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

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Abstract:

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 control and evolved HS bacteria. If the microbiome confers benefits to hosts, then transplant recipients should gain fitness benefits compared to controls. Interestingly, we found that benefits do exist, but 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, mediating 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.

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INTRODUCTION

The microbiome has profound effects on many aspects of organismal biology. From modulating physiology [1] to pathogen protection [2] to social interactions [3], 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 [4–6]. For example, the acquisition of nutrition-provisioning symbionts enables the ecological expansion of sap-feeding insects [7]. For many eukaryotes, though, their microbiomes are environmentally acquired or through other weakly controlled transmission mechanisms [5]. 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 [4, 6, 8]. 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 [9]. The gut microbiome of woodrats facilitates survival on toxic plant diets [10]. In plants, drought-adapted soil communities can increase fitness in drought conditions [11]. 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 [12, 13].

65 The fruit fly, *Drosophila melanogaster*, has emerged as an excellent model to study host-
66 microbe interactions. Flies have relatively simple microbiomes, often composed of fewer than 10
67 bacterial species from acetic acid and lactic acid bacteria families with effects on many different
68 fly traits [14]. The microbiome is largely environmentally acquired through feeding, defecation,
69 and egg smearing during oviposition [15, 16]. After hatching, larvae acquire their microbes
70 through a combination of microbial persistence and colonization and form associations over the
71 lifespan of the fly [17–19]. Many fly-associated microbes can be individually cultured and
72 reconstituted in various combinations to inoculate sterile flies [14, 20]. With the feasibility of
73 microbiome manipulations and rich genetic resources, *Drosophila* is an ideal model to study the
74 interplay between host genetic, microbiome, and environment (i.e., G_H x G_M x E interactions) in
75 shaping host phenotypes that underlie rapid host adaptation.
76 In this study, to investigate whether the microbiome can serve as a reservoir of adaptive
77 potential for hosts, we studied the contribution of the microbiome in the context of experimental
78 evolution. Experimental evolution has a long history of providing fundamental insights into
79 adaptive processes, but has largely neglected the contribution of the microbiome [6, 21, 22].
80 One particularly powerful approach is the Evolve and Resequence (E&R) framework to measure
81 the genomic response to selection [23]. E&R experiments typically begin with large, outbred
82 populations which are then subjected to a selective regime, and in parallel, control populations
83 are maintained in a benign, non-selective environment. After several generations, the control
84 and evolved populations are then sequenced to identify the genomic response to selection.
85 Although traditionally E&R experiments have focused on the organism targeted by selection
86 (e.g., yeast, flies, mice, etc.), the E&R approach can also identify changes in the microbiome
87 associated with the evolved populations [22]. However, to determine whether any changes in
88 the microbiome over generations of selection actually contributed to host adaptation, it is
89 necessary to transplant the microbiome between control and evolved populations. This
90 approach holds great potential to inform how the microbiome shapes host phenotypes and
91 contributes to host adaptation.
92 Here, we leverage ~150 generations of fly and microbial adaptation to a nutritionally
93 stressful, high sugar (HS) diet. HS diets exert stress on flies, and flies exhibit similar obesity-like
94 phenotypes as humans with elevated triglycerides, insulin resistance, and shortened lifespans
95 [24–27]. In turn, the different bacteria in the fly microbiome have a range of effects on fly
96 phenotypes, shaping life history traits [28, 29] and metabolism [28, 30], and these microbiome
97 effects on host phenotype can depend on host genetics [31, 32]. Together, these findings
98 suggest that the microbiome could potentially contribute to host 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. 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.

MATERIALS AND METHODS

Experimental design

We first identified if the microbiome responded to selection from experimental evolution to the HS diet. Then, 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.

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 [33], 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 8% glucose, 8% 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 12% sucrose and at lower population size (>5,000 flies/cage). 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, which is a common approach in experimental evolution studies in *Drosophila*. We consider the two populations, C and HS, to be two different genotypes, though note they are outbred and retain genetic diversity.

133 *Identifying the microbiome response to selection in the HS diet*

134 We first identified how the microbiome changed between C and HS flies through 16S
135 rRNA amplicon sequencing. In brief, DNA was extracted from single, age-matched (7-10 days
136 post eclosion adults) using Zymo Quick-DNA extraction kit (Zymo D3012). The 16S V1-V2
137 region was amplified, pooled, and digested with BstZ17I (NEB R3954) to deplete Wolbachia
138 reads [34]. Libraries were sequenced using 300 bp paired-end reads using the Illumina MiSeq
139 platform at the Princeton University Genomics Core. We used QIIME2 v2019.4 [35] to process
140 reads, cluster into amplicon sequence variants (ASVs) with DADA2, and assign taxonomy with
141 Greengenes classifier trimmed to the 16S rRNA V1-V2 regions. Data was imported into
142 phyloseq for visualization [36]. Flies with fewer than 500 reads/individual were discarded before
143 the analyses.

144 To identify differentially abundant bacteria between the C and HS flies, we used ANCOM
145 (analysis of composition of microbiomes) [37] implemented in QIIME2. We cultured the most
146 differentially abundant bacteria from HS flies, *A. pasteurianus*, and the significantly differentially
147 abundant bacteria within the *Acetobacter* genus and enriched in C flies, *A. indonesiensis*. We
148 confirmed 16S rRNA sequence identity using Sanger sequencing. For simplicity, we refer to *A.*
149 *indonesiensis* as C *Acetobacter* and *A. pasteurianus* as HS *Acetobacter*.

150

151 *Transplanting microbiomes to test for adaptive significance*

152 We first performed both a complete microbiome transplant. We then performed a
153 focused transplant of the dominant C and HS *Acetobacter* strains identified through ANCOM to
154 measure the impact of host genetic, microbial, and environment interactions on fitness-
155 associated phenotypes.

156 First, to transplant the complete microbiome, we collected frass for ~ 5 hours from 300 C
157 and HS donor flies (age matched 7-10 days post eclosion). Flies were placed in sterile 50 ml
158 tubes, lined with a plastic sheet. Frass was then washed off the plastic using sterile water and
159 filtered through fine mesh to remove particulates and any eggs. Preliminary tests suggested that
160 the HS microbiome had much lower bacterial abundance, and so the C microbiome was
161 resuspended in 6 ml sterile water and 3 ml for the HS microbiome. 50 µl of the frass wash was
162 used to inoculate axenic eggs in sterile diets following standard protocols [20], and flies were
163 reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the lifespan of the
164 recipient flies. At 7-10 days post eclosion, recipient flies were stored at -80ºC and DNA
165 subsequently extracted from individuals. The microbiome of recipients was assessed through
166 16S rRNA V1-V2 profiling as described above and rarified to 500 reads/fly. To assess the

167 fidelity of the microbiome transplant, we tested for the effects of host genotype and microbiome
168 on Bray-Curtis dissimilarity for each diet separately using the ADONIS2 implementation of
169 PERMANOVA in vegan [38].
170 To perform the *Acetobacter* transplant, both *Acetobacter* strains were cultured in liquid
171 MRS, density was normalized to OD_{600} = 0.1, and 50 µl of bacteria (or PBS for sterile
172 treatments) was used to inoculate axenic eggs in sterile diets [20]. For each treatment, 4-12
173 replicate populations were maintained. Flies were then reared in a biosafety cabinet at 25ºC and
174 12:12 light:dark cycle through the lifespan of the recipient flies. For the *Acetobacter* transplant,
175 we measured two fitness-associated phenotypes: developmental time and fecundity.
176 To measure developmental time, we counted the number of new pupae every 6 hours
177 during their daytime (i.e., 8am, 2pm, 8pm) until all treatments began eclosing (4-12
178 replicates/treatment, see Supp. Table 1 for sample sizes and replication). For developmental
179 time, we used the survival [39] and survminer [40] packages in R to analyze development data.
180 We used Cox proportional hazards to identify how interactions between fly genotype,
181 microbiome, and diet influence development time.
182 For fecundity, we counted the number of eggs laid by individual 8-10 day old females
183 (N=644 total, see Supp. Table 2 for sample sizes). Females were placed in a 24-well plate with
184 oviposition media. Oviposition media was the same as the fly food media, but with yeast extract,
185 which makes the diet transparent and easier to count eggs. Females laid eggs on both C and
186 HS media; media did not affect egg lay (Mann-Whitney-Wilcoxon test, W=50964, p=0.09), and
187 data were pooled from both oviposition media for analysis. To minimize plate effects, two
188 treatments were assayed on each plate, and treatments were randomly assigned across plates.
189 Females laid eggs for ~4-6 hrs at 25ºC during the late afternoon (2pm-8pm) to capture similar
190 circadian rhythms in egg laying across all treatments. We used a zero-inflated Poisson
191 distribution implemented in glmmTMB [41] testing for the effects of interactions between fly
192 genotype, *Acetobacter* strain, and diet with oviposition plate (N=29 plates) as the random effect.
193
194 *Characterizing the genomic differences between C and HS Acetobacter*
195 To further characterize the mechanisms underlying the fitness-associated benefits from
196 the C and HS *Acetobacter* strains, we performed whole genome sequencing on these bacteria.
197 Libraries were prepared following the manufacturer instructions using the Illumina PCR-free
198 Library Prep kit, and then 150-bp paired end reads were sequenced on an Illumina NovaSeq
199 platform. Genomes were assembled using SPAdes [42] with --isolate flag and annotated using
200 the Rapid Annotation using Subsystem Technology (RAST) server [43].

201 We first compared average nucleotide identity (ANI) between the C and HS *Acetobacter*
202 using OrthoANIu [44]. OrthoANIu computationally fragments bacterial genomes into 1020 bp
203 (contigs <1020 bp are discarded), performs reciprocal BLASTn searches for all fragments, and
204 then computes ANI for all reciprocal BLASTn hits. Then to identify functional changes, we
205 manually compared the RAST annotations to identify pathways unique to either C or HS
206 *Acetobacter*. We also generated the RAST annotations from two other abundant bacteria in the
207 C microbiome and compared them with the HS *Acetobacter* annotations: *Algoriella xinjiangensis*
208 (NCBI accession FOUZ01000040.1) and *Acetobacter persici* (NCBI accession
209 JOPC00000000.1). To confirm the differences between C and HS *Acetobacter* strains, we
210 identified orthologs between the two *Acetobacter* genomes using OrthoFinder v2.5.2 [45]. Then
211 to gain insight into the function of the putative genes, we performed BLASTP v2.10.1+ searches
212 against the NCBI non-redundant protein sequence database [46].
213 The genomic analysis performed above suggested that the HS *Acetobacter* genome
214 encoded several pathways for urea degradation, but were not present in the C *Acetobacter*
215 genome. To validate this observation, we measured the ability of the *Acetobacter* strains to
216 degrade uric acid in fly food. Uric acid was added at 50 µmol concentration to 30 µl of C and HS
217 diet. Then 10 µl of C or HS Acetobacter (or sterile PBS control), normalized to OD_{600}=0.1, were
218 added to the diet and incubated for 4 days at 25ºC to degrade the uric acid. Uric acid
219 concentration was measured using the Amplex Red Uric Acid/Uricase kit (ThermoFisher
220 A22181) following the manufacturer instructions. ANOVA was used to analyze the uric acid
221 degradation by bacteria. We tested for the effects of bacteria (C or HS *Acetobacter* strain), diet,
222 and the interaction between bacteria and diet.
223
224 ***RESULTS***
225 *Fly microbiome responds to HS selection*
226 First, we examined if the microbiome differed between C and HS flies following ~150
227 generations of adaptation to the C or HS diets (Fig. 2A). HS flies were dominated by
228 *Acetobacter pasteurianus* (86.7% +/- 1.8 SE) and unclassified Acetobacteraceae strain (5.1%
229 +/- 1.8 SE), while C flies were more diverse with *Algoriella xinjiangensis* (37.3% +/- 5.3 SE),
230 *Acetobacter persici* (16.9% +/-2.6 SE), and *Acetobacter indonesiensis* (14.8% +/- 2.1 SE). The
231 most differentially abundant bacteria were *A. pasteurianus* and Acetobacteraceae strain for the
232 HS flies, while *A. xinjiangensis* and *A. indonesiensis* were associated with C flies (Fig. 2B,
233 Supp. Table 3). These bacteria are unique to either C or HS flies and found across individual

234 flies, demonstrating that the HS diet selected for a different fly microbiome, dominated by *A.*
235 *pasteurianaus*.
236
237 *Transplanting Acetobacter influences fitness-related traits*
238 To test if transferring the microbiome could transfer adaptive phenotypes to naive hosts,
239 we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. We
240 first transplanted a complete microbiome. On the C diet, the difference in C and HS
241 microbiomes were maintained in recipients (Fig. 2C, Supp. Table 4, PERMANOVA, $R^2 = 0.38$, p
242 = 0.001). However, the microbiome was not successfully transplanted for flies on the HS diet, as
243 the variance explained by microbiome was much lower in recipients (Fig. 2C, Supp. Table 5,
244 PERMANOVA, $R^2 = 0.04$, p = 0.004). This suggests that the HS diet was highly selective and as
245 we discuss later, likely limits bacterial survival.
246 Instead, given the simplicity of the microbiome associated with HS flies, we performed
247 another microbial transplant using the *Acetobacter* strains identified as differentially abundant
248 between C and HS flies: *A. indonesiensis* (C *Acetobacter*) and *A. pasteurianus* (HS
249 *Acetobacter*). While not the complete bacterial community from the C flies, we reasoned that
250 because both community complexity (i.e., single dominant species in the HS microbiome versus
251 several in the C microbiome) and phylogenetic diversity (i.e., single dominant *Acetobacter* in the
252 HS microbiome versus two *Acetobacter* species, *Algoriella*, *Commensalibacter*, etc. in the C
253 microbiome) differ, using only C *Acetobacter* still provides an ecologically relevant substitute,
254 while controlling for differences in diversity between the C and HS microbiome. We then
255 measured higher order, fitness-associated traits (developmental timing and fecundity), which
256 reflect cumulative adaptations to the multitude of selective pressures in the HS diet.
257 Development was shaped by interactions between fly genotype, *Acetobacter*, and diet
258 (Fig. 3A, Supp. Table 6). On the C diet, fly genotype influenced the response to the *Acetobacter*
259 transplant. HS flies with the C *Acetobacter* developed more rapidly (avg. 140.8 hrs +/- 0.54 SE)
260 than with the HS *Acetobacter* (avg. 147.6 hrs +/- 0.60 SE). There was no effect for the C fly.
261 However, on the HS diet, the HS *Acetobacter* accelerated development (HS *Acetobacter* avg.
262 176.5 hrs +/- .50 SE, C *Acetobacter* avg. 186.8 hrs +/-0.60 SE). The three-way interaction
263 between fly genotype, *Acetobacter*, and diet significantly affected development time (log-
264 likelihood =-58500, $X^2 = 47.625$, $p < 0.0001$).
265 Fecundity responded to interactions between fly genotype, *Acetobacter*, and diet (Fig.
266 3B, Supp. Table 7). On average, treatments were more fecund on the C diet, while treatments
267 on HS diets laid 54.3% fewer eggs (C avg. 13.0 eggs +/-0.6 SE, HS, avg. 5.9 eggs +/- 0.4 SE).

268 Sterile treatments substantially lowered fecundity, only on the HS diet (+microbes avg. 7.45
269 eggs +/- 0.5 SE, sterile avg. 1.67 eggs +/- 0.4 SE). Notably, the response to *Acetobacter*
270 treatment depended on fly genotype for the HS diet. On the HS diet, HS flies with HS
271 *Acetobacter* (avg. 8.4 eggs +/-1.4 SE) were almost twice as fecund as flies with the C
272 *Acetobacter* (avg. 4.5 eggs +/- 1.0 SE). The interaction between fly genotype, microbiome, and
273 diet significantly affected fecundity (interaction X^2 = 16.4, df = 2, p=0.0003).
274
275 *HS Acetobacter uniquely encodes uric acid degradation pathway*
276 To better understand how the HS *Acetobacter* may benefit flies in the HS diet, we
277 sequenced and compared the genomes of the C and HS *Acetobacter* strains. ANI between the
278 strains was 73.68% (Supp. Table 8). Annotation by RAST showed that strains shared a majority
279 of predicted functions (>90% for both strains, Supp. Fig. 1). One key difference between the two
280 genomes was that HS *Acetobacter* encodes for several genes that degrade uric acid, but C
281 *Acetobacter* does not (Supp. Table 9). Further, the other dominant bacteria in the C microbiome
282 (*Algoriella xinjiangensis* and *Acetobacter persici*) also lack uric acid degradation genes (Supp.
283 Table 9). Indeed, we found that interactions between *Acetobacter* and the diet shape uric acid
284 degradation (Fig. 4, Supp. Table 10). Only on the HS diet does HS *Acetobacter* reduce the
285 concentration of uric acid by ~50% compared to C *Acetobacter*, with only negligible changes to
286 uric acid concentration on the C diet.
287
288 ***DISCUSSION***
289 Here, we explored how the microbiome influences host fitness in stressful environments.
290 Because all combinations of G_H x G_M x E interactions were produced in the transplant
291 experiment, the relative benefits of C and HS *Acetobacter* across the different host genetic
292 (selected or unselected) and environmental conditions (HS or control diet) could be evaluated.
293 Through reciprocal host x microbiome x diet transplant experiments, we show that G_H x G_M x E
294 interactions shape host fitness by modifying developmental timing and fecundity. The HS
295 *Acetobacter* genome encoded uric acid degradation genes and, *in vitro*, lowered uric acid on the
296 HS diet, while the C *Acetobacter* did not. Our results highlight the complex interactions
297 generated by the microbiome, and we discuss important future directions to further dissect
298 these relationships.
299
300 *Acetobacter dominates HS microbiome*

301 The HS microbiome was dominated by a single species of bacteria, *Acetobacter*
302 *pasteurianus* (Fig. 2). *Acetobacter* species are common in flies, both in laboratory settings and
303 wild environments [18, 47–51]. While *Acetobacter* are typically sugar specialists [52], one major
304 axis of variation separating the C and HS microbiome was the ability to degrade uric acid. We
305 did not find an effect of C or HS *Acetobacter* strains in shaping triglyceride accumulation (Supp.
306 Fig. 2); triglyceride accumulation is often a benefit associated with *Acetobacter* in *Drosophila*
307 [28, 31]. In the context of *Drosophila* biology, uric acid accumulation is commonly associated
308 with high sugar diets in flies [53]. The accumulation of uric acid may also exacerbate the
309 deleterious effects of HS diet, altering oxidative stress and inflammation that can promote
310 obesogenic phenotypes [54, 55]. Uric acid is normally excreted by flies, but high concentrations
311 slow development [56] and shorten lifespan [53, 57]. Not all *Acetobacter* species can degrade
312 uric acid, including the C *Acetobacter*, but the ability to degrade is more common in fly-
313 environment than in other free-living *Acetobacter* species [55]. Here, comparative analyses
314 between the C and HS *Acetobacter* genomes showed that HS *Acetobacter* encoded for several
315 genes in the uric acid degradation pathway, while the C *Acetobacter* genome did not. Uric acid
316 degradation may be the key fitness benefit of the HS *Acetobacter* over the C *Acetobacter* in the
317 HS diet.
318 The fitness advantage of uric acid degradation was likely direct for the HS *Acetobacter*,
319 but indirect for the fly. Over the course of experimental evolution, both fly and microbiome were
320 exposed to the HS diet, and in turn, the HS microbiome was dominated by *A. pasteurianus*,
321 which could degrade uric acid. *Acetobacter* species that degrade uric acid can outcompete
322 *Acetobacter* species that cannot in the fly environment [55]. For the HS *Acetobacter*, uric acid
323 degradation may underlie its persistence in the stressful HS diet, and thus indirectly benefiting
324 flies through reductions of uric acid in the diet. The benefit of uric acid degradation may be
325 especially important during development. Larvae have limited endogenous abilities to detoxify
326 uric acid, and the primary mechanism of uric acid tolerance is by decreasing exposure through
327 decreased uptake or increased excretion [56, 58, 59]. For adults, it is unclear if HS *Acetobacter*
328 also degrades uric acid in the fly gut or simply benefiting from the reduction in the diet. While
329 these questions require further characterization of fly physiology, uric acid degradation by HS
330 *Acetobacter* is similar to a handful of examples of environmentally acquired microbiomes that
331 facilitate adaptation through detoxifying the environment, such as insecticide resistance in bean
332 bugs [9] or terpene detoxification in woodrats [10]. Our results contribute to a growing body of
333 literature that suggests that the microbiome can encode novel functions that hosts can leverage
334 the microbiome during adaptation to stressful environments [6].

335

336 *Adaptive potential of the microbiome depends on evolutionary and ecological context*

337 Many have predicted that the microbiome could accelerate local adaptation in the host,
338 facilitating phenotypic changes to buffer local stressors [6, 7, 60, 61]. If so, transplanting the
339 locally adaptive microbiomes should confer adaptive phenotypes to naive individuals. However,
340 the benefit may depend on interactions between host genotype and environment. G_H x G_M x E
341 interactions have the potential to facilitate or impede evolution in challenging environments [12,
342 13]. Through combining experimental evolution with reciprocal transplants, our data allowed us
343 to test if and how ecological context (i.e., diet) and evolutionary context (i.e., fly genotype)
344 shape the adaptive potential of the microbiome.

345 To shape adaptive potential, the microbiome must contribute to host phenotypic
346 variation, not unlike traditional (host) additive genetic variance [6]. The G_H x G_M x E interaction
347 for development illustrates how diet (the ecological context), when matched with host genotype
348 (the evolutionary context) may increase the fitness benefit of locally adaptive microbiomes (Fig.
349 3A). The HS *Acetobacter* accelerated development on the HS diet (a match between microbe
350 and diet, independent of fly genotype) and slowed development compared to the C *Acetobacter*
351 on the C diet for only the HS fly (a mismatch between microbe and environment, but dependent
352 on fly genotype). The mismatch only mattered for the HS fly; C fly development was similar
353 between the C and HS *Acetobacter* on the C diet. However, the G_H x G_M x E interaction that
354 shaped fecundity is suggestive of how genetic interactions may impede the transfer of adaptive
355 potential. The naive host, C flies, did not receive a fecundity increase from the HS *Acetobacter*
356 in the HS diet, but HS flies did (Fig. 3C). The presence of G_H x G_M x E interactions for both
357 development and fecundity suggests that the microbiome contributed to fly evolution. HS flies
358 responded differently to the *Acetobacter* variation from the C flies, and more so, responsiveness
359 depended on the diet. Because host genotypes may vary in their ability to leverage beneficial
360 microbes in stressful environments, genetic interactions may limit the benefits of locally adaptive
361 microbiomes. Understanding these G_H x G_M interactions will be especially important in using
362 evolution to engineer beneficial microbiomes to solve pressing problems in public health and
363 agriculture [6, 12, 13, 62]. While longitudinal sampling of the evolutionary trajectory is necessary
364 to determine to what extent the HS fly evolved or selected for beneficial *Acetobacter* traits, our
365 results clearly demonstrate how the ecological context and host evolution can shape the
366 adaptive potential of the microbiome.

367

368 *Research priorities*

369 Here, we leveraged host and microbial evolution from ~150 generations of adaptation to
370 a stressful diet through the E&R approach. Such experiments have illuminated many important
371 findings about the role of demography, population genetics, and genetic architecture in shaping
372 adaptive traits [23, 63]. However, missing from this framework has been the role of the
373 microbiome. In a recent survey of ten E&R experiments, the microbiome also frequently
374 responded to experimental evolution, particularly in traits associated with metabolism [22].
375 Taken together with our reciprocal transplant experiment, our data also highlight how the
376 microbiome may contribute to host adaptation – effects previously unseen when focusing on the
377 *Drosophila* genome. Recent work on the *Drosophila* microbiome linked to natural variation in
378 wild populations suggested that a single inoculation of two different bacteria linked to different
379 life-history strategies, can lead to rapid phenotypic change and genetic differentiation in one fly
380 population [29, 64]. Our current study advances these findings by comparing differences in
381 ecologically relevant *Acetobacter* evolution to differences in fly evolution, providing one of the
382 first experimental dissections of host-microbiome interactions in the E&R context. Through
383 reciprocal transplants, we identified how interactions between fly genotype, microbiome, and
384 diet shape fitness related traits.
385 The G_H x G_M x E interactions observed here highlight how evolution in both the host and
386 microbiome occurred during adaptation to the HS diet. In particular, the ability of HS
387 *Acetobacter* to degrade uric acid in the HS diet reflects the potential for diffuse coevolution [65].
388 The HS diet likely led to an increase in uric acid production by flies, and then the *Acetobacter*
389 that could exploit the new niche increased, which the HS flies benefited from the reduction in
390 toxicity. Ultimately, in our system and perhaps many others, the evolutionary benefits between
391 host and microbiome are intertwined. Future work utilizing experimental evolution will
392 demonstrate how the joint evolution of host and microbiome shapes the response to selection,
393 providing fundamental insights into how natural selection operates on complex systems.
394

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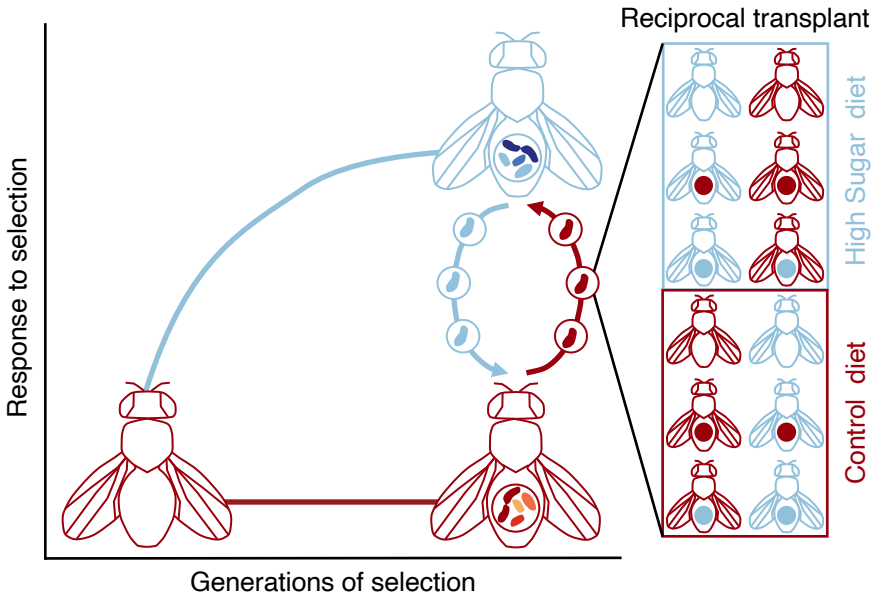
399

400 ***DATA AVAILABILITY***

401 Genome sequencing will be uploaded to NCBI [upon acceptance]. Phenotypic data will be
402 deposited in Dryad [upon acceptance]. Code used to analyze data will be posted on github
403 [upon acceptance].
404
405 ***FIGURES***



406
407
408 Figure 1: Experimental design to leverage ~150 generations of fly adaptation to the high sugar
409 (HS) diet to study the adaptive potential of the microbiome. The conceptualized evolutionary
410 trajectory shows control (C) flies and microbiome in red and HS flies and microbiome in blue. By
411 the end of experimental evolution, the C fly population (red) has a different microbiome than the
412 HS fly population (blue). After the ~150 generations of selection, we performed a fully reciprocal
413 host x microbiome x diet transplant experiment for all combinations of fly genotypes (C or HS),
414 microbes (sterile, C, or HS), and diets (C, or HS). Together, this approach allows for the
415 assessment of the benefit of adapted relative to non-adapted microbes in both host genetic and
416 environmental contexts.
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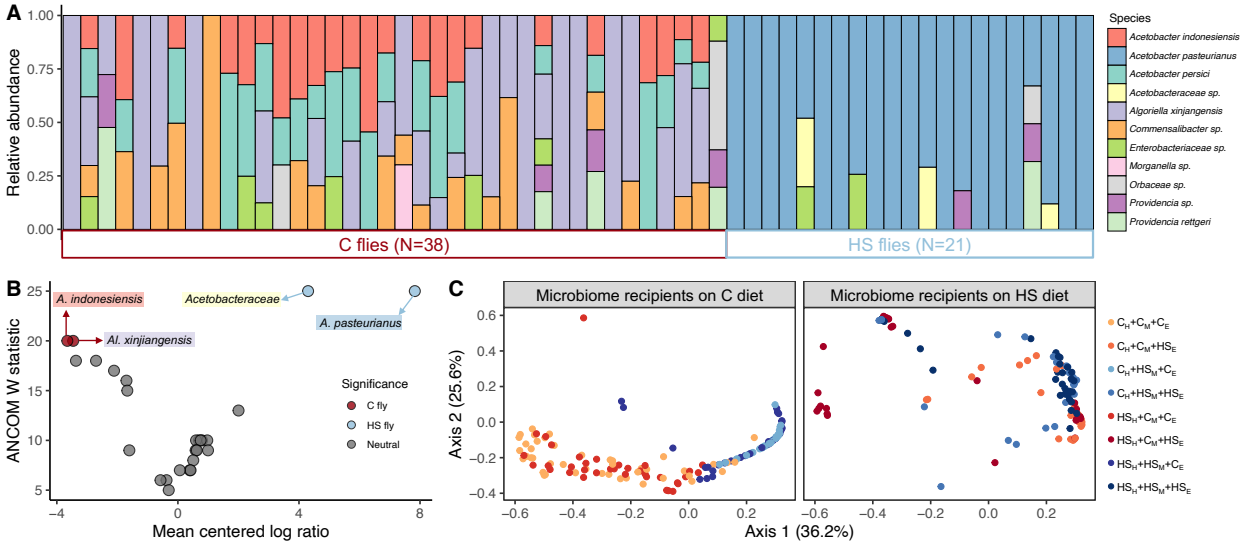

Figure 2: The microbiome in HS flies is different from C flies. A) Relative abundance of bacteria determined using 16S rRNA sequences. Each bar represents an individual fly (N=38 C flies, 21 HS flies). The C fly microbiome is enriched for *A. indonesiensis*, *A. persici*, and *Algoriella xinjiangensis*. The HS fly microbiome is enriched for *A. pasteurianus* and unclassified *Acetobacteraceae*. 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. C) PCoA plots based on Bray-Curtis dissimilarity for recipients in the whole microbiome transplant. Plot is faceted by diet, and colors represent the different combination of host genotype, microbiome, and diet. The difference between C and HS microbiomes was maintained for recipients on the C diet, but not on the HS diet.

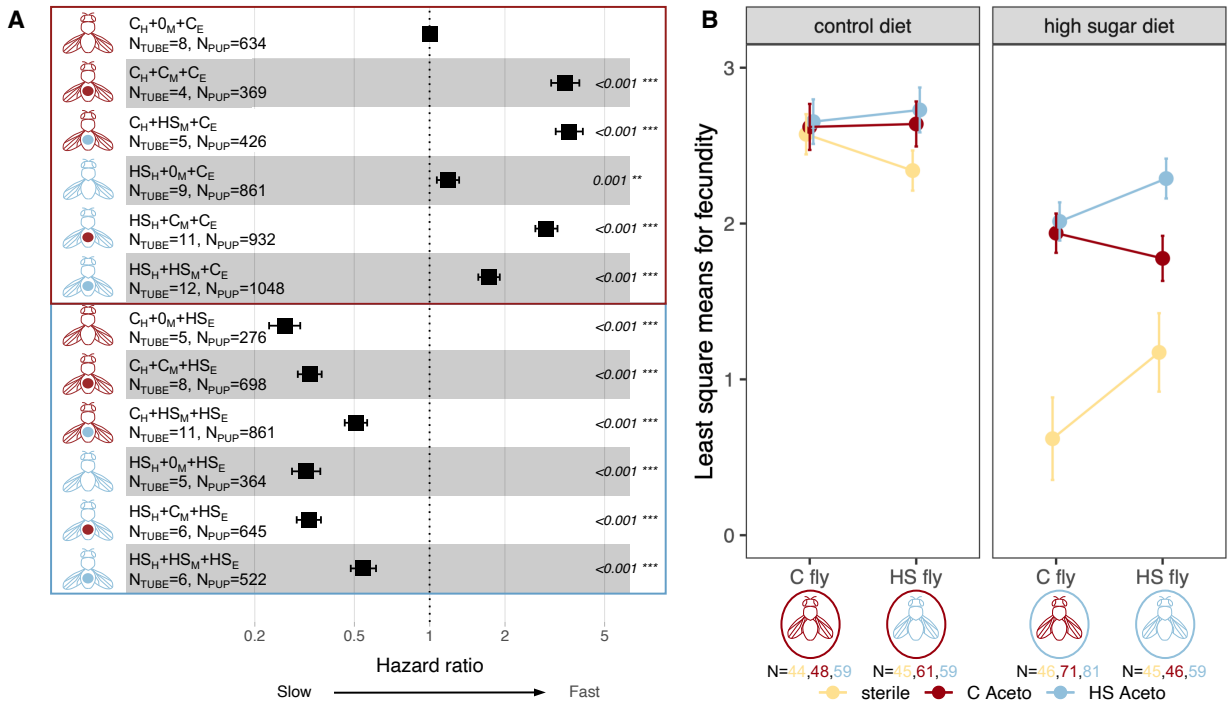

Figure 3: Effects of the *Acetobacter* transplant on fitness-related traits. A) Hazard ratio plot for *Acetobacter* effects on development time. Treatment is shown with the fly diagram, where fly color represents fly genotype, circle represents *Acetobacter* (no circle = sterile), and box represents 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 replicates (N_{TUBE}) and pupae (N_{PUP}) 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 diet, 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.

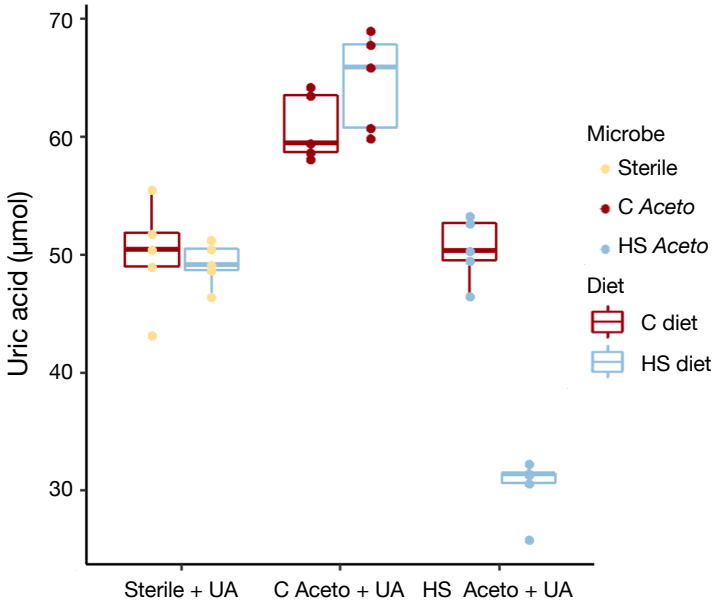

Figure 4: Uric acid degradation by HS *Acetobacter*. Boxplots show uric acid in the *in vitro* diet assay. Boxplots are colored by diet with points colored by *Acetobacter* treatment. Only in the HS diet, HS *Acetobacter* degraded significantly more uric acid than C *Acetobacter*.

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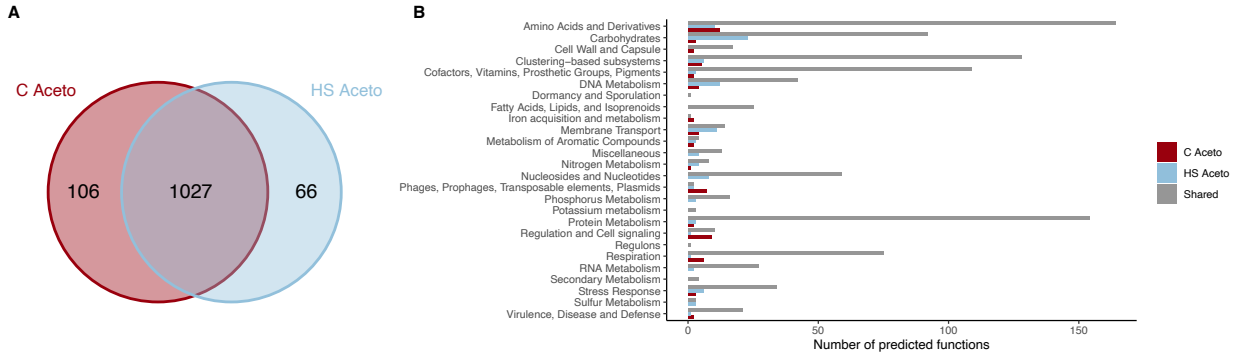
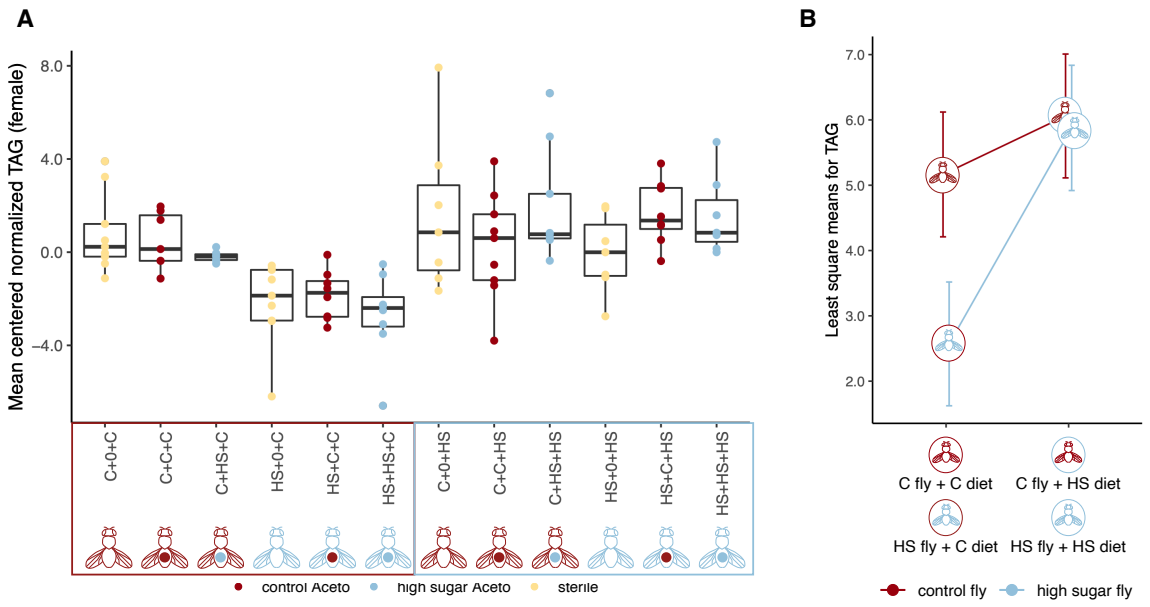


Supp. Fig. 1: Summary of the RAST annotation for the C and HS Acetobacter. A) Venn diagram showing the number of functions unique to C Aceto (106/1133 total), unique to HS Aceto (66/1093 total), or shared (1027). B) Number of predicted functions per category colored by either unique to C Acetobacter (red), unique to HS Acetobacter (blue), or shared (grey). In general, both genomes encoded similar functions, but small differences are observed across the different categories.



Supp. Fig. 1: Metabolic effects of the *Acetobacter* transplant. Triglyceride levels were assessed from pools of three, age-matched (7-10 days old) females. Tryglycerides were measured using colorimetric assay test (Sigma TR0100-1KT) and then normalized to protein using a Bradford assay (BioRad 5000205). 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.

SUPPLEMENTARY TABLES

Supp. Table 1: Sample size and replication for development measured during the *Acetobacter* transplant experiment.

Fly genotype	Microbiome	Diet	No. tubes	Avg larvae/tube	SE avg larvae/tube
control	sterile	control	8	79.2	7.88
control	sterile	high sugar	5	55.2	11.1
control	control	control	4	92.2	10.1
control	control	high sugar	8	87.2	8.37
control	high sugar	control	5	85.2	5.89
control	high sugar	high sugar	11	78.3	4.6
high sugar	sterile	control	9	95.7	7.01
high sugar	sterile	high sugar	5	72.8	10.8
high sugar	control	control	11	84.7	9.44
high sugar	control	high sugar	6	108	9.21
high sugar	high sugar	control	12	87.3	10.9
high sugar	high sugar	high sugar	6	87	6.93

Supp. Table 2: Sample sizes for fecundity in the *Acetobacter* transplant experiment .

Fly genotype	Microbiome	Diet	N Females
control	sterile	control	44
control	sterile	high sugar	46
control	control	control	48
control	control	high sugar	71
control	high sugar	control	59
control	high sugar	high sugar	81
high sugar	sterile	control	45
high sugar	sterile	high sugar	45
high sugar	control	control	61
high sugar	control	high sugar	46
high sugar	high sugar	control	59
high sugar	high sugar	high sugar	59

Supp. Table 3: 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 4: Comparison of microbial community composition (Bray-Curtis dissimilarity) for recipients on C diets using PERMANOVA with 999 permutations.

Category	Df	SS	R^2	F	*P* value
Fly genotype	1	0.683	0.021	5.220	0.007
Microbiome	1	12.415	0.380	94.938	0.001
Fly genotype x Microbiome	1	0.506	0.015	3.871	0.018
Residual	146	19.092	0.584		
Total	149	32.695			

Supp. Table 5: Comparison of microbial community composition for recipients on HS diets using PERMANOVA with 999 permutations.

Category	Df	SS	R^2	F	*P* value
Fly genotype	1	1.827	0.065	8.5721	0.001
Microbiome	1	1.159	0.041	5.439	0.003
Fly genotype x Microbiome	1	1.261	0.045	5.915	0.004
Residual	112	23.869	0.849		
Total	115	28.116			

Supp. Table 6A: 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 6B: 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 7A: 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 7B: 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 8: Average nucleotide identity (ANI) between C and HS *Acetobacter* strains.

Metric	**Value**
ANI value (%)	73.68
C Acetobacter genome length (bp)	3,436,380
HS Acetobacter genome length (bp)	2,895,396
Average aligned length (bp)	1,055,729
C Acetobacter coverage (%)	30.72
HS Acetobacter coverage (%)	37.10

Supp. Table 9: 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 10: 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*