

1
2
3 **Gut microbiomes and reproductive isolation in *Drosophila*.**
4

5
6
7 **Philip T. Leftwich^{a,b}, Naomi V. E. Clarke^a, Matthew I. Hutchings^a and Tracey**
8 **Chapman^{a,1}**
9

10
11 ^aSchool of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich
12 NR4 7TJ, UK.
13

14 ^bThe Pirbright Institute, Ash Road, Pirbright, Surrey, GU24 0NF, UK.
15

16
17
18 ¹To whom correspondence should be addressed: tracey.chapman@uea.ac.uk
19

20
21
22 Running title: Experimental evolution of reproductive isolation
23

24
25
26
27 **Keywords:** Ecological adaptation | diet | holobiome | gut microflora | assortative mating
28
29
30
31

32 Experimental studies of the evolution of reproductive isolation (RI) in real time are a
33 powerful way in which to reveal fundamental, early processes that initiate
34 divergence. In a classic ‘speciation experiment’, populations of *Drosophila*
35 *pseudoobscura* were subjected to divergent dietary selection and evolved
36 significant positive assortative mating by diet. More recently, a direct role for the
37 gut microbiome in determining this type of RI in *D. melanogaster* has been
38 proposed. Manipulation of the diet, and hence gut microbiome, was reported to
39 result in immediate assortative mating by diet, which could be eliminated by
40 reducing gut microbes using antibiotics, and recreated by ‘adding back’
41 *Lactobacillus plantarum*. We suggest that the evolutionary significance of this result
42 is unclear. For example, in *D. melanogaster*, the microbiome is reported as flexible
43 and largely environmentally-determined. Therefore, microbiome-mediated RI would
44 be transient and would break down under dietary variation. In the absence of
45 evolutionary co-association or recurrent exposure between host and microbiome,
46 there are no advantages for the gut bacteria or host in effecting RI. To explore these
47 puzzling effects and their mechanisms further, we repeated the tests for RI
48 associated with diet-specific gut microbiomes in *D. melanogaster*. Despite
49 observing replicable differences in the gut microbiomes in flies maintained on
50 different diets, we found no evidence for diet-associated RI, for any role of gut
51 bacteria, or for *L. plantarum* specifically. The results suggest that there is no
52 general role for gut bacteria in driving the evolution of RI in this species, and
53 resolve an evolutionary riddle.

54

55

56 Significance

57

58 The evolutionary significance of assortative mating by diet, mediated by gut bacteria is a
59 puzzle, but it has had a huge impact and has provided a keystone to support increasing
60 interest in the ‘holobiome’. However, in species such as *D. melanogaster* that have flexible
61 gut microbiomes, any reproductive isolation mediated by gut bacteria specific to host diets
62 can only be transient. Here, we replicated and extended tests of this idea. Despite
63 differences in gut microbiomes, we failed to recover previously observed patterns of non-
64 random mating, and found no evidence that mating preferences were associated with diet
65 or gut bacteria. This suggests that the evolutionary importance of gut microbiomes in host
66 divergence needs careful consideration on a case by case basis.

67 Introduction

68 The experimental study of key elements of incipient reproductive isolation (RI) in the
69 laboratory has provided important insights into the underlying evolutionary processes
70 involved (1, 2). Such data show that key components of the initiation of reproductive
71 divergence can be observed and studied in real time (3-9). A classic study of the evolution
72 of incipient RI, arising as a side effect of natural selection to different diets, is Dodd's (10)
73 experiment on replicated populations of *Drosophila pseudoobscura*. In this, 4 populations
74 were each placed onto maltose- or starch-based diets and maintained for a period of
75 approximately 1 year. Mating tests were then conducted within and between replicates
76 maintained on each of the regimes, and significant assortative mating by diet was
77 observed. This has become a text book example of a 'speciation experiment' (1), relevant
78 to understanding speciation by host shifts (11-13).

79 Many aspects of the mechanisms underlying divergence associated with ecological
80 adaptation or host shifts remain unknown (14, 15). Hence, recent studies that have
81 described mechanistic insights into our understanding of how mate choice is associated
82 with dietary divergence have had a wide impact. For example, there has been much
83 interest prompted by a study that suggested a role for gut bacteria in driving assortative
84 mating in *Drosophila melanogaster* (16, 17). Flies placed on different diets were reported
85 to show instant assortative mating by diet. This was abolished following antibiotic
86 treatment of the adults and re-established by bacterial replacement experiments -
87 specifically by add-back of *Lactobacillus plantarum*. The proposed mechanism was via
88 differential effects of gut bacteria on cuticular hydrocarbons that affect attractiveness (16,
89 18-20).

90 These results stimulated intense interest in the wider role of the gut microbiome in
91 mate choice and, potentially, speciation (21-24). They also provided a keystone for the
92 upsurge of interest in the 'holobiome' concept (e.g. (25)), in which the unit of selection is
93 seen as the sum total of the host plus its microbiome. However, the recent interest in gut
94 microbiomes and their potential role in speciation in fruitflies, presents a significant
95 evolutionary puzzle. Selection at the level of the holobiome, or a causal role for
96 microbiomes in host speciation, requires coevolutionary associations, microbiome stability
97 or recurrent exposure between hosts and microbiomes (26). In many situations in which
98 the holobiome is thought important, these conditions may not exist. For example, natural
99 populations of *D. melanogaster* are reported to exhibit fairly flexible, environmentally-
100 acquired gut microbiomes (e.g. (27-33)). Hence the composition of the gut bacterial
101 community seems to depend largely on the ingested diet (32). Strong, and potentially co-

102 associated, evolutionary relationships between *D. melanogaster* hosts and their gut
103 bacteria have not been reported. Hence, a general role for gut bacteria in the maintenance
104 of RI seems unlikely, given the degree of dietary flexibility exhibited by this species. In
105 addition, it is not clear that there can be any benefit to either host or gut bacteria in the
106 absence of any recurrent, potentially coevolved association. Hence the evolutionary
107 significance of this type of association between gut bacteria and host is unclear (26, 34,
108 35).

109 These reasons may explain the lack of consistency in tests that have investigated a
110 general role for gut bacteria in mating associations and mate choice in *D. melanogaster*
111 (16, 18, 36-38). In order to try to resolve these differences, and to investigate the potential
112 mechanisms underlying the role of gut microbes in assortative mating, we repeated the
113 experiments of Sharon et al. 2010 (16) (Table S1). We used two independent wild type
114 strains of *D. melanogaster* (including two strains of Oregon R, the original background
115 tested) for three test populations in total. We first described the gut microbiomes, on the
116 basis that a precondition for assortative mating mediated by diet and / or gut microbiota, is
117 that the microbiomes should be at least partially distinct between flies maintained on
118 different diets. Conversely, if microbiomes are distinct, but assortative mating by diet is
119 absent, then a role for gut bacteria would not be supported. We then conducted mate
120 choice trials following 5, 30 and 35 generations of maintenance on 'CMY' or 'Starch' diets
121 and manipulated gut microbiome composition by using antibiotic and *L. plantarum* add-
122 back treatments. The results revealed that, although there were replicated differences in
123 the gut microbiomes in flies maintained on the different diets, there was no evidence for
124 assortative mating associated with diet, with gut bacteria or with *L. plantarum* in particular.

125

126

127 **Results and Discussion**

128 *Composition of the gut microbiomes of CMY and Starch flies*

129 A principal coordinate analysis (PCoA) showed that the bacterial gut microbiomes of the
130 three populations of flies maintained on the same CMY and Starch media as in (16) for 30
131 generations exhibited significant, tight clustering according to CMY or Starch diet ($F_{1,11}$
132 =1.52, $P < 0.001$, Table 1, Fig. 1A). Independent biological replicates were generally
133 consistent, but more variable among lines on starch (Fig. 1A). Acetobacteraceae
134 comprised over 50% of the microbiome across all populations reared on the CMY diet,
135 with the next most abundant group being the Lactobacillaceae (Fig. 1B). Flies reared on
136 CMY showed a stable abundance of these core microbes across groups and independent

137 biological replicates. There was a log-fold reduction in the abundance of these same
138 groups of bacteria maintained on Starch (Table S2). Instead, species of Rickettsiaceae
139 were found in much greater abundance, particularly in both replicates of the OR2376 line
140 and one replicate of OR25211 (Fig. 1B). This may reflect a reduction in acquisition of
141 environmental microbes in flies reared on Starch (16, 32). The identity and relative
142 abundances of gut microbes from the guts of flies maintained on the different diets were
143 consistent with previous descriptions. Notably, species in the family Enterobacteriaceae
144 were largely absent and, as reported previously, this was associated with a high frequency
145 of Acetobacteraceae (27-29). Overall, the results showed replicated, significant differences
146 in the gut microbiomes of the flies maintained on different diets.

147

148 *Assortative mating by diet*

149 We tested the mating preferences of each of the wild type lines after 5, 30 or 35
150 generations of maintenance on CMY or Starch diets (Fig. 2). There was no significant
151 deviation from random mating across the experiment for two of the lines (OR25211:
152 Mantel-Haenszel (MH) test statistics $\chi^2_1 = 1.35$, $P = 0.24$; Dahomey: MH $\chi^2_1 = 0.35$, $P =$
153 0.55). OR2376 showed a single significant deviation from random mating in one test (MH
154 $\chi^2_1 = 18.15$, $P < 0.001$), but in a diet disassortative direction. There were no significant
155 differences in the number of homogamic vs. heterogamic matings occurring across all
156 three generations of testing (Fig. S1A; Table S3). The tests for reproductive isolation
157 showed a weak signal for reproductive outbreeding (preference for mating with flies of the
158 opposite diet type) at generation 5 (Table S3). However this was not evident at any
159 subsequent time-point (Fig. S1B). Overall, the results from the mating tests on the wild
160 type lines tested following 3 timepoints of maintenance on the different diets showed no
161 evidence for significant assortative mating by diet.

162

163 *Effect of antibiotic treatment and Lactobacillus plantarum 'add-back' on assortative mating* 164 *by diet*

165 To account for the possibility that differences in the composition of microbiomes between
166 this study and (16) could affect mating responses, we also tested whether the elimination
167 of gut bacteria followed by *L. plantarum* add-back could recreate the proposed pattern of
168 assortative mating (16). We first treated the adults with antibiotics, which effectively
169 eliminated gut microbiomes (SI) and then retested the flies for mating preferences at 3
170 timepoints, as above. The results showed a pattern of random assortment of matings with
171 respect to diet of origin and no evidence of sexual isolation (Fig. S2, S3, Table S3). *L.*

172 *plantarum* isolated from fly guts of each strain was then fed back to a subset of antibiotic
173 treated adults from the same strains prior to testing mating preferences (Fig. S4). No
174 significant mating preferences were generated by *L. plantarum* add-back for any of the
175 three lines tested (Fig. 3; MH $\chi^2_1 = 0.004$, $P = 0.95$). There were again no differences in
176 the number of homogamic vs. heterogamic matings and the sexual isolation indices
177 showed no deviation from random mating across any of the three wild type lines (Fig. S5;
178 Table S3). Hence there was no evidence that add-back of *L. plantarum* could create a diet
179 assortative pattern of mating.

180

181 *Statistical power*

182 An analysis of the statistical power of the experiments presented here revealed that the
183 power of our analyses exceeds that necessary to detect the effect sizes previous reported
184 ((16, 17); full results in SI). Hence the null results presented are statistically robust and
185 show that the previous published results (16, 17) were not replicated here.

186

187 **Conclusions**

188 The composition of the gut microbiomes of flies held on the different diets were distinct,
189 which is consistent with the observations of a relatively flexible microbiota in this species
190 (27-29, 31-33). However, the mating preferences of the flies were not associated with
191 these microbiome differences. The results showed no evidence for assortative mating by
192 diet or gut microbiome, no excess of homogamic pairings and no evidence for significant
193 sexual isolation between any of the wild type strains maintained for the short or long-term
194 on different diets that were previously reported to drive significant positive assortative
195 mating (16). The one example of significant sexual isolation was attributable to an excess
196 of disassortative mating by diet (fewer Starch with Starch fly matings than expected) in the
197 OR2376 line at generation 5. The pattern of random mating was not altered by antibiotic
198 treatment, which successfully removed culturable bacteria from the fly guts. The pattern of
199 matings remained random after *L. plantarum* add-back to axenic flies (i.e. there was no
200 excess of matings between the add-back treated flies). Tested across three populations
201 and over multiple generations of maintenance on the different diets, our results contrast
202 with the results of (16, 17) and provide no evidence of either assortative mating by diet or
203 that mating preference is associated with gut microbiota.

204 Our results suggest that any effects of gut microbes in mate choice or assortative
205 mating in this species are highly variable and represent proximate effects, or
206 epiphenomena derived from an as yet unidentified origin. They resolve a puzzle, as they

207 support the assertion that, in this scenario, the different parties (host and microbiome)
208 have limited evolutionary interests in common. Hence, gut bacteria that exhibit flexible and
209 transient associations with their hosts are unlikely to play a general role in host RI. In other
210 species in which there is obligate or recurrent exposure of hosts and their microbiomes or
211 symbionts, such effects can be important (e.g. (39-41)).

212 We found no evidence for assortative mating by diet in any of the three lines tested
213 in any of our experiments. The reason for the difference in comparison to the original Dodd
214 study conducted on *D. pseudoobscura* (10) is unclear. The time scale of the maintenance
215 on the different diets is comparable, so the number of generations available for the
216 emergence of assortative mating was similar. It is possible that the strength of selection
217 exerted by the diets on the respective host microbiota differed. In addition, the nature and
218 transmission pattern of the microbiome of *D. pseudoobscura* has not yet been described
219 and hence a role for gut microbes in mating preferences in this species remains a
220 possibility (e.g. if there were stable, vertical transmission of the gut microbiome). We
221 suggest that an understanding of the co-associations and transmission dynamics of
222 microbiomes within and across hosts is essential in order to (i) understand the ultimate
223 significance of the effects of gut microbes, and (ii) critically evaluate the likely strength of
224 selection at the level of the holobiome. Hence, assessments of the evolutionary
225 importance of the holobiome, and the role of gut microbiomes in host adaptation and
226 divergence, need careful consideration on a case by case basis (26, 35).

227

228

229 **Materials and Methods**

230 *Stocks and cultures*

231 We used two wild type strains - Dahomey and two lines of Oregon-R (the wild type used in
232 (16)) (OR 2376, OR 25211; Bloomington Stock Centre). Dahomey wild-type flies were
233 from a large laboratory population originally collected in the 1970s in Dahomey (Benin)
234 and served as an additional, independently-derived wild type to Oregon-R. All flies were
235 originally maintained on a standard sugar–yeast-agar (SYA) medium (50g sugar, 100g
236 yeast, 15g agar, 30ml Nipagin (10% w/v solution) and 3ml propionic acid per liter).

237

238 *Generation and maintenance of lines on CMY and Starch diets*

239 We placed populations of Dahomey and the two lines of Oregon-R onto the same Starch
240 and CMY diets as used in (16) (CMY: 0.65% agar, 7.6% cornmeal, 7.6% molasses, 5%
241 inactivated brewer's yeast, 0.1% methyl-4-hydroxybenzoate, 0.76% ethanol and 4%

242 propionic acid; Starch: 3% starch, 5% inactivated brewer's yeast, 1% agar, 0.5% propionic
243 acid). We then tested for assortative mating by diet after 5, 30 and 35 generations of
244 rearing on these diets, with the lines maintained in bottle culture with discrete generations.
245 All experiments and culturing were conducted at 25°C, 50% relative humidity on a 12:12
246 light: dark photoperiod. At emergence for each new generation, a group of 200 females
247 and 200 males were placed into a new bottle containing 70ml of the appropriate diet.
248 Adults were allowed to lay eggs for 48-72h before being removed in order to maintain
249 discrete generations. Each of the CMY and Starch lines were maintained in two
250 independent lines of bottle culture.

251

252 *Composition of the gut microbiomes of CMY and Starch flies, using 16S rDNA sequencing*
253 We examined whether the composition of the microbiomes of the Starch and CMY flies
254 differed, using 16S rDNA sequencing. We compared samples at generation 30 from each
255 of the three lines of *Drosophila* on both CMY and Starch media by using Illumina
256 sequencing of 16S rRNA genes. We first extracted the DNA by collecting n=5 adults per
257 sample, followed by surface sterilization. The extracted gut tissue was homogenized by
258 grinding with plastic pestles inside 2ml microcentrifuge tubes and using three freeze/thaw
259 cycles in liquid nitrogen. Samples were then incubated with 180µl lysis buffer (20m M Tris-
260 HCl, pH 8.0, 2mM sodium EDTA, 1.2% Triton-X 100, 20mg/ml lysozyme) and incubated at
261 37°C for 90 minutes, with brief bead beating at 45 minutes in a bead beater with 0.1mm
262 glass beads (Fisher UK) for 3 minutes. 20µl extraction buffer (2 M Tris-HCl, pH 8.5, 2.5 M
263 NaCl, 0.25M EDTA, 5% w/v SDS) and 15µl of Proteinase K (20mg/ml) were added and
264 samples were incubated overnight at 55°C. After this lysis, 30µl of 3M sodium acetate was
265 added, and the samples allowed to sit for 30 minutes, inverting tubes every 10 minutes for
266 mixing. The samples were then centrifuged at 11,000g for 10 mins. 300 µl of 100% ice-
267 cold isopropanol was added to each sample and incubated at room temperature for 30
268 mins, followed by centrifuging at 18000g for 30 mins. The supernatant was then discarded
269 and the pellet washed in 70% ice cold EtOH, before air drying and resuspension in 20 µl
270 10 mM Tris-Cl, pH 8.5.

271 Approximately 100ng of DNA was used per sample as template for amplification of
272 the 16s rDNA gene. Bacterial universal primers 515F and 806R were used to amplify a
273 291bp fragment (515F: 5' -GTG CCA GCM GCC GCG GTA A-3', 806R: 5' - GGA CTA
274 CHV GGG TWT CTA AT-3), the reverse PCR primer was barcoded with a 12-base error-
275 correcting Golay code to facilitate multiplexing (42). PCR conditions were: initial
276 denaturation at 98°C for 3 mins, 35 cycles at 98°C for 30 secs, 60°C for 30 secs and 72°C

277 for 60 secs; final extension for 10 mins at 72°C. Products were pooled at equimolar ratios,
278 and the pool cleaned with an Agencourt AMPure XP kit (Beckman Coulter). Sequencing
279 was conducted on the Illumina MiSeq 2 × 250 platform (Earlham Institute provider)
280 according to protocols described by (42).

281 Sample reads were assembled with mothur v1.32 (43). Chimeric sequences were
282 removed using the USEARCH software based on the UCHIME algorithm (44). Operational
283 Taxonomic Units (OTUs) were selected using *de novo* OTU picking protocols with a 97%
284 similarity threshold. Taxonomy assignment of OTUs was performed by comparing
285 sequences to the Silva database. PERMANOVA with 1000 permutations was used to first
286 identify whether differences in OTU abundances between samples were described most
287 accurately by diet or genotype (45). Linear discriminant analysis coupled with effect size
288 (LEfSe) was performed to identify the bacterial taxa differentially represented between the
289 two diets at Family or higher taxonomic levels (46). Jack-knifed beta diversity of
290 unweighted Unifrac distances was calculated with 10x subsampling, and these distances
291 were visualized by Principal Coordinate Analysis (PCoA). The R packages Phyloseq and
292 ggplot2 were used for data analysis and visualizing the results, respectively (47, 48).

293

294 *Testing for assortative mating by diet*

295 To test for significant assortative mating by diet, we examined the different wild type
296 strains following 5, 30 and 35 generations of maintenance on CMY or Starch diets.
297 Assortative mating tests were performed as in (16) using quartets of flies comprising 1
298 male and 1 female from the CMY and Starch diets. As noted in the correction to the 2010
299 study (17), only the first mating in any such quartet represents a 'choice' (the second
300 mating being constrained because only one female and male remain). Hence we used the
301 identity of the first pair to mate as the data for tests of assortative mating. For each mating
302 assay experiment, each population was grown for one generation on CMY medium as in
303 (16) and larvae were raised at a standard density of 100 individuals per vial, to both
304 remove any proximate effects of nutrition on mating preference and minimize
305 environmentally-determined differences in body size that might have impacted upon
306 mating success. At eclosion, flies were collected and the sexes separated using light CO₂
307 anesthesia. Virgin males and females were stored 10 per vial on CMY medium until 1 day
308 prior to mating. All flies were then anaesthetized using light CO₂ anesthesia. Half of the
309 vials from each treatment were then selected at random and the flies within them given a
310 small wing clip for identification.

311 For the mating tests, quartets of flies were aspirated into vials, a single male and
312 female from the CMY treatment and a single male and female from the Starch treatment.
313 Wing clipping was used to identify the males and females during the experiment, and was
314 rotated in a factorial design (i.e. in half of all tests the CMY males and females were
315 clipped and in half the Starch were clipped). Hence, the clipping itself was distributed
316 equally across all tests, diet treatments and sexes such that it could not introduce any
317 systematic confound. The setup of the mating quartets and the observations of the
318 matings were carried out using a team of researchers who were blind to strain identity. On
319 the day of the mating tests the two males were placed in each mating vial (empty vials
320 each containing a moist filter paper strip) followed directly afterwards by the two females.
321 The identity of the first pair to mate was then recorded according to the identity of the wing
322 clips of the mating pairs. The clip patterns were decoded after the completion of the mating
323 tests into group / treatment identity. Mating tests were conducted for 5h from the start of
324 lights on. Pairs were given 2h to mate and those that did not mate within this time were
325 discarded. Any vials that contained individuals that died or were immobile during the
326 experiment were discarded. Full sample sizes of initial test numbers, number of matings
327 and non-matings are detailed in [Table S3](#).

328

329 *Effect of microbiome removal and Lactobacillus plantarum 'add-back' on assortative*
330 *mating by diet*

331 In order to rule out the effects of variation in gut microbiome composition, we also tested
332 the effect of gut microbiome removal and *L. plantarum* add-back on assortative mating by
333 diet (SI). We treated the adults prior to the mating tests with a cocktail of antibiotics for 48h
334 (50 µg/mL tetracycline, 200 µg/mL rifampicin, 100 µg/mL streptomycin) to remove their gut
335 bacteria. The effectiveness of this was verified as described in the SI. The mating tests on
336 the microbiome-removed flies were then conducted at generations 5, 30 and 35 and *L.*
337 *plantarum* add-back experiments at generation 38. For the add-back experiment, we
338 isolated *L. plantarum* from each of the three lines (identified to species level by BLAST
339 matching to *L. plantarum*) and tested whether we could generate assortative mating
340 artificially, in the manner proposed (16), by exposing half of the flies from within the same
341 CMY or Starch diet background to \pm *L. plantarum*, and testing for assortative mating as
342 before (for full methods, see SI).

343

344 *Statistical analysis of assortative mating*

345 We used the Mantel-Haenszel test for repeated tests of independence in order to
346 determine whether repeated observations of mating pairs showed any deviation from that
347 of random mating. In addition, the number of observed and total possible pairings for each
348 pair type was calculated for each replicate. This was analyzed using JMATING v.1.0 (49)
349 to calculate the IPSI a joint isolation index. IPSI varies from -1 to +1, with +1 being total
350 assortative mating, and -1 dissassortative mating. Hence, a value of 0 denotes random
351 mating. Following (50) we used IPSI to describe reproductive isolation at each of the three
352 generational time points. Significance of the coefficient was calculated as the bootstrap
353 probability of rejecting the null hypothesis of random distribution after 10,000 iterations of
354 resampling. All bootstrapping was conducted in JMATING, all other statistical analyses
355 were conducted in R v3.1.1 (51). The statistical power of our analyses in comparison to
356 the previous study (16, 17) was then analyzed (for full details, see SI).

357

358

359

360 **ACKNOWLEDGEMENTS**

361 We thank Will Nash, Michael Connelly, Rachel Urquhart, Lucy Friend, Damian Smith,
362 Elizabeth Duxbury, Janet Mason, Wayne Rostant, Michael Bolton and Irina Mohorianu for
363 help with analyses and experiments and the reviewers for insightful comments and the
364 BBSRC (BB/K000489/1) for funding (research grant to TC, PTL and MIH). This work was
365 funded by the Biotechnology and Biological Sciences Research Council Research Grant
366 BB/K000489/1 (to T.C., P.T.L., and M.I.H.).

367

368 **Data Archiving**

369 The individual raw 16S sequences are available in the NCBI sequence read archive (SRA) under
370 BioProject: [PRJNA415376](#).

371

372 All chimera-checked 16S rRNA gene sequences of representative OTUs, the de-replicated, quality
373 filtered Illumina MiSeq data set file, metadata mapping file and the final OTU distribution table,
374 along with raw sanger sequencing reads of *L. plantarum* are deposited at:
375 10.6084/m9.figshare.5469316.

376

377 The chimera-checked 16S consensus sequence for *L. plantarum* used for bacterial add-back has
378 been deposited in the NCBI GenBank with accession MG066537.

379

380 **Author Contributions**

381 PTL, MIH and TC conceived the study, PTL, NVEC and TC conducted the experiments,
382 PTL analyzed the data and PTL and TC wrote the paper, with input from NVEC and MIH.

383

384

385

386 **References**

- 387 1. Coyne JA & Orr HA (2004) *Speciation* (Sinauer Associates Sunderland, MA).
- 388 2. Nosil P (2012) *Ecological Speciation* (Oxford University Press, Oxford).
- 389 3. Kiliyas G, Alahiotis SN, Pelecanos M (1980) A multifactorial genetic investigation of
390 speciation theory using *Drosophila melanogaster*. *Evolution* 34:730-737.
- 391 4. Markow TA (1981) Mating preferences are not predictive of the direction of
392 evolution in experimental populations of *Drosophila*. *Science* 13:1405-1407.
- 393 5. Nadeau NJ, et al. (2012) Genomic islands of divergence in hybridizing *Heliconius*
394 butterflies identified by large-scale targeted sequencing. *Phil Trans Roy Soc*
395 367:343-353.
- 396 6. Feder JL, Egan SP, Nosil P (2012) The genomics of speciation-with-gene-flow.
397 *Trends Genet* 28:342-350.
- 398 7. Etges WJ, De Oliveira CC, Noor MA, & Ritchie MG (2010) Genetics of incipient
399 speciation in *Drosophila mojavensis*. III. Life-history divergence in allopatry and
400 reproductive isolation. *Evolution* 64:3549-3569.
- 401 8. Martin SH, et al. (2013) Genome-wide evidence for speciation with gene flow in
402 *Heliconius* butterflies. *Genome Res* 23:1817-1828.
- 403 9. Seehausen O, et al. (2014) Genomics and the origin of species. *Nat Rev Genet*
404 15:176-192.
- 405 10. Dodd DMB (1989) Reproductive isolation as a consequence of adaptive divergence
406 in *Drosophila pseudoobscura*. *Evolution* 43:1308-1311.
- 407 11. Feder JL, et al. (2003) Allopatric genetic origins for sympatric host-plant shifts and
408 race formation in *Rhagoletis*. *Proc Natl Acad Sci USA* 100:10314-10319.
- 409 12. Via S, Bouck AC, Skillman S (2000) Reproductive isolation between divergent races
410 of pea aphids on two hosts. II. Selection against migrants and hybrids in the
411 parental environments. *Evolution* 54:1626-1637.
- 412 13. Nosil P (2007) Divergent host plant adaptation and reproductive isolation between
413 ecotypes of *Timema cristinae* walking sticks. *Am Nat* 169:151-162.
- 414 14. Butlin R, et al. (2012) What do we need to know about speciation? *Trends Ecol Evol*
415 27:27-39.
- 416 15. Janz N, Nylin SI (2008) In: *Specialization, speciation, and radiation*. The
417 evolutionary biology of herbivorous insects. Ed K. Tilmon (Univ California Press,
418 Berkeley) pp203-215.
- 419 16. Sharon G, et al. (2010) Commensal bacteria play a role in mating preference of
420 *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 107:20051-20056.
- 421 17. Sharon G, et al. (2013) Correction for "Commensal bacteria play a role in mating
422 preference of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 110:4853.
- 423 18. Pavković-Lučić S (2009) Is there ethological isolation among *Drosophila*
424 *melanogaster* strains reared for more than 35 generations on different food? *Arch*
425 *Biol Sci* 61:105-112.
- 426 19. Pavković-Lučić S, et al. (2016) Does my diet affect my perfume? Identification and
427 quantification of cuticular compounds in five *Drosophila melanogaster* strains
428 maintained over 300 generations on different diets. *Chem Biodivers* 13:224-232.
- 429 20. Trajković J, Miličić D, Savić T, Pavković-Lučić S (2017) Sexual selection, sexual
430 isolation and pheromones in *Drosophila melanogaster* strains after long-term
431 maintaining on different diets. *Behav Process* 140:81-86.
- 432 21. Ringo J, Sharon G, Segal D (2011) Bacteria-induced sexual isolation in *Drosophila*.
433 *Fly* 5:310-315.
- 434 22. Brucker RM, Bordenstein SR (2012) Speciation by symbiosis. *Trends Ecol Evol*
435 27:4430451.
- 436 23. Lizé A, McKay R, Lewis Z (2014) Kin recognition in *Drosophila*: the importance of
437 ecology and gut microbiota. *ISME J* 8:469-477.

- 438 24. Shropshire JD, Bordenstein SR (2016) Speciation by symbiosis: the microbiome
439 and behavior. *mBio* 7:e01785-01715.
- 440 25. Sharon G, Segal D, Zilber-Rosenberg I, Rosenberg E (2011) Symbiotic bacteria are
441 responsible for diet-induced mating preference in *Drosophila melanogaster*,
442 providing support for the hologenome concept of evolution. *Gut Microbes* 2:190-
443 192.
- 444 26. Moran NA, Sloan DB (2015) The hologenome concept: helpful or hollow? *PLoS Biol*
445 13:e1002311.
- 446 27. Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A (2011) Bacterial
447 communities of diverse *Drosophila* species: ecological context of a host–microbe
448 model system. *PLoS Genetics* 7:e1002272.
- 449 28. Wong CNA, Ng P, Douglas AE (2011) Low-diversity bacterial community in the gut
450 of the fruitfly *Drosophila melanogaster*. *Env Microbiol* 13:1889-1900.
- 451 29. Wong AC, Chaston JM, Douglas AE (2013) The inconstant gut microbiota of
452 *Drosophila* species revealed by 16S rRNA gene analysis. *ISME J* 7:1922-1932.
- 453 30. Staubach F, Baines JF, Künzel S, Bik EM, Petrov DA (2013) Host species and
454 environmental effects on bacterial communities associated with *Drosophila* in the
455 laboratory and in the natural environment. *PLoS One* 8:e70749.
- 456 31. Wong ACN, Dobson AJ, Douglas AE (2014) Gut microbiota dictates the metabolic
457 response of *Drosophila* to diet. *J Exp Biol* 217:1894-1901.
- 458 32. Blum JE, Fischer CN, Miles J, Handelsman J (2013) Frequent replenishment
459 sustains the beneficial microbiome of *Drosophila melanogaster*. *mBio* 4:e00860–
460 00813.
- 461 33. Early AM, Shanmugarajah N, Buchon N, Clark AG (2017) *Drosophila* genotype
462 influences commensal bacterial levels. *PLoS One* 12:e0170332.
- 463 34. Chandler JA, Turelli M (2014) Comment on "The hologenomic basis of speciation:
464 Gut bacteria cause hybrid lethality in the genus *Nasonia*". *Science* 345:1011.
- 465 35. Douglas AE, Werren JH (2016) Holes in the hologenome: why host-microbe
466 symbioses are not holobionts. *mBio* 7:e02099-02015.
- 467 36. Koukou K, et al. (2006) Influence of antibiotic treatment and *Wolbachia* curing on
468 sexual isolation among *Drosophila melanogaster* cage populations. *Evolution*
469 60:87-96.
- 470 37. Najarro MA, Sumethasorn M, Lamoureux A, Turner TL (2015) Choosing mates
471 based on the diet of your ancestors: replication of non-genetic assortative mating in
472 *Drosophila melanogaster*. *PeerJ* 3:e1173.
- 473 38. Arbuthnott D, Levin TC, Promislow DEL (2016) The impacts of *Wolbachia* and the
474 microbiome on mate choice in *Drosophila melanogaster*. *J Evol Biol* 29:461-468.
- 475 39. Douglas AE (1998) Nutritional interactions in insect-microbial symbioses: aphids
476 and their symbiotic bacteria *Buchnera*. *Ann Rev Entomol* 43:17-37.
- 477 40. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of
478 heritable bacterial symbionts. *Ann Rev Genet*, 42:165-190.
- 479 41. Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of
480 invertebrate biology. *Nat Rev Microbiol* 6:741-751.
- 481 42. Caporaso JG, et al. (2011) Global patterns of 16S rRNA diversity at a depth of
482 millions of sequences per sample. *Proc Natl Acad Sci USA* 108:4516-4522.
- 483 43. Schloss PD, et al. (2009) Introducing mothur: open-source, platform-independent,
484 community-supported software for describing and comparing microbial
485 communities. *Appl Environ Microbiol* 75:7537-7541.
- 486 44. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves
487 sensitivity and speed of chimera detection. *Bioinform* 27:2194-2200.
- 488 45. Kelly BJ, et al. (2015) Power and sample-size estimation for microbiome studies
489 using pairwise distances and PERMANOVA. *Bioinform* btv183.

- 490 46. Segata N, et al. (2011) Metagenomic biomarker discovery and explanation.
491 *Genome Biol* 12:R60.
- 492 47. Wickham H (2011) ggplot2. *Wiley Interdisciplin Rev: Comp Stat* 3:180-185.
- 493 48. McMurdie PJ, Holmes S (2012) Phyloseq: a bioconductor package for handling and
494 analysis of high-throughput phylogenetic sequence data. *Pac Symp Biocomput NIH*
495 *Public Access*:235.
- 496 49. Carvajal-Rodriguez A, Rolan-Alvarez E (2006) JMATING: a software for the
497 analysis of sexual selection and sexual isolation effects from mating frequency data.
498 *BMC Evol Biol* 6:40.
- 499 50. Coyne JA, Elwyn S, Rolán-Alvarez E (2005) Impact of experimental design on
500 *Drosophila* sexual isolation studies: direct effects and comparison to field
501 hybridization data. *Evolution* 59:2588-2601.
- 502 51. R Development Core Team (2015) R: A Language and Environment for Statistical
503 Computing, ver 3.3.2. In. *R Foundation for Statistical Computing* (Vienna, Austria).
504
505

506 **Figure Legends**

507

508 **Fig. 1. Gut microbiome composition of CMY and Starch lines at generation**

509 **30. A:** Principal Coordinate Analysis (PCoA) of the gut bacterial community of the wild
510 type strains maintained on the CMY or Starch diets. Each symbol represents a single
511 biological replicate comprised of a pool of five individuals, there were two independent
512 biological replicates for each treatment. Wild type strains are indicated by the different
513 colors, circles and triangles the CMY and Starch diets, respectively. **B:** Stacked barplot of
514 community composition and distribution of dominant bacterial taxa (>5% abundance,
515 collapsed to Family level) for the gut microbiomes in **A**.

516

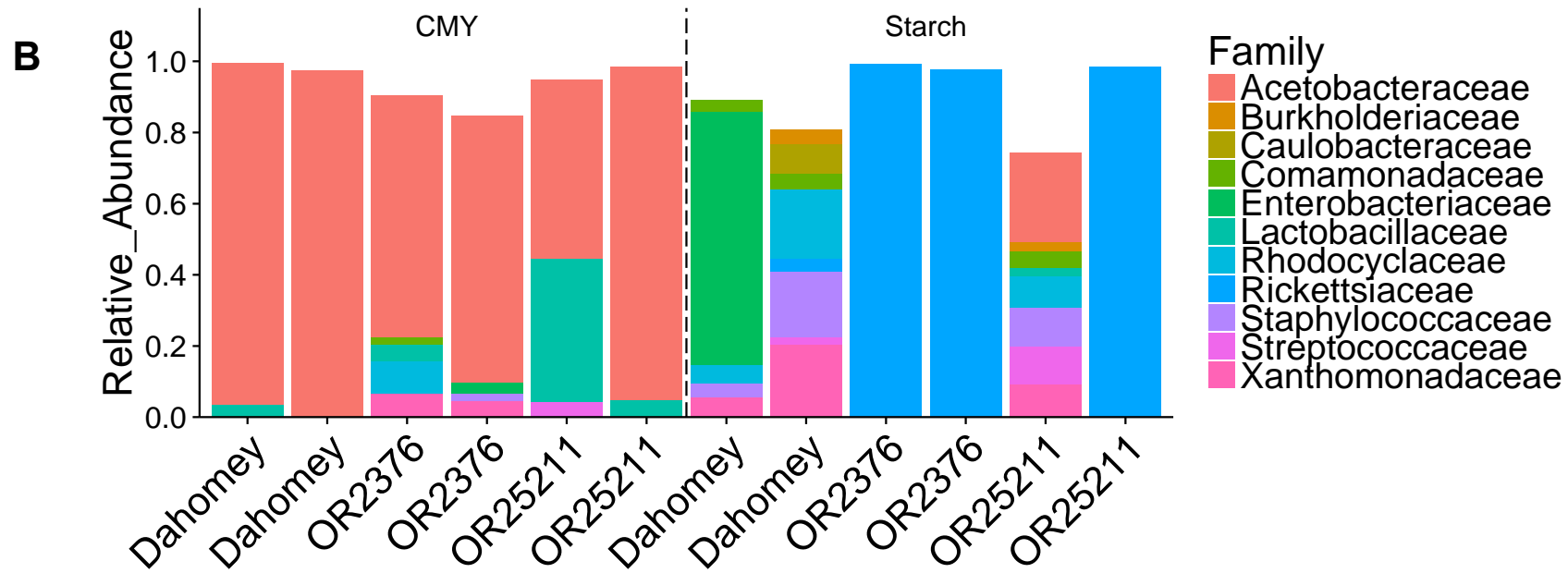
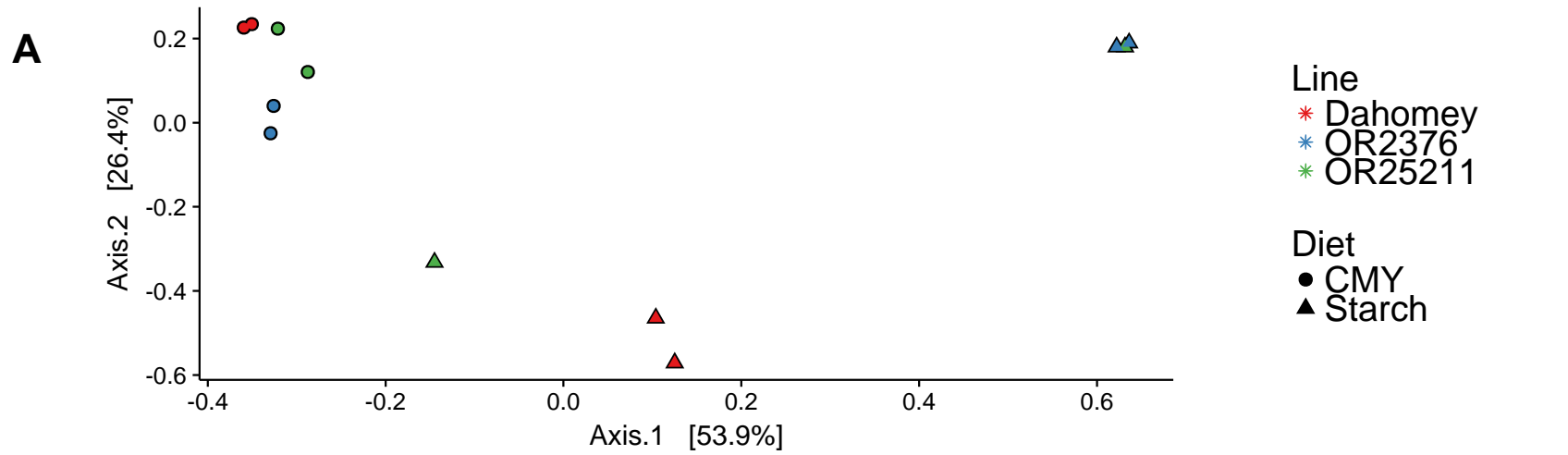
517 **Fig. 2. Number of matings between wild type lines maintained on CMY or**

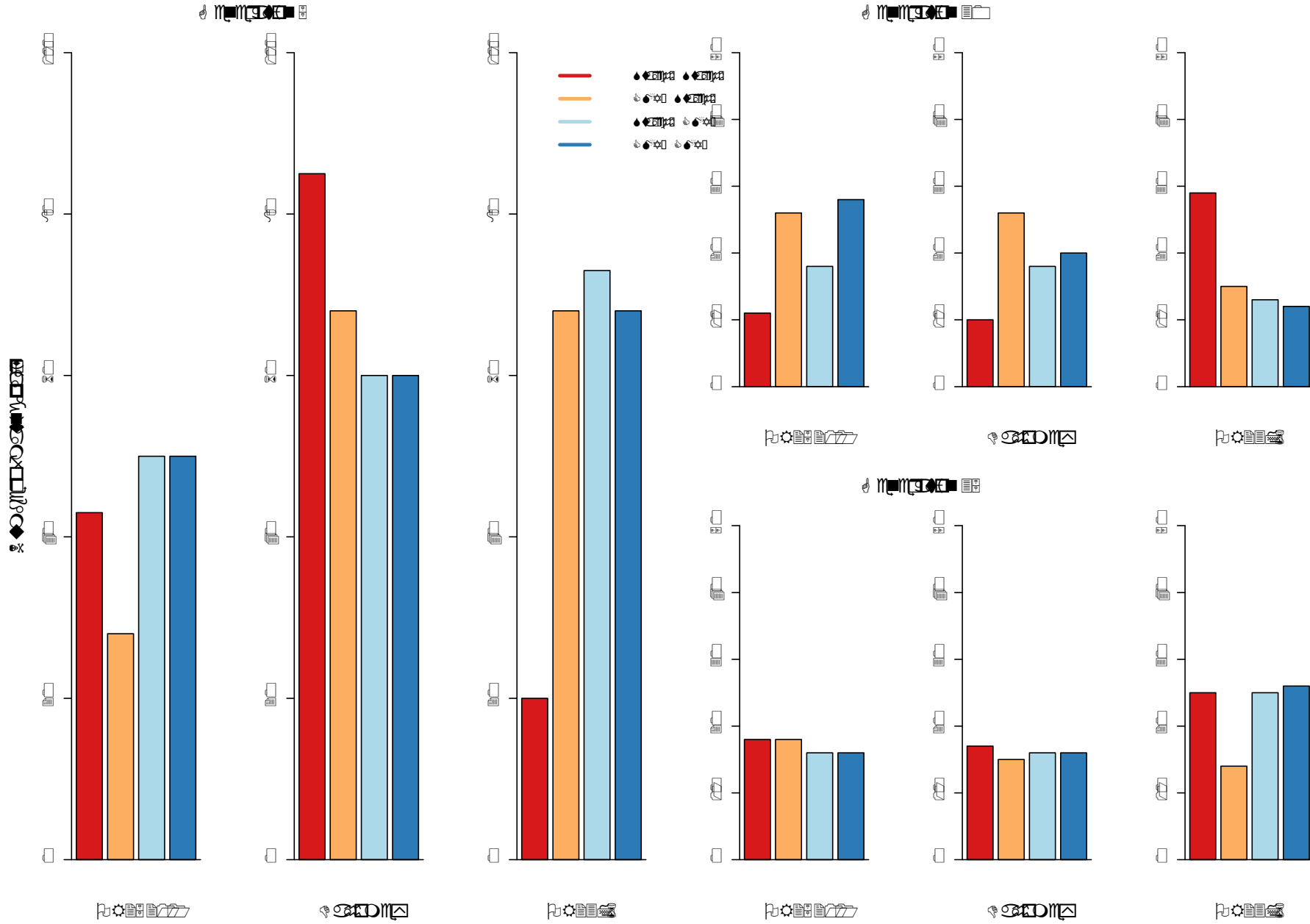
518 **Starch diets.** Barplots represent the number of mating pairs formed in quartet mating
519 tests between CMY and Starch diet lines derived from each wild type population. Matings
520 were scored at generation 5, 30 and 35 of selection of the lines on the two diets. Prior to
521 mating tests, all flies were reared for one generation on the CMY diet (as in (16)).

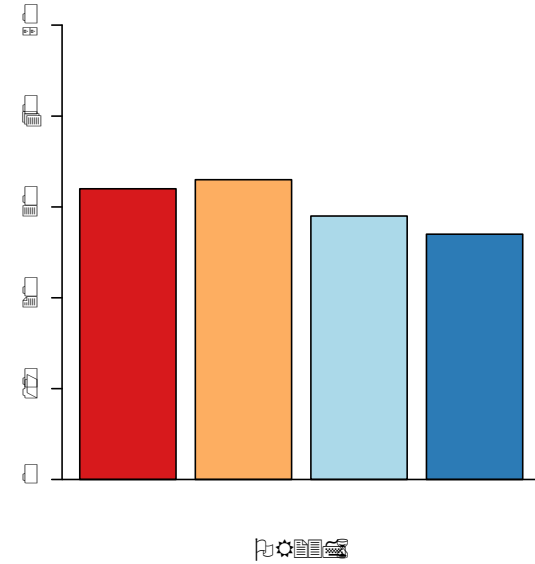
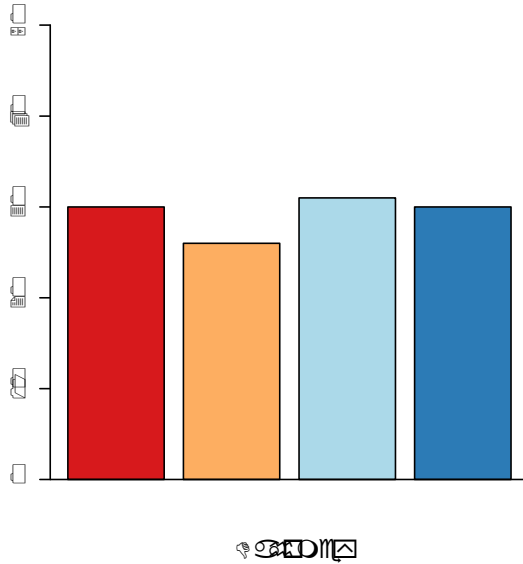
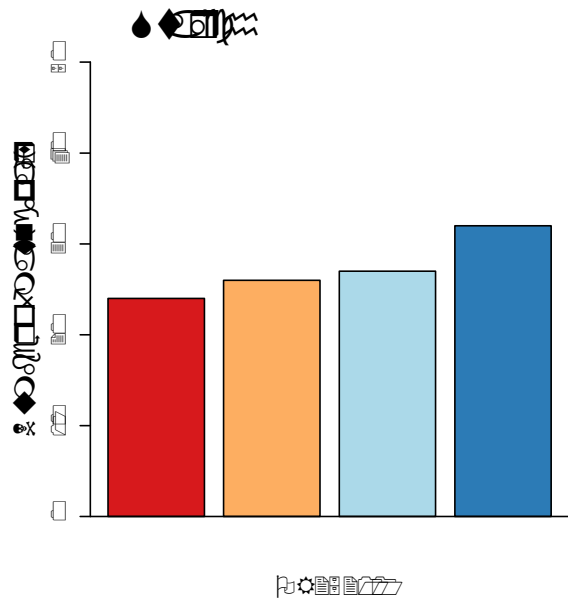
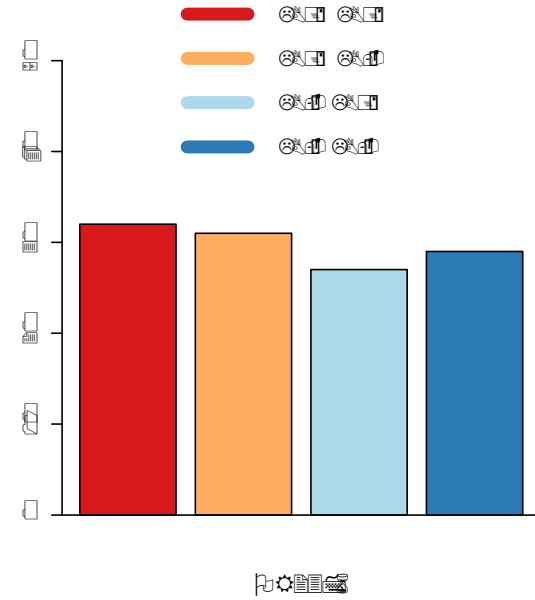
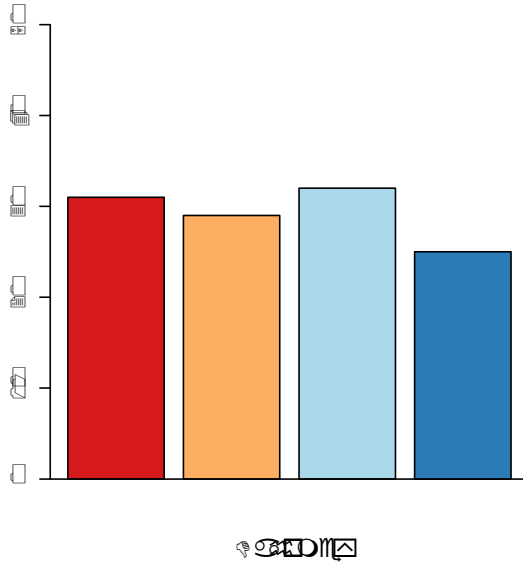
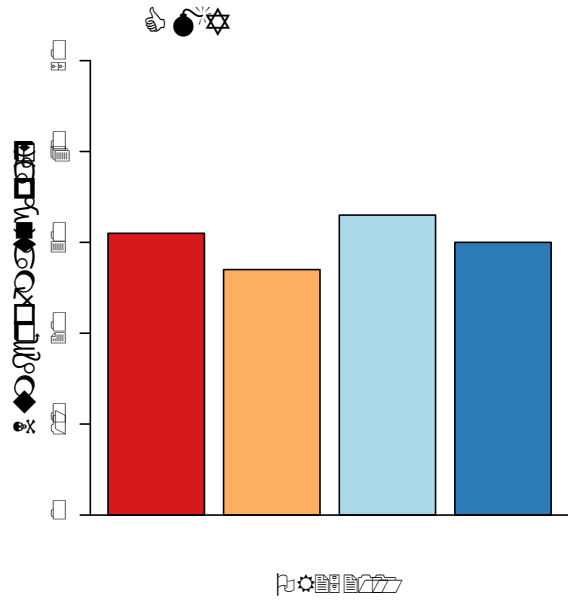
522

523 **Fig. 3. Number of matings between wild type lines maintained on CMY or**

524 **Starch diets following *L. plantarum* 'add-back'.** Barplots represent the number of
525 mating pairs formed in quartet mating tests between CMY and Starch diet lines
526 (generation 38) derived from each wild type population subjected to an antibiotic cocktail
527 to eliminate gut bacteria (as in [Fig. S2](#)) then to *L. plantarum* 'add-back' (LB+), versus 'non
528 add-back' axenic control (LB-).







532 **Table 1.** Results of permutational multivariate analysis of variance (PERMANOVA) analysis of gut microbiome composition between ea
 533 ch of the wild type lines maintained on CMY or Starch diets for 30 generations.

534
 535

Variable	DF	Sum of Squares	Mean Squares	<i>F</i>	<i>R</i> ²	<i>P</i>
Line	2	0.36	0.18	1.1	0.102	0.38
Diet	1	1.52	1.52	9.34	0.43	<0.001
Line * Diet	2	0.64	0.32	1.98	0.18	0.12
Residuals	6	0.97	0.16		0.28	
Total	11	3.5			1	

536

537 There was a highly significant difference in gut microbiome composition in CMY versus Starch diets. Number of permutations was 999,
 538 with terms added sequentially (first to last). *R*² = coefficient of determination.

539