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Environmental temperature is a strong driver of subspecies competition in the *Drosophila* microbiome

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eLife Assessment

This **valuable** study explores changes in the Drosophila microbiome in response to environmental temperature over more than ten years. The evidence showing that temperature leads to diversification of bacterial clades is **solid**, but additional information would help clarify how subspecies competition impacts microbiome composition and the host. The work will interest researchers working with microbiomes, microbial ecology, and evolutionary biology.

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Abstract

Most microbiome research focusses on the taxonomic composition at the species level to understand the impact of environmental factors, but intraspecific diversity has largely been ignored. To address this significant knowledge gap, we took advantage of the simple, culturable microbiome of *Drosophila*. First, we documented that natural populations of *D. simulans* harbor three diverged clades of *Lactiplantibacillus plantarum*, a key nutritional symbiont. We studied the distinct ecological roles of these three clades by exposing flies with their native microbiome to two temperature regimes in the laboratory. Tracking the three clades within the complete *Drosophila* microbiome over a period of more than 10 years at two temperatures, we identified strikingly distinct dynamics in response to the selection regime. We confirmed the functional differentiation of the three clades using *in vitro* growth dynamics and *in vivo* mono-association assays. Our results highlight that environmental selection operates at the subspecies level. Therefore, we conclude that the functional diversification of the microbiome can only be understood when intra– and interspecific diversity is considered.

Introduction

Intraspecific diversity has largely been overlooked in microbial ecology surveys. This can be in part attributed to the success of 16S rRNA profiling, which allows the characterization of species diversity with a very high efficiency (1,2), but cannot identify intraspecific diversity (3). Although environmental variation has been identified as one of the major factors driving species composition, its role for intraspecific adaptation has been largely overlooked (3). This blind spot of microbiome research is particularly surprising as it is known that conspecific strains, i.e. strains from the same species, can display large phenotypic variation (4). Clinically relevant species like *Escherichia coli*, in which different subspecies can be either pathogenic or commensal are a particularly illustrative example (5).

Although a few studies in environmental microbial communities have provided evidence for the functional importance of intraspecific variation (6,7), such analyses are considerably more difficult. Therefore, descriptive correlative studies are more common than functional assays (8). In

the gut-dwelling bacterium *B. fragilis* conspecific strains compete for the same niche leading to the mutual exclusion of toxicogenic and non-toxicogenic strains in the gut environment (9). Not only in the human gut, but also in lakes, soil, and the sea conspecific strains frequently co-occur in sympatry (10–12). It has even been even reported that strain richness, i.e. sympatric strain diversity within the same species, stabilizes microbial communities in fluctuating environments (13,14).

In this work, we further explored the functional implications of intraspecific diversity within the same microbial population. We focused on the species *Lactiplantibacillus plantarum*, an extremely versatile lactic acid bacterium that has been associated with a variety of different habitats, including plants, the gastro-intestinal tracts of mammals and insects, as well as food such as meat, dairy, and pickled products (15). *L. plantarum* is a facultative symbiont of *Drosophila* (16–19). *Drosophila* larvae can enhance the growth of *L. plantarum*, which in response releases essential nutrients for the larvae under poor diet conditions (18). As a result of this nutritional symbiosis, *L. plantarum* is a prevalent taxon in the *Drosophila* microbiome (17).

To study the implications of intraspecific diversity of *L. plantarum* in fruit flies, we took advantage of an experimentally evolved *Drosophila simulans* population that has been adapting to two novel temperature regimes, hot and cold, for more than ten years. We detected three clades of *L. plantarum* co-occurring within the native microbiome of *D. simulans* population before the experimental temperature was modified. However, the exposure of the fly population to the novel temperature regimes altered the intraspecific composition of *L. plantarum,* revealing functional differences between the co-occurring clades. These functional differences were experimentally validated *in vitro* and *in vivo*. Our results provide a clear example for functional diversification of intraspecific diversity and demonstrate how experimental evolution can uncover this otherwise hidden functional diversity.

Results

Genome sequencing reveals the presence of three *L. plantarum* clades in the experimentally evolving populations

We sampled 33 isolates from the Florida experiment (See Materials and Methods for details) to characterize the diversity of *L. plantarum* (Figure 1, Table S1). The isolates formed three clades based on the pairwise average nucleotide identity (ANI). Between-group ANI values were 98.4-99.17%, below the ~99.5% threshold for conspecific strain identity (20). On the other hand, the identity within groups was 99.48-99.99% (Figure 2). The clustering was consistent irrespective of whether we used SNPs in the core genome or presence/absence patterns of accessory genes. This indicates that the three clades are old with very limited genetic exchange.

Since the isolates were sampled either from the unevolved, hot-evolved or cold-evolved populations (Figure 1), we tested whether the clades were randomly distributed across the experimental treatments and observed a non-random distribution (Fisher's exact test, 3×3 contingency table, $p = 5.5e-10$). In combination with the clustering of clades (Figure 2), we conclude that each of the three clades is more prevalent either in the novel experimental populations or in the unevolved founder population, at the beginning of the experiment. We therefore coined the terms H (hot-evolved), C (cold-evolved), and U (unevolved) clades, that will be used throughout the manuscript.

In order to further investigate the association between *L. plantarum* genotype and temperature, we sampled from two additional experiments, obtaining seven isolates from hot-evolved *Drosophila simulans* Portugal and two isolates from constant-cold-evolved *Drosophila simulans* South Africa (Table S1). In a phylogenetic tree, the hot-evolved isolates and the constant-cold-evolved isolates grouped into clades H and C, respectively (Figure S1). These results do not only support the association between *L. plantarum* clade composition and environmental temperature (Fisher's exact test, 3×3 contingency table, $p = 2.6e-12$), but also suggest that clade C is favored irrespective of whether the temperature is fluctuating (20 °C during the day, 10 °C at night) or

Figure 1. **Experimental setup to study the long-term evolution of *L. plantarum* in *Drosophila simulans* populations.**

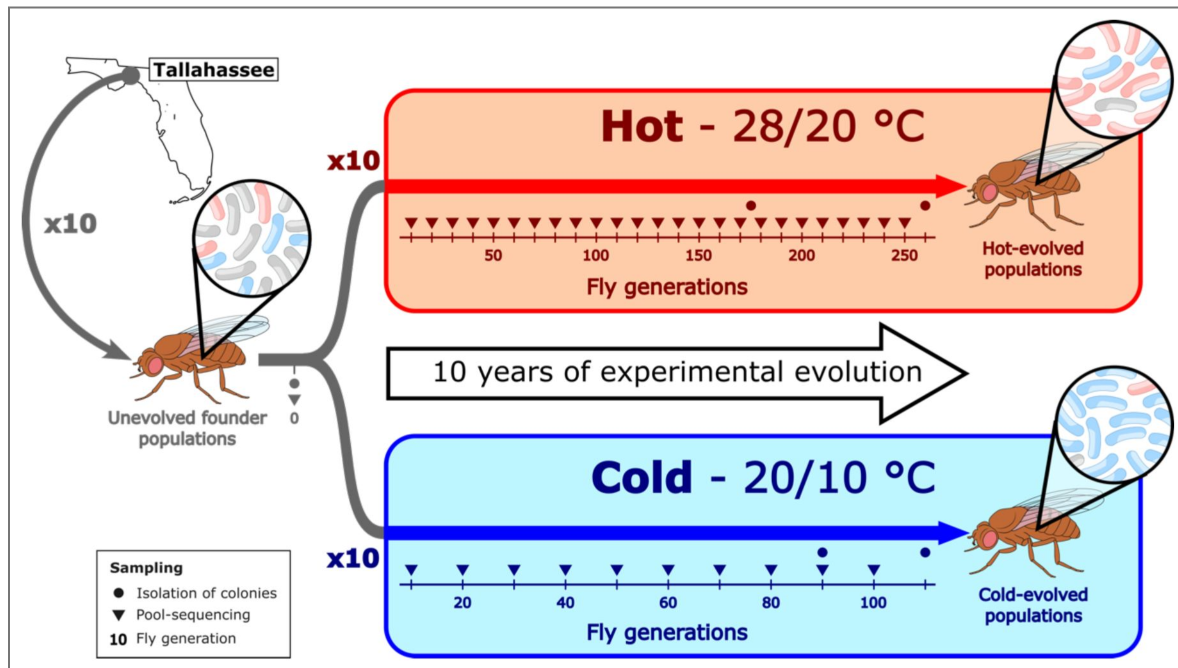
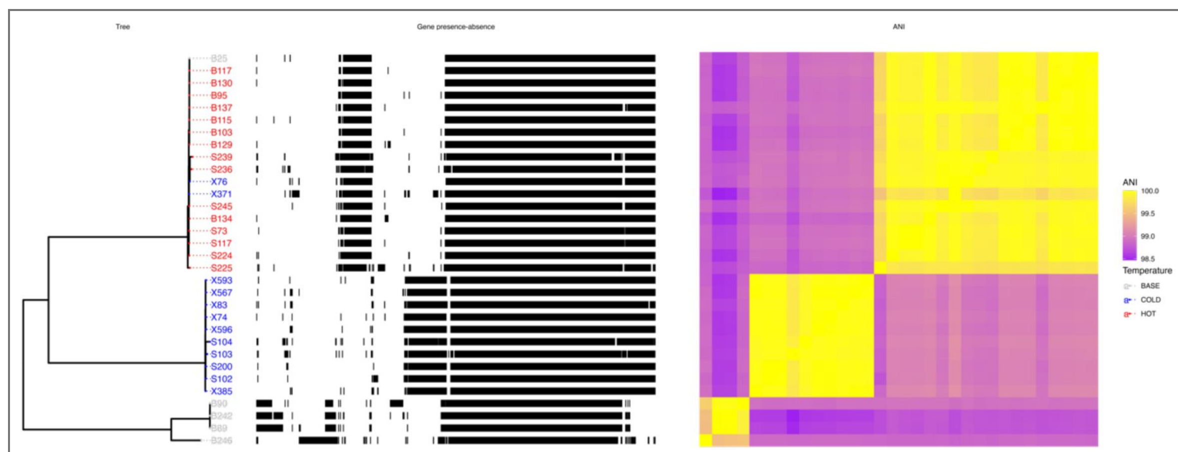


Figure 2. **Pangenome of *L. plantarum* genomes isolated from the Florida experiment (Table S1), sorted by phylogeny.**

Left panel: Maximum likelihood tree built based on the core genome alignment. Tree leaves correspond to the name of the isolate and are coloured based on isolation origin: ancestral population (grey), hot-evolved population (red) or cold-evolved population (blue). Numbers at nodes represent bootstrap support values based on 1000 replicates. Middle panel: Pattern of genes presence (black)/absence (white) in each genome. Right panel: pairwise average nucleotide identity between the isolates. The colour ranges between 98.5% (purple) and 100% (yellow).



constant (15 °C). Furthermore, the grouping of isolates originating from different experiments to the same clades indicates that these are not restricted to a single natural *D. simulans* population, but represent global diversity.

The three sympatric clades independently adapted to their host

Our analyses suggest that all three clades are shared among natural *D. simulans* populations, but it is not clear whether they diverged in *D. simulans* or independently colonized their *Drosophila* host. We used 79 publicly available *L. plantarum* genomes from several environments and geographic locations (Table S2) to reconstruct the phylogeny based on the core genome.

All three *L. plantarum* clades are more closely-related to genomes from other sources than to each other, which implies that they diverged before colonizing *D. simulans* (Figure 3). Moreover, the genomes from the clade H are almost identical to public sequences isolated from global collections of *Drosophila melanogaster* (Figure 3, Table S2). The clade U isolate B246 is closely related to the *Drosophila*-associated isolate LpWF originating from a wild *D. melanogaster* individual (21). Clade C did not have a close relative in the collection. The high similarity of the *L. plantarum* sequences isolated from *D. melanogaster* and *D. simulans* highlights that both *Drosophila* species share this component of the microbiome. Most likely a deeper sampling of the *D. melanogaster* microbiome will also detect clade C.

Despite being phylogenetically divergent, the three sympatric clades could have converged functionally in the process of adaptation to a common environment. In order to test this, we built the *L. plantarum* pangenome including all the available isolates and performed hierarchical clustering based on the presence/absence of accessory genes. We found no grouping of the three sympatric clades (Figure S2), which suggests that they are not only phylogenetically diverged, but have the potential to be functionally different based on their pool of accessory genes.

Clade dynamics are temperature-specific and consistent between population replicates

Up to now, our analyses were restricted to isolates sampled at specific time points of the experiment, but since genomic DNA from flies with their microbiome was sequenced throughout the entire experiment (Figure 1), a metagenomic analysis could track the frequency trajectories of the three clades to shed more light on the adaptation process. We calculated the proportion of reads assigned to each clade in the reads from the population at a given time point. At the beginning of the experiment, the populations were dominated by clade U (54.5% relative abundance on average), followed by clade C (31.9%), and then clade H (13.6%) (Figure 4). However, when the fly populations were subjected to the experimental treatments, clade H became dominant under the hot regime and clade C became dominant under the cold regime. Clade U greatly decreased in relative abundance in both regimes. This suggests that changes in environmental temperature alter the fitness landscape of the population and lead to a new adaptive state, in which the relative fitness of the clades change.

A strong asset of the experimental evolution set up is the availability of 10 replicate populations that were generated from the same founder population and independently maintained throughout the entire experiment under the same conditions. Hence, the comparison of replicates provides an estimate for the strength of deterministic and stochastic forces during the experiment. In the cold regime, all ten replicates exhibited a rapid takeover of clade C (Figure S3). In contrast, the hot-evolved replicates were more variable in the rate at which clade H increased in abundance. The most extreme cases were replicates 8 and 10, in which clade C dominated the population for 90 generations before being replaced by clade H (Figure S3). Thus, the populations subjected to the hot regime took longer to reach a new equilibrium, which is also less reproducible than that of the cold regime. Overall, the temperature-specific dynamic is consistent across replicates in both regimes, confirming the association between environmental temperature and clade frequency that was observed during the isolation of the clones (Figures 1, 2 and S1).

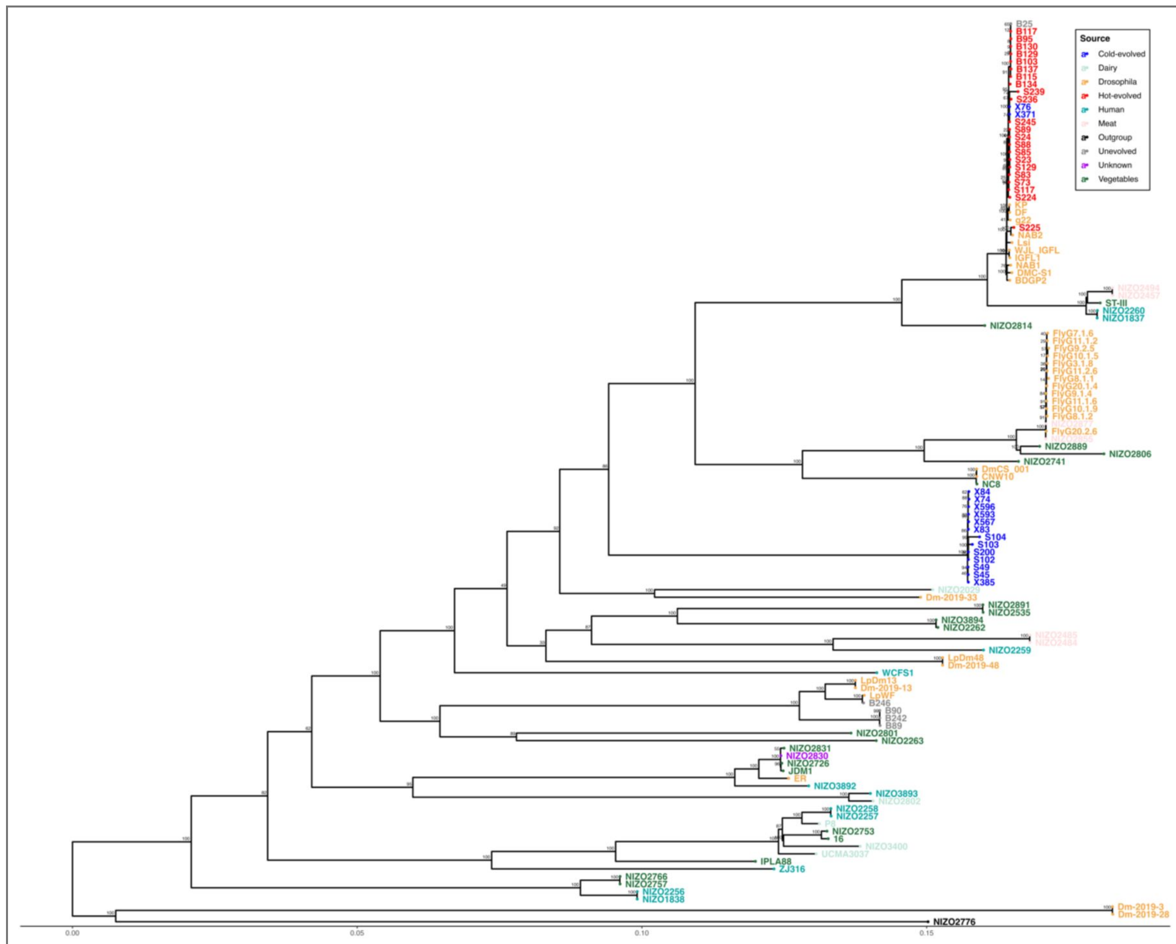


Figure 3. **Maximum likelihood tree of 92 *L. plantarum* genomes.**

Tree leaves are coloured by source of isolation. The genomes from our lab are coloured in according to experimental treatment: unevolved in grey, hot-evolved in red, and cold-evolved in blue. Numbers at nodes represent bootstrap support values based on 1000 replicates.

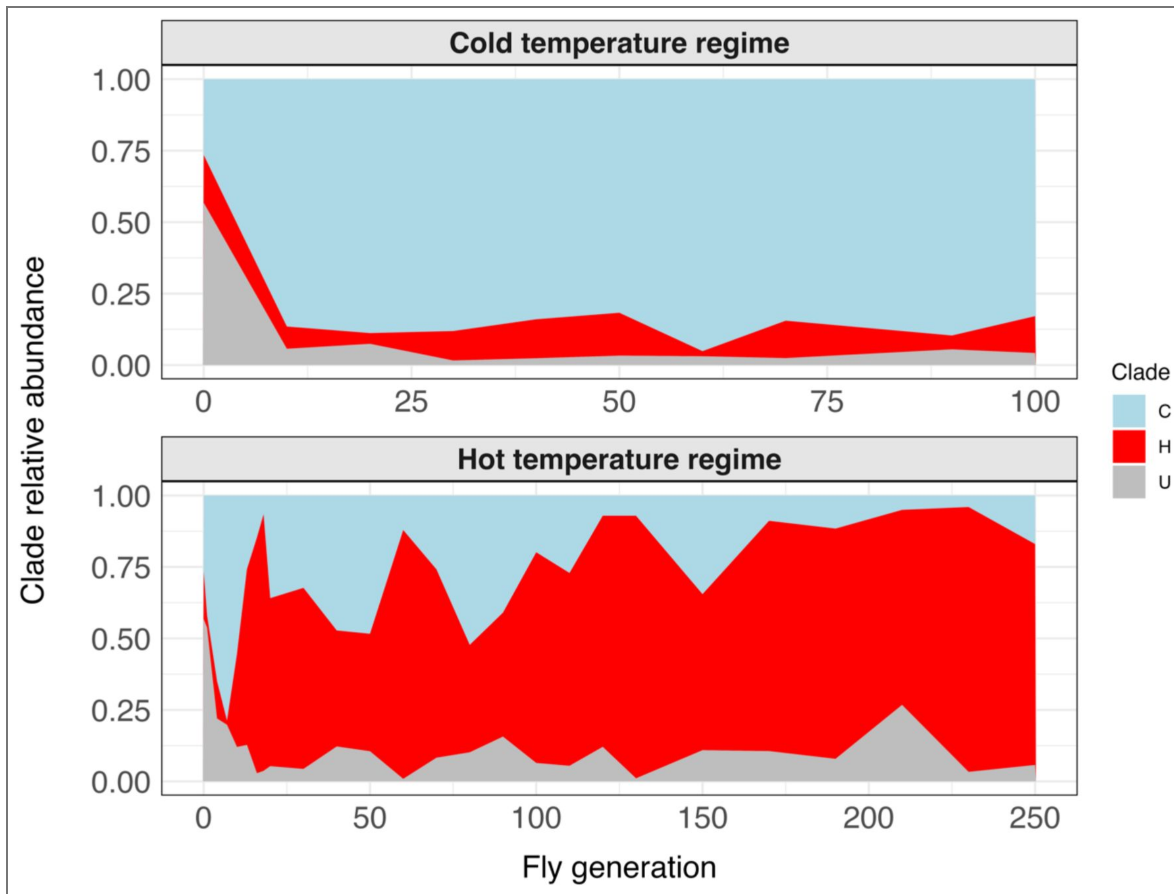


Figure 4. **Clade composition over time in each temperature regime depicted as the relative abundance of each *L. plantarum* clade (top: cold, bottom: hot).**

Clade C in light blue, clade H in red and clade U in grey. For each temperature regime we summed the reads from the ten replicate populations.

Phenotypic differences in liquid culture

The consistent temperature-specific dynamics of *L. plantarum* in the replicate populations resulted in a different clade dominating each novel temperature regime. The fitness differences may arise from an increased growth rate at a given temperature. Alternatively, traits such as toxin-antitoxin systems or host interaction may cause temperature-specific fitness (22). To determine if the observed clade dynamics were driven by differences in temperature-dependent growth rates, we measured the growth rate of 29 isolates from the three clades at hot temperature conditions. These isolates represented all sampling events across the two temperature regimes (hot and cold) as well as the unevolved population (Table S1). The three groups of isolates differed significantly in growth rate, carrying capacity, and inflection time, i.e. the time it takes an isolate to reach the log phase (Figure 5; Kruskal-Wallis test, $p < 0.001$ for all parameters). Isolates belonging to clade C took on average more than 12 h longer to reach the log growth phase. Clade H had the highest growth rate, followed by U, and C. Clade U reached the highest density, followed by H, and C.

These results reveal the functional diversification of the three clades, but do not support the hypothesis that temperature-specific growth rates caused the shifts in composition. In liquid culture, clade U outperformed clade C in growth rate, carrying capacity, and inflection time despite the latter clade prevailed in the *Drosophila* populations for much longer (Figure 4, Figure S3). Nevertheless, we caution that the growth conditions in the *Drosophila* environment are radically different to the growth measured in liquid MRS (23). Furthermore, inside the fly gut *L. plantarum* lives in a complex community in which the different clades interact with each other, with the other components of the microbiome, and finally also with the host. Bacteria can persist in the community by thriving in the food or by stably colonizing the fly gut. The former strategy might be controlled by growth rate and carrying capacity, but the latter is affected by additional factors such as death rate in the gut and defecation rate, that cannot be assessed in liquid medium (21). Moreover, the resource availability is different in the solid fly food and in liquid MRS medium. Thus, observed differences in growth could indicate different strategies to persist in the community.

Fitness effects of *L. plantarum* strains on their host

Although growth dynamics differ significantly among the three clades in liquid culture, they do not explain the observed changes in abundance during experimental evolution. Therefore, we turned to the *Drosophila* host to explore the fitness consequences of colonization by each clade. *L. plantarum* has been shown to increase larval fitness of *Drosophila melanogaster* relative to germ-free flies (18,24,25). We hypothesized that the fitness advantage conferred by *L. plantarum* to its host could depend on the genotype of the symbiont and the environmental temperature, favoring a different clade in each regime. To test this, we designed a set of inoculation experiments in both focal temperatures using two host species, *D. melanogaster* and *D. simulans*, and the three *L. plantarum* clades. While we could produce axenic *D. melanogaster*, attempts to produce axenic *D. simulans* were unsuccessful. For this reason, inoculation experiments involving *D. simulans* were conducted using conventionally reared flies.

In axenic *D. melanogaster* none of the *L. plantarum* clades provided a fitness advantage to the host relative to germ-free controls, contrary to the effects reported in the literature. In the case of clades U and H, the number of offspring and developmental time did not differ significantly from the germ-free control (Figure 6 and S6; Dunn's test, $p > 1$ for all pairs). This discrepancy could be explained by the supply of essential amino acids during larval growth by *L. plantarum*, (18,24–26). The high content of dried yeast 24.3 g/l in the fly food used in our experiment likely provided already sufficient amounts of essential amino acids, which negated the growth-promoting effects of *L. plantarum*. Even more surprising was the significantly reduced fitness in the presence of clade C in both temperature regimes (Figure 6; Dunn's test, $p < 0.05$ for all significant comparisons). A mixture of all three clades resulted in a similar loss in fitness. The fitness reduction was stronger in the first transfer of flies, likely due to a higher bacterial load. This finding suggests an inverse relationship between bacterial abundance and host fitness.

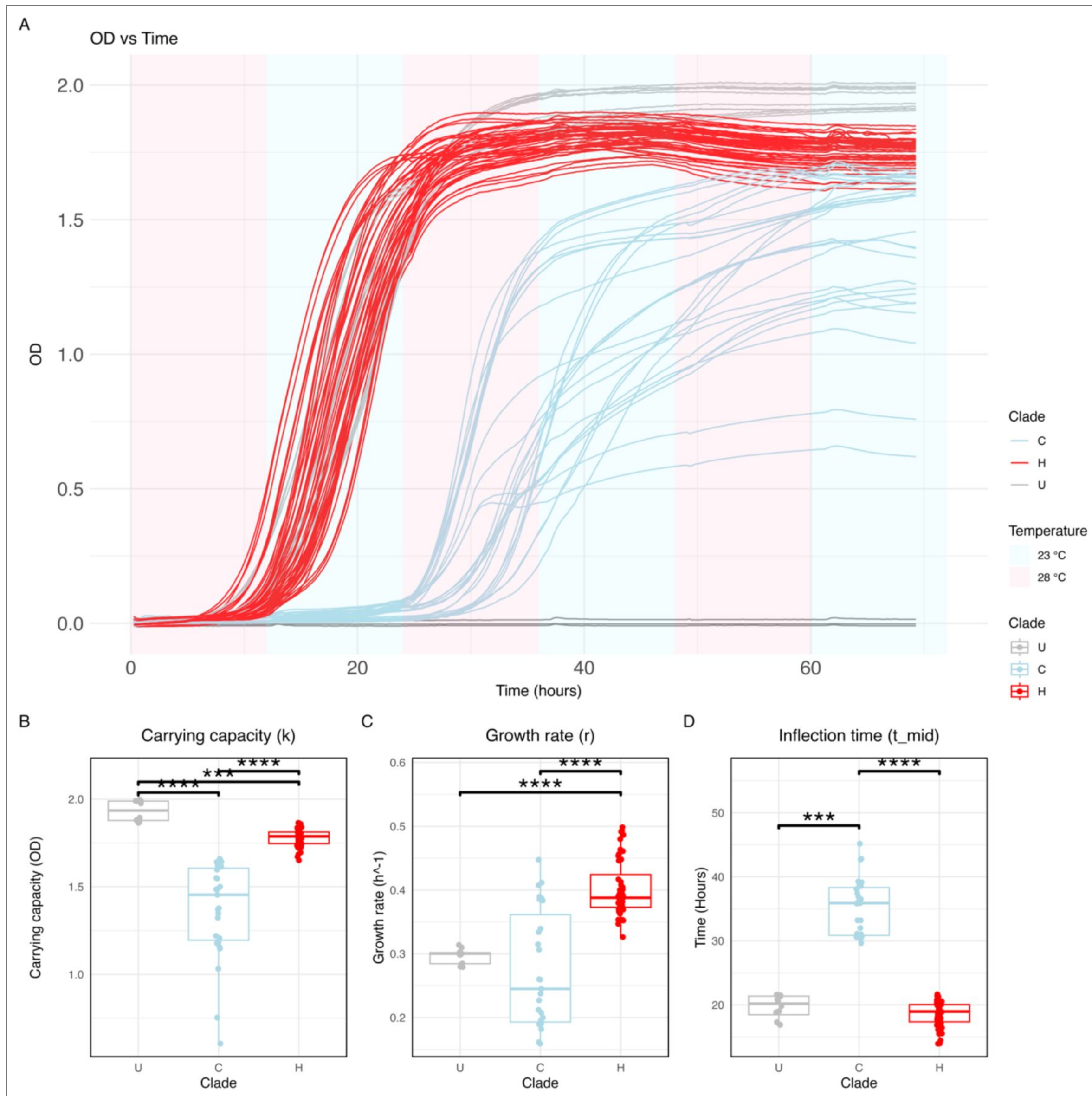


Figure 5. **Clade-specific growth dynamics.**

(A) *L. plantarum* growth curves overlapped. Lines are coloured by isolate's clade. Background colour represents the growth temperature in the fluctuating environment. (B-D) Boxplots depicting the carrying capacity, growth rate, and inflection time of each clade. Each dot corresponds to a technical replicate. Data is grouped and coloured by clade. Statistical significance was determined using Dunn's test with Holm-adjusted p-values. Only significant comparisons are indicated. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

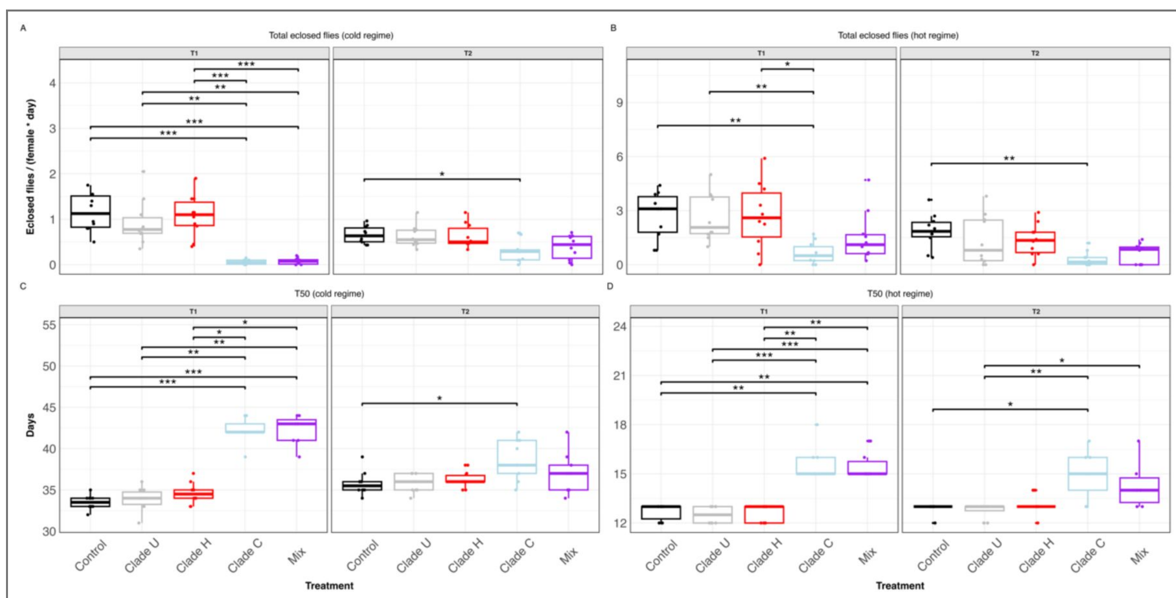


Figure 6. Fitness effect of ***L. plantarum*** inoculation in axenic ***D. melanogaster***.

(A, B) Total number of F1 flies eclosed normalised by day and female under the cold (A) and hot (B) regime. (C, D) Developmental time, estimated as the number of days it takes 50% of the offspring to eclose. Measurements were grouped by inoculation treatment and transfer. Each dot corresponds to a biological replicate (n = 10) Statistical significance was determined using Dunn's test with Holm-adjusted p-values. Only significant comparisons are indicated. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

In conventionally reared *D. simulans* we did not detect differences in the number of eclosed flies (Supplementary figure S6; ANOVA). However, the developmental time was significantly extended after inoculation with clade C at cold temperature (Dunn's test, $p < 0.05$ 05 for all significant comparisons). We attribute the weaker effects in the non-axenic flies to a reduced *L.* plantarum load due to the presence of other taxa (21), or to higher-order interactions with other members of the microbiome (27–29).

In summary, we observed that clade C, which is dominant in the cold-evolved populations, decreases host fitness when axenic flies are inoculated. The magnitude of this effect varies depending on the environmental temperature, the bacterial load, and the presence of other microbial taxa. Clade-specific effects of *L. plantarum* on the *D. melanogaster* host have been reported (19). In a low-protein diet, strains that were not isolated from *Drosophila*, enhanced larval growth relative to germ-free individuals, whereas another *Drosophila*-associated strain did not have any effect. Even a reduction of *Drosophila* lifespan has been reported in mono-association with *L. plantarum* (30), but the reduction of adult viability and developmental rate relative to the germ-free control is a novel finding in our study. We can conclude that the outcome of the *L. plantarum-Drosophila* symbiosis is clade-specific and can be detrimental to the host.

Functional divergence on the genomic level

Our analyses clearly indicate functional divergence between the three clades at genetic and phenotypic levels (see Figures S2 and 4-6). Therefore, we were interested in identifying the genes that contribute to this functional differentiation. A comparative KEGG pathway analysis shows that clade C contains the most KEGG Orthologs (KOs), 1163 KOs, followed by clade H (1157 KOs), and clade U (1131 KOs). A subset of 111 orthologs is unique to one or two clades. Of these, 41 were shared by clades H and C (Figure S7). Among these differential orthologs fell into 53 KEGG metabolic pathways.

Interestingly, we found a larger repertoire of sugar-related genes in the two lab-selected clades, C and H, than in clade U (Table S3, Figure S7). One of these genes encodes the enzyme hexosaminidase (EC: 3.2.1.52) and confers clades C and H the ability to use chitobiose as carbon source. This oligomer is the main product of chitin degradation, that makes up the flies' peritrophic matrix and exoskeleton (31). Therefore, we speculate that the ability to exploit this ubiquitous source of carbon and nitrogen in the lab-maintained fruit flies, could be a strong target of selection in the lab environment. This could explain why clade U, which displayed a high growth rate and carrying capacity in liquid culture (Figure 5), is rapidly outcompeted in the fly populations (Figures 4 and S3).

Furthermore, clade H and C (Table S3) harbor different sugar-related functions, indicating that, despite their overlapping functions, these strains possess distinct sugar metabolic capacities. We also identified functional differences in cofactor biosynthesis and respiratory metabolism between clades. These differences do not explain the clade-specific selection, but reflect the different evolutionary histories of the clades (Table S3, figures S8-S11). A detailed description of the functional diversification can be found in the supplement.

Discussion

Intraspecific diversity within the same environment has been reported to be ecologically important to maintain the stability of the entire microbial community (11,13,14). This parameter has been studied in lakes, soils, seas, and the human gut (10–12). However, little attention has been given to intraspecific variation in *Drosophila*, and insect microbiomes in general (8). Based on our results in experimentally evolved fruit flies, we propose that within-species competition, thus far largely overlooked, could contribute to ecological adaptation and evolution of the host, as it has been observed at the species level in *Drosophila* (32) and other animals (33–35).

Our finding of three co-occurring subspecies of *L. plantarum* in our populations is particularly surprising, given the low microbiome richness of *Drosophila* at species level, which is estimated to be orders of magnitude lower than in humans (36,37). However, the intraspecific richness of *L.*

plantarum in our flies was three times higher than that estimated in human gut microbiomes (11). This discrepancy between inter– and intra-species diversity suggests that the *Drosophila* microbiome is more complex than previously thought (16,27,38). However, this diversity exists below the species level and has therefore remained cryptic due to the limitations of amplicon sequencing (3).

By altering the experimental temperature at which the populations were maintained, we discovered consistent shifts in the composition of *L. plantarum* subspecies, that inevitably led to the dominance of a single clade. Further inoculation experiments revealed that clade composition significantly impacts the fitness of the host. *L. plantarum* genotypes differ in the fitness advantage that they provide to the host (19). However, to our knowledge, no *L. plantarum* genotype has yet been reported to negatively affect *Drosophila* development. Our finding shows that the well-characterized nutritional symbiosis between *Drosophila* and *L. plantarum* depends on the bacterial genotype and cannot be generalized to the entire species. Furthermore, clade C outcompeted the other two clades in the cold populations despite negatively affecting the host. This demonstrates that the benefits of parasitism or mutualism in the *Drosophila* microbiome are context-dependent, as already observed in humans (39).

Finally, we also gained insight into how the microbiome of the fly could have adapted to laboratory conditions. Unlike the non-selected clade U, the two lab-selected clades, C and H, had the unique genetic potential to exploit the byproducts of chitin degradation. Our results align with previous research on the "domestication" of the *Drosophila* microbiome. Comparative genomics in *Acetobacter* have revealed that bacteria associated with lab-reared *Drosophila* were selected for the presence of uricase genes and the loss of flagella, relative to their wild-isolated counterparts (40). In *Acetobacter*, the ability to degrade uric acid excreted by the host provides a significant competitive advantage (40). We hypothesize that the same may apply to the ability to metabolize host-derived chitin. We conclude that our findings are consistent with laboratory adaptation, and extends previous observations from *Acetobacter* to *Lactiplantibacillus*.

Overall, our work emphasizes the importance of intraspecific diversity in maintaining the stability of microbial communities and the host's ability to adapt to environmental changes. The current climate crisis has sparked a significant interest in how environmental temperature affects microbial communities at the species level (41–43). Our study highlights that even subspecies diversity plays a key role in adaptation to environmental temperature, and probably other abiotic factors. Therefore, we propose that a comprehensive understanding of adaptive processes and ecological dynamics in the case of host-microbiome interactions depends critically on including diversity at the subspecies level.

Materials and Methods

Experimental evolution

Twenty replicate populations were set up using 202 isofemale lines from a natural *D. simulans* population collected in Florida (44). The populations were maintained in a 12 h photoperiod and at two different novel temperature regimes: ten replicates were kept in a fluctuating hot environment (28 °C during the day and 18 °C at night), whereas the other ten were kept in a fluctuating cold environment (20 °C during the day and 10 °C at night). The census population size of the replicates was 1000-1250 with a 50:50 sex ratio. The flies in each replicate were equally distributed across five 300 ml bottles containing 60 ml of standard *Drosophila* medium (300 g Agar + 990 g sugar beet syrup + 1000 g malt syrup + 2,310 g corn flour + 390 g soy flour + 900 g yeast in 37.5 L water) (45). The evolved *D. simulans* populations were sequenced in 10 generation intervals using Pool-Seq (46,47). These sequencing reads have been used to study the evolutionary dynamics of fruit flies (47). Similar pool-seq time series data have been previously used to analyze long-term endosymbiont and microbiome dynamics in *D. melanogaster* populations evolving in the same experimental conditions (48–50).

We also isolated *L. plantarum* from two other experimental evolution experiments, one originated from a South African population reared at constant 15 °C and the other from Portugal, which was reared in the fluctuating hot regime as described earlier (51). Both experimental evolution studies followed the same culturing regime that was used for the Florida populations, with temperature being the only experimentally altered variable (52).

Bacterial isolation

We primarily obtained data from the experimental evolution studies using the Florida *D. simulans* founder population and collected 33 *L. plantarum* genomes from five different time points; two time points from each experimental temperature and one time point corresponding to the ancestral flies (Table S1). In addition, we obtained *L. plantarum* genomes from two other experimental evolution studies. Two genomes from the South African experiment and seven genomes from the Portugal experiment.

Around 100 flies from each population were homogenized in sterile PBS using an autoclaved pestle. The homogenate was diluted to avoid physical contact between colonies and streaked on agar plates with three different media: De Man, Rogosa, and Sharpe (MRS), mannitol and tryptic soy. The plates were incubated at 28 °C for 48 h. We identified *L. plantarum* using PCR with custom *Lactiplantibacillus*-specific primers (in 5'-3' orientation, LacF: GATGGGCGCTTACCCGATTA, LacR: CTGCCCCGCAAATTGTTTCA). The proportion of *L. plantarum* colonies in the plates, relative to other taxa, varied across growth media and fly populations. Colonies which could successfully be amplified were picked and regrown to stationary phase in the same medium from which they were isolated, but in liquid format. A glycerol stock of each isolate was made by mixing the culture with glycerol (50% v/v) in 1:1 proportion.

DNA extraction and sequencing

Genomic DNA was extracted from all the replicate populations using the high salt method (53). The fly populations were sampled in 10 generations intervals starting from generation 0. At sampling the age of the flies varied between four and eight days for the hot environment and between nine and 16 days for the cold environment. Pools of flies were sequenced at various time points, using a range of library kits, insert sizes, and read lengths (47).

For sequencing, *the L. plantarum* isolates were grown to stationary phase in MRS medium. gDNA was isolated with the high salt extraction method (53). Additionally, a lysozyme pre-treatment was used to degrade Gram-positive cell wall (54). Briefly, the pellet was resuspended in 480 µl of EDTA 50 mM. The suspension was treated with 120 µl of lysozyme (20 mg/ml dissolved in NaCl 30 mM – EDTA 2mM) and incubated for two hours at 37 °C. After this, the samples were centrifuged at maximum speed, the supernatant was discarded, and the pellet was further treated following the high salt gDNA extraction. Genomic DNA was quality-controlled on an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) and subsequently used to prepare DNBSEQ short-read libraries, and 2×150bp reads were sequenced on a DNBSEQ-G400 (MGI Tech Co., Ltd., Shenzhen, China).

Genomic assemblies

Sequencing reads were processed using BBDuk (*BBMap*, 2025 v38.90) to remove adapters, ΦX174 sequences and low-quality bases with the following parameters: ktrim=r, k=23, mink=11, hdist=1, tbo, tpe. Reads were assembled using SPAdes v4 (56) with kmer sizes 21, 33, 55, 77, 99, and 127. Subsequently, CheckM2 v1.0.1 (57) was used to estimate the completeness and contamination of each assembly, and the taxonomy was determined with GTDB-Tk v2.1.1 (58). Ten assemblies had more than 5% contamination, probably due to accidentally picking two adjacent colonies. We used Centrifuge v1.0.4 (59) to classify and exclude contigs with exogenous origin from these assemblies.

Comparative genomics and phylogeny

We built the pangenome and phylogenetic tree of the collection of genomes isolated from our lab (Table S1). First, we used Prokka v1.14.6 (60) to predict the genes in all the genomes. Roary v3.13 (61) was used to construct the isolates' pangenome and extract the aligned genes belonging to the core genome, i.e. those that are present in all genomes. SNP-sites v2.5.1 (62) was used to extract the variable positions from the concatenated core genes. A maximum likelihood tree of the aforementioned variable positions was built using the model GTR+ASC and 1000 bootstraps with IQ-TREE v3.0.1 (63). Additionally, pairwise average nucleotide identity between all the isolates was computed using the program PyANI v0.2.13.1 (64), with ANIb as alignment method using the whole genomes. This workflow was carried out for the subset of 33 genomes isolated from the Florida experiment and for the whole dataset of 42 genomes, including those isolated from South Africa and Portugal experiments. Unless specified otherwise, subsequent analyses were conducted using the whole dataset pangenome.

The same phylogenetic and pangenomic methods were used for the extended *L. plantarum* genomes collection, including 79 publicly available genomes (Table S2). In order to root the tree, an additional tree was made including the reference genome from the sister species *Lactiplantibacillus paraplantarum* (GCA_003641145.1) as outgroup. The *L. plantarum* isolate that was most closely related to *L. paraplantarum*, NIZO2776, was used to root the tree.

In order to investigate which metabolic functions were differentially encoded in each clade, we followed the Anvi'o v7.1 pangenomic workflow (65,66) to identify "gene clusters" that are unique and ones that are shared across the genomes isolated from our lab. These gene clusters were further annotated against the KEGG database using the built-in Anvi'o function 'anvi-run-kegg-kofams'. The script 'anvi-compute-functional-enrichment' (67) was then used to screen for KEGG functions that are differentially enriched between the clades. Briefly, this script estimates the fraction of genomes from each clade that encode each KEGG Ortholog (KO) and tests whether they are significantly associated to one or more groups. We used the R package ggKegg v1.4.1 (68) to visualize the KEGG pathways in which at least one gene was enriched.

Competitive mapping and clade relative abundance calculation

First, we pre-filtered the *Drosophila* pool-seq reads by removing the reads that have eukaryotic or *Wolbachia* origin. We used Bowtie v2.5.4 (69) with stringent settings (-D 500 –R 40 –N 0 –L 20 –I S,1,0.50 —no-mixed —no-discordant) to map each pool-seq data set against a collection of reference genomes that included *D. simulans* (GCF_016746395.2), *D. melanogaster* (GCF_000001215.4), *D. mauritiana* (GCF_004382145.1), *H. sapiens* (GRCh38), *M. musculus* (GCF_000001635.27), *A. thaliana* (GCF_000001735.4), *S. cerevisiae* (GCF_000146045.2), *C. lupus* (GCF_011100685.1) and a several insect-infecting *Wolbachia* strains (GCF_000008025.1, GCF_000022285.1, GCF_000376585.1, GCF_000376605.1, GCF_000475015.1). We kept the read pairs in which none of the reads mapped to any of the genomes in the collection, which should predominantly represent prokaryotic sequences.

Then, we used SuperPang v0.9.6 (70) to build the reference graph pangenome of each *L. plantarum* clade using all the genomes belonging to the clade as input (Table S1, Figure 3). In order to calculate the clades relative abundance in each pre-filtered pool-seq, we used BBMap (*BBMap*, 2025 v38.90) with "perfect mode" settings to competitively map each read against the three concatenated clade pangenomes. For each sample, the number of reads that mapped uniquely to each clade was counted and normalized to Reads Per Million (RPM) in order to account for differences in genome size and sample depth.

We benchmarked our approach to confirm the relative abundance estimates from our competitive mapping pipeline. Briefly, we simulated 200k HiSeq reads from each reference graph pangenome using InSilicoSeq v2.0.1 (71), and used 'seqkit sampl' v2.3.0 (72) to mix them in different ratios, maintaining the original size of 200k reads: 1:0:0, 0:1:0, 0:0:1, 1:1:1, 2:1:7, and 8:1:1. Each of the combined read sets was randomly subsampled from 150k to 10 reads (10, 20, 40, 60, 80, 100, 200,

300, 400, 500, 600, 700, 800, 900, 1k, 2k, 5k, 10k, 150k reads) and mapped competitively to the three clades. We confirmed that the relative abundances were correctly estimated with as few as 100-1000 reads uniquely mapping the references (Figure S4).

Growth assays

The glycerol stocks from all the available *L. plantarum* isolates were re-grown in agar MRS. A single colony per isolate was grown in liquid MRS and passaged daily for five days in order to remove the influence of freezing. On the fifth day, each suspension was normalized to an optical density (OD_{600}) of 0.01, and serially diluted 1:100 in MRS. We selected 31 isolates that represented samples from all the available time points and temperature regimes, and plated them in triplicates in a flat-bottom 96-wells plate. Three wells were filled with sterile MRS medium as blank controls. Later, we detected contamination in two of the isolates, leaving us with 29 isolates. The plate was incubated for 72 h and OD_{600} was measured every 15 minutes in a Synergy H1 plate reader. The temperature regime was chosen to be similar to the hot conditions of the fly populations, but due to limitations of the spectrophotometer's cooling capacity instead of 28 °C during the day and 18 °C at night, we set it to 28 °C during the day and 23 °C at night. Orbital shaking was set to 425 cpm. The R package GrowthCurver v0.3.1 (73) was used to infer the growth parameters from the growth curves. All statistical analyses were conducted in R v4.4.2 (74) using the package rstatix v0.7.2 (75). We used the Kruskal-Wallis test to assess overall differences in growth parameters among the clades. For post-hoc pairwise comparisons, Dunn's test was applied, with p-value adjustments based on the Holm method. Differences were considered significant at $p < 0.05$.

Production of axenic flies

We generated germ-free flies, using a modified version of the dechorionation protocol described by Kietz *et al.* (76). Specifically, we used 2.8% active chlorine bleach instead of 1% and supplemented bleach, EtOH, and deionized H_2O with TritonX (1% v/v). The addition of this detergent prevents dechorionated eggs from sticking to the walls of the tubes, thus facilitating their transfer to bottles. The whole process was carried out in sterile conditions using a laminar flow hood. Axenic flies were maintained via periodic transfers to new sterile food inside the laminar flow hood. We first carried out this procedure with several *D. simulans* isofemale lines, but it was not possible to fully eliminate the residual microbiome despite several rounds of treatment. However, we could produce and maintain axenic *D. melanogaster* Oregon-R flies. For this reason, we decided to use *D. melanogaster* as axenic host in the inoculation experiments.

Inoculation experiments

A representative isolate from each clade (Table S1, B89 for ancestral, S103 for cold, and S239 for hot) was re-grown from the glycerol stocks in MRS medium. After three transfers, OD_{600} was normalized to 0.05. Autoclaved vials with axenic fly food were inoculated with 50 µl of each bacterial suspension (10 vials per treatment). In total five treatments were used: the pure culture from each genotype, a mixture of the three genotypes in equal proportions ("mix"), and control, in which the vials were inoculated with 50 µl of fresh MRS medium. After overnight absorption of the liquid, twenty axenic flies (ten female and ten male flies) were added to each vial and allowed to lay eggs for either 24 h (hot conditions) or 48 h (cold conditions). After this step, the flies were transferred to a new set of vials with axenic, uninoculated food, and allowed to lay eggs for another 24 h (hot conditions) or 72 h (cold conditions). The adults were discarded and the vials were incubated at hot (28 °C during the day and 18 °C at night) or cold conditions (20 °C during the day and 10 °C at night) until the new generation eclosed. The number of eclosed flies was recorded daily. We estimated the total number of flies per vial and the developmental time as proxies of fitness. We calculated developmental time as the day from the start of the experiment until the day at which 50% of the flies in the vial were eclosed. The inoculation experiment was carried out with axenic *D. melanogaster* Oregon-R and non-axenic *D. simulans* from Florida. We used the Kruskal-

Wallis test to assess overall differences in the two fitness phenotypes among the treatments. For post-hoc pairwise comparisons, Dunn's test was applied, with p-values were adjusted using the Holm method. Differences were considered significant at $p < 0.05$.

Supporting Information

Extended clade-specific differences in KEGG metabolic pathways

Clades C and H encode a shared genetic repertoire related to sugar metabolism that is lacking in clade U. In contrast, the latter does not encode any unique sugar-related function. Clade U is unable to hydrolyze chitobiose into N-acetyl-glucosamine monomers because it lacks the enzyme hexosaminidase (EC: 3.2.1.52). It also lacks transaldolase activity (EC: 2.2.1.2), that connects the Embden–Meyerhof–Parnass pathway with the non-oxidative pentose phosphate pathway (77). Finally, it cannot use sorbitol as a carbon source, since it does not encode the PTS transporter to internalize it (EC: 2.7.1.198; Supplementary figure S8) nor the enzyme sorbitol dehydrogenase (EC: 1.1.1.140), that converts sorbitol into fructose. These metabolic capacities, specially chitobiose degradation, could play a role in the rapid decrease in abundance of clade U observed in both temperature regimes (Supplementary Table S3). Chitobiose is the main product of chitin degradation, that makes up the flies' exoskeleton (78). We have detected the presence of microbial chitinases in the metagenomic reads (unpublished observation), which suggests that chitobiose might be available in the community. Thus, the ability to exploit this ubiquitous source of carbon and nitrogen could be very advantageous in the fly microbiome context, but would not affect the fitness in liquid culture (Figures 4 and 5). We also found other sugar-related functions are differentially present between clades C and H (Supplementary Table S3), suggesting that, despite having overlapping functions, these clades also encode unique sugar-related functions.

We also found differences in cofactor biosynthesis pathways. Although all the analyzed genomes can import riboflavin, the capacity to produce it *de novo* is enriched in clades C and H (Supplementary Table S3; Supplementary figure S9). This vitamin is essential in many physiological processes (79). Therefore, the possibility to synthetize it *de novo* under low extracellular riboflavin conditions is an advantage. In contrast, clade U has the unique capacity to synthesize *de novo* guanylyl molybdenum cofactor (Supplementary figure S10), that is essential in molybdenum-dependent enzymes (80). One of such enzymes is the nitrate reductase system Nar. Interestingly, clade U also harbors the operons *nreABC* and *narGHIJ*, that encode for genes that sense anoxic conditions and use nitrate as terminal electron donor instead of oxygen, respectively (81–83). The presence of this molybden-dependent alternative respiratory system exclusively in clade U suggests a different evolutionary history, in which this clade was exposed to anaerobic conditions before adapting to *Drosophila* (Supplementary figure S11). Similarly, the enzyme sulfur oxidoreductase (EC: 1.8.1.18) is highly enriched in clade C, which suggests that it can potentially use sulfur as terminal electron acceptor in absence of oxygen (Supplementary figure S11).

Among the genes that were uniquely present in clade C, we found *sbnA* and *sbnB*, that mediate the synthesis of L-2,3-diaminopropionic acid (84). This unusual amino acid serves as precursor of antibiotics and siderophores (84), but the rest of the biosynthesis pathways are absent in the genomes. However, by itself is also a potent enzymatic inhibitor (85,86). Although it goes beyond the scope of this work, we hypothesize that the synthesis of this compound by clade C could be responsible for the observed dose-dependent toxicity upon inoculation in the axenic host (Figure 6). As a sanity check, we blasted the two protein sequences against the clustered non-redundant NCBI database. The best matches had identities of maximum 60%, and belonged to *Streptococcus*, *Bacillus* and *Chitinophaga*. No publicly available *L. plantarum* encodes these genes, which further supports the exceptionality of clade C observed in the phylogeny (Figure 3).

Supplementary Figures

Fig. S1. Pangenome of all L. plantarum genomes (Table S1), sorted by phylogeny.

Left panel: Maximum likelihood tree built based on the core genome alignment. Tree leaves correspond to the name of the isolate and are coloured based on isolation origin: unevolved populations (grey), hot-evolved populations (red) or cold-evolved populations (blue). Tree tips are coloured based on the experiment from which the isolate was obtained: Florida (black), Portugal (pink) or South Africa (green). Numbers at nodes represent bootstrap support values based on 1000 replicates. Middle panel: Pattern of genes presence (black)/absence (white) in each genome. Right panel: heat map depicting the pairwise average nucleotide identity between the isolates. The colour ranges between 98.5% (purple) and 100% (yellow).

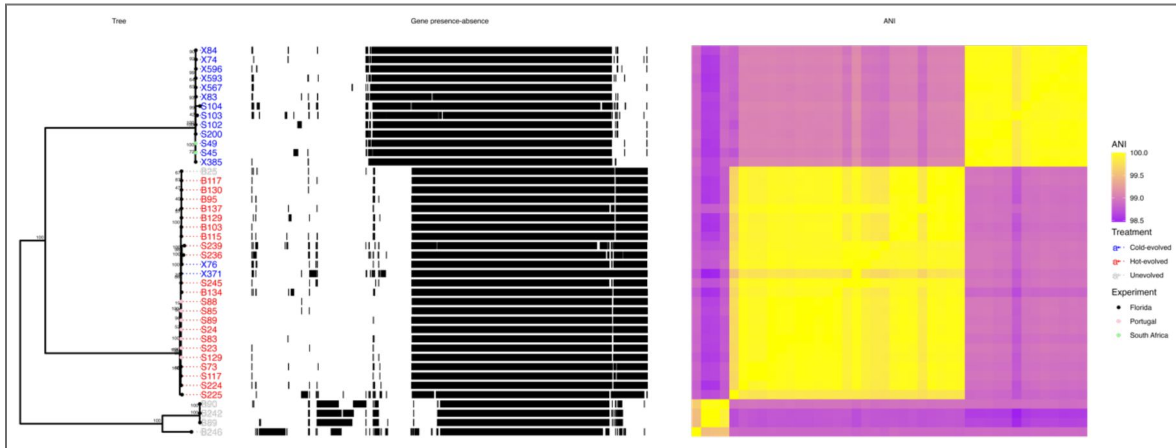


Fig. S2. Hierarchical clustering of 92 *L. plantarum* genomes based on the presence-absence of accessory genes.

Tree leaves are coloured by source of isolation. The genomes from our lab are coloured in according to experimental treatment: unevolved in grey, hot-evolved in red, and cold-evolved in blue. Red values in each branch indicate the p-value for each cluster's robustness based on 1000 bootstrap iterations.

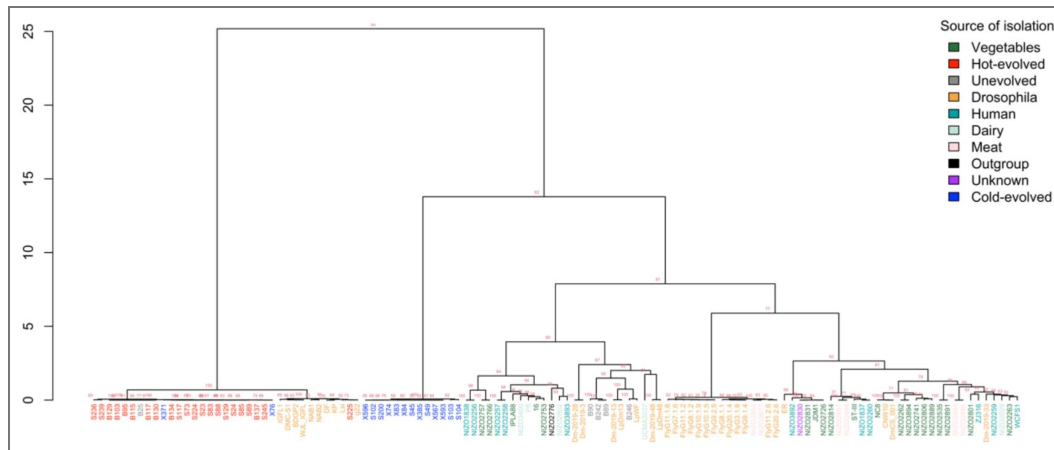


Fig. S3. Clade composition over time per replicate population and temperature regime depicted as the relative abundance of each L. plantarum clade.

Clade C in light blue, clade H in red and clade U in grey.

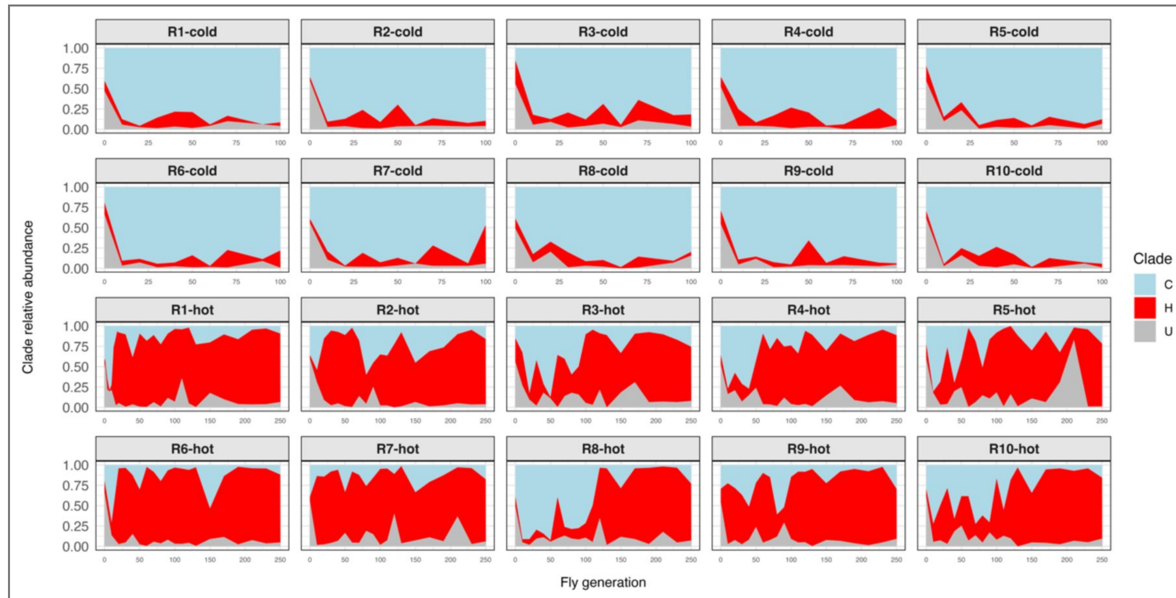
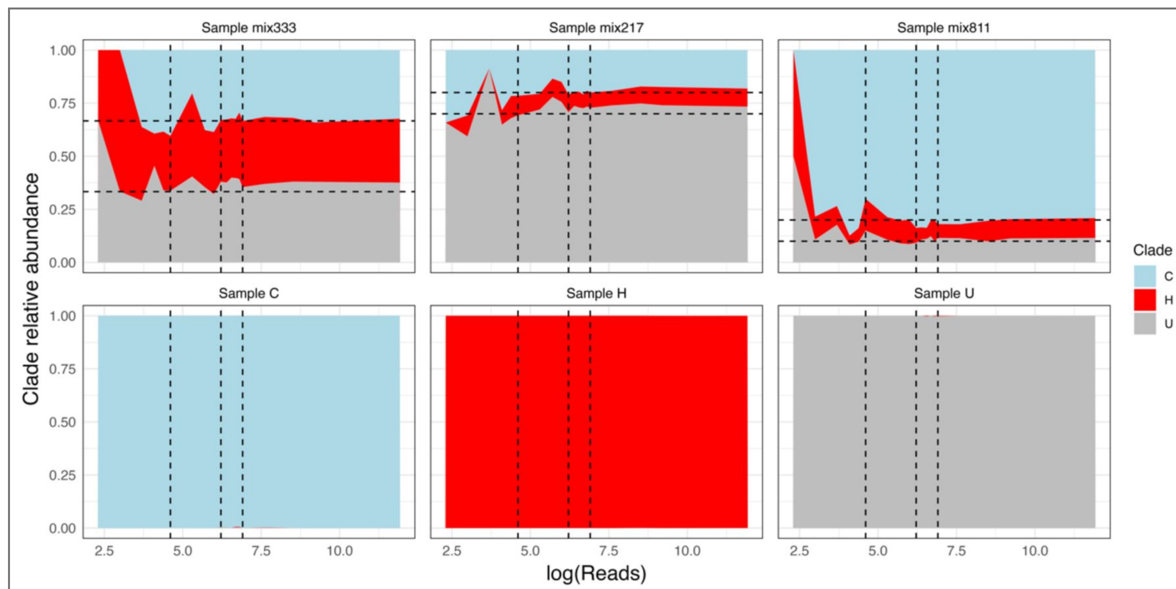


Fig. S4. Relative abundance of each L. plantarum clade across different reads depths in simulated reads sets with known clade composition.

Dashed vertical lines show the values of log(100), log(500), and log(1000) reads. Dashed horizontal lines show the true simulated relative abundances of each clade in the reads set. The top row titles reference to the ratio of reads from each clade following descending order: C:H:U. The bottom plots depict scenarios in which all the reads belong to a single taxon.



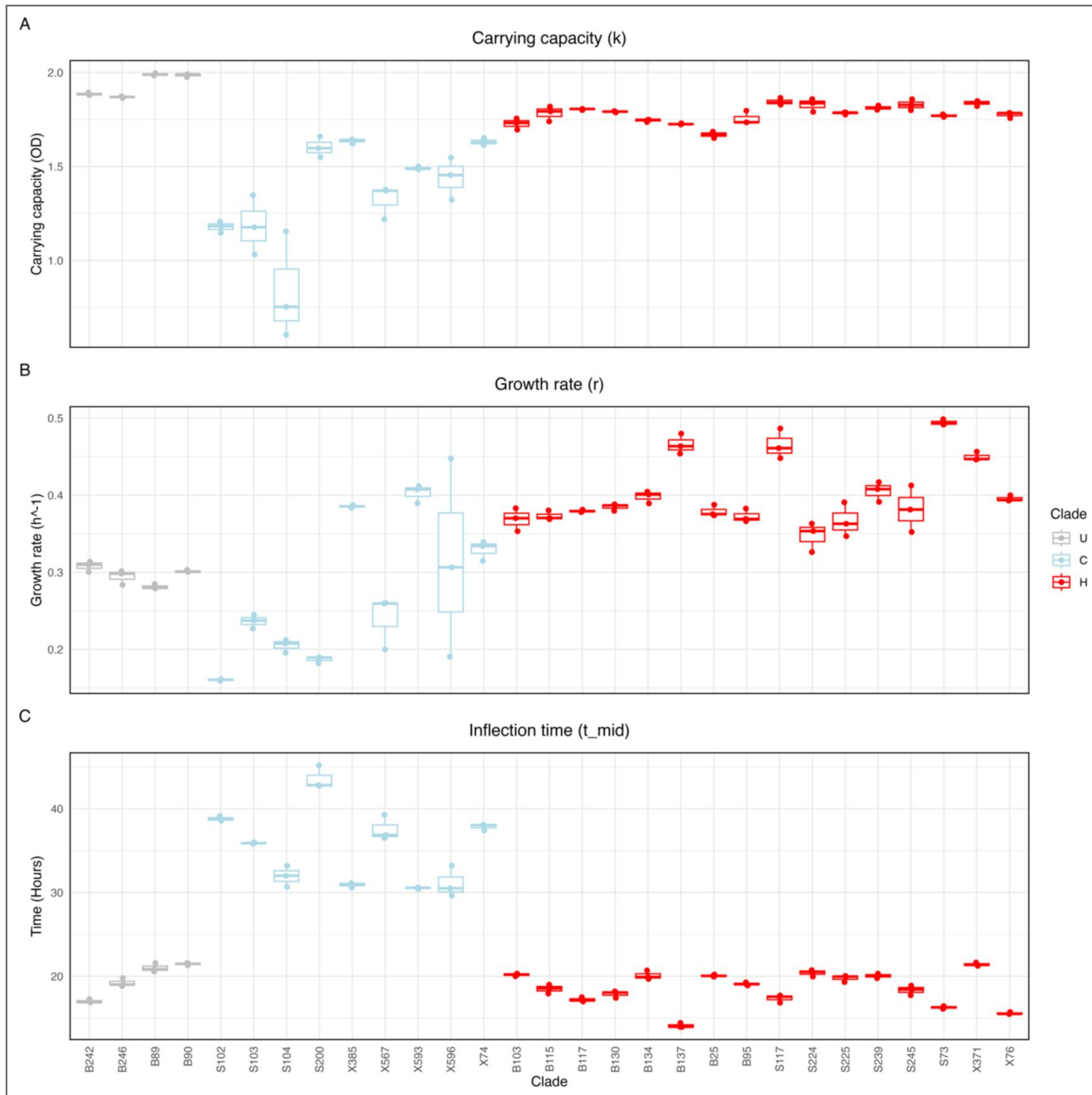


Fig. S5. **Boxplots depicting the carrying capacity (A), growth rate (B) and inflection time (C) of each isolate.**

The boxplots were coloured by clade. Measurements were obtained from three independent technical replicates for each isolate.

Fig. S6. **Fitness effect of *L. plantarum* inoculation in conventionally reared *D. simulans*.**

(A, B) Total number of F1 flies eclosed normalized by day and female under the cold (A) and hot (B) regime. (C, D) Developmental time, estimated as the number of days it takes 50% of the offspring to eclose. Measurements were grouped by inoculation treatment and transfer. Each dot corresponds to a biological replicate (n = 10) Statistical significance was determined using Dunn's test with Holm-adjusted p-values. Only significant comparisons are indicated. **** p < 0.0001; *** p < 0.001; ** p < 0.01; * p < 0.05.

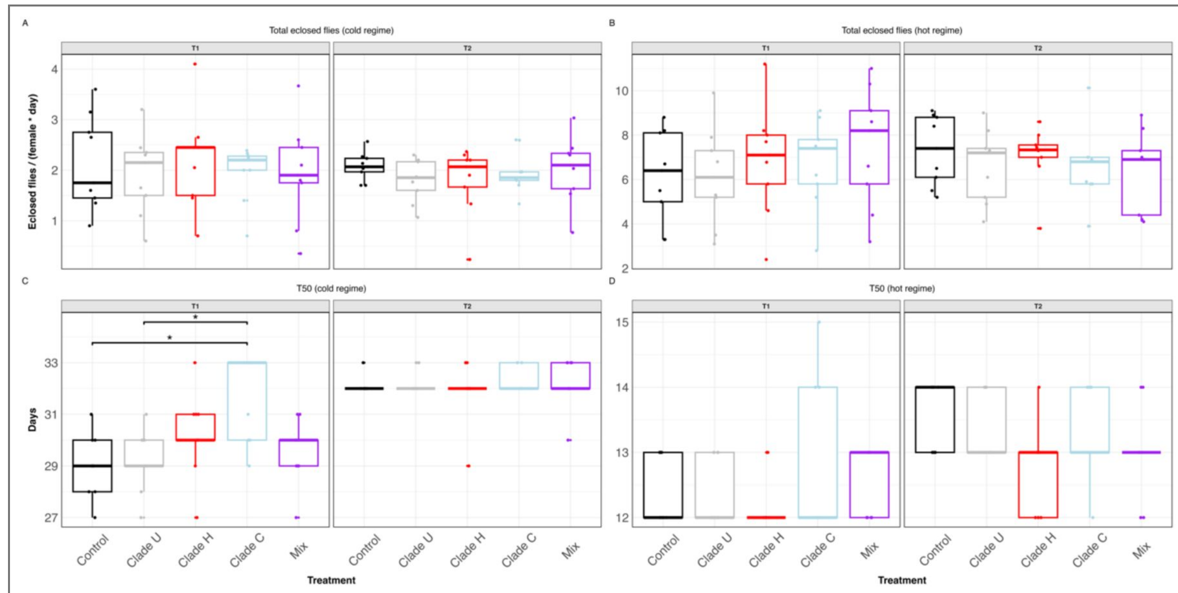
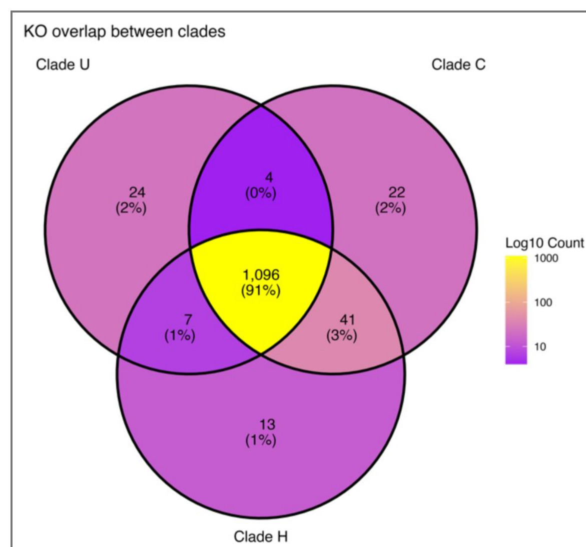


Fig. S7. **Venn diagram depicting the overlap in KEGG Orthologs between the three clades.**

Segments were coloured by number of KOs.



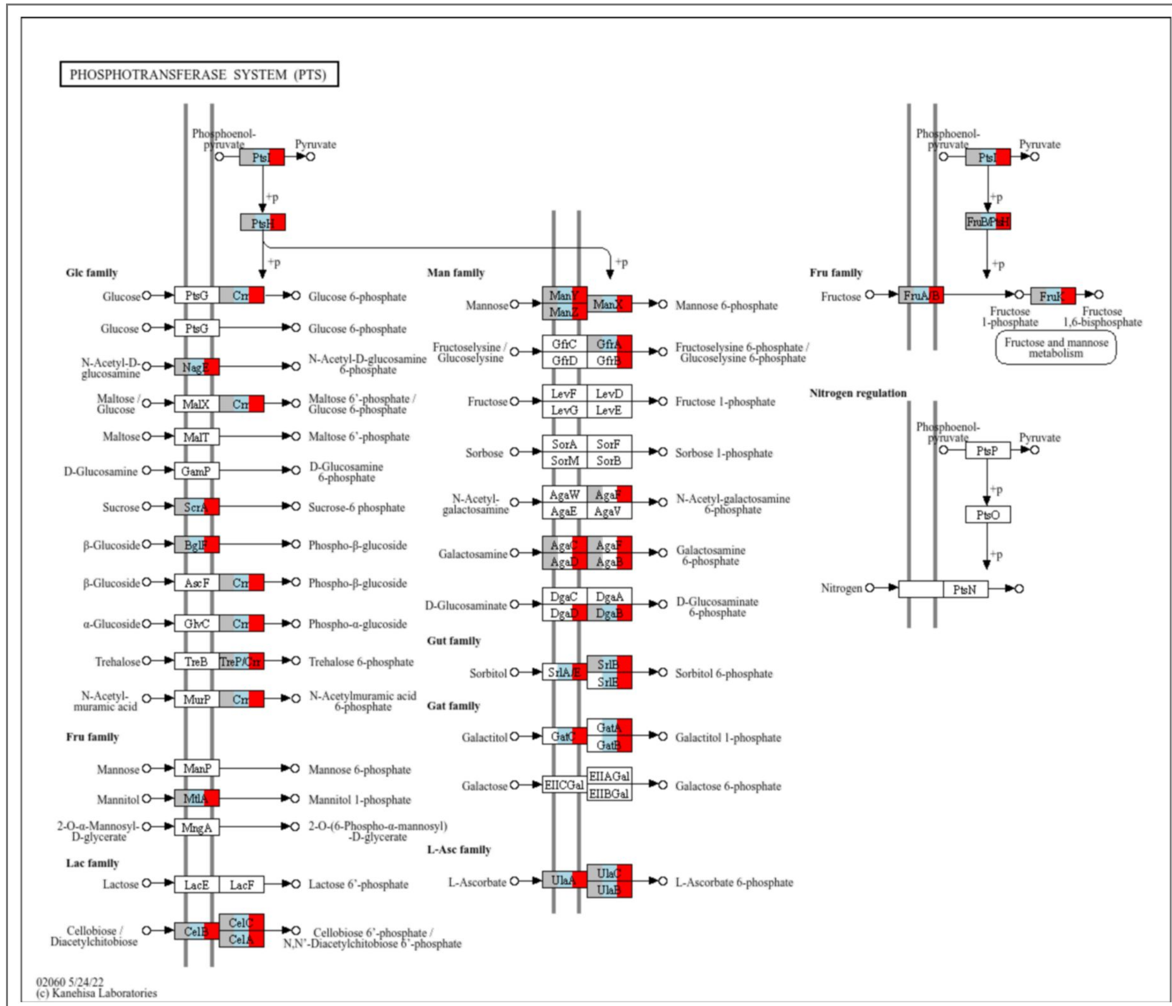


Fig. S8. **Graphical representation of the KEGG pathway map02060 (Phosphotransferase system).**

Each rectangle represents a KEGG Ortholog (KO). Orthologs that are enriched in each clade are coloured in grey (present in clade U), blue (present in clade C) and/or red (present in clade H). The three clades differ in the set of sugar-related PTS transporters. Clades C and H have the capacity to internalize sorbitol and galactitol, whereas clades H and U have the ability to import galactosamine.

Fig. S9. Graphical representation of the KEGG pathway map00740 (Riboflavin metabolism).

Each rectangle represents a KEGG Ortholog (KO). Orthologs that are enriched in each clade are coloured in grey (present in clade U), blue (present in clade C) and/or red (present in clade H). Clade U lacks the capacity to produce de novo FAD from GTP, since it is lacking several genes from the pathway.

Fig. S10. Graphical representation of the KEGG pathway map00790 (Folate biosynthesis).

Each rectangle represents a KEGG Ortholog (KO). Orthologs that are enriched in each clade are coloured in grey (present in clade U), blue (present in clade C) and/or red (present in clade H). Clade U has the unique capacity to synthesize de novo guanylyl molybdenum cofactor.

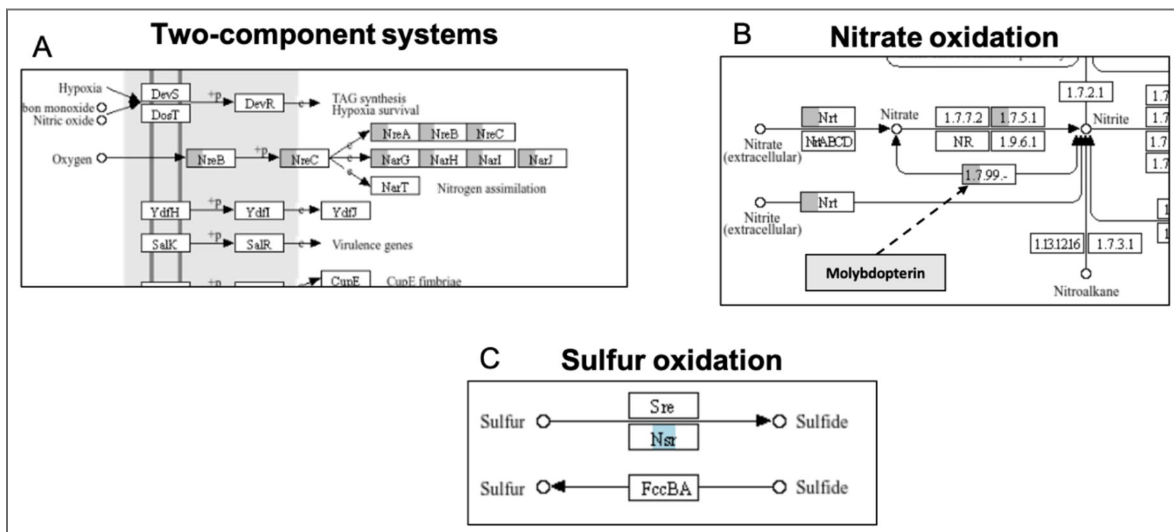


Fig. S11. **Subset of the KEGG pathways map02020 (Two-component system; A), map00910 (Nitrogen metabolism; B), and map00920 (Sulfur metabolism; C).**

Each rectangle represents a KEGG Ortholog (KO). Orthologs that are enriched in each clade are coloured in grey (present in clade U), blue (present in clade C) and/or red (present in clade H). Clade U encodes a unique alternative respiratory system. The operons nreABC and narGHIJ encode for genes that sense anoxic conditions and use nitrate as terminal electron donor instead of oxygen (A, B). The enzyme sulfur oxidoreductase (Nsr) is highly enriched in the clade C, which suggests that it can potentially use sulfur as terminal electron acceptor in absence of oxygen (C).

Data availability

The sequencing raw data used in this study are deposited in the European Nucleotide Archive (ENA) under the BioProject accessions: PRJEB96332, PRJEB29281, PRJEB20780, PRJEB20533, and PRJEB15225 (See supplementary tables S1 and S4). All codes and material needed for data processing and reproduction can be found in *https://github.com/bosco-gracia-alvira/Lpla_intraspecific_diversity*.

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Additional information

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Bosco Gracia-Alvira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft. Stefanie Migotti: Resources, Writing – review and editing. Xiaomeng Tian: Resources, Writing – review and editing. Viola Nolte: Data curation, Resources. Christian Schlötterer: Conceptualization, Project administration, Funding acquisition, Resources, Supervision, Writing – review and editing.

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Additional files

Tables S1-S4.

References

1. **Langille MGI,** Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* **31**:814-21 https://doi.org/10.1038/nbt.2676 | PubMed

2. **Douglas GM,** Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. (2020) PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* **38**:685-8 https://doi.org/10.1038/s41587-020-0548-6 | PubMed
3. **Caro-Quintero A,** Ochman H (2015) Assessing the Unseen Bacterial Diversity in Microbial Communities. *Genome Biol Evol* **7**:3416-25 https://doi.org/10.1093/gbe/evv234 | PubMed
4. **Van Rossum T,** Ferretti P, Maistrenko OM, Bork P (2020) Diversity within species: interpreting strains in microbiomes. *Nat Rev Microbiol* **18**:491-506 https://doi.org/10.1038/s41579-020-0368-1 | PubMed
5. **Ohnishi M,** Kurokawa K, Hayashi T (2001) Diversification of Escherichia coli genomes: are bacteriophages the major contributors?. *Trends Microbiol* **9**:481-5 https://doi.org/10.1016/s0966-842x(01)02173-4 | PubMed
6. **Ansorge R,** Romano S, Sayavedra L, Porras MÁG, Kupczok A, Tegetmeyer HE, et al. (2019) Functional diversity enables multiple symbiont strains to coexist in deep-sea mussels. *Nat Microbiol* **4**:2487-97 https://doi.org/10.1038/s41564-019-0572-9 | PubMed
7. **Gould AL,** Donohoo SA, Román ED, Neff EE (2023) Strain-level diversity of symbiont communities between individuals and populations of a bioluminescent fish. *ISME J* **17**:2362-9 https://doi.org/10.1038/s41396-023-01550-6 | PubMed
8. **Lange C,** Boyer S, Bezemer TM, Lefort MC, Dhami MK, Biggs E, et al. (2023) Impact of intraspecific variation in insect microbiomes on host phenotype and evolution. *ISME J* **17**:1798-807 https://doi.org/10.1038/s41396-023-01500-2 | PubMed
9. **Hecht AL,** Casterline BW, Earley ZM, Goo YA, Goodlett DR, Bubeck Wardenburg J (2016) Strain competition restricts colonization of an enteric pathogen and prevents colitis. *EMBO Rep* **17**:1281-91 https://doi.org/10.15252/embr.201642282 | PubMed
10. **Wolff R,** Shoemaker W, Garud N (2023) Ecological Stability Emerges at the Level of Strains in the Human Gut Microbiome. *mBio* **14**:e0250222 https://doi.org/10.1128/mbio.02502-22 | PubMed
11. **Chen-Liaw A,** Aggarwala V, Mogno I, Haifer C, Li Z, Eggers J, et al. (2025) Gut microbiota strain richness is species specific and affects engraftment. *Nature* **637**:422-9 https://doi.org/10.1038/s41586-024-08242-x | PubMed
12. **Kashtan N,** Roggensack SE, Rodrigue S, Thompson JW, Biller SJ, Coe A, et al. (2014) Single-Cell Genomics Reveals Hundreds of Coexisting Subpopulations in Wild Prochlorococcus. *Science* **344**:416-20 https://doi.org/10.1126/science.1248575 | PubMed
13. **García-García N,** Tamames J, Linz AM, Pedrós-Alió C, Puente-Sánchez F (2019) Microdiversity ensures the maintenance of functional microbial communities under changing environmental conditions. *ISME J* **13**:2969-83 https://doi.org/10.1038/s41396-019-0487-8 | PubMed
14. **Meziti A,** Tsementzi D, Rodriguez-R LM, Hatt JK, Karayanni H, Kormas KA, et al. (2019) Quantifying the changes in genetic diversity within sequence-discrete bacterial populations across a spatial and temporal riverine gradient. *ISME J* **13**:767-79 https://doi.org/10.1038/s41396-018-0307-6 | PubMed
15. **Martino ME,** Bayjanov JR, Caffrey BE, Wels M, Joncour P, Hughes S, et al. (2016) Nomadic lifestyle of Lactobacillus plantarum revealed by comparative genomics of 54 strains isolated from different habitats. *Environ Microbiol* **18**:4974-89 https://doi.org/10.1111/1462-2920.13455 | PubMed
16. **Erkosar B,** Storelli G, Defaye A, Leulier F (2013) Host-Intestinal Microbiota Mutualism: "Learning on the Fly". *Cell Host Microbe* **13**:8-14 https://doi.org/10.1016/j.chom.2012.12.004 | PubMed
17. **Martino ME,** Joncour P, Leenay R, Gervais H, Shah M, Hughes S, et al. (2018) Bacterial Adaptation to the Host's Diet Is a Key Evolutionary Force Shaping Drosophila-Lactobacillus Symbiosis. *Cell Host Microbe* **24**:109-119. https://doi.org/10.1016/j.chom.2018.06.001 | PubMed
18. **Storelli G,** Strigini M, Grenier T, Bozonnet L, Schwarzer M, Daniel C, et al. (2018) Drosophila Perpetuates Nutritional Mutualism by Promoting the Fitness of Its Intestinal Symbiont Lactobacillus plantarum. *Cell Metab* **27**:362-377. https://doi.org/10.1016/j.cmet.2017.11.011 | PubMed
19. **Lee J,** Han G, Kim JW, Jeon CO, Hyun S (2020) Taxon-Specific Effects of Lactobacillus on Drosophila Host Development. *Microb Ecol* **79**:241-51 https://doi.org/10.1007/s00248-019-01404-9 | PubMed

20. **Rodriguez-R LM,** Conrad RE, Viver T, Feistel DJ, Lindner BG, Venter SN, et al. (2023) An ANI gap within bacterial species that advances the definitions of intra-species units. *mBio* **15**:e02696-23 https://doi.org/10.1128/mbio.02696-23 | PubMed

21. **Obadia B,** Güvener ZT, Zhang V, Ceja-Navarro JA, Brodie EL, Ja WW, et al. (2017) Probabilistic Invasion Underlies Natural Gut Microbiome Stability. *Curr Biol* **27**:1999-2006. https://doi.org/10.1016/j.cub.2017.05.034 | PubMed

22. **Larkin AA,** Martiny AC (2017) Microdiversity shapes the traits, niche space, and biogeography of microbial taxa. *Environ Microbiol Rep* **9**:55-70 https://doi.org/10.1111/1758-2229.12523 | PubMed

23. **Fink JW,** Manhart M (2023) How do microbes grow in nature? The role of population dynamics in microbial ecology and evolution. *EcoEvoRxiv* https://doi.org/10.1016/j.coisb.2023.100470

24. **Storelli G,** Defaye A, Erkosar B, Hols P, Royet J, Leulier F (2011) *Lactobacillus plantarum* Promotes *Drosophila* Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing. *Cell Metab* **14**:403-14 https://doi.org/10.1016/j.cmet.2011.07.012 | PubMed

25. **Téfit MA,** Leulier F (2017) Lactobacillus plantarum favors the early emergence of fit and fertile adult Drosophila upon chronic undernutrition. *J Exp Biol* **220**:900-7 https://doi.org/10.1242/jeb.151522 | PubMed

26. **Erkosar B,** Storelli G, Mitchell M, Bozonnet L, Bozonnet N, Leulier F (2015) Pathogen Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via Inhibition of Protein Digestion. *Cell Host Microbe* **18**:445-55 https://doi.org/10.1016/j.chom.2015.09.001 | PubMed

27. **Douglas AE** (2019) Simple animal models for microbiome research. *Nat Rev Microbiol* **17**:764-75 https://doi.org/10.1038/s41579-019-0242-1 | PubMed

28. **Jones EW,** Carlson JM, Sivak DA, Ludington WB (2022) Stochastic microbiome assembly depends on context. *Proc Natl Acad Sci* **119**:e2115877119 https://doi.org/10.1073/pnas.2115877119 | PubMed

29. **Ludington WB** (2022) Higher-order microbiome interactions and how to find them. *Trends Microbiol* https://doi.org/10.1016/j.tim.2022.03.011 | PubMed

30. **Fast D,** Duggal A, Foley E (2018) Monoassociation with Lactobacillus plantarum Disrupts Intestinal Homeostasis in Adult Drosophila melanogaster. *mBio* **9** https://doi.org/10.1128/mbio.01114-18 | PubMed

31. **Beier S,** Bertilsson S (2013) Bacterial chitin degradation—mechanisms and ecophysiological strategies. *Front Microbiol* **4** https://doi.org/10.3389/fmicb.2013.00149 | PubMed

32. **Rudman SM,** Greenblum S, Hughes RC, Rajpurohit S, Kiratli O, Lowder DB, et al. (2019) Microbiome composition shapes rapid genomic adaptation of *Drosophila melanogaster*. *Proc Natl Acad Sci* **116**:20025-32 https://doi.org/10.1073/pnas.1907787116 | PubMed

33. **Montaño-Salazar S,** Quintanilla E, Sánchez JA (2023) Microbial shifts associated to ENSO-derived thermal anomalies reveal coral acclimation at holobiont level. *Sci Rep* **13**:22049 https://doi.org/10.1038/s41598-023-49049-6 | PubMed

34. **Petersen C,** Hamerich IK, Adair KL, Griem-Krey H, Torres Oliva M, Hoeppner MP, et al. (2023) Host and microbiome jointly contribute to environmental adaptation. *ISME J* **17**:1953-65 https://doi.org/10.1038/s41396-023-01507-9 | PubMed

35. **Zhang S,** Song F, Wang J, Li X, Zhang Y, Zhou W, et al. (2024) Gut microbiota facilitate adaptation of invasive moths to new host plants. *ISME J* **18**:wrae031 https://doi.org/10.1093/ismejo/wrae031 | PubMed

36. **Wong CNA,** Ng P, Douglas AE (2011) Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environ Microbiol* **13**:1889-900 https://doi.org/10.1111/j.1462-2920.2011.02511.x | PubMed

37. **Ludington WB,** Ja WW (2020) Drosophila as a model for the gut microbiome. *PLOS Pathog* **16**:e1008398 https://doi.org/10.1371/journal.ppat.1008398 | PubMed

38. **Matos RC,** Leulier F (2014) Lactobacilli-Host mutualism: 'learning on the fly'. *Microb Cell Factories* **13**:S6 https://doi.org/10.1186/1475-2859-13-s1-s6 | PubMed

39. **Nysten J,** Sofras D, Van Dijck P (2024) One species, many faces: The underappreciated importance of strain diversity. *PLOS Pathog* **20**:e1011931 https://doi.org/10.1371/journal.ppat.1011931 | PubMed

40. **Winans NJ,** Walter A, Chouaia B, Chaston JM, Douglas AE, Newell PD (2017) A genomic investigation of ecological differentiation between free-living and Drosophila-associated bacteria. *Mol Ecol* **26**:4536-50 https://doi.org/10.1111/mec.14232 | PubMed

41. **Moghadam NN,** Thorshauge PM, Kristensen TN, de Jonge N, Bahrndorff S, Kjeldal H, et al. (2018) Strong responses of Drosophila melanogaster microbiota to developmental temperature. *Fly* **12**:1-12 https://doi.org/10.1080/19336934.2017.1394558 | PubMed

42. **Li J,** Bates KA, Hoang KL, Hector TE, Knowles SCL, King KC (2023) Experimental temperatures shape host microbiome diversity and composition. *Glob Change Biol* **29**:41-56 https://doi.org/10.1111/gcb.16429 | PubMed

43. **Bååth E,** Kritzberg ES (2024) Temperature Adaptation of Aquatic Bacterial Community Growth Is Faster in Response to Rising than to Falling Temperature. *Microb Ecol* **87**:38 https://doi.org/10.1007/s00248-024-02353-8 | PubMed

44. **Barghi N,** Tobler R, Nolte V, Schlötterer C (2017) Drosophila simulans: A Species with Improved Resolution in Evolve and Resequence Studies. *G3* **7**:2337-43 https://doi.org/10.1534/g3.117.043349 | PubMed

45. **Lai WY,** Schlötterer C (2022) Evolution of phenotypic variance in response to a novel hot environment. *Mol Ecol* **31**:934-45 https://doi.org/10.1111/mec.16274 | PubMed

46. **Schlötterer C,** Tobler R, Kofler R, Nolte V (2014) Sequencing pools of individuals — mining genome-wide polymorphism data without big funding. *Nat Rev Genet* **15**:749-63 https://doi.org/10.1038/nrg3803 | PubMed

47. **Barghi N,** Tobler R, Nolte V, Jakšić AM, Mallard F, Otte KA, et al. (2019) Genetic redundancy fuels polygenic adaptation in Drosophila. *PLOS Biol* **17**:e3000128 https://doi.org/10.1371/journal.pbio.3000128 | PubMed

48. **Versace E,** Nolte V, Pandey RV, Tobler R, Schlötterer C (2014) Experimental evolution reveals habitat-specific fitness dynamics among *W olbachia* clades in *D rosophila melanogaster*. *Mol Ecol* **23**:802-14 https://doi.org/10.1111/mec.12643 | PubMed

49. **Mazzucco R,** Nolte V, Vijayan T, Schlötterer C (2020) Long-Term Dynamics Among Wolbachia Strains During Thermal Adaptation of Their Drosophila melanogaster Hosts. *Front Genet* **11**:482 https://doi.org/10.3389/fgene.2020.00482 | PubMed

50. **Mazzucco R,** Schlötterer C (2021) Long-term gut microbiome dynamics in Drosophila melanogaster reveal environment-specific associations between bacterial taxa at the family level. *Proc R Soc B Biol Sci* **288**:20212193 https://doi.org/10.1098/rspb.2021.2193 | PubMed

51. **Mallard F,** Nolte V, Tobler R, Kapun M, Schlötterer C (2018) A simple genetic basis of adaptation to a novel thermal environment results in complex metabolic rewiring in Drosophila. *Genome Biol* **19**:119 https://doi.org/10.1186/s13059-018-1503-4 | PubMed

52. **Otte KA,** Nolte V, Mallard F, Schlötterer C (2021) The genetic architecture of temperature adaptation is shaped by population ancestry and not by selection regime. *Genome Biol* **22**:211 https://doi.org/10.1186/s13059-021-02425-9 | PubMed

53. **Miller SA,** Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**:1215 https://doi.org/10.1093/nar/16.3.1215 | PubMed

54. **Alimolaei M,** Golchin M (2016) An Efficient DNA Extraction Method for Lactobacillus casei, a Difficult-to-Lyse Bacterium. *Int J Enteric Pathog* **4**:e32472 https://doi.org/10.17795/ijep32472

55. **Bushnell B** (2025) BBMap. SourceForge. https://sourceforge.net/projects/bbmap/

56. **Prjibelski A,** Antipov D, Meleshko D, Lapidus A, Korobeynikov A (2020) Using SPAdes De Novo Assembler. *Curr Protoc Bioinforma* **70**:e102 https://doi.org/10.1002/cpbi.102 | PubMed

57. **Chklovski A,** Parks DH, Woodcroft BJ, Tyson GW (2023) CheckM2: a rapid, scalable and accurate tool for assessing microbial genome quality using machine learning. *Nat Methods* **20**:1203-12 https://doi.org/10.1038/s41592-023-01940-w | PubMed

58. **Chaumeil PA,** Mussig AJ, Hugenholtz P, Parks DH (2022) GTDB-Tk v2: memory friendly classification with the genome taxonomy database. *Bioinformatics* **38**:5315-6 https://doi.org/10.1093/bioinformatics/btac672 | PubMed

59. **Kim D,** Song L, Breitwieser FP, Salzberg SL (2016) Centrifuge: rapid and sensitive classification of metagenomic sequences. *Genome Res* https://doi.org/10.1101/gr.210641.116 | PubMed

60. **Seemann T** (2014) Prokka: rapid prokaryotic genome annotation. *Bioinforma Oxf Engl* **30**:2068-9 https://doi.org/10.1093/bioinformatics/btu153 | PubMed

61. **Page AJ,** Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. (2015) Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **31**:3691-3 https://doi.org/10.1093/bioinformatics/btv421 | PubMed

62. **Page AJ,** Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, et al. (2016) SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb Genomics* **2**:e000056 https://doi.org/10.1099/mgen.0.000056 | PubMed

63. **Minh BQ,** Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, et al. (2020) IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol* **37**:1530-4 https://doi.org/10.1093/molbev/msaa015 | PubMed

64. **Pritchard L,** Glover RH, Humphris S, Elphinstone JG, Toth IK (2015) Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal Methods* **8**:12-24 https://doi.org/10.1039/c5ay02550h

65. **Eren AM,** Esen ÖC, Quince C, Vineis JH, Morrison HG, Sogin ML, et al. (2015) Anvi'o: an advanced analysis and visualization platform for 'omics data. *PeerJ* **3**:e1319 https://doi.org/10.7717/peerj.1319 | PubMed

66. **Delmont TO,** Eren AM (2018) Linking pangenomes and metagenomes: the Prochlorococcus metapangenome. *PeerJ* **6**:e4320 https://doi.org/10.7717/peerj.4320 | PubMed

67. **Shaiber A,** Willis AD, Delmont TO, Roux S, Chen LX, Schmid AC, et al. (2020) Functional and genetic markers of niche partitioning among enigmatic members of the human oral microbiome. *Genome Biol* **21**:292 https://doi.org/10.1186/s13059-020-02195-w | PubMed

68. **Sato N,** Uematsu M, Fujimoto K, Uematsu S (2023) Imoto S. ggkegg: analysis and visualization of KEGG data utilizing the grammar of graphics. *Bioinformatics* **39**:btad622 https://doi.org/10.1093/bioinformatics/btad622 | PubMed

69. **Langmead B,** Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**:357-9 https://doi.org/10.1038/nmeth.1923 | PubMed

70. **Puente-Sánchez F,** Hoetzinger M, Buck M, Bertilsson S (2023) Exploring environmental intra-species diversity through non-redundant pangenome assemblies. *Mol Ecol Resour* https://doi.org/10.1111/1755-0998.13826 | PubMed

71. **Gourlé H,** Karlsson-Lindsjö O, Hayer J, Bongcam-Rudloff E (2019) Simulating Illumina metagenomic data with InSilicoSeq. *Bioinformatics* **35**:521-2 https://doi.org/10.1093/bioinformatics/bty630 | PubMed

72. **Shen W,** Sipos B, Zhao L (2024) SeqKit2: A Swiss army knife for sequence and alignment processing. *iMeta* **3**:e191 https://doi.org/10.1002/imt2.191 | PubMed

73. **Sprouffske K,** Wagner A (2016) Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinformatics* **17**:172 https://doi.org/10.1186/s12859-016-1016-7 | PubMed

74. **R Core Team** (2024) R: A Language and Environment for Statistical Computing. https://www.R-project.org/

75. **Kassambara A.** (2023) rstatix: Pipe-Friendly Framework for Basic Statistical Tests. https://rpkgs.datanovia.com/rstatix/

76. **Kietz C,** Pollari V, Meinander A (2018) Generating Germ-Free Drosophila to Study Gut-Microbe Interactions: Protocol to Rear Drosophila Under Axenic Conditions. *Curr Protoc Toxicol* **77**:e52 https://doi.org/10.1002/cptx.52 | PubMed

77. **Samland AK,** Sprenger GA (2009) Transaldolase: From biochemistry to human disease. *Int J Biochem Cell Biol* **41**:1482-94 https://doi.org/10.1016/j.biocel.2009.02.001 | PubMed

78. **Beier S,** Bertilsson S (2013) Bacterial chitin degradation—mechanisms and ecophysiological strategies. *Front Microbiol* **4** https://doi.org/10.3389/fmicb.2013.00149 | PubMed

79. **García-Angulo VA** (2017) Overlapping riboflavin supply pathways in bacteria. *Crit Rev Microbiol* **43**:196-209 https://doi.org/10.1080/1040841x.2016.1192578 | PubMed

80. **Schwarz G,** Mendel RR, Ribbe MW (2009) Molybdenum cofactors, enzymes and pathways. *Nature* **460**:839-47 https://doi.org/10.1038/nature08302 | PubMed

81. **Moreno-Vivián C,** Cabello P, Martínez-Luque M, Blasco R, Castillo F (1999) Prokaryotic Nitrate Reduction: Molecular Properties and Functional Distinction among Bacterial Nitrate Reductases. *J Bacteriol* **181**:6573-84 https://doi.org/10.1128/jb.181.21.6573-6584.1999 | PubMed

82. **Fedtke I,** Kamps A, Krismer B, Götz F (2002) The Nitrate Reductase and Nitrite Reductase Operons and the narT Gene of Staphylococcus carnosus Are Positively Controlled by the Novel Two-Component System NreBC. *J Bacteriol* **184**:6624-34 https://doi.org/10.1128/jb.184.23.6624-6634.2002 | PubMed

83. **Sohaskey CD,** Wayne LG (2003) Role of narK2X and narGHJI in Hypoxic Upregulation of Nitrate Reduction by Mycobacterium tuberculosis. *J Bacteriol* **185**:7247-56 https://doi.org/10.1128/jb.185.24.7247-7256.2003 | PubMed

84. **Kobylarz MJ,** Grigg JC, Takayama S ichi J, Rai DK, Heinrichs DE, Murphy MEP (2014) Synthesis of L-2,3-Diaminopropionic Acid, a Siderophore and Antibiotic Precursor. *Chem Biol* **21**:379-88 https://doi.org/10.1016/j.chembiol.2013.12.011 | PubMed

85. **Garcia-Hernandez M,** Kun E (1957) Inhibition of enzymic transamination of aspartic acid by hydroxyaspartate, 2,3-diaminosuccinate and 2,3-diaminopropionate. *Biochim Biophys Acta* **24**:78-82 https://doi.org/10.1016/0006-3002(57)90148-8 | PubMed

86. **Kalyani JN,** Ramachandra N, Kachroo AH, Mahadevan S, Savithri HS (2012) Functional Analysis of the Genes Encoding Diaminopropionate Ammonia Lyase in Escherichia coli and Salmonella enterica Serovar Typhimurium. *J Bacteriol* **194**:5604-12 https://doi.org/10.1128/jb.01362-12 | PubMed

Gracia-Alvira B, Migotti S, Tian X, Nolte V, Schlötterer C (2026) Intraspecific diversity of Lactiplantibacillus plantarum reflects functional diversification in the microbiome of Drosophila simulans. ENA. ID PRJEB96332 https://www.ebi.ac.uk/ena/browser/view/PRJEB96332

Barghi N, Tobler R, Nolte V, Jakšić AM, Mallard F, Otte KA, Dolezal M, Taus T, Kofler R, Schlötterer C (2019) Genetic redundancy fuels polygenic adaptation in Drosophila. ENA. ID PRJEB29281 https://www.ebi.ac.uk/ena/browser/view/PRJEB29281

Barghi N, Tobler R,Nolte V, Schlötterer C (2018) Drosophila simulans: a species with improved resolution in Evolve and Resequence studies. ENA. ID PRJEB20780 https://www.ebi.ac.uk/ena/browser/view/PRJEB20780

Kofler R, Senti KA, Nolte V, Tobler R, Schlötterer C (2018) P-element invasion in Drosophila simulans. ENA. ID PRJEB20533 https://www.ebi.ac.uk/ena/browser/view/PRJEB20533

Nouhaud P, Tobler R, Nolte V, Schlötterer C (2016) Ancestral population reconstitution from isofemale lines as a tool for experimental evolution. ENA. ID PRJEB15225 https://www.ebi.ac.uk/ena/browser/view/PRJEB15225

Peer reviews

Reviewer #1 (Public review):

Summary:

The factors that create and maintain diversity in host-associated microbiomes remain poorly understood. A better understanding of these factors will help in the efforts to leverage the adaptive potential of the microbiome to help solve pressing problems in health and agriculture.

Experimental evolution provides a promising path forward as we can track the causes and consequences in the emergence of novel variants, but experimental evolution remains underutilized in host-microbiome interactions. Here, Gracia-Alvira utilizes a long-term experimental evolution study in Drosophila simulans under hot and cold temperature regimes to identify strain-level variation in an important fly bacterium, Lactiplantibacillus plantarum. They identify three strains of L. plantarum, which are most prevalent in their respective three temperature regimes, suggesting that these are locally adapted bacteria. Then, using a combination of genomics, in vitro, and in vivo, Gracia-Alvira et al attempt to understand the factors that led to the differentiation of the hot and cold L. plantarum and their impacts on the fly host.

Strengths:

This is an excellent use of experimental evolution to track the emergence of novelty in the microbiome. The genomic analyses are all solid and appropriate for the data sets. It is especially striking that the comparisons with the other, independent experimental evolution studies in different labs (and across continents between Portugal and South Africa) show a consistent response to temperature. Many have disregarded the microbiome as it is something that is too sensitive to seemingly innocuous variables (particularly in the fly microbiome), such that we cannot find generalities. However, this finding highlights the potential for experimental evolution to uncover these dynamics. The question of how strains emerge and are maintained is timely and is one of the key open questions in host-microbiome evolution currently.

Weaknesses:

(1) The framing in the title and throughout the discussion about "subspecies competition" does not match the data that was collected. The subspecies competition requires actually tracking the competitive outcomes between the hot, cold, and unevolved L. plantarum. In the in vivo work, I can see that mixes of the strains were made, but they did not track whether the cold strain outcompeted the hot strain in vivo under cold conditions, for example. While Figure 4 is suggestive that there is ongoing competition in the hot temperature regime, this is not necessarily shown in the cold, which is dominated by the C clade. It could also be that the bacteria cannot survive in the flies at the different temperatures. The growth curve assays hint that the bacteria can grow, but the plate reader couldn't actually maintain the 18 {degree sign}C temperature (line 455). So all of this evidence is very indirect and insufficient to say that strain competition is driving these patterns.

(2) The in vivo results are interesting in that there appears to be a fitness cost of clade C, but the explanation is underdeveloped. I say under-developed because in Figure 4, the cold L. plantarum remains much higher throughout adaptation to the hot temperature regime than the hot L. plantarum in the cold regime. The hot L. plantarum is low abundance throughout the cold regime. I felt like this observation was not explained, but it seems relevant to understanding the strain dynamics.

I will also note that this is not the first time that L. plantarum or other Lactobacillus have been shown to exert fitness costs to Drosophila. Gould, PNAS, 2018, shows that both Lactobacillus plantarum and Lactobacillus brevis in mono-association have lower fitness (measured through Leslie matrix projections using lifespan and fecundity) than axenic flies. Many studies of wild Drosophila fail to find Lactobacillus, or it is low abundance (e.g., Chandler, PLoS Genetics, 2014; Wang, Environmental Microbiology Reports, 2018; Henry & Ayroles, Molecular Ecology, 2022; Gale, AEM, 2025). This might help provide useful context for the in vivo results.

(3) The data in Figure 4 are compelling to focus on the L. plantarum variants. However, I can see from the methods that the competitive mapping included only other strains of Wolbachia. It is not clear how other members of the microbiome changed in response to the temperature regimes. As I note in point #2, given that Lactobacillus is often rare, it is not clear what the rest of the microbiome looks like over the course of adaptation. Indeed, it seems like Mazzucco & Schlotterer, PRSB, 2021 did a broader analysis of the microbiome and found that Acetobacter is by far the most common bacterium (I think this data is also part of the data shown here?). Expanding on why or why not in this context is important and will improve this study, particularly if the focus is on connecting these evolutionary dynamics to ecological competition to explain the emergence of strain diversity.

https://doi.org/10.7554/eLife.110808.1.sa3

Reviewer #2 (Public review):

Summary:

In this manuscript, Gracia-Alvira et al. investigated how environmental temperature affects competition among members of the microbiome, with a focus on intraspecific diversity, using the Drosophila model.

Notably, the authors identified three clades of Lactiplantibacillus plantarum from a natural population of Drosophila simulans collected in Florida. They tracked the dynamics of these three bacterial clades under two temperature conditions over the course of more than ten years. Using comparative genomics and phylogeny, they showed that these three bacterial clades likely adapted to their host independently in a temperature-specific manner. Further, by combining in vitro culture and in vivo mono-association assays, they demonstrated the functional divergence of these three bacterial clades phenotypically, including their growth dynamics and effects on host fitness. Lastly, they performed pathway analysis and speculated on key genomic variance supporting such functional divergence.

Strengths:

The laboratory evolutionary experiment in response to cold or hot environmental temperature is impressive, given its more than ten years of experimental time period. This collection of achieved microbiome samples paired with the fly host data can be a valuable resource for the field.

Weaknesses:

The laboratory evolutionary experiment can be limited due to its artificial experimental setup. For example, wild flies rely on a more diverse set of food sources and are constantly exposed to new bacterial inoculations, whereas under laboratory conditions, flies live in a more restricted ecosystem. In addition, environmental temperatures differ among different locations, but they also involve seasonal changes within the same region. This manuscript can be strengthened with further discussions that elaborate on these limitations.

Moreover, the extent of host effects involved in these experiments remains ambiguous, because it is unclear whether these Lactiplantibacillus plantarum mostly reside within fly guts or on Drosophila medium. The laboratory evolutionary experiment possibly favored better colonizers on Drosophila medium under either cold or hot temperatures, which subsequently can saturate fly guts. As fully dissociating these variables can be experimentally tedious, the authors may want to comment more on these aspects in the discussion. Or they may want to consider some measurements. For example, measuring the growth rate of these bacteria on Drosophila medium under different temperatures, in addition to the current MRS culture experiments, or measuring the portion of the Lactiplantibacillus on Drosophila medium versus these stably colonizing fly guts.

https://doi.org/10.7554/eLife.110808.1.sa2

Reviewer #3 (Public review):

Summary:

The study presents an analysis of 297 pangenomes derived from 20 populations of Drosophila simulans, at 19 time points for fast-reproducing individuals in a hot environment, or at 10 time points for slow-reproducing individuals in a cold environment, over a period of more than 10 years. The authors select a particular microbial component of the pangenomes and study the dynamics of Lactiplantibacillus plantarum strains in two environments. They discover that the revealed operational taxonomic units could be divided into three phylogenetic clades, which have their own genomic and genetic features, different adaptive capabilities that depend on the environment, and have a distinct impact on the fitness of the host.

Strengths:

The authors prove that bacterial microbiome components are sensitive to the environment and could rapidly (years) be fixed in eukaryotic populations. This study establishes a tractable model that potentially enables the study of variability of the physiological influence of distinct strains of an important commensal species, Lactiplantibacillus plantarum, on the Drsosophila host. It is clearly shown that this single species consists of several phylogenetically and functionally diverse strains. The authors did not limit their interest to their own model, but rather they have integrated a comparative approach by analysing phylogenetic relationships among 92 described L.plantarum strains.

Overall, the study is novel and delivers important discoveries of a longitudinal, well-replicated experiment, generating a substantial amount of genomic data. It highlights an important dimension of research that environmental selection operates at the subspecies level.

Weaknesses:

Even though the authors show only one particular example by conducting their longitudinal experiment, they honestly acknowledge failures important for interpretation of the biological significance of the results (gnotobiotic mono-association experiments was done with D.melanogaster, but not D. simulans) and therefore they state limitations of their conclusions (weaker effects in the non-axenic flies are due to the presence of other taxa or to higher-order interactions with other members of the microbiome). These interactions could significantly affect bacterial growth, metabolism, and physiological influence on the host.

The authors exploit the results of their experiment to speculate about a wide range of evolutionary phenomena, like within-species competition, ecological adaptation and evolution of the host, fitness advantage of bacteria to the host, the benefits of parasitism or

mutualism, the domestication of the microbiome, etc. At the end, they conclude that their study "highlights that even subspecies diversity plays a key role in adaptation to environmental temperature". However, the potential mechanisms of such adaptation are barely discussed, so that the focus of the study shifts from the temperature-induced changes in microbial population structures toward metabolism-related adaptations of clade representatives that enable them to diversify their carbon and nitrogen sources. The role of the temperature factor remains elusive.

In addition to that, the paper has a clearly minimalistic experimental approach to address functional properties of the revealed L.plantarum strains, so that their own fitness, or their relationship with the Drosophila host, is characterised superficially. Therefore, the authors' discourse can be speculative rather than factual (especially when the authors use the expression "likely" to share their guesses in the "Results" section). Nevertheless, these minor drawbacks do not underscore the novelty of the discovered phenotypes and the importance of their further investigation.

https://doi.org/10.7554/eLife.110808.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The factors that create and maintain diversity in host-associated microbiomes remain poorly understood. A better understanding of these factors will help in the efforts to leverage the adaptive potential of the microbiome to help solve pressing problems in health and agriculture.*
>
> *Experimental evolution provides a promising path forward as we can track the causes and consequences in the emergence of novel variants, but experimental evolution remains underutilized in host-microbiome interactions. Here, Gracia-Alvira utilizes a long-term experimental evolution study in Drosophila simulans under hot and cold temperature regimes to identify strain-level variation in an important fly bacterium, Lactiplantibacillus plantarum. They identify three strains of L. plantarum, which are most prevalent in their respective three temperature regimes, suggesting that these are locally adapted bacteria. Then, using a combination of genomics, in vitro, and in vivo, Gracia-Alvira et al attempt to understand the factors that led to the differentiation of the hot and cold L. plantarum and their impacts on the fly host.*
>
> *Strengths:*
>
> *This is an excellent use of experimental evolution to track the emergence of novelty in the microbiome. The genomic analyses are all solid and appropriate for the data sets. It is especially striking that the comparisons with the other, independent experimental evolution studies in different labs (and across continents between Portugal and South Africa) show a consistent response to temperature. Many have disregarded the microbiome as it is something that is too sensitive to seemingly innocuous variables (particularly in the fly microbiome), such that we cannot find generalities. However, this finding highlights the potential for experimental evolution to uncover these dynamics. The question of how strains emerge and are maintained is timely and is one of the key open questions in host-microbiome evolution currently.*
>
> *Weaknesses:*

> *(1) The framing in the title and throughout the discussion about "subspecies competition" does not match the data that was collected. The subspecies competition requires actually tracking the competitive outcomes between the hot, cold, and unevolved L. plantarum. In the in vivo work, I can see that mixes of the strains were made, but they did not track whether the cold strain outcompeted the hot strain in vivo under cold conditions, for example.*

We thank the reviewer for the honest concern and take this opportunity to defend our claim of "subspecies competition used across the manuscript. As the reviewer states, subspecies competition requires tracking the competitive outcomes between the three clades, and this is what we did by sampling and sequencing across ten years of experimental evolution (Figures 4 and S3). For this reason, we point that the subspecies competition assessment comes from the direct observation of changes in relative abundance across the time series, and not from the follow-up experiments *in vivo* or *in vitro*.

> *While Figure 4 is suggestive that there is ongoing competition in the hot temperature regime, this is not necessarily shown in the cold, which is dominated by the C clade. It could also be that the bacteria cannot survive in the flies at the different temperatures. The growth curve assays hint that the bacteria can grow, but the plate reader couldn't actually maintain the 18 {degree sign}C temperature (line 455). So all of this evidence is very indirect and insufficient to say that strain competition is driving these patterns.*

We thank the reviewer for the alternative hypothesis that could explain the observed subspecies dynamic. We rule out that dominance of clade C in the cold occurs because the other two clades cannot grow in this regime based on three pieces of evidence:

(1) In the time series, clades H and U decrease, but never disappear (Figures 4 and S3), even showing some peaks of abundance in specific replicate populations (Figure S3).

(2) We isolated individuals belonging to clade H in the cold-evolved populations, as shown in figure 2. This is a direct evidence that clade H prevails in the cold-evolved populations, although in low abundance.

(3) We did grow the three taxa in fly food petri dishes incubated at both temperature regimes, observing growth in all cases.

We will include the food growth experiment in the revised manuscript as further supporting evidence for growth in both regimes.

> *(2) The in vivo results are interesting in that there appears to be a fitness cost of clade C, but the explanation is underdeveloped. I say under-developed because in Figure 4, the cold L. plantarum remains much higher throughout adaptation to the hot temperature regime than the hot L. plantarum in the cold regime. The hot L. plantarum is low abundance throughout the cold regime. I felt like this observation was not explained, but it seems relevant to understanding the strain dynamics.*

We acknowledge that a strong fitness cost of clade C is observed in axenic *D. melanogaster*. In the native host, *D. simulans*, with reduced microbiome, we observed delayed development that could even be an advantage depending on the situation, as pointed out by reviewer 3 in the recommendations.

Even if we assume that flies colonized with clade C are less fit in the experimental evolution, another caveat is whether the flies can actively select for the *L. plantarum* clade. Under this assumption, a clade that imposes a fitness cost to the fly (clade C) should be selected against over time because the flies colonized by this clade will have less offspring, or develop later than the rest. Alternatively, as the microbiome is shared among all the individuals in the

population, the host might not be able to "purge" the pernicious clade, and *L. plantarum* dynamics might be controlled solely by the relative fitness between clades in the given experimental treatment. We will discuss this hypothesis in the revision as a way to explain the relationship between the abundance of each clade and the effect on the host.

> *I will also note that this is not the first time that L. plantarum or other Lactobacillus have been shown to exert fitness costs to Drosophila. Gould, PNAS, 2018, shows that both Lactobacillus plantarum and Lactobacillus brevis in mono-association have lower fitness (measured through Leslie matrix projections using lifespan and fecundity) than axenic flies. Many studies of wild Drosophila fail to find Lactobacillus, or it is low abundance (e.g., Chandler, PLoS Genetics, 2014; Wang, Environmental Microbiology Reports, 2018; Henry & Ayroles, Molecular Ecology, 2022; Gale, AEM, 2025). This might help provide useful context for the in vivo results.*

We thank the reviewer for the references. These observations will be compared to our phenotypic results and discussed in the revised version of the manuscript.

> *(3) The data in Figure 4 are compelling to focus on the L. plantarum variants. However, I can see from the methods that the competitive mapping included only other strains of Wolbachia.*

We appreciate the thorough reading of the methods by the reviewer. The competitive mapping comprised two steps: first we discarded the reads that mapped to *Drosophila*, *Wolbachia* and additional potential contaminants from sequencing facitilies (human, dog...). This step leaves the reads originated from whole the external microbiome of the flies, including *L. plantarum*. The second competitive mapping step recruits the reads that map any clade of *L. plantarum*.

> *It is not clear how other members of the microbiome changed in response to the temperature regimes. As I note in point #2, given that Lactobacillus is often rare, it is not clear what the rest of the microbiome looks like over the course of adaptation. Indeed, it seems like Mazzucco & Schlotterer, PRSB, 2021 did a broader analysis of the microbiome and found that Acetobacter is by far the most common bacterium (I think this data is also part of the data shown here?). Expanding on why or why not in this context is important and will improve this study, particularly if the focus is on connecting these evolutionary dynamics to ecological competition to explain the emergence of strain diversity.*

We acknowledge that the rest of the *Drosophila* microbiome is not addressed in this study, as we wanted to focus the storyline around the intraspecific dynamics found in *L. plantarum*. We consider that a complete characterization of the whole *Drosophila* microbiome would unnecessarily elongate the paper and thus we treat it as a constant biotic factor.

We must point out that our dataset is not the one reported by Mazzucco & Schlötterer, which was done in *D. melanogaster*, rather than *D. simulans*. Nevertheless, both experiments share the same infrastructure, temperature regimes and fly maintenance.

We will include a list of taxa that were isolated from the populations, as well as to report *L. plantarum* prevalence and abundance across the experiment in order to provide context of the microbiome, beyond *L. plantarum*, to the readership.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *In this manuscript, Gracia-Alvira et al. investigated how environmental temperature affects competition among members of the microbiome, with a focus on intraspecific*

> *diversity, using the Drosophila model. Notably, the authors identified three clades of Lactiplantibacillus plantarum from a natural population of Drosophila simulans collected in Florida. They tracked the dynamics of these three bacterial clades under two temperature conditions over the course of more than ten years. Using comparative genomics and phylogeny, they showed that these three bacterial clades likely adapted to their host independently in a temperature-specific manner. Further, by combining in vitro culture and in vivo mono-association assays, they demonstrated the functional divergence of these three bacterial clades phenotypically, including their growth dynamics and effects on host fitness. Lastly, they performed pathway analysis and speculated on key genomic variance supporting such functional divergence.*
>
> *Strengths:*
>
> *The laboratory evolutionary experiment in response to cold or hot environmental temperature is impressive, given its more than ten years of experimental time period. This collection of achieved microbiome samples paired with the fly host data can be a valuable resource for the field.*
>
> *Weaknesses:*
>
> *The laboratory evolutionary experiment can be limited due to its artificial experimental setup. For example, wild flies rely on a more diverse set of food sources and are constantly exposed to new bacterial inoculations, whereas under laboratory conditions, flies live in a more restricted ecosystem. In addition, environmental temperatures differ among different locations, but they also involve seasonal changes within the same region. This manuscript can be strengthened with further discussions that elaborate on these limitations.*

As the reviewer has correctly noted, our experimental setting is not exempt from limitations. Lab-reared flies are fed with a defined standard diet. Furthermore, although the system is not completely close to bacterial migration, this is limited as replicate populations are not allowed to mix during the maintenance of the flies. For this reason, we consider our laboratory setting as a compromise between observing wild populations, which undergo all biotic and abiotic stresses but cannot be manipulated, and evolving the bacteria in absence of the host, or in gnobiotic hosts, in which biotic interactions are not fully considered. We will extend on this in the new version of the manuscript.

> *Moreover, the extent of host effects involved in these experiments remains ambiguous, because it is unclear whether these Lactiplantibacillus plantarum mostly reside within fly guts or on Drosophila medium. The laboratory evolutionary experiment possibly favored better colonizers on Drosophila medium under either cold or hot temperatures, which subsequently can saturate fly guts. As fully dissociating these variables can be experimentally tedious, the authors may want to comment more on these aspects in the discussion. Or they may want to consider some measurements. For example, measuring the growth rate of these bacteria on Drosophila medium under different temperatures, in addition to the current MRS culture experiments, or measuring the portion of the Lactiplantibacillus on Drosophila medium versus these stably colonizing fly guts.*

The reviewer's point was briefly addressed in the Results chapter: "Phenotypic differences in liquid culture".

> ***Reviewer #3 (Public review):***
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> *Summary:*
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> *The study presents an analysis of 297 pangenomes derived from 20 populations of Drosophila simulans, at 19 time points for fast-reproducing individuals in a hot*

> *environment, or at 10 time points for slow-reproducing individuals in a cold environment, over a period of more than 10 years. The authors select a particular microbial component of the pangenomes and study the dynamics of Lactiplantibacillus plantarum strains in two environments. They discover that the revealed operational taxonomic units could be divided into three phylogenetic clades, which have their own genomic and genetic features, different adaptive capabilities that depend on the environment, and have a distinct impact on the fitness of the host.*
>
> *Strengths:*
>
> *The authors prove that bacterial microbiome components are sensitive to the environment and could rapidly (years) be fixed in eukaryotic populations. This study establishes a tractable model that potentially enables the study of variability of the physiological influence of distinct strains of an important commensal species, Lactiplantibacillus plantarum, on the Drsosophila host. It is clearly shown that this single species consists of several phylogenetically and functionally diverse strains. The authors did not limit their interest to their own model, but rather they have integrated a comparative approach by analysing phylogenetic relationships among 92 described L.plantarum strains.*
>
> *Overall, the study is novel and delivers important discoveries of a longitudinal, well-replicated experiment, generating a substantial amount of genomic data. It highlights an important dimension of research that environmental selection operates at the subspecies level.*
>
> *Weaknesses:*
>
> *Even though the authors show only one particular example by conducting their longitudinal experiment, they honestly acknowledge failures important for interpretation of the biological significance of the results (gnotobiotic mono-association experiments was done with D.melanogaster, but not D. simulans) and therefore they state limitations of their conclusions (weaker effects in the non-axenic flies are due to the presence of other taxa or to higher-order interactions with other members of the microbiome). These interactions could significantly affect bacterial growth, metabolism, and physiological influence on the host.*

We agree with the reviewer in that the use gnobiotic animals is a limitation, as by "tuning" the flies' microbiome we are modifying the interactions between members, which can potentially change the phenotypic outcome. Nevertheless, we use it as a complementary approach, rather than the only inference in our study.

> *The authors exploit the results of their experiment to speculate about a wide range of evolutionary phenomena, like within-species competition, ecological adaptation and evolution of the host, fitness advantage of bacteria to the host, the benefits of parasitism or mutualism, the domestication of the microbiome, etc. At the end, they conclude that their study "highlights that even subspecies diversity plays a key role in adaptation to environmental temperature". However, the potential mechanisms of such adaptation are barely discussed, so that the focus of the study shifts from the temperature-induced changes in microbial population structures toward metabolism-related adaptations of clade representatives that enable them to diversify their carbon and nitrogen sources. The role of the temperature factor remains elusive.*

We acknowledge that our study does not fully resolve the mechanism by which a different clade ends up dominating each temperature regime. The MRS liquid experiment was an attempt to answer whether differences in optimal growth temperature could explain the temperature-specific abundance of the two clades. Our experiments showed, however,

thatthis was not the case. Beyond this point, it is hard to disentangle the role of the temperature, as it could also act indirectly on the bacteria, for example, through the host or the food.

A second observation in our time series was that a third clade, U, was unfit in both regimes despite starting the experiment in high abundance. For this reason we also studied what made this clade less fit. Based on our analyses, we propose that the decrease of clade U was driven by the shift to a laboratory diet, shared by all experimental populations.

> *In addition to that, the paper has a clearly minimalistic experimental approach to address functional properties of the revealed L.plantarum strains, so that their own fitness, or their relationship with the Drosophila host, is characterised superficially. Therefore, the authors' discourse can be speculative rather than factual (especially when the authors use the expression "likely" to share their guesses in the "Results" section). Nevertheless, these minor drawbacks do not underscore the novelty of the discovered phenotypes and the importance of their further investigation.*

We consider the reviewer's concern and will tone down the phrasing when reporting our findings in the revised version of the manuscript.

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