Title: Host evolutionary history and ecological context modulate the adaptive potential of the microbiome

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Abstract (186 words/200):
Can the microbiome serve as a reservoir of adaptive potential for hosts? To address this question, we leveraged ~150 generations of experimental evolution in Drosophila melanogaster in a stressful high sugar (HS) diet. We performed a fully reciprocal transplant experiment using the dominant control and HS bacteria and measured life-history and metabolic traits. If the microbiome can indeed confer benefits to hosts, then transplant recipients should gain fitness benefits compared to controls. Interestingly, we found that these benefits do exist, but are non-linear. The benefits depend on specific interactions between host genotype and the microbiome, and these interactions are modulated by the environment. Mismatches between fly evolution and microbiome exerted fitness costs by slowing development and reducing fecundity, especially in the stressful HS diet. The dominant HS bacteria (*Acetobacter pasteurianus*) uniquely encoded several genes to enable uric acid degradation, mediated the toxic effects of uric acid accumulation due to the HS diet for flies. Our study demonstrates that host genotype x microbiome x environment interactions have substantial effects on host phenotype, highlighting how host evolution and ecological context together shape the adaptive potential of the microbiome.

Introduction

The microbiome has profound effects on many aspects of organismal biology. From modulating physiology (Sommer and Bäckhed, 2013) to pathogen protection (Selosse et al., 2014) to social interactions (Archie and Tung, 2015), the microbiome is frequently implicated as a key regulator of many host phenotypes--but can the microbiome also influence host adaptation? For hosts where microbes are tightly controlled and faithfully transmitted across generations, microbes can have clear influence on host evolution (Henry et al., 2021; Koskella et al., 2017; Moran and Sloan, 2015). For example, the acquisition of nutrition-provisioning symbionts enables the ecological expansion of sap-feeding insects (Sudakaran et al., 2017). For many eukaryotes, though, their microbiomes are environmentally acquired or through other weakly controlled transmission mechanisms (Koskella et al., 2017). Weak control over microbial transmission disrupts the links between the microbiome and host over generations. Without these links, the microbiome may have limited effects on host evolution (Douglas and Werren, 2016; Henry et al., 2021; Moran and Sloan, 2015). Microbiomes may still have the potential to influence host evolution because environmentally acquired microbes must survive and adapt to external environments. In doing so, microbiomes may acquire beneficial traits that hosts can leverage. Thus, microbiomes have the potential to precede and facilitate rapid host adaptation, but this process is poorly understood.

Environmentally acquired microbiomes appear to facilitate rapid host adaptation in several systems. For example, bean bugs acquire pesticide resistance from their soil-dwelling *Burkholderia* symbiont (Kikuchi et al., 2012). The gut microbiome of woodrats facilitates survival on toxic plant diets (Kohl and Dearing, 2016). In plants, drought-adapted soil communities can increase fitness in drought conditions (Lau and Lennon, 2012). These studies suggest that microbes can adapt and evolve to stressful conditions in natural environments and buffer the stressor for the host, but much about the evolution of these interactions remains unclear—for example, we do not know to what extent these microbial effects depend on host genotypes.

Host genetic variation may influence the responsiveness to microbial variation such that some combinations of host and microbiome differentially affect host phenotypes within species. The effects on host phenotype will also likely depend on the environmental context, where mismatches between the environment, host genotype, and microbiome may negatively affect host fitness. In other words, host genotype x microbial genotype x environment (G_H x G_M x E) interactions may shape how host-microbiome systems evolve. Such complex G_H x G_M x E interactions are likely to be common, and yet their contribution in shaping host phenotypic variation and adaptation are underexplored. As the study of microbiomes moves beyond descriptive attributes, experimentally assessing the interactions between host and microbial evolution are a key research priority for better understanding the interplay in host-microbiome interactions (Busby et al., 2017; Mueller and Sachs, 2015).

The fruit fly, *Drosophila melanogaster*, has emerged as an excellent model to study host-microbe interactions. Flies have relatively simple microbiomes, often composed of fewer than 10 bacterial species from acetic acid and lactic acid bacteria families with effects on many different fly traits (Douglas, 2018). The microbiome is largely environmentally acquired through feeding, defecation, and egg smearing during oviposition (Blum et al., 2013; Wong et al., 2015). After hatching, larvae acquire their microbes through a combination of microbial persistence and colonization and form associations over the lifespan of the fly (Bakula, 1969; Broderick and Lemaitre, 2012; Obadia et al., 2017). Many fly-associated microbes can be individually cultured and reconstituted in various combinations to inoculate sterile flies (Douglas, 2018; Koyle et al., 2016). With the feasibility of microbiome manipulations and rich genetic resources, *Drosophila* is an ideal model to study the interplay between host genetic, microbiome, and environment in shaping host phenotypes that underlie rapid host adaptation.

In this study, to investigate whether the microbiome can serve as a reservoir of adaptive potential for hosts, we studied the contribution of the microbiome in the context of experimental evolution. Experimental evolution has a long history of providing fundamental insights into

adaptive processes, but has largely neglected the contribution of the microbiome (Henry et al., 2021; Hoang et al., 2016). One particularly powerful approach is the Evolve and Resequence (E&R) framework to measure the genomic response to selection (Long et al., 2015). E&R experiments typically begin with large, outbred populations which are then subjected to a selective regime, and in parallel, control populations are maintained in a benign, non-selective environment. After several generations, the control and evolved populations are then sequenced to identify the genomic response to selection. Although traditionally E&R experiments have focused on the organism targeted by selection (e.g., yeast, flies, mice, etc.), the E&R approach can also identify changes in the microbiome associated with the evolved populations (Henry and Ayroles, 2021). However, to determine whether any changes in the microbiome over generations of selection actually contributed to host adaptation, it is necessary to transplant the microbiome between control and evolved populations. This approach holds great potential to inform how the microbiome shapes host phenotypes and contributes to host adaptation.

Here, we leverage ~150 generations of fly and microbial adaptation to a nutritionally stressful, high sugar (HS) diet. HS diets exert stress on flies, and flies exhibit similar obesity-like phenotypes as humans with elevated triglycerides, insulin resistance, and shortened lifespans (Musselman et al., 2011; Na et al., 2013; Pallares et al., 2021; Rovenko et al., 2015). In turn, the different bacteria in the fly microbiome have a range of effects on fly phenotypes, shaping life history traits (Newell and Douglas, 2013; Walters et al., 2020) and metabolism (Newell and Douglas, 2013; Shin et al., 2011), and these microbiome effects on host phenotype can depend on host genetics (Chaston et al., 2016; Dobson et al., 2015). Together, these findings suggest that the microbiome contributes to host traits during adaptation to the HS diet. Our goal was to determine whether transplanting the adapted microbiome into non-adapted fly genotype would confer adaptive traits in the stressful HS diet. Through a fully reciprocal host x microbiome x diet transplant experiment (Fig. 1), we evaluate how each component contributes to fitness

associated traits in flies. The power of our approach is to compare the benefit of adapted microbes relative to non-adapted microbes under different host genetic and environmental contexts (i.e., HS or control). Our results point to the important role microbiomes may play in influencing host adaptation but also highlight the complexities of environmental context dependence on host-microbiome interactions.

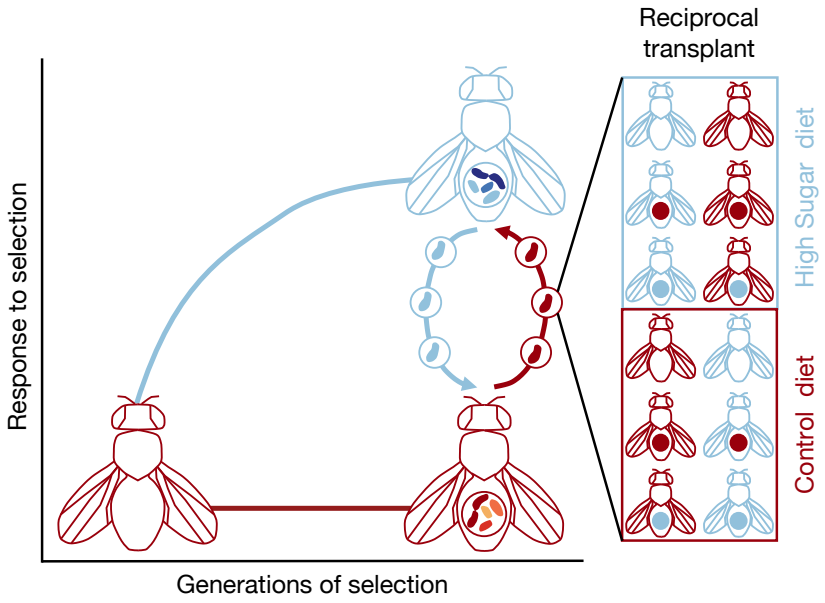

Figure 1: Experimental design to leverage ~150 generations of fly adaptation to the high sugar (HS) diet to study the adaptive potential of the microbiome. The conceptualized evolutionary trajectory shows control (C) flies and microbiome in red and HS flies and microbiome in blue. By the end of experimental evolution, the C fly population (red) has a different microbiome than the HS fly population (blue). After the ~150 generations of selection, we performed a fully reciprocal host x microbiome x diet transplant experiment for all combinations of fly genotypes (C or HS), microbes (sterile, C, or HS), and diets (C, or HS). Together, this approach allows for the assessment of the benefit of adapted relative to non-adapted microbes in both host genetic and environmental contexts.

RESULTS

Fly microbiome responds to HS selection

Here, we leveraged ~150 generations of fly and microbial adaptation to the HS diet to understand how the microbiome can contribute to host evolution. Flies were reared in both the non-selective control (C) diet (10% glucose, 10% yeast, 1.2% agar) and the HS diet (same base

diet supplemented with an additional 13% sucrose; full details in methods). Both C and HS populations were maintained in parallel over the course of the experiment, and the data here resulted from the C and HS populations after ~150 generations.

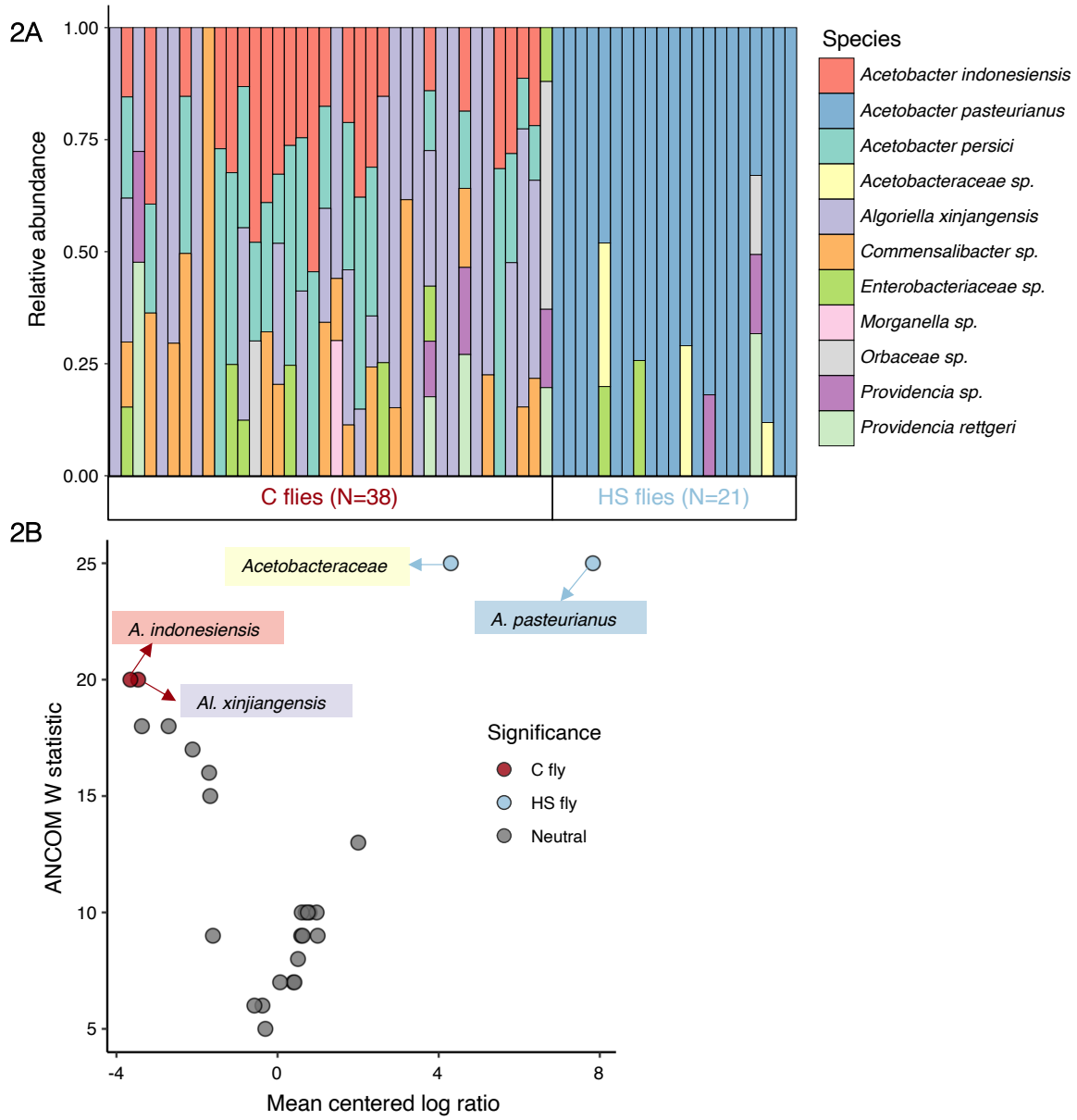

Figure 2: The microbiome in HS flies is different from C flies. A) Relative abundance of bacteria determined using 16S rRNA sequences. Each bar represents an individual fly (N=38 C flies, 21 HS flies). The C fly microbiome is enriched for *A. indonesiensis*, *A. persici*, and *Algoriella xinjiangensis*. The HS fly microbiome is enriched for *A. pasteurianus*. B) ANCOM results for differentially abundant bacteria between C and HS flies. Each point represents a different ASV, colored by whether significantly enriched for C (red) or HS flies (blue). Labels denote the

taxonomic classification for significant ASVs and are highlighted to match the color from the relative abundance in 2A.

First, we established that the microbiome changed between C and HS flies (Fig. 2A). HS flies were dominated by *Acetobacter pasteurianus* (86.7% +/- 1.8 SE) and unclassified Acetobacteraceae strain (5.1% +/- 1.8 SE), while C flies were more diverse with *Algoriella xinjiangensis* (37.3% +/- 5.3 SE), *Acetobacter persici* (16.9% +/-2.6 SE), and *Acetobacter indonesiensis* (14.8% +/- 2.1 SE). The most differentially abundant bacteria were *A. pasteurianus* and Acetobacteraceae strain for the HS flies, while *A. xinjiangensis* and *A. indonesiensis* were associated with C flies (Fig. 2B, Supp. Table 1). These bacteria are unique to either C or HS flies and found across individual flies, demonstrating that the HS diet selected for a different fly microbiome, dominated by *A. pasteurianaus*.

Transplanting Acetobacter influences fitness-related traits

To test if transferring the microbiome could transfer adaptive phenotypes to naive hosts, we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. For the microbiome, we transplanted the differentially abundant *Acetobacters* from the C and HS flies identified above. While not the complete bacterial community from the C flies, we reasoned that because both community complexity (i.e., single dominant species in the HS microbiome versus several in the C microbiome) and taxonomic diversity (i.e., single dominant *Acetobacter* in the HS microbiome versus two *Acetobacter* species, *Algoriella*, *Commensalibacter*, etc. in the C microbiome), we would focus the transplant on the two differentially abundant *Acetobacters*: *A. indonesiensis* (C *Acetobacter*) and *A. pasteurianus* (HS *Acetobacter*).

We hypothesized that transplanting the HS *Acetobacter* should confer fitness benefits to non-adapted C flies in the HS diet. We note that the effect of HS diet on flies has been well documented and likely exerted several selective pressures on the fly, from the metabolic stress of insulin resistance (Musselman et al., 2011), dehydration (van Dam et al., 2020), mating

preferences (Schultzhaus et al., 2017), and sexually dimorphic responses to high sugar diets (Pallares et al., 2021). Therefore, we primarily focused on higher order traits associated with fitness: developmental timing and fecundity, which may reflect the cumulative adaptations to the multitude of selective pressures in the HS diet. Because all combinations of G_H x G_M x E interactions were produced in the transplant experiment, the relative benefits of C and HS *Acetobacter* across the different host genetic (selected or unselected) and environmental conditions (HS or control) could be evaluated.

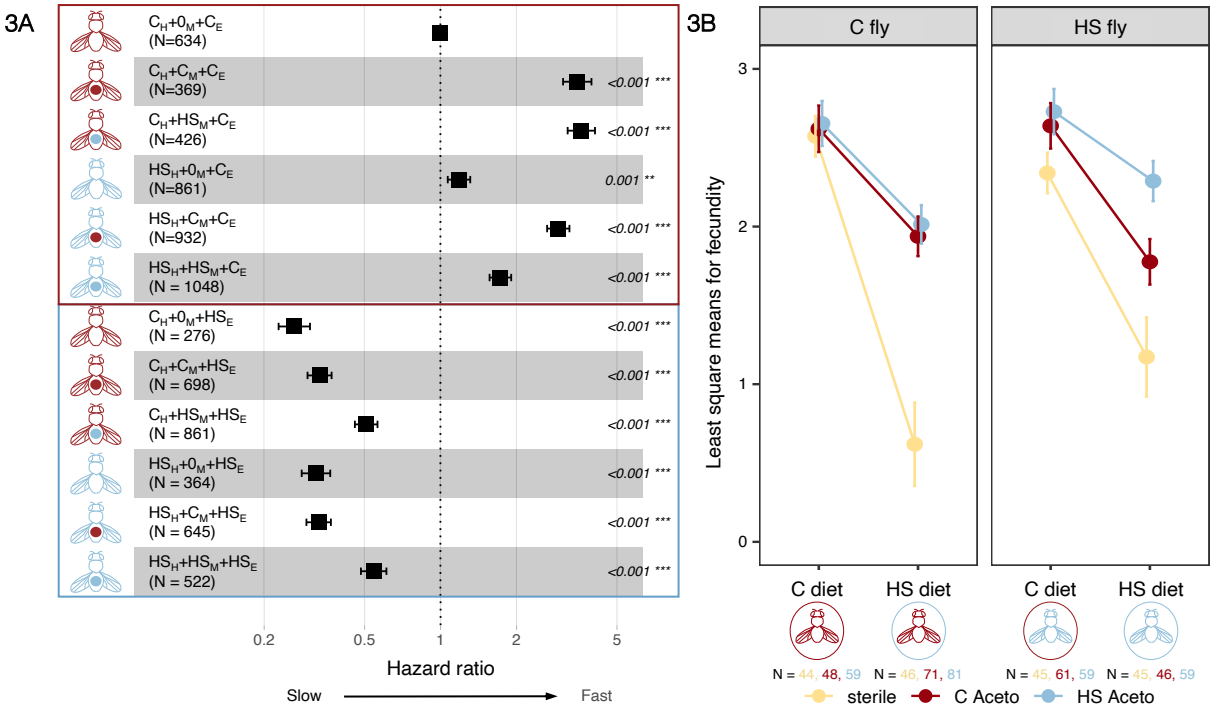

Figure 3: Effects of the *Acetobacter* transplant on fitness-related traits. A) Hazard ratio plot for *Acetobacter* effects on development time. Treatment is shown with the fly diagram, where fly color represents fly genotype, circle represents *Acetobacter* (no circle = sterile), and box represents diet. The full treatment is written out (i.e., $C_H+C_M+C_E$ = C fly with C microbiome on the C diet) with the total number of larvae phenotyped across replicates shown below. Boxplots show hazard ratios and 95% confidence intervals for each treatment relative to $C_H+0_M+C_E$. Mismatches between diet and microbiome slowed development. In the C diet, HS flies with C *Acetobacter* developed faster than the HS flies with HS *Acetobacter*. However, on the HS diet, C *Acetobacter* slowed, while HS *Acetobacter* accelerated development. B) Least square means (LSM) from fecundity, modeled with a zero-inflated Poisson distribution show fly genotype x *Acetobacter* x diet interactions shaped fecundity. Plots are faceted by fly genotype, where colors represent *Acetobacter* treatment, and the number of age-matched females assayed below the

fly diagram. Error bars show standard error for LSMs. For the HS fly genotype on the HS diet, the HS *Acetobacter* increased fecundity relative to the C *Acetobacter*, suggesting a fecundity benefit with the adapted microbiome.

Development was shaped by interactions between fly genotype, *Acetobacter*, and diet (Fig. 3A, Supp. Table 2). On the C diet, fly genotype influenced the response to the *Acetobacter* transplant. HS flies with the C *Acetobacter* developed more rapidly (avg. 140.8 hrs +/- 0.54 SE) than with the HS *Acetobacter* (avg. 147.6 hrs +/- 0.60 SE). There was no effect for the C fly. However, on the HS diet, the HS *Acetobacter* accelerated development (HS *Acetobacter* avg. 176.5 hrs +/- .50 SE, C *Acetobacter* avg. 186.8 hrs +/-0.60 SE). The three-way interaction between fly genotype, *Acetobacter*, and diet significantly affected development time (log-likelihood =-58500, X^2 = 47.625, $p < 0.0001$).

Fecundity responded to interactions between fly genotype, *Acetobacter*, and diet (Fig. 3B, Supp. Table 3). On average, treatments were more fecund on the C diet, while treatments on HS diets laid 54.3% fewer eggs (C avg. 13.0 eggs +/-0.6 SE, HS, avg. 5.9 eggs +/- 0.4 SE). Sterile treatments substantially lowered fecundity, only on the HS diet (+microbes avg. 7.45 eggs +/- 0.5 SE, sterile avg. 1.67 eggs +/- 0.4 SE). Notably, the response to *Acetobacter* treatment depended on fly genotype for the HS diet. On the HS diet, HS flies with HS *Acetobacter* (avg. 8.4 eggs +/-1.4 SE) were almost twice as fecund as flies with the C *Acetobacter* (avg. 4.5 eggs +/- 1.0 SE). The interaction between fly genotype, microbiome, and diet significantly affected fecundity (interaction X^2 = 16.4, df = 2, p=0.0003).

To gain insight into how these interactions shape host metabolism, we also assayed flies for triglyceride (TAG) normalized to protein levels. TAG levels were also shaped by interactions between fly genotype and diet (Supp. Fig. 1A, Supp. Table 4). On the C diet, HS flies had lower TAG levels than C flies, but this relationship was not observed on the HS diet (Supp. Fig. 1B, fly genotype x diet interaction, $F_{1,78.0}$ = 9.00, p = 0.004). *Acetobacter*, alone or through interactions, did not influence TAG levels. Thus, fly metabolism was primarily shaped by host genetics and

diet, but not by differences between *Acetobacters*.

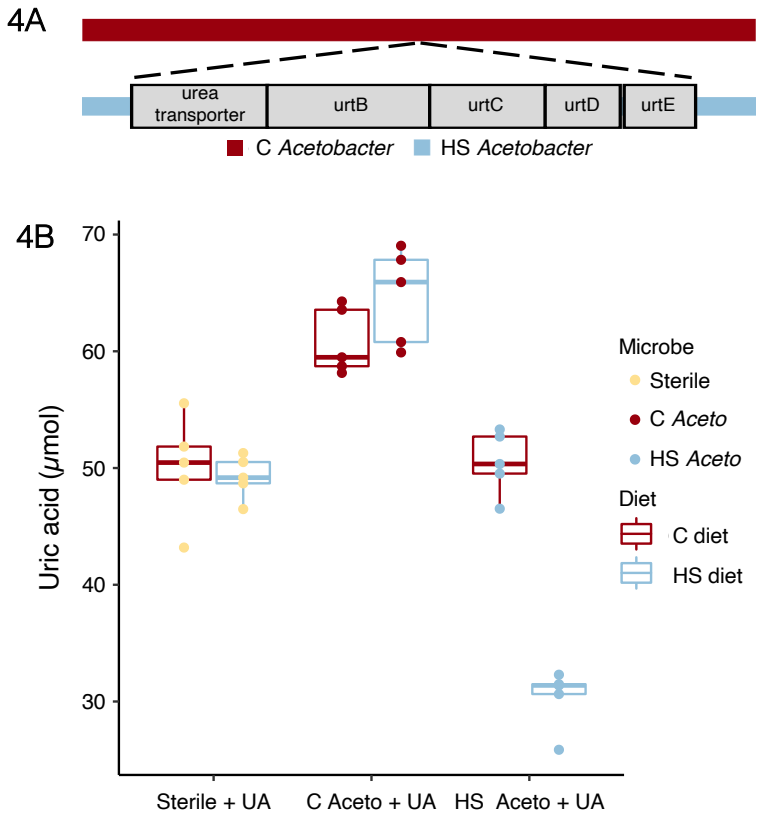

Figure 4: Genomic analysis of the C and HS *Acetobacters*. A) Schematic showing the genes in the uric acid degradation pathway encoded only by HS *Acetobacter*. B) Boxplots show uric acid in the *in vitro* diet assay. Boxplots are colored by diet with points colored by *Acetobacter* treatment. Only in the HS diet, HS *Acetobacter* degraded significantly more uric acid than C *Acetobacter*.

HS Acetobacter uniquely encodes uric acid degradation pathway

To better understand how the HS *Acetobacter* may benefit flies in the HS diet, we sequenced and compared the genomes of the C and HS *Acetobacters*. One key difference between the two genomes was that HS *Acetobacter* encodes for several genes that degrade uric acid, but C *Acetobacter* does not (Fig. 4A). Further, the other dominant bacteria in the C microbiome (*Algoriella xinjiangensis* and *Acetobacter persici*) also lack uric acid degradation genes (Supp. Table 5). Indeed, we found that interactions between *Acetobacter* and the diet shape uric acid degradation (Fig. 4B, Supp. Table 6). Only on the HS diet does HS *Acetobacter* reduce the concentration of uric acid by ~50% compared to C *Acetobacter*, with only negligible

changes to uric acid concentration on the C diet.

DISCUSSION

Here, we explored how the microbiome influences host fitness in stressful environments. Through reciprocal host x microbiome x diet transplant experiments, we show that G_H x G_M x E interactions shape host fitness by modifying developmental timing and fecundity. The HS *Acetobacter* genome encoded uric acid degradation genes and, in vitro, lowered uric acid on the HS diet, while the C *Acetobacter* did not. Our results highlight the complex interactions generated by the microbiome, and we discuss important future directions to further dissect these relationships.

Acetobacter dominates HS microbiome

The HS microbiome was dominated by a single species of bacteria, *Acetobacter pasteurianus* (Fig. 2). *Acetobacter* species are common in flies, both in laboratory settings and wild environments (Adair et al., 2018; Broderick and Lemaitre, 2012; Chandler et al., 2011; Staubach et al., 2013; Wang et al., 2020). While *Acetobacter* are typically sugar specialists (Kersters et al., 2006), here, one major axis of variation separating the C and HS microbiome was the ability to degrade uric acid. In the context of *Drosophila* biology, uric acid accumulation is commonly associated with high sugar diets in flies (van Dam et al., 2020). The accumulation of uric acid may also exacerbate the deleterious effects of HS diet, altering oxidative stress and inflammation that can promote obesogenic phenotypes (Sautin and Johnson, 2008; Winans et al., 2017). Uric acid is normally excreted by flies, but high concentrations slow development (Henry et al., 2018) and shorten lifespan (Lang et al., 2019; van Dam et al., 2020). Not all *Acetobacter* species can degrade uric acid, including the C *Acetobacter*, but the ability to degrade is more common in fly-environment than in other free-living *Acetobacter* species (Winans et al., 2017). Here, comparative analyses between the C and HS *Acetobacter* genomes

showed that HS *Acetobacter* encoded for several genes in the uric acid degradation pathway, while the C *Acetobacter* genome did not. Uric acid degradation may be the key fitness benefit of the HS *Acetobacter* over the C *Acetobacter* in the HS diet.

The fitness advantage of uric acid degradation was likely direct for the HS *Acetobacter*, but indirect for the fly. Over the course of experimental evolution, both fly and microbiome were exposed to the HS diet, and in turn, the HS microbiome was dominated by *A. pasteurianus*, which could degrade uric acid. *Acetobacter* species that degrade uric acid outcompete *Acetobacter* species that cannot in the fly environment (Winans et al., 2017). For the HS *Acetobacter*, uric acid degradation may underlie its persistence in the stressful HS diet, and thus indirectly benefiting flies through reductions of uric acid in the diet. The benefit of uric acid degradation may be especially important during development. Larvae have limited endogenous abilities to detoxify uric acid, and the primary mechanism of uric acid tolerance is by decreasing exposure through decreased uptake or increased excretion (Etienne et al., 2001; Henry et al., 2018; Pierce et al., 1999). For adults, it is unclear if HS *Acetobacter* also degrades uric acid in the fly gut or simply benefiting from the reduction in the diet. While these questions require further characterization of fly physiology, uric acid degradation by HS *Acetobacter* is similar to a handful of examples of environmentally acquired microbiomes that facilitate adaptation through detoxifying the environment, such as insecticide resistance in bean bugs (Kikuchi et al., 2012) or terpene detoxification in woodrats (Kohl and Dearing, 2016). Our results contribute to a growing body of literature that suggests that the microbiome can encode novel functions that hosts can leverage the microbiome during adaptation to stressful environments (Henry et al., 2021).

Adaptive potential of the microbiome depends on evolutionary and ecological context

Many have predicted that the microbiome could accelerate local adaptation in the host, facilitating phenotypic changes to buffer local stressors (Alberdi et al., 2016; Carrier and Reitzel,

2017; Henry et al., 2021; Sudakaran et al., 2017). If so, transplanting the locally adaptive microbiomes should confer adaptive phenotypes to naive individuals. However, the benefit may depend on interactions between host genotype and environment. G_H x G_M x E interactions have the potential to facilitate or impede evolution in challenging environments (Busby et al., 2017; Mueller and Sachs, 2015). Through combining experimental evolution with reciprocal transplants, our data allowed us to test if and how ecological context (i.e., diet) and evolutionary context (i.e., fly genotype) shape the adaptive potential of the microbiome.

To shape adaptive potential, the microbiome must contribute to host phenotypic variation, not unlike traditional (host) additive genetic variance (Henry et al., 2021). The G_H x G_M x E interaction for development illustrates how diet (the ecological context), when matched with host genotype (the evolutionary context) may increase the fitness benefit of locally adaptive microbiomes (Fig. 3A). The HS *Acetobacter* accelerated development on the HS diet (a match between microbe and diet, independent of fly genotype) and slowed development compared to the C *Acetobacter* on the C diet for only the HS fly (a mismatch between microbe and environment, but dependent on fly genotype). The mismatch only mattered for the HS fly; C fly development was similar between the C and HS *Acetobacter* on the C diet. However, the G_H x G_M x E interaction that shaped fecundity is suggestive of how genetic interactions may impede the transfer of adaptive potential. The naive host, C flies, did not receive a fecundity increase from the HS *Acetobacter* in the HS diet, but HS flies did (Fig. 3C). The presence of G_H x G_M x E interactions for both development and fecundity suggests that the microbiome contributed to fly evolution. HS flies responded differently to the *Acetobacter* variation from the C flies, and more so, responsiveness depended on the diet. Because host genotypes may vary in their ability to leverage beneficial microbes in stressful environments, genetic interactions may limit the benefits of locally adaptive microbiomes. Understanding these G_H x G_M interactions will be especially important in using evolution to engineer beneficial microbiomes to solve pressing problems in public health and agriculture (Busby et al., 2017; Henry et al., 2021; Mueller et al.,

2020; Mueller and Sachs, 2015). While longitudinal sampling of the evolutionary trajectory is necessary to determine to what extent the HS fly evolved or selected for beneficial *Acetobacter* traits, our results clearly demonstrate how the ecological context and host evolution can shape the adaptive potential of the microbiome.

We acknowledge that despite the ~150 generations of adaptation to the HS diet, the HS diet still comes at a fitness cost to the HS flies. For example, development time was still slower on the HS diet compared to the C diet (Fig. 3A). HS flies were less fecund on the HS diet than on the C diet (Fig. 3B). For metabolic indices, genotypic differences were only observed on the C diet (Supp. Fig. 1). One possibility for these unexpected results is the use of dechorionation to manipulate the microbiome. Dechorionation, which removes the chorion that protects eggs as well as the microbes smeared during egg laying, is a common tool for manipulating the *Drosophila* microbiome (Douglas, 2018; Koyle et al., 2016). Dechorionation has conflicting effects on different aspects of fly physiology. One study found no significant effect on metabolic indices, like protein and TAG levels, compared to conventionally reared flies (Ridley et al., 2013). However, another study found that dechorionation increased overall mortality, though improved starvation resistance (Heys et al., 2018). It is possible that dechorionation affected our results, but we did not test for this effect. We raise this point as one challenging aspect of using experimentally evolved hosts for microbiome manipulations, as adaptation to novel environments may change host biology in unexpected ways.

Conclusions and research priorities

Here, we leveraged host and microbial evolution from ~150 generations of adaptation to a stressful diet through the E&R approach. Such experiments have illuminated many important findings about the role of demography, population genetics, and genetic architecture in shaping adaptive traits (Long et al., 2015; Schlötterer et al., 2015). However, missing from this framework has been the role of the microbiome. In a recent survey of ten E&R experiments, the

microbiome also frequently responded to experimental evolution, particularly in traits associated with metabolism (Henry and Ayroles, 2021). Taken together with our reciprocal transplant experiment, our data also highlight how the microbiome may contribute to host adaptation – effects previously unseen when focusing on the *Drosophila* genome. Recent work on the *Drosophila* microbiome linked to natural variation in wild populations suggested that a single inoculation of two different bacteria linked to different life-history strategies, can lead to rapid phenotypic change and genetic differentiation in one fly population (Rudman et al., 2019; Walters et al., 2020). Our current study advances these findings by comparing differences in ecologically relevant *Acetobacter* evolution to differences in fly evolution, providing one of the first experimental dissections of host-microbiome interactions in the E&R context. Through reciprocal transplants, we identified how interactions between fly genotype, microbiome, and diet shape fitness related traits.

The G_H x G_M x E interactions observed here highlight how evolution in both the host and microbiome occurred during adaptation to the HS diet. In particular, the ability of HS *Acetobacter* to degrade uric acid in the HS diet reflects the potential for diffuse coevolution (Koskella and Bergelson, 2020). The HS diet likely led to an increase in uric acid production by flies, and then the *Acetobacter* that could exploit the new niche increased, which the HS flies benefited from the reduction in toxicity. Ultimately, in our system and perhaps many others, the evolutionary benefits between host and microbiome are intertwined. Future work utilizing experimental evolution will demonstrate how the joint evolution of host and microbiome shapes the response to selection, providing fundamental insights into how natural selection operates on complex systems.

MATERIALS AND METHODS

Fly populations

All fly populations were maintained at 25ºC with 12 hour light:dark cycles. The base

ancestral population was derived by round-robin crossing a subset of the Global Diversity lines [38], which were then maintained at large population size (>10,000 flies) and allowed to interbreed freely for ~50 generations before HS selection began. The control (C) diet was composed of 10% glucose, 10% yeast, 1.2% agar, with 0.04% phosphoric acid and 0.4% propionic acid as preservatives. Three replicate populations for HS selection were derived from the base population and maintained on the same diet, but with an additional 13% sucrose and at lower population size (>5000 flies/cage until generation 100 and ~2000 flies/cage after). The control population was the ancestral population maintained at large population size (>10,000 flies) in a parallel on the C diet during the entirety of the HS selection. The approach used here allowed for natural selection on HS diets; we did not guide or select for particular traits as is often used in experimental evolution in *Drosophila*. We consider the two populations, C and HS, to be two different genotypes, though note they are not inbred and retain genetic diversity.

Transplanting microbiomes to test for adaptive significance

To test if the microbiome can transfer adaptive traits, we performed a reciprocal microbiome transplant experiment (Fig. 1). All host genotypes x microbiome x diet interactions were generated in the reciprocal transplant. Both genotypes (C and HS flies) were inoculated with microbiomes (C, HS) or kept sterile and raised in both diets (C and HS) for a total of 12 treatments. This experimental design allowed us to evaluate how host x microbiome x environment interactions influence the transfer of adaptive phenotypes.

We first identified how the microbiome changed between C and HS flies through 16S rRNA amplicon sequencing. In brief, DNA was extracted from single, age-matched (7-10 days post eclosion adults) using Zymo Quick-DNA extraction kit (Zymo D3012). The 16S V1-V2 region was amplified, pooled, and digested with BstZ17I (NEB R3954) to deplete Wolbachia reads (Simhadri et al., 2017). Libraries were sequenced using 300 bp paired-end reads using the Illumina MiSeq platform at the Princeton University Genomics Core. We used QIIME2

v2019.4 (Bolyen et al., 2019) to process reads, cluster into amplicon sequence variants (ASVs) with DADA2, and assign taxonomy with Greengenes classifier trimmed to the 16S rRNA V1-V2 regions. Data was imported into phyloseq for visualization (McMurdie and Holmes, 2013). Flies with fewer than 500 reads/individual were discarded before the analyses.

To identify differentially abundant bacteria between the C and HS microbiomes, we used ANCOM (analysis of composition of microbiomes) implemented in QIIME2 (Mandal et al., 2015). We cultured the most differentially abundant bacteria from HS flies, *A. pasteurianus*, and the significantly differentially abundant bacteria within the *Acetobacter* genus and enriched in C flies, *A. indonesiensis*. We confirmed 16S rRNA sequence identity using Sanger sequencing. Because the 16S rRNA phylogeny is discordant with a multilocus phylogeny in the Acetobacteraceae (Winans et al., 2017) and for simplicity, we refer to *A. indonesiensis* as C *Acetobacter* and *A. pasteurianus* as HS *Acetobacter*.

To perform the transplant, both *Acetobacters* were cultured in liquid MRS, density was normalized to OD_{600} = 0.1, and 50 µl of bacteria (or PBS for sterile treatments) was used to inoculate axenic eggs in sterile diets (Koyle et al., 2016). For each treatment, 6-10 replicates were maintained. Flies were then reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the lifespan of the recipient flies.

Phenotypic effects of the Acetobacter transplant

To measure development time, we counted the number of new pupae every 6 hours during their daytime (i.e., 8am, 2pm, 8pm) until all treatments began eclosing. We used the survival (Therneau and Lumley, 2015) and survminer (Kassambara, 2018) packages in R to analyze development data. We used Cox proportional hazards to identify how interactions between fly genotype, microbiome, and diet influence development time.

For fecundity, we counted the number of eggs laid by individual 8-10 day old females (N=644). Females were placed in a 24-well plate with oviposition media. To minimize plate

effects, two treatments were assayed on each plate, and treatments were randomly assigned across plates. Females laid eggs for ~4-6 hrs at 25ºC during the late afternoon (2pm-8pm) to capture similar circadian rhythms in egg laying across all treatments. We analyzed fecundity using a zero-inflated Poisson distribution implemented in glmmTMB (Magnusson et al., 2017) with oviposition plate (N=29 plates) as the random effect.

For nutritional indices, we assayed pools (N=95) of three age-matched (8-10 days old) females for total triglycerides. Triglycerides were normalized to protein amount (Tennessen et al., 2014). Protein serves as a proxy for size and was measured using a Bradford assay (BioRad 5000205). Total triglycerides represent fat stored for flies and were assayed using a colorimetric assay (Sigma TR0100-1KT). The biological replicates of treatments were assayed across different assay plates. We analyzed the triglyceride data using linear mixed models, testing for the effect of fly genotype, *Acetobacter*, and diet with assay plate (N=14) as the random effect using lme4 package in R (Bates et al., 2007).

Characterizing the genomic differences between C and HS Acetobacter

To further characterize the mechanisms underlying the fitness-associated benefits from the C and HS Acetobacters, we performed whole genome sequencing on these bacteria. Libraries were prepared following the manufacturer instructions using the Illumina PCR-free Library Prep kit, and then 150-bp paired end reads were sequenced on an Illumina NovaSeq platform. Genomes were assembled using SPAdes (Prjibelski et al., 2020) with --isolate flag and annotated using the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al., 2008).

To identify functional changes, we compared the RAST annotations to identify pathways unique to either C or HS *Acetobacter*. We also downloaded two other abundant bacteria in the C microbiome, generated annotations with RAST, and compared them with the HS *Acetobacter* annotations: *Algoriella xinjiangensis* (NCBI accession FOUZ01000040.1) and *Acetobacter*

persici (NCBI accession JOPC00000000.1). To confirm the differences between C and HS *Acetobacters*, we identified orthologs between the two *Acetobacter* genomes using OrthoFinder v2.5.2 (Emms and Kelly, 2019). Then to gain insight into the function of the putative genes, we performed BLASTP v2.10.1+ searches against the NCBI non-redundant protein sequence database (Altschul et al., 1990).

The genomic analysis performed above suggested that the HS *Acetobacter* genome encoded several pathways for urea degradation, but were not present in the C *Acetobacter* genome. To validate this observation, we measured the ability of the *Acetobacters* to degrade uric acid in fly food. Uric acid was added at 50 μmol concentration to 30 μl of C and HS diet. Then 10 μl of C or HS Acetobacter (or sterile PBS control), normalized to OD_{600}=0.1, were added to the diet and incubated for 4 days at 25ºC to degrade the uric acid. Uric acid concentration was measured using the Amplex Red Uric Acid/Uricase kit (ThermoFisher A22181) following the manufacturer instructions.

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DATA AVAILABILITY

Genome sequencing will be uploaded to NCBI [upon acceptance]. Phenotypic data will be deposited in Dryad [upon acceptance]. Code used to analyze data will be posted on github [upon acceptance].

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SUPPLEMENTARY TABLES

Supp. Table 1: ANCOM results for differentially abundant ASVs from C and HS fly microbiomes. Taxonomy was characterized to the lowest level an assignment could be made, and most ASVs were classified to species level. CLR=centered log ratio, W=test statistic, Sig=significant difference between groups (N.S. is nonsignificant).

ASV ID	CLR	W	Sig	Taxonomy
d84f3e1a7dbb97bc4695b954c8a2d2b2	4.30129864	25	HS fly	*Acetobacteraceae*
fb59adcbdd838dc7853f7388afb62b5a	7.82830283	25	HS fly	*Acetobacter pasteurianus*
9329896cd08f88a88296d921369cd8ea	-3.4609509	20	C fly	*Algoriella xinjiangensis*
f0d11f9f8ba452d5485a79e0b31b538f	-3.6539534	20	C fly	*Acetobacter indonesiensis*
3f2be3d57ac38f60b0b9c56fa5857f63	-2.7057494	18	N.S.	*Algoriella xinjiangensis*
70e118808cdc81b5b2670837c7833d8d	-3.3688051	18	N.S.	*Acetobacter persici*
dd4826376c1de49b8687c4f882d3bcf3	-2.1106948	17	N.S.	*Enterobacteriaceae*
606aa8c7117f801dc5ccc8c2a78c68c3	-1.7000214	16	N.S.	*Commensalibacter*
1c735b8a08dcbd5ca158987733234114	-1.6719837	15	N.S.	*Algoriella xinjiangensis*
da74ac9885f0aa2d29ec4338b46aa051	2.00310762	13	N.S.	*Orbaceae*
4043baca9447b9860efabab56603efc0	0.79635173	10	N.S.	*Acetobacter species*
61fd19eda2dee9c93613432491556a99	0.69392446	10	N.S.	*Alphaproteobacteria*
7070daee4850cf801719eab99ce88a82	0.96974134	10	N.S.	*Commensalibacter*
b3ccbf1d4fc85411f948429591f371cf	0.59968784	10	N.S.	*Pseudomonas*
ca7279bfdcb977287193c567f96b025c	0.75112887	10	N.S.	*Acetobacter indonesiensis*
0a04cded1e993516d5cdc851bfe07180	-1.6048832	9	N.S.	*Algoriella xinjiangensis*
6e4961fe480153e97ebdcf8fca4913fb	0.99515931	9	N.S.	*Providencia*
8dc187c9810636b7199c030caaaf0694	0.58697562	9	N.S.	*Morganella*
9f9bb5e1a63c5a0820d1571839534a2f	0.62165362	9	N.S.	*Commensalibacter*
677d95c5a0bc1c25435861fe262cb487	0.50699766	8	N.S.	*Alphaproteobacteria*
4af26492bbe6d0d4980bc82c2f08f32d	0.3940931	7	N.S.	*Providencia*
ad8b60b9428bc76f3b50e428f4f5a29f	0.41862229	7	N.S.	*Stenotrophomonas*
f72576c61dde7b9b6ad683dea71b105f	0.06411428	7	N.S.	*Enterobacteriaceae*
3c3c65e801e07d1be83bfa6d962b5627	-0.3759816	6	N.S.	*Commensalibacter*
681de033774ada405895dd81abc7593f	-0.5750105	6	N.S.	*Providencia rettgeri*
97b76e52dafa224603888fb56ef0b831	-0.3031253	5	N.S.	*Commensalibacter*

Supp. Table 2A: Analysis of deviance for Cox model with interactions between fly genotype, microbiome, and diet for developmental timing in the *Acetobacter* transplant.

Category	Log-likelihood	X^2	Df	*P* value
Null	-60640			
Fly genotype	-60584	111.642	1	<2.20E-16***
Acetobacter	-60517	134.874	2	<2.20E-16***
Diet	-58732	3568.585	1	<2.20E-16***
Fly x Acetobacter	-58675	115.244	2	<2.20E-16***
Fly x Diet	-58658	34.288	1	4.75E-09***
Acetobacter x Diet	-58523	268.613	2	<2.20E-16***
Fly x Acetobacter x Diet	-58500	47.625	2	4.55E-11***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Supp. Table 2B: Model selection statistics for developmental timing in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two and three-way interactions.

Model	Log-likelihood	X^2	X^2 Df	P(>|Chi|)
Model 1	-58732			
Model 2	-58500	465.77	7	<2.20E-16***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Supp. Table 3A: Fixed effects for fecundity in the *Acetobacter* transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	X^2	Df	*P value*
Intercept	637.8446	1	<2.20E-16***
Fly genotype	4.0333	1	0.0446106*
Acetobacter	39.7839	2	2.30E-09***
Diet	53.9716	1	2.03E-13***
Fly x Acetobacter	7.787	2	0.0203736*
Fly x Diet	8.4003	1	0.0037517**
Acetobacter x Diet	14.4492	2	0.0007285***
Fly x Acetobacter x Diet	16.3387	2	0.0002832***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Supp. Table 3B: Model selection statistics for fecundity in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two and three-way interactions.

Model	Df	AIC	BIC	logLik	deviance	X^2	X^2 Df	*P* value
Model 1	7	6333.5	6365	-3159.8	6319.5			
Model 2	14	6296.8	6359.8	-3134.4	6268.8	50.753	7	1.03E-08***

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Supp. Table 4A: Fixed effects for triglyceride accumulation in the *Acetobacter* transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	F	Df	Df.res	*P* value
Intercept	30.7704	1	7.002	0.0008616***
Fly genotype	12.0201	1	78.037	0.0008591***
Diet	27.3194	1	78.039	1.40E-06***
Acetobacter	0.028	2	78.008	0.9724439
Fly x Diet	8.9996	1	78.043	0.0036234**
Fly x Acetobacter	1.7817	2	78.041	0.1751283
Diet x Acetobacter	1.771	2	78.098	0.1769287

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

Supp. Table 4B: Model selection statistics for triglyceride in the *Acetobacter* transplant. Model 1 is without interactions, while Model 2 includes all two-way interactions. The three-way interaction was not significant and removed from the analysis.

Model	Df	AIC	BIC	logLik	deviance	X^2	X^2 Df	*P* value
Model 1	7	440.31	458.19	-213.16	426.31			
Model 2	12	433.09	463.73	-204.54	409.09	17.226	5	0.00409**

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*

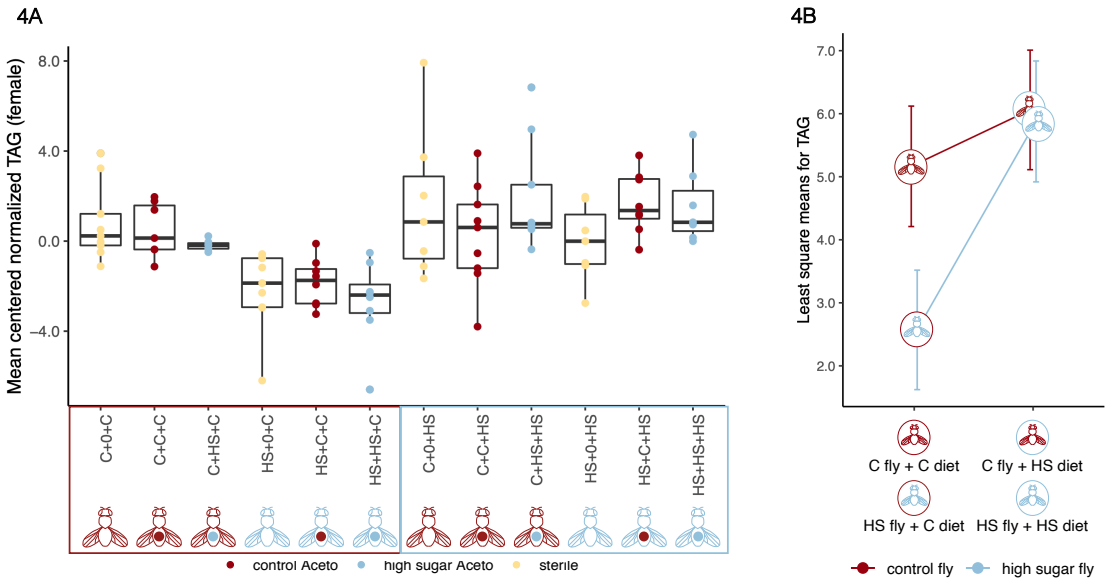
Supp. Table 5: Uric acid degradation presence/absence in the most abundant bacteria from the C and HS microbiomes. Annotation was performed using RAST, and each were compared to the HS *Acetobacter* (Y= present, N= not present). Only HS *Acetobacter* encoded for uric acid degradation pathways.

Role	HS Aceto	C Aceto	A. persici	Algoriella
Urea ABC transporter, ATPase protein UrtD	Y	N	N	N
Urea ABC transporter, ATPase protein UrtE	Y	N	N	N
Urea ABC transporter, permease protein UrtB	Y	N	N	N
Urea ABC transporter, permease protein UrtC	Y	N	N	N
Urease accessory protein UreE	Y	N	N	N
Urease accessory protein UreF	Y	N	N	N
Urease accessory protein UreG	Y	N	N	N
Urease alpha subunit (EC 3.5.1.5)	Y	N	N	N
Urease beta subunit (EC 3.5.1.5)	Y	N	N	N
Urease gamma subunit (EC 3.5.1.5)	Y	N	N	N

Supp. Table 6: ANOVA results for uric acid degradation by *Acetobacters*.

Category	Sum Sq	Df	F value	Pr(>F)
Intercept	12504.2	1	1188.4315	<2.2E-16***
Acetobacter	374.6	2	17.8035	1.82E-05***
Diet	1.5	1	0.1442	0.7075
Acetobacter x Diet	810.9	2	38.534	3.22E-08***
Residuals	252.5	24		

Significance codes: *'***' <0.001; '**' <0.01; '*' <0.05*



Supp. Fig. 1: Metabolic effects of the *Acetobacter* transplant. A) Boxplot for triglycerides (TAG) normalized to protein. TAG amount reflects fat storage in *Drosophila*. Each point represents a pool of three age-matched female flies. TAG levels were mean centered to control for each batch assayed. Interactions between fly genotype and diet shaped TAG levels ($F_{1,78.1}$ = 9.00, p=0.004), but *Acetobacter* had no effect. B) LSM from the mixed model shows the significant interaction between fly genotype and diet. Error bars show standard error for LSM. TAG levels responded to diet for HS flies, but not for C flies.