

1 **Adaptation to chronic malnutrition leads to reduced dependence on microbiota in**

2 ***Drosophila***

3 Berra Erkosar^{1,2*}, Sylvain Kolly¹, Jan R. van der Meer², Tadeusz J. Kawecki¹

4 ¹Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne,
5 Switzerland

6 ²Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne,
7 Switzerland

8 ***Corresponding author:** Berra Erkosar, Department of Ecology and Evolution, Department
9 of Fundamental Microbiology, University of Lausanne, CH 1015 Switzerland;

10 berra.erkosarcombe@unil.ch, phone +41 21 692 4255, fax +41 21 692 4165

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16 **Abstract**

17 Numerous studies have shown that animal nutrition is tightly linked to gut
18 microbiota, especially under nutritional stress. In *Drosophila*, microbiota are known to
19 promote juvenile growth, development and survival on poor diets, mainly through enhanced
20 digestion leading to changes in hormonal signaling. Here we show that this reliance on
21 microbiota is greatly reduced in replicated *Drosophila* populations that adapted to a poor
22 larval diet in the course of over 170 generations of experimental evolution. Protein and
23 polysaccharide digestion in these malnutrition-adapted populations became much less
24 dependent on colonization with microbiota. This was accompanied by changes in at least
25 some targets of dFOXO transcription factor, which is a key regulator of cell growth and
26 survival. Our study suggests that some metazoans have retained the evolutionary potential to
27 adapt their physiology such that association with microbiota may become optional rather than
28 essential.
29

30 Introduction

31 Nutrient availability is a major factor limiting survival, growth and reproduction of
32 many animal species¹, resulting in natural selection for adaptation to cope with nutritional
33 stress. Yet, little is known about evolutionary adaptations that help juvenile animals not only
34 to survive, but also grow, develop and reach maturity under chronic nutrient shortage.
35 However, recent studies point to a particular importance of gut microbiota in coping with
36 such chronic malnutrition. For example, mono-colonization with *Lactobacillus plantarum*
37 buffers the growth of infant mice against the effects of nutrient shortage through a mechanism
38 involving Insulin-like Growth Factor (IGF) signaling². Important insights about the
39 mechanisms of microbiota-mediated enhancement of fitness under nutrient shortage have
40 recently emerged from studies in *Drosophila*. As other insects that feed on a variety of
41 sources, *Drosophila* have a rather simple and transient gut microbiota consisting of a
42 subsample of ambient bacteria growing on their food (decomposing fruits)³. Nonetheless, as
43 the more specialized commensals of mammals, these microbes provide a number of
44 nutritional and metabolic benefits to their hosts^{4,5}. The same strain of *L. plantarum* that
45 alleviated the effect of nutrient limitation on growth of mice² promotes the growth of
46 *Drosophila* larvae on protein-poor diet, an effect mediated through upregulation of host's
47 proteolytic enzymes leading to enhanced digestion and modulation of Insulin and TOR
48 pathways^{6,7}. Another study using *Drosophila* also pointed to the commensal *Acetobacter*
49 *pomorum* controlling larval growth by modulating Insulin/IGF-like signaling (IIS); this
50 phenotype was again particularly pronounced on poor diets⁸. Based on these findings, one
51 might hypothesize that animal populations often exposed to chronic malnutrition would adapt
52 by evolving an improved ability to benefit from their microbiota.

53 We address this hypothesis with experimental evolution⁹. To study evolutionary
54 adaptation to chronic juvenile malnutrition, we have maintained six outbred *Drosophila*
55 *melanogaster* populations ("Selected" populations) for over 170 generations on an extremely
56 poor larval diet (containing only 0.3% w/v of yeast). The nutrient content of the poor diet is
57 so low that non-adapted larvae take twice as long to develop as on a standard diet and the

58 resulting adults are only half the normal size¹⁰. Compared to six “Control” populations
59 maintained in parallel on a standard diet, the Selected populations evolved increased egg-to-
60 adult survival, smaller critical size for metamorphosis initiation and faster development on the
61 poor diet^{10,11}.

62 Here we test how this enhanced performance of the malnutrition-adapted Selected
63 populations depends on interactions with microbiota and study the underlying physiological
64 mechanisms. By manipulating the microbiota colonization status of larvae we demonstrate
65 that, contrary to our expectations, these malnutrition-adapted populations became largely
66 independent from microbiota for growth and survival on the poor diet. We show that protein
67 and carbohydrate digestion in Selected larvae is much less affected by microbiota than in
68 Controls, in spite of both types of larvae carrying microbiota of similar composition and
69 abundance. Finally, our populations exhibit differential expression of some targets of the
70 major cell growth regulator dFOXO¹². This indicates that site-specific function of dFOXO
71 contributes to the physiological changes that result from adaptation to malnutrition, which
72 compensates the microbiota effect in the non-adapted Control populations.

73

74 **Results**

75 *Effect of microbiota on development and survival of experimentally evolved populations*

76 We have maintained experimentally evolving Selected and Control populations on,
77 respectively, a poor and a standard diet under a discrete-generation density-controlled regime
78 for over 170 generations (i.e., over 10 years, see *Experimental Procedures*). This culture
79 regime hindered vertical transmission of microbiota within populations from one generation
80 to the next, and was conducive to exchange of microbes between populations as well as with
81 the general environment of the climate room. Therefore, we did not expect one-to-one
82 coevolution between the populations and their specific microbial communities. For this
83 reason, to test the effects of microbiota we used a “common” microbiota inoculum collected
84 from the feces of adults of all 12 populations (see *Experimental Procedures* for details). The

85 "germ-free" (GF) flies were fed heat killed inoculum to control for potential effect of bacteria
86 as food.

87 Larvae of our Selected populations had previously been reported to develop faster
88 and survive better than Control larvae on poor diet (but not on standard diet)^{10,11}, a
89 manifestation of their evolutionary adaptation to the poor diet; however, in those studies the
90 colonization of the larvae by microbiota was not controlled and not assessed. We
91 hypothesized that the improved performance of Selected larvae on the poor diet is at least in
92 part mediated by an improved ability to benefit from interactions with microbiota. If so, one
93 would predict that their superiority over Control larvae would diminish if they were deprived
94 of the help of microbiota, i.e., in a GF state. To test this prediction, we compared the length of
95 larval development and survival of Selected and Control populations in a GF state and when
96 experimentally colonized with microbiota collected from adult feces. On the poor food, while
97 Control larvae colonized with microbiota developed 40% faster and were three times more
98 likely to survive than their GF siblings, the corresponding effect of microbiota treatment on
99 Selected larvae was much smaller (**Fig 1A, B**). On the standard food, the effect of microbiota
100 on development and survival was markedly smaller (**Fig S1A, B**). Thus, while in the GF state
101 the Selected larvae took 30% less time to pupate on poor diet and were about three times as
102 likely to survive as the Controls, the advantage of the Selected over Control populations for
103 both traits diminished when the larvae were colonized with microbiota (**Fig 1A, B**). In
104 particular, the developmental time became statistically indistinguishable between the Selected
105 and Control larvae in the colonized state (even though Selected still tended to develop about
106 one day faster than Controls). These results are opposite to our prediction. They imply that, in
107 the course of their evolutionary adaptation to the poor diet, the Selected populations became
108 less dependent on microbiota and much better able to cope with nutrient shortage without
109 their help.

110

111 *Protein digestion in Selected and Control populations*

112 Recently, it has been shown that upon nutrient scarcity, one of the members of
113 *Drosophila* microbiota, *Lactobacillus plantarum*, promotes intestinal protease expression,
114 leading to enhanced dietary protein digestion and increased amino acid concentrations in the
115 host tissues ⁷. We therefore hypothesized that the weaker effect of microbiota on the survival
116 and developmental time of Selected than Control populations could be mediated by a
117 differential effect on protein digestion efficiency. To test this prediction, we measured
118 protease activity (relative to the total protein content) in whole GF and microbiota-colonized
119 larvae at different time points during the 3rd instar (**Fig 2A**). This relative protease activity
120 declined over time, which could reflect changes in protease secretion as well as an increase in
121 the amount of protein accumulated in the larval body relative to the size of the digestive
122 system. Irrespective of this apparent decline over time, inoculation with microbiota strongly
123 enhanced proteolysis in Control larvae, but had a significantly smaller effect on proteolysis in
124 Selected larvae. Thus, while GF Selected larvae exhibited (marginally significantly, $p =$
125 0.083) higher levels of protease activity than GF Controls (particularly in mid- to late 3rd
126 instar), the trend went in the opposite direction in microbiota-associated larvae. Thus, the
127 pattern of proteolytic activity of the Selected and Control larvae in the absence and presence
128 of microbiota matches the pattern of larval performance reported above. Apparently, being
129 supplemented with microbiota helps Control populations to recover from low digestive
130 activity, helping them grow and survive.

131 As previously reported, seven serine proteases, including five Jonah proteases are
132 transcriptionally induced upon colonization with *L. plantarum* ⁷. To determine if differences
133 in expression of the same proteases may be responsible for the pattern of proteolytic activity
134 in our populations, we dissected the intestines of GF and colonized larvae at early and late 3rd
135 instar, and carried out a qRT-PCR analysis on 11 proteases including Trypsins, Jonah
136 proteases and a few others known to have serine type protease activity. Consistent with the
137 previous report ⁷, we detected an elevated expression in all populations upon colonization
138 with microbiota in all five Jonah proteases and three other serine proteases (*CG18179*,
139 *CG18180*, *CG8299*; **Fig 2C**). However, trypsin superfamily proteases (α -Try, β -Try, ε -Try)

140 (which are clustered together in the genome and reported to have a very localized expression
141 in the gut¹³) exhibited the opposite pattern, i.e., were downregulated by microbiota (**Fig 2C**).
142 Out of the 11 proteases, we identified two (*Jon66Cii*, *CG18180*) whose mRNA levels were
143 consistently higher in Selected populations compared to Controls; we also observed that
144 *CG8299* had higher expression in Control than selected populations (**Fig 2C**). Trends for
145 differences between Selected and Control populations could also be observed for several
146 other proteases (all three Trypsins, *CG18179*, *Jon65Ai*, *Jon44E*, *Jon99Ci*, **Fig 2C**), but they
147 were not sufficiently consistent between time points or replicate populations to be statistically
148 significant. The digestive proteases are likely to some degree functionally redundant, and thus
149 it is conceivable that evolution would achieve functionally similar changes in digestion by
150 targeting different genes in different replicate populations, making detection of a signature of
151 evolution in a gene-by-gene analysis difficult. Therefore, we analyzed the entire protease
152 expression dataset with multivariate analysis of variance (MANOVA) and Principal
153 Component Analysis (PCA). The correlation circle clearly confirmed that the levels of
154 expression of the three Trypsins were positively correlated and well separated from other
155 proteases (**Fig 2B right**). This suggests that these two groups of proteases are regulated by
156 different processes and/or may have a different function within the gut. GF and microbiota-
157 colonized larvae were clearly separated by the 1st PC, with Selected and Control populations
158 somewhat less distinctly separated along the 2nd PC (**Fig 2B left**). Given that the 1st PC
159 explains more than twice as much variance as the 2nd, this implies microbiota are a major
160 factor that changes protease levels, with a greater impact than the evolutionary history.
161 Importantly, in spite of highly significant main effects of both microbiota and evolutionary
162 regime in the MANOVA, there was no interaction between them (**Table S2**). Thus, even
163 though these results suggest that evolutionary adaptation to poor food was in part mediated by
164 changes in protein digestion, changes in the expression of digestive proteases cannot fully
165 account for the differential effects of microbiota on the developmental time and survival.

166

167 *Carbohydrate digestion in Selected and Control populations*

168 Given that our poor diet is low in carbohydrate as well as protein content, we next
169 asked if carbohydrate digestion is also different between Selected and Control populations
170 and if it is differentially influenced by microbiota. About 30 % of carbohydrates in both poor
171 and standard diet consist of polysaccharides (starch) from the cornmeal (the rest are sucrose
172 and glucose). Polysaccharide digestion occurs as a two-step process whereby starches are first
173 broken-down to disaccharides by amylases before being hydrolyzed to monosaccharides.
174 Alpha-amylase activity is under direct negative regulation by glucose concentration in
175 *Drosophila* larvae, which occurs at the transcriptional level. Amylase activity is therefore
176 expected to be lower in larvae with higher glucose concentration^{14,15}. We quantified amylase
177 activity rates in Selected and Control larvae in both colonized and GF states. Microbiota had a
178 striking effect on how amylase activity (again normalized to total larval protein content)
179 changed over time: while it declined between an early and late 3rd stage in the microbiota-
180 colonized larvae, it increased sharply during the corresponding developmental period in GF
181 larvae (slope difference $p < 0001$, **Fig 3A**). Because no such increase is observed for protease
182 activity (**Fig 2A**), it implies that GF larvae upregulate their investment in polysaccharide
183 digestion relative to protein digestion towards the end of their development. Irrespective of
184 these temporal changes, GF Selected larvae consistently showed three-fold lower amylase
185 activity than GF Control larvae of the same stage (blue symbols in **Fig 3A**); this difference is
186 much smaller and non-significant in microbiota-colonized larvae (orange symbols in **Fig 3A**).
187 Thus, we again observed a pattern of interaction such that the difference due to evolutionary
188 history was more pronounced in germ free than in microbiota-colonized state. However, fast
189 development and high survival on poor diet (**Fig 1**) were associated with lower amylase
190 activity. This implies that increased amylase activity is a sign of nutritional stress. Given the
191 negative regulation of amylase activity by glucose concentration^{14,15}, these results suggest
192 that Control larvae may have lower glucose levels than Selected larvae under GF conditions.

193 To verify if the pattern we observed is regulated at the transcriptional level we
194 quantified amylase transcript levels in the guts. We analyzed expression of two amylases.
195 Both gene transcripts were significantly reduced in colonized larvae in all populations (**Fig3**

196 C). Under GF condition, *Amy-P* levels were higher in Control populations than in Selected
197 populations, but no significant difference was detected in *Amy-D* levels (**Fig 3C**). Given that
198 relative expression abundance of *Amy-P* is much higher than *Amy-D* (roughly 20 times, **Fig**
199 **3C**), *Amy-P* is likely to be the major gene contributing to the amylase activity pattern that we
200 observed earlier (**Fig 3A**). Even though *Amy-P* expression is reduced by microbiota and is
201 expressed at lower levels in Selected than Control populations, the expression pattern does
202 not fully explain what we observe for amylase activity, and other regulatory mechanisms (e.g.
203 cAMP levels¹⁴) may also play a role in regulating amylase activity.

204 In the gut, glucose is generated through the hydrolysis of maltoses by maltases. If
205 amylase activity is lower in Selected populations and upon microbiota colonization because
206 of glucose concentration in the gut and/or hemolymph, maltase activity is predicted to be
207 higher in these conditions. To check this we also analyzed expression of four *maltase* genes.
208 In agreement with this prediction, we observed a high expression of maltases in Selected
209 populations for *Mal-A1*, *-A3* and *-A4*, although not for *Mal-A8* (**Fig 3C**). A consistent
210 decrease in expression can be observed upon colonization only in Control populations for
211 *Mal-A1*, *-A8* (**Fig 3C**). *Mal-A4* exhibits this trend only at late 3rd instar but this is not
212 statistically significant due to high variation among populations (**Fig 3C**). *Mal-A3* expression
213 is rather induced in Selected populations upon colonization, and remains unchanged in the
214 Control ones (**Fig 3C**). To spot the general trend among these carbohydrate-digesting
215 enzymes we performed multivariate analyses. We observed a clear separation between the
216 evolutionary regime, colonization status and developmental stage (**Fig 3B** left, **Table S3**).
217 However, we observed only a marginally significant interaction between the evolutionary
218 regime and developmental stage, and no interactions between other factors (**Fig 3B** left,
219 **Table S3**). Furthermore, PCA correlation circle on carbohydrate digesting enzymes shows
220 that amylase and maltase expression patterns are uncorrelated (**Fig 3B** right). Altogether,
221 although Selected and Control populations exhibit different levels of carbohydrate-digesting
222 enzymes, transcriptional differences of digestive enzymes cannot fully explain the interaction
223 between evolutionary history and colonization status.

224

225 *Characterization of the microbiota*

226 To identify the bacterial taxa that might be involved in the digestive enzyme
227 induction in our experiments, we performed a microbial community profiling using 16S
228 rRNA gene sequencing. All our populations carry the endosymbiont *Wolbachia* (data not
229 shown), which would dominate the reads if the 16S sequencing were performed on DNA
230 extracted from whole flies or from dissected guts. Therefore, we performed this analysis first
231 using the feces of the adults pooled from the 12 populations, i.e., the microbial community
232 used for experimental inoculations described above. This community was dominated by a
233 single *Acetobacter sp.* that contributed ~82% reads, with ~14% reads attributable to
234 Pseudomonadaceae, ~2% to other Acetobacteraceae and ~2% other less abundant taxa
235 (**Fig4A, lowermost bar**).

236 To check if association with Control versus Selected larvae promoted different
237 members of this microbial community, we used this feces suspension to inoculate poor-diet
238 larval cultures of each population upon hatching, and collected samples of the medium at the
239 end of larval development (this was done in the same experiment that provided larvae for the
240 gene expression experiment described above). 16S sequencing of these samples revealed that,
241 irrespective of evolutionary history of the populations, they all consisted almost exclusively
242 (> 99% of the community) of the single *Acetobacter spp* already prevalent in the inoculum
243 (**Fig4A**).

244 Could then the differences between Selected and Control populations in the effects of
245 microbiota inoculation on larval performance and digestive enzymes be mediated by
246 differential colonization of their guts by this dominant *Acetobacter* strain? To address this
247 question, we mono-colonized freshly hatched GF larvae of all twelve populations with this
248 strain, allowed them to develop on the poor diet, and estimated the amount of bacteria inside
249 the larval gut at the end of larval development. This was done by using qPCR to quantify
250 bacterial DNA (using primers specific to Acetobacteraceae 16S rRNA gene) relative to host
251 genomic DNA (using primers for *Actin*). We found no systematic difference between these

252 experimentally colonized Selected and Control larvae in the amount of bacterial DNA relative
253 to host DNA (**Fig 4B** orange symbols), nor in the absolute Ct values for the bacterial DNA
254 (**Fig S1**). The latter indicates that the amount of bacterial DNA in these samples was about
255 1000-fold above the detection threshold; based on preliminary data (not shown) this roughly
256 corresponds to 600-900 CFUs per larvae. Analogous Ct values for GF larvae were
257 comparable to what was observed in a mock sample only containing sterilized water, which
258 sets the detection limit (black line in **Fig S1**). This assures that our procedure of generating
259 GF animals was effective.

260 The above results indicate that Selected and Control populations become similarly
261 colonized by the dominant *Acetobacter* strain upon experimental inoculation followed by
262 development on the poor diet. This implies that adaptation of Selected populations to poor
263 diet did not cause any changes in the gut that would affect its colonization by commensals.
264 However, this does not preclude a difference in the amount of bacteria they normally harbor
265 under their respective evolutionary regimes (in their “conventional” environment), given that
266 the regimes differ in diet and does not involve experimental inoculation. To address this issue,
267 we used the same approach to quantify bacterial colonization by *Acetobacter* in the main
268 cultures used to propagate these populations under the experimental evolution that is ongoing
269 in the lab (i.e., on poor diet for Selected and on standard diet for Control populations).
270 Interestingly, despite the difference in diet, these larvae reared in their respective
271 conventional environments were colonized with comparable levels of Acetobacteraceae (**Fig**
272 **4B** green symbols). This suggests that the ability of Selected lines to become largely
273 independent of microbiota (i.e. their ability to cope with being GF) is a physiological result of
274 being adapted to malnutrition and not of being maintained GF by coincidence.

275

276 ***Growth rate and activation of dFOXO targets***

277 *Acetobacter pomorum* has been shown to promote larval growth through induction of
278 Insulin/IGF-like signaling (IIS) by acetic acid secretion, evidenced by cytoplasmic retention
279 of dFOXO in larval fat body⁸. *Acetobacter sp.* in our system is also likely to secrete acetic

280 acid since we observe a clear reduction from pH 3.5 to pH 2.0 in the media of all 12
281 populations upon colonization. We thus hypothesized that microbiota would promote larval
282 growth in Control populations, but less so in Selected populations. However, adult size is thus
283 not a good proxy for larval growth rate in these populations: because Selected populations
284 evolved a smaller critical size for metamorphosis initiation, they reach a smaller adult size
285 than Controls despite growing faster on the poor diet^{10,11}. Therefore, we combined adult body
286 size (dry weight) of freshly emerged adults (**Fig S2**) with developmental time data (**Fig 1A**)
287 to estimate mean larval growth rate of each population under both microbiota conditions,
288 following the approach described in¹⁰. As expected, we found that inoculation with
289 microbiota increased larval growth rate, but this effect was significantly greater in Control
290 than in Selected populations (**Fig 5A**), suggesting that IIS and/or target of rapamycin (TOR)
291 pathways may respond differently to microbiota (**Fig 5A**).

292 In *Drosophila*, TOR and IIS pathways control systemic larval growth and dFOXO is
293 the key mediator of IIS in regulating ribosome biogenesis and cellular growth¹². dFOXO is a
294 transcription factor that has >900 direct or indirect targets, a part of which respond to nutrient
295 sensing¹⁶. To study IIS-dFOXO activity, we analyzed the transcription of three established
296 dFOXO targets, namely *d4EBP*, *dInR*, and *l(2)efl*¹⁷⁻¹⁹ in late third stage whole larvae upon
297 mono-association with the *Acetobacter* strain isolated from our populations. We observed a
298 significant reduction in *InR* mRNA levels upon colonization by bacteria in Control
299 populations but not in Selected ones (**Fig5B**). Since *InR* is negatively regulated by insulin-
300 like peptides¹⁹, this suggests that Control populations do switch from “low nutrition” to “high
301 nutrition” mode physiologically whereas Selected populations are rather insensitive to
302 inoculation with *Acetobacter* and keep their metabolic state as it is. However, we observed no
303 significant difference in *d4EBP* (**Fig 5B**), indicating that differential dFOXO activity in
304 Selected and Control populations does not occur on all dFOXO targets.

305 Interestingly, we also saw that the stress response gene *l(2)efl*, known to be involved
306 in lifespan regulation¹⁷, was reduced in all populations when they were GF and significantly
307 induced in *Acetobacter* colonized larvae only in Selected populations (**Fig 5B**). This might

308 suggest that Selected populations perceive colonization by *Acetobacter* as a stress signal;
309 however this gene might also play a hitherto unknown role in larval development or nutrition.
310 Taken together these data suggest that dFOXO activates the transcription of a selection of its
311 target differentially in Selected and Control populations in response to colonization by
312 *Acetobacter*.

313

314 **Discussion**

315 We set out to study physiological bases of experimental evolutionary adaptation to
316 chronic juvenile malnutrition, expecting that they will involve an improved ability of the
317 animal host to exploit its microbiota. Instead, we found that our experimentally evolved
318 Selected populations became much less dependent on microbiota for their survival and
319 growth in less than 200 generations of evolution on a nutrient-poor larval diet. This is rather
320 surprising, given the well-documented dependence of non-adapted *Drosophila* larvae facing
321 nutrient shortage on benefits provided by gut microbiota⁶⁻⁸. This dependence on microbiota
322 remains strong in our Control populations, which originated from the same base population as
323 Selected populations but do not have a history of laboratory evolution on the poor diet.

324 It has previously been described in *Drosophila* adults and larvae that *L. plantarum*
325 (mono-associated or as a part of a microbiota community) induces transcription of a set of
326 digestive enzymes^{7,20}. Our data from the non-adapted Control populations support the notion
327 that microbiota promote protein digestion and indicate that this effect is not specific to
328 microbiota containing *Lactobacilli* but it also occurs in association with *Acetobacter*. In 2011,
329 Shin et al. showed that benefit conferred by their *Acetobacter* strain arises through the
330 induction of IIS by acetic acid production; yet, adding only acetic acid to the medium does
331 not bring any growth benefit, indicating that other bacterial factors are involved in growth
332 promotion⁸. Our data complements this view and suggest that enhanced digestion
333 (presumably resulting in improved nutrient acquisition) may be one of the mechanisms
334 mediated by *Acetobacter*, in addition to acetic acid secretion. Interestingly, our data also
335 reveal that even though they are all serine proteases, Trypsins and Jonah proteases respond

336 quite differently to *Acetobacter* colonization, indicating that these two sets may have
337 functional differences.

338 Enhanced Jonah protease activity has been shown to be sufficient for promoting
339 larval growth upon malnutrition⁷. This, in combination with our data where GF Selected
340 populations show a higher proteolytic activity mid-3rd larval stage and lower amylase activity
341 throughout the third stage suggests enhancing digestion can be an evolutionary mechanism to
342 insure growth under nutritional stress. In addition to the basal differences that occur at the GF
343 state, we have also shown that microbiota affect digestion differently in Selected and Control
344 populations. In Control populations, we observe a large increase in proteolytic activity as well
345 as a clear decrease in amylase activity upon colonization. This is then accompanied by a
346 significant reduction in InR levels, which is an indication of higher levels insulin-like
347 peptides in the system and thus higher nutrient availability¹⁹. In contrast, microbiota have
348 little effect on the protease and amylase activity of Selected populations, whose InR levels
349 appear insensitive to *Acetobacter* colonization. This suggests that Selected populations
350 probably keep their metabolism in a “nutrient shortage” mode in order to continue high
351 nutrient uptake rate. This might also be associated with changes in mitochondrial function and
352 oxidative phosphorylation levels in Selected populations. Previously it was described that
353 larvae that were grown in low (1%) yeast has shown reduced mitochondrial abundance and
354 respiration activity in their fat body²¹. It remains to be determined if our Selected populations
355 have overcome this defect and if microbiota has a direct influence on mitochondrial
356 abundance and function in evolved and wild type populations.

357 We found an increase in stress response gene *l(2)efl* transcript levels between control
358 and selected populations upon *Acetobacter* colonization. *l(2)efl* has been described to be
359 upregulated under oxidative stress, heat shock and ionizing radiation and shown to be
360 regulated by a detoxifying ABC-transporter dMRP4 and JNK pathway^{17,22}. However the link
361 between stresses induced by larval malnutrition and growth remains to be elucidated.
362 Together with *InR* data, these colonization and regime specific differences in different genes
363 indicate that dFOXO acts differently in control and selected populations; but since this is not

364 true for translation inhibitor *4EBP*²³, differences in upstream signaling mechanisms or
365 different transcription factor partners must be involved. A comprehensive transcriptome
366 analysis would give a more precise picture on the transcriptional changes that occur during
367 adaptation to chronic malnutrition and upon microbiota association.

368 The genetic basis of natural variation in microbiota-dependent nutritional response
369 was previously studied using *Drosophila* Genetic Reference Panel (DGRP) lines^{24,25}. These
370 studies identified key genes involved in nutritional allocation by microbiota. As expected,
371 genes related to IIS/TOR pathways, as well as JAK-STAT or Notch pathways, were shown to
372 be important for microbiota dependent nutrition²⁴. These genes were identified by genome-
373 wide association studies upon nutritional indices on flies raised on highly rich (10% sugar-
374 yeast diet), which is clearly different than our setup. Despite this, future genomics studies
375 comparing significant SNPs between our Control and Selected populations to the ones
376 identified in these studies will help us understand adaptive forces that shape nutrient
377 acquisition in the presence and absence of microbiota.

378 Irrespective of its physiological basis, the fact that our Selected populations became
379 much less dependent on microbiota for larval growth and survival under strong nutrient
380 limitations is intriguing from an evolutionary viewpoint. Our quantification of microbiota
381 implies that under their culture regime Selected populations are similarly exposed to
382 microbiota as Control populations, and become colonized by a quantity of bacteria
383 comparable to that resulting from our experimental inoculations. This suggests that the
384 reduced dependence of Selected populations on microbiota is not a consequence of being
385 underexposed to bacteria in the course of their experimental evolution, but a direct effect of
386 adaptation to nutrient shortage under the strong selection imposed by the extremely poor diet.
387 As we have reported²⁶ the Selected populations also evolved a greater susceptibility to the
388 gram-negative intestinal pathogen *Pseudomonas entomophila*. Thus, evolutionary adaptation
389 to nutritional stress may affect interactions between the host and both beneficial and harmful
390 gut microbes.

391 The relationship between animals and gut microbiota likely goes back hundreds of
392 millions of years, and during this evolutionary time most animals became dependent on gut
393 bacteria for nutritional benefits and various metabolic tasks^{5,27}. In *Drosophila* (and
394 presumably in many other insects with diverse diets) this host-microbiota relationship is less
395 intimate than in mammals or in insects feeding on unbalanced or hard-to-digest diets, such as
396 blood, plant sap or wood^{4,5}. Rather than relying on transmission of specialized gut microbes
397 from mother to offspring, *Drosophila* acquire their gut microbiota from the microbial
398 community living on the food substrate³. However, microbiota still exerts its beneficial effect
399 by supplementing food with vitamin B in poor environments and regulating sugar metabolism
400 in high glucose environments²⁸. In addition, *Drosophila* microbiota also stimulates the host
401 immune system, interferes with pathogens, and provides signals to key pathways to which
402 regulate growth and tissue homeostasis^{29,30}. And, given that the natural food for *D.*
403 *melanogaster* is decomposing fruit, the larvae are likely never deprived of those beneficial
404 microbes in nature. It is thus remarkable that the species retained the potential to rapidly
405 evolve a markedly reduced dependence on gut microbiota for fitness under nutritional stress.

406

407 **Methods**

408 ***Experimentally evolved fly populations and diet***

409 Six replicate Selected and six replicate Control populations were maintained at 20°C and 70%
410 humidity, with 12/12h dark/light cycle on a 21-day generation cycle. Control populations
411 were cultured on standard cornmeal (5%)-yeast (1.25%)-sugar (3% sucrose, 6% glucose)
412 medium and Selected populations were cultured on poor medium containing 1/4 of the
413 nutrients during larval development¹⁰. Experimental evolution was carried out as described in
414 detail in¹⁰. All 12 populations originated from the same base population. At each generation,
415 eggs were collected on live yeast, leading to contamination of egg surfaces with yeast, which
416 may cause alterations in the gut microbiota of larvae. Eggs were rinsed with tap water to
417 enable egg counting, which dilutes the flies' natural microbiota and causes environmental
418 contamination. Approximately 200 eggs were collected from adults of each population and

419 distributed on their respective media for larval growth. Upon emergence, adults from all
420 populations were transferred to standard medium supplemented with dry yeast. Experiments
421 were carried out between generation 177 and generation 200. Before each experiment,
422 populations were reared on standard medium for >2 generations to avoid maternal effects.
423 To avoid changes in the conventional recipe, we kept the food clean by boiling it. The food
424 used in the experiments was boiled for >10 min and poured at 78°C in autoclaved fly bottles
425 using tools sterilized with 70% ethanol under the hood.

426

427 ***Preparation of gnotobiotic larvae***

428 Embryos were collected from an overnight egg laying on orange juice-agar plates
429 supplemented with yeast. Embryos were washed with tap water, sterilized by soaking in 5%
430 bleach for 3 minutes and were rinsed with autoclaved water. 200 eggs were counted on a
431 mesh, under a stereomicroscope, next to a Bunsen burner to avoid further contamination.
432 Counted eggs were transferred to fly bottles containing standard or poor food medium.
433 For the GF treatment, 300 µl of heat inactivated bacteria (developmental time experiment) or
434 sterile PBS (enzymatic activity assays and RT-qPCR experiments) was added on the sterile
435 embryos.
436 To colonize larvae with microbiota, fecal transplantation was used. Adults (10 males and 10
437 females) were collected from all populations and kept on standard food for five days. They
438 were transferred on a petri dish with a slice of medium and allowed to defecate for 48 hours.
439 Feces were collected after removal of the medium using an ethanol washed brush in sterile
440 PBS. Feces were filtered through a previously bleached and rinsed mesh and remaining
441 solution was adjusted to a culture turbidity (OD) of 1 to have approximately 10⁹ cells. 300 µl
442 were inoculated on the embryos for colonization.
443 To mono-associate larvae with *Acetobacter*, bacteria were grown for 48 hours at 30°C under
444 agitation in Man, Ragosa and Sharpe (MRS) medium (Difco, #288110) supplemented with
445 2.5% D-Mannitol (Sigma, #M1902). Bacteria were harvested by centrifugation at 3000 rpm

446 for 10 minutes and diluted with sterile PBS to reach OD 1. 300 μ l of culture was added on
447 sterile embryos.

448

449 ***Developmental Time and Survival***

450 To measure developmental time and egg-to-pupa survival gnotobiotic animals were prepared
451 as described above. Embryos from 6 Control and 6 selected populations were collected to
452 have 3 biological replicates in each condition (GF vs colonized) and on each food (standard
453 vs poor), resulting in 144 fly bottles to score. Emerging pupae were scored every day to
454 determine larval development time. Three replicate bottles were scored for each population
455 on each condition.

456

457 ***Adult dry weight and growth rate***

458 The first group of adults emerging from standard or poor food were discarded on the day of
459 emergence and newly emerging ones were collected within 48 hours of eclosion. 10 males
460 and 10 females from each bottle were picked randomly, separated and frozen at -20°C. When
461 the number of adults were not sufficient (valid for GF Control populations on poor food) the
462 procedure was repeated and adults emerged on different days were pooled. If the number of
463 adults was less than 10 the sample was discarded. To determine the dry body weight, flies
464 were dried at 80°C for two days and weighted on a precision balance.

465 Following ¹⁰, larval growth rate on poor diet was estimated separately for each sex and
466 population as $\ln(\text{final size}/\text{initial size})/(\text{time available for growth})$. Final size was the mean
467 dry weight of adults, initial size was assumed to be 0.005 mg, the approximate dry weight of
468 an egg (R. K. Vijendravarma et al, unpublished data). Time available for growth was
469 estimated as the egg-to-adult time minus 48 h to account for time needed for egg hatching, the
470 fact that pupae were scored at 24 h intervals, and the time the larvae spend wandering before
471 pupation (which does not differ between the Selected and Control populations ³¹). While this
472 estimate is necessarily approximate, all conclusions about growth rate were robust to
473 changing the time available for growth by ± 24 h.

474

475 ***Nucleic acid extraction and qPCR***

476 RNA extractions were performed from three biological replicates of 10 dissected midguts or
477 10 whole larvae from all six Selected and six Control populations (resulting in 72 gut samples
478 for each time point and 72 whole larval RNA samples) using RNAeasy Mini Kit (Qiagen).

479 Reverse transcription was performed as described in ⁷.

480 DNA extraction was carried from samples containing 10 surface sterilized (upon washing in
481 sterile water and EtOH) larvae using DNeasy Blood & Tissue Kit (Qiagen) following
482 manufacturer's protocol adapted for insect cells. For conventionally reared lines, larvae were
483 collected from two replicate vials per population. Mono-associated and GF groups were
484 collected from one vial per population.

485 qPCR was carried out using gene specific primer sets (available as Supplementary
486 information in ⁷ or upon request), using the Power SYBR Green PCR Master Mix (Life
487 technologies, #4368702) under the following conditions: 95°C 10 min, 40 cycles of 95°C, 15
488 sec and 60°C, 1 min. Melting curve analysis ensured amplification of a single product. Ratios
489 of gene of interest to reference gene ($2^{-\Delta C_t}$) were log transformed for statistical analysis.

490

491 ***Protease activity assay***

492 20-50 Whole larvae (equivalent of a volume of 40 μ l) were collected from 6 Selected and 6
493 Control populations in three biological replicates at different time points resulting in 198
494 individual samples to process. Protease activity was measured using Azocasein assay as
495 described in ⁷, which was optimized for whole larvae.

496

497 ***Amylase activity assay***

498 Amylase activity was measured using the Amylase Activity Assay Kit (Sigma, #MAK009)
499 following manufacturer's instructions and using the same samples as in the *Protease activity*
500 *assay*. 50 μ l of sample was added to the substrate mix on a 96-well plate. Absorbance at 405
501 nm was read every 20 min for 17 hours at 25°C. The rate of the reaction, *k* constant, was

502 calculated using non-linear least squares (nls) models in R using function *wrapnls* in package
503 *nlmrt* with the equation: $y=c+A(1-e^{-kt})$. The rate was normalized to total protein quantity as
504 for protease activity assay.

505

506 ***16S rRNA gene sequencing***

507 Community profiling was from adult feces and poor medium colonizing bacteria during larval
508 stages. Adult feces collection was described in the section "Developmental time and survival"
509 above. Larval medium was washed with 10 ml sterile PBS. The resulting solution was
510 centrifuged for 1 min at 3000 rpm to precipitate the food. The supernatant was re-centrifuged
511 at 13000 rpm for 10 min. Bacterial pellet was resuspended in 1 ml sterile PBS. 5 μ l of this
512 suspension was used directly in the PCR to amplify the V1-V2 regions of the 16S rRNA
513 gene, without any DNA extraction. Regions were amplified using the KAPA HiFi HotStart
514 ReadyMix (Kapa Biosystems # KK2601) and primers 8-27F: 5'-
515 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCMTGGCTCAG-3'
516 and 339-356R: 5'- GTCTCGTGGGCTCGGAGATGTGTATA-
517 AGAGACAGTGCTGCCTCCCGTAGGAG-3' including adapter sequences (underlined) for
518 the second PCR round. Three replicate 25 μ l PCR reactions containing 10 ng μ l⁻¹ DNA and 1
519 μ M of each primer were carried out under following conditions: 95°C 3 min, 25 cycles of
520 95°C 30 sec-56°C 15 sec-72°C 30 sec, followed by a final incubation at 72°C for 5 min.

521 Products were pooled from triplicate reactions and verified for amplicon size on a Fragment
522 Analyzer (Advanced Analytical Technologies, Inc.). Libraries were prepared and sequenced
523 at the Lausanne Genome Technology Facilities of the University of Lausanne according to
524 the Illumina 16S Metagenomic Sequencing Library Preparation protocol. Briefly, first round
525 PCR products were cleaned-up using AMPure XB (Beckman Coulter Genomics #A63881)
526 beads. An index PCR was carried out on the purified fraction using a Nextera XT Index Kit
527 (Illumina #FC-131-1001) to produce sequencing libraries. Libraries were again verified by
528 Fragment analyzer, mixed with 20% PhiX library (Illumina #FC-110-3001), and subjected to
529 Illumina MiSeq paired-end sequencing.

530 All steps of sequence analysis were performed using the QIIME 1.8.0 bioinformatics software
531 ³². Raw 300 bp paired-end reads were filtered by size (minimum 100 bp overlap between
532 paired ends) and quality (phred-scores ≥ 20). Chimeric reads were eliminated using the
533 Usearch algorithm ^{33,34}. Reads were classified into operational taxonomic units (OTUs) using
534 the open reference OTU clustering pipeline, excluding the pre-filtering step and using the
535 *uclust* method ³³. Reads were aligned to the Greengenes database ³⁵ using PyNAST ³⁶ with
536 99% identity threshold, to have specificity down to the species level. Taxonomies were
537 assigned using the RDP classifier ³⁷ and phylogenetic trees were built using FastTree 2.1.3 ³⁸.
538

539 ***Isolation of Acetobacter sp***

540 To isolate *Acetobacter*, media from (randomly chosen) Control #4 and Selected #29 were
541 streaked on MRS-Mannitol plates. A single colony was used to prepare liquid cultures (as
542 described in *Preparation of Gnotobiotic Animals*) and establish glycerol stocks, as well as for
543 16S rRNA gene full-length amplification using universal primers (sequences available upon
544 request) and KAPA HiFi HotStart ReadyMix. The 16S rRNA gene product was sequenced
545 using Sanger sequencing (GATC Biotech). The obtained sequence was assigned to
546 *Acetobacter* using RDP classifier (<https://rdp.cme.msu.edu/classifier/classifier.jsp>). To make
547 sure that we isolated the dominant strain, which was detected during community profiling, we
548 aligned sequences using APE Software.

549

550 ***Statistical Analysis***

551 Univariate analysis was performed using general linear mixed models (GMM) using
552 Satterthwaite approximation for the degrees of freedom (Proc Mixed of SAS v. 9.3).
553 Multivariate analysis was done using “ade4” package in R ³⁹. Evolutionary regime (Selected
554 or Control) and microbiota treatment (germ-free or colonized) were fixed factors; time point
555 was also a fixed factor except for enzyme activity assays, where more than two time points
556 were included. Replicate populations were treated as a random factor nested in evolutionary
557 regimes. A priori pairwise contrasts were performed within the framework of the GMM

558 (using the Slices option of Proc Mixed). Detailed output of all analyses can be found in

559 **Supplementary Tables S1-S5.**

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668

669 **Author Contributions**

670 BE and TJK designed the experiments. BE and SK performed the experiments. BE and TJK
671 analyzed the data. BE, JvdM and TJK wrote the article.

672

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677

678 **Competing and financial interests**

679 We declare no competing or financial interests.

680

681 **Figure Legends**

682 **Fig 1. Microbiota affects development and survival differently in Selected and Control**
683 **populations on poor food.**

684 **A.** Mean egg-to-pupa development time in Selected and Control populations, with or without
685 microbiota. **B.** Mean egg-to-pupa survival rate under the same conditions. Symbols and error
686 bars represent mean \pm SEM for each population (where error bars are not visible, they are
687 smaller than the symbols). Black horizontal bars represent the means of the six replicate
688 populations. Main effect differences analyzed by GMM are represented in the panels.
689 Interaction = Colonization \times Regime. Detailed statistics are presented in Supplementary
690 Table S1.

691

692 **Fig 2. Microbiota affects protein digestion differently in Selected and Control**
693 **populations.**

694 **A.** Protease activity in Selected and Control larvae through the 3rd larval instar in the presence
695 or absence of microbiota. **B.** Projections of protease expression dataset into 1st and 2nd PCs
696 (left) together with correlation circle (right) representing the variables. Light shade: early 3rd
697 instar, dark shade, late 3rd instar. **C.** Relative abundance ($2^{-\Delta Ct}$) of different proteases
698 measured by qRT-PCR from dissected guts of Selected and Control larvae at early and late
699 L3 stage. Symbols represent mean \pm SEM of the six replicate populations, with 3 biological
700 replicates per population. A selection of key statistical results from GMM is represented in
701 the panels. Interaction = Colonization \times Regime. Detailed statistics including pairwise
702 contrasts are presented in Supplementary Table S2.

703

704 **Fig 3. Microbiota affects carbohydrate digestion differently in Selected and Control**
705 **populations.**

706 **A.** Amylase activity in Selected and Control larvae through the 3rd larval instar in the
707 presence or absence of microbiota. Significant pairwise difference between GF Control and
708 GF Selected populations are shown with a black line. **B.** Projections of amylase and maltase

709 expression dataset into 1st and 2nd PCs (left) together with correlation circle (right)
710 representing the variables. Light shade: early 3rd instar, dark shade: late 3rd instar. **C.** Relative
711 abundance ($2^{-\Delta Ct}$) of different amylases and maltases measured by qRT-PCR from dissected
712 guts of Selected and Control larvae at early and late 3rd instar. Symbols represent mean \pm
713 SEM of for the six replicate populations, with three biological replicates each. A selection of
714 A selection of key statistical results from GMM is represented in the panels. Interaction =
715 Colonization \times Regime, Interaction 2 = Time \times Colonization. Detailed statistics including
716 pairwise contrasts are presented in Supplementary Table S3.

717

718 **Fig 4. Microbiota of Selected and Control populations**

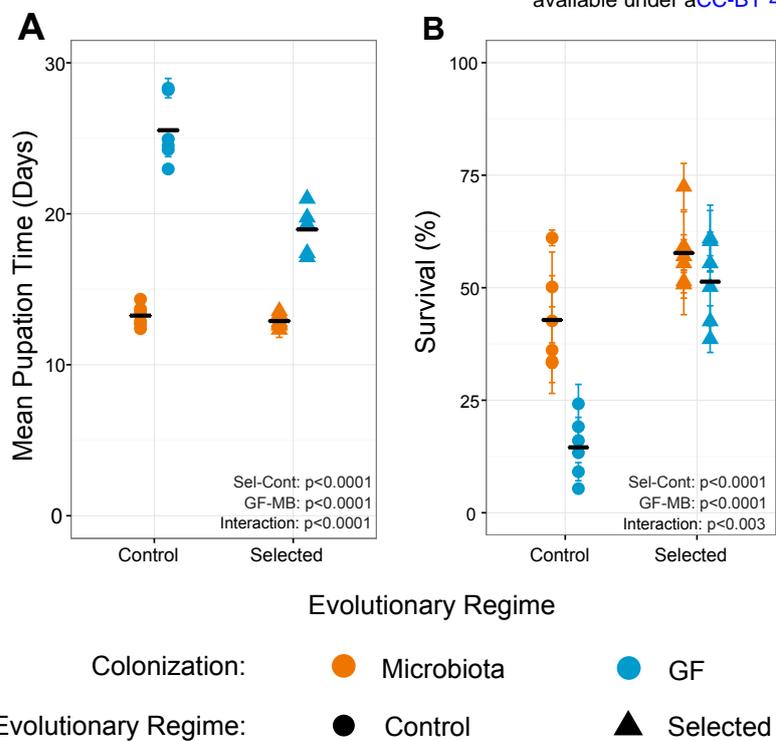
719 **A.** The identities and relative abundances of 5 most abundant taxa in the mixed adult feces
720 (used as the source of inoculum), and from the larval poor medium of Selected and Control
721 populations previously colonized with that inoculum, assigned by 16S rRNA gene amplicon
722 sequencing. C: Control, S: Selected Populations. **B.** The abundance of *Acetobacteraceae*
723 relative to the host DNA in GF, *Acetobacter* mono-associated and conventionally reared (by
724 experimental evolution) Selected and Control populations measured by qPCR. Symbols
725 represent mean \pm SEM of for each population. Black bars represent the mean of the six
726 populations within regime. Main effect differences analyzed by GMM are represented in the
727 panel. Interaction refers to colonization \times evolutionary regime. Details including pairwise
728 contrasts are presented in Supplementary Table S4.

729

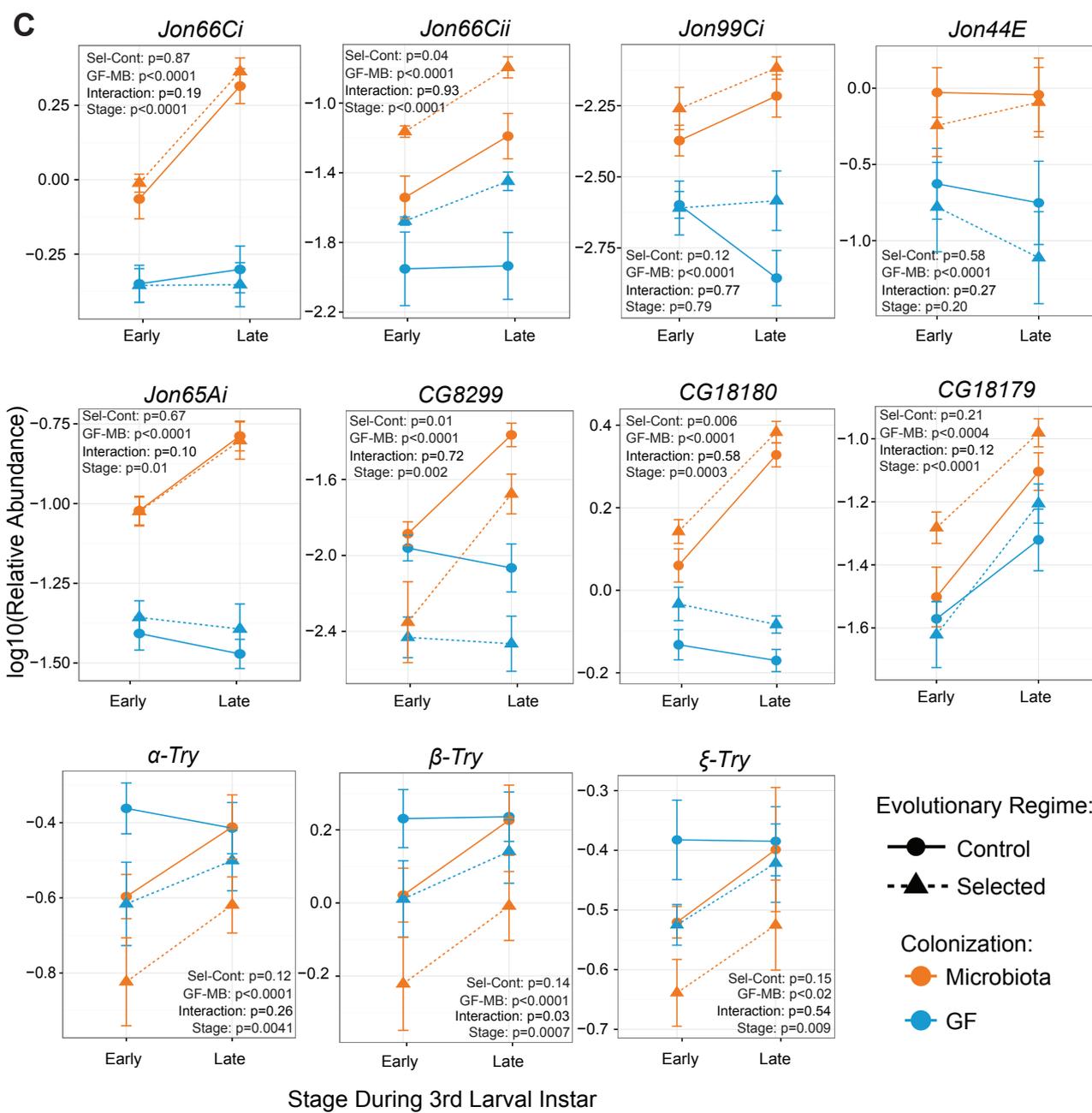
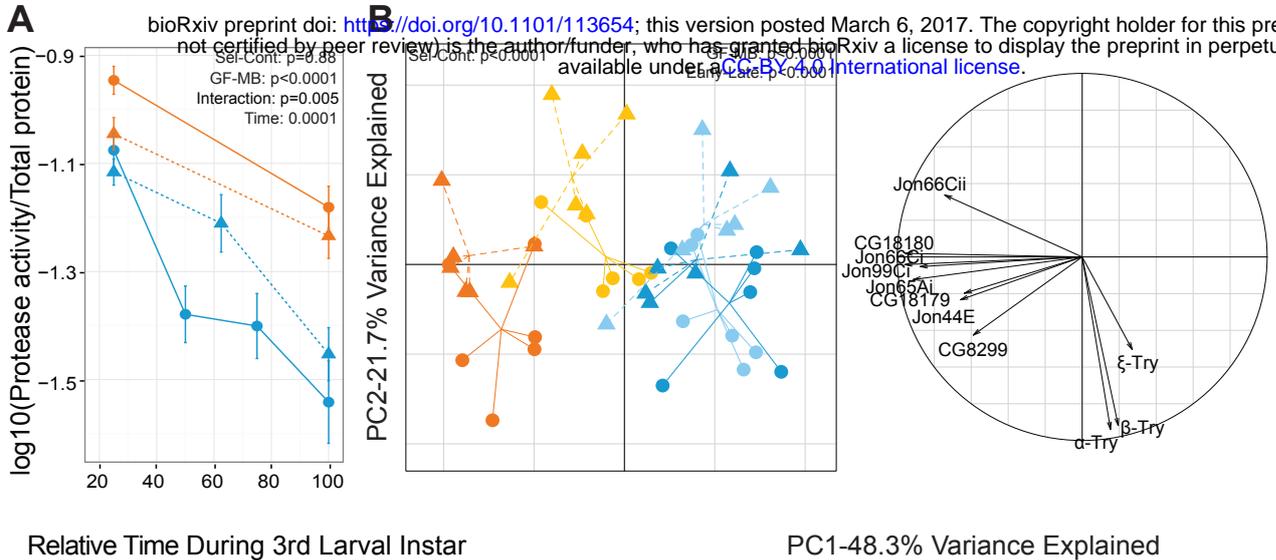
730 **Fig 5. Growth rate and IIS pathway are regulated differently by microbiota association** 731 **in Selected and Control populations.**

732 **A.** Growth rate on poor medium for males and females of Selected and Control populations
733 with and without microbiota. Main effect differences analyzed by GMM are represented in
734 the panel. **B.** Relative abundance ($2^{-\Delta Ct}$) of different dFOXO targets measured by qRT-PCR
735 from whole Selected and Control larvae that are GF or mono-associated with *Acetobacter* at
736 late 3rd larval instar. Points represent mean \pm SEM of for 6 populations. Black bars represent

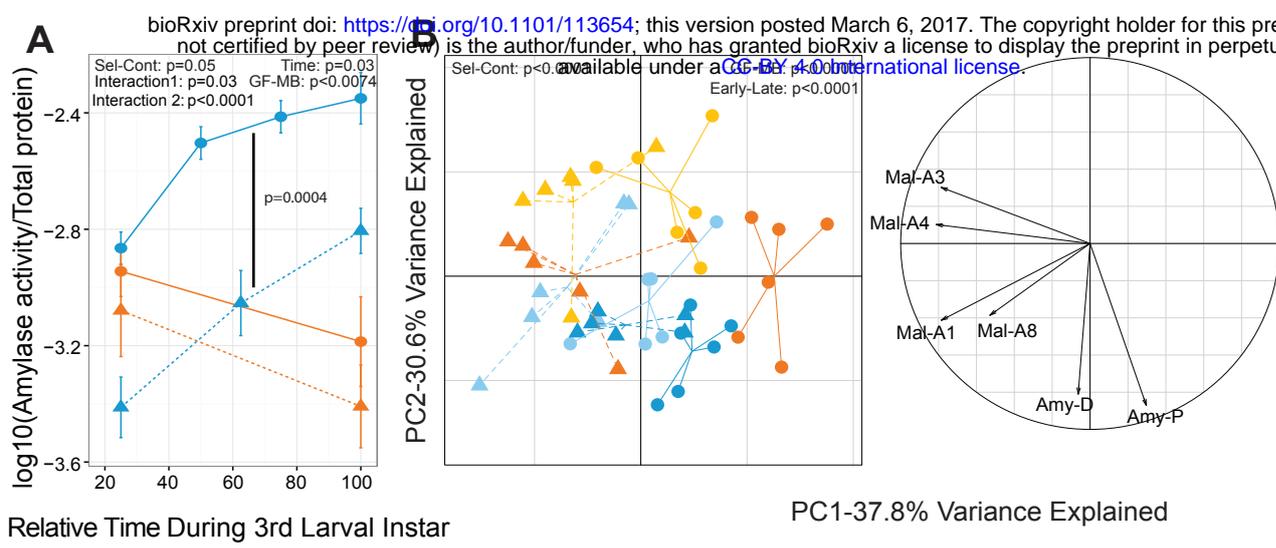
737 the mean for 6 populations. The interaction between colonization and evolutionary regime
738 analyzed by GMM, and significant pairwise contrasts within each regime are represented in
739 the panel. Detailed statistics are presented in Supplementary Table S5.
740
741



Erkosar et al. Fig 1

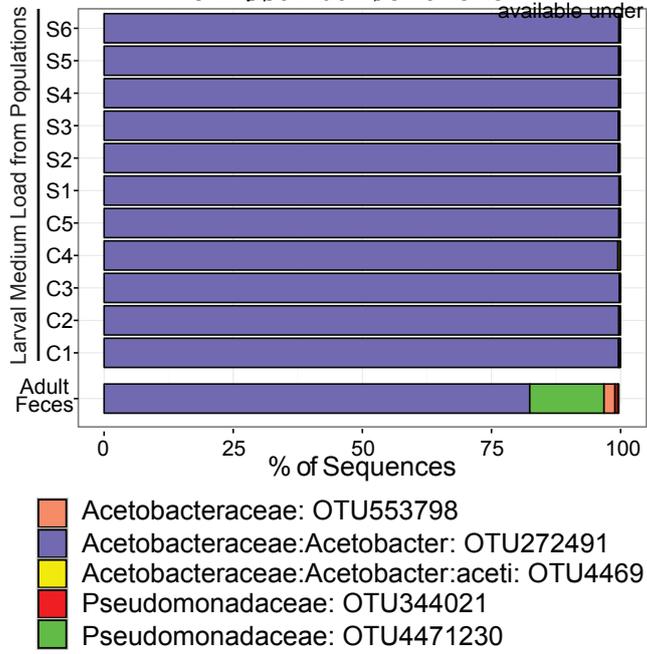


Erkosar et al. Fig 2

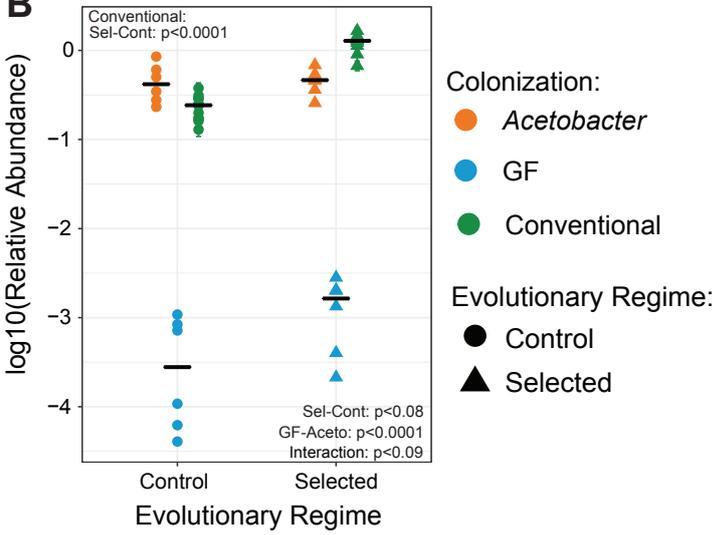


Erkosar et al. Fig 3

A

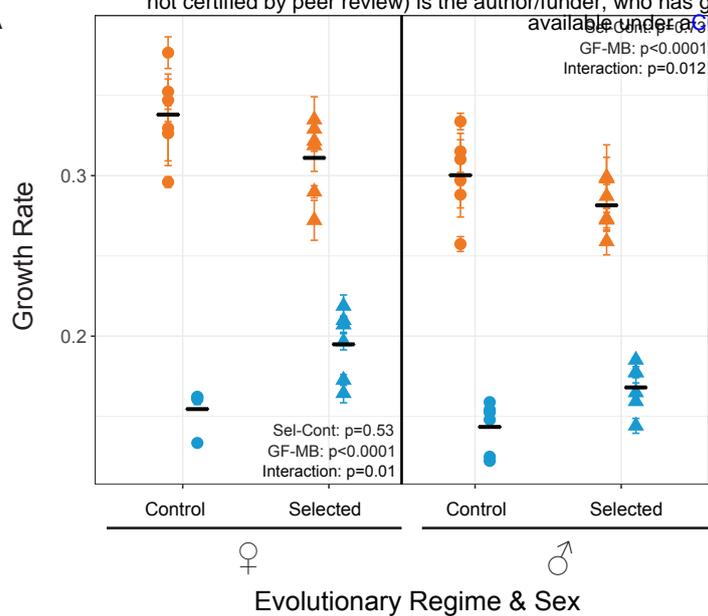


B

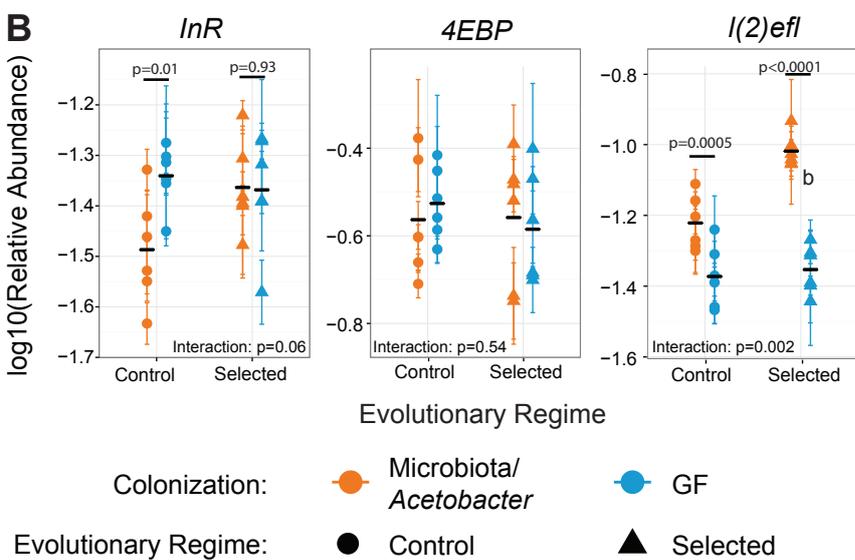


Erkosar et al. Fig 4

A



B



Erkosar et al. Fig 5