Title: The environment modulates the effects of the microbiome on host adaptive potential

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Abstract

Can the microbiome serve as a reservoir of adaptive potential for hosts? To address this question, we leverage ~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, limiting the accumulation of uric acid, a key driver of toxicity for flies in the HS diet. Our study suggests that both ecological context and host evolution shape the adaptive potential of the microbiome demonstrates that host genotype x microbiome x environment interactions can have a substantial effect on host phenotype. We conclude by proposing future directions that highlight the benefit of using experimental evolution to study the contribution of the microbiome to host evolution.

Significance statement

While abundantly clear that the microbiome shapes many aspects of organismal biology, when and how the microbiome shapes the evolution of host traits remains poorly understood. Through leveraging ~150 generations of experimental evolution to a nutritionally stressful environment in fruit flies, we performed reciprocal host x microbiome x environment transplants to identify how microbial evolution can benefit hosts. We find that hosts can leverage adaptation in the microbiome to improve fitness, but the effects depend on the environmental context. Primarily, the evolved microbiome detoxifies uric acid, mitigating the accumulation of a toxic metabolic byproduct for flies living in the nutritionally stressful environment. Overall, our results suggest that both host and microbe evolution is intertwined in stressful environments.

38 ***Introduction***

39 The microbiome has profound effects on many aspects of organismal biology. From
40 modulating physiology (1) to pathogen protection (2) to social interactions (3), the microbiome is
41 frequently implicated as a key regulator of many host phenotypes--but can the microbiome also
42 influence host adaptation? For hosts where microbes are tightly controlled and faithfully
43 transmitted across generations, microbes can have clear influence on host evolution (4–6). For
44 example, the acquisition of nutrition-provisioning symbionts enables the ecological expansion of
45 sap-feeding insects (7). For many eukaryotes, though, their microbiomes are environmentally
46 acquired or through other weakly controlled transmission mechanisms (5). Weak control over
47 microbial transmission disrupts the links between the microbiome and host over generations.
48 Without these links, the microbiome may have limited effects on host evolution (4, 6, 8).
49 Microbiomes may still have the potential to influence host evolution because environmentally
50 acquired microbes must survive and adapt to external environments. In doing so, microbiomes
51 may acquire beneficial traits that hosts can leverage. Thus, microbiomes have the potential to
52 precede and facilitate rapid host adaptation, but this process is poorly understood.

53 The environmentally acquired microbiome appears to facilitate rapid host adaptation in
54 several systems. For example, bean bugs acquire pesticide resistance from their soil-dwelling
55 *Burkholderia* symbiont (9). The gut microbiome of woodrats facilitates survival on toxic plant
56 diets (10). In plants, drought-adapted soil communities can increase fitness in drought
57 conditions (11). These studies suggest that microbes can adapt and evolve to stressful
58 conditions in natural environments and buffer the stressor for the host, but much about the
59 evolution of these interactions remains unclear—for example, we do not know to what extent
60 these microbial effects depend on host genotypes. Host genetic variation may influence the
61 responsiveness to microbial variation such that some combinations of host and microbiome
62 differentially affect host phenotypes within species. The effects on host phenotype will also likely
63 depend on the environmental context, where mismatches between the environment, host

64 genotype, and microbiome may negatively affect host fitness. In other words, host genotype x
65 microbial genotype x environment (G_H x G_M x E) interactions may shape how host-microbiome
66 systems evolve. Such complex G_H x G_M x E interactions are likely to be common, and yet their
67 contribution in shaping host phenotypic variation and adaptation are underexplored. As the
68 study of microbiomes moves beyond descriptive attributes, experimentally assessing the
69 interactions between host and microbial evolution are a key research priority for better
70 understanding the interplay in host-microbiome interactions (12, 13).

71 The fruit fly, *Drosophila melanogaster*, has emerged as an excellent model to study host-
72 microbe interactions. Flies have relatively simple microbiomes, often composed of fewer than 10
73 bacterial species from acetic acid and lactic acid bacteria families (14). The microbiome is
74 largely environmentally acquired through feeding, defecation, and egg smearing during
75 oviposition (15, 16). After hatching, larvae acquire their microbes through a combination of
76 microbial persistence and colonization and form associations over the lifespan of the fly (17–
77 19). Thus, the microbiome is mostly environmentally acquired, and these microbes also must
78 persist independently in the habitat. Many fly-associated microbes can be individually cultured
79 and reconstituted in various combinations to inoculate sterile flies (14, 20). With the feasibility of
80 microbiome manipulations and rich genetic resources, *Drosophila* is an ideal model to study the
81 interplay between host genetic, microbiome, and environment in shaping host phenotypes.

82 In this study, we take advantage of the *Drosophila* system to study how the microbiome
83 can serve as a reservoir of adaptive potential for hosts under stressful environments by
84 leveraging ~150 generations of fly and microbial adaptation to a high sugar (HS) diet. HS diets
85 exert stress on flies, and flies exhibit similar obesity-like phenotypes as humans with elevated
86 triglycerides, insulin resistance, and shortened lifespans (21–24). In turn, the different bacteria
87 in the fly microbiome have a range of effects on fly phenotypes, shaping life history traits (25,
88 26) and metabolism (25, 27), and these microbiome effects on host phenotype can depend on
89 host genetics (28, 29). Together, these findings suggest that the microbiome may contribute to

90 host traits during adaptation to the HS diet. Our goal here was to determine whether
91 transplanting the adapted microbiome into non-adapted fly genotype would confer adaptive
92 traits in the stressful HS diet. Through a fully reciprocal host x microbiome x diet transplant
93 experiment (Fig. 1), we evaluate how each component contributes to fitness associated traits in
94 flies. The power of our approach is to compare the benefit of adapted microbes relative to non-
95 adapted microbes under different host genetic and environmental contexts. Our results highlight
96 the complexities of environmental context dependence on host-microbiome interactions.
97

98 ***RESULTS***

99 *Fly microbiome responds to HS selection*

100 Here, we leveraged ~150 generations of fly and microbial adaptation to HS diets to
101 understand how the microbiome can contribute to host evolution. We note that HS diets likely
102 exerted several selective pressures on the fly, from the metabolic stress of insulin resistance
103 (21), dehydration (30), mating preferences (31), and sexually dimorphic responses to high sugar
104 diets (24). First, we established that the microbiome changed between C and HS flies (Fig. 2A).
105 HS flies were dominated by *Acetobacter pasteurianus* (86.7% +/- 1.8 SE) and unclassified
106 Acetobacteraceae strain (5.1% +/- 1.8 SE), while C flies were more diverse with *Algoriella*
107 *xinjiangensis* (37.3% +/- 5.3 SE), *Acetobacter persici* (16.9% +/-2.6 SE), and *Acetobacter*
108 *indonesiensis* (14.8% +/- 2.1 SE). The most differentially abundant bacteria were *A.*
109 *pasteurianus* and Acetobacteraceae strain for the HS flies, while *A. xinjiangensis* and *A.*
110 *indonesiensis* were associated with C flies (Fig. 2B, Supp. Table 1). These bacteria are unique
111 to either C or HS flies and found across individual flies (Supp. Fig. 1), demonstrating that the HS
112 diet selected for different fly microbiomes.
113

114 *Transplanting Acetobacter influences life history traits*

115 To test if transferring the microbiome could transfer adaptive phenotypes to naive hosts,

116 we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. We
117 first performed a fecal transplant in flies, but found that the transplant was not faithful (Supp.
118 Fig. 2), as is common for fecal transplants in other taxa (32–34). We then transplanted the
119 dominant *Acetobacters* from C and HS diets identified above in a fully reciprocal experiment and
120 measured life history and metabolic traits. We reasoned that transplanting the HS *Acetobacter*
121 should confer fitness benefits to non-adapted C flies in the HS diets. Because all combinations
122 of G_H x G_M x E interactions were produced in the transplant experiment, the relative benefits of
123 C and HS *Acetobacter* across the different host genetic and environmental conditions could be
124 evaluated.

125 Development was shaped by interactions between fly genotype, *Acetobacter*, and diet
126 as was observed in the whole community transplant (Fig. 3A, Supp. Table 2). Importantly, like in
127 the microbiome transplant, all sterile treatments developed slowest. However, flies responded
128 differently to the *Acetobacter* transplant. On the C diet, fly genotype influenced the response to
129 the *Acetobacter* transplant. HS flies with the C *Acetobacter* developed more rapidly (avg. 140.8
130 hrs +/- 0.54 SE) than with the HS *Acetobacter* (avg. 147.6 hrs +/- 0.60 SE). There was no effect
131 for the C fly. However, on the HS diet, the HS *Acetobacter* accelerated development (HS
132 *Acetobacter* avg. 176.5 hrs +/- .50 SE, C *Acetobacter* avg. 186.8 hrs +/-0.60 SE). The three-way
133 interaction between fly genotype, *Acetobacter*, and diet significantly affected development time (
134 log-likelihood =-58500, X^2 = 47.625, $p < 0.0001$).

135 Fecundity responded to interactions between fly genotype, *Acetobacter*, and diet (Fig.
136 3B, Supp. Table 3). On average, treatments were more fecund on the C diet, while treatments
137 on HS diets laid 54.3% fewer eggs (C avg. 13.0 eggs +/-0.6 SE, HS, avg. 5.9 eggs +/- 0.4 SE).
138 Sterile treatments substantially lowered fecundity, only on the HS diet (+microbes avg. 7.45
139 eggs +/- 0.5 SE, sterile avg. 1.67 eggs +/- 0.4 SE). Notably, the response to *Acetobacter*
140 treatment depended on fly genotype for the HS diet. On the HS diet, HS flies with HS
141 *Acetobacter* (avg. 8.4 eggs +/-1.4 SE) were almost twice as fecund as flies with the C

142 *Acetobacter* (avg. 4.5 eggs +/- 1.0 SE). The interaction between fly genotype, microbiome, and
143 diet significantly affected fecundity (interaction X^2 = 16.4, df = 2, p=0.0003).
144 To gain insight into how these interactions shape host metabolism, we assayed flies for
145 triglyceride (TAG) normalized to protein levels. TAG levels were also shaped by interactions
146 between fly genotype and diet (Supp. Fig. 3A, Supp. Table 4). On the C diet, HS flies had lower
147 TAG levels than C flies, but this relationship was not observed on the HS diet (Supp. Fig. 3B, fly
148 genotype x diet interaction, $F_{1,78.0}$ = 9.00, p = 0.004). *Acetobacter*, alone or through interactions,
149 did not influence TAG levels. Thus, fly metabolism was primarily shaped by host genetics and
150 diet, but not by differences between *Acetobacters*.
151
152 *HS Acetobacter uniquely encodes uric acid degradation pathway*
153 To better understand how the HS *Acetobacter* may benefit flies in the HS diet, we
154 sequenced and compared the genomes of the C and HS *Acetobacters*. One key difference
155 between the two genomes was that HS *Acetobacter* encodes for several genes that degrade
156 uric acid, but C *Acetobacter* does not (Fig. 4A). Indeed, we found that interactions between
157 *Acetobacter* and the diet shape uric acid degradation (Fig. 4B, Supp. Table 5). Only on the HS
158 diet, HS *Acetobacter* reduced the concentration of uric acid by ~50% compared to C
159 *Acetobacter*, but only negligible changes to uric acid on the C diet.
160 Finally, we reasoned that if the match between *Acetobacter* and diet benefits flies, then
161 we should see enrichment for the matched *Acetobacter* in our experimentally evolving fly
162 populations. Using the assembled C and HS *Acetobacter* genomes, C *Acetobacter* comprised
163 87.0% of *Acetobacter* reads in the C fly population, and HS *Acetobacter* comprised 84.2% in the
164 HS fly population (Fig. 4C).
165
166 ***DISCUSSION***
167 Here, we explored how the microbiome influences host fitness in stressful environments.

168 Through reciprocal host x microbiome x diet transplant experiments, we show that G_H x G_M x E
169 interactions shape host fitness by modifying developmental timing and fecundity. The HS
170 *Acetobacter* genome encodes uric acid degradation genes and, in vitro, lowers uric acid on the
171 HS diet, while the C *Acetobacter* does not. Our results highlight the complex interactions
172 generated by the microbiome, and we discuss important future directions to further dissect
173 these relationships.
174
175 *Acetobacter dominates HS microbiome*
176 *Acetobacter* was the most abundant genus in the HS flies (Fig. 2). *Acetobacter* are
177 common in flies, both in laboratory settings and wild environments(18, 35–38). Furthermore,
178 *Acetobacter* are sugar specialists and tolerate highly acidic environments, like during vinegar
179 fermentation (39). In the context of *Drosophila* biology, some strains of *Acetobacter* reduce
180 glucose levels and enhance oxidation of ethanol, which promotes larval development (27, 40).
181 Additionally, reducing glucose levels is critical for flies to survive HS diets (41). Over the course
182 of experimental evolution, both fly and microbiome were exposed to HS diets, and in turn, the
183 HS microbiome was dominated by *A. pasteurianus* (Fig. 2B). Taken together, these results
184 indicate that HS selection also changed the fly microbiome, selecting for bacteria that could
185 survive HS stress.
186 Comparative analyses between the C and HS *Acetobacter* genomes showed that HS
187 *Acetobacter* encoded for several genes in the uric acid degradation pathway, while the C
188 *Acetobacter* genome did not. *In vitro* testing also showed that HS *Acetobacter* reduced uric acid
189 in the HS diet, but C *Acetobacter* did not (Fig. 4B). Uric acid accumulation is associated with
190 high sugar diets in flies, particularly when extra water is not provided to ameliorate water stress
191 associated with HS diets (30). The accumulation of uric acid may also exacerbate the effects of
192 HS diet, altering oxidative stress and inflammation that can promote obesogenic phenotypes
193 (42, 43). Uric acid is normally excreted by flies, but high concentrations slow development (44)

194 and shorten life spans (30, 45). Not all *Acetobacter* species can degrade uric acid, including the
195 C *Acetobacter*, but the ability to degrade is more common in fly-environment than in other free-
196 living *Acetobacter* species (43). Uric acid degradation may be the key fitness benefit of the HS
197 *Acetobacter* over the C *Acetobacter* in the HS diet.

198 The fitness advantage of uric acid degradation was likely direct for the HS *Acetobacter*,
199 but indirect for the fly. *Acetobacter* species that degrade uric acid outcompete *Acetobacter*
200 species that cannot in the fly environment (43). For the HS *Acetobacter*, uric acid degradation
201 may underlie its persistence in the stressful HS diet, and thus indirectly benefiting flies through
202 reductions of uric acid in the diet. The benefit of uric acid degradation may be especially
203 important during development. Larvae have limited endogenous abilities to detoxify uric acid,
204 and the primary mechanism of uric acid tolerance is through decreasing exposure through
205 decreased uptake or increased excretion (44, 46, 47), which likely impacts resource
206 accumulation in the larvae during development on the HS diet. For adults, it is unclear if HS
207 *Acetobacter* also degrades uric acid in the fly gut or simply benefiting from the reduction in the
208 diet. While these questions require further characterization of fly physiology, uric acid
209 degradation by HS *Acetobacter* is similar to other examples of environmentally acquired
210 microbiomes facilitating adaptation through detoxifying the environment, such as terpene
211 detoxification in woodrats (10) or insecticide resistance in bean bugs (9). Our results contribute
212 to a growing body of literature that suggests that the microbiome can encode novel functions
213 that hosts can leverage the microbiome during adaptation to stressful environments (48).
214

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

216 Many have predicted that the microbiome could accelerate local adaptation in the host,
217 facilitating phenotypic changes to buffer local stressors (6, 7, 49, 50). If so, transplanting the
218 locally adaptive microbiomes should confer adaptive phenotypes to naive individuals. However,
219 the benefit may depend on interactions between host genotype and environment. G_H x G_M x E

220 interactions have the potential to facilitate or impede evolution in challenging environments (12,
221 13). Through combining experimental evolution with reciprocal transplants, our data allowed us
222 to test if and how ecological context (i.e., diet) and evolutionary context (i.e., fly genotype)
223 shape the adaptive potential of the microbiome.

224 In our attempt to transplant the complete microbiome community (Supp. Fig. 2), the
225 ecological context likely limited transplant success. While we expected the HS diet to promote
226 differentiation between C and HS microbiome recipients, we found the opposite (Supp. Table 6,
227 8). Low fidelity in microbiome transplants is common in both flies (51, 52) and mammals (32–
228 34), and much remains unclear about how ecological and evolutionary conditions promote or
229 impede microbiome transplants. For example, Rudman et al. (51) inoculated fly diets with either
230 *Acetobacter tropicalis* or *Lactobacillus brevis*. Even at the start of the experiment, the highest
231 relative abundance the inoculated bacteria reached was less than 20%, and over the growing
232 season declined to negligible detection. Nevertheless, significant genetic divergence was
233 detected that matched the clinal nature of the fly-bacteria associations. Future work should
234 further investigate how the combination of other bacterial traits, like absolute abundance
235 compared to variation in microbiome composition, influences the effects on host evolution.

236 To shape adaptive potential, the microbiome must contribute to host phenotype, not
237 unlike traditional (host) additive genetic variance (6). The G_H x G_M x E interaction for
238 development illustrates how diet, the ecological context, when matched with evolutionary
239 context, may increase the fitness benefit of locally adaptive microbiomes (Fig. 3A). The HS
240 *Acetobacter* accelerated development on the HS diet (a match between microbe and diet,
241 independent of fly genotype) and slowed development compared to the C *Acetobacter* on the C
242 diet for only the HS fly (a mismatch between microbe and environment, but dependent on fly
243 genotype). The mismatch only mattered for the HS fly; C fly development was similar between
244 the C and HS *Acetobacter* on the C diet. However, the G_H x G_M x E interaction that shaped
245 fecundity is suggestive of how genetic interactions may impede the transfer of adaptive

246 potential. The naive host, C flies, did not receive a fecundity increase from the HS *Acetobacter*
247 in the HS diet, but HS flies did (Fig. 3C). The presence of G_H x G_M x E interactions suggests that
248 the microbiome contributed to fly evolution. HS flies responded differently to the *Acetobacter*
249 variation from the C flies, and more so, responsiveness depended on the diet. Because host
250 genotypes may vary in their ability to leverage beneficial microbes in stressful environments,
251 genetic interactions may limit the benefits of locally adaptive microbiomes. Understanding these
252 G_H x G_M interactions will be especially important in using evolution to engineer beneficial
253 microbiomes to solve pressing problems in public health and agriculture (6, 12, 13, 53). While
254 longitudinal sampling of the evolutionary trajectory is necessary to determine to what extent the
255 HS fly evolved or selected for beneficial *Acetobacter* traits, our results clearly demonstrate how
256 the ecological context and host evolution can shape the adaptive potential of the microbiome.
257 We acknowledge that despite the ~150 generations of adaptation to the HS diet, the HS
258 diet still comes at a fitness cost to the HS flies. For example, developmental time was still
259 slower on the HS diet compared to the C diet (Fig. 3A). HS flies were less fecund on the HS diet
260 than on the C diet (Fig. 3C). For metabolic indices, genotypic differences were only observed on
261 the C diet (Supp. Fig. 3). One possibility for these unexpected results is the use of
262 dechorionation to manipulate the microbiome. Dechorionation, which removes the chorion that
263 protects eggs as well as the microbes smeared during egg laying, is a common tool for
264 manipulating the *Drosophila* microbiome (14, 20). Dechorionation has conflicting effects on
265 different aspects of fly physiology. One study found no significant effect on metabolic indices,
266 like protein and TAG levels, compared to conventionally reared flies (54). However, another
267 study found that dechorionation increased overall mortality, though improved starvation
268 resistance (55). It is possible that dechorionation affected our results, but we did not test for this
269 effect. We raise this point as one challenging aspect of using experimentally evolved hosts for
270 microbiome manipulations, as adaptation to novel environments may change host biology in
271 unexpected ways.

272

273 *Conclusions and research priorities*

274 Here, we leveraged host and microbial evolution from ~150 generations of adaptation to
275 a stressful diet. The fly populations used in this experiment were originally conceived as an
276 Evolve & Resequence (E&R) experiment. The E&R approach in *Drosophila* uses outbred
277 populations to identify signatures of selection across the genome and has illuminated many
278 important findings about the role of demography, population genetics, and genetic architecture
279 in shaping adaptive traits (56, 57). However, missing from this framework is the role of the
280 microbiome. In a survey of ten E&R experiments, the microbiome also frequently responded to
281 experimental evolution, particularly in traits associated with metabolism (58). Taken together,
282 our data here also highlight how the microbiome may contribute to host adaptation – effects
283 previously unseen when focusing on the *Drosophila* genome. Recent work on the microbiome
284 linked to natural variation in wild populations suggested that a single inoculation of two different
285 bacteria linked to different life-history strategies, can lead to rapid phenotypic change and
286 genetic differentiation in one fly population (26, 51). Our current study advances these findings
287 by comparing differences in ecologically relevant *Acetobacter* evolution to differences in fly
288 evolution, providing one of the first experimental dissections of host-microbiome interactions in
289 the E&R context. Through reciprocal transplants, we identified how interactions between fly
290 genotype, microbiome, and diet shape fitness related traits.

291 The G_H x G_M x E interactions observed here highlight how evolution in both the host and
292 microbiome occurred during adaptation to the HS diet. In particular, the ability of HS
293 *Acetobacter* to degrade uric acid in the HS diet reflects the potential for diffuse coevolution (59).
294 The HS diet likely led to an increase in uric acid production by flies, and then the *Acetobacter*
295 that could exploit the new niche increased, which the HS flies benefited from the reduction in
296 toxicity. However, this hypothetical scenario can only be addressed through longitudinal
297 sampling of the evolutionary trajectory for both the host and microbiome. Ultimately, in our

298 system and perhaps many others, the evolutionary benefits between host and microbiome are
299 intertwined. Future work utilizing experimental evolution will demonstrate how the joint evolution
300 of host and microbiome shapes the response to selection, providing fundamental insights into
301 how natural selection operates on complex systems.
302
303 ***METHODS***
304 Full details of methods can be found in the Supplementary Materials and Methods.
305 *Fly populations*
306 All fly populations were maintained at 25ºC with 12 hour light:dark cycles. The base
307 population was derived by round-robin crossing a subset of the Global Diversity lines [38], which
308 were then maintained at large population size (>10,000 flies) and allowed to interbreed freely for
309 ~50 generations before HS selection began. Control diet composed of 10% glucose, 10% yeast,
310 1.2% agar, with 0.04% phosphoric acid and 0.4% propionic acid as preservatives. Three
311 replicate populations for HS selection were derived from the base population and maintained on
312 the same diet, but with an additional 13% sucrose and at lower population size (>5000
313 flies/cage until generation 100 and ~2000 flies/cage after). The approach used here allowed for
314 natural selection on HS diets; we did not guide or select for particular traits as is often used in
315 experimental evolution in *Drosophila*. In parallel, three replicate populations were reared on
316 control diets as is standard for experimental evolution studies, but these are not the focus of this
317 study. Because we were interested in the microbiome transferring adaptive traits to naive hosts,
318 we defined the outbred base population as our control, non-evolved population. We consider the
319 two populations, C and HS, to be two different genotypes, though note they are not inbred and
320 retain genetic diversity.
321 *Transplanting microbiomes to test for adaptive significance*
322 To test if the microbiome can transfer adaptive traits, we performed a reciprocal
323 microbiome transplant experiment (Fig. 1). All host genotypes x microbiome x diet interactions

324 were generated in the reciprocal transplant. Both genotypes (C and HS flies) were inoculated
325 with microbiomes (C, HS) or kept sterile and raised in both diets (C and HS) for a total of 12
326 treatments. This experimental design allowed us to evaluate how host x microbiome x
327 environment interactions influence the transfer of adaptive phenotypes.

328 We first identified how the microbiome changed between C and HS flies. We performed
329 16S amplicon sequencing and used ANCOM (analysis of composition of microbiomes)
330 implemented in QIIME2 (60). We cultured the most differentially abundant bacteria from HS
331 flies, *A. pasteurianus*, and the significantly differentially abundant bacteria within the
332 *Acetobacter* genus and enriched in C flies, *A. indonesiensis*. We confirmed 16S rRNA sequence
333 identity using Sanger sequencing. Because the 16S rRNA phylogeny is discordant with a
334 multilocus phylogeny in the Acetobacteraceae (43) and for simplicity, we refer to *A.*
335 *indonesiensis* as C *Acetobacter* and *A. pasteurianus* as HS *Acetobacter*.

336 To perform the transplant, both *Acetobacters* were cultured in liquid MRS, density was
337 normalized to OD_{600} = 0.1, and 50 µl of bacteria (or PBS for sterile treatments) was used to
338 inoculate axenic eggs in sterile diets (20). For each treatment, 6-10 replicates were maintained.
339 Flies were then reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the
340 lifespan of the recipient flies.

341 *Phenotypic effects of the Acetobacter transplant*

342 To measure developmental time, we counted the number of new pupae every 6 hours
343 during their daytime (i.e., 8am, 2pm, 8pm) until all treatments began eclosing. We used the
344 survival (61) and survminer (62) packages in R to analyze development data. We used Cox
345 proportional hazards to identify how interactions between fly genotype, microbiome, and diet
346 influence development time.

347 For fecundity, we counted the number of eggs laid by individual 8-10 day old females
348 (N=644). Females were placed in a 24-well plate with oviposition media. To minimize plate
349 effects, two treatments were assayed on each plate, and treatments were randomly assigned

350 across plates. Females laid eggs for ~4-6 hrs at 25ºC during the late afternoon (2pm-8pm) to
351 capture similar circadian rhythms in egg laying across all treatments. We analyzed fecundity
352 using a zero-inflated Poisson distribution implemented in glmmTMB (63) with oviposition plate
353 (N=29 plates) as the random effect.

354 For nutritional indices, we assayed pools (N=95) of three age-matched (8-10 days old)
355 females for total triglycerides. Triglycerides were normalized to protein amount(64). Protein
356 serves as a proxy for size and was measured using a Bradford assay (BioRad 5000205). Total
357 triglycerides represent fat stores for flies and were assayed using a colorimetric assay (Sigma
358 TR0100-1KT). The biological replicates of treatments were assayed across different assay
359 plates. We analyzed the triglyceride data using linear mixed models, testing for the effect of fly
360 genotype, *Acetobacter*, and diet with assay plate (N=14) as the random effect using lme4
361 package in R (65).

362 *Characterizing the genomic differences between C and HS Acetobacter*

363 To further characterize the mechanisms underlying the fitness-associated benefits from
364 the C and HS Acetobacters, we performed whole genome sequencing on these bacteria.
365 Libraries were prepared following the manufacturer instructions using the Illumina PCR-free
366 Library Prep kit, and then 150-bp paired end reads were sequenced on an Illumina NovaSeq
367 platform. Genomes were assembled using SPAdes (66) with --isolate flag and annotated using
368 the Rapid Annotation using Subsystem Technology (RAST) server (67).

369 To identify functional changes, we compared the RAST annotations to identify pathways
370 unique to either C or HS Acetobacter. We identified orthologs between the two Acetobacter
371 genomes using OrthoFinder v2.5.2 (68). Then to gain insight into the function of the putative
372 genes, we performed BLASTP v2.10.1+ searches against the NCBI non-redundant protein
373 sequence database (69).

374 The genomic analysis performed above suggested that the HS Acetobacter genome
375 encoded several pathways for urea degradation, but were not present in the C Acetobacter

376 genome. To validate this observation, we measured the ability of the Acetobacters to degrade
377 uric acid in fly food. Uric acid was added at 50 μmol concentration to 30 μl of C and HS diet.
378 Then 10 μl of C or HS Acetobacter (or sterile PBS control), normalized to OD_{600}=0.1, were
379 added to the diet and incubated for 4 days at 25ºC to degrade the uric acid. Uric acid
380 concentration was measured using the Amplex Red Uric Acid/Uricase kit (Sigma) following the
381 manufacturer instructions.

382 We then leveraged whole genome sequencing in the fly to determine if the C and HS fly
383 populations from the original E&R experiment were enriched for their respective C or HS
384 Acetobacter. Fly genomes were sequenced on the Illumina NovaSeq S4 using 150 bp paired-
385 end reads (full details in the supplement). Reads were mapped to Wolbachia (NCBI accession
386 NC_002978.6), C and HS Acetobacter genomes using BWA (70), discarding reads that were
387 non-uniquely mapped as well as those mapped to Wolbachia. The relative abundance of each
388 Acetobacter was then compared between C and HS flies.

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393 ***DATA AVAILABILITY***

394 Genomes and amplicon sequencing will be uploaded to NCBI [upon acceptance]. Phenotypic
395 data will be deposited in Dryad [upon acceptance]. Code used to analyze data will be posted on
396 github [upon acceptance].

397

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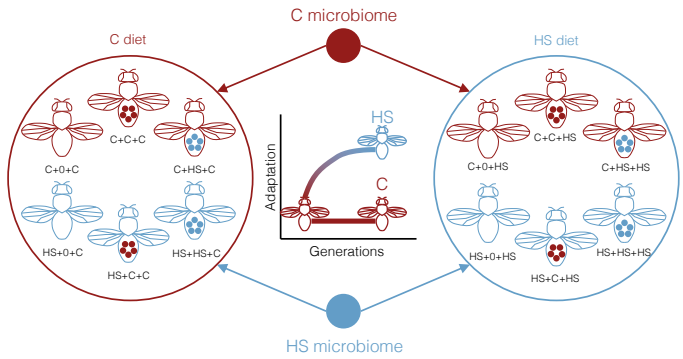
550

1A



1B

ID	Treatment (fly genotype + microbe + diet)	Symbol
C+0+C	C fly + sterile + C diet	
C+C+C	C fly + C micro + C diet	
C+HS+C	C fly + HS micro + C diet	
HS+0+C	HS fly + sterile + C diet	
HS+C+C	HS fly + C micro + C diet	
HS+HS+C	HS fly + HS micro + C diet	
C+0+HS	C fly + sterile + HS diet	
C+C+HS	C fly + C micro + HS diet	
C+HS+HS	C fly + HS micro + HS diet	
HS+0+HS	HS fly + sterile + HS diet	
HS+C+HS	HS fly + C micro + HS diet	
HS+HS+HS	HS fly + HS micro + HS diet	

Fig. 1. We leveraged ~150 generations of fly adaptation to HS diets to study the role of the microbiome in adaptation. A) Experimental design for fully reciprocal host x microbiome x diet transplant to test the role of microbial evolution to fly adaptation. The inset diagrams experimental evolution that generated the flies and microbes. Fly genotypes (C or HS) were inoculated with microbes (none, C, or HS) in two diets (C or HS). The same experimental design was used in two iterations: community transplant or *Acetobacter* transplant. B) The table shows the abbreviated treatment codes used throughout this study, and it is colored to emphasize the microbiome treatment. The symbols represent the same information visually diagrammed as flies. Red colors represent control, blue colors represent high sugar, and yellow (or empty flies) represent sterile treatments.

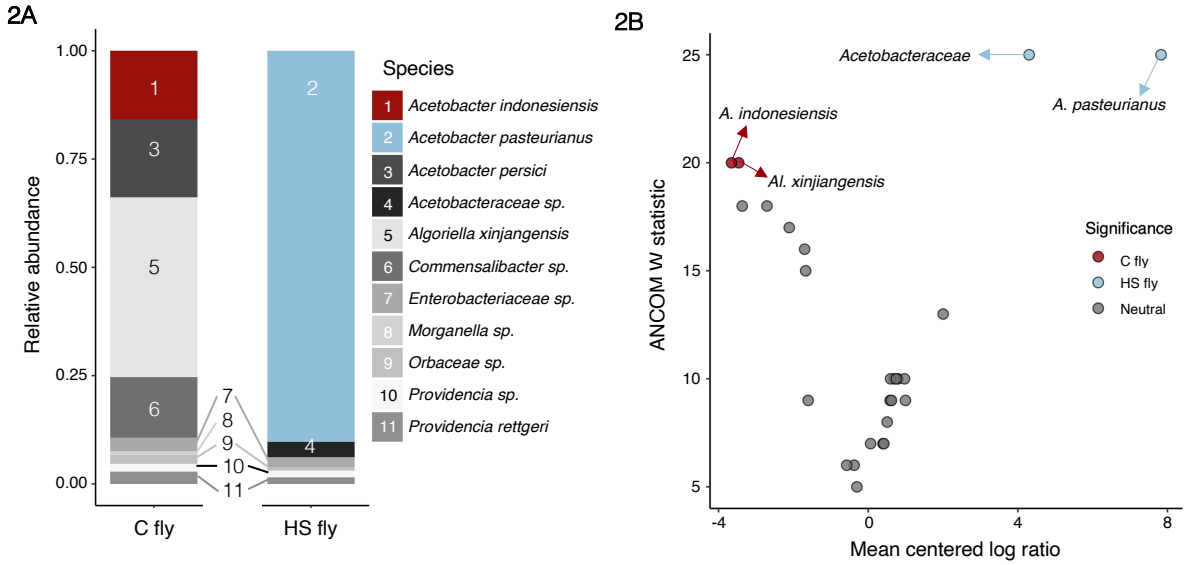


Fig. 2: The microbiome in HS flies is different from C flies. A) Relative abundance of microbiome determined using 16S rRNA sequencing. The C fly microbiome is enriched for *A. indonesiensis*, *A. persici*, and *Algoriella xinjiangensis*. The HS fly microbiome is enriched for *A. pasteurianus*. B) ANCOM results for differentially abundant bacteria between C and HS flies. Colored points represent significantly different ASVs and arrows denote taxonomic classification.

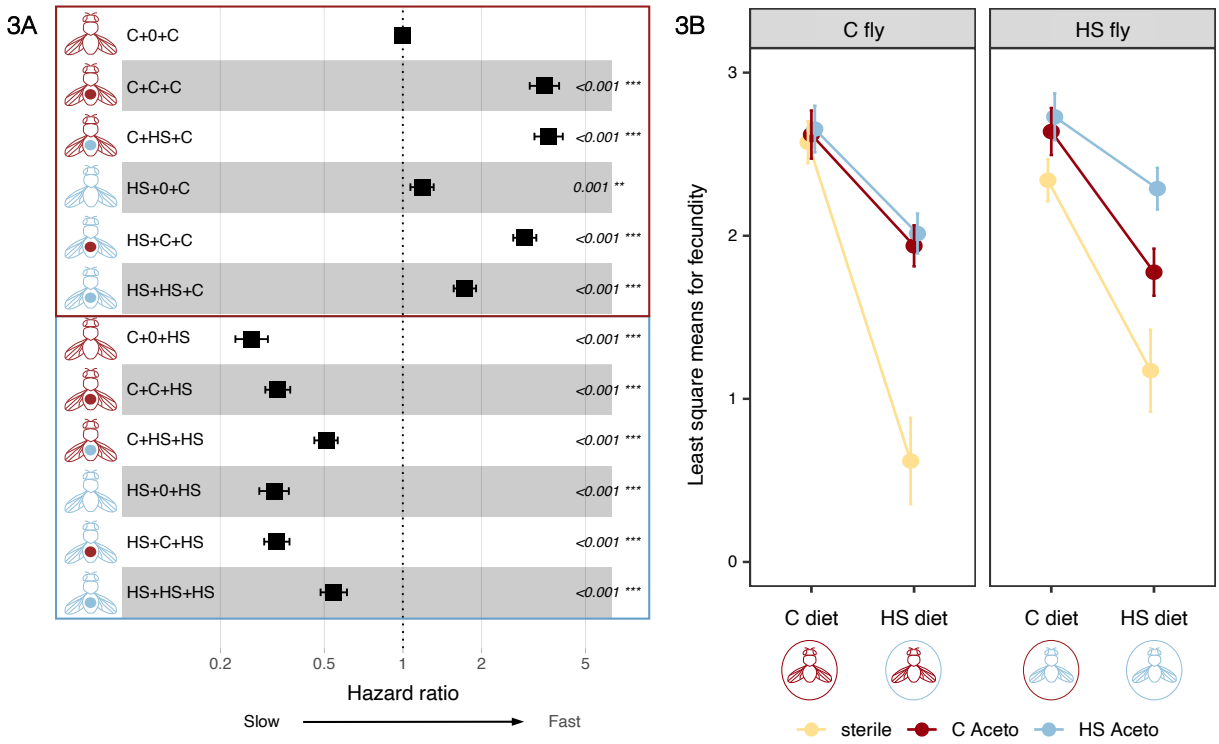


Fig. 3: Life history effects of the *Acetobacter* transplant. A) Hazard ratio plot for *Acetobacter* effects on development time. Boxplots show hazard ratios and 95% confidence intervals for each treatment relative to C+0+C. Mismatches between diet and microbiome slowed development. In C diet, HS flies with C *Acetobacter* developed faster than HS flies with HS *Acetobacter*. However, on 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. Colors represent Acetobacter treatment. Error bars show standard error for LSMs. For the HS fly genotype, the HS *Acetobacter* increased fecundity relative to the C *Acetobacter*, suggesting a fecundity benefit with the adapted microbiome.

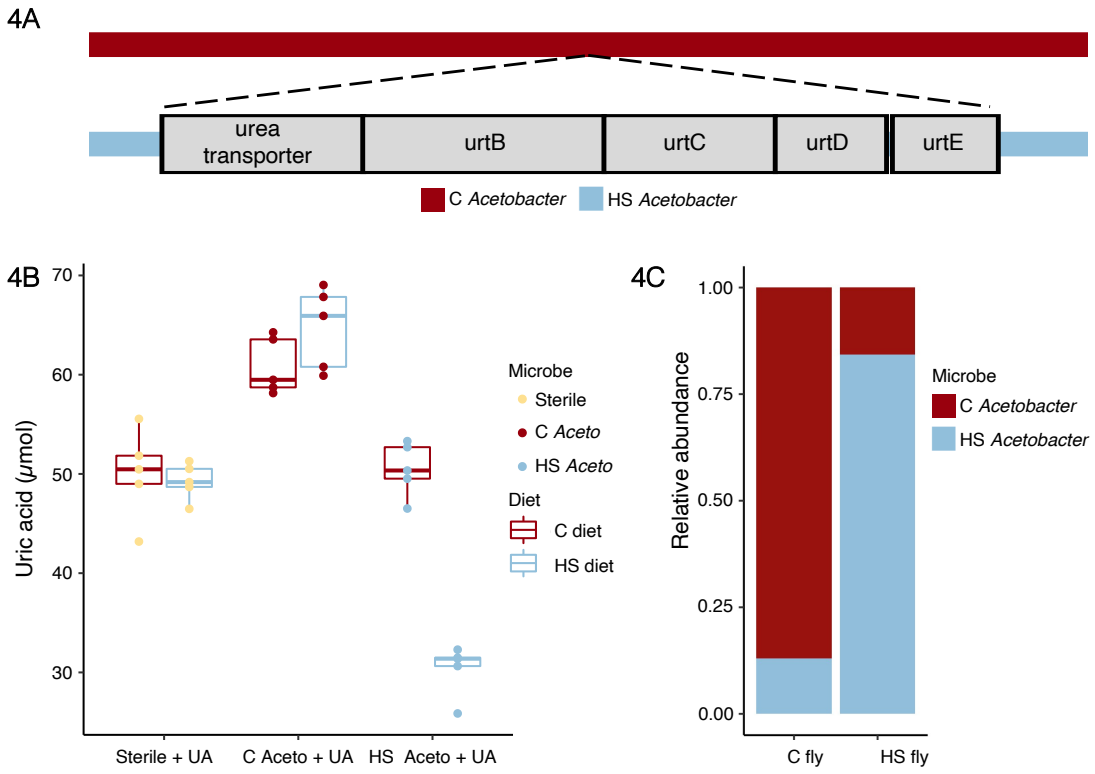
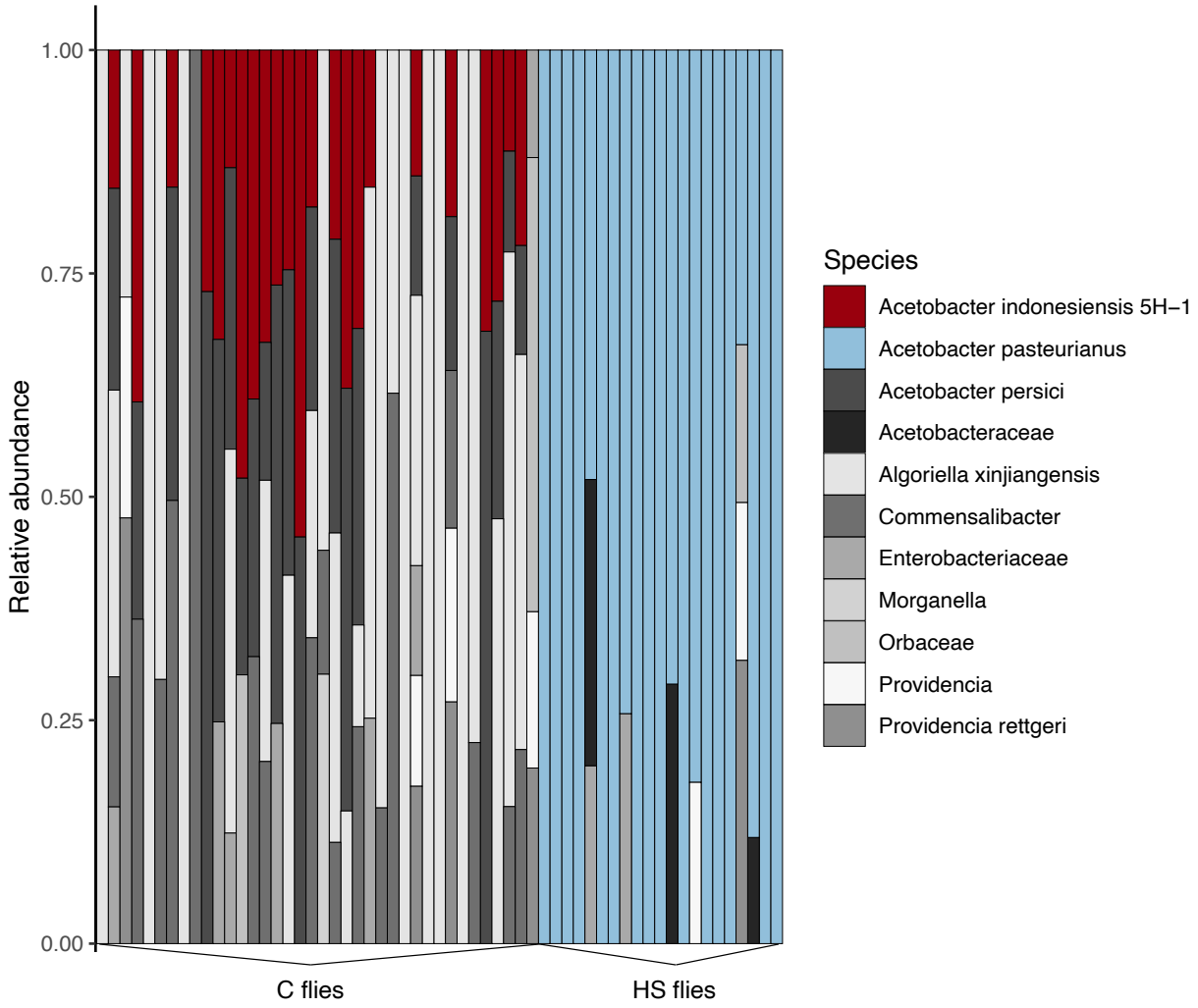
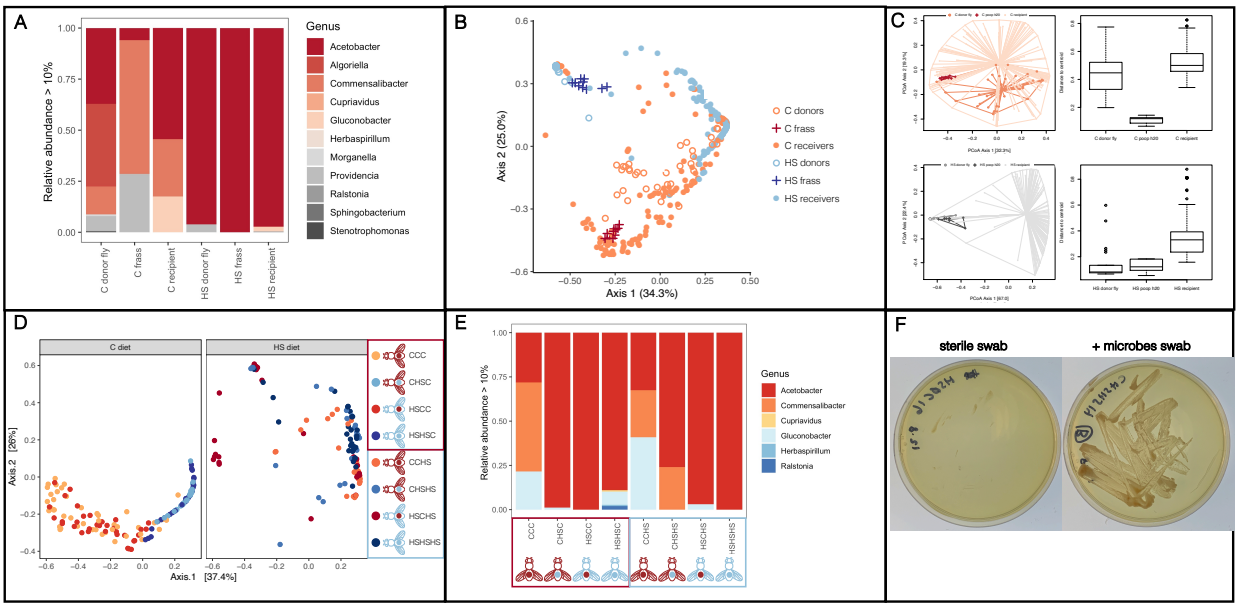


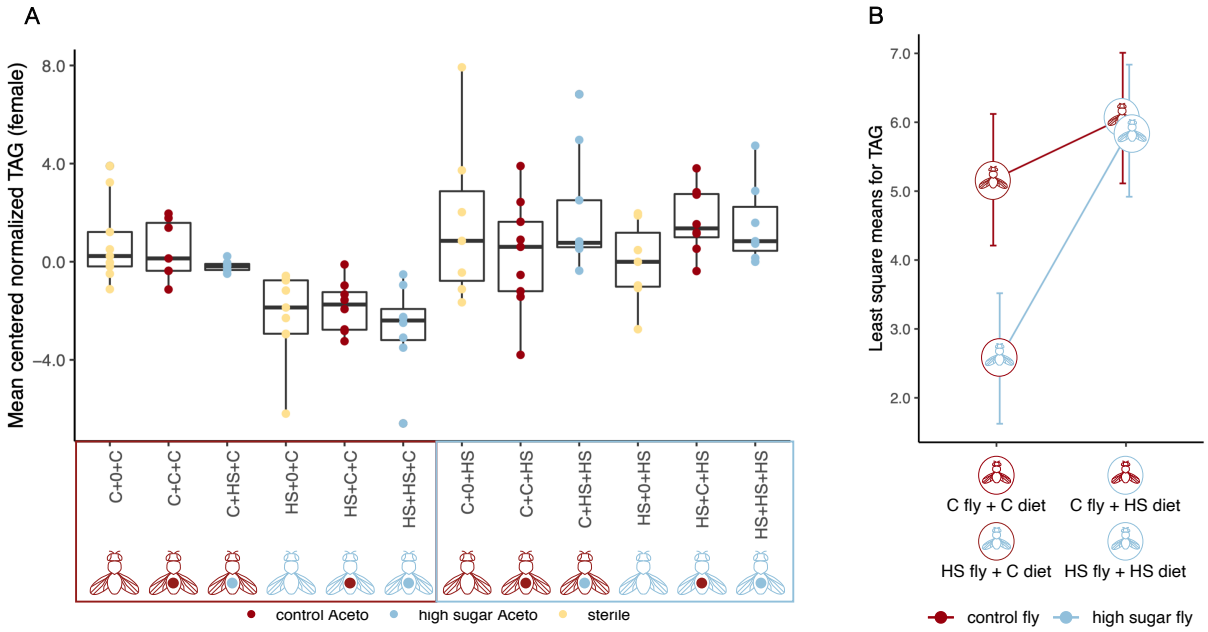
Fig 4: Genomic analysis of the C and HS Acetobacter. A) Schematic showing the genes in the uric acid degradation pathway encoded by only HS *Acetobacter*. B) Boxplots are colored by diet with points colored by microbe treatment. Only the HS diet, the HS Acetobacter degraded significantly more uric acid. C) Relative abundance of the C and HS Acetobacter in the selection lines. C Acetobacter was more abundant in the C flies, and the HS Acetobacter was more abundant in the HS flies.



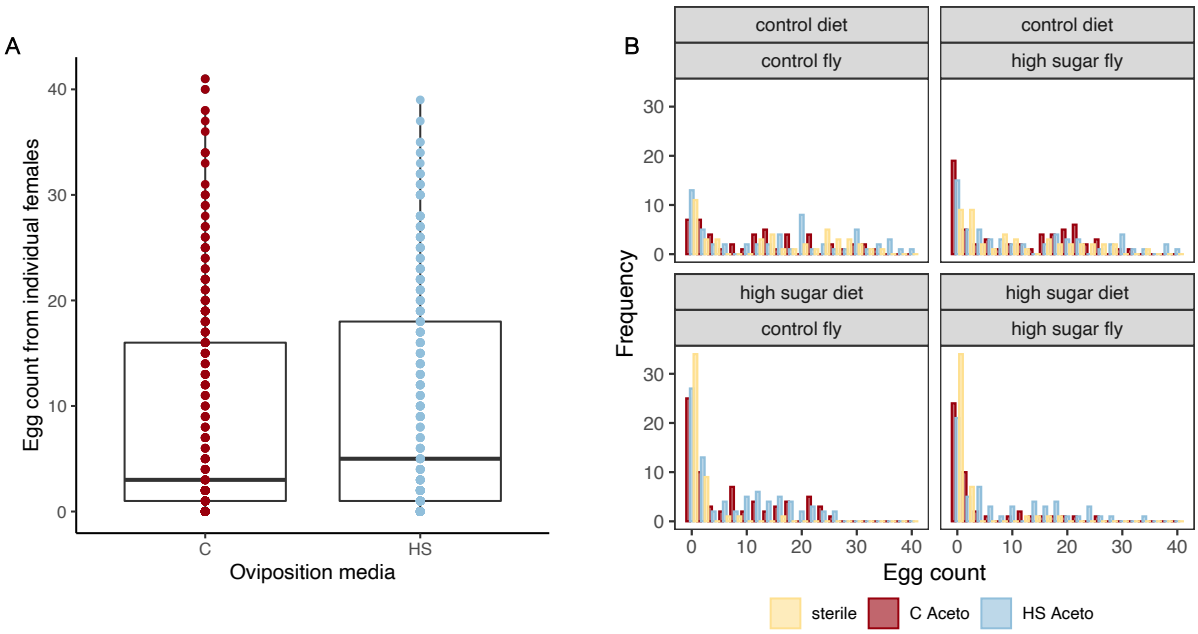
Supp. Fig. 1: Relative abundance for individual C and HS flies. Each bar represents an individual fly. Color denotes species. Individuals are grouped by the fly genotype. These flies were used as the donors in the frass microbiome transplant.



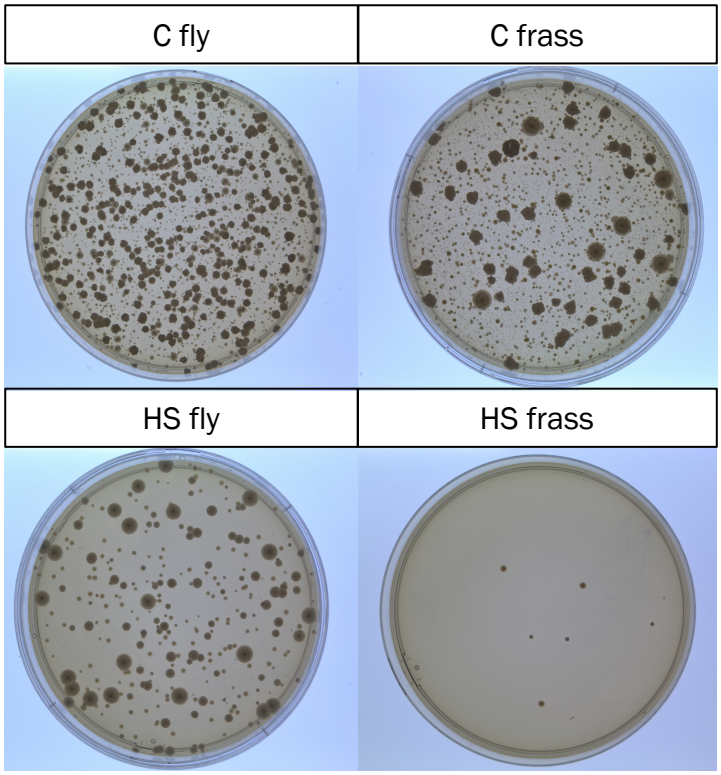
Supp. Fig 2: 16S rRNA characterization of microbiome transplant. A: Relative abundance at the genus level for donors, frass, and recipients in microbiome transplant. Each step of the transplant (i.e., donor, frass, recipient) shows overlap in only a few species. B: PCoA plots for Bray-Curtis distance based on relative abundance of ASVs for the microbiome transplant. Points represent individual flies, except for the frass (which was pooled from 250 flies and 6-8 replicates sequenced). Axis 1 mostly separates between frass and flies, while Axis 2 shows some separation between C and HS microbiomes. C: The relationship between donor, frass, and recipient separated by C (in red) and HS microbiomes (in grey). Each point represents a fly, while the lines show the distance to mean value for each group. Boxplots show the average difference in centroid dispersion. D: PCoA plots for Bray-Curtis distance for only the recipients, separated by diet. On the C diet, flies that received the C microbiome were distinct from those with the HS microbiome, suggesting fidelity of the microbiome transplant. However, on the HS diet, the microbiome transplant did not reconstitute differences between C and HS microbiomes in the recipient. E) Relative abundance of genera for each treatment in the recipients. The C recipients were composed of primarily *Acetobacter* and *Commensalibacter* genera, while only *Acetobacter* genera in HS recipients, especially on HS diets. F) Images representative of sterility checks. Food was swabbed during development and streaked on MRS plates. Sterile swab shows no growth and only food smudged. Microbes swab shows microbial growth.



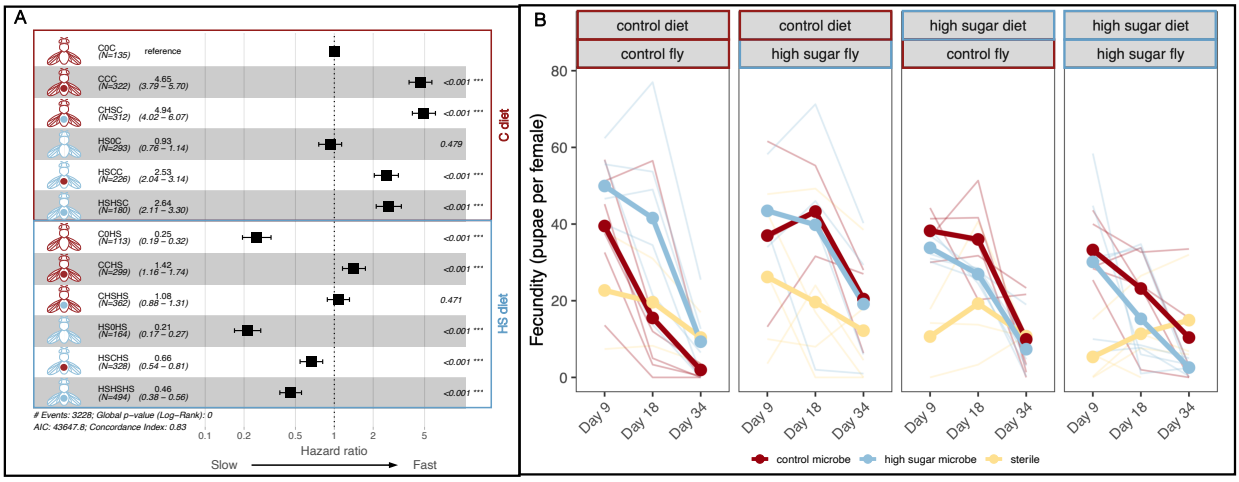
Supp. Fig. 3: Metabolic effects of the *Acetobacter* transplant. A) Boxplot for triglycerides (TAG) normalized to protein. TAG amount reflects fat storage in *Drosophila*. Each point represents a pool of three age-matched female flies. TAG levels were mean centered to control for each batch assayed. 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.



Supp. Fig. 4: A) No difference for egg counts between C and HS oviposition media (Kruskall-Wallis X^2 = 2.85, df=1, p = 0.09). B) Histogram for egg counts from each treatment faceted by diet and fly genotype. Bars represent the number of females that laid eggs and colored by microbiome treatment.



Supp. Fig. 5: Differences in bacterial abundance between flies and frass for the C and HS fly genotypes. Figures show cultured bacteria (50 µl of 500 µl homogenate of 5 female flies or 50 µl of frass from ~200 flies) on MRS media incubated for 3 days. C flies harbor higher amounts of bacteria than HS flies. Bacteria in HS frass is especially low abundance.



Supp. Fig. 6: Life history effects of the microbiome transplant. A) Hazard ratio plot for microbiome effects on developmental time. Boxplots show hazard ratios and 95% confidence intervals for each treatment relative to C0C. On the C diet, both fly genotypes with either C or HS microbiomes developed faster than sterile flies. However, on HS diet, and the C microbiome accelerated while HS microbiome slowed development. The interaction effects on the Cox proportional hazards model best explained variation in development (log-likelihood= -21807, df=7, X^2=78.026, p<0.001), suggesting that fly evolution and environment shape phenotypic effects of the microbiome. B) Fecundity effects of the microbiome transplant at three timepoints over 34 days. Plots are faceted by fly genotype and diet. Thick lines with dots represent the average fecundity for the microbiome treatment. Thinner lines represent the replicates. Fecundity declined over time ($F_{2,105.3}$ = 49.4, p<0.0001) and was shaped by both diet ($F_{2,49}$ = 4.35, p=0.02) and microbiome ($F_{1,50}$ = 5.41, p=0.02).

SUPPLEMENTARY TABLES

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: 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. Table 6: Comparison of centroid dispersion for donors, frass, and recipients in the microbiome transplant with 999 permutations.

Category	Df	SS	MS	F	*P* value
Microbiome source	5	5.715	1.143	36.282	0.001
Residuals	334	10.522	0.0315		

Supp. Table 7: Comparison of microbial community composition for recipients on C diets using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	0.6954	0.6954	6.129	0.02344	0.004
Microbiome	1	12.6455	12.6455	111.45	0.42627	0.001
Fly genotype x Microbiome	1	0.4395	0.4395	3.874	0.01482	0.01
Residuals	140	15.8849	0.1135	0.5354		
Total	143	29.6653	1			

Supp. Table 8: Comparison of microbial community composition for recipients on HS diets using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	1.8347	1.83472	8.5998	0.06509	0.001
Microbiome	1	1.1485	1.14846	5.3831	0.04074	0.006
Fly genotype x Microbiome	1	1.3113	1.3113	6.1464	0.04652	0.003
Residuals	112	23.8945	0.21334	0.84765		
Total	115	28.189	1			

Supp. Table 9: Comparisons of microbial community composition for all recipients using PERMANOVA with 999 permutations.

Category	Df	SS	MS	F	R^2	*P* value
Fly genotype	1	1.189	1.1891	22.026	0.0172	0.001
Microbiome	1	9.302	9.3019	172.301	0.13455	0.001
Diet	1	10.577	10.5766	195.913	0.15299	0.001
Fly genotype x Microbiome	1	0.339	0.3387	6.274	0.0049	0.001
Fly genotype x Diet	1	1.656	1.6562	30.678	0.02396	0.001
Microbiome x Diet	1	4.959	4.9593	91.863	0.07173	0.001
Fly genotype x Microbiome x Diet	1	1.163	1.163	21.543	0.01682	0.001
Tube (random effect)	39	28.451	0.7295	13.513	0.41153	0.001
Residuals	213	11.499	0.054	0.16633		
Total	259	69.135	1			

Supp. Table 10: Analysis of deviance for Cox model with interactions between fly genotype, microbiome, and diet in the microbiome transplant.

Category	Log-likelihood	X^2	Df	*P* value
Null	-22850			
Fly genotype	-22716	268.8227	1	<2.20E-16
Microbiome	-22475	480.3245	2	<2.20E-16
Diet	-21846	1258.5088	1	<2.20E-16
Fly genotype x Microbiome	-21819	53.3452	2	2.61E-12
Fly genotype x Diet	-21817	4.2178	1	0.04
Microbiome x Diet	-21807	20.0385	2	4.45E-05
Fly genotype x Microbiome x Diet	-21807	0.4244	2	0.8088

Supp. Table 11: Fixed effects from the mixed model of lifetime fecundity in the microbiome transplant. Significance was evaluated using Type III Wald F tests with Kenward-Roger degrees of freedom.

Category	F	Df	Df.res	*P* value
Intercept	46.6596	1	60.701	4.62E-09
Fecundity time point	49.4043	2	105.269	7.38E-16
Fly genotype	0.0266	1	48.831	0.87103
Microbiome	4.3529	2	49.038	0.01819
Diet	5.4115	1	48.952	0.02419

SUPPLEMENTARY MATERIALS AND METHODS

Fly populations

All fly populations were maintained at 25ºC with 12 hour light:dark cycles. The base population consisted of an outbred population created by crossing 15 inbred lines initially collected in the Netherland (a subset of the Global Diversity Lines) (1). Briefly, we crossed each inbred line to another using a round-robin design. The resulting F1s were crossed in a similar pattern to ensure parental representation and that no chromosome would be lost in the first round of crosses. The resulting F2 individuals were placed in large population cages, maintained at large population size (>10,000 flies) and allowed to interbreed freely for ~50 generations before high sugar (HS) selection began. Flies were maintained on the control diet 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 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*. In parallel, three replicate populations were reared on control diets as is standard for experimental evolution studies, but these are not the focus of this study. Because we were interested in the microbiome transferring adaptive traits to naive hosts, we defined the outbred base population as our control, non-evolved population. We consider the two populations, C and HS, to be two different genotypes. We note that the flies are not traditional inbred lines and still retain genetic diversity, but for the purposes of this study, we consider their evolutionary history to have generated two distinct genotypes. Fly generations over the course of adaptation were discrete, where we allowed flies to lay eggs in bottles, and then bottles seed the next generation in a new cage. Microbial transfer between generations primarily occurred during egg lay as bottles are closed and incubated separately from the previous generation. Microbial transmission was not

guided in any way, which allows for a naturalistic understanding of how the microbiome adapted to the HS diet along with fly adaptation.

Transplanting Acetobacters 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.

To better understand the host-microbiome interaction, we identified the differentially abundant bacteria between C and HS flies through ANCOM (analysis of composition of microbiomes) implemented in QIIME2 (2). For ANCOM, analysis was performed on ASVs (amplicon sequence variants). Only ASVs present in >5 fly samples were included. 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 (3) and for simplicity, we refer to *A. indonesiensis* as C *Acetobacter* and *A. pasteurianus* as HS *Acetobacter*.

To perform the transplant, *Acetobacter* was cultured in liquid MRS media at 25ºC for 2-3 days. Bacterial 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(4). 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. At 8-10 days post eclosion, a subset of 4-6 flies from all replicates were homogenized in 100 µl PBS and plated on MRS media.

Bacterial growth was visually confirmed to be the original *Acetobacter* isolates and sterility for sterile treatments.

Phenotypic effects of the Acetobacter transplant

To measure developmental 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 (5) and survminer (6) 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 with the following model:

$$DT = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + e$$

Where DT = developmental time (pupation), y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), and eas residual error.

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 C and HS oviposition media. Oviposition media was similar to the fly diets, but with yeast extract to make the media transparent for ease of egg counting. We did not find a difference in egg counts between C and HS oviposition media (Supp. Fig. 4, Kruskall-Wallis $X^2 = 2.85$, df=1, p = 0.09), and for the subsequent analyses, pooled together egg counts from both 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(7) with the following model:

$$F = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + d + e$$

Where F = egg count from individual females, y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), where $d \sim N(0, \sigma_o)$ = random effect of oviposition plate (N=29 plates), and e as residual error. We tested for significance of fixed

effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package in R (8). Least square means were used to visualize the interactions with the emmeans package in R (9).

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 (10). Protein serves as a proxy for size and was measured using a Bradford assay (BioRad 5000205). Total triglycerides represent fat stores for flies and were assayed using a colorimetric assay (Sigma TR0100-1KT). Triglycerides were normalized by protein amount (10). Each assay was performed in triplicate in 96 well plates with all 12 treatments in each plate to minimize batch effects. Triplicate values were averaged; values with standard deviation > 0.02 were removed from analysis. The replicates of treatments were assayed across different assay plates. We visualized the triglyceride data by regressing out the effect of the assay plates to show mean-centered values. We modeled the response as:

$$TG = y + \beta_1 G + \beta_2 M + \beta_3 E + \beta_4(G*M) + \beta_5(M*E) + \beta_6(G*E) + \beta_7(G*M*E) + d + e$$

Where TG= triglyceride amount, y = grand mean, G = fly genotype (C or HS), M = *Acetobacter* treatment (C, HS, or sterile), E = diet (C or HS), where $d \sim N(0, \sigma_o)$ = random effect of assay plate (N=14 assay plates), and eas residual error. The three way interaction between fly genotype, Acetobacter, and diet was not significant and was removed from the model. We tested for significance of fixed effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package (8). Least square means were visualized to show interaction effects with the emmeans package in R (9).

Characterizing 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. Adapters and low quality sequences were removed using Trimmomatic (11), resulting in ~53.9 million and 54.6 million reads for the C and HS *Acetobacter*, respectively. Genomes were assembled using SPAdes (12) with --isolate flag. Low coverage contigs (less than 2X) and shorter than 500 bp were removed. Assembly statistics were generated using Quast (13). The genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (14) and compared to identify pathways unique to either C or HS *Acetobacter*. We identified orthologs between the two Acetobacter genomes using OrthoFinder v2.5.2 (15). 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 (16). We confirmed that the unique functions identified using RAST annotation were also unique among the orthologs identified with OrthoFinder.

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 (Sigma) following the manufacturer instructions. Duplicates for a subset of samples (4/40 samples) were run to determine coefficient of variation (CV) in the measurement; because CV was on average ~3.3%, duplicates were averaged and combined with the other single estimates (36/40 samples). All measurements were on the same assay plate. We tested for interactions between Acetobacter and diet in degrading uric acid using ANOVA in R.

We then leveraged whole genome sequencing in the fly to determine if the C and HS fly populations from the original E&R experiment were enriched for their respective C or HS

Acetobacter. Fly genomes were sequenced on the Illumina NovaSeq S4 using 150 bp paired-end reads. For each of the three replicate C populations and three replicate HS populations, 192 flies were sequenced at ~0.5X coverage of the fly genome. Reads were cleaned using Trimmomatic as above, and then mapped to *Wolbachia* (NCBI accession NC_002978.6), C and HS *Acetobacter* genomes using BWA (17), discarding reads that were non-uniquely mapped as well as those mapped to *Wolbachia*. The relative abundance of each *Acetobacter* was then compared between C and HS flies.

Frass Microbiome transplant

To test if the whole community could transfer adaptive potential, we performed a microbiome transplant using frass using a similar design as outlined in Fig. 1. The frass was intended to represent the community more completely than the specific isolates used in the Acetobacter transplant. However, as we discuss in the Supplementary Results, the microbiome transplant was not faithful, and thus, we focused on the results from the Acetobacter transplant.

To transplant the microbiome, we collected fly frass from 300 C and 300 HS age-matched flies (7-10 days old). Donor flies were placed in sterile 50 ml tubes lined with a plastic sheet and were allowed to defecate for ~ 5hrs. After 5 hrs, flies were immobilized at 4ºC and the plastic sheet removed. Fly frass was washed off the plastic using sterile water to make a fecal solution. Because of large differences in the abundance of bacteria between C and HS flies (Supp. Fig. 5), C frass was resuspended in 6 ml, while HS frass was resuspended in 3 ml. The fecal solution was passed through a fine mesh to remove any fly eggs and other miscellaneous fly material. 50 µl of fecal solution (or sterile water for the sterile treatments) was applied to axenic C and HS fly eggs onto sterile C and HS diets (7.5 ml food in 50 ml tube). Axenic eggs were prepared following standard procedures(4). Sterile diets were prepared like the normal maintenance diets, but without acid and autoclaved. Flies were then reared in a biosafety cabinet at 25ºC and 12:12 light:dark cycle through the lifespan of the recipient flies. 5-6

replicates of each treatment were maintained and assessed for microbiome and phenotypic effects.

Assessing fidelity of the frass microbiome transplant

To determine the fidelity of the microbiome transplant, we performed 16S rRNA sequencing on the donor flies, frass, and adult recipient flies. All flies were age matched 7-10 days. Individual flies were sequenced. In brief, DNA was extracted using Zymo Quick DNA extraction kits, and then the 16S V1-V2 region was amplified, pooled, and digested with BstZ17I to deplete Wolbachia reads (18). 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 to cluster into amplicon sequence variants (ASVs) with DADA2 and assign taxonomy with Greengenes classifier trimmed to the 16S rRNA V1-V2 region. Data was imported into phyloseq for visualization and statistical analyses (19).

Flies with fewer than 500 reads were discarded from the analysis. Relative abundance of genera was compared across levels of the transplant (i.e., donor vs. frass vs. recipient). To assess how community structure varied in response to the microbiome treatment, beta-diversity measures were calculated with subsampling down to 500 reads/fly. Microbiomes were visualized with principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity from ASVs. We tested if dispersion differed between the levels of transplant using a permutation test for homogeneity of multivariate dispersion. Because of significant differences in variance between the transplant levels, we focused on differences between groups within the recipients. We performed an ADONIS test to assess how interactions between fly genotype, microbiome, and diet influence the microbiome of recipients.

Effects of the frass microbiome transplant on fly phenotypes

First, we measured development time in the recipients. We counted the number of new pupae every 6 hours during their day time (i.e., 8am, 2pm, 8pm) until all treatments began eclosing. We used the survival (5) and survminer (6) 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.

Second, we measured fecundity over the lifespan of recipients. Five females and five males from each replicate were transferred to new tubes. Flies were allowed to lay eggs for 24 hours and then flipped onto new food. Fecundity was measured at three timepoints over 34 days. Flies were flipped every 3-4 days in between fecundity measurements to avoid crowding and competition with developing larvae. Pupae were counted as a measure for fecundity and then divided by the number of females that survived to each fecundity measurement. We then analysed the effects of time, fly genotype, microbiome, and diet on fecundity with tube as a random effect using lme4 (20). We tested the significance of fixed effects using Type III ANOVA with Kenward-Roger degrees of freedom using the car package in R (8). We tested if including interactions significantly improved the model fit by using a likelihood ratio test.

SUPPLEMENTARY RESULTS

Microbiome transplant results

To test if transferring the microbiome could transfer adaptive phenotypes to non-evolved hosts, we performed a fully reciprocal, host genotype x microbiome x diet transplant experiment. We assessed the fidelity of the microbiome transplant using 16S rRNA sequencing of the donors, frass, and recipients. For both C and HS flies, the frass (which inoculated the diet of the recipients) was composed of fewer bacterial genera than found in donors (Supp. Fig. 2). The HS donor, frass, and recipient were dominated by Acetobacter species.

To compare how bacterial communities were structured across donor, frass, and recipient, we performed ordination analysis on Bray-Curtis distance matrix (Supp. Fig. 2B). The different steps of the transplant have different centroid dispersion (Supp. Table 6, N_{perm}=999, $F_{5,334}$=36.862, p<0.001). Broadly, the dispersion was greatest for recipients overall (Supp. Fig. 2C). For C flies, dispersion in frass microbial communities is greatly reduced compared to both donors and recipients. However, on HS flies, dispersion in frass and donors is similar, but lower than for recipients. The results from the dispersion analysis suggest that a subset of the microbiome was transferred through the frass, and for the recipients, differences in the structure of microbiome communities accumulated through their lifespan.

Both fly genotype and diet shaped the fidelity of the microbiome transplant in the recipients. Using Bray-Curtis distance to examine microbiome differences in recipients, we find that both microbiome and diet strongly impact beta-diversity in the recipients (Supp. Fig. 2D). On the C diet, recipients cluster by microbiome received (Supp. Table 7, $F_{1,143}$=111.45, r^2=0.43, p=0.001), suggesting a successful microbiome transplant. However, on the HS diet, the microbiome transplant breaks down and only had weak, though significant effects (Supp. Table 8, $F_{1,115}$ = 4.35, r^2 = 0.04, p=0.003). Overall, diet explained 14% and microbiome explained 15% of variation in microbial communities, while interactions between fly, microbiome, and diet had significant, but weak effects (r^2 < 8%, Supp. Table 9). For example, C flies that receive the C

microbiome contained *Acetobacter*, *Commensalibacter*, and *Gluconobacter*, whereas HS flies only contained *Acetobacter* (Supp. Fig. 2D). Thus, the HS diet potentially selected for associations between fly and *Acetobacter*. Overall, despite the deviation from the donor microbiomes (Supp. Fig. 2C), the microbiome transplant led to different microbiomes between C and HS recipients, especially on the C diet.

Microbiome transplant shapes life history traits, but depends on the environment

The microbiome significantly shaped developmental time, but depended on fly genotype and diet (Supp. Fig. 6A, concordance = 0.825, log-rank test $p < 0.0001$). On the C diet, both fly genotypes responded to the microbiome in a similar way: sterility slowed development (avg. 136.9 +/-0.41 hrs), while C and HS microbiomes accelerated development similarly (C avg. 119.8 +/- 0.49 hrs, HS avg. 121.2 +/- 0.49 hrs). However, on the HS diet, the microbiome shaped development in different ways. The C microbiome (avg. 135.7 +/- 0.59 hrs) accelerated development compared to the HS microbiome (avg. 142.26 +/- 0.52 hrs) and the sterile treatments were the slowest (avg. 167.8 +/- 0.73 hrs). Two-way interactions between fly genotype, microbiome, and diet significantly shaped development time, but the three-way interaction did not (Supp. Table 10). The model with interactions best explained development data compared to one without interactions (log-likelihood = -21807, $X^2 = 78.02$, df =7, $p < 0.0001$). Overall, the results from development suggest that the microbiome and diet contribute to fly development.

To approximate fitness consequences of the microbiome transplant, we measured fecundity over the lifespan of the recipients (Supp. Fig. 6B). Fecundity declined over time ($F_{2,101.4} = 46.6$, $p < 0.0001$). The decline in fecundity was modified by both the diet and microbiome, but not by fly genotype (Supp. Table 11). Diet reduced fecundity by 26% in the HS diet (C diet avg. 26.3 +/- 2.40, HS diet avg. 19.8 +/- 1.69 pupae/female). The microbiome also affected fecundity, but only with the sterile flies having significantly lower fecundity (sterile avg.

15.4 +/-2.25, C avg. 24.5 +/- 2.42, HS avg. 26.1 +/- 2.56). The interaction between microbiome and time was significant ($F_{4,101.3}$ = 5.84, p = 0.0003), driven by the slower decline in fecundity for the sterile treatments. The model that included this interaction best explained variation in fecundity (log-likelihood = -644.3, X^2 = 22.44, p = 0.0002). The results from the fecundity trials suggest that the presence of a microbiome can modify fly fitness on the HS diet, but little difference was observed between the C and HS microbiomes.

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