Gut microbiomes and reproductive isolation in *Drosophila*.

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

Significance

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

Introduction

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

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

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

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

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

Results and Discussion

Composition of the gut microbiomes of CMY and Starch flies

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

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

Assortative mating by diet

We tested the mating preferences of each of the wild type lines after 5, 30 or 35 generations of maintenance on CMY or Starch diets (Fig. 2). There was no significant deviation from random mating across the experiment for two of the lines (OR25211: Mantel-Haenszel (MH) test statistics χ^2_1 = 1.35, P = 0.24; Dahomey: MH χ^2_1 = 0.35, P = 0.55). OR2376 showed a single significant deviation from random mating in one test (MH χ^2_1 = 18.15, P < 0.001), but in a diet disassortative direction. There were no significant differences in the number of homogamic vs. heterogamic matings occurring across all three generations of testing (Fig. S1*A*; Table S3). The tests for reproductive isolation showed a weak signal for reproductive outbreeding (preference for mating with flies of the opposite diet type) at generation 5 (Table S3). However this was not evident at any subsequent time-point (Fig. S1*B*). Overall, the results from the mating tests on the wild type lines tested following 3 timepoints of maintenance on the different diets showed no evidence for significant assortative mating by diet.

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

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

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

Statistical power

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

Conclusions

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

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

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

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

Materials and Methods

Stocks and cultures

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

Generation and maintenance of lines on CMY and Starch diets

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

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

Composition of the gut microbiomes of CMY and Starch flies, using 16S rDNA sequencing

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

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

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

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

Testing for assortative mating by diet

To test for significant assortative mating by diet, we examined the different wild type strains following 5, 30 and 35 generations of maintenance on CMY or Starch diets. Assortative mating tests were performed as in (16) using quartets of flies comprising 1 male and 1 female from the CMY and Starch diets. As noted in the correction to the 2010 study (17), only the first mating in any such quartet represents a ‘choice’ (the second mating being constrained because only one female and male remain). Hence we used the identity of the first pair to mate as the data for tests of assortative mating. For each mating assay experiment, each population was grown for one generation on CMY medium as in (16) and larvae were raised at a standard density of 100 individuals per vial, to both remove any proximate effects of nutrition on mating preference and minimize environmentally-determined differences in body size that might have impacted upon mating success. At eclosion, flies were collected and the sexes separated using light CO_2 anesthesia. Virgin males and females were stored 10 per vial on CMY medium until 1 day prior to mating. All flies were then anaesthetized using light CO_2 anesthesia. Half of the vials from each treatment were then selected at random and the flies within them given a small wing clip for identification.

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

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

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

Statistical analysis of assortative mating

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

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Data Archiving

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

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

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

Author Contributions

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

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. Kilias 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.

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

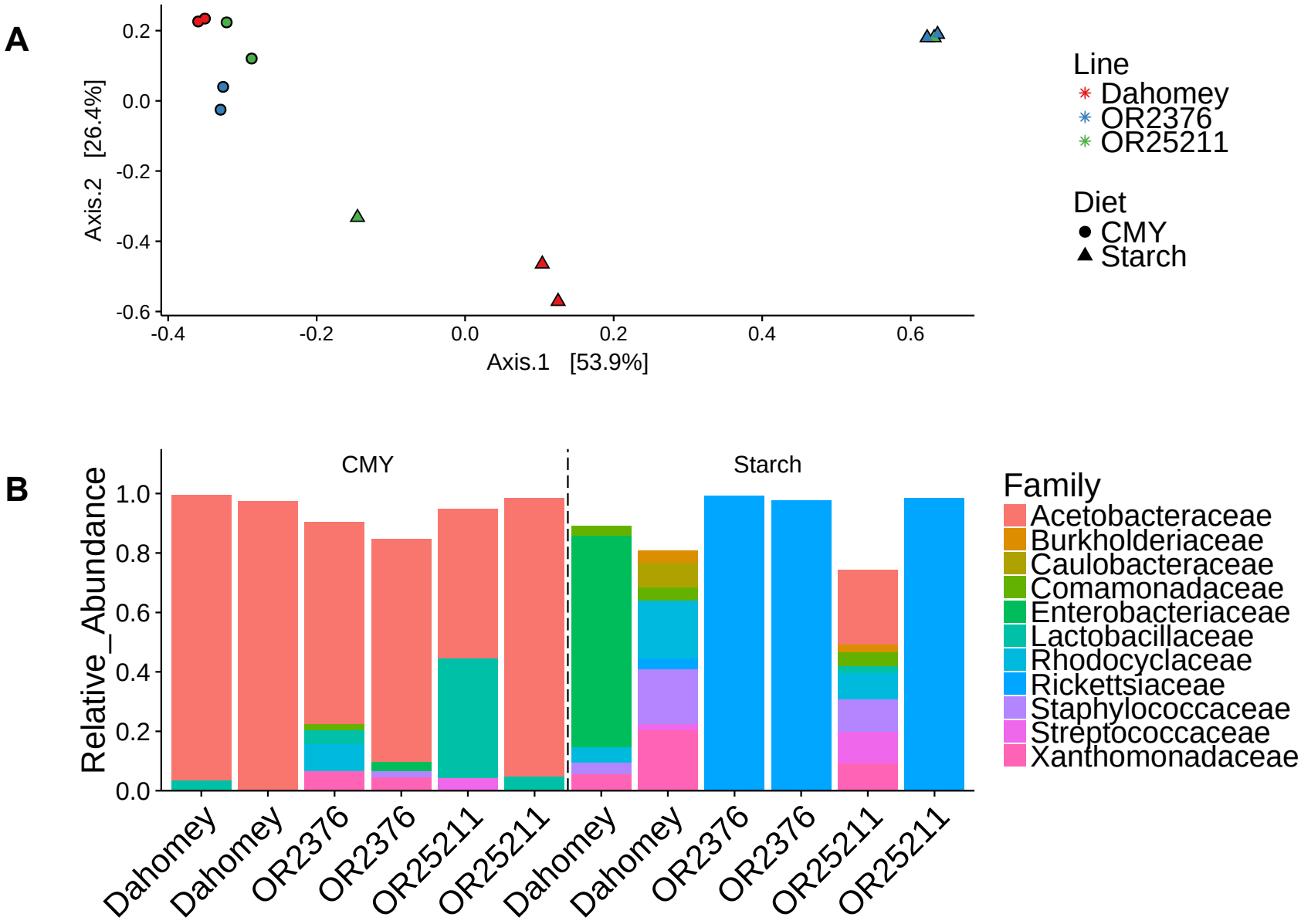
46. Segata N, et al. (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* 12:R60.
47. Wickham H (2011) ggplot2. *Wiley Interdisciplin Rev: Comp Stat* 3:180-185.
48. McMurdie PJ, Holmes S (2012) Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic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

Figure Legends

Fig. 1. Gut microbiome composition of CMY and Starch lines at generation 30. *A*: Principal Coordinate Analysis (PCoA) of the gut bacterial community of the wild type strains maintained on the CMY or Starch diets. Each symbol represents a single biological replicate comprised of a pool of five individuals, there were two independent biological replicates for each treatment. Wild type strains are indicated by the different colors, circles and triangles the CMY and Starch diets, respectively**.** ***B****:* Stacked barplot of community composition and distribution of dominant bacterial taxa (>5% abundance, collapsed to Family level) for the gut microbiomes in ***A***.

Fig. 2. Number of matings between wild type lines maintained on CMY or Starch diets. Barplots represent the number of mating pairs formed in quartet mating tests between CMY and Starch diet lines derived from each wild type population. Matings were scored at generation 5, 30 and 35 of selection of the lines on the two diets. Prior to mating tests, all flies were reared for one generation on the CMY diet (as in (16)).

Fig. 3. Number of matings between wild type lines maintained on CMY or Starch diets following *L. plantarum* 'add-back'. Barplots represent the number of mating pairs formed in quartet mating tests between CMY and Starch diet lines (generation 38) derived from each wild type population subjected to an antibiotic cocktail to eliminate gut bacteria (as in Fig. S2) then to *L. plantarum* 'add-back' (LB+), versus 'non add-back' axenic control (LB-).


A
0.2
0.0
-0.2
-0.4
-0.6
Axis.2 [26.4%]
-0.4
-0.2
0.0
0.2
0.4
0.6
Axis.1 [53.9%]
Line
Dahomey
OR2376
OR25211
Diet
CMY
Starch
B
CMY
Starch
Relative_Abundance
1.0
0.8
0.6
0.4
0.2
0.0
Dahomey
Dahomey
OR2376
OR2376
OR25211
OR25211
Dahomey
Dahomey
OR2376
OR2376
OR25211
OR25211
Family
Acetobacteraceae
Burkholderiaceae
Caulobacteraceae
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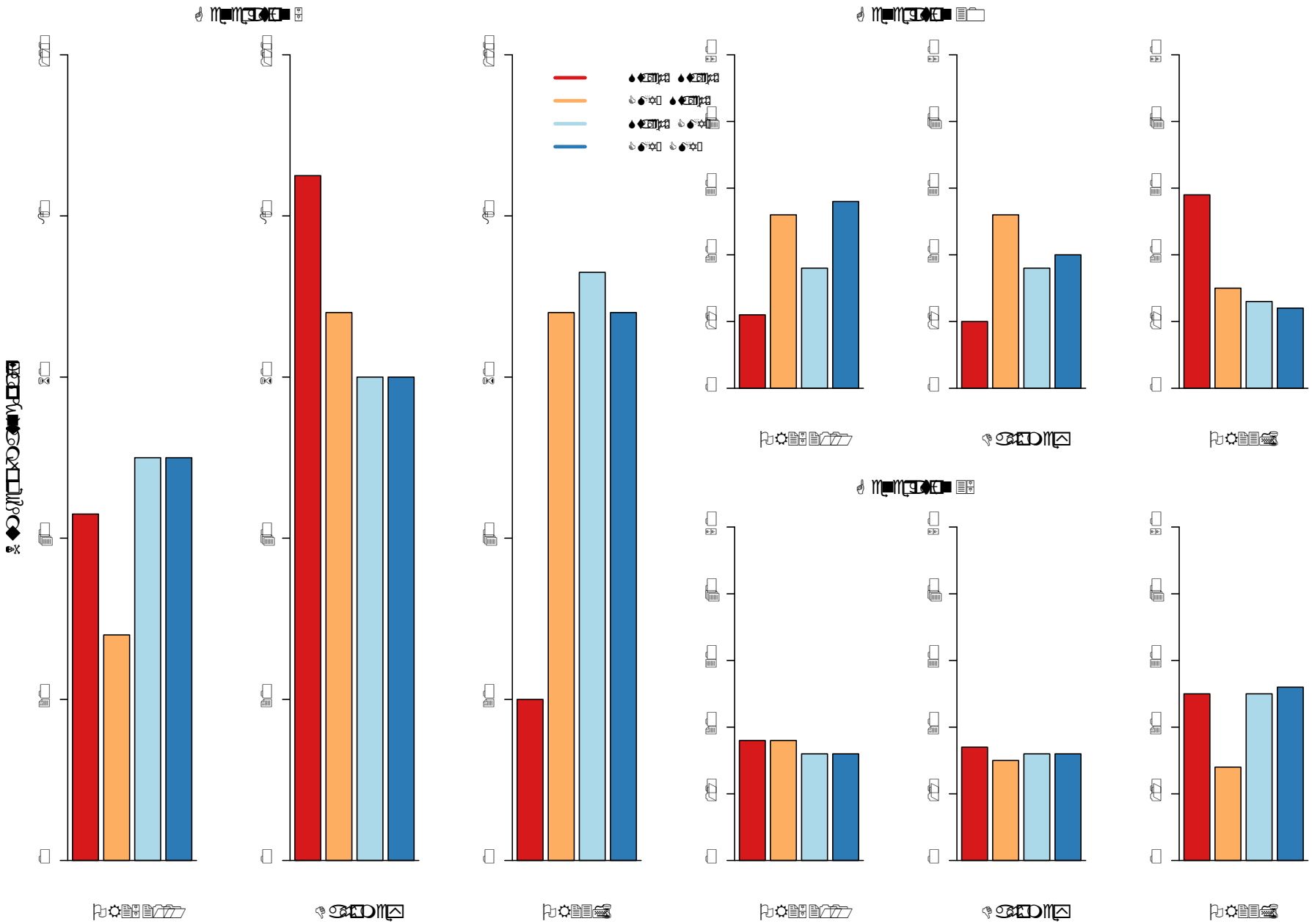
Streptococcaceae
Xanthomonadaceae

529



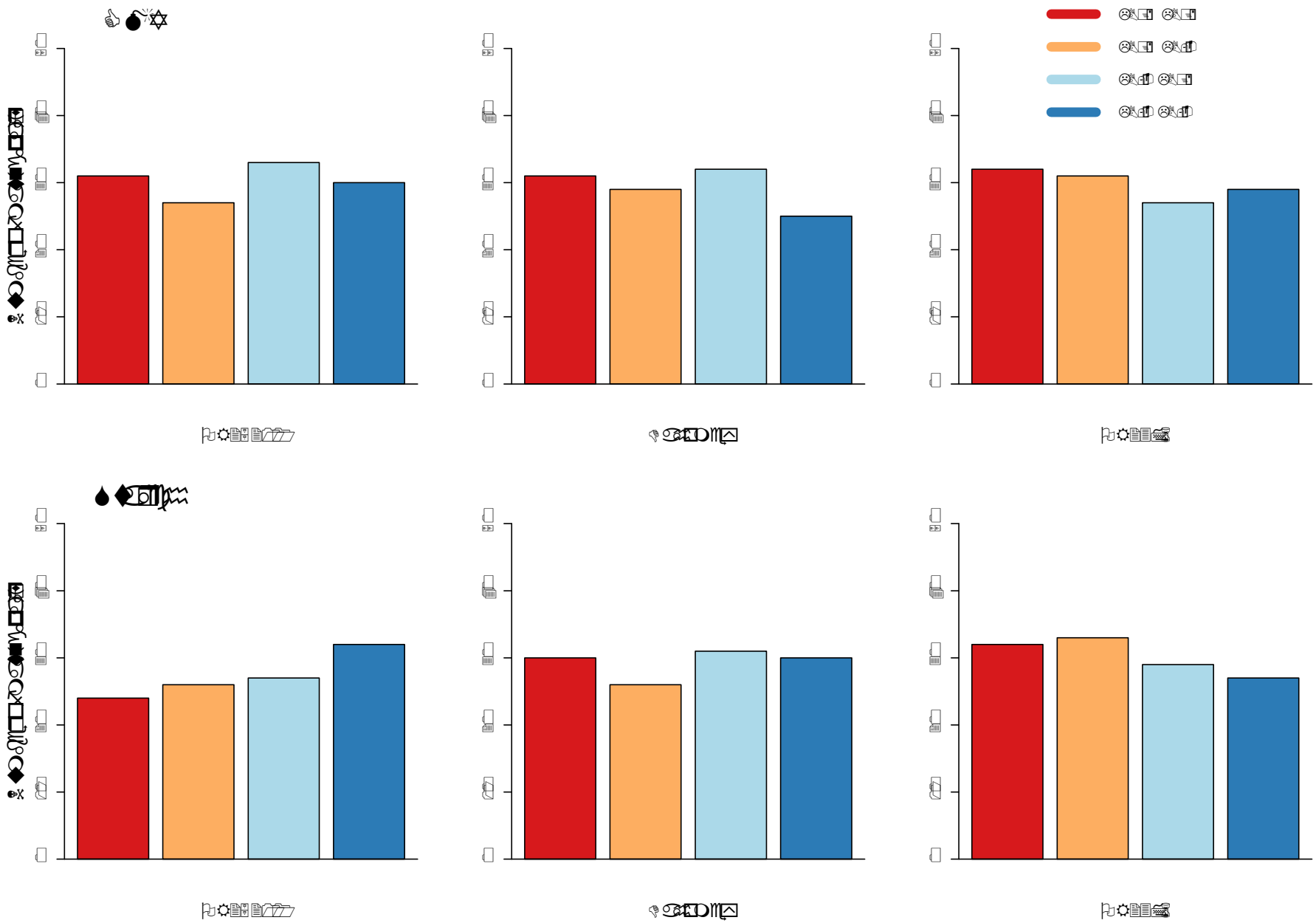


Table 1. Results of permutational multivariate analysis of variance (PERMANOVA) analysis of gut microbiome composition between each of the wild type lines maintained on CMY or Starch diets for 30 generations.

Variable	DF	Sum of Squares	Mean Squares	*F*	R^2	*P*
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	

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