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1 **Title**

2 *Drosophila melanogaster* establishes a species-specific mutualistic interaction with
3 stable gut-colonizing bacteria

4

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15

16 **Abstract**

17 Animals live together with diverse bacteria that can impact their biology. In *Drosophila*
18 *melanogaster,* gut-associated bacterial communities are relatively simple in composition but
19 also have a strong impact on host development and physiology. However, it is still unknown
20 if bacteria can proliferate and stably associate with the gut of *D. melanogaster*. In fact, it is
21 generally assumed that bacteria are transient and their constant ingestion with food is required
22 to maintain their presence in the gut. Here, we identify bacterial species from wild-caught *D.*
23 *melanogaster* that stably associate with the host independently of continuous inoculation.
24 Moreover, we show that specific *Acetobacter* wild isolates can proliferate in the gut. We
25 further demonstrate that the interaction between *D. melanogaster* and the wild isolated
26 *Acetobacter thailandicus* is mutually beneficial and that the stability of the gut association is
27 key to this mutualism. The stable population in the gut of *D. melanogaster* allows continuous
28 bacterial spreading into the environment, which is advantageous to the bacterium itself. The
29 bacterial dissemination is in turn advantageous to the host since the next generation of flies
30 develops in the presence of this particularly beneficial bacterium. *Ac. thailandicus* leads to a
31 faster host development and higher fertility of emerging adults, when compared to other
32 bacteria isolated from wild-caught flies. Furthermore, *Ac. thailandicus* is sufficient and
33 advantageous when *D. melanogaster* develops in axenic or freshly collected figs,
34 respectively. This isolate of *Ac. thailandicus* colonizes several genotypes of *D. melanogaster*
35 but not of the closely related *Drosophila simulans*, indicating that the stable association is
36 host specific. This work establishes a new conceptual model to understand *D. melanogaster*-

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37 gut microbiota interactions in an ecological context; stable interactions can be mutualistic
38 through microbial farming, a common strategy in insects. Moreover, these results develop the
39 use of *D. melanogaster* as a model to study gut microbiota proliferation and colonization.
40

41 **Author summary**

42 Animals, including humans, live together with complex bacterial communities in their
43 gut that influence their physiology and health. The fruit fly *Drosophila melanogaster* has
44 been an excellent model organism to study host-microbe interactions and harbours a relative
45 simple gut bacterial community. However, it is not known which of these bacteria can
46 proliferate and form stable communities in the gut, and the current hypothesis is that these
47 bacteria are only transiently associated with the gut. Here, we show that in *D. melanogaster*
48 collected from a natural population stable gut bacteria do exist. We isolated specific species
49 that can proliferate in the gut and form a stable association. This is beneficial to the bacteria
50 since they can be constantly spread by the flies as they move around. On the other hand, this
51 is a form of farming as the next generation of flies benefit from the association with these
52 particular bacteria during development. They become adults faster and are more fertile than if
53 they develop with other bacteria encountered in nature. These advantages are also observed
54 when flies develop in figs, a natural food source. Our findings show that *D. melanogaster* has
55 stable colonizing bacteria in the gut and establish a new framework to study host-gut bacteria
56 interactions.
57

58 **Introduction**

59 Animals live with microbial communities that have a strong impact on their
60 physiology, including their development, nutrition, immunity and behavior [1]. These effects
61 may be partially explained by adaptation of animals to the ubiquitous presence of microbes
62 and integration of this cue in their developmental and physiological programs. However,
63 association with specific microbes may increase their fitness in the environment they live or
64 provide the capacity to explore new niches. For instance, many endosymbionts in insects
65 provide essential metabolites, allowing hosts to explore food sources deficient in some
66 nutrients, as plant sap and blood [2-6].

67 A primary organ for animal-microbe interactions is the gut, which is an interface
68 between the external environment and the animal body. The gut microbiota can be very
69 complex and comprised of up to one thousand different bacterial species, as in humans [7]. Its
70 composition varies to different degrees between and within host species. Moreover, even
71 within the same host it can be very dynamic and fluctuate with host age and health, diet, and
72 other environmental conditions [8-11]. Understanding the composition of the gut microbiota,

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73 which factors regulate it, and how these interactions impact both the host and the microbes
74 are, therefore, major research questions.
75 *Drosophila melanogaster* has been used as model system to study host interaction with
76 gut bacteria [12,13]. Besides the host genetics, it has the advantage of having a simpler
77 bacterial community, when compared with mammals, and being relatively simple to produce
78 axenic and gnotobiotic animals. *D. melanogaster* raised in axenic conditions have a delayed
79 development, and are not viable under certain nutritional conditions, and bacteria can rescue
80 these developmental problems [14-16]. Bacteria also affect the fly lifespan, gut homeostasis,
81 interaction with pathogens, and behavior [17-23]. All of these phenotypes demonstrate the
82 importance of bacteria to this host and the need to understand these interactions for a
83 comprehensive view of *D. melanogaster* biology.
84 Despite the recognized importance of gut-associated bacteria to *D. melanogaster* what
85 constitutes its gut microbiota is still an open question. Laboratory *D. melanogaster* is
86 associated with few bacterial species, which belong mainly to *Acetobacter* and *Lactobacillus*
87 genera [20,22,24-27]. This contrasts with data from flies sampled in their natural
88 environment, which have a more diverse population of bacteria. In addition to *Acetobacter*
89 and *Lactobacillus*, they are also enriched in bacteria from other families and genera [25,28].
90 Because *D. melanogaster* feeds on fermenting and rotten fruits containing many microbes, it
91 is, however, difficult to understand which of the identified bacteria are colonizing the host gut
92 and which are transiently passing with the food. Likewise, a similar problem is present in
93 laboratory conditions, where flies live in a relatively closed environment. The bacteria found
94 in their gut could simply correspond to food growing bacteria ingested by the flies. This
95 hypothesis is supported by the fact that frequent transfer of adult flies to clean food vials
96 strongly reduces their gut bacterial loads [20,27]. Consequently, the current working model is
97 that the gut-associated bacteria in *D. melanogaster* are environmentally acquired and do not
98 constitute *bona fide* gut symbionts.
99 Most functional studies in *D. melanogaster*, however, have been performed with
100 bacterial isolates from lab stocks. The properties of bacterial isolates from wild-caught *D.*
101 *melanogaster* could differ. Bacteria found in the gut of some other *Drosophila* species differ
102 from the bacteria present in their food source, suggesting that they can be gut symbionts
103 [29,30], and raising the possibility of these also existing in *D. melanogaster*. Moreover, a
104 recent study compared the ability to colonize the gut of different *Lactobacillus plantarum*
105 strains and found that one wild strain was able to colonize flies more frequently that strains
106 isolated from laboratory flies [31]. Therefore, it is possible that natural populations of *D.*
107 *melanogaster* have stable colonizing bacterial communities in their guts.
108 Here we analyzed bacterial isolates from the gut of wild-caught *D. melanogaster* and
109 compared it to bacteria from lab stocks. Using a protocol that avoids re-infection of flies with

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110 bacteria growing on the food, we identified bacterial species that are stably associated with
111 the gut of wild *D. melanogaster*. Moreover, these isolates can stably associate and proliferate
112 in the gut of lab flies. We further analyze the specificity of these interactions and fitness
113 advantage of stable associations. Our results lead to the identification of gut symbionts in *D.*
114 *melanogaster* and demonstrate fitness advantages for both partners in an ecological context.
115
116 **Results**
117
118 **Wild caught flies have stable gut-colonizing bacteria**
119 In order to analyze the diversity and stability of gut bacteria in *Drosophila*
120 *melanogaster* we used culture-dependent techniques. We plated single gut homogenates in
121 agar plates of five different culture media (Luria Broth (LB), Mannitol, Brain Heart Infusion
122 (BHI), MRS Broth (MRS), and Liver Broth Infusion (LBI)). This approach allowed us to
123 determine absolute number of bacteria present in each gut and isolate bacteria for follow up
124 experiments.
125 We started by analyzing levels of bacteria in the gut of flies from our standard
126 laboratory stock $w^{1118}$ DrosDel isogenic strain ($w^{1118}$ *iso*) [32,33]). We assessed these levels in
127 young conventionally raised flies (Day 0) and after these flies were maintained singly for ten
128 days either in the same vial or passed to a new vial twice a day (similarly to the protocol in
129 [20]). The latter protocol was designed to decrease the probability of flies getting re-infected
130 with their own bacteria or bacteria growing on fly food and, therefore, allowed us to test if
131 there was a resident gut bacterial microbiota in this *D. melanogaster* lab stock (stability
132 assay). In flies kept in the same vial for ten days, bacterial levels in the gut increased
133 approximately 17-fold (Fig 1A and S1A Fig, linear mixed model fit (lmm), $p < 0.001$). In
134 contrast, flies that were passed twice a day to a new vial, during these ten days, had an
135 approximately 2,200-fold decrease in their gut bacterial levels (Fig 1B and S1A Fig, lmm, $p <$
136 $0.001$). A sharp decrease in bacterial loads was confirmed by quantitative PCR (qPCR), a
137 culture independent method, using universal primers for the 16S rRNA gene (Fig 1C and S1B
138 Fig, lmm, $p < 0.001$). These results show that bacterial levels in the gut of these flies are
139 dependent on fly husbandry and suggest that these bacteria are transient, similarly to what
140 was previously shown with a different laboratory stock [20]. Since these bacteria are
141 associated with the lab stock and bacterial loads in the gut of these flies actually increase over
142 time if they are kept in the same vials for ten days, we tested their growth on fly food (Fig
143 1D). We placed single flies per vial (Day 0), discarded them after 24 hours (Day 1), and kept
144 the vials for a further nine days (Day 10). Bacterial levels on the surface of the fly food
145 increased 7.6 x $10^8$ fold, from Day 1 to Day 10, clearly showing their capacity to grow on fly

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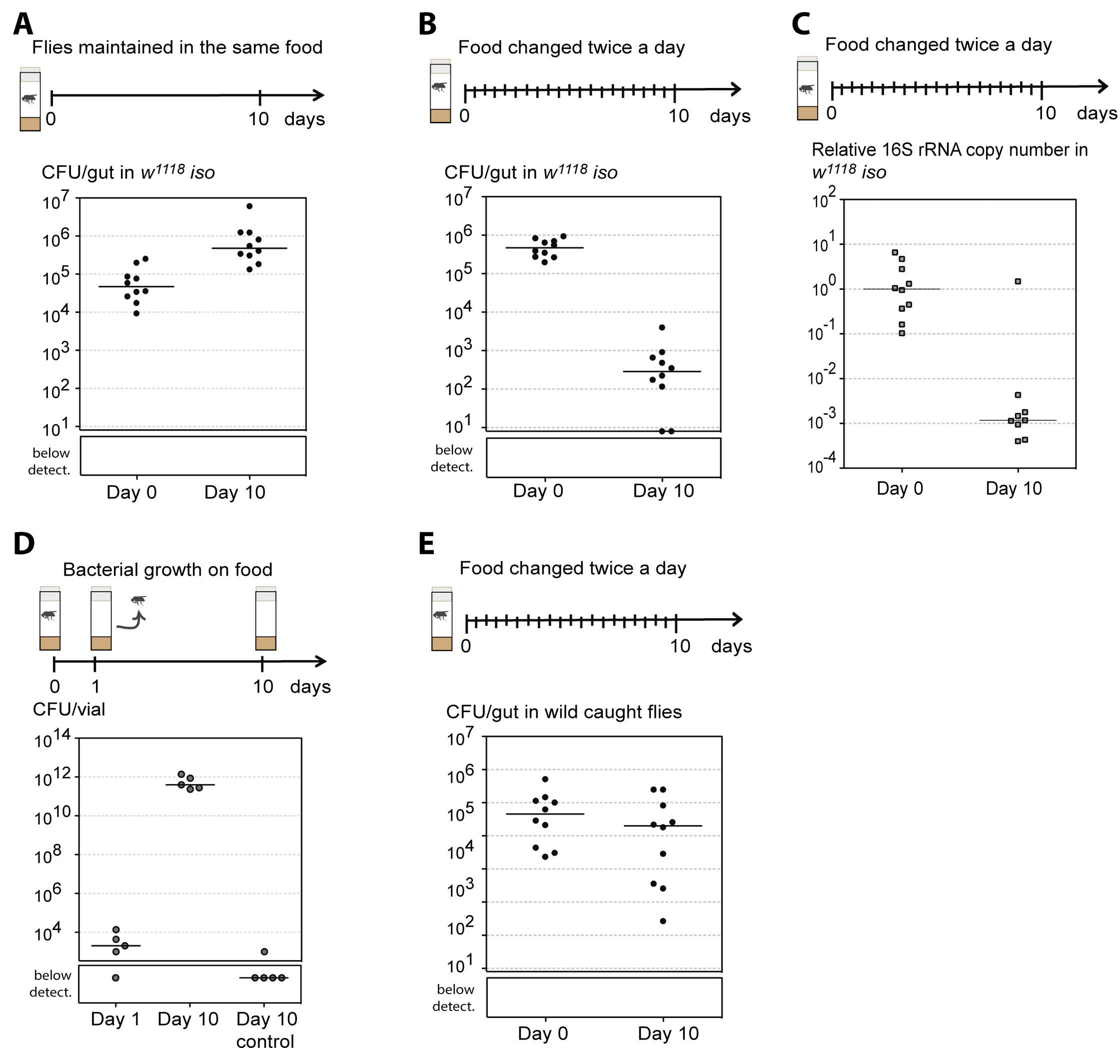
146 food (Fig 1D, lm, $p < 0.001$). Therefore, the bacteria associated with this lab stock grow on
147 the fly food and are only transiently associated with the gut of adult flies.
148




149
150

151 **Fig 1 – Wild-caught *D. melanogaster* have a stable gut microbiota.** Single 3-6 days old $w^{1118}$
152 *iso* males were kept in the same vial during ten days (A) or exposed to a stability protocol by being
153 passed to new vials twice a day (B, C). (A, B) Ten individuals were analyzed at each day and total
154 number of CFUs per gut determined by bacterial plating. Bacterial levels between day 0 and day 10
155 increase in (A) and decrease in (B) (lmm, $p < 0.001$ for both). (C) Relative amount of 16S rRNA
156 bacterial gene was measured by quantitative-PCR in ten individual guts from each day, using the host
157 gene *Rpl32* as a reference gene. Relative amount of 16S rRNA gene decreases between days (lmm, $p <$
158 $0.001$). (D) Single 3-6 days old $w^{1118}$ *iso* males were placed in food vials for 24 hours and then
159 discarded. Bacterial levels on the food were determined at this point (Day 1) and after incubating the
160 vials for further nine days (Day 10). Bacterial levels were also assessed in control vials, not exposed to
161 flies (Day 10 control). Five vials were analyzed for each condition and total number of CFUs per vial
162 determined by bacterial plating. Bacterial levels increase between Day 1 and Day 10 (lm, $p < 0.001$).
163 (E) Bacterial levels from wild-caught flies at the day of collection (Day 0) and after 10 days of the
164 stability protocol (Day 10). Ten individuals were analyzed for each day and total number of CFUs per
165 gut determined by plating. Bacterial levels on the flies significantly decrease with time (lmm, $p =$
166 $0.004$). (A-E) Each dot represents an individual gut or vial and lines represent medians. Statistical
167 analyses were performed together with replicate experiments shown in S1 Fig.

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168
169 We next asked if we could find stable bacteria in the gut of *D. melanogaster* collected
170 from natural populations. We captured *D. melanogaster* from a population growing on fallen
171 figs and quantified their gut bacterial levels at the time of collection (Day 0) and ten days
172 after using the same stability assay designed to avoid re-infection (Day 10) (Fig 1E, S1C and
173 S1D Fig). Although there is a statistical significant change in the bacterial levels in the gut
174 with time (lmm, $p = 0.004$), the bacterial levels only decreased 4.8 fold in ten days. Moreover,
175 at Day 10 wild flies maintained 2.9 x $10^4$ CFU per gut, while $w^{1118}$ *iso* flies only had 100 CFU
176 per gut. Also, even after 20 days of this protocol wild flies still maintained approximately 6.1
177 x $10^3$ CFU per gut (S1D Fig), showing a long-term stability of their microbiota. These results
178 show that wild flies carry bacteria that are stably associated with their gut.
179 In order to identify and isolate the bacteria that can stably interact with the gut of *D.*
180 *melanogaster*, we analysed the bacterial composition of the cultured gut extracts of $w^{1118}$ *iso*
181 and wild flies represented in Fig 1B and 1E. For each fly gut homogenate, in each of the five
182 media, we distinguished colonies by morphology, determined CFUs per gut of each
183 morphological type, and isolated two colonies of each morphological type. For each isolate
184 we sequenced by Sanger a fragment of the 16S rRNA gene, which included the V2 to V4
185 hypervariable regions. After sequencing we classified morphological types into operational
186 taxonomic units (OTUs), based on Greengenes alignment tool and database [34], and
187 determined the number of colony forming units (CFUs) of each OTU in each fly gut (Fig 2).
188 In general we could assign each morphological type to one OTU. However, in samples from
189 wild flies we could not distinguish by morphology the colonies of different *Lactobacillus*
190 species, different Acectobacteraceae (*Acetobacter* and *Gluconobacter* species), and several
191 genera of Enterobacteriaceae. We therefore calculated CFUs per fly for each of these groups
192 of bacteria and not individual OTUs (Fig 2). The frequencies of the different OTUs belonging
193 to these groups, in the different conditions, are shown in Fig 3B, 3D, 3F, 3H and S3 Fig.

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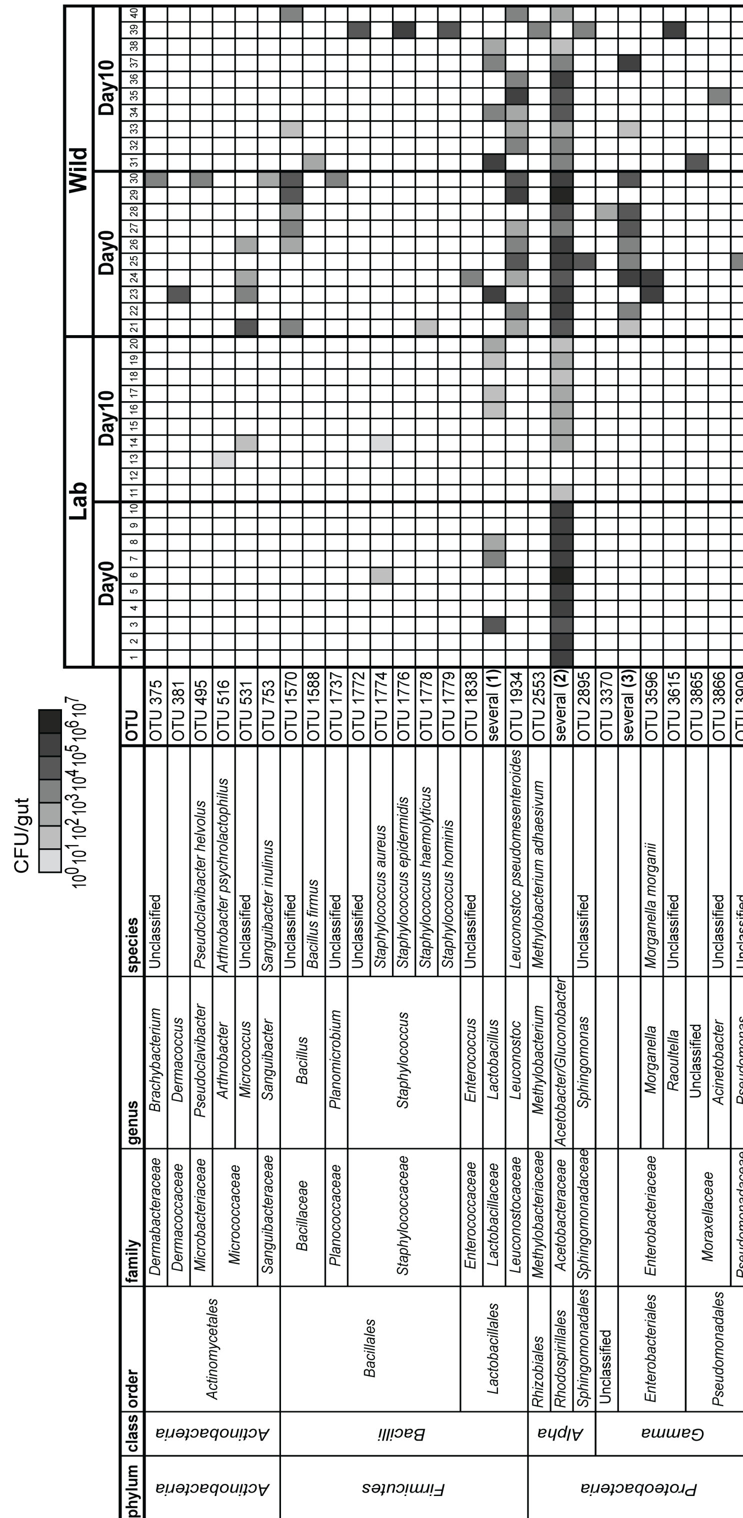


194

195 **Fig 2 – Higher diversity of gut bacterial communities in wild-caught *D. melanogaster*.**
196 Bacterial OTUs present in the gut of laboratory (1-20) and wild caught (21-40) flies before (Day 0) and
197 after being exposed to the stability protocol (Day 10). Gut homogenates from flies represented in Fig
198 1B and E were plated in different culture media and representative colonies of each morphological type
199 were sequenced. Each column represents one individual gut. Bacterial levels are represented on a grey-
200 scale from $10^0$ to $10^7$ CFUs per gut. Colonies of different *Lactobacillus, Acetobacteriaceae* or
201 *Enterobactereaceae* were not possible to distinguish by morphological type and are grouped together.

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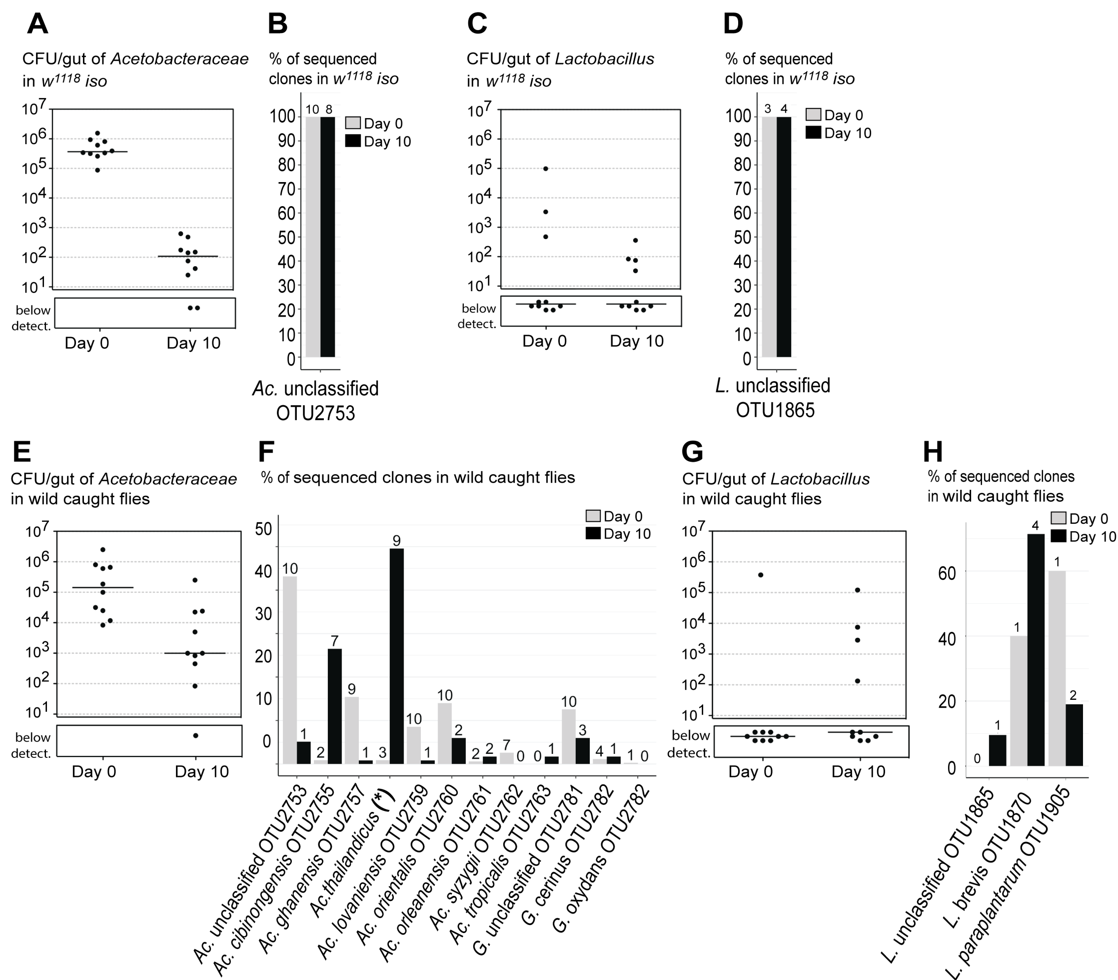
202 The presence of *Lactobacillus* species and *Leuconostoc pseudomesenteroides* in wild-caught flies is not
203 independent (Pearson's Chi-squared test, $p = 0.014$). Frequencies of the different OTUs in these groups
204 are represented on Fig 3B, D, F, H and S3B Fig.
205




206
207 **Fig 3 – Wild-caught flies maintain in the gut particular *Acetobacter* species .** Total levels of
208 *Acetobactereaceae* (A, E) and *Lactobacillus* (C, G) in laboratory $w^{1118}$ *iso* (A, C) and in wild-caught
209 flies (E, G) before (Day 0) and after 10 days of the stability protocol (Day 10). Each dot represents one
210 individual gut and lines represent medians. Levels of *Acetobactereaceae* decrease between days in both
211 types of flies (lm, $p <= 0.002$ for both). Changes in levels of *Lactobacillus* are not significant in both
212 (lm, $p >= 0.302$). Frequencies of sequenced colonies of *Acetobactereaceae* (B, F) and *Lactobacillus*
213 (D, H) in $w^{1118}$ *iso* (B, D) and in wild-caught flies (F, H). *Ac.- Acetobacter*, *G. - Gluconobacter* and *L. -*
214 *Lactobacillus*. Numbers on the top of the bars correspond to the number of flies carrying each OTU,
215 from a total of 10 flies (B, D, F, H). * *Ac. thailandicus* was initially identified as *Ac. indonesiensis*
216 OTU2758 based on partial sequence of 16S rRNA gene.
217

218 Laboratory flies presented very low diversity in their gut bacterial community, as
219 previously reported [25,26,28]. From each gut of laboratory flies we could isolate one to two
220 different OTUs at Day 0, and zero to three different OTUs at Day 10. In total, we isolated
221 from these flies three and five different OTUs at Day 0 and Day 10, respectively (S2 Fig).
222 Also, the accumulation curves indicate that we sampled most of the diversity present in
223 laboratory flies possible with our approach (S2 Fig). Laboratory flies were mainly found

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224 associated with two OTUs, *Acetobacter* OTU2753 and *Lactobacillus* OTU1865 (Fig 2, Fig
225 3A-3D). On Day 0, all the flies were associated with high levels of *Acetobacter* OTU2753
226 (Fig 3A, 3B), while *Lactobacillus* OTU1865 was only present in some individuals (Fig 3C,
227 3D). After 10 days of the stability assay, *Acetobacter* levels decrease (lm, $p = 0.001$), while
228 *Lactobacillus* levels are not significantly different ($p = 0.635$) (Fig 3A, 3C). Importantly,
229 when we analyzed the bacterial species that were capable of growing on fly food in Fig 1D,
230 we found these two same OTUs, with *Acetobacter* OTU2753 being the most abundant.
231 Altogether, these results show that this *D. melanogaster* laboratory stock has very low
232 bacterial diversity and is mainly associated with transient bacteria able to grow on fly food.

233 In contrast, wild caught flies were associated with a higher diversity of bacterial
234 species (Fig 2, Fig 3F, 3H, and S3B Fig). From each gut of wild flies we isolated nine to 16
235 different OTUs at Day 0, and three to 14 different OTUs at Day 10. In total, we isolated 35
236 and 31 different OTUs at Day 0 and Day 10, respectively (S2 Fig). Moreover, it seems that
237 we are not close to saturation with these samples and that further sampling would allow the
238 identification of more OTUs associated with the gut of *D. melanogaster* from this wild
239 population (S2 Fig).

240 The individual characterization of bacterial species present in each gut allowed us to
241 discriminate between OTUs that were only present in one or few individuals, albeit at higher
242 levels, and OTUs associated with most individuals. At the day of collection (Day 0) 50% or
243 more of the flies had in their gut *Bacillus* OTU1570, *Leuconostoc pseudomesenteroides*
244 OTU1934, *Acetobacter* OTU2753, *Ac. ghanensis* OTU2757, *Ac. lovaniensis* OTU2759, *Ac.*
245 *orientalis* OTU2760, *Ac. syzygii* OTU2762, *Gluconobacter* OTU2781, *Enterobactereaceae*
246 OTU3529, *Tatumella* OTU3635 and *Kluyvera ascorbata* OTU3643 (Fig 2, Fig 3F and S3B
247 Fig). Ten days after the stability assay only a few bacteria remained associated with the gut of
248 most individuals. One of these bacteria was *L. pseudomesenteroides*, which was present in six
249 out of ten flies and did not show a significant reduction in levels between Day 0 and Day 10
250 (Fig 2, S4 Fig lm, $p = 0.372$). Bacteria from the *Acetobacteraceae* family also remained
251 associated with the gut of most wild flies, at an estimated $1.3 \times 10^3$ CFU per gut at Day 10,
252 despite a significant reduction of approximately 100-fold in their levels between Day 0 and
253 Day 10 (lm, $p = 0.002$) (Fig 2, Fig 3E). However, the frequencies of different OTUs of
254 *Acetobacteraceae* changed significantly between Day 0 and Day 10 (Fig 3F, Pearson's Chi-
255 square with Monte Carlo simulation, $p < 0.001$). At Day 10, all the OTUs that were dominant
256 at Day 0 become present at lower frequencies and *Acetobacter cibinongensis* OTU2755 and
257 *Acetobacter indonesiensis* OTU2758 (later re-identified as *Acetobacter thailandicus*, see
258 below) became the dominant bacteria (Fig 3F). These two bacteria were present in at least
259 seven and nine individuals out of ten, respectively, and together represented 76% of the
260 sequenced colonies.

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261 We isolated clones of these gut bacteria to further characterize them. Analysis of the
262 full 16S rRNA gene sequence (S1 Text) confirmed the identity of the wild isolates
263 *Leuconostoc pseudomesentoroides* OTU1934 and *Acetobacter cibinongensis* OTU2755,
264 based on Greengenes [34]. We also confirmed the identity of the transient laboratory isolate
265 as *Acetobacter* OTU2753. However, the analysis of the full 16S rRNA gene from the
266 previously identified *Ac. indonesiensis* OTU2758 isolate matched several different
267 *Acetobacter* OTUs with 98% identity. Therefore, we used BLAST to analyze the full
268 sequence against the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database
269 [35]. *Ac. thailandicus* 16S rRNA gene was the best hit and was 99% identical to the sequence
270 of this isolate [36].

271 Overall, this analysis identified three species that seem to be stably associated with the
272 gut of wild flies in this population: *L. pseudomesenteroides*, *Ac. cibinongensis* and *Ac.*
273 *thailandicus*.

274

275 ***Acetobacter thailandicus* and *Ac. cibinongensis* proliferate in the gut of *D.***
276 ***melanogaster* and are stably associated with it.**

277 To study the interaction of these bacteria with *D. melanogaster* we generated stocks of
278 $w^{1118}$ *iso* flies monoassociated with each of these bacteria and we tested their persistence using
279 the stability assay. In agreement with our previous observations, the laboratory isolate of *Ac.*
280 OTU2753 did not persist in the gut and disappeared from the majority of the flies (lmm, $p <$
281 $0.001$) (S5A and S5E Fig). On the other hand, the wild isolates of *Ac. cibinongensis*, *Ac.*
282 *thailandicus* and *L. pseudomesenteroides* persisted in the gut of flies until Day 10, showing a
283 more stable association with the host (S5B-S5D and S5F-S5H Fig). *L. pseudomesenteroides*
284 levels did not significantly change with treatment ($p = 0.96$) and, although *Ac. cibinongensis*
285 and *Ac. thailandicus* levels significantly decreased in the ten days ($p < 0.001$ for both), both
286 remained in the gut at approximately 100 and 3,800 CFUs, respectively.

287 To better assess the bacterial dynamics within the gut, we developed a more strict
288 protocol to avoid re-infection. We maintained single flies in cages with a larger food surface
289 (356.7 $cm^2$ compared with 3.8 $cm^2$ in vials), which was changed daily (Fig 4A). We assessed
290 gut bacterial levels at the beginning of the experiment and after one, two, five and ten days of
291 this treatment. In accordance with previous data, *Ac.* OTU2753 levels rapidly decreased and
292 most flies had no detectable bacteria in their gut after five days of treatment (Fig 4B). *Ac.*
293 *cibinongensis* and *Ac. thailandicus* also presented an initial decrease in bacterial levels in the
294 gut, but these seemed to stabilize after two days of treatment, confirming their stability in the
295 gut (Fig 4C and D). However, and contrary to what was observed in vials, *L.*
296 *pseudomesenteroides* was not stable when the protocol was performed in cages (Fig 4E).
297 After two days, approximately 50% of flies lost *L. pseudomesenteroides* from their gut. An

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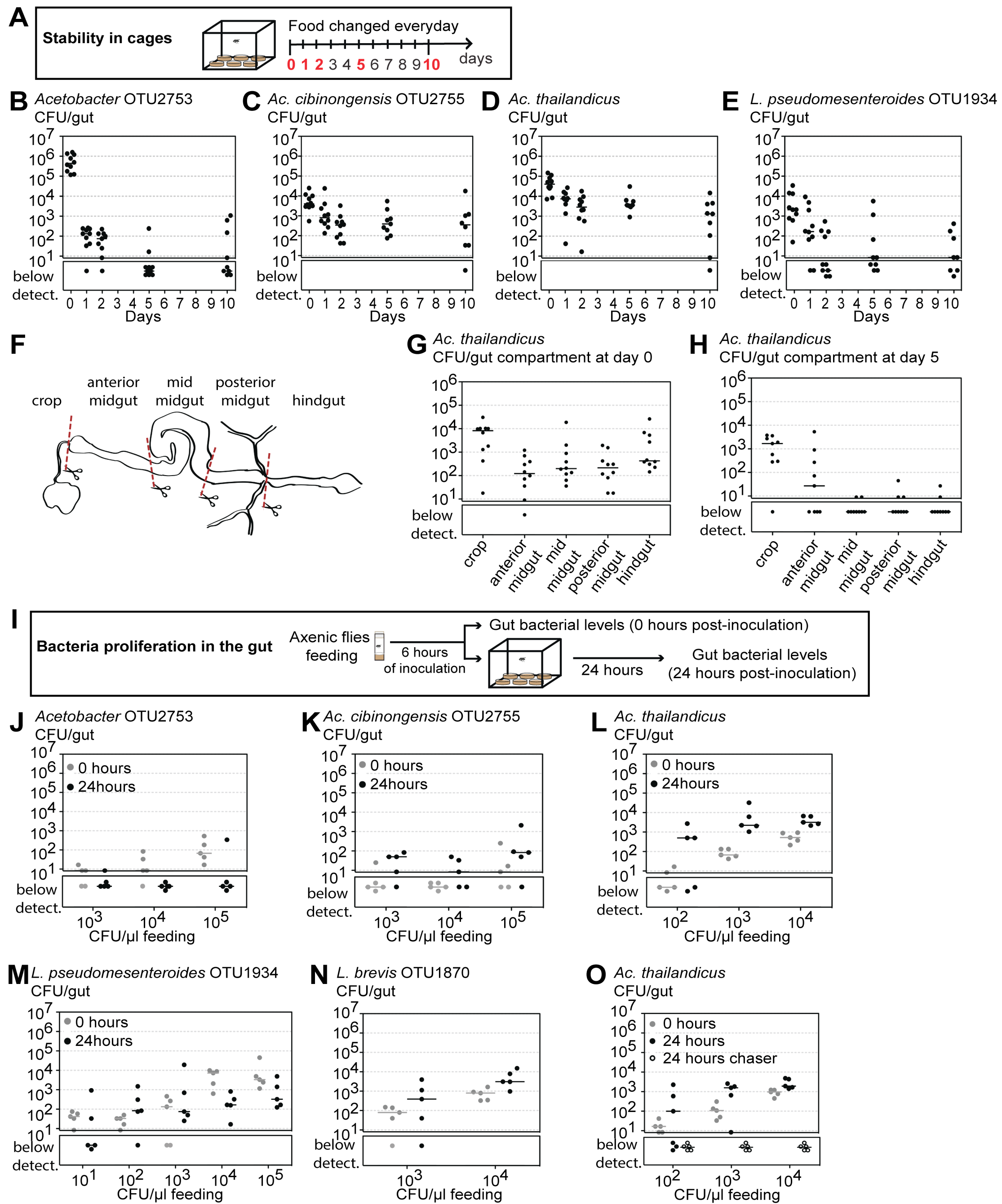
298 independent replicate with data from only Day 0 and Day 5 showed similar results for all
299 bacteria (S5E-S5H Fig).




300

301 **Fig 4 – *Ac. thailandicus* and *Ac. cibinongensis* proliferate and stably colonize the gut of *D.*
302 *melanogaster*.** (A-E) Stability of different bacteria in monoassociation. Single 3-6 days old $w^{1118}$ *iso*
303 males from monoassociated stocks with *Ac.* OTU2753 (B), *Ac. cibinongensis* (C), *Ac. thailandicus* (D)
304 or *L. pseudomesenteroides* (E) were exposed to the stability protocol in cages, as shown in the scheme
305 (A). Number of CFUs in individual guts was assessed by plating at days 0, 1, 2, 5 and 10 of the
306 protocol. Stability of different bacteria was analyzed by fitting the data to an exponential decay model
307 represented in S5I Fig. (F-H) Distribution of *Ac. thailandicus* in the gut. Scheme of gut regions
308 analysed (F). Number of CFUs in each gut compartment from $w^{1118}$ *iso* males monoassociated with *Ac.*
309 *thailandicus* before (G) and after (H) five days of the stability protocol. (I-O) Proliferation of different
310 bacteria in the gut of *D. melanogaster*. 3-6 days old axenic $w^{1118}$ *iso* males were inoculated for 6 hours
311 with different concentrations of *Ac.* OTU2753 (J), *Ac. cibinongensis* (K), *Ac. thailandicus* (L, O), *L.*

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312 *pseudomesenteroides* (M), and *L. brevis* (N). Bacterial levels were assessed 0 and 24 hours post-
313 inoculation. During this period males were singly placed in cages as shown in the scheme (I). In (O)
314 axenic chaser males were placed in cages together with males inoculated with *Ac. thailandicus*. At 24
315 hours bacterial levels were assessed for both males. Bacterial levels between 0 and 24 hours decrease
316 in flies inoculated with *Ac.* OTU2753 (lmm, $p < 0.001$), increase in flies inoculated with *Ac.*
317 *cibinongensis*, *Ac. thailandicus*, and *L. brevis* ($p = 0.024$, $p < 0.001$, and $p = 0.046$, respectively) and
318 do not significantly change in flies inoculated with *L. pseudomesenteroides* ($p = 0.158$). Ten (B-E and
319 G-H) or five (J-O) individuals were analyzed for each condition, per replicate, and total number of
320 CFUs per gut determined by plating. Each dot represents one gut or one gut fragment and lines
321 represent medians. Statistical analyses were performed together with replicate experiments shown in
322 S5 and S6 Fig.
323

324 We compared the dynamics of the gut levels of the four bacteria by fitting the data of
325 Fig 4B-4E to an exponential decay model (S5I Fig). This model estimates the exponential
326 decay rate which corresponds to the rate of bacterial loss from the gut and an asymptote that
327 corresponds to the levels at which the bacteria tend to stabilize after this loss. The simplest
328 model that explains the data has the same estimate for the exponential decay rate for all the
329 bacteria. There are, however, significant differences between the asymptotes of all the
330 bacteria (Contrasts between nonlinear least-square estimates, $p < 0.014$), except between *Ac.*
331 OTU2753 and *L. pseudomesenteroides* ($p = 0.395$). Overall, an interpretation of this fit is that
332 in all cases most of the bacterial population is in an unstable compartment, at the beginning of
333 the experiment, from where they tend to disappear with similar dynamics. However, *Ac.*
334 *cibinongensis* and *Ac. thailandicus* are also present in a stable compartment, at levels that
335 correspond to the calculated asymptotes (approximately 300 and 1,300 CFU per gut,
336 respectively).

337 In order to identify in which gut region bacteria could be stably associated with the
338 host, we analyzed *Ac. thailandicus* levels present in different gut regions before (Day 0) and
339 after 5 days of the stability protocol in cages (Day 5) (Fig 4F). At Day 0, *Ac. thailandicus* was
340 distributed along the gut, being present at lower levels in the midgut, compared with crop and
341 hindgut (Fig 4G and S5J Fig). After 5 days, bacteria were found in two anterior gut sections,
342 one comprising the crop and the other comprising the anterior midgut and the proventriculus
343 (Fig 4H and S5K Fig). Therefore, the niche for the stable population of *Ac. thailandicus* is the
344 anterior part of the gut.

345 We next asked if these bacteria had the capacity to proliferate in the gut of *D.*
346 *melanogaster*, since stability in the gut could be achieved through other mechanisms (e.g.
347 bacteria could be simply attaching to the gut and avoiding elimination). Thus, we developed a
348 protocol to analyze proliferation based on giving a small inoculum of bacteria and test if
349 bacterial loads increase over 24h. We raised flies in axenic conditions and exposed 3-6 days
350 old males to different doses of bacteria. After 6 hours of feeding on the bacteria inoculum,
351 flies were either collected to dissect and assess bacterial levels in the gut (0h) or placed singly

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352 in cages, as described above, and collected 24 hours later (Fig 4I). In this assay, *Ac.*
353 OTU2753 did not colonize the gut of adult flies and at the higher inoculum titers the levels
354 decreased between 0h and 24h (lmm, $p < 0.001$) (Fig 4J, S6A, S6E Fig), indicating that these
355 bacteria cannot proliferate in the gut of *D. melanogaster*. On the other hand, the levels of *Ac.*
356 *cibinongensis* and *Ac. thailandicus* increased in 24h ($p = 0.024$ and $p < 0.001$, respectively)
357 (Fig 4K, 4L, S6B, S6C, S6F, S6G Fig), showing that these bacteria can proliferate in the gut
358 of *D. melanogaster. Ac. thailandicus* proliferate more and reached higher levels than *Ac.*
359 *cibinongensis* ($p = 0.019$). Interestingly, in flies exposed to *Ac. thailandicus* inoculums
360 superior to $10^2$ CFU/μl, these bacteria reach between 600 and 1,900 CFU per gut (Fig 4L,
361 S6C and S6G Fig). These levels are similar to the stable compartment population size
362 estimated above (1,300 CFU per gut), indicating that *Ac. thailandicus* can rapidly colonize a
363 fly.

364 *L. pseudomesenteroides* levels did not significantly increase or decrease over 24h
365 (lmm, $p = 0.158$) (Fig 4M and S6D Fig). At inoculums superior to $10^2$ CFU/μl, *L.*
366 *pseudomesenteroides* levels at 24h are between 150 and 550 CFU per gut. These results fail to
367 show proliferation of *L. pseudomesenteroides* but indicate that this bacterium is not
368 eliminated at the same rate as the unstable *Ac.* OTU2753.

369 Since *Lactobacillus* species are commonly found associated with *D. melanogaster* and
370 shown to impact its physiology [25,26,28,37-39], we also tested isolates of *Lactobacillus*
371 *paraplantarum* OTU1905 and *Lactobacillus brevis* OTU1870 in this assay (Fig 4N, S6H-S6J
372 Fig). These *Lactobacillus* were isolated from the gut of wild flies at day 0 of the stability
373 assay (Fig 3G and 3H) and the isolates identity confirmed by sequencing the full 16S rRNA
374 gene (S1 Table). *L. paraplantarum* levels do not change over 24h (lmm, $p = 0.65$) and can be
375 sustained at 200 to 800 CFU per gut (similarly to *L. pseudomesenteroides*) (S6H and S6I Fig).
376 On the other hand, the levels of *L. brevis* increase in 24h ($p = 0.046$), showing that this
377 bacterium proliferates in the gut of *D. melanogaster* (Fig 4N and S6J Fig).

378 Overall, these assays show that *Ac. cibinongensis*, *Ac. thailandicus*, and *L. brevis*
379 isolates proliferate in the gut of *D. melanogaster*. On the contrary, the transient *Ac.* OTU2753
380 cannot proliferate and is rapidly lost. *L. pseudomesenteroides* and *L. paraplatarum* have an
381 intermediate phenotype where proliferation is not shown but the bacteria can sustain
382 themselves in the gut over a period of 24h after oral inoculation.

383 As all these *Acetobacter* species were able to grow on fly food (S7 Fig), it was still
384 possible that the increase in the levels of *Ac. thailandicus* in the proliferation assay (Fig 4L,
385 S6C Fig) was due to a very fast growth on the fly food and re-acquirement by feeding. To test
386 this possibility we placed axenic (chaser) flies in cages simultaneously with the flies that had
387 fed on *Ac. thailandicus,* at time 0h of the experiment. At 24 hours none of the axenic chaser
388 flies had bacteria in their gut (Fig 4O and S6G Fig). This demonstrates that the levels

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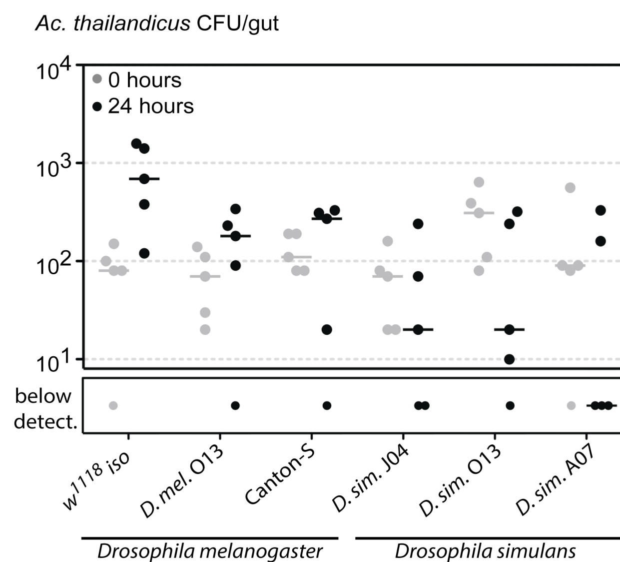
389 measured in the inoculated fly were due to proliferation in the gut and not due to bacteria
390 acquired from the food.
391
392 ***Ac. thailandicus* gut proliferation is species specific**
393 To test if proliferation of *Ac. thailandicus* in the gut is host specific we compared its
394 proliferation in *D. melanogaster* and *Drosophila simulans*. These two species share the same
395 habitat, feed on the same material and are frequently captured together [40]. We used a
396 proliferation protocol similar to the one described above (see figure legend, S8A, S8B Fig) to
397 test three different genetic backgrounds of each host species. These included one isofemale
398 line of each species that were collected simultaneously, from the same place as the initial
399 collection of wild *D. melanogaster*. There is a significant difference in the colonization by *Ac.*
400 *thailandicus* in these two host species (Fig 5, S8C-S8E Fig, lmm, $p < 0.001$), with the levels
401 increasing over 24h in *D. melanogaster* but decreasing in *D. simulans*. These results suggest
402 that *D. melanogaster* and *Ac. thailandicus* interaction is host specific. Interestingly, although
403 *Ac. thailandicus* colonizes all strains of *D. melanogaster* tested (Fig5, S8C-S8E Fig), there is
404 variation in the growth at 24h, indicating modulation of this process by the host genotype
405 (lmm, $p = 0.002$).
406




407
408 **Fig 5 - *Ac. thailandicus* proliferates specifically in *D. melanogaster* but not in *D. simulans*.**
409 Axenic 3-6 days old *D. melanogaster* or *D. simulans* males were inoculated for 6 hours with *Ac.*
410 *thailandicus* ($10^4$ CFU/µl). Bacterial levels were assessed 0 and 24 hours post-inoculation. During this
411 period males were singly placed in bottles. Three different genetic backgrounds for *D. melanogaster*
412 ($w^{1118}$ *iso*, *D. mel*. O13 and Canton-S) and for *D. simulans* (*D. sim.* J04, *D. sim.* O13 and *D. sim*. A07)
413 were tested. Bacterial levels in the gut increase in *D. melanogaster* and decrease in *D. simulans* (lmm,
414 $p < 0.001$). Five individuals were analyzed for each condition and total number of CFUs per gut
415 determined by plating. Each dot represents one gut and the lines represent medians. Statistical analysis
416 was performed together with replicate experiments shown in S8C-E.
417

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418 ***Ac. thailandicus* stable association with *D. melanogaster* is mutually beneficial**

419 Symbiotic associations can range from pathogenic to mutualistic. As *Acetobacter*
420 species have been previously described as beneficial to *D. melanogaster* [16] we tested if the
421 stable association between *D. melanogaster* with *Ac. thailandicus* could be advantageous for
422 both. We started to test this hypothesis by comparing fitness parameters of flies
423 monoassociated with *Ac. thailandicus*, *Ac.* OTU2753 and axenic flies by measuring time to
424 pupariation and adulthood and total number of its progeny. Both *Ac. thailandicus* and *Ac.*
425 OTU2753 monoassociated stocks had a much higher fertility than axenic flies and there was
426 no significant difference between them (S9A, S9B Fig, lm, $p < 0.001$ for the comparisons of
427 each *Acetobacter* monoassociation with axenic flies, in number of pupae or adults, $p > 0.968$
428 for the comparisons between *Acetobacter* monoassociated stocks). Flies monoassociated with
429 either *Acetobacter* also developed until pupariation or adulthood approximately 3 days faster
430 than axenic flies (S9C, S9D Fig, lm, $p < 0.001$ for each *Acetobacter* monoassociation
431 comparison with axenic flies). Flies monoassociated with *Acetobacter* OTU2753 developed
432 slightly faster to pupae (0.38 days) and adults (0.57 days) ($p < 0.001$ for each comparison).
433 These results show that in this setup the association with either *Acetobacter* is clearly
434 advantageous when comparing with axenic conditions and that the stable *Ac. thailandicus*
435 does not provide a greater benefit than the lab isolate *Ac.* OTU2753.

436 However, the advantage of a stable association may not be revealed by directly
437 studying monoassociated *D. melanogaster* stocks. In these conditions the bacteria are
438 continuously associated with *D. melanogaster*, even if it only present in the food or transiting
439 through the gut. But in the wild *D. melanogaster* adults freely move in space and can explore
440 a continuously changing environment, a situation in which a stable association could be
441 important. Therefore, we established a protocol to test the fitness benefits of the stable
442 interaction in a scenario that simulates this changing environment. The protocol is similar to
443 the proliferation protocol outlined above. After six hours of feeding on an inoculum of
444 bacteria, one female and two males were placed per cage and maintained there for ten days,
445 with food being changed daily (Fig 6A). After ten days of this protocol males exposed to *Ac.*
446 *thailandicus* have a median of 6,800 CFU per gut (Fig 6B and S10A Fig), showing that
447 colonization can be sustained for a long time. In females, *Ac. thailandicus* grows in the gut
448 between the beginning of the experiment and ten days in the cage (Wilcoxon rank sum test, $p$
449 $< 0.001$) and reaches a median of 17,500 CFU per gut. These results show that *Ac.*
450 *thailandicus* also colonizes and proliferates in female *D. melanogaster*. On the other hand,
451 *Ac.* OTU2753 levels decrease between the beginning of the experiment and day ten in
452 females ($p = 0.048$) and they have a median of 0 CFU per gut at day ten in both sexes,
453 confirming that flies are not colonized by these bacteria (Fig 6B and S10A Fig).
454

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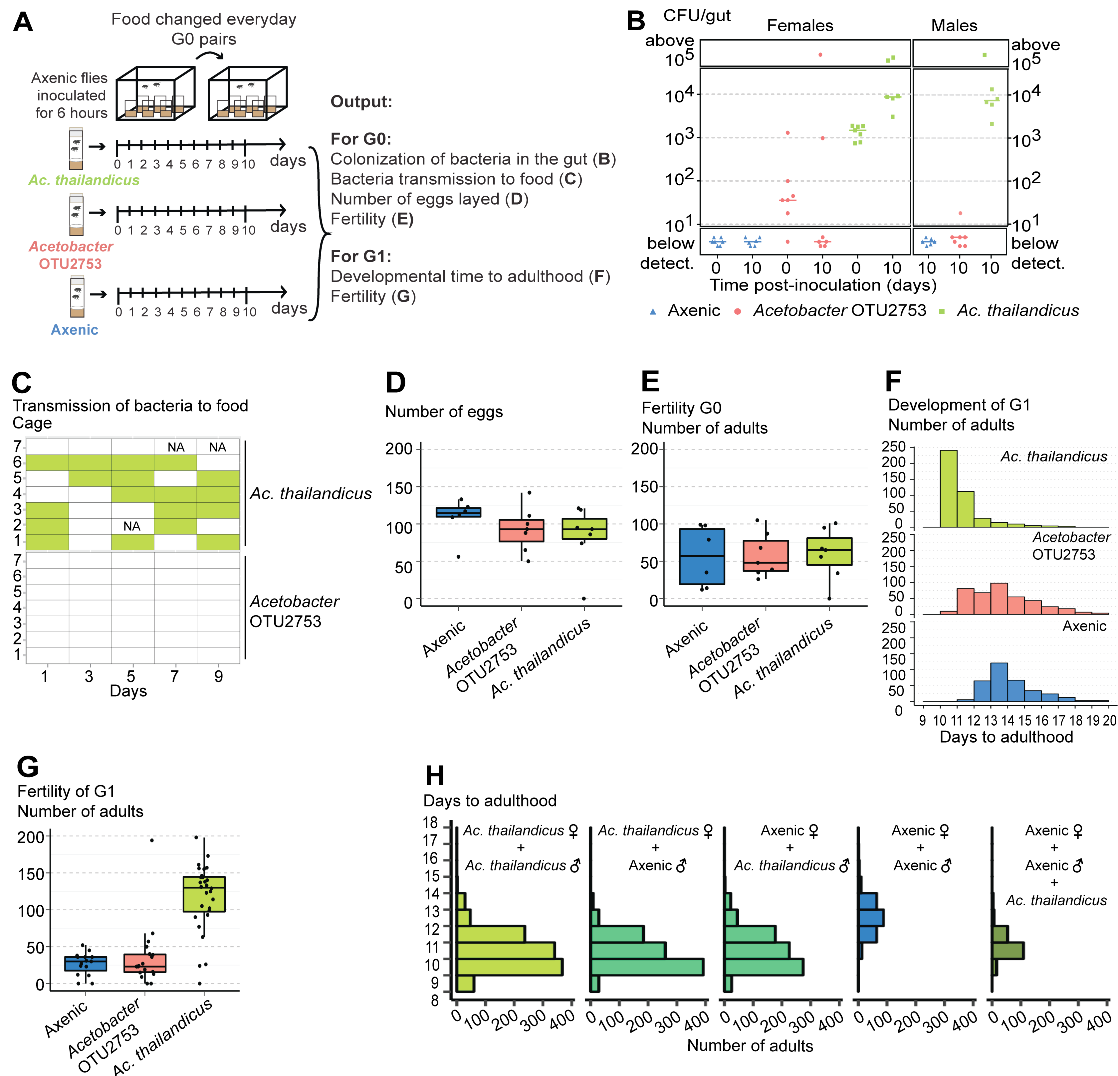


**Fig 6 - *Ac. thailandicus* stable association with *D. melanogaster* is mutualistic.** (A) Axenic 1-3 days old $w^{1118}$ *iso* males and females (G0) were in contact with an inoculum of $10^5$ CFU/µl of *Ac.* OTU2753, *Ac. thailandicus,* or sterile Mannitol (Axenic), for 6 hours. Two males and one female were placed per cage, with 6-7 cages for each condition, during 10 days with daily changed food. This experimental setup corresponds to data shown in panels B-G. (B) Bacterial levels in single guts of females at time 0 (0 days) and 10 days post-inoculation and in males 10 days post-inoculation, analyzed by plating. Bacterial levels between the two time-points increased in females inoculated with *Ac. thailandicus* and decreased in females inoculated with *Ac.* OTU2753 (Mann-Whitney test, $p < 0.001$ and $p = 0.048$ respectively). (C) Presence of bacteria on the food collected from cages at days 1, 3, 5, 7 and 9 of the protocol, analyzed by plating. Filled rectangles represent presence of bacteria. NA stands for samples that were not analyzed. *Ac. thailandicus* is transmitted to the food with higher frequency than *Ac.* OTU2753 (glm-binomial, $p < 0.001$). (D-G) Effect of bacterial association on the fitness of *D. melanogaster*. Total number of eggs laid by flies inoculated, or not, with different *Acetobacte*r (D) and total number of adults that emerged from these eggs (E). Total number of eggs or adults is not different between conditions (lmm, $p > 0.484$ for all comparisons). (F) Developmental time to adulthood of the progeny (G1) of flies inoculated or not with different *Acetobacter*. Developmental time to adulthood is faster in progeny from flies inoculated with *Ac. thailandicus* than in the other two conditions and in progeny from flies inoculated with *Ac.* OTU2753 compared to progeny from axenic flies (lmm, $p < 0.001$, for these comparisons). (G) Fertility of G1 was assessed by placing two males and one female of G1 per vial, flipping them every other day for 10 days, and analyzing total number of emerged adults. Fifteen or more couples were made per condition. Fertility is

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477 higher in progeny from flies inoculated with *Ac. thailandicus* compared with the other two conditions
478 (lmm, $p < 0.001$, for both comparisons) and not different in the comparison between the progeny of
479 flies inoculated with *Ac.* OTU2753 or axenic ($p = 0.592$). (H) One male and one female 1-2 days old
480 $w^{1118}$ *iso*, either axenic or monoassociated with *Ac. thailandicus,* were placed in vials and flipped every
481 other day for 10 days. To one set of vials with axenic parents *Ac. thailandicus* was added on the eggs
482 after passing the parents. Developmental time to adulthood of the progeny was assessed. Ten couples
483 were made per condition. There are no differences on developmental time to adulthood if either or both
484 parents are monoassociated with *Ac. thailandicus* (lmm, $p > 0.412$ for all these comparisons). Progeny
485 from couples where either or both parents are monoassociated and progeny from axenic flies where *Ac.*
486 *thailandicus* culture is added on the eggs develop faster than progeny from axenic flies (lmm, $p <$
487 $0.001$, for all these comparisons). (B) Each dot represents one gut and lines represent medians. (D, E
488 and G) Each dot represents the total progeny of one female. All statistical analyses were done together
489 with replicate experiments shown in S10 and S11.
490

491 As a measure of the fitness benefit for the bacteria, in being stably associated with *D.*
492 *melanogaster,* we tested if they could be transmitted to the food. We analyzed bacterial
493 transmission by flies during the experiment, at days one, three, five, seven and nine. Flies
494 associated with *Ac. thailandicus* transmitted bacteria to the food with a much higher
495 frequency than flies associated with *Acetobacter* OTU2753, where transmission occurred
496 only once (Fig6C, S10B Fig, generalized linear model with binomial distribution (glm-
497 binomial), $p < 0.001$). Moreover, the probability of transmission of *Ac. thailandicus* to the
498 food was independent of the day of the experiment (anova on glm-binomial models, $p =$
499 $0.811$). These results show that upon gut colonization *Ac. thailandicus* can be continuously
500 transmitted by *D. melanogaster*. This may be advantageous to the bacteria and mediate their
501 dispersal in the environment.

502 To compare the effect of this association on the host fitness, we started by analyzing
503 the fertility of the flies in terms of number of eggs laid and adult progeny, during the
504 experiment. The number of eggs or adult progeny were not significantly different between
505 axenic flies and flies exposed to either bacteria (Fig 6D, 6E, S10C, S10D Fig, lmm, $p > 0.484$
506 for all comparisons). However, the time that these embryos took to reach adulthood was
507 different. Progeny from flies colonized by *Ac. thailandicus* developed two or three days faster
508 than progeny from flies previously exposed to *Ac.* OTU2753 or axenic flies, respectively (Fig
509 6F, S10E, lmm, $p < 0.001$ for both comparisons). However, the progeny of flies exposed to
510 *Ac.* OTU2753 developed only 0.6 days faster than axenic flies ($p < 0.001$). Moreover, the
511 fertility of this progeny was strongly influenced by the interaction of their parents with
512 bacteria. The progeny from flies previously colonized by *Ac. thailandicus* had a much higher
513 fertility than the progeny from flies previously exposed to *Ac.* OTU2753 or axenic flies (Fig
514 6G, S10F Fig, lmm, $p < 0.001$ for both comparisons), while there was no difference between
515 the progeny of flies exposed to *Ac.* OTU2753 or axenic flies ($p = 0.592$). These data show
516 that the interaction of adult flies with stable bacteria does not affect their fertility but has a
517 strong influence on the development and fertility of its progeny.

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518 This trans-generational effect could be due to an effect of the stable *Ac. thailandicus*
519 gut population on the parents, and a subsequent indirect effect on the progeny, or through the
520 transmission of the bacteria to the next generation and its effect during larval development.
521 We tested if the developmental time of the progeny was dependent on the bacterial
522 association with either parent by analysing the four possible couple combinations of flies
523 raised axenically or monoassociated with *Ac. thailandicus* (Fig 6H, S11 Fig). There is no
524 difference in developmental time to pupariation or adulthood if either or both parents are from
525 the monoassociated stock (lmm, $p > 0.412$ for all these comparisons). The progeny of these
526 three crosses develop, on average, 2.7 to 2.8 days faster than the progeny of crosses with both
527 parents axenic ($p < 0.001$ for all comparison). These results show that the trans-generational
528 effect on developmental time is not specifically associated with the mother or the father. Also,
529 adding *Ac. thailandicus* to the progeny of axenic flies rescues the developmental delay. When
530 bacteria are added these flies develop approximately two days faster ($p < 0.001$). This is not a
531 full rescue since axenic eggs plus *Ac. thailandicus* still develop, on average, 0.5 to 0.8 days
532 slower than flies with either or both parents from monoassociated stocks ($p < 0.001$ for all
533 comparisons). This may be explained by the fact that the bacteria are only added when the
534 parents are removed from the vial, after two days of egg laying. These data is compatible with
535 a scenario where flies associated with *Ac. thailandicus*, either male or female, can transmit
536 the bacteria to the next generation, which then plays an important role in its development. In
537 agreement with this hypothesis, we have shown above that *Ac. thailandicus* can be
538 continuously transmitted to the environment (Fig 6C, S10B Fig). Moreover, we detected
539 bacteria in the surface of twenty out of twenty eggs laid by flies monoassociated with *Ac.*
540 *thailandicus,* by testing bacterial growth in medium. This demonstrates that *Ac. thailandicus*
541 is efficiently transmitted from mothers to their progeny.
542 We also observed that *Ac. thailandicus* affected the fertility of *D. melanogaster* in this
543 assay. Similarly to the results above, there is no difference in total number of progeny if either
544 or both parents are from the monoassociated stock (pupae or adult number, lm, $p > 0.180$ for
545 all these comparisons). However, if both parents are axenic the number of pupae or adults
546 total progeny is lower ($p < 0.001$ for all comparisons). This lower number of pupae or adults
547 is not rescued by adding *Ac. thailandicus* to the axenic eggs ($p = 0.998$), indicating that these
548 bacteria are not affecting egg to pupae or adult survival. Since exposing axenic adults to *Ac.*
549 *thailandicus* does not alter their fertility (Fig 6D, 6E), this fertility effect may be dependent
550 on either parent development in the presence of *Ac. thailandicus* or in the presence of *Ac.*
551 *thailandicus* in the fly food for the two days of the egg laying.
552 The results above suggest that a stable association with gut bacteria is beneficial to
553 adult *D. melanogaster*, because it allows continuous transmission to the next generation,
554 promoting its faster development and higher fertility. Unstable interactions lead to loss of the

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555 bacterial population and non-transmission to the next generation. However, these experiments
556 were performed by providing axenic food to flies, and in a natural scenario flies are bound to
557 encounter many other bacteria present in the food substrates. If all bacteria were equally
558 beneficial for fly development this stable association could be irrelevant. Therefore, we tested
559 if different bacteria naturally encountered by *D. melanogaster* confer different fitness benefits
560 to the flies. We sterilized eggs of $w^{1118}$ *iso* and associated them with different bacteria found
561 in the gut of flies from a natural population (sampled from the isolates of Fig 2, Fig 7A). We
562 determined total number of adults that developed from these eggs, their developmental time,
563 and their fertility. The number of adults that emerged (G0) was not different between
564 associations with different bacteria or in germ-free conditions (S12A, S12B Fig, lmm, $p >$
565 $0.282$ for all pairwise comparisons). However, we did observe differences in the
566 developmental time and fertility of these adults associated with different bacterial isolates,
567 and found a negative correlation between these parameters (Pearson correlation -0.91, $p <$
568 $0.001$) (Fig 7B, S12C-S12F Fig, S13 Fig). Flies associated with *Ac. thailandicus* developed
569 faster than axenic flies and flies associated with 11 out of the other 15 bacteria (lmm, $p <$
570 $0.038$ for all these pairwise comparisons). These flies are also more fertile than axenic flies
571 and flies associated with 11 out of the other 15 bacteria (lmm, $p < 0.018$). Flies associated
572 with *Ac*. OTU2753, *Lactobacillus brevis*, and *Lactobacillus paraplantarum* developed as fast
573 and are as fertile as *Ac. thailandicus* ($p > 0.200$ for these pairwise comparisons). While flies
574 associated with *Ac. cibinongensis* developed slower than with *Ac. thailandicus* ($p = 0.023$),
575 the developmental time of flies with *L. pseudomesenteroides* is not significantly different ($p =$
576 $0.224$). However, both have lower fertility than flies with *Ac. thailandicus* ($p < 0.001$). On
577 average, flies associated with *L. pseudomesenteroides* or *Ac. cibinongensis* develop faster and
578 have a higher fertility than axenic flies but these differences are not statistically significant ($p$
579 $> 0.082$, for all these comparisons). On the other hand, flies associated with *Bacillus flexus*
580 OTU1589 were not different from axenic flies in terms of developmental time or fertility ($p =$
581 $0.878$). Overall, these data demonstrate that different bacteria have a variable effect on the
582 development and fertility of *D. melanogaster,* with some not conferring any advantage to the
583 flies development or fertility. *Ac. thailandicus* seems particularly beneficial to *D.*
584 *melanogaster* and, therefore, the stable association may be advantageous to the host.

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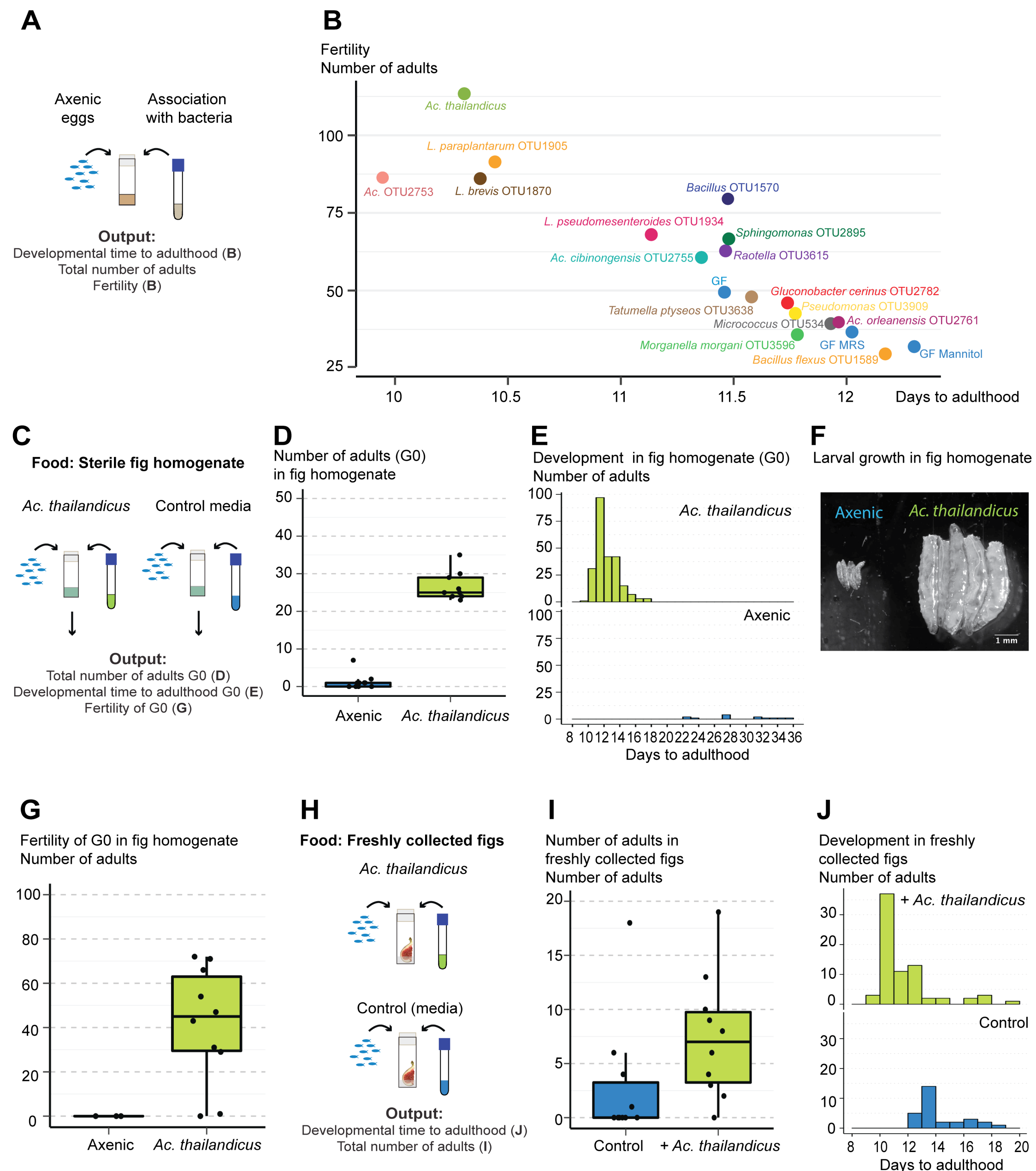


585

586 **Fig 7 – *Ac. thailandicus* is beneficial in the context of other wild bacteria and natural food**
587 **substrates.** (A) $w^{1118}$ *iso* eggs were associated with different bacteria isolated from the gut of wild-
588 caught *D. melanogaster*. As controls, axenic eggs that had no treatment (GF) or in which sterile media
589 were added (GF MRS and GF Mannitol) were used. (B) For each bacterium, estimates of
590 developmental time to adulthood of these eggs are plotted against estimates of their fertility. These
591 estimates derive from the statistical analysis of data presented in S12C-F and S13 Fig. There is a
592 negative correlation between developmental time and fertility (Pearson correlation -0.91, $p < 0.001$).
593 (C) Fifty axenic $w^{1118}$ *iso* eggs were placed in vials containing sterilized fig homogenate. *Ac.*
594 *thailandicus* or sterile culture media were added on the top of the eggs. Ten vials were used per
595 condition. Total number of adults that emerged (D) and developmental time to adulthood (E) was
596 determined. More eggs inoculated with *Ac. thailandicus* developed to adulthood and faster than axenic
597 eggs (lmm, $p < 0.001$ for both comparisons). (F) Larvae five days post inoculation with either
598 condition in fig homogenate. (G) Fertility of flies developed in fig homogenate with and without the
599 addition of *Ac. thailandicus*. Two males and one female were collected from G0 and placed per vial
600 containing fig homogenate for 10 days, with vials flipped every other day. The *Ac. thailandicus*
601 condition has ten replicates but only three from axenic eggs were possible to perform. Adults from

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602 eggs inoculated with *Ac. thailandicus* were more fertile than axenic adults (lmm, $p = 0.003$). (H) Fifty
603 axenic $w^{1118}$ *iso* eggs were placed in vials containing freshly collected non-sterile figs. *Ac. thailandicus*
604 culture or sterile media (Control) was added on the top of the eggs. The total number of adults that
605 emerged (I) and their developmental time to adulthood (J) was analyzed. Ten vials were analyzed per
606 condition. There were more adults emerging from vials inoculated with *Ac. thailandicus* (lmm, $p =$
607 $0.010$). Developmental time to adulthood was faster in eggs inoculated with *Ac. thailandicus* in this
608 experimental replicate but not significantly different in the other replicate represented on S14E Fig
609 (lmm, $p < 0.001$ and $p = 0.557$, respectively). Statistical analyses from (D-J) were done together with
610 replicate experiments shown in S14.
611
612 We also analyzed the impact of *Ac. thailandicus* on *D. melanogaster* fitness when they
613 develop in fruit, a more natural food substrate, instead of standard fly food. We compared
614 development from eggs to adults on a sterile fig homogenate with or without adding *Ac.*
615 *thailandicus* (Fig 7C). The association with *Ac. thailandicus* strongly influenced the number
616 of emerging adults, with very few flies reaching adulthood in axenic conditions (Fig 7D,
617 S14A Fig, lmm, $p < 0.001$). Moreover, while *Ac. thailandicus* associated flies develop, on
618 average, in 11.5 days, the few axenic flies that reach adulthood are slower and take 28 days
619 (Fig 7E, S14B Fig, lmm, $p < 0.001$). This reflects a delay in growth since five days old larvae
620 in axenic conditions were much smaller than larvae with *Ac. thailandicus* (Fig 7F). We
621 subsequently tested the fertility of the adult flies that developed in these two conditions.
622 Adults that developed on figs in the presence of *Ac. thailandicus* were also more fertile (Fig
623 7G, S14C Fig, lmm, $p = 0.003$). In fact, the few flies that developed in axenic conditions were
624 all sterile. These results show that *Ac. thailandicus* benefit for the development and fertility of
625 flies is even more pronounced in a natural food substrate.
626 However, in nature, fruits are not sterile but exposed to many environmental
627 organisms. Therefore, the advantage we observed of *Ac. thailandicus* could be absent in
628 normal non-sterilized fruit. Thus, we further tested the potential benefit of *Ac. thailandicus* by
629 comparing the development of *D. melanogaster*, from axenic eggs, in non-cleaned, freshly
630 collected figs, in the presence or absence of these bacteria (Fig 7H). Flies grown in the
631 presence of *Ac. thailandicus* had approximately the double of the survival rate to adulthood
632 than control flies with no bacteria added (Fig 7I, S14D Fig, lmm, $p = 0.010$). This is similar to
633 the effect seen in sterile figs. The effect of *Ac. thailandicus* on the time to reach adulthood
634 varies with replicate (Fig 7J, S14E Fig, lmm, $p < 0.001$). In one replicate the bacteria
635 presence does not affect time of development (S14E Fig, $p = 0.557$), while in the other
636 replicate *Ac. thailandicus* decreases time of development by 3.5 days (Fig 7J, $p < 0.001$). This
637 difference between replicates may reflect the variable bacteria consortiums in the figs
638 collected at different times. These results support that the stable association between *D.*
639 *melanogaster* and *Ac. thailandicus* is beneficial for the flies in their natural environment.
640

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641 **Discussion**

642 Here, we identify bacterial isolates from a natural population of *Drosophila*
643 *melanogaster* that can proliferate and stably colonize the gut of their host. These results
644 demonstrate that *D. melanogaster* has *bona fide* gut bacterial symbionts in the wild. We
645 further show that the association with one of these gut bacterial symbionts, *Acetobacter*
646 *thailandicus*, can be mutually beneficial. On one hand, stable colonization of *D. melanogaster*
647 gut permits continuous bacterial shedding to the environment, and, therefore, potentially
648 increasing bacterial dispersion in the wild. On the other hand, transmission of *Ac.*
649 *thailandicus* to the food substrate, concomitant with egg laying, benefits *D. melanogaster*
650 larval development. These bacteria shorten developmental time and increase fertility of *D.*
651 *melanogaster*. This stable interaction may be particularly important for *D. melanogaster* since
652 different bacteria affect differentially its development and *Ac. thailandicus* is more beneficial
653 than most bacteria sampled from the gut of wild flies. Moreover, *Ac. thailandicus* is still
654 beneficial when larvae develop in non-sterile fruit collected from nature.

655

656 **Diversity and stability of gut bacteria in wild and laboratory *D. melanogaster***

657 In this study, one of our main concerns was to quantify, in different scenarios, absolute
658 levels of live bacteria in the gut of *D. melanogaster*. Therefore, the several protocols we
659 developed were mainly based in culture dependent techniques. This approach also allowed us
660 to isolate bacteria for further functional characterization. Moreover, gut bacteria of *D.*
661 *melanogaster* previously identified through 16S rRNA gene sequencing [20,26,28,41-46]
662 belong to genera that can also be identified by culture dependent techniques. However, it is
663 possible that our approach missed gut bacteria that do not grow in the media or conditions
664 that we used. Additionally, our approach mainly identifies the bacterial strains that are more
665 frequent in the gut as there is a limited number of colonies in the plates analyzed. Because of
666 these limitations our analysis may be incomplete. Nonetheless, our approach managed to
667 quantify overall gut bacterial numbers in different husbandry conditions, and, when tested,
668 the results were confirmed by quantitative PCR. Moreover, we were able to identify, isolate,
669 and analyze bacteria that can stably associate with *D. melanogaster* gut.

670 Our results show a striking difference in gut bacterial diversity between lab and wild
671 caught flies. Lab flies carry mainly two bacterial species corresponding to *Acetobacter*
672 OTU2753 and *Lactobacillus* OTU1865. This low diversity and dominance of *Acetobacter* and
673 *Lactobacillus* species is in agreement with several previous studies on the gut associated
674 bacteria in lab flies [20,22,24-27]. On the other hand, wild caught flies have a much higher
675 diversity of bacteria. We were able to identify 35 different OTUs in the ten individual flies
676 freshly collected from the wild, and the sampling did not seem close to saturation. This higher
677 diversity is also in agreement with previous reports [25,28]. The characterization of individual

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678 flies allowed us to identify *Enterobacteriaceae*, *Acetobacteriaceae* (mainly *Acetobacter* and
679 *Gluconobacter* species), *Leuconostocaceae*, and *Bacillaceae* as the most prevalent families,
680 present in over 50% of the flies. These families of bacteria have been identified before in wild
681 caught *D. melanogaster*, although *Bacillaceae* are found less frequently [25,28,41-43,46].
682 *Lactobacillus* was found in only one out of ten freshly collect wild flies analysed. Although
683 the low prevalence of *Lactobacillus* could be a characteristic of this specific population, it is a
684 general trend observed in other published surveys [25,28,41-43,46].

685 We tested persistence of bacteria in the gut of *D. melanogaster* by regularly changing
686 individual flies to fresh axenic food and, therefore, reducing the potential intake of bacteria
687 from contaminated food. This protocol is alike the one used in Blum et al. 2013 [20].
688 Similarly to that paper, we also found that the *Acetobacter* and *Lactobacillus* species
689 associated with this laboratory stock cannot stably persist in the gut. Moreover, we show that
690 these bacteria can grow in the fly food. Thus, these bacteria are only transiently passing
691 through the gut. This result highlights how husbandry conditions can affect *D. melanogaster*
692 gut bacterial levels and that these measured levels can be unrelated with gut colonization (also
693 shown in [20,27]).

694 In contrast to lab flies, wild caught flies carry bacteria that, following this protocol,
695 persist in the gut of *D. melanogaster*. This shows that in its natural state *D. melanogaster*
696 lives with gut colonizing bacteria. *L. pseudomesenteroides*, *Ac. cibinongensis* and *Ac.*
697 *thailandicus* were each present in more than 50% of wild flies at the end of the stability
698 protocol. They are, therefore, interesting bacteria to further characterize in their interaction
699 with *D. melanogaster*.

700 Several bacteria were present in 50% or more of the flies when they were caught, but
701 were severely reduced in frequency after the stability protocol. These include *Bacillus*
702 OTU1570, the *Enterobactereaceae* OTU3529, *Tatumella* OTU3635, and *Kluyvera ascorbata*
703 OTU3643, and the *Acetobacteraceae Ac.* OTU2753, *Ac. ghanensis* OTU2757, *Ac. lovaniensis*
704 OTU2759, *Ac. orientalis* OTU2760, and *Gluconobacter* OTU2781. These species may be
705 transient gut bacteria that were acquired from the environment. However, it is also possible
706 that they are stable gut bacteria that cannot be sustained in the particular lab environment we
707 used. For instance, in the fly food we used there may be nutritional requirements missing for
708 their maintenance or there could be toxic compounds to them (e.g. methylparaben). In the
709 future, this protocol could be repeated using other food source, as for example the fruit
710 matching the source of capture. However, it will be difficult to assert that a particular
711 bacterial strain cannot persist in the gut even if it fails to show that property under more
712 natural conditions. The natural environment of *D. melanogaster* is very complex and includes
713 decomposing and fermenting fruit replete with different microorganisms. This will be hard to
714 replicate and study in a controlled lab setup.

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715 At the end of the stability protocol there was still a high diversity of bacteria in the gut
716 of *D. melanogaster* even if most were present in less than 50% of the flies. These may
717 represent rare but stable gut bacteria of *D. melanogaster*, as the case of *Lactobacillus* species.
718 A particular fly (fly 39 in Fig 2) has an interesting pattern of microbiota composition after the
719 stability protocol. It is the only wild caught fly that has no *Lactobacillales* or
720 *Acetobacteriaceae*. Instead it carries six rare OTUs at relatively high levels. This gut
721 microbiota composition may represent a disease-related dysbiosis and some of these bacteria
722 could be pathogenic.
723
724 **Gut colonization by *Ac. thailandicus* and *Ac. cibinongensis***
725 To further characterize the interaction of the bacteria that persist in the gut of wild flies,
726 we studied them in monoassociation with lab flies. In contrast to the lab isolate of *Ac.*
727 OTU2753, both *Ac. cibinongensis* and *Ac. thailandicus* persist in the gut of lab flies until the
728 end of the stability protocol. However, the levels of both bacteria decreased significantly in
729 the first two days of this assay. These results indicate that the majority of the bacteria found in
730 the gut of these flies at the beginning of the experiment were transient and lost with the same
731 dynamics as unstable bacteria, but a certain part of these two bacterial populations are stably
732 associated with the host. These results, in monoassociation, demonstrate that either of these
733 bacteria have the autonomous property to persist in the host, independently of other
734 microbiota members. Moreover, this property seems largely independent of host background
735 since it is observed in the $w^{1118}$ *iso* lab flies and in several individuals of the natural outbred
736 population.
737 Both *Ac. thailandicus* and *Ac. cibinongensis* are able to proliferate in the gut of *D.*
738 *melanogaster*. Interestingly, *Ac. thailandicus* seems to proliferate faster and reaches higher
739 levels in 24h, which is coherent with higher bacterial levels in the stability protocol. The
740 stability and proliferation assays show that these bacteria are *bona fide D. melanogaster* gut
741 colonizers.
742 The niche of the stable population of *Ac. thailandicus* is the anterior gut of *D.*
743 *melanogaster* since it is present in the crop and anterior midgut samples and absent from the
744 mid midgut to the hindgut. The crop is a diverticulum of the oesophagus that can store liquid
745 food [47]. In our analysis, the anterior midgut sample also included the proventriculus, which
746 is part of the foregut. This raises the possibility that *Ac. thailandicus* stable population is
747 restricted to the foregut. The epithelium in the foregut region has a cuticular lining, which
748 could provide a surface for the bacteria to attach. Also, the crop lumen is not subject to the
749 same linear flux as the rest of the gut lumen, which might facilitate bacterial persistence. A
750 similar argument is made for the appendix and cecum, in humans and other mammals, as a
751 reservoir of microbiota [48,49].

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752 The midgut has a different structure and kinetics to the foregut. In the midgut the
753 peritrophic matrix separates the gut lumen from the epithelial cells. This barrier is
754 continuously secreted at the proventriculus and moves through the midgut with food [50,51],
755 which may hamper stable bacterial colonization. Moreover, the foregut-midgut border may
756 work as a physical or immunological barrier for microorganisms in insects [27,52-54], and
757 the acidic region in the anterior midgut may also contribute to bacteria killing [38,55]. A
758 reduction in bacterial loads after this transition is evident for *Ac. thailandicus* even before the
759 stability protocol, and was previously observed in *D. melanogaster* gut [27,38].
760 The anterior gut may be a common location for bacterial colonization in *D.*
761 *melanogaster*. *Pseudomonas aeruginosa*, a pathogenic bacteria, also colonizes the crop,
762 where it forms a biofilm [56], and it was also suggested that the anterior gut could be a site
763 for stable attachment of *Lactobacillus plantarum* [31]. Moreover, the crop was identified as
764 the region where yeasts proliferate in flies, 130 years ago [57]. In the future it will be
765 interesting to investigate where other *D. melanogaster* bacterial gut colonizers reside (e.g. *Ac.*
766 *cibinongensis, L. brevis*). In other insects gut bacteria are known to colonize diverse
767 locations, including the proventriculus, the posterior midgut, and the hindgut [58-63].
768

769 ***D. melanogaster* and *Ac. thailandicus* mutualism**

770 Given the stable association between *D. melanogaster* and *Ac. thailandicus*, we asked
771 if there was any advantage for either partner in this interaction. Symbiosis between a host and
772 a microbe does not necessarily signifies mutualism and the effect of host-association on the
773 microbial partner has been less frequently studied [64,65]. Our results indicate that the stable
774 association of *Ac. thailandicus* to the gut of the adult fly is advantageous to this bacterium
775 since it can promote its dispersal.
776 The interaction with *Ac. thailandicus* is also advantageous to *D. melanogaster* in
777 several scenarios. *Ac. thailandicus* shortens larvae developmental time to pupariation and
778 adulthood when compared to axenic conditions. This effect does not necessarily increase
779 fitness but it may, if there are no associated trade-offs, as shown with *L. plantarum* [37].
780 Interestingly, adult flies that developed in the presence of *Ac. thailandicus* are also more
781 fertile, a clear measure of fitness, when compared with flies that developed axenically. These
782 phenotypes demonstrate the benefit of *Ac. thailandicus* during *D. melanogaster* development.
783 Other bacteria have been shown before to shorten development time of *D. melanogaster*
784 [15,16,66-69] and increase adult fertility when associated in larval stages [70]. Moreover,
785 adding the unstable lab isolate *Ac.* OTU2753 to axenic eggs also had a similar effect to
786 adding *Ac. thailandicus*, in terms of developmental time and later adult fertility. So the direct
787 developmental benefit conferred by these *Acetobacter* does not seem dependent on the
788 capacity to colonize the gut of *D. melanogaster*. However, the most interesting aspect of this

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789 result is that, out of the 15 bacteria isolated from wild flies, *Ac. thailandicus* induced the
790 shorter development time and higher fertility. Therefore, out of the set of bacteria interacting
791 with *D. melanogaster* in the wild, this stable gut symbiont is particularly beneficial.

792 We do not know the mechanism through which *Ac .thailandicus,* or the other bacteria
793 we tested, benefit *D. melanogaster*. The negative correlation that we observed between
794 developmental time and fertility, suggests a similarity on the mechanisms behind these
795 phenotypes. Microorganisms have for long been recognized as important for *Drosophila*
796 development and as a source of food [14,71]. In fact the standard *Drosophila* food used in the
797 lab is partly composed of dead *Saccharomyces cerevisiae* [72], which, in this diet, is required
798 and sufficient for *Drosophila* development. Moreover, in lab diets the bacterial influence on
799 host development is generally stronger the less yeast extract the food contains [15,16]. A
800 recent study with *L. plantarum* also shows that heat-killed bacteria can rescue growth in
801 germ-free conditions almost to the same extent as live bacteria [38]. In adults, constant supply
802 of heat-killed yeast *Issatchenkia orientalis* can also extend the lifespan of *D. melanogaster* to
803 the same extent as live yeast [19]. The nutritional value of these microorganisms may be
804 based on supplying aminoacids or vitamins to the host [14,19,71,73]. Other evidence
805 indicates that the effect of microorganisms on development of *D. melanogaster* could also be
806 independent of its nutritional value. Bacteria can directly impact host physiology by
807 activating the insulin pathway, via acetic acid production in the case of an *Acetobacter*
808 *pomorum*, or gut proteases in the case of *L. plantarum* [16,39,74].

809 The benefit of *Ac. thailandicus* for *D. melanogaster* becomes even more evident when
810 larvae develop in figs, a natural food substrate. On sterile figs homogenates very few larvae
811 reach adulthood in axenic conditions, and those that do are severely delayed in growth and are
812 infertile as adults. These results show the insufficiency of fruit, or figs in this particular case,
813 to support normal *D. melanogaster* development. *Ac. thailandicus* rescues these phenotypes
814 and is, therefore, sufficient for *D. melanogaster* development on fruit, indicating a nutritional
815 basis for the interaction.

816 An alternative hypothesis is that bacteria are detoxifying some toxic components
817 present on the food. Detoxifying symbiosis is known to occur in many insects [75]. However,
818 the fact that *Ac. thailandicus* is beneficial both in lab food and figs indicate that to a large
819 extent its benefit is independent of food toxins.

820 Although we saw that *D. melanogaster* benefits when it develops with *Ac. thailandicus*,
821 we did not see a direct effect when flies are exposed to the bacteria only during adulthood.
822 When we associated this bacterium to axenic adults, and they maintained a stable bacterial
823 population for several days, their fertility did not change. However, direct effects of bacteria
824 on adults have been previously reported on oocyte development or fertility [70,76]. Many
825 factors may explain the different results, including the identity of the bacteria tested. Another

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826 explanation could be that the relatively small bacterial stable population in the gut, as in our
827 assay, does not have an impact on host fertility, but higher levels of *Ac. thailandicus* would.
828 The positive effect of *Ac. thailandicus* on the progeny of adults seem to be only due to being
829 transmitted to the next generation and not to any effect on the adult itself. This is
830 demonstrated by the fact that adding *Ac. thailandicus* to axenic eggs has the same effect, in
831 terms of development, as having parents associated with the bacterium. Nonetheless, it will be
832 interesting in the future to determine if the stable *Ac. thailandicus* population has any other
833 effect on the adult physiology.
834
835 **Gut colonization by *Leuconostoc and Lactobacillus***
836 Analysis of *L. pseudomesenteroides* stability and proliferation in *D. melanogaster* gut
837 produced ambiguous results. This bacterium seemed very stably associated with the gut of
838 wild and monoassociated lab flies when the stability protocol was performed in vials. When
839 we implemented the protocol using cages, however, it disappeared from 50% of the flies.
840 These results illustrate how sensitive to experimental conditions is this assay, and that
841 stringency is crucial. The proliferation assay did not clearly show an increase or decrease in
842 *L. pseudomesenteroides* at 24h, when compared to the beginning of experiment. These results
843 could be the consequence of this bacterium being able to very rapidly proliferate in the gut of
844 the fly but unable to attach to the host and, therefore, require a constant cycle of re-
845 inoculation. Maybe this cycle could be kept in vials but broke down in cages. Further
846 experiments will be required to test this hypothesis and elucidate the interaction of *L.*
847 *pseudomesenteroides* with *D. melanogaster*.
848 *Lactobacillus* species were still present in wild flies at the end of stability protocol,
849 although less frequently than *Ac. thailandicus*, *Ac. cibinongensis*, and *L.*
850 *pseudomesenteroides.* Interestingly, the data indicate a negative interaction between
851 *Lactobacillus* and *Leuconostoc* presence. Both are lactic acid bacteria (order *Lactobacilalles*)
852 and they may occupy the same niche and compete for resources. Of the many bacterial
853 isolates from the gut of wild flies, *L. brev*is, and *L. paraplantarum* are the most beneficial in
854 terms of development time and fertility of *D. melanogaster*, together with *Ac. thailandicus*.
855 This contrasts with previous reports indicating a small or null effect of lab *Lactobacillus*
856 isolates on fecundity [37,70]. *L. brev*is is present in four out of ten wild flies after the stability
857 protocol and proliferates in the gut of *D. melanogaster*. So, *L. brev*is may also be a beneficial
858 *bona fide* gut symbiont of *D. melanogaster*, although not as frequent as *Ac. thailandicus* in
859 this population.
860
861 **Ecological advantage of a stable gut association with beneficial bacteria**

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862 Our results indicate that the interaction between *D. melanogaster* and the gut symbiont
863 *Ac. thailandicus* is especially beneficial for both partners in the wild (Fig 8). The small stable
864 bacterial population in the gut serves as a reservoir for the inoculation of the environment that
865 the adult fly explores and exploits. This is beneficial to the bacteria since it leads to their
866 continuous dissemination. On the other hand, transmission of *Ac. thailandicus* to the food
867 substrate of the next generation, concomitant with egg laying, benefits *D. melanogaster*
868 development. This association is therefore a form of farming, a strategy adopted by several
869 insects, including ants, termites and ambrosia beetles with fungi [77]. The stability of the *D.*
870 *melanogaster–Ac. thailandicus* interaction provides the host some independence from the
871 local bacterial populations and enables it to explore and modulate bacterial populations in
872 new locations.

873

874 **Fig 8 – Model for an ecological advantage of a stable association between *D. melanogaster***
875 **and beneficial gut bacteria.** (A) In the absence of stable gut bacteria, the fitness of *D. melanogaster* is
876 dependent on the presence of more (red) or less (blue) beneficial bacteria in the food substrate. (B)
877 Carrying a stable population of beneficial bacteria (green) in the gut allows constant bacterial
878 inoculation of food substrate and consequent association with the next host generation. This leads to a
879 higher fitness of this next generation.
880

881 Besides the interaction with these stable bacteria in the wild, *D. melanogaster* also
882 interacts with a plethora of environmental bacteria that are transiently associated with the gut.
883 Many of these non-colonizing bacteria probably positively impact on *D. melanogaster*
884 biology, and vice-versa. *D. melanogaster* are attracted to feed on, or oviposit in substrates
885 with specific potential benefiting bacteria [76,78-81]. Attraction to fermenting fruits enriched
886 with beneficial bacteria may be a strategy adopted by *D. melanogaster* to increase interactions
887 with these bacteria. Furthermore, *D. melanogaster* most likely disperses bacteria that transit

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888 through its gut. By attracting flies certain bacteria could, therefore, increase their probability
889 of being dispersed. However, if bacteria are not stably associated with the flies, this would be
890 a transient phenomenon, as evident in the rapid loss of *Ac.* OTU2753 in our experimental
891 system involving a short exposure to bacteria and continuous change of food in cages. *D.*
892 *melanogaster* may also benefit bacteria by promoting their growth in the food substrate [38],
893 which could be advantageous for the host if biased towards beneficial bacteria. Despite all
894 these potential mechanisms promoting beneficial interactions, relying on the immediate
895 environmental and local bacterial community may be suboptimal for *D. melanogaster* (Fig 8).
896 *Ac. thailandicus* belongs to the acetic acid bacteria, a group of bacteria that oxidise the
897 ethanol present on fermenting fruits to acetic acid. These bacteria are found associated with
898 many *Drosophila* species and a wide range of other insect species, which normally rely on
899 high-sugar diets [82,83]. Several *Drosophila* species are attracted by acetic acid bacteria and
900 this is probably related with the production of acetic acid [79,80,84]. In addition, the aerobic
901 environment and acidic pH of digestive tracts of most insects are suitable for acetic acid
902 bacteria growth [82,85-87], and these bacteria produce extracellular matrixes, which can be
903 involved in host adherence [83,88]. *Ac. thailandicus* interaction with *D. melanogaster* is
904 another contribution to the understanding of the association of this group of bacteria with
905 insects in an ecological context.
906 In the future it will be interesting to address some questions relevant for this model. For
907 instance, we do not know how stable is *Ac. thailandicus* in the gut of larvae or if this stability
908 is important. It may be sufficient for the bacteria to grow on the food substrate since larvae
909 are less mobile and they will be in constant contact with the local external population of
910 bacteria. Another important aspect is to understand how adult flies acquire *Ac. thailandicus*.
911 This could be through constant association throughout the developmental stages, including
912 from larvae to pupae to adult, or *de novo* acquisition after adult eclosion [66].
913 This farming interaction model may extend to other bacteria, including *L. brevis*.
914 Moreover, our study focused on the gut colonizing bacterial species in one *D. melanogaster*
915 population. It will be important to analyze other natural populations and determine to what
916 extent there is conservation of stably colonizing species or if different *D. melanogaster*
917 populations harbor different gut bacterial symbionts. This analysis could elucidate if there is a
918 core gut microbiota of *D. melanogaster* based on stable symbionts. Diet has been shown
919 before to influence the composition of the total bacteria associated with *D. melanogaster*
920 [25,28,89]. Thus, it would be interesting to investigate how diet, or geography, determines the
921 stable gut bacterial community. Moreover, additional studies need to be performed to identify
922 other types of microbes that can stably associate with *D. melanogaster*. Particularly, it would
923 be important to identify natural yeasts isolates that would colonize *Drosophila* intestine,
924 given that flies are constantly exposed to different yeasts in the natural habitat.

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925 Interactions between microbes may affect their colonization and their influence on host
926 phenotypes. These may happen with other colonizing bacteria or with environmental bacteria
927 on the food substrate or while in transit through the gut. Our analysis of wild-caught flies
928 incorporates, to a certain degree, this complexity. For instance, *Ac. thailandicus* that stably
929 colonizes in monoassociation is also present in the gut of the majority of wild flies of the
930 population we analyzed, showing that its association is robust in the face of rich bacterial
931 communities. Moreover, the beneficial effect of this bacterium observed in monoassociation
932 is also present in the context of complex and natural microbial communities of figs. On the
933 other hand, the analysis of wild-caught flies also indicates a negative interaction between
934 *Lactobacillus* and *Leuconostoc* species.
935
936 **Specificity of gut symbionts**
937 *Ac. thailandicus* can colonize the gut of *D. melanogaster* but not of *D. simulans.* On the
938 other hand, *Ac. thailandicus and Ac. cibinongensis* seem to be the only stable *Acetobacter*
939 species in the population we analyzed and *Ac.* OTU2753 from the lab cannot colonize the gut
940 of *D. melanogaster*. This indicates that these stable interactions are specific from both host
941 and symbiont perspectives. Subtle differences in the bacteria associated with *D. melanogaster*
942 and *D. simulans* in the wild have been found before [28] but differences may be clearer when
943 looking into the stable gut symbionts of different *Drosophila* species.
944 The presence of these species-specific mutualistic interactions of gut bacteria with *D.*
945 *melanogaster* raises the possibility that these are long-term interactions and the result of
946 adaptation. Therefore, they may be a good system to study host-symbiont evolution and even
947 address questions of co-evolution and co-speciation [30,90-92].
948 We do not know the cause of the specificity of these colorizations. The interaction
949 between the host immune system and different bacteria could be one of the mechanisms
950 involved in this selection. Pathogenic bacteria can down-regulate or escape from the host
951 immune system to establish infection [93]. In *D. melanogaster* alterations in immunity have
952 an impact on gut bacterial compositions or load [22,27,94]. In mosquitoes the expression of
953 host C-type lectins protects gut bacteria from antimicrobial peptides (AMPs) action in the gut
954 and therefore modulate the gut bacterial community [95]. Many innate immune genes in
955 *Drosophila* species are under fast positive selection [96-98] and differences in these genes
956 could mediate association of different *Drosophila* species with different stable gut bacteria.
957
958 **Stable gut bacteria in *D. melanogaster* as an experimental system**
959 Although the perspective of a transient microbiota has been dominant in most analyses
960 of gut bacteria in *Drosophila* [20,27,38,43,99], there is some evidence of stable gut bacteria
961 in these flies. Recently it was shown that a wild isolate of *Lactobacillus plantarum* has a

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962 higher frequency of gut colonization than a lab isolate [31]. These results are in agreement
963 with a tendency for wild isolates of bacteria being better at colonizing *D. melanogaster.*
964 However, in this study once bacterial colonization was established, titres were constant over
965 time in wild and lab isolates [31]. It will be interesting to also test these isolates with the
966 proliferation and stability protocols that we describe here. On a different approach, analysis of
967 wild-caught individuals from other mushroom and cactus-feeding *Drosophila* species have
968 identified bacterial strains highly enriched in the gut but very poorly represented in matched
969 substrate samples [29,30]. This indicates that these enriched bacteria are gut symbionts and it
970 will be also interesting to study them in more detail.
971 The presence of stable associations in the wild raises the question of why these seem to
972 have been lost in laboratory stocks. Part of the answer may be related with the fact that
973 association with non-colonizing bacteria can be as beneficial as with colonizing bacteria in
974 the lab (e.g. *Ac.* OTU2753 *vs Ac. thailandicus*). Fly husbandry conditions in the lab normally
975 ensure transmission of bacteria from generation to generation even if they do not stably
976 colonize the gut. Therefore, under laboratory conditions, there may be a loss of selective
977 pressure for stability. This can lead to loss of the capacity to stably colonize the gut by the
978 bacteria either by drift or by selection if there is a cost associated with this capacity.
979 Alternatively, colonizing bacteria may be replaced by non-colonizing strains in the lab. The
980 lab diet is relatively uniform and different from the natural diet, therefore, bacteria better
981 adapted to these conditions may outcompete wild isolates [100]. Moreover, use of antifungal
982 antimicrobials, and sometimes antibiotics, may constantly or occasionally severely disrupt
983 bacterial communities associated with the flies that are then replaced with local bacterial
984 strains that do not have the capacity to colonize *Drosophila*. One or combinations of these
985 factors may over the long periods of time that flies are kept in the lab lead to the loss of the
986 original microbiota. From our experience, wild bacterial isolates seem to be easily
987 outcompeted in lab conditions and replaced by other bacteria, since we needed to carefully
988 handle the fly stocks to keep the monoassociations with wild isolates.
989 Exploring the interactions between hosts and its natural colonizing symbionts can
990 uncover new phenotypes missed in laboratory experiments. Previous studies with other
991 organisms have shown that indeed this can be the case. For instance, in the nematode
992 *Caenorhabditis elegans,* bacteria isolated from natural habitats conferred higher fitness when
993 compared with the standard *E. coli* used in the laboratory [101,102]. Also, wild collected
994 mice harbor a different microbiota to laboratory mice, which decreases inflammation and is
995 protective upon infection and tumorigenesis [103]. The capacity to colonize and proliferate in
996 the gut of *D. melanogaster* described in this study, demonstrates different properties from lab
997 and wild bacterial isolates. Moreover, other phenotypes associated with this wild isolates may
998 yet be identified.

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999 The stable interaction we found between *D. melanogaster* and gut bacteria will be
1000 useful to address important questions in the gut microbiota field using this model system.
1001 This includes identifying and characterizing from the host and bacteria perspective genes
1002 required for colonization and for the control of this interaction. Moreover, it will allow
1003 understanding determinants of specificity, which are largely unknown, although adhesion and
1004 biofilm formation are important in this process [104,105]. These questions are also relevant to
1005 specifically understand better and manipulate insect gut symbionts. The release of insects
1006 with specific gut bacteria in interventions may be useful against pests (e.g. by increasing the
1007 fitness of sterile males [106]) and against vectors of disease (e.g. by increasing resistance to
1008 pathogens [107,108]. Knowing what regulates gut stability may be important for the success
1009 of these approaches.
1010 Our work defines a new paradigm for the association between *D. melanogaster* and gut
1011 bacteria in which stable associations exist and contribute to the fitness of both partners in an
1012 ecological context. Therefore this new conceptual and experimental framework to study gut
1013 stable symbionts will contribute to the growing field of *Drosophila*-microbe interactions.
1014

1015 ## Materials and Methods

1016

1017 ### Wild fly collection, stocks source, and maintenance

1018 Wild flies were collected with traps, with fallen figs as bait, placed for 24h under a fig
1019 tree in Oeiras, Portugal (GPS coordinates 38°41'32.1"N, 9°18'59.4"W). *D. melanogaster* and
1020 *D. simulans* males were identified according to [40]. All the material to collect and sort wild
1021 flies was sterilized prior to use.
1022 DrosDel $w^{1118}$ isogenic stock ($w^{1118}$ *iso*) [33] was used as a laboratory stock, unless
1023 otherwise indicated. The female lines *D. melanogaster* O13 and *D. simulans* O13 were
1024 established from single wild females collected in 2013, and latter identified to the species
1025 level. Other stocks used were *D. melanogaster* Canton-S (Bloomington Drosophila Stock
1026 Center at Indiana University, stock #1), and *D. simulans* A07 and J04 (Drosophila Species
1027 Stock Center from California University, stocks #14021-0251.260, and #14021-0251.187,
1028 respectively). Unless otherwise indicated flies were 3-6 days in the beginning of experiments.
1029 The age of wild-caught flies is uncontrolled.
1030 Stocks were kept and experiments were performed at 25ºC in standard *Drosophila* food
1031 composed of 1.05L water, 80g molasses, 22g beet syrup, 8g agar, 10g soy flour, 80g
1032 cornmeal, 18g yeast, and 30ml of a solution containing 0.2g of carbendazim (Sigma) and
1033 100g of methylparaben (Sigma) in 1L of absolute ethanol. Food was autoclaved before
1034 dispensing it into vials.
1035

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1036 **Bacterial culture**

1037 Analysis of bacteria present in the gut was performed by culture dependent methods in
1038 order to isolate bacteria for further manipulations. From each fly the gut (including crop,
1039 midgut, and hindgut) together with the Malpighian tubules was dissected in Tris-HCl 50mM,
1040 pH 7.5, and homogenized with a plastic pestle in an 1.5mL microcentrifuge tube with 250µL
1041 Luria Broth (LB). Each sample was serially diluted (1:10 factor) and 30µL from each dilution
1042 were plated in five different culture media: LB (GRiSP), MRS (Merck), Liver Infusion Broth
1043 (Becton Dickinson), Brain heart infusion (BHI) (Sigma-Aldrich) and Mannitol (3g of Bacto
1044 Peptone (Becton Dickinson), 5g of Yeast Extract (Sigma-Aldrich), 25g of D-Mannitol
1045 (Sigma-Aldrich), 1L of Milli-Q water). Plates were incubated at 25ºC for six days and
1046 dilutions containing 30-300CFUs were used to count and isolate bacteria.

1047 To analyze flies or food associated with only specific bacterial isolates samples were
1048 plated on specific media to grow the correspondent bacteria (Mannitol for *Acetobacter* and
1049 MRS for *Leuconostoc or Lactobacillus*). Plates were incubated at 25ºC for 4 days.

1050

1051 **Quantification, isolation and identification of gut-associated bacteria**

1052 For quantification of total bacteria in each gut sample we selected the data from the
1053 medium that presented the highest number of colonies.

1054 For a detailed analysis bacterial colonies were assigned, in each culture medium plate,
1055 per sample, to distinct morphological types and determined their number. Two colonies of
1056 each morphological type, per culture medium plate, per sample, were re-streaked and, after
1057 growth, colonies were picked, dissolved in 500µL LB containing 15% glycerol (v/v) and
1058 frozen at -80ºC.

1059 To identify each bacterial isolate a PCR was performed to amplify the 16S rRNA gene.
1060 For most samples a bacterial colony, or part of it, was directly placed in the PCR reaction
1061 tube (colony PCR). In the few cases where amplification was unsuccessful by colony PCR,
1062 DNA extraction was performed with ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo
1063 Research according to the manufacturer's instructions). Primers used were: 27f (5'-
1064 GAGAGTTTGATCCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTTACGA-3')
1065 with the following PCR conditions: 94ºC for 4min; 30 cycles of 95ºC for 30sec, 58ºC for
1066 1min, and 72ºC for 2min; 72ºC for 10min. PCR products were sequenced at Source
1067 Biosciences Sequencing Center. Sequences were trimmed to 800bp of each sequence
1068 including V2 to V4 hypervariable regions. These sequences were aligned against a core set
1069 aligned fasta file from Greengenes [34] using PyNAST [109], and classified into operational
1070 taxonomic units (OTUs) according to Greengenes taxonomy [34]. Sequences that matched
1071 *Ralstonia* OTU3005, *Novosphingobium stygium* OTU2886, and *Novosphingobium* OTU2881

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1072 were removed from the analysis since they were occasionally present on negative controls for
1073 PCR.
1074 In most cases each morphological type corresponded to one OTU. However, three
1075 groups of bacteria had different OTUs commonly assigned to the same morphological type.
1076 Thus, these bacteria could not be distinguished, within their group, based on colony
1077 morphology. These groups are composed of bacteria belonging to the *Lactobacillus* genus,
1078 the *Acectobacteraceae* family (*Acetobacter* and *Gluconobacter* genera), or the
1079 *Enterobacteriaceae* family. The frequencies of the sequenced colonies from each group are
1080 represented in Fig 3 and S3 Fig.
1081 To determine CFUs per gut for each OTU, or group of bacteria, the data from the
1082 medium that presented the highest number of colonies was selected.
1083 Bacterial isolates used for phenotypic analysis (*Ac.* OTU2753, *Ac. thailandicus*, *Ac.*
1084 *cibinongensis, L. pseudomesenteroides* and all isolates used in Fig 7B and S12) were
1085 sequenced with both 27f and 1495r primers, and analyzed at least from V2 to V8 hyper
1086 variable regions of the 16S rRNA sequence. Sequences were automatically edited with
1087 PhredPhrap and consensus sequences were generated using BioEdit Sequence Alignment
1088 Editor Software. Sequences are in S1 text and deposited in GenBank with the following
1089 accession numbers: MG808351.1, MG808350.1, MG808352.1, MG808353.1, MG808354.1,
1090 MG808355.1, MG808356.1, MG808357.1, MG808358.1, MG808359.1, MG808360.1,
1091 MG808361.1, MG808362.1, MG808363.1, MG808364.1, MG808365.1.
1092
1093 **Real-time quantitative PCR for 16S rRNA gene**
1094 DNA was extracted from dissected single guts with QIAamp DNA Micro kit (Qiagen)
1095 as described in the protocol "isolation of Genomic DNA from Tissues". To facilitate DNA
1096 extraction from Gram-positive bacteria the guts were homogenized in 180μL of enzymatic
1097 lysis buffer with Lysozyme (from DNeasy Blood & Tissue Kit, QIAGEN) and incubated for
1098 1 hour at 37ºC, before starting the protocol. DNA concentrations were determined with a
1099 NanoDrop ND-1000 Spectrophotometer. Quantitative-PCR reactions were carried out in
1100 CFX384 Real-Time PCR Detection System (BioRad). For each reaction in 384-well plate
1101 (BioRad), 6 μL of iQ SYBR Green supermix (BioRad), 0.5 μL of each primer solution at 3.6
1102 mM and 5 μL of diluted DNA were used. Each plate contained three technical replicates of
1103 every sample for each set of primers. Primers used to amplify the 16 S rRNA gene were: 8FM
1104 (5'–AGAGTTTGATCMTGGCTCAG-3') and Bact515R (5'-
1105 TTACCGCGGCKGCTGGCAC-3') [110]. Primers used to amplify *Rpl32* were: Rpl32
1106 forward (5'-CCGCTTCAAGGGACAGTATC-3') and Rpl32 reverse (5'-
1107 CAATCTCCTTGCGCTTCTTG-3'). The thermal cycling protocol for the amplification was:
1108 initial 50ºC for 2 min, denaturation for 10 min at 95ºC followed by 40 cycles of 30 sec at

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1109 95ºC, 1 min at 59ºC and 30 sec at 72ºC. Melting curves were analyzed to confirm specificity
1110 of amplified products. Ct values for manual threshold of 10 were obtained using the program
1111 SDS 2.4 or with Bio- Rad CFX Manager with default threshold settings. 16S rRNA gene
1112 levels were calculated relative to Day 0 sample with the Pfaffl method [111] using
1113 *Drosophila Rpl32* as a reference gene.
1114
1115 **Generation of axenic and monoassociated flies**
1116 To develop axenic flies, embryos were sterilized with 2% sodium hypochlorite during
1117 10 minutes, followed by 70% ethanol during 5 minutes and washed with sterile water.
1118 Embryos were placed in sterilized food vials and maintained in axenic conditions or
1119 monoassociated with 40µL of overnight bacterial culture of specific isolates. Monoassociated
1120 stocks were kept at 25ºC and flipped every 20 days, using sterile gloves. We waited at least
1121 two generations in monoassociation before performing experiments.
1122
1123 **Analysis of bacterial stability in the gut**
1124 The gut stability protocol in vials was based on placing a single fly per vial, with a food
1125 surface of 3.8 $cm^2$, and changing it twice a day to new vials. The stability protocol in cages
1126 was based on placing a single fly per cage with six petri dishes with a total fly food surface of
1127 486 $cm^2$, and changing them daily. Bacterial levels were analyzed in single guts.
1128 To analyze the gut region where stable bacteria are present, individual guts were
1129 dissected into 5 different regions - crop, anterior midgut, mid midgut, posterior midgut and
1130 hindgut. The proventriculus was included in the anterior midgut sample. Each gut region from
1131 a single fly was homogenized, plated, and quantified as described above.
1132
1133 **Analysis of bacterial proliferation in the gut**
1134 The proliferation assay was based on providing an inoculum of bacteria to axenic male
1135 flies for 6h and measure gut bacterial levels, by plating, immediately at the end of this period
1136 (time 0h), and 24h later. Bacteria were grown in the Mannitol (*Acetobacter*) or MRS
1137 (*Leuconostoc* or *Lactobacillus*) liquid media in a shaker at 28ºC overnight. Bacterial
1138 concentrations (cell/ml) were calculated based on OD600 using a spectrophotometer
1139 (SmartSpec 3000 from Biorad) using the formula OD1 = 5 x $10^8$ cell/ml. The inoculum was
1140 provided in vials by adding 180µL of bacterial solution in 2.5% sucrose to a round filter paper
1141 placed on top of the fly food. After the inoculation period, flies were placed singly in cages or
1142 in bottles (food surface: 486 $cm^2$ and 28 $cm^2$, respectively) for 24h. Bacterial levels were
1143 analysed in single guts by plating.
1144 To confirm that the 24h data corresponded to bacteria growing in the gut and not
1145 bacteria growing on the fly food and in transit we added an axenic fly to the cage or bottle at

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1146 time 0, in some experiments. Bacterial levels in the gut of these chaser flies were determined
1147 at time 24h, simultaneously with the co-habiting experimental fly.
1148
1149 **Analysis of bacterial proliferation on fly food**
1150 To analyze bacterial growth on food from bacteria associated with flies, conventionally
1151 reared 3-6 days old males were placed singly in vials for 24h, in order to contaminate the food
1152 with bacteria. After that period, flies were discarded and vials were incubated for 9 days at
1153 25ºC. Bacterial levels were determined after discarding the flies (Day 1) and after the 9 days
1154 of incubation (Day 10). Vials that never contained flies before were used as control vials and
1155 incubated also for 9 days (Day 10 control). 2.9g of top layer of food were homogenized in
1156 10mL LB. This homogenate was plated in the five different media.
1157 To analyze growth of *Acetobacter* species on the fly food, 3-6 days-old males
1158 monoassociated with the different *Acetobacter* were singly placed in vials with 4ml of fly
1159 food for 16 hours. After that period, males were discarded and bacterial levels were assessed
1160 at that time-point (Day 0) and after 1 or 5 days of incubating the vials at 25ºC. All the food
1161 from the vial was homogenized in 4ml LB. Mannitol plates were incubated at 25ºC for 4
1162 days.
1163
1164 **Fitness parameters determination**
1165 To determine fitness parameters in monoassociated stocks (S9 Fig) one virgin female
1166 and three 0-3-days old males were placed per vial for 3 days and then discarded. Time to
1167 pupariation and to adulthood was daily assessed, as well as total number of pupae and adults.
1168 To analyze fitness parameters of flies in a changing environment (Fig 6, S10 Fig),
1169 axenic 1-3 days old females and males were in contact for 6h with an inoculum of $10^5$
1170 CFU/µL *Acetobacter OTU 2753*, *Ac. thailandicus* or with sterile Mannitol. After this period,
1171 one female and two males were placed per cage for 10 days. Each cage contained six bottles
1172 with food that were changed everyday (total food surface of $170cm^2$). Single gut bacterial
1173 loads were analyzed in females 0 hours and 10 days post-inoculation and in males 10 days
1174 after inoculation.
1175 From each cage, all the six bottles were daily collected, number of eggs was counted
1176 and bottles were kept to daily assess adult emergence (Fertility G0 and Development of G1).
1177 Transmission of bacteria to the food was analyzed in bottles without eggs at days 1, 3, 5, 7,
1178 and 9. The food surface was washed with 1000µL of Mannitol and 100µl of this suspension
1179 was plated in Mannitol. As a control, food from axenic flies was also tested at days 1 and 9
1180 and no bacteria were detected.
1181 To analyze fertility of G1, bottles from day 9 and 10 from each condition were used to
1182 collect flies. One female and one male from the same condition were placed per vial and

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1183 flipped to new ones every other day, during 10 days. Adult emergence was daily assessed to
1184 determine total number of adults (Fertility of G1).

1185 To analyze if the benefit of *Ac. thailandicus* was dependent on the association with
1186 either parent we compared the four possible pairs of males and females from an axenic stock
1187 and a stock monoassociated with *Ac. thailandicus* (Fig 6H, S11). We placed one female and
1188 one male, both 1-2 days old, per vial and flies were passed to new vials every other day
1189 during 10 days. We also tested a condition in which 30μL of an overnight *Ac. thailandicus*
1190 culture was added to the progeny of axenic parents immediately after emptying it of parents.
1191 We daily assessed developmental time to pupariation and adulthood.

1192 To analyze fitness parameters conferred by different natural bacterial isolates (Fig 7B,
1193 S12 Fig), 50 sterilized eggs were placed per vial and inoculated with 40μL of an overnight
1194 bacterial culture. All isolates were grown at 28ºC in Mannitol, except *L. brevis*, *L.*
1195 *paraplantarum* and *L. pseudomesenteroides* that were grown in MRS. As controls we
1196 analyzed sterilized eggs associated with only Mannitol or MRS, or with no medium added.
1197 Developmental time to adulthood (Number of adults (G0), days to adulthood (G0)) was
1198 assessed. One male and female of the first adults emerging from each condition were placed
1199 per vial and flipped every other day during 8 or 10 days. Adult emergence was daily assessed
1200 to determine total number of adults (Fertility of G0).

1201 To analyze the impact of *Ac. thailandicus* on fitness parameters in sterile figs
1202 homogenate (Fig7 C-G), 50 sterilized eggs were placed per vial and inoculated with 40μL of
1203 an overnight culture of *Ac. thailandicus* or sterile Mannitol. Adult emergence was daily
1204 assessed. For the analysis of this G0 fertility, one male and one female adults that emerged
1205 from these vials were placed per vial and flipped every other day during 8 or 10 days. Adult
1206 emergence was daily assessed to determine total number of adults (Fertility of G0). The fig
1207 food homogenate was produced with 300mL homogenized commercial frozen figs, 600mL
1208 water, and 4.8g agar. After autoclave, food was poured to each vial in sterile conditions,
1209 inside a laminar flow hood.

1210 To analyze the fitness impact of *Ac. thailandicus* in fresh figs, we collected these at the
1211 same location where the wild flies were collected. Figs were cut in quarters and placed them
1212 in vials with sterilized agar (0.8% agar in water) at the bottom to fix the fig. Thirty sterilized
1213 embryos were placed on the top of these figs and inoculated with 40μL of an overnight
1214 culture of *Ac. thailandicus* or sterile Mannitol. Quarters originated from the same fig were
1215 distributed to the two conditions. Adult emergence was daily assessed. As a control, figs
1216 without the addition of eggs were kept and no flies emerged from those ones. Also, all flies
1217 that emerged from the experimental conditions had white eyes, confirming that they
1218 developed from the sterilized eggs and not from a possible contamination with wild flies
1219 present in the figs.

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1220

1221 **Statistical analysis**

1222 The statistical analysis was performed in R [112] and graphs were generated using the
1223 package ggplot2 [113] and GraphPad. The script of all the analyses is provided in S2 Text,
1224 where details can be found.

1225 Bacterial levels, number of eggs, pupae and adults, and time to pupariation and
1226 adulthood were analyzed using linear models (*lm*), or linear mixed-effect models (*lmer*
1227 package *lme4* [114]) if there were random factors. Significance of interactions between
1228 factors was tested by comparing models fitting the data with and without the interactions
1229 using analysis of variance (*anova*). Models were simplified when interactions were not
1230 significant. Pairwise comparisons of the estimates from fitted models were analyzed using
1231 *lmerTest* [115], *lsmeans* [116], and *multcomp* [117] packages.

1232 Timecourse analysis of bacterial stability in cages was performed fitting a non-linear
1233 least-squared model with the parameters of an exponential decay curve. Model simplification
1234 was achieved through analysis of variation (*anova*) and Akaike information criterion (*AIC*) of
1235 fitted models.

1236 Bacterial levels in flies in the changing environment cage assay were analysed with the
1237 non-parametric Mann-Whitney test (*wilcox.test*) because some data points were high and not
1238 estimated precisely.

1239 Bacteria transmission to bottles in the changing environment cage assay was analysed
1240 with a generalized linear mixed-effects (*lme4* package) with a binomial distribution.

1241 Independence of *Lactobacillus* and *Leuconostoc,* or different *Acetobactereaceae,*
1242 presence in wild-caught flies was tested with the Pearson's Chi-squared test *chisq.test*.

1243 Correlation between developmental time and fertility of flies that developed associated
1244 with different bacteria was tested through the Pearson correlation (*cor.test)* of the means of
1245 these parameters.

1246

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1259

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1577

1578 ## Supporting information

1579

1580 **S1 Fig - Wild-caught *D. melanogaster* have a stable gut microbiota.** Single 3-6 days old $w^{1118}$
1581 *iso* males were kept in the same vial during ten days (A) or exposed to a stability protocol by being
1582 passed to new vials twice a day (A, B). (A) Five individuals were analyzed at each day and total
1583 number of CFUs per gut determined by bacterial plating. Bacterial levels increase in the flies
1584 maintained in the same vials and decrease in the flies flipped to new vials twice a day (lmm, $p < 0.001$
1585 for both). (B) Relative amount of 16S rRNA bacterial gene was measured by quantitative-PCR in five
1586 individual guts from each day, using the host gene *Rpl32* as a reference gene. Relative amount of 16S
1587 rRNA gene decreases between days (lmm, $p < 0.001$). (C, D) Bacterial levels from wild-caught flies on
1588 the day of collection (Day 0) and after 5, 10 or 20 days of the stability protocol. Bacterial levels on the
1589 flies significantly decrease with time (lmm, $p = 0.004$). Each dot represents an individual gut and the
1590 lines represent medians. Statistical analyses were performed together with replicate experiments shown
1591 in Fig 1.

1592

1593 **S2 Fig - Higher diversity of gut bacterial communities in wild-caught *D. melanogaster*.**
1594 Accumulation curve of the different bacterial OTUs present in wild-caught and laboratory flies before
1595 (Day 0) and after (Day 10) being exposed to the stability protocol.

1596

1597 **S3 Fig - Total levels and diversity of *Enterobacteriaceae* in wild-caught *D. melanogaster*.**
1598 (A) Levels of *Enterobacteriaceae* in the gut of wild caught flies before (Day 0) and after 10 days of the
1599 stability protocol (Day 10). Each dot represents one gut and lines represent medians. Levels of
1600 *Enterobacteriaceae* decrease between days (lm, $p = 0.01$). (B) Frequencies of sequenced colonies of
1601 *Enterobacteriaceae* for Day 0 and Day 10, represented as several in Fig 2. Numbers on the top of the
1602 bars correspond to the number of flies carrying that specific OTU, from a total of 10 flies.

1603

1604 **S4 Fig – *Leuconostoc pseudomesenteroides* stably associates with the gut of wild *D.***
1605 ***melanogaster*.** Total *L. pseudomesenteroides* levels in the gut of wild caught flies in the day of
1606 collection (Day 0) and after 10 days of the stability protocol (Day 10). Levels of *L.*
1607 *pseudomesenteroides* are not significantly different between days (lm, $p = 0.372$). Each dot represents
1608 one gut and the line represents the median.

1609

1610 **S5 Fig - *Ac. thailandicus* and *Ac. cibinongensis* stably colonize the gut of *Drosophila***
1611 ***melanogaster*.** (A-I) Single 3-6 days old $w^{1118}$ *iso* males from monoassociated stocks with *Ac.*
1612 OTU2753 (A, E), *Ac. cibinongensis* OTU2755 (B, F), *Ac. thailandicus* (C, G), or *L.*
1613 *pseudomesenteroides* (D, H) were exposed to the stability protocol for ten days in vials (A-H) or five
1614 days in cages (E-H). Number of CFUs in individual guts was assessed by plating before and after five

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1615 or ten days of the stability protocol. Ten flies were analyzed for each condition. *Acetobacter* OTU2753,
1616 *Ac. cibinongensis*, and *Ac. thailandicus* levels decrease between day 0 and day 10 in vials (lmm, $p <$
1617 $0.001$ for all), but *Leuconostoc pseudomesenteroides* levels do not significantly change ($p = 0.96$). (I)
1618 Data from Fig 4 B-E was fitted to an exponential decay model that estimates the exponential decay
1619 rate, which corresponds to the rate of bacterial loss from the gut, and an asymptote, that corresponds to
1620 the levels at which the bacteria levels tend to stabilize after this loss. The rate of decay is the same for
1621 all the bacteria but there are differences between the asymptotes of all bacteria (contrasts of nonlinear
1622 least-square model estimates, $p < 0.014$), except between *Ac*. OTU2753 and *L. pseudomesenteroides* ($p$
1623 $= 0.395$). (J, K) Number of CFUs in each gut region from $w^{1118}$ *iso* males monoassociated with *Ac.*
1624 *thailandicus* before (F) and after (G) five days of the stability protocol in cages. Statistical analyses
1625 were performed together with replicate experiments shown in Fig 4B-G.
1626
1627 **S6 Fig - *Ac. thailandicus, Ac. cibinongensis* and *L. brevis* proliferate in the gut of**
1628 ***Drosophila melanogaster.*** Three to six days old axenic $w^{1118}$ *iso* males were inoculated for 6 hours
1629 with different concentrations of *Ac.* OTU2753 (A, E), *Ac. cibinongensis* OTU2755 (B, F), *Ac.*
1630 *thailandicus* (C, G), *L. pseudomesenteroides* (D), *L. paraplantarum* (H, I) and *L. brevis* (J). Bacterial
1631 levels were assessed by plating 0 and 24 hours post-inoculation. During this period males were singly
1632 placed in cages. In (G) axenic chaser males were placed in cages together with males inoculated with
1633 *Ac. thailandicus*. At 24 hours bacterial levels were assessed in both males. Bacterial levels between 0
1634 and 24 hours decrease in flies inoculated with *Ac.* OTU2753 (lmm, $p < 0.001$), increase in flies
1635 inoculated with *Ac. cibinongensis*, *Ac. thailandicus*, and *L. brevis* (lmm, $p = 0.024$, $p < 0.001$, and $p =$
1636 $0.046$, respectively) and do not significantly change in flies inoculated with *L. pseudomesenteroides*
1637 and *L. paraplantarum* (lmm, $p = 0.158$ and $p = 0.65$, respectively). Four to five males were used per
1638 condition, except in (B) where three males were used at one time-point and in (D) where two males
1639 were used on the inoculation $10^4$ CFU/µl. Each dot represents one gut and lines represent medians.
1640 Statistical analyses were performed together with replicate experiments shown in Fig 4J-O.
1641
1642 **S7 Fig - *Acetobacter* species grow on the fly food media.** Single 3-6 days old $w^{1118}$ *iso* males
1643 from a monoassociated stock with *Ac.* OTU2753 (A, D), *Ac. thailandicus* (B, E) or *Ac. cibinongensis*
1644 (C, F) were placed per vials for a period of 16 hours and then discarded. Bacterial levels on the food
1645 were determined by plating after discarding the flies (Day 0) and after one or five days of incubating
1646 these vials. Levels of *Acetobacter* on the food increase for all conditions between Day 0 and Day 5
1647 (lmm, $p < 0.001$). Five vials were used per condition. Each dot represents the bacterial levels on the
1648 food of one vial and lines represent medians.
1649
1650 **S8 Fig - *Ac. thailandicus* proliferates in the gut of *D. melanogaster* and not in *D. simulans.***
1651 (A, B) Optimization of proliferation protocol in bottles. Axenic 3-6 days old $w^{1118}$ *iso* were inoculated
1652 for 6 hours with different concentrations of *Ac*. OTU2753 (A) or *Ac. thailandicus* (B). Bacterial levels
1653 were assessed 0 and 24 hours post-inoculation. During this period males were singly placed in bottles
1654 (food surface of $28.27cm^2$) together with an axenic chaser male, from which bacterial levels were also

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1655 assessed at 24h. Levels of *Ac*. OTU2753 decrease between days (lmm, $p < 0.001$). Levels of *Ac.*
1656 *thailandicus* increase when flies are inoculated with the lowest concentration ($p < 0.001$) and are
1657 maintained when flies are inoculated with the highest concentration ($p = 0.426$). (C-E) Axenic 3-6 days
1658 old *D. melanogaster* or *D. simulans* males were inoculated for 6 hours with $10^3$ CFU/µl (C, D) or $10^4$
1659 CFU/µl (E) of *Ac. thailandicus*. Bacterial levels were assessed 0 and 24 hours post-inoculation. During
1660 this period males were singly placed in bottles. Three different genetic backgrounds for *D.*
1661 *melanogaster* (*$w^{1118}$ iso*, *D. mel*. O13 and Canton-S) and for *D. simulans* (*D. sim.* J04, *D. sim.* O13 and
1662 *D. sim*. A07) were used. Bacterial levels in the gut increase in *D. melanogaster* and decrease in *D.*
1663 *simulans* ($p < 0.001$). Five individuals were analyzed for each condition and total number of CFUs per
1664 gut determined by plating. Each dot represents one gut and the line represents medians. Statistical
1665 analyses were performed together with replicate experiment shown in Fig 5.
1666
1667 **S9 Fig - Flies monoassociated with *Acetobacter* develop faster and are more fertile than**
1668 **axenic flies in a constant environment.** (A-D) Total number of pupae (A), total number of adults (B),
1669 developmental time to pupariation (C) and developmental time to adulthood (D) was analyzed in flies
1670 from a monoassociated stock with *Ac.* OTU2753 or *Ac. thailandicus,* or in axenic flies. One female and
1671 three males from each condition were placed per vials for three days and then discarded. Number of
1672 pupae or emerged adults was daily assessed. Ten vials were used per condition. Flies monoassociated
1673 with either *Acetobacter* species develop faster and have higher fertility than axenic flies (lm, $p <$
1674 $0.001$). (A, B) Each dot represents the total progeny of one female.
1675
1676 **S10 Fig - *Ac. thailandicus* stable association with *D. melanogaster* is mutualistic.** Axenic 1-
1677 3 days old *$w^{1118}$ iso* males and females (G0) were in contact with an inoculum of $10^5$ CFU/µl of *Ac.*
1678 OTU2753 or *Ac. thailandicus*, for 6 hours. Two males and one female were placed per cage, with 5
1679 cages for each condition, during 10 days with daily changed food. (A) Bacterial levels in single guts of
1680 females 0 hours and 10 days post-inoculation and in males 10 days post-inoculation, analyzed by
1681 plating. Bacterial levels between the two time-points increased in females inoculated with *Ac.*
1682 *thailandicus* and decreased in females inoculated with *Ac.* OTU2753 (Mann-Whitney test, $p < 0.001$
1683 and $p = 0.048$ respectively). (B) Presence of bacteria on the food collected from cages at days 1, 3, 5, 7
1684 and 9 of the protocol, analyzed by plating. Filled rectangles represent presence of bacteria. *Ac.*
1685 *thailandicus* is transmitted to the food with higher frequency than *Ac.* OTU2753 (glm-binomial, $p <$
1686 $0.001$). (C-F) Effect of bacterial association on the fitness of *D. melanogaster*. Total number of eggs
1687 laid by flies inoculated with different *Acetobacte*r (C) and total number of adults that emerged from
1688 these eggs (D). Total number of eggs or adults is not different between conditions (lmm, $p > 0.484$).
1689 (E) Developmental time to adulthood of the progeny (G1) of flies inoculated with different
1690 *Acetobacter*. Developmental time to adulthood is faster in progeny from flies inoculated with *Ac.*
1691 *thailandicus* than in progeny from flies inoculated with *Ac.* OTU2753 (lmm, $p < 0.001$). (F) Fertility of
1692 G1. Two males and one female of G1 were placed per vial and flipped every other day for 10 days.
1693 Five couples were made per condition. Total number of emerged adults was analyzed. Fertility is
1694 higher in progeny from flies inoculated with *Ac. thailandicus* compared than in progeny from flies

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1695 inoculated with *Ac.* OTU2753 (lmm, $p < 0.001$). Statistical analyses were performed together with
1696 replicate experiments shown in Fig 6B-G.
1697
1698 **S11 Fig - Both parents transmit the beneficial effect of *Ac. thailandicus* to their progeny.**
1699 Combinations of one male and one female 1-2 days old $w^{1118}$ *iso*, either axenic or monoassociated with
1700 *Ac. thailandicus* (Bact.), were placed in vials and flipped every other day for 10 days. To one set of
1701 vials with axenic parents *Ac. thailandicus* was added on the eggs after passing the parents. Ten couples
1702 were made per condition. Developmental time to pupariation (A, E), to adulthood (C), total number of
1703 pupae (B, F) and total number of adults (F, G) was assessed. (A-D) correspond to one experimental
1704 replicate and (E-G) correspond to another experimental replicate, together with data from Fig 6H.
1705 Progeny from couples where either or both parents are monoassociated and progeny from axenic flies
1706 where *Ac. thailandicus* culture is added on the eggs develop faster than progeny from axenic flies
1707 (lmm, $p < 0.001$, for all these comparisons). Total number of progeny (pupae or adults) from couples
1708 where either or both parents are monoassociated with *Ac. thailandicus* is higher than in progeny from
1709 axenic flies (lmm, $p < 0.001$). (B, D, F, G) Each dot represents the total progeny of one female.
1710 Statistical analyses were performed together with replicate experiment shown in Fig 6H.
1711
1712 **S12 Fig - Different bacterial species have different impact on host developmental time and**
1713 **fertility.** Fifty $w^{1118}$ *iso* eggs were associated with different bacteria isolated from the gut of wild-
1714 caught *D. melanogaster.* As controls, axenic eggs that had no treatment (GF) or in which sterile media
1715 were added (GF MRS and GF Mannitol) were used. Ten vials were used for each condition. Total
1716 number of emerged adults (A, B) and their developmental time to adulthood was daily assessed (C, D).
1717 Number of emerged adults is not significantly different between conditions (lmm, $p > 0.282$ for all
1718 pairwise comparisons). Flies from eggs associated with *Ac. thailandicus* developed faster than from
1719 axenic eggs or eggs associated with 11 out of the other 15 bacteria (lmm, $p < 0.038$ for these pairwise
1720 comparisons). (E, F) Fertility of G0 was assessed. Two males and one female that developed in the
1721 presence of different bacteria (G0) were placed per vial and flipped every other day for 8 (E) or 10 (F)
1722 days. Five couples were made per condition. Total number of emerged adults was analyzed. Flies
1723 associated with *Ac. thailandicus* are more fertile than axenic flies or flies associated with 11 out of the
1724 other 15 bacteria (lmm, $p < 0.018$). (A, C, E) and (B, D, F) correspond to two experimental replicates.
1725 Correlation between developmental time and fertility is represented in Fig 7B. Each dot represents the
1726 total progeny of one female (A, B, E, F) and the size of the circle represents the mean number of adults
1727 that emerged per day (C, D). Statistical groups of significance for C, D, E, F are shown in S13 Fig.
1728
1729 **S13 Fig - Statistical groups of significance for developmental time and fertility of flies**
1730 **associated with different bacterial isolates.** Developmental time to adulthood (A) and fertility (B) of
1731 flies associated with different bacterial isolates from S12 Fig was analyzed with Tukey's pairwise
1732 comparisons on the lmm estimates. Statistical groups of significance were generated with *cld* function
1733 in R. Groups with the same letter are not significantly different from each other.
1734

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1735 **S14 Fig - *Ac. thailandicus* is beneficial for *D. melanogaster* in a natural food source.** (A, B)
1736 Thirty axenic $w^{1118}$ *iso* eggs were placed in vials containing sterilized fig homogenate. *Ac. thailandicus*
1737 or sterile culture media were added on the top of the eggs. Four to six vials were used per condition.
1738 Total number of adults that emerged (A) and developmental time to adulthood (B) was determined.
1739 More eggs inoculated with *Ac. thailandicus* developed to adulthood and faster than axenic eggs (lmm,
1740 $p < 0.001$ for both comparisons). (C) Fertility of flies developed in fig homogenate with and without
1741 the addition of *Ac. thailandicus*. Two males and one female were collected from G0 and placed per vial
1742 containing fig homogenate for 10 days, with vials flipped every other day. The *Ac. thailandicus*
1743 condition has ten replicates but only one from axenic eggs was possible to perform. Adults from eggs
1744 inoculated with *Ac. thailandicus* were more fertile than axenic adults (lmm, $p = 0.003$). (D, E) Fifty
1745 axenic $w^{1118}$ *iso* eggs were placed in vials containing freshly collected non-sterile figs. *Ac. thailandicus*
1746 culture or sterile media (Control) was added on the top of the eggs. The total number of adults that
1747 emerged (D) and their developmental time to adulthood (E) was analyzed. Ten vials were analyzed per
1748 condition. There were more adults emerging from vials inoculated with *Ac. thailandicus* (lmm, $p =$
1749 $0.010$). Developmental time to adulthood was not significantly different in this experimental replicate
1750 but faster in eggs inoculated with *Ac. thailandicus* in the other replicate represented on Fig 7J (lmm, $p$
1751 $= 0.557$ and $p < 0.001$, respectively). Statistical analyses were performed together with replicate
1752 experiments shown in Fig 7D-J.
1753
1754 **S1 Text - Sequences of the full 16S rRNA gene of the bacteria used in the phenotypic**
1755 **assays.** Sequence obtained by amplifying the gene with the primers 27F and 1495r. Code corresponds
1756 to code of laboratory isolate. It is also shown results of analysis on Greengenes, and of the BLAST
1757 analysis against the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database.
1758
1759 **S2 Text – R script for data analysis.** Text is in R Markdown format.
1760
1761 **S1 Data - Bacterial levels from $w^{1118}$ *iso* before and after 10 days in the same vial.** Bacterial
1762 numbers calculated per gut from each culture media used (BHI, LB, MRS, Mannitol or Liver) at day 0
1763 or day 10 of the protocol. Data for Fig 1A and S1A Fig.
1764
1765 **S2 Data - Bacterial levels from $w^{1118}$ *iso* before and after 10 days of being flipped to new**
1766 **vials twice a day.** Bacterial numbers calculated per gut from each culture media used (BHI, LB, MRS,
1767 Mannitol or Liver) at day 0 or day 10 of the protocol. Data for Fig 1B and S1A Fig.
1768
1769 **S3 Data - Relative 16S rRNA copy number $w^{1118}$ *iso* before and 10 days after being flipped**
1770 **to new vials twice a day.** Data for Fig 1C and S1B Fig.
1771
1772 **S4 Data - Bacterial levels on the food after inoculating the food with flies.** Bacterial numbers
1773 calculated per food vial from each culture media used (BHI, LB, MRS, Mannitol or Liver) one or ten
1774 days after placing one fly. Data for Fig 1B and S1A Fig.

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1775

1776 **S5 Data - Bacterial levels from wild caught flies before and 10 days after being flipped to**
1777 **new vials twice a day.** Bacterial numbers calculated per gut from each culture media used (BHI, LB,
1778 MRS, Mannitol or Liver) at day 0 or day 10 of the protocol. Data for Fig 1E, S1C and S1D Fig.
1779
1780 **S6 Data - Database for bacterial isolates that were sequenced and classified.** Source -
1781 sample origin. Day - day of the stability protocol. Media and Dilution - culture media and respective
1782 dilution from where colonies were isolated. Fly –gut sample number. Cfu_plate and Cfu_gut - Number
1783 of colonies analyzed in the plate and calculated per gut. morphotype - morphological type for one
1784 medium and one dilution. There is no correspondence with the same morphotype number in different
1785 media or flies. Bact_Code - code of laboratory isolate. greengenes_tax_string - list of taxonomic
1786 assignment according to Greengenes taxonomy. greengenes_prokMSA_id - identifier for the nearest
1787 neighbor sequence in the Greengenes database. greengenes_Simrank_id - percent of 7mers shared
1788 between the query sequence and the nearesr neighbor sequence. greengenes_DNAML_id - identity
1789 between the query and the nearest neighbor sequences. greengenes_DNAML_columns - number of
1790 bases compared between the query and the nearest neighbor sequences. sequence – 16S rRNA gene
1791 partial sequence. Fly_ID - Concatenation of Source, Day and Fly information. Unique_morpho -
1792 concatenation of Source, Day, Fly, Media and morphotype information. Data for Fig 2, Fig 3, S2, S3
1793 and S4 Fig.
1794
1795 **S7 Data - Stability of *$w^{1118}$ iso* monoassociated with different bacteria before and after**
1796 **being exposed to the stability protocol in vials and in cages.** Data for Fig 4B-E, S5A- S5I Fig.
1797
1798 **S8 Data - Stability of *Ac. thailandicus* in monoassociated *$w^{1118}$ iso* with in different gut**
1799 **regions.** Data for Fig 4G, H and S5J, S5K Fig.
1800
1801 **S9 Data - Proliferation of different *Acetobacter* species and *Leuconostoc* in *$w^{1118}$ iso*.** Data
1802 for Fig 4J-M and S6A-S6F Fig.
1803
1804 **S10 Data - Proliferation of *Lactobacillus* species in *$w^{1118}$ iso*.** Data for Fig 4N, and S6H, S6J
1805 Fig.
1806
1807 **S11 Data - *Acetobacter* growth on the food.** Data for S7 Fig.
1808
1809 **S12 Data - Proliferation of *Ac. thailandicus* in *$w^{1118}$ iso* during 24h in bottles with chaser**
1810 **GF flies.** Data for S8A, S8B Fig.
1811
1812 **S13 Data - Proliferation of *Ac. thailandicus* in *D. melanogaster* and *D. simulans*.** Data for
1813 Fig 5 and S8C-S8E Fig.
1814

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1815 **S14 Data - Developmental time to pupariation and to adulthood of *w*$^{1118}$ *iso*
1816 monoassociated with *Ac. thailandicus*, *Ac.* OTU2753 and axenic.** Column D-P correspond to
1817 number of new pupae or adults on the days 6-18 after egg laying. Data for S9 Fig.
1818
1819 **S15 Data - Colonization of *Ac. thailandicus* and *Ac.* OTU2753 in *w*$^{1118}$ *iso* males and
1820 females at 0 days and 10 days after inoculation with the bacteria and being exposed to the
1821 stability protocol.** nc – Growth of bacteria in lowest dilution plate is too high to determine precisely
1822 CFUs. This data is represented as "above $10^5$ CFU/gut" in figures. Data for Fig 6B and S10A Fig.
1823
1824 **S16 Data - Transmission of *Ac. thailandicus* to food.** Column D-H correspond to assessment
1825 of bacteria in the food on days 1, 3, 5, 7, and 9 of the experiment. No data – no data collected. nc –
1826 Growth of bacteria in lowest dilution plate is too high to determine precisely CFUs. Data for Fig 6C
1827 and S10B Fig.
1828
1829 **S17 Data - Number of eggs laid by *w*$^{1118}$ *iso* inoculated with *Ac. thailandicus*, *Ac.* OTU2753
1830 or control, over the 10 days in cages.** Column D-M correspond to number of eggs on days 1-10 of
1831 experiment. Data for Fig 6D and S10C Fig.
1832
1833 **S18 Data - Developmental time of progeny from *w*$^{1118}$ *iso* inoculated with *Ac. thailandicus*,
1834 *Ac.* OTU2753 or control, over the 10 days in cages.** Columns E-O correspond to number of new
1835 emerged adults on vials corresponding to days 10-20 after egg laying. Data for Fig 6E, F and S10D,
1836 S10E Fig.
1837
1838 **S19 Data - Fertility from progeny from *w*$^{1118}$ *iso* inoculated with *Ac. thailandicus*, *Ac.*
1839 OTU2753 or control.** cagepair - cage from where the pairs were collected. daypair – pairs were
1840 collected from bottles of day 9 or 10 of the experiment. vialday – vial date; pairs were placed in new
1841 food vials every other day until day 8. Columns H-V correspond to number of new emerged adults on
1842 days 8-22 after egg laying. Data for Fig 6G and S10F Fig.
1843
1844 **S20 Data - Developmental time to pupariation, adulthood and respective total number of
1845 progeny from one or both parents monoassociated with *Ac. thailandicus*.** Conditions used were:
1846 53F + 53M – both parents associated with *Ac. thailandicus*. GFF + GFM – both parents axenic. 53F +
1847 GFM – female with *Ac. thailandicus,* male axenic. GFF + 53M – female axenic, male with *Ac.*
1848 *thailandicus.* GFF + GFM + Bact – both parents axenic and *Ac. thailandicus* added. vialday – vial date;
1849 pairs were placed in new food vials every other day until day 8. Columns F-W correspond to number of
1850 new pupae or emerged adults on days 5-22 after egg laying. Data for Fig 6H and S11 Fig.
1851
1852 **S21 Data - Developmental time to adulthood from *w*$^{1118}$ *iso* associated with different
1853 bacterial isolates.** Columns E-O correspond to number of new emerged adults on days 9-19 after egg
1854 laying. Data for Fig 7B, S12A-S12D Fig and S13B Fig.

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1855

1856 **S22 Data - Fertility of $w^{1118}$ *iso* developed with different bacterial isolates.** Data for Fig 7B,
1857 S12E, S12F Fig and S13A Fig.

1858

1859 **S23 Data - Developmental time of axenic $w^{1118}$ *iso* or associated with *Ac. thailandicus* in**
1860 **sterilized fig food.** Columns D-AD correspond to number of new emerged adults on days 9-35 after
1861 egg laying. Data for Fig 7D, E and S12A-S12D Fig.

1862

1863 **S24 Data - Fertility of axenic $w^{1118}$ *iso* or associated with *Ac. thailandicus* in fig food.** Data
1864 for Fig 7G and S13C Fig.

1865

1866 **S25 Data - Developmental time of $w^{1118}$ *iso* with and without the addition of *Ac.***
1867 ***thailandicus* in freshly collected figs.** Columns E-U correspond to number of new emerged adults on
1868 days 9-25 after egg laying. Data for Fig7I, J and S14D, S14E Fig.

1869