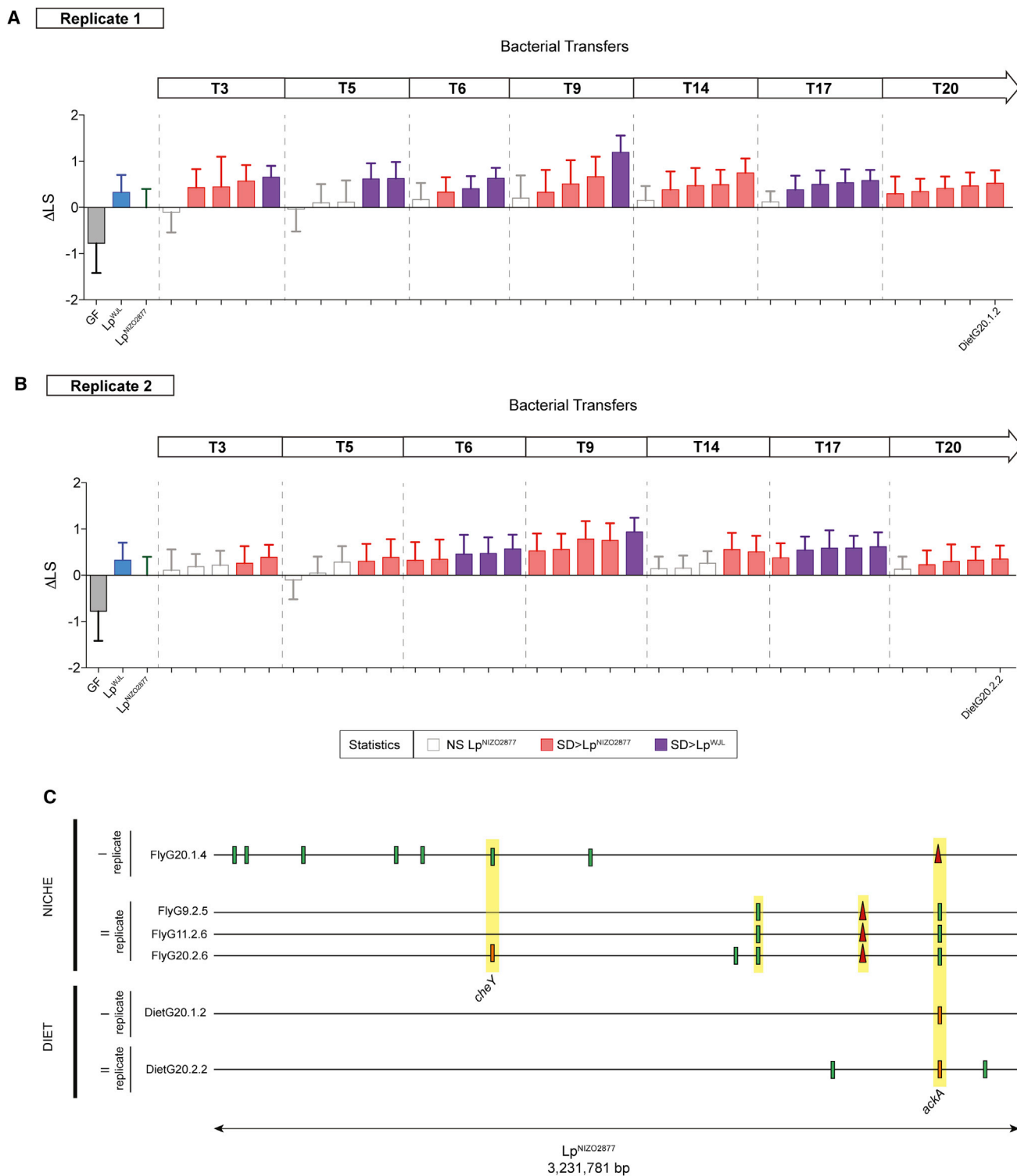


Figure 5. $Lp^{NIZO2877}$ Adaptation to the Diet Increases Its Host's Growth

(A and B) Longitudinal size of larvae (LS) measured 7 days AED on poor-nutrient diet. Larvae were kept germ-free (GF) or associated with $Lp^{NIZO2877}$, Lp^{WJL} and with $Lp^{NIZO2877}$ -evolved strains evolved in poor-nutrient diet in the absence of *Drosophila*. The Delta in larval size (ΔLS) shows the difference between the size of larvae associated with $Lp^{NIZO2877}$ -evolved strains and the size of larvae associated with $Lp^{NIZO2877}$ from transfer 3 (T3) to transfer 20 (T20) for the first replicate (A) and the second replicate (B) of evolution. $Lp^{NIZO2877}$ -evolved strains that exhibited a significant difference (improved) at promoting larval growth compared with the ancestor strain (Student's t test: $p < 0.05$) are shown in red. $Lp^{NIZO2877}$ -evolved strains that exhibited a significant difference (improved) at promoting larval growth compared with the beneficial *L. plantarum* Lp^{WJL} strain are shown in purple. The evolved strains that have been selected for further analyses are labeled on the x axis.

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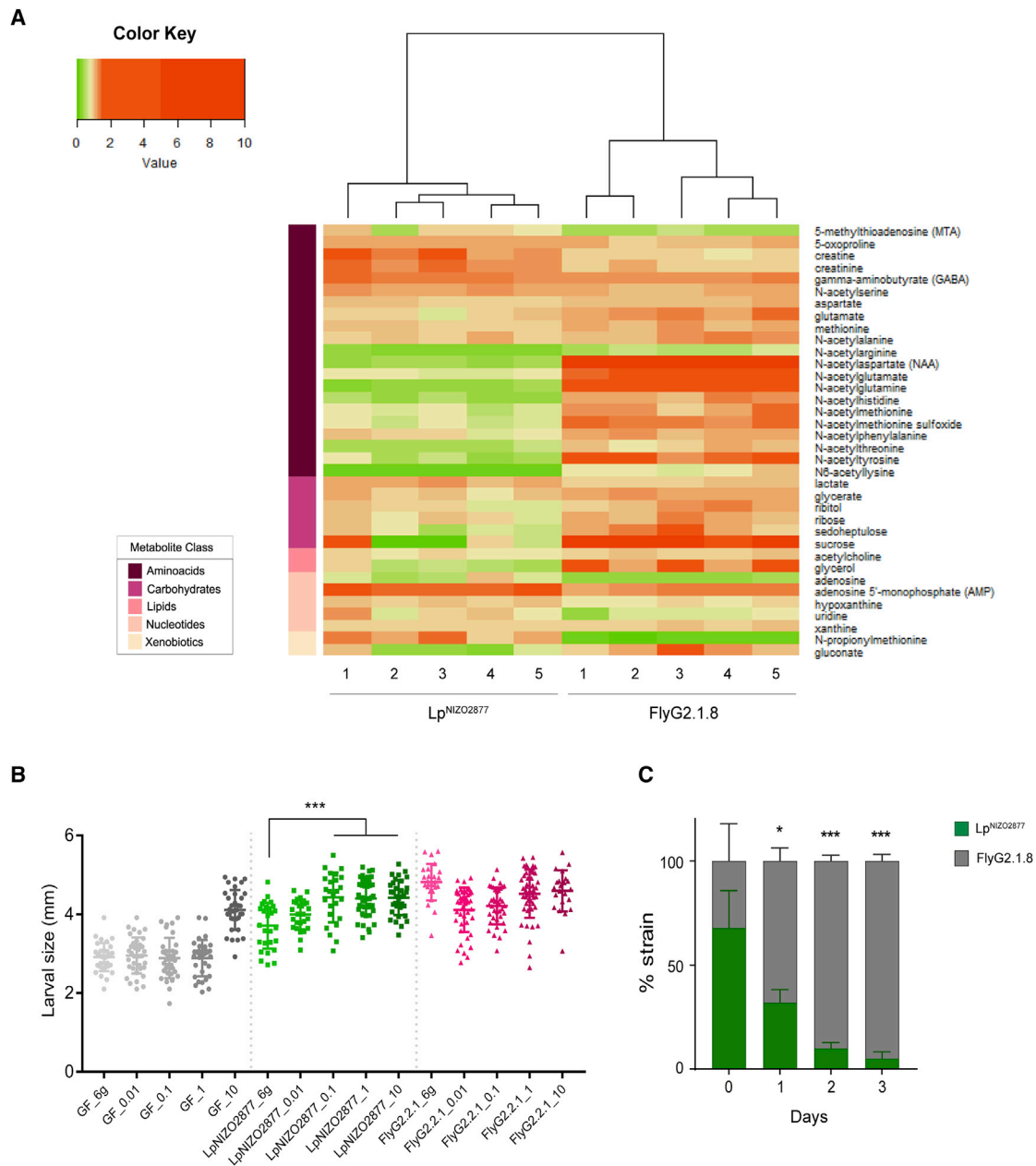


Figure 6. N-Acetyl-Glutamine Recapitulates the Beneficial Effect of FlyG2.1.8 on $Lp^{NIZO2877}$ -Associated Larvae

(A) Heatmap showing the metabolites that differ significantly between experimental groups ($Lp^{NIZO2877}$ and FlyG2.1.8) (two-sided t tests, $p < 0.05$). The heatmap 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 AED on poor-nutrient diet supplemented with different concentrations (g/L) of N-acetyl-glutamine (x axis). Larvae were kept germ-free (GF) or associated with $Lp^{NIZO2877}$ (ancestor) and with Fly.G2.1.8 (evolved strain). Larval size is shown as mean \pm SEM. *** $p < 0.01$.

(C) 1:1 competitive assay between $Lp^{NIZO2877}$ and $Lp^{NIZO2877}$ -evolved strain (FlyG2.1.8) in poor-nutrient diet supplemented with 0.1 g/L of N-acetyl-glutamine. Error bars represent the percentage of each strain detected by qPCR. * $p < 0.05$, *** $p < 0.01$, obtained by Student's t test.

the fly diet (Figure 4D); this conferred a strong competitive advantage to the evolved strains bearing these mutations and led to their fixation (Figure 4). The appearance of the *ackA* variants pro-

duced a significant modification of *L. plantarum* metabolite production, leading to the accumulation of N-acetyl-glutamine. This molecule does not per se improve bacterial fitness, so it

(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 show SNPs. Different colors 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 labeled (*cheY*, *ackA*).

remains elusive how *ackA* variants confers competitive advantage to *L. plantarum* cells on the fly diet. Nevertheless, N-acetyl-glutamine is directly involved in *L. plantarum/Drosophila* symbiosis, as it is sufficient to improve *L. plantarum* benefit on fly growth (Figure 6B). Our results indicate that *ackA* mutations possibly cause a shift in the metabolism of *L. plantarum* by modifying the usage of cellular acetyl groups, which would confer benefits to *L. plantarum* growth on the fly diet, thus improving its symbiotic effect. *ackA* participates in the reversible conversion of acetate to acetyl-phosphate; *ackA* variants might impede this reaction, and 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. These interpretations stem from the hypothesis that all the *ackA* variants obtained along *L. plantarum* experimental evolution likely generate inactive proteins (Figures S2 and S5B). Nevertheless, further work is needed to establish that all the variants lead to strict loss of function of *ackA*. Generating an *ackA* knockout in the ancestral *Lp*^{NIZO2877} strain, measuring the activity of *ackA* protein variants, and probing the metabolic consequences of *ackA* variants in all the ancestral and evolved strains will likely provide insights into the specific molecular mechanisms underlying our findings.

Our results identify *ackA* as the first target of selection exerted by the nutritional environment on *Lp*^{NIZO2877}. Of note, *Lp*^{WJL}, a potent growth-promoting strain isolated from *D. melanogaster* gut (Martino et al., 2015b; Ryu et al., 2008; Storelli et al., 2011), shows two nucleotide substitutions in the *ackA* gene, compared with *Lp*^{NIZO2877} (Figure S6), which might concur with its high beneficial effect in our experimental settings. Yet, due to the high genetic diversity of *L. plantarum* strains (Martino et al., 2016), we posit that such a genetic target hinges upon the genomic background of *Lp*^{NIZO2877}. According to their specific network of genetic polymorphisms, other non-beneficial isolates might fix mutations affecting other genes in order to adapt to the host environment and improve their fitness.

Understanding how evolutionary forces shape host-microbe symbiosis is essential to comprehend the mechanisms of their functional influence. Using the facultative nutritional mutualism between *Drosophila* and *L. plantarum* as a model, our results reveal that the primary selection pressure acting on *L. plantarum* originates from the nutritional substrate alone, which is strong enough to drive the rapid fixation of a *de novo* mutation. The resulting genetic changes confer a fitness advantage to the evolved bacteria and trigger a metabolic adaptation in bacterial cells, which is quickly capitalized by *Drosophila* as a physiological growth advantage, hence symbiosis can be perpetuated. This is a clear example of by-product mutualism, whereby animal hosts enjoy benefits from the by-products of the self-serving traits of their microbial symbionts (Bronstein, 1994; Connor, 1995; Holland and Bronstein, 2008). By showing that bacterial adaptation to the host nutritional medium results in a higher microbial competitive advantage and improvement of symbiotic benefit, we posit that such a process represents the first step in the emergence and evolution of facultative mutualism. Our results do not rule out the possibility that the animal host might exert additional selection pressure on its bacterial partners. Indeed, *Drosophila* is also known to directly affect the

fitness of its own microbiota through the activity of innate immune effectors (Guo et al., 2014; Ryu et al., 2008) or the secretion of bacterial maintenance factors (Storelli et al., 2018). Nevertheless, our findings demonstrate the utmost importance of the shared nutritional substrate in the evolution of *Drosophila-L. plantarum* symbiosis.

Symbiosis is an evolutionary imperative and facultative symbioses are widespread in nature. Despite their unequivocal diversity, animal-microbe symbioses share striking similarities (Foster et al., 2017) and nutrition often plays a major role in shaping the composition of symbiotic microbial communities (Conlon and Bird, 2015; David et al., 2015; Groussin et al., 2017; Hacquard et al., 2015; Lozupone et al., 2012; Muegge et al., 2011). Our results provide direct experimental evidence that nutrition drives the evolution of a bacterial symbiont and, given that other animal and microbe partners have likely faced nutritional challenges over time, common evolutionary trajectories might have occurred. We therefore posit that bacterial adaptation to the diet can be the first step in the emergence and perpetuation of facultative animal-microbe symbioses. Our work provides another angle from which to help unravel the complex adaptive processes in the context of evolving symbiosis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and six tables and can be found with this article online at <https://doi.org/10.1016/j.chom.2018.06.001>.

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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.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.

DECLARATION OF INTERESTS

M.E.M. and F.L. are inventors of a pending patent application (INPI-n° FR1757717) which applies to bacterial strains presented in this article.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>L. plantarum</i> : Lp ^{NIZO2877}	Martino et al., 2015a	LKHZ01000000
<i>L. plantarum</i> : Lp ^{WJL}	Martino et al., 2015a, 2015b	LKLZ00000000
<i>L. plantarum</i> : FlyG2.1.8	This paper	PEBE00000000
<i>L. plantarum</i> : FlyG3.1.8	This paper	PEGI00000000
<i>L. plantarum</i> : FlyG7.1.6	This paper	PEGJ00000000
<i>L. plantarum</i> : FlyG8.1.1	This paper	PEGK00000000
<i>L. plantarum</i> : FlyG8.1.2	This paper	PEGL00000000
<i>L. plantarum</i> : FlyG9.1.4	This paper	PEGM00000000
<i>L. plantarum</i> : FlyG10.1.5	This paper	PEGN00000000
<i>L. plantarum</i> : FlyG10.1.9	This paper	PEGO00000000
<i>L. plantarum</i> : FlyG11.1.2	This paper	PEGP00000000
<i>L. plantarum</i> : FlyG11.1.6	This paper	PEGQ00000000
<i>L. plantarum</i> : FlyG20.1.4	This paper	PEGR00000000
<i>L. plantarum</i> : FlyG2.1.8Rev	This paper	N/A
<i>L. plantarum</i> : FlyG9.2.5	This paper	PEGS00000000
<i>L. plantarum</i> : FlyG11.2.6	This paper	PEGT00000000
<i>L. plantarum</i> : FlyG20.2.6	This paper	PEGU00000000
<i>L. plantarum</i> : DietG20.1.2	This paper	PEGV00000000
<i>L. plantarum</i> : DietG20.2.2	This paper	PEGW00000000
<i>E. coli</i> E135	Zhang et al., 2012	N/A
Chemicals, Peptides, and Recombinant Proteins		
Inactivated Dried Yeast	Bio Springer	Springaline BA95/0-PW
Cornmeal	Westhove	Farigel maize H1
Agar	VWR	#20768.361
Methylparaben Sodium Salt	MERCK	#106756
Propionic Acid	Sigma-Aldrich	P1386
Man, Rogosa and Sharpe (MRS) Broth Medium	Difco	#288130
Man, Rogosa and Sharpe (MRS) Agar Medium	Difco	#288210
N-Acetyl Glutamine	Sigma-Aldrich	A9125-25G
PBS	Dutscher	NA.25
Glycerol	Sigma-Aldrich	G5516
SuperScript™ II Reverse Transcriptase	Invitrogen, ThermoFisher Scientific	18064014
SYBR GreenER™ qPCR SuperMix Universal	Invitrogen, ThermoFisher Scientific	1176202K
Rifampicin	Sigma-Aldrich	R3501
Chloramphenicol	Sigma-Aldrich	C0378
Erythromycin	Sigma-Aldrich	E1300000
Ampicillin	Sigma-Aldrich	A9393
Glycine	Sigma-Aldrich	67419
Sucrose	Sigma-Aldrich	S7903
Magnesium chloride	Sigma-Aldrich	M8266
Critical Commercial Assays		
NucleoSpin RNA Isolation Kit	Macherey-Nagel	740955.240C
UltraClean Microbial DNA Isolation Kit	MOBIO Laboratories, Inc.	12224-250

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ion Xpress™ Plus Fragment Library Kit	Ion Torrent	4471269
Gibson Assembly Master Mix	NEB	E2611S
T4 Polynucleotide Kinase	NEB	M0201S
T4 DNA Ligase	NEB	M0202S
Deposited Data		
Metabolomic Dataset of Diet	This paper	Table S3
<i>L. plantarum</i> FlyG2.1.8 genome	This paper	PEBE00000000
<i>L. plantarum</i> FlyG3.1.8 genome	This paper	PEGI00000000
<i>L. plantarum</i> FlyG7.1.6 genome	This paper	PEGJ00000000
<i>L. plantarum</i> FlyG8.1.1 genome	This paper	PEGK00000000
<i>L. plantarum</i> FlyG8.1.2 genome	This paper	PEGL00000000
<i>L. plantarum</i> FlyG9.1.4 genome	This paper	PEGM00000000
<i>L. plantarum</i> FlyG10.1.5 genome	This paper	PEGN00000000
<i>L. plantarum</i> FlyG10.1.9 genome	This paper	PEGO00000000
<i>L. plantarum</i> FlyG11.1.2 genome	This paper	PEGP00000000
<i>L. plantarum</i> FlyG11.1.6 genome	This paper	PEGQ00000000
<i>L. plantarum</i> FlyG20.1.4 genome	This paper	PEGR00000000
<i>L. plantarum</i> FlyG9.2.5 genome	This paper	PEGS00000000
<i>L. plantarum</i> FlyG11.2.6 genome	This paper	PEGT00000000
<i>L. plantarum</i> FlyG20.2.6 genome	This paper	PEGU00000000
<i>L. plantarum</i> DietG20.1.2 genome	This paper	PEGV00000000
<i>L. plantarum</i> DietG20.2.2 genome	This paper	PEGW00000000
Experimental Models: Organisms/Strains		
<i>D.melanogaster</i> : y,w (reference strain for this work)		N/A
Oligonucleotides		
Primer: 16S_UniF: GTGSTGCAYGGYTGTGCGTCA	Packey et al., 2013	N/A
Primer: 16S_UniR: ACGTCRTCCMCACCTTCCTC	Packey et al., 2013	N/A
Primers for SNP verification, see Table S4	This paper	N/A
Primers for competition tests, see Table S4	This paper	N/A
Primers for engineering <i>L. plantarum</i> with CRISPR-Cas9, see Table S6	This paper	N/A
Recombinant DNA		
Plasmids used to engineer <i>L. plantarum</i> with CRISPR-Cas9, see Table S5	This paper	N/A
pJP005		CB651
pMSP3545		Addgene 46888
pCas9	Jiang et al., 2013	Addgene 42876
Software and Algorithms		
ImageJ	NIH Image	https://imagej.net/ImageJ
Leica application suite (LAS)	Leica	N/A
Scan 1200 Automatic HD colony counter and Software	Intersciences	Ref. 437 000
Breseq	Deatherage and Barrick, 2014	http://barricklab.org/twiki/bin/view/Lab/ToolsBacterialGenomeResequencing
R Studio	RStudio Team, 2015	https://www.rstudio.com/
PROVEAN	Choi et al., 2012	http://provean.jcvi.org
Geneious	Kearse et al., 2012	https://www.geneious.com
Gibson assembly	Choi et al., 2012	http://provean.jcvi.org

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, François Leulier (francois.leulier@ens-lyon.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

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 ([Martino et al., 2015a](#)). 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. \(2011\)](#).

Fly Diets Used in This Study

Poor Yeast Diet: 8g inactivated dried yeast, 80g cornmeal, 7.2g Agar, 5.2g methylparaben sodium salt, 4 mL 99% propionic acid for 1 litre.

PYD + N-acetyl-Glutamine. Fly food is prepared by mixing 8g of inactive dried yeast, 80g of cornmeal, 7.2g of agar, 5.2g of methylparaben sodium salt, 4 mL of 99% propionic acid in 800 mL water. After cooking and before solidification, fly food is mixed with serial dilutions of N-acetyl-Glutamine solution (prepared from a stock solution at 1g N-acetyl-Glutamine/L sterile water). Fly food is then mixed vigorously by vortexing, and then poured in microtubes.

Fly food was poured in petri dishes (diameter=55mm; fly food volume ≈ 7ml) to grow larvae used for larval longitudinal length analysis. Fly food was poured in 50 ml tubes for Experimental Evolution setup. Fly food was poured in 1.5 ml microtubes (fly food volume=100µl) for metabolites profiling.

Colonization and Infection of Larvae

40 embryos collected from GF females were transferred to a fresh poor nutrient GF medium in a 55 mm petri dish. 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 x10⁷ CFU) of the respective bacteria. Emerging larvae were allowed to develop on the contaminated medium at 25°C.

METHOD DETAILS

Experimental Evolution Design

The experimental “Adaptive Evolution” (AE) model of *L. plantarum* in *Drosophila* Niche (*Drosophila* + Diet) was designed as follows: for the first generation of the AE model, 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 in MRS broth to stationary phase (18h). The culture was washed in sterile PBS and 300 µl of PBS-washed culture containing 10⁸ CFU of *L. plantarum*^{NIZO2877} were added directly on the embryos and the fly food (bacterial load = 10⁷ CFU/ml) ([Figure S1A](#)). No further inoculation of the ancestor strain *L. plantarum*^{NIZO2877} has been performed after the beginning of the first generation until the end of the experimental evolution. Emerging larvae were allowed to develop on the medium inoculated with the bacterial culture 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 10⁶ CFU/ml) was also indirectly transferred to the new medium by inoculation 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 ancestral 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 (10⁶ CFU) were added directly on 100 µl of poor nutrient GF diet (bacterial load = 10⁷ CFU/ml) ([Figure S5A](#)) 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 μ l of PBS using glass beads and 10 μ l of the crushed medium (10^5 CFU) were used to inoculate 100 μ l 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. (Schwarzer et al., 2016).

Generation Time of *L. plantarum*

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 (Poulsen et al., 1995). *L. plantarum* strains were cultured to stationary phase (18h) and washed in sterile PBS. Serial dilutions have been prepared and 5 μ l containing a total of 10^3 colony-forming units (CFUs) were added to 100 μ l 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 μ l on a Biorad CFX96 apparatus (Biorad) using SYBR GreenER qPCR Supermix (Invitrogen, USA). The reaction mixture consisted of 0.5 μ l of each primer (10 μ M each), 12.5 μ l of SYBR GreenER mix, 10 μ l of water and 1.5 μ l 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/ μ l of cDNA extracted from *L. plantarum*^{NIZO2877} culture) were quantified by Real-time PCR using universal 16S primers (forward primer, UniF 5'-GTGSTGCAYGGYTGTCTCA-3' and reverse primer, UniR 5'-ACGTCRTCCMCACCTTCCTC-3', Table S4) (Packey et al., 2013). 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 et al. (Widdel, 2007).

Larval Size Measurements

Larvae ($n \geq 120$) 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 stereomicroscope M205FA and larval longitudinal size (length) was measured using ImageJ software (Schneider et al., 2012).

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 (Deatherage and Barrick, 2014) 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 (RStudio Team, 2015) 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) (Choi et al., 2012) 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 (Table S5) and the Cas9+tracrRNA from pCas9 with oligos oRL1-oRL4 (Table S6). These PCR fragments were stitched together using Gibson assembly (Gibson et al., 2009) (NEB CN#E2611S) 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 (Figure S3A). The targeting sgRNA was synthesized as a repeat-spacer-repeat array under a constitutive *Lactobacillus* promoter (P_{pgm}) (Duong et al., 2011). 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 (Figure S3B).

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 (Table S5). Colony PCR was used to screen for successful clones using oligos oRL09-10. Colony PCR was performed by picking a single colony into 20 μ L of 20 mM NaOH and incubating at 98°C for 20 minutes. These tubes were then microwaved for 1 minute with the cap open, and 5 μ L 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 and MRS agar and incubated at 37°C. Antibiotic concentrations for *L. plantarum* were as follows: rifampicin (25 μ g/mL), chloramphenicol (10 μ g/mL), and erythromycin (10 μ g/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 37°C. Plasmids were maintained at the following antibiotic concentrations: erythromycin (50 μ g/mL), chloramphenicol (34 μ g/mL), and ampicillin (50 μ g/mL). Electroporation of *L. plantarum* was adapted from numerous protocols (Spath et al., 2012; Teresa Alegre et al., 2004; Thompson and Collins, 1996). 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 to prevent aeration of the bacteria. These tubes were cultured at 37°C and 250 RPM for ~3 hours, or until the OD₆₀₀ was approximately 0.85. Cells were centrifuged at 5,000 RPM for 10 minutes at 4°C. The pelleted cells were kept on ice, then washed twice with ice-cold 10 mM MgCl₂ followed by a wash in SacGly (10% glycerol with 0.5 M sucrose). The washed cell pellet was then re-suspended in 1 mL of SacGly and centrifuged at 20,000 RPM for 1 minute and the final pellet was re-suspended in 500 μ L of SacGly. For all transformations, 60 μ L of this suspension was added to a 1-mm gap cuvette and transformed at 1.8kV, 200 Ω resistance, and 25 μ F capacitance. Following electroporation, cells were resuspended in 1 mL of MRS broth and transferred to a sterile tube and incubated at 37°C without shaking for 3 hours. 250 μ L 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 5 μ g of p3545_Cas9 or pCas+RSR isolated from a methylation-free *E. coli* strain (EC135) (Zhang et al., 2012). Transformation efficiencies of the Cas9 plasmid into Fly.G2.1.8 were vastly improved in the presence of the homologous-recombination template (Figure S3C). 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 (Figure S3D). Seven colonies harbored the inserted CCT triplet (FlyG2.1.8^{Rev}, Figure S3D), 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 (Gomaa et al., 2014). To clear the plasmids from the edited strains, colonies were subjected to multiple rounds of non-selective outgrowths in liquid medium (Figure S3E). 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 (Table S1).

Bacterial 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 10⁴ 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 μ L 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 *ackA* gene sequence using Geneious 9 (Kearse et al., 2012). Quantitative PCR was performed in a total of 20 μ l 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*; Table S4). The reaction mixture consisted of 0.5 μ l of each primer (10 μ M each), 12.5 μ l of SYBR GreenER mix, 10 μ l of water and 1.5 μ l 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/ μ l. Relative quantification of each bacterial strain has been performed using 16SrRNA as reference gene (UniF-UniR primers, Table S4).

Metabolite Profiling

Microtubes containing axenic poor nutrient diet were inoculated with bacterial suspension (10³ 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.

Information Related to Experimental Design

Blinding was not used in the course of our study. No data or subjects were excluded from our analyses.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data representation and statistical analysis were performed using Graphpad PRISM 6 software (www.graphpad.com). For metabolite profiling, we performed Student's t test with Welch correction to determine if differences in metabolites levels between two conditions are statistically significant. For all the other pairwise comparisons throughout our study, we performed Mann Whitney's test. We applied Kruskal Wallis test to perform statistical analyses of multiple ($n > 2$) conditions. No particular method was used to determine whether the data met assumptions of the statistical approach.

DATA AND SOFTWARE AVAILABILITY

The accession number for *FlyG2.1.8* genome reported in this paper is NCBI: PEBE00000000. The accession number for *FlyG3.1.8* genome reported in this paper is NCBI: PEGI00000000. The accession number for *FlyG7.1.6* genome reported in this paper is NCBI: PEGJ00000000. The accession number for *FlyG8.1.1* genome reported in this paper is NCBI: PEGK00000000. The accession number for *FlyG8.1.2* genome reported in this paper is NCBI: PEGL00000000. The accession number for *FlyG9.1.4* genome reported in this paper is NCBI: PEGM00000000. The accession number for *FlyG10.1.5* genome reported in this paper is NCBI: PEGN00000000. The accession number for *FlyG10.1.9* genome reported in this paper is NCBI: PEGO00000000. The accession number for *FlyG11.1.2* genome reported in this paper is NCBI: PEGP00000000. The accession number for *FlyG11.1.6* genome reported in this paper is NCBI: PEGQ00000000. The accession number for *FlyG20.1.4* genome reported in this paper is NCBI: PEGR00000000. The accession number for *FlyG9.2.5* genome reported in this paper is NCBI: PEGS00000000. The accession number for *FlyG11.2.6* genome reported in this paper is NCBI: PEGT00000000. The accession number for *FlyG20.2.6* genome reported in this paper is NCBI: PEGU00000000. The accession number for *DietG20.1.2* genome reported in this paper is NCBI: PEGV00000000. The accession number for *DietG20.2.2* genome reported in this paper is NCBI: PEGW00000000.