

# An altered microbiome in a Parkinson's disease model *Drosophila melanogaster* has a negative effect on development

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3

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12

## 13 **Abstract**

14 It is well-established that there are differences in the fecal microbiota composition between Parkinson's  
15 disease (PD) patients and control populations, but the mechanisms underlying these differences are not  
16 yet fully understood. To begin to close the gap between description and mechanism we studied the  
17 relationship between the microbiota and PD in a model organism, *Drosophila melanogaster*. First, fecal  
18 transfers were performed with a *D. melanogaster* model of PD that had a mutation in the *parkin* (*park<sup>25</sup>*)  
19 gene. Results indicate that the PD model feces had a negative effect on both pupation and eclosion in  
20 both control and *park<sup>25</sup>* flies, with a greater effect in PD model flies. Analysis of the microbiota  
21 composition revealed differences between the control and *park<sup>25</sup>* flies, consistent with many human  
22 studies. Conversely, gnotobiotic treatment of axenic embryos with feces-derived bacterial cultures did  
23 not affect eclosion. We speculate this result might be due to similarities in bacterial prevalence  
24 between mutant and control feces. Further, we confirmed a bacteria-potentiated impact on mutant and  
25 control fly phenotypes by measuring eclosion rate in *park<sup>25</sup>* flies that were mono-associated with  
26 members of the fly microbiota. Both the fecal transfer and the mono-association results indicate a host  
27 genotype-microbiota interaction. Overall, this study concludes functional effects of the fly microbiota on  
28 PD model flies, providing support to the developing body of knowledge regarding the influence of the  
29 microbiota on PD.

30

## 31 Introduction

32            Parkinson's disease (PD) is the second most common neurodegenerative disease affecting more  
33 than ten million people worldwide<sup>1</sup>. Most cases of PD are idiopathic, while a small percentage is genetic  
34 in origin<sup>2</sup>. Of these genetic cases, mutation of the *PRKN* gene contributes to approximately 50% of all  
35 autosomal recessive juvenile parkinsonism<sup>3</sup>. Mutation of the *Drosophila melanogaster* ortholog of the  
36 *PRKN* gene, *parkin* (*park*), leads to a tenable model of PD that has many similarities to PD patients:  
37 selective loss of dopaminergic neurons, decreased motor function, loss of olfaction, reduced lifespan,  
38 mitochondrial dysfunction and others<sup>4-7</sup>. The relative ease of use of flies and powerful genetic tools  
39 available in this fly PD model has contributed greatly to the study and understanding of PD<sup>8</sup>.

40            Gastrointestinal (GI) dysfunctions are among the most common non-motor symptoms  
41 associated with PD, with constipation being the most common premotor symptom, affecting more than  
42 70% of PD patients<sup>9</sup>. There have been a number studies that have demonstrated that the gut  
43 microbiota is altered in PD patients compared to healthy control individuals<sup>10</sup> and it has been  
44 hypothesized that this altered microbiota is largely responsible for many of the GI disorders observed.  
45 Beyond this, the altered PD microbiota has been hypothesized to play a role in non-GI PD symptoms,  
46 specifically related to the gut-brain axis. In support of this, PD patient fecal transplant into germ-free PD  
47 model mice produced an increase in motor deficits compared to PD model mice with a healthy donor  
48 fecal transplant<sup>11</sup>. Taken together, the results from these previous studies prompted our investigation  
49 of the microbiota in the *park* mutant fly model.

50            Relative to humans and other mammals, the *D. melanogaster* microbiota is low-abundance and  
51 low-diversity, making it simpler and easier to study microbiota interactions. Laboratory and wild flies are  
52 typically colonized by  $10^4$  -  $10^5$  microorganisms, and the 2-5 most abundant isolates often represent >  
53 90% of the microbial community<sup>12-14</sup>. The most represented bacteria in the gut are usually acetic acid

54 (AAB) and lactic acid bacteria (LAB), especially members of the genera *Acetobacter* and *Lactobacillus*,  
55 respectively, and *Enterobacteriaceae*. Similar to mammals, the fly microbiota composition is determined  
56 by both fly genotype and diet<sup>14-17</sup>. Fly larvae possess no gut microbes upon hatching and thus obtain and  
57 develop their microbiota from both the environment and food source. Because there is no evidence of  
58 high-fidelity host-mediated acquisition or retention of specific microorganism within or across  
59 generations, the *Drosophila* microbiota is ‘inconstant’; although some bacterial isolates colonize and  
60 persist within the gut better than others<sup>18-20</sup>. A previous analysis of the microbiota in a PD fly model  
61 revealed differences in the diversity, but not specific taxonomic changes, between the microbiota of  
62 control and PD-model flies<sup>21</sup>. The intracellular endosymbiont *Wolbachia* is also a common inhabitant of  
63 the reproductive tract of *Drosophila* and, unlike the gut microbiota, is transmitted from mother to  
64 offspring within the egg<sup>22</sup>.

65         The association between *Drosophila* and its microbiota is experimentally tractable: bacteria-free  
66 embryos are readily derived by bleach treatment and members of the *Drosophila* microbiota can be  
67 isolated in pure culture in the laboratory. Inoculating bacteria-free fly embryos with a defined microbial  
68 species or community is called gnotobiotic culture and permits exquisite dissection of the contributions  
69 of individual microorganisms to specific fly phenotypes<sup>23</sup>. Adding back one or more bacterial species to  
70 the same genotype of sterile fly embryos permits the detection of the magnitude of variation in host  
71 traits that is due to the microbiota<sup>24</sup>. Unlike the gut microbiota, bleach treatment does not eliminate  
72 *Wolbachia* from the fly embryos.

73         In this study we sought to better define the relationship between the microbiota and a *D.*  
74 *melanogaster* PD model by addressing two major questions: 1) Does microbiota manipulation, including  
75 via fecal transfer, bacterial-elimination, or gnotobiotic culture affect development, an early and  
76 fundamental biologic process, in a PD-fly model? 2) Does the microbiota vary between control and PD

77 model flies? This study aims to address these questions by measuring fly pupation, eclosion, and/or  
78 microbiota composition under a variety of conventional and gnotobiotic culture conditions.

79

## 80 **Results**

### 81 **Fecal transfer from *park<sup>25</sup>* flies reduces total pupation rates**

82 To determine whether differences in the microbiome between control and *park<sup>25</sup>* flies might  
83 contribute to variation in fly phenotypes we compared the pupation rate of flies that received fecal  
84 transfers from control and *park<sup>25</sup>* donors. Fecal transfers were performed by allowing males to defecate  
85 on cooked but not autoclaved diet vials for 3 days before transferring fly embryos to the feces-seeded  
86 diet. The *park<sup>25</sup>* feces reduced the total pupation rate of all fly genotypes when compared to the  
87 embryos that were placed on food that contained control feces (post-hoc Tukey test:  $P < 0.0001$  for all  
88 three; **Fig. 1**). Each of the pupation rates are based off the 60 embryos placed on the food, such that  
89 both the *park<sup>25</sup>* heterozygous and homozygous pupae numbers come from the same 60 embryos. Two-  
90 way ANOVA analysis revealed that 86% of the variation is due to genotype ( $P < 0.0001$ ), which is  
91 expected given the effects of the *park<sup>25</sup>* mutation. Additionally, 3.6% of the variation is due to the fecal  
92 transfer effect ( $P < 0.0001$ ). When analyzing daily pupation rates, the homozygous *park<sup>25</sup>* flies were the  
93 only genotype that had reduced pupation rates on two consecutive days when they received feces from  
94 *park<sup>25</sup>* mutants, suggesting that the *park<sup>25</sup>* homozygous flies are more susceptible to the detrimental  
95 effects of the *park<sup>25</sup>* fecal transfer (**Supplementary Fig. S1**). Together, these results identify a negative  
96 effect on flies of multiple genotypes when they received a fecal transfer from *park<sup>25</sup>* versus control flies.

### 97 **The *park<sup>25</sup>* feces reduces fly eclosion rates**

98            Eclosion rates for each fly genotype-fecal transfer combination were determined by dividing the  
99            number of flies that eclosed by the total number of pupae that had developed for that genotype. **Figure**  
100            **2A** shows that all three fly genotypes experienced a reduced eclosion rate when placed on the *park*<sup>25</sup>  
101            feces compared to the control feces (control and homozygous *park*<sup>25</sup>,  $P < 0.0001$ ; heterozygous *park*<sup>25</sup>,  $P$   
102            = 0.0101). As with pupation, the majority of variation identified was due to fly genotype (54%,  $P <$   
103            0.0001), which is reflected by the reduced eclosion rates of both the *park*<sup>25</sup> heterozygous and  
104            homozygous flies on control feces compared to the control flies on control feces (both  $P < 0.0001$ ). In  
105            agreement with pupation, a smaller amount of variation was due to the feces (11.9%,  $P < 0.0001$ ) and  
106            there was also a significant interaction between the fly genotype and the feces transfer, indicating that  
107            there might be a specific effect of the fecal transfer in the *park*<sup>25</sup> fly ( $P = 0.0042$ , 1.4% of variation).

108            Regardless of the source of the fecal inoculum, both the heterozygous and homozygous *park*<sup>25</sup>  
109            flies had reduced eclosion rates compared to the control flies, indicating that the *park*<sup>25</sup> genotype likely  
110            has a reduced eclosion rate due to the *park*<sup>25</sup> mutation (**Fig. 2A**). However, it appears that the *park*<sup>25</sup>  
111            fecal transfer had an additional negative impact on the *park*<sup>25</sup> flies. This feces-dependent differential  
112            effect, based on fly genotype, of eclosion reduction due to the *park*<sup>25</sup> feces becomes more apparent  
113            when observing the number of pupae that failed to eclose. This measurement indicates that the control  
114            flies did not have an increased number of failed eclosures ( $P = 0.1423$ ), while the heterozygous and  
115            homozygous *park*<sup>25</sup> pupae did ( $P = 0.0023$ ,  $P < 0.0001$ , respectively; **Supplementary Fig. S2**).

116            Further, the detrimental effects of the *park*<sup>25</sup> feces on fly eclosion were of larger magnitude for  
117            the homozygous *park*<sup>25</sup> flies than other genotypes. When we calculated the percent eclosion rates of  
118            each genotype on *park*<sup>25</sup> feces relative to control feces, there was no difference in the eclosion rates of  
119            the heterozygous mutants and control flies ( $P = 0.1211$ ), but both genotypes had higher eclosion rates  
120            than the *park*<sup>25</sup> homozygous flies ( $P < 0.0001$  vs both, **Fig. 2B**). Further support of differential feces-  
121            genotype interaction is provided by observing the eclosion rate over time. **Supplementary Figure S3**

122 shows that the control flies experienced a reduction in eclosion due to *park*<sup>25</sup> feces on day 10 (P <  
123 0.0001), while heterozygous *park*<sup>25</sup> flies had no significant reduction on any day in the experiment.  
124 However, homozygous *park*<sup>25</sup> flies experienced a reduction in eclosion due to *park*<sup>25</sup> feces on days 9, 10  
125 and 11 (P = 0.0001, P = 0.0012, P = 0.0005, respectively).

## 126 **The whole-body microbiota varies between conventional PD model and control flies**

127 Our observations suggest that the composition of *park*<sup>25</sup> and control fly microbiomes are  
128 different and cause different developmental effects on the flies tested. Thus, as an extension of these  
129 results we measured the bacterial microbiota of whole-body conventionally reared *park*<sup>25</sup> and control  
130 flies. The samples for sequencing were collected at a different time than the experiments above and,  
131 because of the inconstant microbiota<sup>25</sup>, the sequencing results should not be conflated as measuring the  
132 microbiota of the flies that deposited feces in the previous experiments. Sequencing of the V4 region of  
133 the 16S rRNA gene revealed significant differences in the whole-body microbial communities of our  
134 stocks. The most notable difference between the mutant and control populations was the presence of  
135 the reproductive tract endosymbiont *Wolbachia* in the control flies (**Supplementary Fig. S4** and  
136 **Supplementary Table S1**); however, *Wolbachia* are not likely to be transferred between flies via  
137 ingestion and therefore are not likely candidates for the effects observed with fecal transfer. After  
138 *Wolbachia* were removed from the analysis (to focus on non-reproductive tract microorganisms), beta-  
139 diversity metrics that factor microbial abundance reported significant differences in the microbiota  
140 composition of the different fly stocks with fly genotype, but not with the sex of the flies (**Fig. 3**,  
141 **Supplementary Fig. S5**, and **Table 1**). Also, there was not a significant genotype \* sex interaction,  
142 indicating that both males and females showed the same genotype-dependent changes in microbiota  
143 composition (**Table 1**). Amplicon sequence variants assigned to the LAB (more abundant in controls) and  
144 AAB (less abundant in controls) were significantly different in relative abundance between flies of  
145 different genotypes (**Supplementary Fig. S6**). The decreased abundance of AAB in the control flies,

146 which also bore *Wolbachia*, is consistent with previous reports that *Wolbachia* prevalence is negatively  
147 associated with AAB abundance<sup>26</sup>. Overall, the data reveal a consistent difference in the microbiota  
148 composition of *Wolbachia*-discordant control and *park*<sup>25</sup> mutant flies that were reared side-by-side  
149 under conventional laboratory conditions.

#### 150 **Axenic preparation of *park*<sup>25</sup> homozygous embryos has a dramatic effect on eclosion**

151 To determine if the different effects of fecal transfer from *park*<sup>25</sup> or control flies could be  
152 attributed to variation in the bacterial microbiota we measured eclosion rates in flies that were  
153 inoculated as sterile embryos with cultured feces from control and *park*<sup>25</sup> adult flies. We observed two  
154 major differences between the different treatment approaches: fecal transfer vs fecal bacterial culture  
155 inoculation. First, the process of generating axenic embryos dramatically decreased the eclosion rates of  
156 homozygous, but not heterozygous, *park*<sup>25</sup> mutants relative to controls. (**Fig. 4**,  $P < 0.0001$ ). Second,  
157 there was no effect related to feces source (control or *park*<sup>25</sup>) that were used to create the bacterial  
158 cultures on eclosion rate regardless of the genotype that received the bacterial culture. These results  
159 were not due to a limited number of homozygous pupae present in the tubes, as all experimental vials  
160 with *park*<sup>25</sup> pupae contained approximately 30% *park*<sup>25</sup> homozygous pupae, while the axenic  
161 experimental vials had the most pupae/vial (**Supplementary Table S2**). The most significant source of  
162 variation was fly genotype ( $P < 0.0001$ ), accounting for 65% of the variation, with the bacterial status of  
163 the fly contributing only 0.23% to variation ( $P = 0.0082$ ). No difference in the eclosion rates were  
164 observed between the axenic and the two gnotobiotic treatments regardless of fly genotype (all  $P >$   
165 0.52). Together, these results suggest that variation in the cultured bacteria in fly feces does not  
166 contribute to the variation in the eclosion rates observed in *park*<sup>25</sup> mutant and control flies when reared  
167 on fly feces-seeded vials.

168 In contrast to whole-body flies, the major difference in the microbiota of fecal samples collected  
169 from *park*<sup>25</sup> mutant and control flies was attributed to microbial identity, not abundance (**Table 2** where

170 unweighted Unifrac, but not weighted Unifrac or Bray-Curtis distance metrics showed significant  
171 variation in the community composition with host genotype; corresponding PCoA plots are in **Figure 5**  
172 and **Supplementary Figure S7**. Analyzed feces were collected from independent experiments and,  
173 because of the inconstant microbiota, the results cannot be directly compared to other experiments  
174 here. Analysis of the fecal samples and siblings of the fecal donors revealed that the microbiota  
175 composition varied with respect to both the host genotype and the sample type (fly or feces). For  
176 example, the feces was dominated by an *Enterococcus* ASV (**Fig. 5A**) and the four AAB ASVs that were  
177 most abundant in the flies (**Fig. 3, Supplementary Table S3 and Supplementary Fig. S8**) were detected  
178 at very low levels. The low level of AAB reads in the feces suggests that AAB DNA in living or dead cells  
179 persists poorly between the location of abundant bacterial cells in the flies and collection of < 24 h old  
180 feces. Additionally or alternatively, *Enterococcus* cells may grow rapidly in the feces since there is little  
181 evidence of their abundance in live flies, or *Enterococcus* DNA may survive gut transit well. Together,  
182 these results suggest that the role of P generation defecation in establishing the F1 adult microbiota in  
183 the flies in our study, and perhaps flies generally, is incompletely understood.

#### 184 **Variation in the bacterial microbiota of *D. melanogaster* influences eclosion success in *park*<sup>25</sup> mutants**

185 To understand the extent to which bacterial microbiota of *D. melanogaster* influence eclosion  
186 success in homozygous and heterozygous *park*<sup>25</sup> mutants, we compared eclosion rates of mono-  
187 associated flies. Previous gnotobiotic experiments provided no direct evidence that variation in the  
188 bacterial communities of the flies influenced fly eclosion in these *park*<sup>25</sup> mutants. In contrast, fecal  
189 transfer experiments suggested that microbiome changes did affect fly eclosion. Similar to our results  
190 with cultured feces, we observed dramatically reduced eclosion in homozygous *park*<sup>25</sup> flies compared to  
191 heterozygous *park*<sup>25</sup> flies (**Fig. 6**). Unlike with cultured feces, there were differences in fly eclosion rates  
192 when they were axenic or colonized with a combination of 4 bacterial species cultured from flies in  
193 Ithaca, NY<sup>27</sup> (heterozygous *park*<sup>25</sup>: P = 0.0025, homozygous *park*<sup>25</sup>: P < 0.0001) or individually with

194 *Acetobacter tropicalis* (heterozygous *park*<sup>25</sup>: P = 0.0088, homozygous *park*<sup>25</sup>: P = 0.0456). Additionally,  
195 there was a significant fly genotype \* bacterial treatment interaction (two-way ANOVA, P < 0.0001): the  
196 combination and *A. tropicalis* treatments led to higher eclosion survival than the axenic treatment in  
197 *park*<sup>25</sup> homozygous flies; but lower survival than axenic flies for *park*<sup>25</sup> heterozygous flies. Taken  
198 together, these results confirm that variation in the bacterial microbiota of *park*<sup>25</sup> flies can contribute to  
199 variation in a key survival phenotype, eclosion success.

200

## 201 **Discussion**

202         There have been many studies showing alterations in the fecal microbiota of PD patients  
203 compared to control populations<sup>10</sup>. To our knowledge, there has only been a single study that has  
204 looked at the microbiota in a fly PD model (*PINK1*<sup>B9</sup>) that also identified differences between the  
205 microbiome of the PD model and control flies<sup>21</sup>. Under condition-matched conventional rearing, our  
206 *park*<sup>25</sup> PD model fly microbiota was considerably different from the control fly (**Fig. 3**), with significant  
207 differences in the abundance of AAB and LAB. Fly sex was not a determinant of variation in microbiota  
208 composition. Alterations in microbiota were observed in conventionally-reared flies and therefore  
209 factors such as inconstant exposure to or acquisition of distinct sets of microbes in the different vials  
210 from which samples were drawn could contribute to the observed effects; though the level of  
211 replication and matched rearing conditions of the flies suggests potential influences of host genotype on  
212 fly microbiota composition. Future experiments with gnotobiotic flies could conclusively rule out  
213 environmental effects but could be challenging because of the low survival rates of the homozygous  
214 *park*<sup>25</sup> flies (**Fig. 4**).

215         Presence of the endosymbiont *Wolbachia* has been associated with lower counts of *Acetobacter*  
216 *spp.* in other flies<sup>28</sup> and is positively correlated with worsening phenotypes in a fly model of Alzheimer's

217 disease. In agreement with this, a recent report linked *Wolbachia* and neurodegenerative disease  
218 severity in *Drosophila* by showing that administration of a *Lactobacillus* probiotic increased *Acetobacter*  
219 abundance, lowered *Wolbachia* titers, and ameliorated Alzheimer's disease phenotypes<sup>29</sup>. Thus, the  
220 presence of *Wolbachia* could be a factor contributing to differences between the microbiota of our  
221 control and *park*<sup>25</sup> flies. Our analysis identified that the *Wolbachia* status of our control and mutant  
222 stocks was not congruent. In consideration of the fecal transfer, it is important to recognize that  
223 *Wolbachia* are intracellular endosymbionts that are transmitted between generations via the germ line  
224 and not fecal transfer<sup>22</sup>. This was validated by sequence analysis which demonstrated that *Wolbachia*  
225 were not represented in the sequenced fecal samples, confirming their absence and irrelevance to  
226 observed functional effects of the *park*<sup>25</sup> fecal transfer (**Fig. 5**). Our data indicate that *Acetobacter spp.*  
227 are reduced in our control flies both in diversity and abundance (**Fig. 3** and **Supplementary Fig. S6**). The  
228 data do raise the question if the differences in the eclosion of control and *park*<sup>25</sup> flies is due to their  
229 discordant *Wolbachia* status. While some influence on eclosion is possible, *Wolbachia* is unlikely to be  
230 the sole contributor to this observed difference as PD phenotypes have been detected in another  
231 laboratory using the same *park*<sup>25</sup> mutant and a *Wolbachia*-concordant control strain (<sup>5</sup>, unpublished  
232 data). The potential direct and indirect (through the microbiota) influence(s) *Wolbachia* has on *park*<sup>25</sup>  
233 mutant development requires further analysis.

234 We used fecal transfers to assess whether the microbiota influences *park*<sup>25</sup> mutant eclosion  
235 success. Microbiota studies typically rear dechorionated embryos free of bacteria or with a defined  
236 bacterial inoculum<sup>23</sup>, but we adopted an alternate fecal transfer approach for two reasons. First, fecal  
237 transfers have successfully identified microbiota effects in other studies (e.g., <sup>30</sup>) and provided a  
238 straightforward method to use in initial functional explorations. Second, as shown in **Figures 4** and **6**, the  
239 viability of axenic and gnotobiotic *park*<sup>25</sup> flies is very low, which makes this process extremely difficult  
240 and impedes experimentation. We do not know the cause of this high mortality rate, but it appears to

241 be related to the dechoriation process. Alternative approaches that avoid the dechoriation step  
242 are available, but these approaches also have limitations. For example, while raising axenic fly stocks for  
243 several generations after dechoriation the stocks are vulnerable to bacterial contamination, requiring  
244 the use of antibiotics which can alter but not necessarily eliminate all colonizing microorganisms. One  
245 successful recent approach fed bacteria to newly eclosed (and presumably bacteria-depleted) *PINK1*  
246 mutant flies<sup>21</sup>, which might be a more high-throughput approach.

247         The exposure of hatching larvae to *park*<sup>25</sup> fly feces led to dramatically reduced fly eclosion  
248 success than did exposure to control feces. Conversely, when we inoculated flies with cultured feces  
249 there was no difference in the effect on fly eclosion. The difference in outcome between the two  
250 experiments suggests that different effectors are transmitted, or possibly diluted, when the feces is  
251 cultured first versus when it is directly deposited. We hypothesized that the fecal microbiota would  
252 largely reflect the adult fly microbiota and that culturing feces versus direct deposition would lead to  
253 similar outcomes. However, we detected no difference between the fecal microbiota of mutant and  
254 control flies even though adult mutant and control flies had a different microbiota composition. In this  
255 regard, the outcomes of the fecal transfer vs culture experiments were congruent: there was only a  
256 difference in recipient phenotypes when there was a difference in the source's microbiota composition.  
257 Altered phenotypes following the transfer of direct but not cultured feces could be also be due to an  
258 effector that is abiotic or non-bacterial (e.g., fungal); or it may be that the culture step abates the effect.  
259 Culture in standard laboratory medium may select for certain strains in ways that does not occur in the  
260 fly diet, leading to differences in identity and abundance of key microbiota members. To address these  
261 potential limitations, the bacterial mono-association experimental approach was critical to  
262 understanding whether variation in bacterial microbiota can alter the eclosion success of *park*<sup>25</sup> flies in a  
263 genotype-dependent (heterozygous vs. homozygous) manner.

264 We found that there is a functional consequence with the feces transfer, in that the *park*<sup>25</sup> feces  
265 had a negative impact on pupation and eclosion on both the control and *park*<sup>25</sup> flies; however, the  
266 homozygous *park*<sup>25</sup> flies appeared to be affected the most. It is possible that homozygous *park*<sup>25</sup> flies  
267 are more susceptible to *park*<sup>25</sup> feces due to their general weak state. It is established that *park*<sup>25</sup> flies  
268 have reduced mitochondrial function and deficiency in energy production<sup>5,7</sup>. Additionally, axenic flies  
269 have disrupted insulin-like signaling and glucose regulation compared to microbiota-colonized  
270 flies<sup>24,31,32</sup>. Therefore, axenic homozygous *park*<sup>25</sup> flies may have compounding, additive deficiencies that  
271 reduce their ATP production during eclosion, which is likely a high energy-requiring process. In support  
272 of this idea, 34.9% of all axenic *park*<sup>25</sup> pupae were found dead and stuck in the process of eclosing,  
273 compared to 1.6% of the axenic control flies or 3.4% of the axenic heterozygous *park*<sup>25</sup> flies. Moreover,  
274 when the *park*<sup>25</sup> homozygous flies had two fecal-derived gnotobiotic treatments, the rate of being stuck  
275 in eclosion reduced to 15.9% with the control bacteria and 13.4% with the *park*<sup>25</sup> bacteria. This reduced  
276 rate of incomplete eclosion in the gnotobiotic populations might have masked the negative effect of the  
277 *park*<sup>25</sup> bacteria on eclosion in these flies compared to the axenic controls, however, since both  
278 gnotobiotic groups had similar reductions in getting stuck, this is unlikely.

279 Despite the large number of studies showing an altered fecal microbiome in PD patients, there  
280 have been very few studies demonstrating that the PD microbiome has functional consequences. The  
281 most compelling functional study utilized PD model mice that had a fecal transplant from PD and  
282 healthy control patients. The PD microbiome transplant mice showed an increase in motor dysfunction  
283 and alpha-synuclein aggregation<sup>11</sup>. Our fecal transfer experiments, like another recent *Drosophila* PD  
284 model functional microbiome study<sup>21</sup>, did not directly manipulate the microbiome to a specifically-  
285 defined microbiota composition but we do use mono-association experiments to demonstrate species-  
286 specific influences of the associated microorganisms. Our study is the first to indicate that there might  
287 be a specific microbiota \* fly genotype effect that might also be occurring with the PD microbiome and

288 homozygous *park*<sup>25</sup> flies. This type of specific microbiota \* fly genotype interaction is known to happen  
289 with *Wolbachia*<sup>33</sup>. This study adds to the small group of publications that indicate that the altered PD  
290 microbiome negatively affects biological processes in the host, which has major implications for PD  
291 patients.

292

## 293 **Methods**

### 294 ***Drosophila* Stocks and Maintenance**

295 Mutant, *park*<sup>25</sup>, flies were provided by Dr. Leo Pallanck at the University of Washington. This  
296 mutant stock was derived from *w*<sup>1118</sup> control flies, which were obtained from the Bloomington  
297 *Drosophila* Stock Center (Indiana University). In all experiments, *w*<sup>1118</sup> flies were used as the control for  
298 the *park*<sup>25</sup> flies. The *park*<sup>25</sup> stock in our laboratory has been backcrossed with the *w*<sup>1118</sup> stock so that all  
299 chromosomes are from the *w*<sup>1118</sup> background. The *park*<sup>25</sup> chromosome is balanced over the TM6C  
300 balancer, allowing for identification of homozygous and heterozygous flies through use of the *Tubby*  
301 gene phenotype. All fly stocks were raised on standard cornmeal-molasses diet at 25°C in a 14/10-hour  
302 light cycle.

### 303 **Fecal transfer**

304 In each of three separate experiments, five separate food vials for each fly genotype were  
305 seeded with forty males of that genotype to allow flies to deposit their feces on the food. Males were  
306 used so that no embryos were laid on the food. All males were over the age of three days to ensure that  
307 they had an established microbiome<sup>34</sup>. A random mix of both homozygous and heterozygous *park*<sup>25</sup>  
308 male flies were used as the fecal donors for the *park*<sup>25</sup> feces. Four days post-seeding, 60 embryos of the

309 specific genotype were placed on the feces-prepared food. The embryos were collected and counted as  
310 described below.

### 311 **Embryo collection**

312 Fly stocks (control and *park*<sup>25</sup>—heterozygous and homozygous) were placed in square  
313 polypropylene fly bottles with a molasses “puck” as the lid. The molasses puck was a 35 mm petri dish  
314 cover filled with a molasses-agar media (200 mL ddH<sub>2</sub>O, 6.24 g drosogagar [Genesee Scientific], 2 mL  
315 Tegosept, and 50 mL of molasses). The bottles were stored upside down, so the puck was at the bottom,  
316 while the top of the bottle had small holes for air transfer. Yeast paste was put on the inside of the  
317 bottle to help stimulate oogenesis. Flies were allowed to lay their embryos for <24 hours at 25°C.  
318 Embryo collection was performed by wetting a paintbrush with ddH<sub>2</sub>O and carefully brushing the  
319 embryos from the molasses-agar. The embryos were then washed off the puck directly into a 1.5 mL  
320 centrifuge tube and were rinsed with ddH<sub>2</sub>O for a total of three washes. After washing, the embryos  
321 were pipetted into a glass spot plate in one of the three wells. Under a stereo microscope, 60 embryos  
322 were counted and placed in one of the empty wells with < 0.5 ml water. A fine-tipped paintbrush was  
323 used to paint the 60 embryos onto the surface of the fecal-prepared food. A different paintbrush was  
324 used for each fly genotype to prevent bacterial transfer between genotypes during embryo deposition.  
325 The embryos were collected for six days, with new parental fly populations being introduced every two  
326 days to produce three biological replicates with each biological replicate having two technical replicates.

### 327 **Axenic and gnotobiotic experiments**

328 We reared flies with bacteria cultured directly from fly feces beginning with axenic fly embryos.  
329 Axenic embryos were derived as described previously<sup>23</sup>. Briefly, control and *park*<sup>25</sup> embryos were  
330 collected as above and suspended in a 0.6% sodium hypochlorite solution for 2.5 minutes. These  
331 embryos were then transferred to fresh 0.6% sodium hypochlorite solution in a sterile hood to

332 dechorionate the embryos. The sterile, dechorionated embryos were collected with a sterile paintbrush  
333 and approximately 60 embryos were brushed onto sterile food. These embryos were either maintained  
334 as axenic or inoculated with  $5 \times 10^5$  CFUs from control or *park<sup>25</sup>* fecal bacterial cultures, or from individual  
335 bacterial strains, including, *Lactobacillus brevis*, *Lactiplantibacillus plantarum*, *Acetobacter pomorum*, or  
336 *Acetobacter tropicalis*.

337 To produce the fecal bacterial cultures, feces were collected from the *park<sup>25</sup>* and control fly  
338 embryo collection bottles by scraping the feces off with a sterile toothpick to inoculate Luria-Bertani (LB)  
339 and modified deMan-Rogosa-Sharpe (mMRS) medium. These cultures were grown at 30°C with aeration  
340 for 16 hrs. To preferentially cultivate aerotolerant microbes, separate MRS cultures were grown in  
341 loosely capped tubes with no shaking for 16 hrs. To generate the microbiome inoculum, each culture  
342 was normalized to  $10^7$  mL<sup>-1</sup>, combined in equivalent ratios, and 50 µl containing  $5 \times 10^5$  total CFUs was  
343 used to inoculate the sterile embryos. Control axenic embryos were collected each day for four days  
344 with a minimum of eight tubes/day, while the axenic *park<sup>25</sup>* embryos were collected each day for five  
345 days with a minimum of 28 tubes/day. The bacterial culture embryos were collected each day for four  
346 days with a minimum of 10 tubes/day for control embryos or 14 tubes/day for the *park<sup>25</sup>* embryos.

347 To confirm axenic flies were truly bacteria-free, pools of five whole-body adult axenic flies from  
348 each axenic control vial were homogenized at the end of each experiment and cultured on LB and  
349 duplicate MRS plates (one incubated with standard atmospheric conditions, one in microoxic conditions  
350 in a sealed, CO<sub>2</sub>-flooded chamber) at 30°C. If > 10 CFU/fly were detected, those flies were deemed non-  
351 axenic and removed from the analysis.

### 352 **Pupation and eclosure measurements**

353 Newly developed pupae were counted on days 5, 6, 7 and 8 post-embryo collection. Due to the  
354 *Tubby* mutation on the TM6C balancer chromosome present in the heterozygous *park<sup>25</sup>* flies,

355 homozygous and heterozygous *park*<sup>25</sup> flies were differentiated. Although analyzed separately, these  
356 two pupal populations account for the full 60 *park*<sup>25</sup> embryos painted in the fecal transfer experiments.  
357 Individual fly eclosion was quantified on days 9, 10, 11 and 12 post-embryo collection.

### 358 **16S sequencing and analysis**

359 We prepared and analyzed DNA samples for 16S rRNA marker gene analysis as done  
360 previously<sup>26,28</sup>. Sequencing libraries were prepared by extracting DNA from pools of 10 flies using the  
361 Zymo Quick-DNA fecal/soil microbe kit (D6011, Zymo, Irvine, CA). Then, the V4 region of the extracted  
362 DNA was amplified and sequenced using a dual-barcoding method described by Kozich<sup>35</sup>, with the  
363 exception of substituting Accuprime PFX DNA polymerase reagents for Accuprime PFX Supermix. The  
364 Invitrogen SequalPrep Normalization kit was used to normalize samples into pools of 96 samples (in  
365 some cases, the samples were normalized as part of a pool with samples not published in this study).  
366 Then, fragments in the size range of 250-450 nucleotides were size-selected using a BluePippin (BYU  
367 DNA Sequencing Center). Finally, samples in this study were sequenced on partial lanes of a MiSeq using  
368 500 cycle chemistry (paired-end 2 x 250, BioDesign Institute at Arizona State University)

369 Sequenced reads were analyzed using QIIME2<sup>36</sup> and R. The reads were trimmed based on quality  
370 scores, denoised and dereplicated using DADA2<sup>37</sup> to call individual amplicon sequence variants (ASVs),  
371 and taxonomy was assigned using the GreenGenes classifier<sup>38,39</sup>. To enable the calculation of Unifrac  
372 beta-diversity metrics<sup>40,41</sup>, a phylogeny of all ASVs was constructed<sup>42</sup> based on mafft alignment<sup>43</sup>. For  
373 some analyses, *Wolbachia* reads were pre-filtered out so that reproductive tract symbionts were not  
374 included in the analysis. Before performing beta-diversity analyses, samples were normalized to varying  
375 read thresholds that maximized the number of reads per sample and the number of samples retained:  
376 350 (**Fig. 3**), 3000 (**Fig. 5**), and 399 (**Supplementary Fig. S8**). Permutational multivariate analysis of  
377 variance (PERMANOVA) of Bray Curtis distances and of unweighted and weighted Unifrac distances

378 were used to test for host genotype and sex-dependent variation in microbiota composition<sup>44</sup>. We also  
379 used Analysis of Composition of Microbiomes (ANCOM) to test for differences in the abundances of  
380 specific individual or groups of ASVs<sup>45</sup>.

381 One sample was removed from **Figure 3** analyses because it was almost exclusively  
382 enterococcus. Removing it did not change the significance of any comparisons but did reduce noise.  
383 Analyses of the data that include this sample are presented in **Supplementary Figure S2**.

### 384 **Development Statistical Analysis**

385 Statistical analyses were performed by using One-Way and Two-Way ANOVA, with post-hoc  
386 Tukey's, Sidak's, or Dunnett's tests to determine differences between the arcsin transformed  
387 percentages of each group by GraphPad Prism 9. All other data analysis was in RStudio version 1.3.1093  
388 using R version 3.6.3 or the terminal. All graphs display the mean  $\pm$  the standard error of the mean.  
389 Details on each test performed and their results are presented in the results section or legends.

390

### 391 **Author contributions**

392 Study conception and design: G.B.C.; Study supervision: G.B.C., J.M.C., and S.B.K.; Data collection: J.P.-C.,  
393 D.R.H., T.B.C., Z.S.P., S.A.T.; Data and statistical analyses: G.B.C., E.M.M., and J.M.C.; Writing and  
394 revision: G.B.C., J.M.C. and S.B.K.

395

### 396 **Data availability**

397 The reads are publicly available at the NCBI SRA under ACCESSION FORTHCOMING.

398

399 **Code availability**

400 Code used for this study can be obtained by contacting the corresponding author.

401

402 **Competing interests**

403 The authors declare no competing interests.

## 404   **References**

- 405   1       *Statistics - Parkinson's Foundation*, <[https://www.parkinson.org/Understanding-](https://www.parkinson.org/Understanding-Parkinsons/Statistics)  
406       [Parkinsons/Statistics](https://www.parkinson.org/Understanding-Parkinsons/Statistics)> (2020).
- 407   2       Hernandez, D. G., Reed, X. & Singleton, A. B. Genetics in Parkinson disease: Mendelian versus  
408       non-Mendelian inheritance. *J Neurochem* **139 Suppl 1**, 59-74, doi:10.1111/jnc.13593 (2016).
- 409   3       Lücking, C. B. *et al.* Association between early-onset Parkinson's disease and mutations in the  
410       parkin gene. *N Engl J Med* **342**, 1560-1567, doi:10.1056/NEJM200005253422103 (2000).
- 411   4       Chambers, R. P. *et al.* Nicotine increases lifespan and rescues olfactory and motor deficits in a  
412       Drosophila model of Parkinson's disease. *Behavioural brain research* **253**, 95-102,  
413       doi:10.1016/j.bbr.2013.07.020 (2013).
- 414   5       Greene, J. C. *et al.* Mitochondrial pathology and apoptotic muscle degeneration in Drosophila  
415       parkin mutants. *Proc Natl Acad Sci U S A* **100**, 4078-4083, doi:10.1073/pnas.0737556100 (2003).
- 416   6       Whitworth, A. J. *et al.* Increased glutathione S-transferase activity rescues dopaminergic neuron  
417       loss in a Drosophila model of Parkinson's disease. *Proc Natl Acad Sci U S A* **102**, 8024-8029,  
418       doi:10.1073/pnas.0501078102 (2005).
- 419   7       Cackovic, J. *et al.* Vulnerable Parkin Loss-of-Function Drosophila Dopaminergic Neurons Have  
420       Advanced Mitochondrial Aging, Mitochondrial Network Loss and Transiently Reduced  
421       Autophagosome Recruitment. *Front Cell Neurosci* **12**, 39, doi:10.3389/fncel.2018.00039 (2018).
- 422   8       Hewitt, V. L. & Whitworth, A. J. Mechanisms of Parkinson's Disease: Lessons from Drosophila.  
423       *Curr Top Dev Biol* **121**, 173-200, doi:10.1016/bs.ctdb.2016.07.005 (2017).
- 424   9       Fasano, A., Visanji, N. P., Liu, L. W., Lang, A. E. & Pfeiffer, R. F. Gastrointestinal dysfunction in  
425       Parkinson's disease. *Lancet Neurol* **14**, 625-639, doi:10.1016/S1474-4422(15)00007-1 (2015).

- 426 10 Boertien, J. M., Pereira, P. A. B., Aho, V. T. E. & Scheperjans, F. Increasing Comparability and  
427 Utility of Gut Microbiome Studies in Parkinson's Disease: A Systematic Review. *J Parkinsons Dis*  
428 **9**, S297-S312, doi:10.3233/JPD-191711 (2019).
- 429 11 Sampson, T. R. *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model  
430 of Parkinson's Disease. *Cell* **167**, 1469-1480.e1412, doi:10.1016/j.cell.2016.11.018 (2016).
- 431 12 Wong, C. N., Ng, P. & Douglas, A. E. Low-diversity bacterial community in the gut of the fruitfly  
432 *Drosophila melanogaster*. *Environ Microbiol* **13**, 1889-1900, doi:10.1111/j.1462-  
433 2920.2011.02511.x (2011).
- 434 13 Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. & Kopp, A. Bacterial communities of diverse  
435 *Drosophila* species: ecological context of a host-microbe model system. *PLoS genetics* **7**,  
436 e1002272, doi:10.1371/journal.pgen.1002272 (2011).
- 437 14 Staubach, F., Baines, J. F., Kunzel, S., Bik, E. M. & Petrov, D. A. Host species and environmental  
438 effects on bacterial communities associated with *Drosophila* in the laboratory and in the natural  
439 environment. *PLoS One* **8**, e70749, doi:10.1371/journal.pone.0070749 (2013).
- 440 15 Chaston, J. M., Dobson, A. J., Newell, P. D. & Douglas, A. E. Host Genetic Control of the  
441 Microbiota Mediates the *Drosophila* Nutritional Phenotype. *Appl Environ Microbiol* **82**, 671-679,  
442 doi:10.1128/AEM.03301-15 (2016).
- 443 16 Carmody, R. N. *et al.* Diet dominates host genotype in shaping the murine gut microbiota. *Cell*  
444 *Host Microbe* **17**, 72-84, doi:10.1016/j.chom.2014.11.010 (2015).
- 445 17 Goodrich, J. K. *et al.* Human genetics shape the gut microbiome. *Cell* **159**, 789-799,  
446 doi:10.1016/j.cell.2014.09.053 (2014).
- 447 18 Winans, N. J. *et al.* A genomic investigation of ecological differentiation between free-living and  
448 *Drosophila*-associated bacteria. *Mol Ecol* **26**, 4536-4550, doi:10.1111/mec.14232 (2017).

- 449 19 Pais, I. S., Valente, R. S., Sporniak, M. & Teixeira, L. *Drosophila melanogaster* establishes a  
450 species-specific mutualistic interaction with stable gut-colonizing bacteria. *PLoS biology* **16**,  
451 e2005710, doi:10.1371/journal.pbio.2005710 (2018).
- 452 20 Gould, A. L. *et al.* Microbiome interactions shape host fitness. *Proc Natl Acad Sci U S A* **115**,  
453 E11951-E11960, doi:10.1073/pnas.1809349115 (2018).
- 454 21 Xu, Y. *et al.* EGCG ameliorates neuronal and behavioral defects by remodeling gut microbiota  
455 and TotM expression in *Drosophila* models of Parkinson's disease. *FASEB J* **34**, 5931-5950,  
456 doi:10.1096/fj.201903125RR (2020).
- 457 22 Werren, J. H., Baldo, L. & Clark, M. E. Wolbachia: master manipulators of invertebrate biology.  
458 *Nat Rev Microbiol* **6**, 741-751, doi:10.1038/nrmicro1969 (2008).
- 459 23 Koyle, M. L. *et al.* Rearing the Fruit Fly *Drosophila melanogaster* Under Axenic and Gnotobiotic  
460 Conditions. *Journal of visualized experiments : JoVE*, doi:10.3791/54219 (2016).
- 461 24 Shin, S. C. *et al.* *Drosophila* microbiome modulates host developmental and metabolic  
462 homeostasis via insulin signaling. *Science* **334**, 670-674, doi:10.1126/science.1212782 (2011).
- 463 25 Wong, A. C., Chaston, J. M. & Douglas, A. E. The inconstant gut microbiota of *Drosophila* species  
464 revealed by 16S rRNA gene analysis. *ISME J* **7**, 1922-1932, doi:10.1038/ismej.2013.86 (2013).
- 465 26 Walters, A. W. *et al.* The microbiota influences the *Drosophila melanogaster* life history strategy.  
466 *Mol Ecol* **29**, 639-653, doi:10.1111/mec.15344 (2020).
- 467 27 Newell, P. D. *et al.* In vivo function and comparative genomic analyses of the *Drosophila* gut  
468 microbiota identify candidate symbiosis factors. *Front Microbiol* **5**, 576,  
469 doi:10.3389/fmicb.2014.00576 (2014).
- 470 28 Rudman, S. M. *et al.* Microbiome composition shapes rapid genomic adaptation of. *Proc Natl*  
471 *Acad Sci U S A* **116**, 20025-20032, doi:10.1073/pnas.1907787116 (2019).

- 472 29 Tan, F. H. P. *et al.* *Lactobacillus* probiotics improved the gut microbiota profile of a *Drosophila*  
473 *melanogaster* Alzheimer's disease model and alleviated neurodegeneration in the eye. *Benef*  
474 *Microbes* **11**, 79-89, doi:10.3920/BM2019.0086 (2020).
- 475 30 Zare, A., Johansson, A. M., Karlsson, E., Delhomme, N. & Stenberg, P. The gut microbiome  
476 participates in transgenerational inheritance of low-temperature responses in *Drosophila*  
477 *melanogaster*. *FEBS Lett* **592**, 4078-4086, doi:10.1002/1873-3468.13278 (2018).
- 478 31 Ridley, E. V., Wong, A. C., Westmiller, S. & Douglas, A. E. Impact of the resident microbiota on  
479 the nutritional phenotype of *Drosophila melanogaster*. *PLoS One* **7**, e36765,  
480 doi:10.1371/journal.pone.0036765 (2012).
- 481 32 Storelli, G. *et al.* *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating  
482 hormonal signals through TOR-dependent nutrient sensing. *Cell Metab* **14**, 403-414,  
483 doi:10.1016/j.cmet.2011.07.012 (2011).
- 484 33 Simhadri, R. K. *et al.* The Gut Commensal Microbiome of *Drosophila melanogaster* Is Modified by  
485 the Endosymbiont *Wolbachia*. *mSphere* **2**, doi:10.1128/mSphere.00287-17 (2017).
- 486 34 Blum, J. E., Fischer, C. N., Miles, J. & Handelsman, J. Frequent replenishment sustains the  
487 beneficial microbiome of *Drosophila melanogaster*. *mBio* **4**, e00860-00813,  
488 doi:10.1128/mBio.00860-13 (2013).
- 489 35 Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a  
490 dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on  
491 the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* **79**, 5112-5120,  
492 doi:10.1128/AEM.01043-13 (2013).
- 493 36 Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science  
494 using QIIME 2. *Nat Biotechnol* **37**, 852-857, doi:10.1038/s41587-019-0209-9 (2019).

495 37 Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data.  
496 *Nature methods* **13**, 581-583, doi:10.1038/nmeth.3869 (2016).

497 38 McDonald, D. *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and  
498 evolutionary analyses of bacteria and archaea. *ISME J* **6**, 610-618, doi:10.1038/ismej.2011.139  
499 (2012).

500 39 Bokulich, N. A. *et al.* Optimizing taxonomic classification of marker-gene amplicon sequences  
501 with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**, 90, doi:10.1186/s40168-018-0470-z  
502 (2018).

503 40 Lozupone, C. & Knight, R. UniFrac: a new phylogenetic method for comparing microbial  
504 communities. *Appl Environ Microbiol* **71**, 8228-8235, doi:10.1128/AEM.71.12.8228-8235.2005  
505 (2005).

506 41 Lozupone, C. A., Hamady, M., Kelley, S. T. & Knight, R. Quantitative and qualitative beta diversity  
507 measures lead to different insights into factors that structure microbial communities. *Appl*  
508 *Environ Microbiol* **73**, 1576-1585, doi:10.1128/AEM.01996-06 (2007).

509 42 Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2--approximately maximum-likelihood trees for  
510 large alignments. *PLoS One* **5**, e9490, doi:10.1371/journal.pone.0009490 (2010).

511 43 Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid multiple sequence  
512 alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059-3066,  
513 doi:10.1093/nar/gkf436 (2002).

514 44 Oksanen, J. *et al.* (Retrieved from <https://CRAN.R-project.org/package=vegan>, 2018).

515 45 Mandal, S. *et al.* Analysis of composition of microbiomes: a novel method for studying microbial  
516 composition. *Microb Ecol Health Dis* **26**, 27663, doi:10.3402/mehd.v26.27663 (2015).

517

518 **Tables**

519 **Table 1. Genotype and sex-dependent differences in microbiota composition of control and *park*<sup>25</sup>**  
 520 **mutants.**

521 Effects of fly genotype (G), fly sex (S), fly vial (V), and the G \* S interaction (GS) are shown along with  
 522 residuals (R) and totals (T) as determined by PERMANOVA. PERMANOVA values are degrees of freedom  
 523 (df), sum of squares (SS), mean squares (MS), F statistic (F), R<sup>2</sup> value (R<sup>2</sup>), and P-value (P).

524

		Weighted Unifrac					Unweighted Unifrac					Bray Curtis				
	df	SS	MS	F	R <sup>2</sup>	P	SS	MS	F	R <sup>2</sup>	P	SS	MS	F	R <sup>2</sup>	P
G	2	0.37	0.19	72.18	0.71	0.001	0.20	0.10	6.12	0.21	0.001	6.71	3.36	111.90	0.79	0.001
S	1	0.00	0.00	1.38	0.01	0.24	0.02	0.02	1.25	0.02	0.29	0.04	0.04	1.33	0.00	0.25
V	3	0.04	0.01	5.02	0.07	0.002	0.04	0.01	0.90	0.05	0.54	0.47	0.16	5.27	0.06	0.001
GS	2	0.00	0.00	0.80	0.01	0.49	0.02	0.01	0.73	0.02	0.70	0.02	0.01	0.41	0.00	0.79
R	41	0.11	0.00	0.20			0.67	0.02	0.70			1.23	0.03	0.15		
T	49	0.53	1.00				0.96	1.00				8.48	1.00			

525

526 **Table 2. Genotype-dependent differences in fecal microbiota composition of control and *park*<sup>25</sup>**  
 527 **mutants.**

528 Effects of fly genotype (G), fly vial (V), residuals (R), and totals (T) as determined by PERMANOVA.

529 PERMANOVA values are degrees of freedom (df), sum of squares (SS), mean squares (MS), F statistic (F),

530 R<sup>2</sup> value (R<sup>2</sup>), and P-value (P).

531

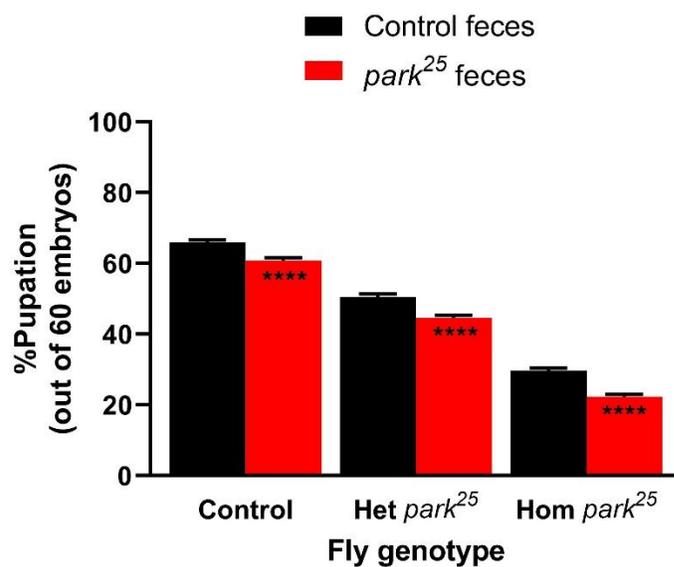
		Weighted Unifrac					Unweighted Unifrac					Bray-Curtis				
	df	SS	MS	F	R <sup>2</sup>	P	SS	MS	F	R <sup>2</sup>	P	SS	MS	F	R <sup>2</sup>	P
G	1	0.02	0.02	2.34	0.09	0.061	0.38	0.38	2.60	0.10	0.003	0.24	0.24	1.82	0.08	0.10
V	10	0.11	0.01	1.24	0.46	0.26	1.54	0.15	1.05	0.42	0.36	1.16	0.12	0.89	0.39	0.61
R	12	0.10	0.01	0.45			1.76	0.15	0.48			1.57	0.13	0.53		
T	23	0.23	1.00				3.69	1.00				2.96	1.00			

532

533 **Figures**

534 **Figure 1. Feces from *park<sup>25</sup>* flies reduces total pupation rate.**

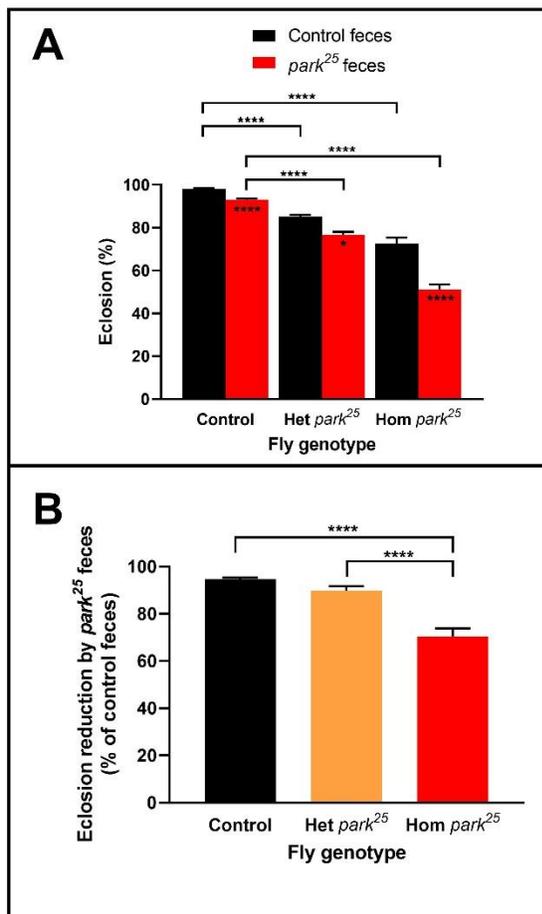
535 Total pupation rates were calculated in control, heterozygous (Het) *park<sup>25</sup>* and homozygous (Hom)  
536 *park<sup>25</sup>* flies from the 60 embryos that were placed on food that had control or *park<sup>25</sup>* feces present. Data  
537 are presented as mean and SEM. Asterisks represent the results of a post-hoc Sidak's multiple  
538 comparisons test comparing the two feces groups within each fly genotype (\*\*\*\* = P < 0.0001). Results  
539 are from 45 separate vials in each group.



540

541 **Figure 2. Eclosion rates are reduced in all fly genotypes but more in homozygous *park*<sup>25</sup> flies with**  
542 ***park*<sup>25</sup> feces transfer.**

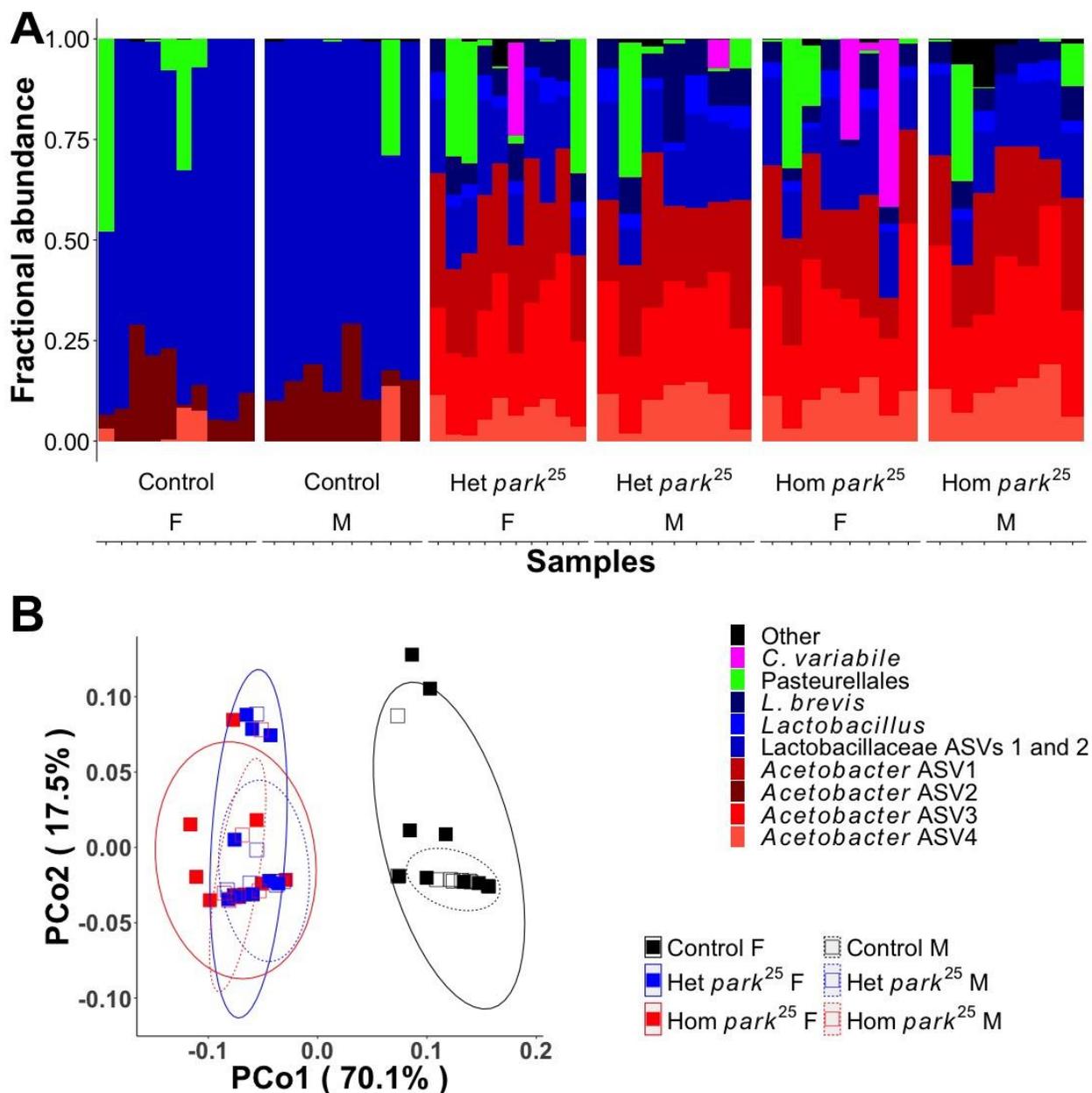
543 **A)** Total eclosion rates were determined in control, heterozygous (Het) *park*<sup>25</sup> and homozygous (Hom)  
544 *park*<sup>25</sup> flies based on the total number of pupae for each genotype. **B)** The relative eclosion reduction  
545 caused by the *park*<sup>25</sup> fecal transfer, calculated as a percentage of the control feces eclosion, was  
546 determined. Data are presented as mean and SEM. Asterisks represent the results of a post-hoc  
547 Tukey's multiple comparisons test. Asterisks inside the bars compare the two feces groups within each  
548 fly genotype. \* = P < 0.05 and \*\*\*\* = P < 0.0001. Results are from 45 separate vials in each group.



549

550 **Figure 3. The microbiota of control and *park*<sup>25</sup> flies.**

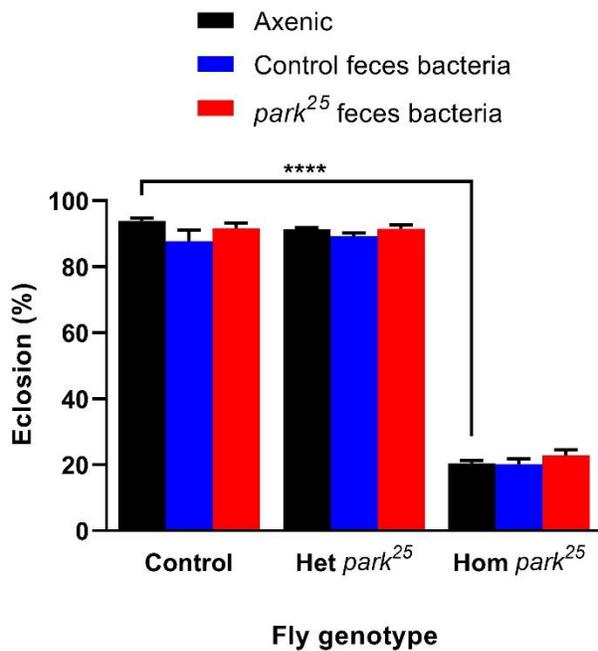
551 **A)** Taxon plot of control and *park*<sup>25</sup> flies, separated by sex. Mutants of *park*<sup>25</sup> were distinguished as  
 552 homozygotes and heterozygotes based on the presence of the Tubby marker. Bars represent distinct  
 553 ASVs. The legend shows the lowest taxonomic level that was assigned to each ASV. **B)** Principal  
 554 coordinates plot, showing the first two coordinates calculated from a weighted Unifrac distance matrix.



555

556 **Figure 4. Axenic preparation of homozygous *park*<sup>25</sup> embryos dramatically reduces eclosion rate**

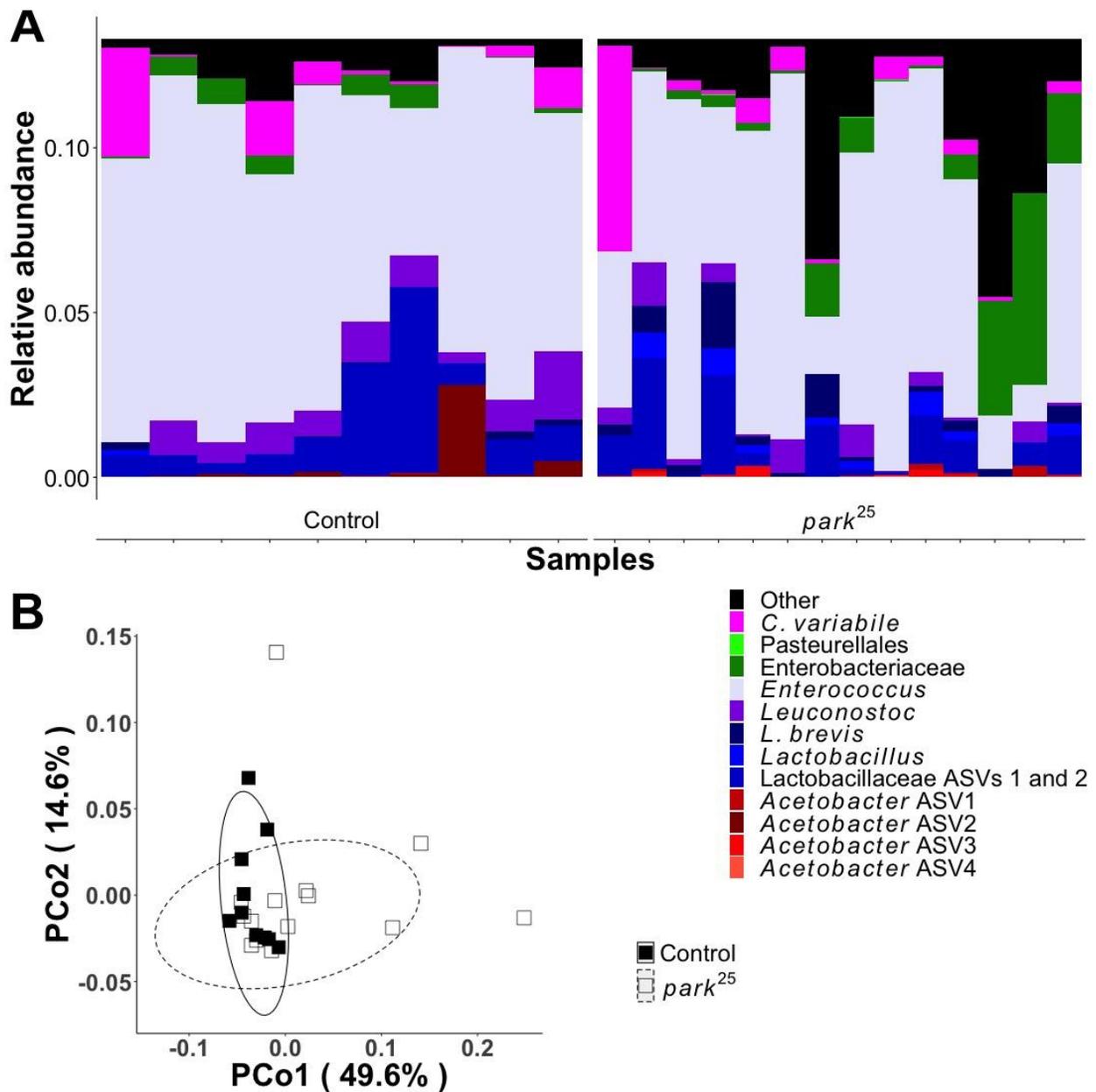
557 Embryos from *park*<sup>25</sup> and control flies were made axenic and gnotobiotic for feces-derived bacteria from  
558 *park*<sup>25</sup> or control flies. Pupae count and eclosion was recorded from each vial. Heterozygous (Het) *park*<sup>25</sup>  
559 pupae were differentiated from the homozygous (Hom) *park*<sup>25</sup> pupae by the presence of the Tubby  
560 marker on the TM6C balancer chromosome. Data are presented as mean and SEM. Post-hoc Tukey's  
561 analysis results are shown: \*\*\*\* = P < 0.0001. Sample (number of vials) sizes: Control (axenic = 40,  
562 control feces = 41, *park*<sup>25</sup> feces = 42), Het & Hom *park*<sup>25</sup> (axenic = 202, control feces = 63, *park*<sup>25</sup> feces =  
563 59).



564

565 **Figure 5. The microbiota of feces from control and *park*<sup>25</sup> flies.**

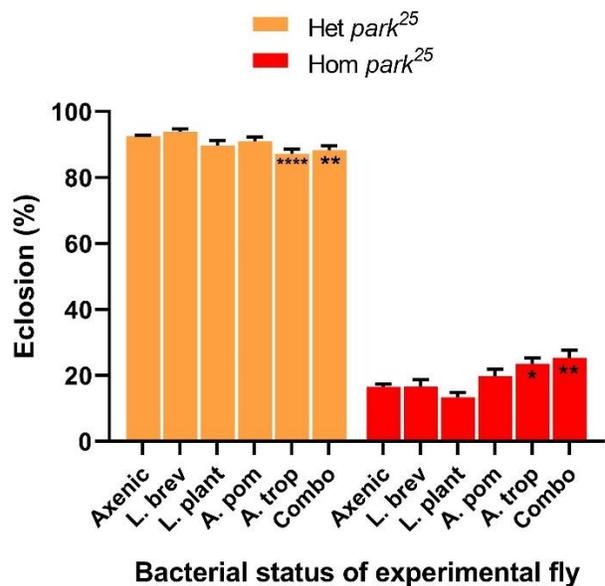
566 Fecal samples were collected from male controls and a mixture of heterozygous and homozygous male  
 567 *park*<sup>25</sup> flies. **A)** A taxon plot with bars representing distinct ASVs. The legend shows the lowest taxonomic  
 568 level that was assigned to each ASV. **B)** Principal coordinates plot, showing the first two coordinates  
 569 calculated from a weighted Unifrac distance matrix.



570

571 **Figure 6. Mono-association with *A. tropicalis* or a combination of bacteria with *park<sup>25</sup>* flies can alter**  
572 **eclosion rate.**

573 Embryos from *park<sup>25</sup>* flies were made axenic and then mono-associated with four different laboratory  
574 bacterial strains, or inoculated with an equal CFU combination of the four strains (Combo). Pupae count  
575 and eclosion was recorded from each vial. Heterozygous (Het) *park<sup>25</sup>* pupae were differentiated from  
576 the homozygous (Hom) *park<sup>25</sup>* pupae by the presence of the Tubby marker on the TM6C balancer  
577 chromosome. Data are presented as mean and SEM. Post-hoc Dunnett's analysis comparing to the  
578 axenic control flies are shown: \*\*\*\* =  $P < 0.0001$ , \*\* =  $P < 0.01$  \* =  $P < 0.05$ . Axenic ( $n=160$ ), *L. brev* =  
579 *Lactobacillus brevis* ( $n=24$ ), *L. plant* = *Lactiplantibacillus plantarum* ( $n=37$ ), *A. pom* =  
580 *Acetobacter pomorum* ( $n=35$ ), and *A. trop* = *Acetobacter tropicalis* ( $n=37$ ), combination ( $n=32$ ).



581

582

## Supplementary Files

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