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1 **Title: Continent-wide structure of bacterial microbiomes of European *Drosophila***
2 ***melanogaster* suggests host-control**
3

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19 **Competing interests**
20 The authors declare no conflict of interest.
21

22 **Abstract**
23 The relative importance of host-control, environmental effects, and stochasticity in the
24 assemblage of host-associated microbiomes has been much debated. With recent
25 sampling efforts, the underpinnings of *D. melanogaster's* microbiome structure have
26 become tractable on larger spatial scales. We analyzed the microbiome among fly
27 populations that were sampled across Europe by the *European Drosophila Population*

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28*Genomics Consortium* (DrosEU). We combined environmental data on climate and food-
29substrate, dense genomic data on host population structure, and microbiome profiling.
30Food-substrate, temperature, and host population-structure correlated with microbiome-
31structure. The microbes, whose abundance was co-structured with host populations, also
32differed in abundance between flies and their substrate in an independent survey,
33suggesting host-control. Patterns of enrichment and depletion of microbes between host
34and substrate were consistent with a model of host-control, where the host manipulates its
35microbiome for its benefit. Putative host-control was bacterial strain specific, supporting
36recent evidence for high specificity of *D. melanogaster*-microbe interaction.
37

38**Introduction**

39Species interactions, such as the interactions between microbes and their hosts are
40expected to be frequently driven by competition for resources that results in evolutionary
41conflict (Queller and Strassmann, 2018). In particular, horizontally transmitted microbes
42can leave an exploited host and move on to the next host, favoring conflict (Ebert, 2013a).
43Nonetheless, microbes that benefit higher organisms are prevalent (e.g. Jaenike et al.,
442010; Ankrah and Douglas, 2018; Bang et al., 2018). The frequent benefits that hosts
45derive from their microbiome are thought to result from strong selection pressure on the
46host to evolve selectivity towards beneficial microbes (Foster and Wenseleers, 2006;
47Schluter and Foster, 2012). This selectivity is also termed host-control. However, evidence
48for strong host-control over microbiome is limited for many organisms and its importance
49compared to environmental factors and stochastic processes in shaping host-associated
50microbiomes is debated.

51 Recent data across a variety of hosts, including mammals and insects, suggest that
52host-control might not be a major driver of microbiome structure. First, environmental
53factors like host-diet often have a dominant effect on microbiome composition (Chandler et

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54al., 2011; Wu et al., 2011; Staubach et al., 2013; Wang et al., 2014; Waidele et al., 2017;
55Rothschild et al., 2018). Second, the discovery of substantial variation of the microbiome
56between individuals of the same species (Ley et al., 2008; The Human Microbiome Project
57Consortium et al., 2012; Linnenbrink et al., 2013; Wang and Staubach, 2018) is also
58difficult to explain under strong host-control. To explain this variation, stochastic,
59ecologically neutral processes have moved into focus. These processes comprise
60ecological drift, dispersal, and colonization history (Hubbell, 2001). A dominance of neutral
61microbiome structuring principles appears plausible for diverse species (Sieber et al.,
622018), including *Drosophila* (Adair et al., 2018).

63 Without strong host control it is difficult to understand why higher organisms receive
64benefits from their microbiome so frequently. This is because host-control is supposed to
65be an important driver of the evolution of microbiome derived benefits in a setting that
66would otherwise frequently result in conflict between host and microbe (Foster and
67Wenseleers, 2006; Schluter and Foster, 2012; Ebert, 2013b; Queller and Strassmann,
682018). A model that resolves this contradiction has recently been proposed by Foster et al.
69(2017). In their 'ecosystem on a leash' model, the microbiome behaves similar to an
70ecosystem that is mainly shaped by microbe-microbe interactions. Host-control (the
71'leash') acts only on a small subset of microbes that affect host fitness. Targeted fostering
72and exclusion of a relatively small set of microbes in the microbiome might suffice to favor
73beneficial microbiome function (Schluter and Foster, 2012; Agler et al., 2016; Foster et al.,
742017).

75 Low but effective levels of host-control as proposed by the 'ecosystem on a leash'-
76model, also holds promise to help us to better understand the interaction of *Drosophila*
77with its microbiome. In *D. melanogaster*, the microbiome has well documented effects on
78somatic growth and reproductive output (Storelli et al., 2011; Shin et al., 2011; Téfit and
79Leulier, 2017; Sannino et al., 2018; Sommer and Newell, 2018). The effects of the

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80microbiome on these traits is caused, at least in part, by an increase in the efficiency of
81nutrient acquisition by the host in the presence of specific microbial taxa. For instance,
82reproductive (Pais et al., 2018) and nutritional (Sannino et al., 2018) benefits as well as
83protection from pathogens (Shin et al., 2011) can be derived from members of the family
84Acetobacteraceae. This family dominates the natural microbiome (Corby-Harris et al.,
852007; Cox and Gilmore, 2007; Chandler et al., 2011; Barata et al., 2012; Staubach et al.,
862013; Adair et al., 2018; Walters et al., 2018). However, the role of host-control in the
87prevalence of these potentially beneficial bacteria is unclear because the ability of *D.*
88*melanogaster* to shape its associated microbiome might be limited (Wong et al., 2013;
89Blum et al., 2013; Broderick et al., 2014). Instead, probabilistic processes contribute to gut
90colonization (Obadia et al., 2017) and community structure in natural populations can be
91explained to a large extent by neutral ecological mechanisms (Adair et al., 2018). As in
92mammals and other organisms, environmental factors, such as the time of collection
93(Behrman et al., 2018; Adair et al., 2018) or diet (Chandler et al., 2011; Staubach et al.,
942013; Erkosar et al., 2018; Wang and Staubach, 2018) have a strong effect on the
95*Drosophila* microbiome. The food-substrate that flies live on can be a more important
96driver of adaptation for the microbes than the host environment in *D. melanogaster*
97(Martino et al., 2018).

98 On the other hand, *D. melanogaster* microbiome structure is associated with host
99genotype (Unckless et al., 2015; Chaston et al., 2016; Behrman et al., 2018), indicating
100genotype dependent host-control. Microbial communities within flies differ from that in their
101stool (Fink et al., 2013), suggesting a selection process inside the fly. Further evidence
102that *D. melanogaster* exerts control over its microbiome comes from a recent study, which
103found that *D. melanogaster* larvae potentially foster *Lactobacillus plantarum* via excretions
104(Storelli et al., 2018). Host-control by *D. melanogaster* can be highly specific and fine-
105tuned. Pais et al. (2018) showed that *Acetobacter thailandicus* can persist in the gut of *D.*

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106*melanogaster*, can be dispersed by the host, and provide a fitness benefit to the host,
107while closely related *Acetobacter* strains cannot. In lab-reared flies, dysregulation of
108antimicrobial effectors leads to highly specific changes in microbiome composition;
109Intriguingly, these changes in community composition can preferentially select for non-
110pathogenic taxa over pathogenic ones, despite a close phylogenetic relationship between
111these microbial species (Ryu et al., 2008).

112 Host-control of the microbiome that increases host fitness is a key parameter of the
113'ecosystem on a leash'- model. The evidence for host-control in *D. melanogaster* suggests
114that this model might help us to understand the prevalence of benefits that *D.*
115*melanogaster* derives from its microbiome in the face of horizontal transmission, high intra-
116specific variation of microbiomes, and strong environmental effects. Given that the model
117was originally developed with the mammalian microbiome in mind (Foster et al., 2017), its
118applicability to *D. melanogaster* would create a common framework to understand
119mammalian and *D. melanogaster* microbiomes. As a consequence, results could become
120more transferable between the systems. Obviously, this is highly desirable because *D.*
121*melanogaster* is one of the best developed model systems in biology with many
122advantages over mammalian models. Together with the relatively simple microbiome
123(Erkosar et al., 2013) that is dominated by bacteria (Kapun et al., 2018) it holds promise
124for unraveling the driving forces of host-microbiome interactions (Erkosar et al., 2013;
125Douglas, 2018).

126 What is currently missing to assess whether the 'ecosystem on a leash' model is
127applicable to *D. melanogaster* is more information on the extent of host-control on larger
128ecological scales and under natural conditions. These are the conditions where potential
129host-control has originated by evolutionary processes. If host-control is important for *D.*
130*melanogaster*, imprints of host-control on the microbiome should also become apparent in
131a natural setting. Such imprints could be reflected by co-structure of host population

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132genetic variation and the microbiome because host-control in *D. melanogaster* varies
133between natural populations (Behrman et al., 2018; Walters et al., 2018) and depends on
134host genotype (Unckless et al., 2015; Chaston et al., 2016; Pais et al., 2018). Furthermore,
135if the natural *D. melanogaster* microbiome is subject to host-control, it should show general
136properties of host-associated microbiomes, for example elevated levels of 16S gene
137copies. Finally, microbes that are subject to host-control should differ in abundance
138between the host and its environment because the effects of host-control outside the host
139should be smaller or absent.

140 In order to test these basic predictions and further assess the role of host-control in
141natural *D. melanogaster* populations, we profiled the bacterial microbiome, in 50 samples
142across Europe, using 16S rRNA gene sequencing (Figure 1 and Table S1). The sampling
143range covered different climates and allowed us to address the effect of environmental
144factors on the microbiome. We combined the 16S profiles with population level allele
145frequency data for more than 20,000 neutral SNPs to test for co-structure of the
146microbiome with host genetic variation. For further exploration of potential host-control, we
147tested whether microbiomes showed typical properties of host-associated communities,
148specifically in terms of increased 16S gene copy number. Finally, we identified bacterial
149taxa that correlated with host population structure. These taxa were analyzed in an
150independent survey, comparing fly-associated microbiomes to that of their substrate, to
151test whether these taxa show different abundance that indicates of host-control.

152

153**Results**

154We analyzed a total of 5,217,762 16S rRNA reads after quality filtering (Table S2).
1552,672,402 *Wolbachia* sequences were removed. In order to make the diversity
156assessment comparable between samples, we rarefied (subsampled) the number of
157sequences per sample to 5,135 per sample for the continental range analysis and to 898

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158 sequences per sample for the fly versus substrate survey. We grouped the sequences into
159 100% identity Operational Taxonomic Units (OTUs) for high resolution analysis, unless
160 otherwise noted. We chose 100% identity to resolve strain level differences because the
161 interaction with the fly host may differ for closely related bacteria (Ryu et al., 2008; Pais et
162 al., 2018). Please note that the sequences were rigorously quality filtered and sequencing
163 errors were removed (see Materials and Methods).
164
165 Community composition and diversity
166 The *Drosophila* microbiome across Europe was dominated by acetic acid bacteria
167 (Acetobacteraceae 63.6%, Figure S1). The three most common genera were *Acetobacter*
168 (26.1%), *Gluconobacter* (17.4%), and *Commensalibacter* (15.4%). Enterobacteriaceae
169 were also common (15.2%). Shannon diversity was 2.61 +/- 0.65 (SD) at the 100%
170 identity OTU level and 1.99 +/- 0.53 (SD) at the 97% identity OTU level. Composition and
171 diversity of the *D. melanogaster* microbiome were similar to those reported previously from
172 natural *D. melanogaster* isolates. For a comparison of alpha diversity between studies see
173 Staubach et al. (2013).
174
175 The natural *D. melanogaster* bacterial microbiome is structured on a continental scale
176 As a first step to better understand the structuring principles of the *D. melanogaster*
177 microbiome, we tested whether the natural *Drosophila* associated microbiome is structured
178 on a continental scale. The absence of continental structure would be consistent with a
179 stochastic distribution of microbes and speak against both, host-control, as well as
180 selection of microbial taxa by other environmental factors.
181 Bray-Curtis-Dissimilarities (BCD) of the bacterial communities increased rapidly with
182 geographic distance (r = 0.196, P = 0.0015, Mantel-Test, Figure 2), indicating geographic

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183structure of microbial communities associated with *D. melanogaster* on a continent-wide
184scale.
185
186Host genetic differentiation and temperature correlate with microbiome structure
187In order to identify the factors underpinning continental structure, we modeled microbiome
188composition in a Redundancy Analysis (RDA) framework. We selected temperature,
189precipitation, and substrate as candidate environmental variables that could affect
190microbiome structure. We chose temperature because it can affect microbial communities
191on geographic scales (Thompson et al., 2017). Substrate is a major determinant of natural
192*Drosophila* microbiomes (Chandler et al., 2011; Staubach et al., 2013; Wang and
193Staubach, 2018). A lack of precipitation might affect microbiome assembly by selecting for
194xerotolerant microbes. Because microbial communities might reflect long-term or short-
195term trends in temperature and precipitation, we included annual, as well as monthly
196means of temperature and precipitation in our model. The inclusion of monthly
197temperature and precipitation at the time of collection allows us to assess seasonal
198variation in these parameters that could affect microbiomes. Finally, we reasoned that
199*Drosophila* host population structure might explain interpopulation differences in microbial
200community composition. This would be expected if potential host-control of the microbiome
201varied between host populations or if microbes and fly host show patterns of co-dispersal
202and parallel demographic histories on a continent-wide scale.
203 The full model (including all factors described above) explained approximately half
204(46.3%) of the variance in the bacterial microbiome, indicating that the model contained
205factors that affect the bacterial microbiome. In order to select the most relevant
206explanatory variables from the full model, we employed a forward model selection
207approach. This resulted in host genetic differentiation (PC1 and PC2), mean annual
208temperature (T(y)), and substrate as relevant factors for *Drosophila* microbiome

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209composition at the 100% OTU level (Table 1). We further reasoned that the microbiome
210might be structured at a higher taxonomic level. In particular, Acetobacteraceae comprise
211many bacteria that are dispersed by *D. melanogaster* and convey benefits to their hosts
212(Shin et al., 2011; Barata et al., 2012; Pais et al., 2018). Conversely, many
213Enterobacteriaceae are *Drosophila* pathogens. Susceptibility and virulence of these
214bacteria varies between natural host populations (Behrman et al., 2018). By applying the
215same model selection approach at the bacterial family level, we also identified host genetic
216differentiation (PC1) and annual mean temperature (T(y)) as relevant factors for
217microbiome composition (Table 2).
218
219The abundance of OTU2 (*Commensalibacter*), and Enterobacteraceae co-vary with host
220population structure
221We were interested to identify bacteria underlying the correlation of microbiome
222composition with host population structure. These bacteria might respond to potential
223differences in host-control between natural host populations. Therefore, we tested whether
224the relative abundance of OTUs correlated with host genetic variation. At the 100% identity
225OTU level, only the abundance of OTU2 (*Commensalibacter*, Acetobacteraceae)
226correlated with PC1 of host genetic variation (Figure 3, P = 0.00017, q = 0.0065, r = -0.52,
227Pearson's Product-Moment correlation). This suggested strain level specificity of host
228effects on the microbiome. At the family level, Enterobacteraceae (P = 0.037, q = 0.17, r =
2290.30, Pearson's correlation), Leuconostocaceae (P = 0.047, q=0.17, r = 0.29, Pearson's
230correlation), and Acetobacteraceae (P = 0.039 , q = 0.17, r = -0.30, Pearson's correlation)
231were structured according to PC1. However, when removing all sequences from OTU2,
232Acetobacteraceae were not significantly correlated with host population structure anymore
233(P = 0.16, q = 0.38), suggesting that OTU2 contributed significantly to the correlation. A
234representative sequence of OTU2 perfectly matched *Commensalibacter intestini* strain

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235A911 (Roh et al., 2008), a previously described commensal of *D. melanogaster*. No
236individual OTU correlated with PC2 of host genetic variation.
237
238No evidence for pronounced dispersal limitation of bacteria that correlate with host
239population structure
240We hypothesized above that the microbiome could be affected by host-control that varies
241between host populations. Alternatively, a correlation of microbiomes with host population
242structure could result from bacteria that are dispersal limited and depend on the fly host for
243dispersal. If the bacteria were severely dispersal limited on a global scale, we would
244expect the occupied geographic range of the bacteria in question to be rather limited.
245However, this is not the case; the bacterial groups that are structured according to host
246population structure in Europe (OTU2, Enterobacteraceae, Acetobacteraceae,
247Leuconostocaceae) can also be found along the East Coast and on the West Coast of the
248USA (Figure 4). Furthermore, these bacterial groups, were also previously found in
249association with wild-caught *D. takahashii* from Hawaii, *D. seychellia* collected from
250morinda fruit on the Seychelles, cactus feeding *D. mojavensis* and even in mushroom
251feeding *Microdrosophila* (Chandler et al., 2011). A representative sequence of OTU2
252matched sequences from these diverse locations and species perfectly (Chandler et al.,
2532011). This suggested that there is no pronounced dispersal limitation on a global scale for
254these bacteria and that the bacteria in question are rather cosmopolitan. Hence, a
255scenario, in which the bacteria are severely dispersal limited and depend on *D.*
256*melanogaster* dispersal on the continental scale, appears implausible.
257
25816S copy number of the natural *Drosophila* bacterial microbiome is typical for host-
259associated communities

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260Because the bacteria that are co-structured with their host populations on the continental 261scale are cosmopolitan, dispersal effects seemed insufficient to explain the co-structure of 262microbiomes and host population genetic variation. Therefore, we reasoned that host-263control might contribute to the co-structure. If the *Drosophila* micrbiomes that we analyzed 264were subject to host-control, they should differ from environmental microbiomes. Analyzing 26516S rRNA gene copy numbers can help to distinguish between environmental and host-266associated microbiomes: host-associated microbiomes have increased 16S rRNA gene 267copy numbers (Thompson et al., 2017) when compared to environmental microbiomes. 268The 16S gene copy number of our samples was in the typical range of host-associated 269communities, and significantly higher than that of non-host associated communities (P < 2702.2e-16, Mann–Whitney U test, Figure 5). In an independent survey, where we compared 271the microbiomes of flies and their immediate substrate, we also found more copies of the 27216S rRNA gene in the flies than in the substrate (P < 0.01, Mann–Whitney U test, one-273sided, Figure 5). This distinguishes the *D. melanogaster* microbiome from purely 274environmental microbiomes and supports host-related structuring.

275

276<u>Host-specificity of microbes that are co-structured with host population genetic variation</u>

27716S gene copy numbers suggested that the natural *D. melanogaster* microbiome is a 278typical host-associated community. This encouraged us to further explore the possibility 279that interactions with the host underlie the co-structure of microbiomes with host genetic 280variation. In order to test this, we analyzed whether the bacteria that were co-structured 281with host genetic variation differed in abundance between flies and their substrate. 282Specifically, we hypothesized that potential host-control would lead to a depletion of 283Enterobacteraceae in the flies because flies might avoid or reduce contact with these 284bacteria for their frequent pathogenicity. For example, Enterobacteraceae of the genera 285*Providencia*, *Serratia*, *Erwinia*, *Pseudomonas* are *Drosophila* pathogens. Indeed,

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286Enterobacteraceae were more abundant in the substrate than in the flies (*P* = 0.026,
287paired Mann-Whitney test, one sided, Figure 6A). Furthermore, we expected to find OTU2
288(*C. intestini*) at higher abundance in the fly than in the substrate because this OTU is a
289common member of the *D. melanogaster* associated community and contributes to healthy
290gut homeostasis (Ryu et al., 2008; Chandler et al., 2011). Indeed, OTU2 was enriched in
291flies (Figure 6B, *P* = 0.022, paired Mann-Whitney test, one-sided). Finally, we expected
292that Acetobacteraceae in general would be enriched in flies over substrate because this
293family contains several members that benefit *D. melanogaster* (Shin et al., 2011; Pais et
294al., 2018). This expectation was also confirmed (Figure 6C, *P* = 0.034, paired Mann-
295Whitney test, one-sided). However, when OTU2 was excluded from the analysis of
296Acetobacteraceae, Acetobacteraceae were not significantly enriched in flies anymore (*P* =
2970.21 paired Mann-Whitney test, one sided), indicating that OTU2 contributed to family level
298differences. We found no difference between flies and substrate for Leuconostocaceae (*P*
299= 0.27, paired Mann-Whitney test, two-sided).

300

301**Discussion**

302We set out to test whether there is evidence for host-control over the microbiome by *D.*
303*melanogaster* in a natural setting. For this purpose, we combined a comprehensive
304analysis of the structuring principles of the *D. melanogaster* microbiome on a continent-
305wide scale (Figure 1 and Table S1) with an independent survey comparing the microbiome
306of flies to that of their substrate. This resulted in several lines of evidence that support the
307idea that *D. melanogaster* exerts limited, but detectable and highly specific control over its
308microbiome.

309

310Co-structure between host genetic variation and the microbiome

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311The correlation of host population genetic differentiation and the differentiation of
312microbiomes can be interpreted as evidence for host-control. This correlation is consistent
313with a model, in which stronger genetic differentiation leads, on average, to larger
314differences in host-control, and hence host-associated microbiomes. Given ample
315evidence for variation in host-control between natural populations that depends on
316genotype (Lazzaro et al., 2008; Corby-Harris and Promislow, 2008; Behrman et al., 2018;
317Walters et al., 2018) this seems a reasonable model.

318 It appears unlikely that co-structure resulted from environmental factors that affect
319both, the microbiome and host genetic variation for two reasons: First, we accounted for
320the most plausible environmental factors that could affect microbiomes and the host at the
321same time in our model (food-substrate, temperature, precipitation). Second, for assessing
322host genetic variation, we used SNPs from small introns that are considered least affected
323by natural selection (Parsch et al., 2010; Lawrie et al., 2013). Therefore, it is unlikely that
324selection exerted by environmental factors that also affect the microbiome strongly affects
325these SNPs and generates co-structure.

326 It is similarly difficult to explain the co-structure by co-dispersal of *Drosophila* and
327bacteria because we found no evidence for pronounced dispersal limitation of the bacteria
328that co-vary with host genetic differentiation on a global scale (Figure 4). Instead, our data
329and previous studies suggest that these bacteria are cosmopolitan (Cox and Gilmore,
3302007; Chandler et al., 2011). Taken together that environmental variation was accounted
331for and that we found no evidence for dispersal limitation, a role for host-control in the
332observed co-structure appears plausible.

333

33416S copy numbers and differences between flies and substrate microbiomes support host-
335control

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336Host effects on the microbiome were further supported by 16S rRNA gene copy numbers
337that were in the typical range for host-associated communities and significantly different
338from that of non-host associated communities (Figure 5). As expected, the copy number in
339the substrate samples was smaller than that in fly samples. Interestingly, the copy number
340in substrate microbiomes was still larger than that of typical non-host associated
341microbiomes. This is consistent with *Drosophila* also affecting the microbiome of its
342immediate environment (Wong et al., 2015; Chaston et al., 2016; Storelli et al., 2018) and
343transforming it to appear more host-like.

344 Besides the increased number of 16S gene copies, host-control was evident from
345differences between the host microbiome and that of its substrate; three of the four
346bacterial groups (Acetobacteraceae, Enterobacteraceae, OTU2) that correlated with host
347genetic variation on a continental scale (Figure 3) also differed in abundance between flies
348and their substrate (Figure 6).

349

350Fitness effects of microbes that show evidence of host structuring support host-control

351The evidence above supports host-related structuring of the microbiome that is consistent
352with host-control. However, the term 'host-control' also implies that the effects of the host
353on the microbiome provide some fitness benefit to the host. The bacteria that are
354structured in the host environment and the direction of the structuring (enrichment or
355depletion) suggest such fitness benefits.

356 It seems reasonable to assume that the reduction of Enterobacteriaceae in the fly
357environment (Figure 6), is likely beneficial for the flies, because this family comprises a
358range of the most important *D. melanogaster* pathogens. Examples are *Providencia*
359(Galac and Lazzaro, 2011), *Serratia* (Flyg et al., 1980; Lazzaro et al., 2006), *Erwinia*
360(Basset et al., 2000), and *Pseudomonas* (Vodovar et al., 2005). A reduction of
361Enterobacteriaceae in the fly gut is in line with results from Ryu et al. (2008). These

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362authors have shown that Enterobacteriaceae, including the highly pathogenic *Erwinia* 363*carotovora carotovora-15* do not persist in the fly gut.

364 In contrast to Enterobacteriaceae, Acetobacteraceae were enriched in the host. 365This pattern was mainly driven by OTU2 (*C. intestini*). This OTU matches sequences from 366previous studies on fruit flies in the natural environment (Blast results Table S3) (Cox and 367Gilmore, 2007; Chandler et al., 2011; Wang and Staubach, 2018) and in the laboratory 368(Ryu et al., 2008). In particular, it perfectly matches *C. intestini strain A911* (Roh et al., 3692008). This strain is sensitive to anti-microbial peptides (AMPs) (Ryu et al., 2008), and 370hence can be subject to host-control. In wild-type flies, it is a dominant member of the 371microbiome. When AMPs are misregulated it is replaced by *Gluconobacter morbifer* that 372has detrimental effects on flies. Thus, in the wild-type gut environment, *C. intestini strain* 373*A911* is favored by the host and has a protective function. Favoring of a protective microbe 374can be considered host-control.

375

376The specificity of host-control

377*G. morbifer* as well as *C. intestini* are Acetobacteraceae, and hence relatively closely 378related. That flies can favor one over the other, points towards highly specific host-control 379in *D. melanogaster*. Our results suggest that host-control can also be highly specific under 380natural conditions*;* Only OTU2 (perfect sequence match with *C. intestini*) was strongly co-381structured with host genetic variation and at the same time enriched in flies over substrate 382(Figure 3 and 6). The evidence for high specificity in the interaction with bacteria that we 383found parallels recent results from Pais et al. (2018). These authors found that 384*Acetobacter thailandicus* colonizes *D. melanogaster* and persists in the gut, while a closely 385related *Acetobacter* strain does not persist. High specificity also fits in with results from 386Adair et al. (2018), who showed that the assembly of the natural bacterial microbiome in 387the *D. melanogaster* gut can be largely explained by neutral processes, except for a

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388specific set of bacteria. This specificity of the interaction of *D. melanogaster* with its
389microbiome is also fully compatible with recent advances in understanding the mechanistic
390principles of *D. melanogaster* immunity. A combination of highly specific regulation of the
391IMD pathway via different peptidoglycan recognition proteins (PGRPs) and specific
392regulation of the duox pathway (Ha et al., 2005; Lhocine et al., 2008; Bosco-Drayon et al.,
3932012; Lee et al., 2013; Guo et al., 2014; Iatsenko et al., 2016; Neyen et al., 2016) can lead
394to highly specific selection processes acting on bacterial communities in the fly gut.

395 While we found support for specific interaction with OTU2 (*C. intestini)*, more
396general mechanisms seemed to be at work for the interaction with Enterobacteriaceae.
397The reduction of Enterobacteraceae in the fly, when compared to the substrate was not
398linked to any specific OTUs from this family. Likewise, the co-structure with host genetic
399variation, was only apparent for the family as a whole. This family level host-control could
400arise in response to signals that are common to Enterobacteriaceae or from their potential
401pathogenicity in the sense of a danger or damage signal (Matzinger, 2002). Alternatively,
402the *Drosophila* gut might be just less favorable in terms of its physical condition (e.g. pH)
403or presence of antimicrobial agents (e.g. AMPs).

404

405Environmental factors and the *D. melanogaster* microbiome

406In addition to host genetic structure, temperature as well as the substrate, the flies were
407collected from, correlated with microbiome structure. While the effects of substrate on the
408fly microbiome are well described (Chandler et al., 2011; Staubach et al., 2013; Wang and
409Staubach, 2018), a continental scale temperature effects on a host-associated microbiome
410has, to our knowledge, not been described before. Temperature affects environmental
411microbiomes on a global scale (Zhou et al., 2016; Thompson et al., 2017) and the effect in
412*Drosophila* might reflect the exposure of flies to different environmental microbiomes.
413Temperature dependence of the microbiome could also be the result of a temperature

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414dependent dietary switch (Brankatschk et al., 2018); small scale structure of the food
415sources might allow flies to acquire selectively more plant or yeast material, which might
416lead to changes in microbiome composition.

417 While there was a significant effect of annual temperature on the microbiome, the
418correlation with monthly temperature at the collection date only showed a trend (P =
4190.065). Because our seasonal sampling was relatively limited (nine locations), more data is
420required to address the question whether seasonal temperature changes affect the
421microbiome. Seasonal variation in *D. melanogaster* associated microbiomes has been
422described by Behrman et al. (2018) and correlates with differences in pathogen
423susceptibility of the host. This points towards the possibility that seasonal changes in the
424microbiome could add to seasonal selective regimes and contribute to seasonal genome
425variation in *D. melanogaster* (Bergland et al., 2014; Machado et al., 2018).

426 The effects of temperature on the microbiome seemed more general as no specific
427OTU nor family was significantly correlated with temperature variation.

428

429**Conclusion**

430*D. melanogaster* lives in a microbe rich environment; rotting fruit. In this environment, it is
431essential for flies to foster beneficial microbes and avoid pathogens. Using continental
432scale data from natural populations, we presented evidence for specific host-control that
433favors a protective bacterium. This adds to the recent notion of high specificity of host-
434microbe interaction in *D. melanogaster* and shows that this specificity unfolds in an
435ecological and evolutionary context. At the same time, our study supports previous findings
436of strong environmental effects on the natural *D. melanogaster* microbiome. Strong
437environmental effects in combination with host-control of a relatively small subset of the
438partners is concordant with the 'ecosystem on a leash' model. Our results support the idea
439that this model might serve as a common framework to understand *D. melanogaster* and

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440mammalian microbiomes. A common framework for mammalian and *D. melanogaster*,
441increases the transferability and generalizability between systems. Hence, we see a bright
442future for the *D. melanogaster* microbiome as a model for other organisms, including
443mammals, in host-microbiome research.

444

445Materials and Methods

446Fly and substrate samples

447European fly samples were collected as described in Kapun et al. (2018). In short, 50
448samples of *D. melanogaster* were collected from 31 locations across the European
449continent with a joint effort of European research groups (Figure 1 and Table S1). Each
450sample contained a pool of 33-40 wild-caught males. We used males only because only
451males can be reliably distinguished from sympatric *D. simulans.* The effects of pooling on
452*D. melanogaster* microbiome profiling were assessed in detail by Wang and Staubach
453(2018). In short, pooling provides a more comprehensive picture of the population
454microbiome than an individual fly. While differences in microbiome structure between
455individuals tend to even out in a pool, differences between populations be well
456differentiated. Because we were interested in variation between populations here, a
457pooling approach is well suited. All 50 samples were included for analyzing *Drosophila*-
458associated bacterial community composition, diversity and dispersal patterns. Because
459data on host genetic differentiation for samples FR_Vil_14_06, UA_Yal_14_17, and
460DK_Kar_14_40 was not available, these samples were excluded from the analysis of
461continental scale community structure. The visualization of fly samples on the map in
462Figure 1 was generated with the R package ‘ggmap' (Kahle and Wickham, 2013).

463 Twelve samples from the East Coast of the USA were collected in the same fashion
464as the European samples and represent population pools of males. Seven of these
465samples were already analyzed in Behrman et al. (2018) (see Table S1 for details). The

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466samples named NY and WI were described in Machado et al. (2018). However, the 16S
467data for these samples was generated here. Because we did not have detailed information
468on the substrate, these samples were collected from, we did not include them in our
469continental scale modeling. We used these samples for evaluating the global range of
470bacteria (Figure 4). For the same purpose, we included 13 fly samples from Wang and
471Staubach (2018) that were primarily collected at the West Coast of the USA (see Table S1
472for details).

473 For the survey of the microbiome of flies and their substrate, pairs of pools of five
474flies and the corresponding substrate for a total of 24 samples were collected. The
475immediate substrate, on which the flies that we collected were sitting and feeding was
476collected with a sterile scalpel and transferred to a sterile microcentrifuge tube. The survey
477spanned 6 different substrates from 4 locations (Table S1).

478

479DNA extraction, PCR and sequencing

480DNA from the DrosEU samples was extracted by standard phenol-chloroform extraction
481after homogenization with 3 minutes of bead beating on QIAGEN TissueLyser II as
482described in Kapun et al. (2018). DNA from population pools from the USA were extracted
483as described in Bergland et al. (2014). DNA extraction for pools of five flies and the
484corresponding substrate was performed using the Qiagen QIAamp DNA extraction kit
485(Qiagen, Carlsbad, CA) combined with bead beating in the same way as for fly samples
486from Wang and Staubach (2018).

487 Barcoded bacterial broad range primers, 515F (5´GTGCCAGCMGCCGCGGTAA3´)
488and 806R (5´GGACTACHVGGGTWTCTAAT3´) from Caporaso et al. (2011) were used to
489amplify the V4 region of the bacterial 16S rRNA gene. DNA was amplified with Phusion®
490Hot Start DNA Polymerase (Finnzymes, Espoo, Finland) under the following conditions: 30
491sec at 98°C; 30 cycles of 9 sec at 98°C, 60 sec at 50°C and 90 sec at 72°C; final

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492extension for 10 min at 72°C. In order to reduce PCR bias, amplification reactions were
493performed in duplicate and pooled. PCR products quantified on an agarose gel and pooled
494in equimolar amounts. Extraction control PCRs were negative and excluded. The resulting
495pool was gel extracted using the Qiaquick gel extraction kit (Qiagen, Carlsbad, CA) and
496sequenced on an illumina MiSeq sequencer reading 2 × 250bp.

497

498Data analysis

499We analyzed sequencing data using MOTHUR v1.40.0 (Schloss et al., 2009). Main
500processing steps in MOTHUR included alignment of paired reads, quality filtering, removal
501of PCR errors, removal of chimeric sequences, subsampling (rarefication), and alpha-
502diversity calculations (Kozich et al., 2013). Sequences were taxonomically classified using
503the SILVA reference database 'Release 132' (Pruesse et al., 2007) as implemented in
504MOTHUR. A detailed step by step analysis script with all commands executed can be
505found in the supplementary File Script1 for full reproducibility. For studying geographical
506microbiome structure, OTUs were clustered at 100% identity. Only for the comparison of
507Shannon diversity to previous studies, we also included clustering at 97% sequence
508identity.

509 To identify factors that shape microbial communities, we applied Redundancy
510Analysis (RDA). Following Borcard, Gillet and Legendre (2018), OTU count data was
511Hellinger transformed to allow analysis in the linear RDA framework. In order to reduce the
512effects of rare species on RDA and assuming that ecologically relevant species should be
513frequent, we focused the analysis on OTUs with more than 1000 reads across samples.
514Our candidate explanatory variables were temperature, precipitation, substrate and host
515genetic differentiation. Data of annual and monthly mean temperature (BIO 1 and tmean)
516and precipitation (BIO 12 and prec) were downloaded from WorldClim (Fick and Hijmans,
5172017, see supplementary File Script2). Host genetic differentiation was represented by the

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518first two principle components of an allele frequency based Principle Components Analysis
519performed by Kapun et al. (2018). In short the data represents allele frequencies from
520more than 20,000 SNPs in short intronic sequences that evolve putatively neutral and best
521represent population structure. In order to select the variables that were most important for
522microbiome structure, we applied forward model selection of additive linear models. This
523was done with the ordistep function from the vegan R package (Oksanen et al., 2018). The
524Ordistep function provides a stepwise approach to select variables based on permutation
525P-values and Akaike's Information Criterion (AIC).

526 In order to test for potential spatial autocorrelation we followed the protocol by
527(Borcard et al., 2018) using the dbmem function. This protocol employs eigenvector
528analysis to detect autocorrelation at different scales. We found no evidence for significant
529autocorrelation in our data (see supplementary File Script3) after removal of the continent-
530wide trend in species distributions that we analyzed here. All algorithms were part of the
531vegan (Oksanen et al., 2018) and adespatial R packages (Dray et al., 2018). Geographic
532distances were computed with the gdist function from the Imap R package (Wallace, 2012,
533see supplementary File Script4).

534 For the correlation of host genetic differentiation with the relative abundance of
535individual OTUs and bacterial families, we calculated q-values with the p.adjust function in
536R to account for multiple testing. Following the recommendation by Efron et al. (2007),
537only significant correlations (P < 0.05) with bacterial groups with q-values smaller than 0.2
538were considered significant.

539 Average community 16S rRNA gene copy number (ACN) was predicted from 16S
540rRNA gene amplicon data using PICRUSt (Langille et al., 2013). The method for
541calculating ACN was adapted from Thompson et al. (2017). We first classified sequences
542using the Greengenes reference database and generated an OTU table in the Biom-format
543using the make.biom function in MOTHUR. The resulting biom-formated table served as

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544input for the normalize_by_copy_number.py command implemented in PICRUSt. The
545output file is a normalized observation table. ACN for each sample were calculated as the
546raw sample sum divided by the normalized sample sum.
547
548**Data availability**
549Raw sequence data is in the submission process to the ncbi short read archive (SRA).
550
551**Acknowledgements**
552We thank the DrosEU consortium for the coordinated sampling and fruitful collaboration.
553We thank the Paul Schmidt (University of Pennsylvania), John Pool (University of
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558408908608) and the Landesgraduiertenfoerterung of the state of Baden-Württemberg.
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570**Table 1** RDA model selection for factors that explain community composition at the 100%
571identity OTU level. The forward selection approach starts with the null model, adding the
572best explanatory factors one by one until adding the next factor fails to significantly
573improve the model. Significant p-values and the best model are in bold. P-values are
574based on permutation tests. PC1 = Axis 1 of host genetic variation, PC2 = Axis 1 of host
575genetic variation, substrate = substrate the flies were collected from, T(y) = mean annual
576temperature, T(m) = mean monthly temperature.

577

model	factors	add	AIC	*P*
M0	NULL		- 20.757	
M1	PC1	+PC1	- 21.802	**0.001**
M2	PC1+substrate	+bait	- 18.470	**0.007**
M3	PC1+substrate+PC2	+PC2	- 19.076	**0.013**
M4	**PC1+substrate+PC2+T(y)**	**+T(y)**	**- 19.905**	**0.011**
M5	PC1+substrate+PC2+T(y)+T(m)	+T(m)	- 20.130	0.065

579

580**Table 2** RDA model selection for factors that explain community composition at the family
581level. The forward selection approach starts with the null model, adding the best
582explanatory factors one by one until adding the next factor fails to significantly improve the
583explanatory power of the model. Significant p-values and the best model are in bold. P-
584values are based on permutation tests. PC1 = Axis 1 of host genetic variation, PC2 = Axis
5851 of host genetic variation, substrate = substrate the flies were collected from, T(y) = mean
586annual temperature.

model	factors	add	AIC	*P*
M0	NULL		- 73.782	
M1	T(y)	+T(y)	- 76.095	0.002
M2	**T(y)+PC1**	**+PC1**	**- 77.237**	**0.012**
M3	T(y)+PC1+PC2	+PC2	- 77.488	0.067

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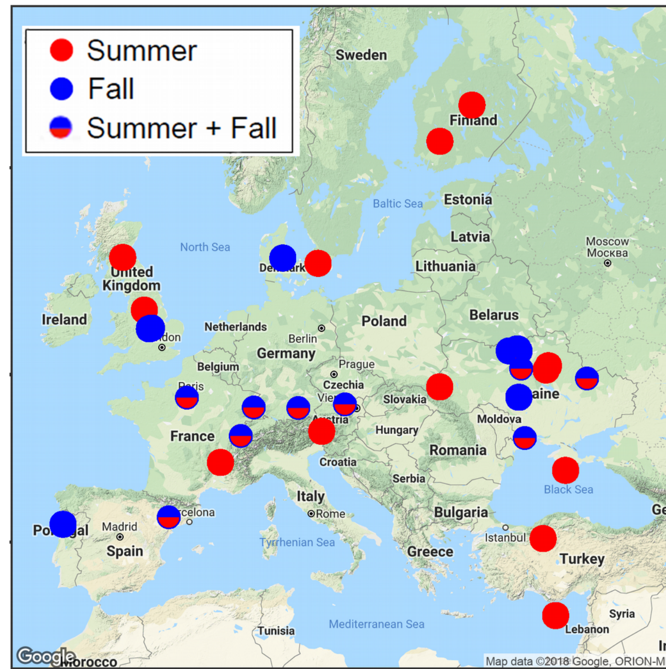

589 **Figure 1** Overview of sampling locations. The map shows the geographic locations of 50
590 samples for bacterial community analysis in the 2014 DrosEU dataset. The color of the
591 circles indicates the sampling season for each location.

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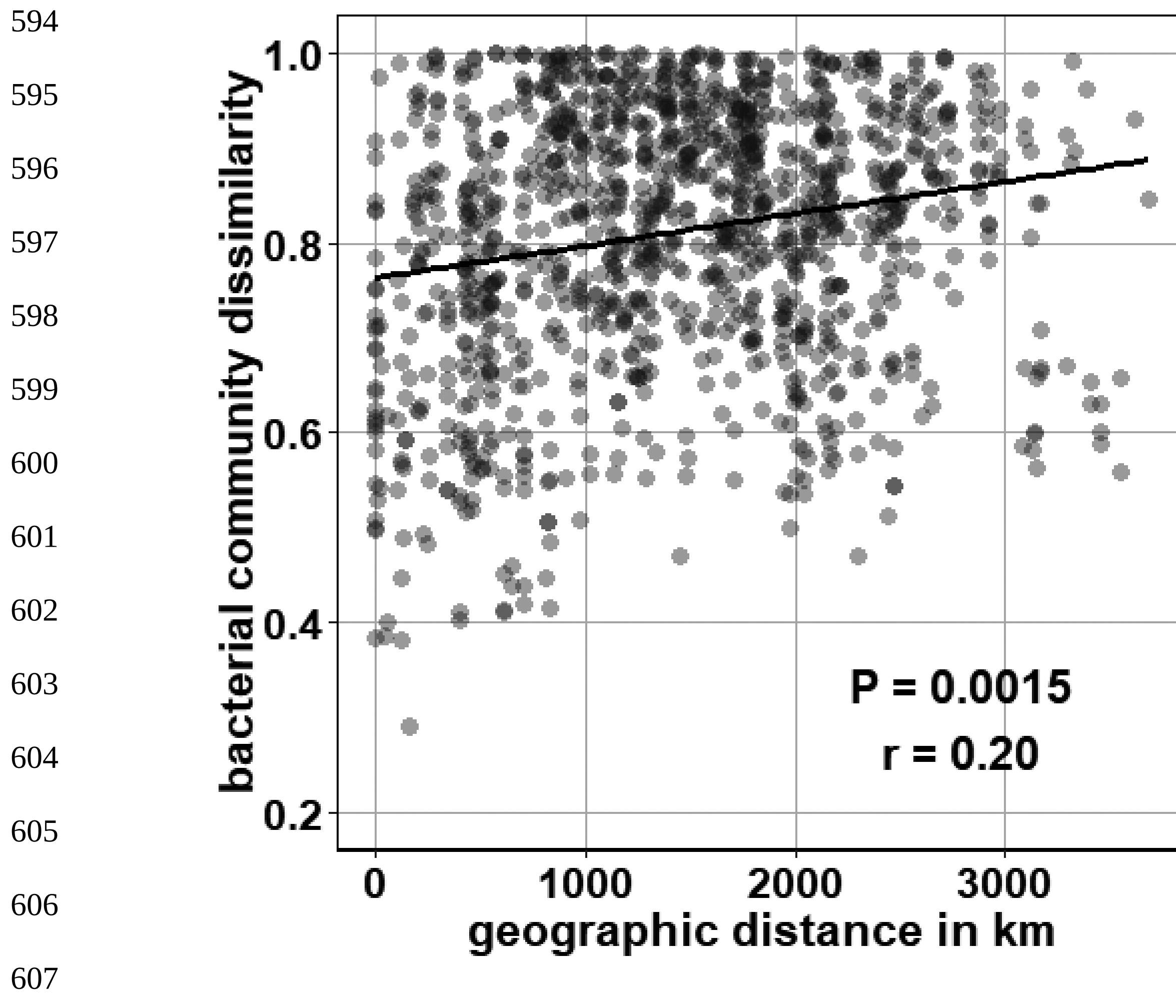

608 **Figure 2** Correlation between pairwise geographic distance and BCD of the bacterial
609 communities. P-value according to Mantel test; r is Pearson's correlation coefficient.

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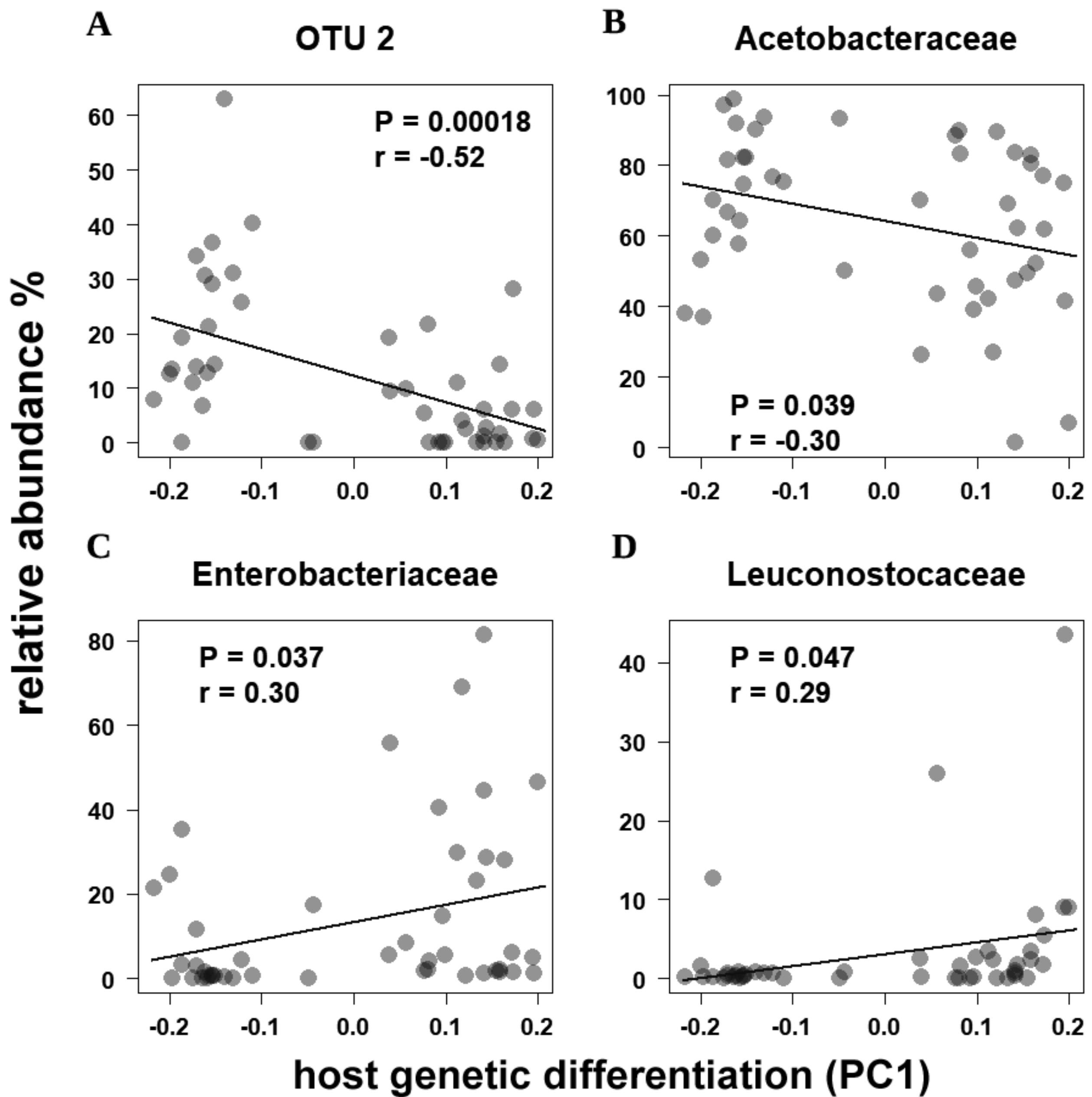

612**Figure 3** OTU2 (A) and three bacterial families (B-D) correlate with host genetic
613differentiation (PC1). P-value and correlation coefficient according to Pearson's Product-
614Moment Correlation.

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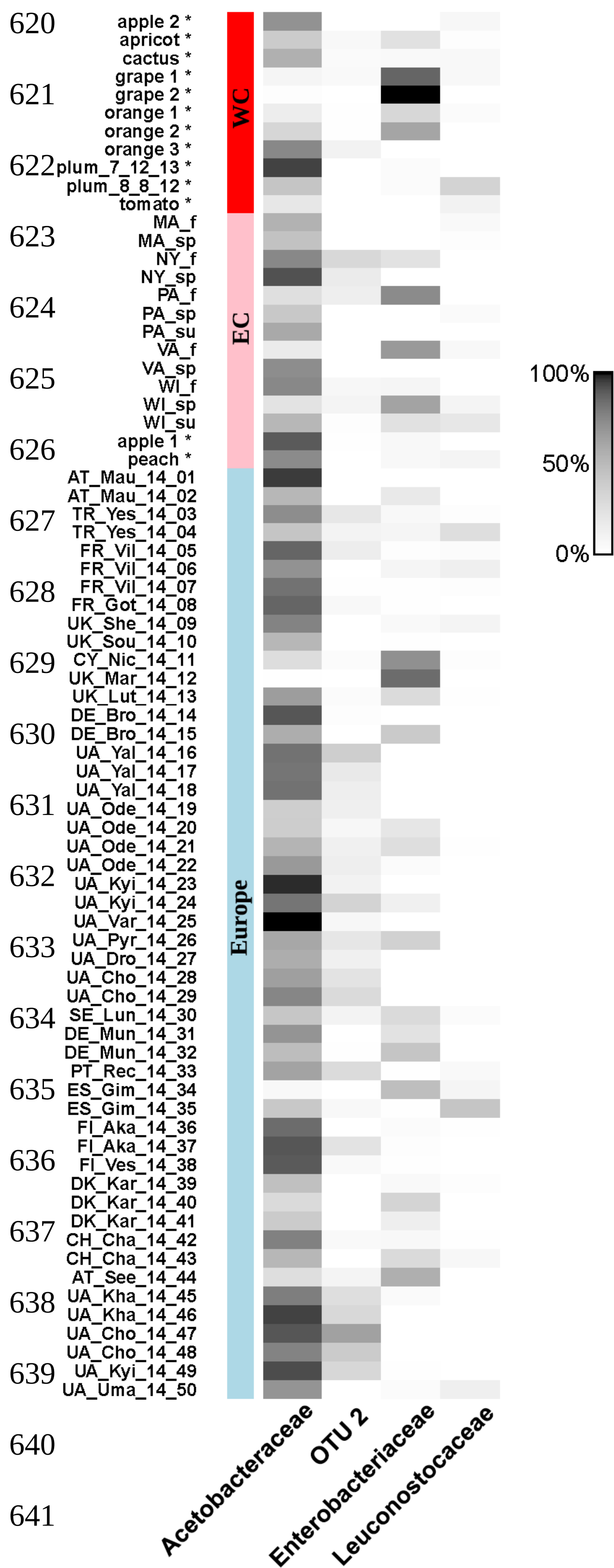

642 **Figure 4** The bacteria that were structured
643 according to host genetic variation, are common in Europe, the East Coast (EC), and the
644 West Coast (WC) of and North America. Gray scale indicates relative abundance.
645 Samples marked with * were described in Wang & Staubach (2018).

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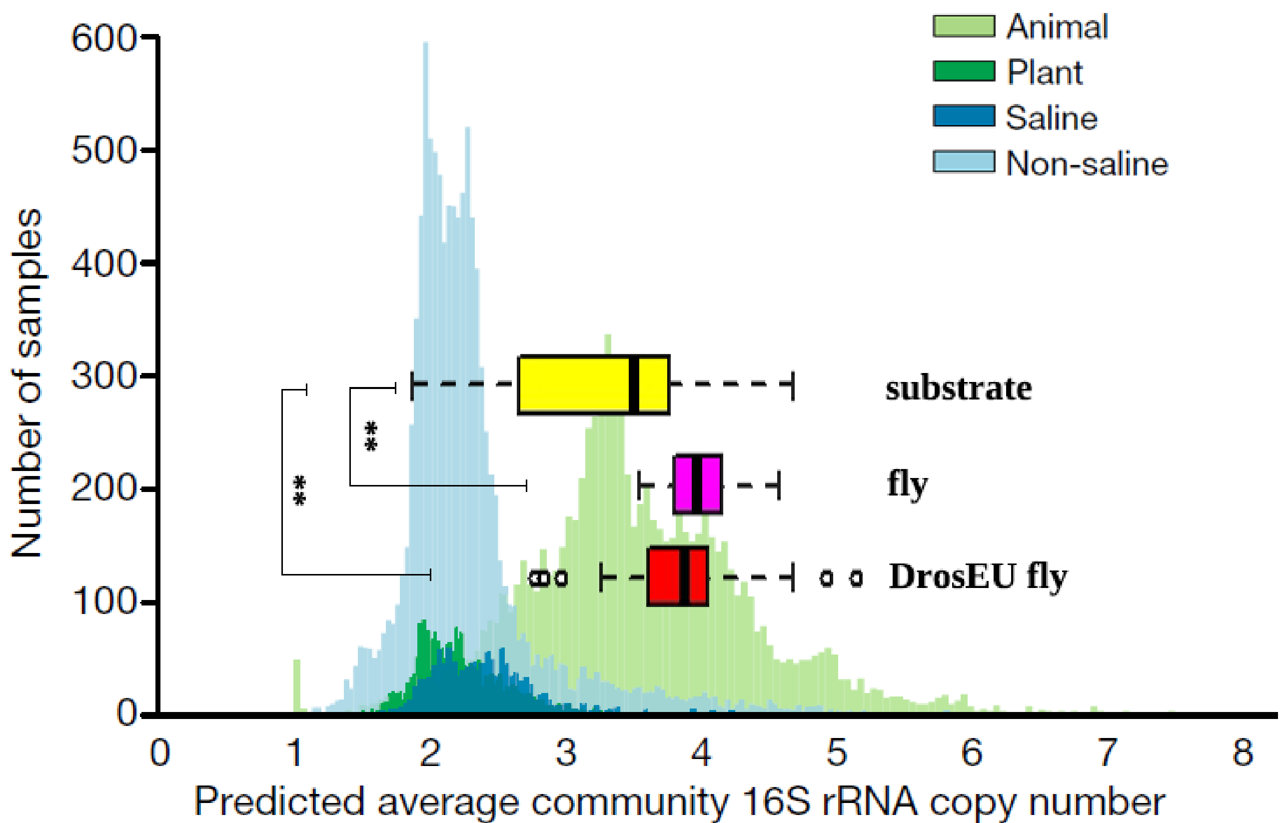

648**Figure 5** Comparison of 16S rRNA gene average copy number (ACN) between flies and 649substrates. ACN was higher in the fly than in the substrate communities (** $P < 0.01$, Mann-650Whitney-U test, one-sided). Barplot (blue and green) in the background shows ACN from 651(Thompson et al. 2017). Fly as well as substrate samples were in the typical range of 652animal-associated microbiomes.

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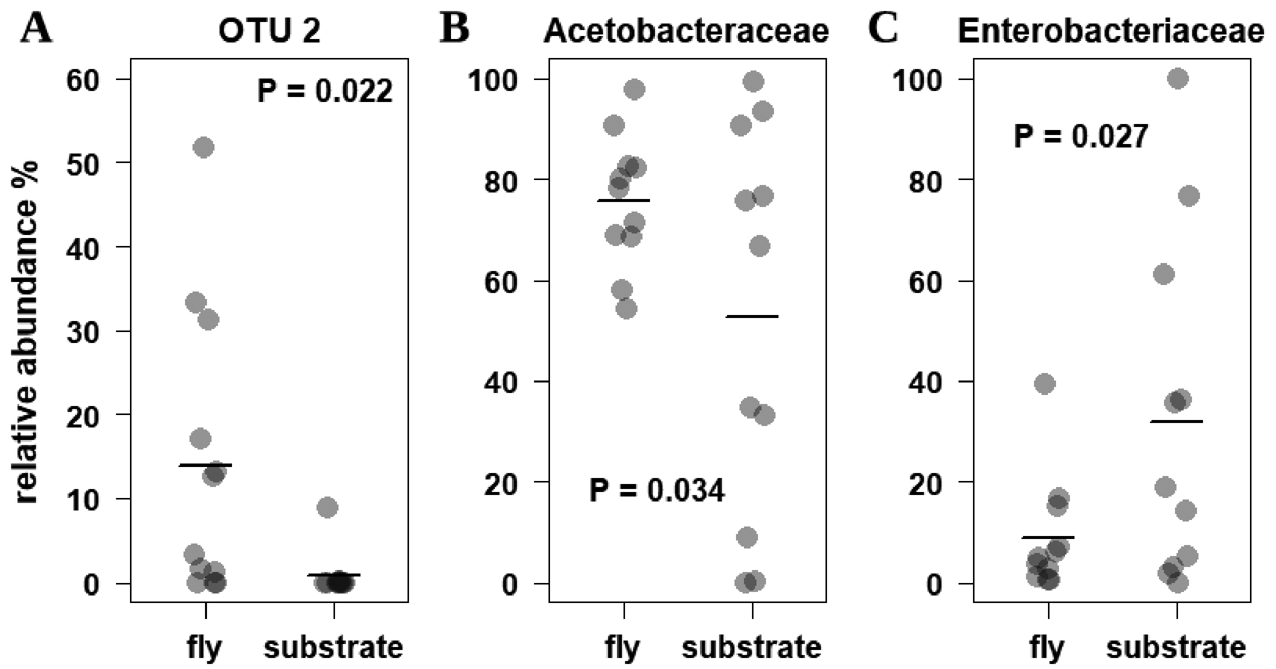

660**Figure 6** Comparison of relative abundance of (A) OTU2, (B) Acetobacteraceae and (C)
661Enterobacteraceae between flies and their substrate. P-values according to paired Mann-
662Whitney-U test, one-sided.

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