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1 **Microbiome derived acidity protects against microbial invasion in *Drosophila***

2

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11

12 **Abstract**

13 Microbial invasions underlie host-microbe interactions that result in microbial

14 pathogenesis and probiotic colonization. While these processes are of broad interest,

15 there are still gaps in our understanding of the barriers to entry and how some microbes

16 overcome them. In this study, we explore the effects of the microbiome on invasions of

17 foreign microbes in *Drosophila melanogaster*. We demonstrate that gut microbes

18 *Lactiplantibacillus plantarum* and *Acetobacter tropicalis* improve survival during invasion

19 of a lethal gut pathogen and lead to a reduction in microbial burden. Using a novel multi-

20 organism interactions assay, we report that *L. plantarum* inhibits the growth of three

21 invasive Gram-negative bacteria, while *A. tropicalis* prevents this inhibition. A series of

22 *in vitro* and *in vivo* experiments revealed that inhibition by *L. plantarum* is linked to its

23 ability to acidify both internal and external environments, including culture media, fly

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24 food, and the gut itself, while *A. tropicalis* diminishes the inhibition by quenching acids.
25 We propose that acid produced by the microbiome serves as an important gatekeeper
26 to microbial invasions, as only microbes capable of tolerating acidic environments can
27 colonize the host. The methods described herein will add to the growing breadth of tools
28 to study microbe-microbe interactions in broad contexts.
29

30 **Introduction**

31 Animals consistently interact with complex communities of microorganisms throughout
32 their lives. The collection of microbes that inhabits a particular niche is known as the
33 microbiome. While many foreign microbes encountered by a host are transient, others
34 have the potential to enter existing communities and establish themselves on or within
35 the host, in a process known as microbial invasion[1,2]. Invasion can affect the host in
36 different ways, depending on the actions of the microbe and the response by the host[3].
37 Positive outcomes of microbial invasion are observed during therapeutic fecal
38 microbiome transplants, in which the microbiome from a healthy individual is transferred
39 to a patient with gut disease, leading to a remodeling of the microbial community and
40 improved health[4]. Conversely, microbial invasion can also produce negative outcomes
41 on a host, as is the case during intestinal pathogenesis of *Escherichia coli* O157:H7[5].
42 Though the host outcomes are different in these cases, there is overlap in that new
43 microbes enter and establish themselves within an existing community structure. The
44 study of microbial invasion as a generalized concept is a relatively recent appreciation.
45 The ability of a microbe to invade a community is generally governed by the
46 introduction, establishment, and growth of foreign microbes in a new niche[1]. Attaining

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47 each phase of invasion requires that a microorganism overcome multiple barriers to
48 entry, including physical and chemical barriers such as temperature and pH as well as
49 biological barriers such as competition with the existing microbial community and
50 actions of the host immune response[1,6].
51
52 Studying invasion in the laboratory can be complicated due to the complexity of many
53 microbial communities. For example, the human gut microbiome consists of hundreds of
54 species, making it difficult to characterize specific microbe-microbe interactions[7,8].
55 *Drosophila melanogaster* is an attractive model for the study of microbial invasion for
56 several reasons. Its intestinal tract is structurally and functionally similar to that of
57 vertebrates, including humans, while having a more simplistic microbiome composition
58 that can be controlled and manipulated in the laboratory[9–13]. Previous studies in *D.*
59 *melanogaster* have examined microbial invasion through the lenses of microbial
60 pathogenesis[14–18], microbiome assembly[10,19], and probiotic colonization[20,21]. In this
61 study, we explore the effect of the gut microbiome on the invasion of three Gram-
62 negative bacteria previously used in *D. melanogaster* studies: the lethal
63 entomopathogen *Pseudomonas entomophila* (Pe), the non-lethal pathogen
64 *Pectobacterium carotovora* (Ecc15), and the human probiotic organism *Escherichia coli*
65 Nissle 1917 (EcN). Here, we report that the microbiome protects *D. melanogaster*
66 during microbial invasion, leading to improved probability of survival and reduced
67 microbial burden compared to axenic (microbiome-free) hosts. We report antimicrobial
68 activity by the gut microbiome member *Lactiplantibacillus plantarum* (Lp) against
69 invasive microbes, which is linked to its ability to acidify the surrounding environment.

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70 Another microbiome member, *Acetobacter tropicalis* (At) reduces the inhibitory capacity
71 of Lp by neutralizing acidity. Overall, this work expands our understanding of microbe-
72 microbe interactions by characterizing acidity from the microbiome as a chemical barrier
73 to microbial invasion, a phenomenon that can be observed in many animals, including
74 humans[22–25].
75
76 **Results**
77 **The gut microbiome protects the host during invasion with a lethal pathogen.**
78 To determine how common members of the *D. melanogaster* microbiome impact host
79 susceptibility to Pe infection, we generated axenic flies without microbiomes by
80 hypochlorite dechorionation of embryos[26]. We then generated gnotobiotic animals by re-
81 associating axenic flies with defined microbial communities: Lp, At, or LpAt (**Figure 1A**).
82 During infection with Pe, each gnotobiotic treatment group experienced improved levels
83 of survival compared to infected axenic flies (**Figure 1B**). Lp mono-colonization resulted
84 in statistically better survival compared to mono-colonization with At. Co-colonization of
85 the two gut microbes resulted in survival comparable to mono-colonization with Lp and
86 At alone. We also assessed the microbial burden of Pe over the course of the infection
87 and found that, while Pe load remained high over 7 days in axenic flies, the presence of
88 Lp in the mono-colonized gnotobiotic condition resulted in lower levels of Pe compared
89 to axenic flies at 72 hours and 7 days (**Figures 1C, S1)**. Pe levels in At mono-colonized
90 flies remained high, similar to axenic conditions. When flies contained both Lp and At
91 prior to infection, Pe load was reduced at 72 hours compared to axenic flies, but it
92 increased again by 7 days. Together, these results demonstrate that the microbiome,

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93 particularly Lp, limits the colonization ability of Pe in the gut, which is consistent with the

94 reduction in mortality observed in gnotobiotic flies.

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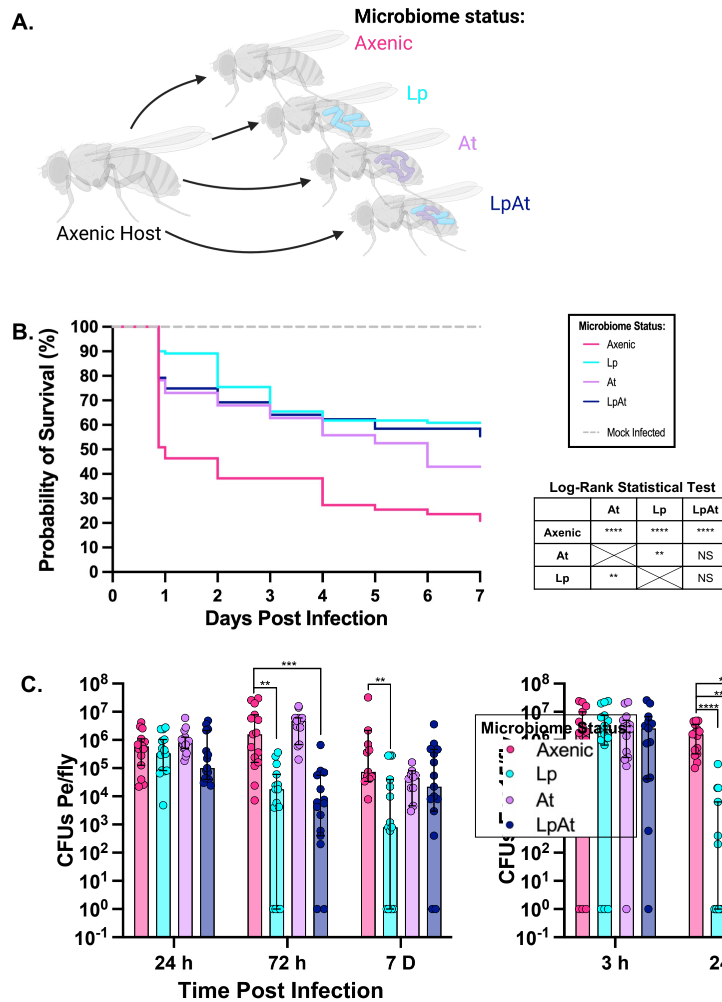


96

97 **Figure 1. Presence of microbiome members reduces host susceptibility**

98 **to *P. entomophila*. A)** Scheme describing the generation of gnotobiotic flies.

99 **B)** Kaplan-Meier survival analysis of female flies infected with *P. entomophila* (Pe) via

100 feeding (feeding occurred from t=0 to t=1 day); fly microbiome treatments included no

101 bacteria (Axenic), *L. plantarum* (Lp), *A. tropicalis* (At), or a combination

102 of Lp and At (Lp/At). The mock infected group shows survival of axenic flies fed LB

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103 media; survival of all gnotobiotic conditions fed LB were also recorded but were not
104 significantly different to axenic control and are not shown. Log-rank statistical analyses
105 of each infection condition are compared to the other conditions. Significance is
106 expressed as follows: NS, not significant; **, P ≤ 0.01; ****, P ≤ 0.0001. n=60 flies per
107 control & 110-160 flies per Pe-infected treatment over 3 independent replicates. **C)**
108 Number of colony forming units (CFUs) of bacteria per fly infected with Pe screened in
109 axenic flies and gnotobiotic flies colonized with Lp, At, and Lp/At. Flies were sacrificed
110 to determine Pe load at 24 hours, 72 hours, and 7 days post feeding infection. Each
111 point represents bacterial load from an individual fly; bars and error bars represent the
112 median and 95% confidence intervals; limit of detection is $2x10^2$ CFUs/Fly. n=9 flies per
113 control & 10-15 flies (depending on availability of living flies) per infected treatment per
114 time point over 3 biological replicates. Statistical significance was determined between
115 flies containing different microbiomes by the Kruskal-Wallis method with Dunn's multiple
116 comparisons analysis. P-values are represented as follows: *, P ≤ 0.05; **, P ≤ 0.01; ***,
117 P ≤ 0.001; ****, P ≤ 0.0001.
118

119 **Microbial invasion by Gram-negative microbes is reduced in flies associated with**
120 ***L. plantarum***

121 Previous studies in *D. melanogaster* have demonstrated that the microbiome protects
122 the host during challenge with lethal pathogens[27]. However, the role of the microbiome
123 during the introduction of non-lethal invasive microbes has largely gone unaddressed.
124 We selected two such Gram-negative bacteria: *Pectobacterium carotovora* (Ecc15), a
125 plant pathogen that has adapted to cause gut disease in flies[15], and *Escherichia coli*

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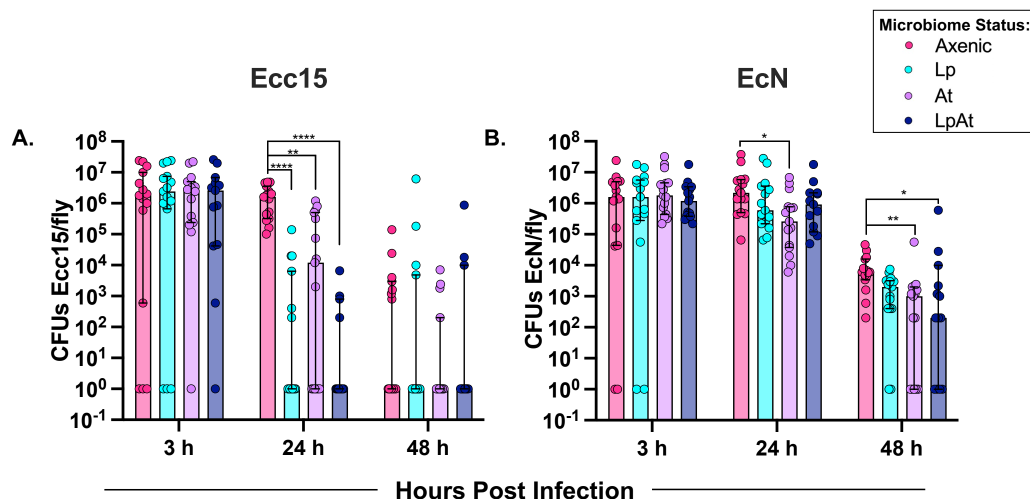
126 Nissle 1917 (EcN), which we previously identified as a colonization proficient strain of *E.*
127 *coli* that does not cause obvious disease in flies[20]. We administered both organisms to
128 axenic and gnotobiotic flies and assessed microbial burden over the following 48 hour
129 period to probe the impacts of the microbiome on the outcome of infection. We found
130 that the Ecc15 microbial load was reduced in all three microbiome treatments compared
131 to axenic flies at 24 hours post-infection, with Lp-associated flies exhibiting the greatest
132 diminution (**Figure 2A**). Surprisingly, EcN bacterial load was not greatly affected by the
133 presence of Lp, but slight differences in bacterial load were observed at 24 hours
134 between axenic and At-gnotobiotes and at 48 hours between axenic and gnotobiotes
135 harboring At alone or the combination of Lp and At **(Figure 2B)**. Taken together with the
136 Pe infection data, these results strongly suggest that the presence of Lp protected the
137 host during invasion of pathogenic microbes, and we sought to uncover the mechanism
138 of this protection.
139




140

141 **Figure 2. Microbiome composition alters the microbial load of invasive organisms**
142 **during infection.** Number of colony forming units (CFUs) of bacteria per fly infected

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143 with Ecc15 (**A**), and EcN (**B**). Each invasive organism was screened in axenic flies and
144 gnotobiotic flies colonized with Lp, At, and Lp/At. Bacterial load was determined at 3
145 hours, 24 hours, and 48 hours post feeding infection. Each point represents bacterial
146 load from an individual fly; bars and error bars represent the median and 95%
147 confidence intervals; limit of detection is $2x10^2$ CFUs/Fly. n=15 flies per treatment per
148 time point over 3 biological replicates. Statistical significance was determined between
149 flies containing different microbiomes by the Kruskal-Wallis method with Dunn's multiple
150 comparisons analysis. P-values are represented as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***,
151 $P \leq 0.001$; ****, $P \leq 0.0001$.
152

153 ***L. plantarum* inhibits invasive organisms *in vitro***

154 Traditionally, the "cross-streak" assay has been used as an *in vitro* culture-based
155 method to study binary microbe-microbe interactions. To expand this method to
156 examine the combined effects of two microbiome organisms on the growth of invasive
157 microbes, we developed the multi-organism interactions assay **(Figure 3A)**. In this
158 assay, intersecting lines of Lp and At were spread at a 90˚ angle in the middle of a Petri
159 dish and allowed to grow for three days, giving enough time for the microbes to secrete
160 metabolites into the environment (**Figure S2**). The invasive organism (Pe, Ecc15, or
161 EcN) was then streaked into the adjacent quadrants and grown for an additional 1-2
162 days. These assays revealed that Lp, but not At, inhibited the growth of Pe, Ecc15, and
163 EcN, as evidenced by a zone of inhibition near the Lp streak and growth close to the At
164 streak (**Figure 3B,** see dashed regions). Interestingly, our assay also captured that At
165 modulated the inhibitory effects of Lp on all three organisms, as the zones of inhibition

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166 diminish away from the distal end of the Lp streak, practically disappearing at the
167 intersection point of Lp and At (**Figure 3B**). We observed the same patterns of inhibition
168 when Pe, Ecc15, and EcN were co-cultured with fly microbiome isolates in liquid media.
169 Co-culturing with At did not impact growth of Pe, Ecc15, or EcN, co-culturing with Lp
170 alone inhibited their growth, and the addition of At reduced this inhibition by Lp (**Figure**
171 **3C**). Interestingly, EcN was more resilient during co-culture with Lp than Pe and Ecc15,
172 which were completely inhibited, but populations were still ~1000x less abundant than
173 when grown on its own. Altogether, these results demonstrate that Lp has an
174 antimicrobial effect on the growth of the three invasive organisms, which At can
175 ameliorate by creating an environment more favorable for their growth.
176

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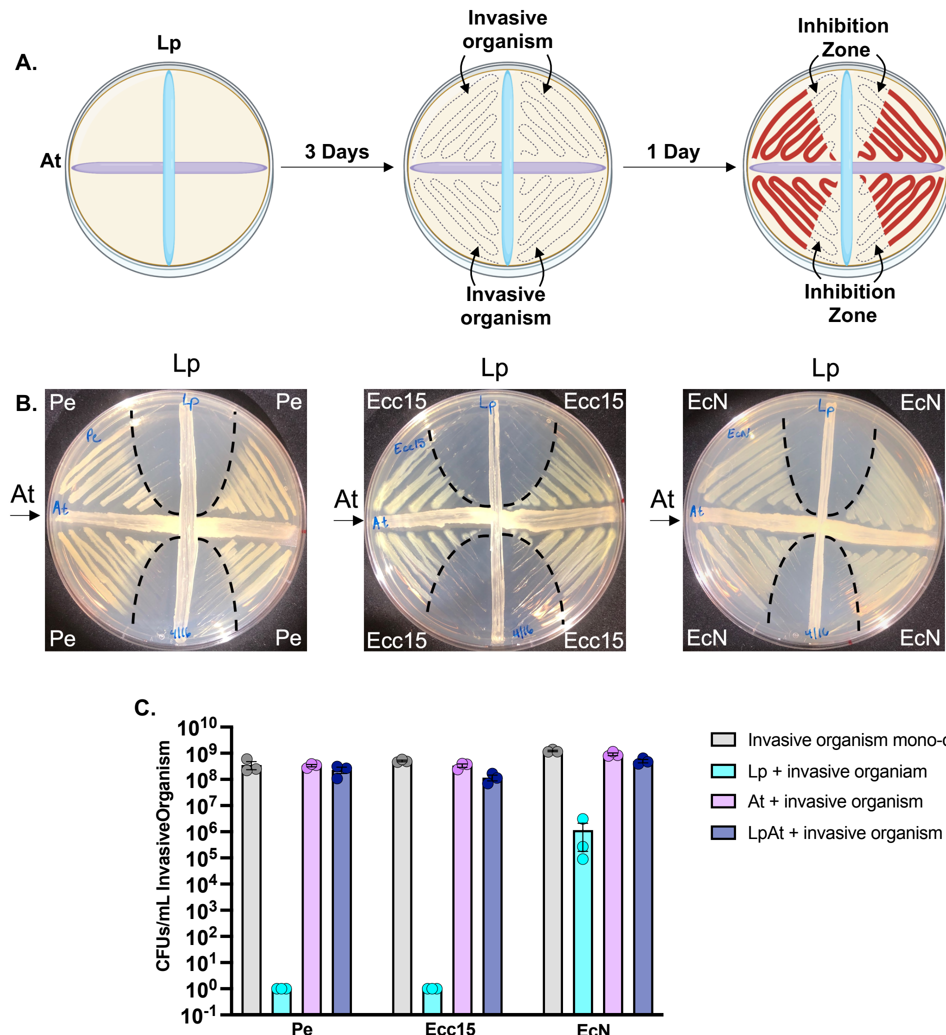


177

178 **Figure 3. *In vitro* growth assays reveal inhibition of Gram-negative invasive**
179 **organisms by Lp. A)** Scheme describing the multi-organism interaction assay
180 procedure. **B)** Multi-organism interaction assays display growth effects of gut
181 microbes Lp and At on invasive organisms Pe, Ecc15, and EcN. Microbiome members
182 were grown in perpendicular streaks for 3 days, and invasive organisms were added to
183 the adjacent quadrants and allowed to grow for an additional day. Zones of inhibition
184 are indicated by dashed lines. **C)** Co-culture analysis of invasive organisms with
185 microbiome members. Pe, Ecc15, and EcN were grown in mono-culture or in media

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186 previously inoculated with Lp, At, or a mixture of both (LpAt). Bars and error bars
187 represent the mean concentration of invasive organisms in CFUs/mL ±SEM. N=3
188 biological replicates per condition.
189
190 ***L. plantarum*-mediated inhibition is driven by environmental acidification.** Lactic
191 acid (and its conjugate base, lactate), a major Lp metabolite, has recently been
192 implicated as an important factor in microbe-microbe interactions and microbe-host
193 interactions in *D. melanogaster* [28–30]. To determine if the secretion of lactic acid is a
194 potential mechanism by which Lp inhibits the growth of the selected invasive organisms,
195 we measured the pH of the media surrounding Lp in various growth conditions. We first
196 observed the pH of media in which Lp and At were grown at the same time (streaked
197 perpendicular to one another as in **Figure 3A**) using media plates containing the pH
198 indicator bromophenol blue, and saw distinct acidic regions (as indicated by a yellow
199 color) surrounding Lp, with larger acid penetrance further away from the interaction
200 point with At (**Figure 4A**). Introduction of the invasive organisms yielded zones of
201 inhibition overlapping with the zones of acidity (**Figures 4A, S3**). Interestingly, while Pe
202 was inhibited as in **Figure 3B**, the acidic zone surrounding Lp returned to a blue color,
203 indicating that the pH had risen back above 4.6 (**Figure S3**). This experiment suggested
204 that the ability of Lp to acidify the surrounding media is linked to its inhibition of invasive
205 microbes and that At reduces its inhibitory capacity by neutralizing acidity. To mimic the
206 growth conditions of the microbiome members *in vitro*, we determined the pH of
207 overnight cultures of Lp and At mono-cultures and Lp/At co-cultures (**Figure 4B**). The
208 Lp culture was found to be pH 4.0, consistent with its known capacity to secrete lactic

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209 acid. *A. tropicalis* had a pH of 7.0, and the Lp/At co-culture had a pH of 6.0. To test the
210 effect of low pH on the growth of the invasive microbes, culture media was adjusted to
211 pH 4.0 with either lactic acid or hydrochloric acid. Invasive microbes were grown alone
212 in standard media and acidified media and in co-culture with At in acidified media. The
213 lactic acid media completely prevented the growth of the Pe, Ecc15, and EcN (**Figure**
214 **4C**). Media acidified with HCl reduced (but did not eliminate) the culture densities of Pe
215 and Ecc15, while having little effect on the growth of EcN (**Figure S4).** Co-culturing with
216 At in both acidified media treatments restored the growth of each organism to normal
217 levels, while also raising the pH of each culture to >5.0 (data not shown). To
218 complement this analysis, we co-cultured each invasive organism with Lp in standard
219 media (starting pH 6.5) and media buffered to pH 6.0 with 60 mM phosphate buffer
220 (**Figure 4D**). As in **Figure 3C**, Pe and Ecc15 were completely inhibited by Lp, and EcN
221 was partially inhibited in standard media. However, all three organisms grew to a normal
222 level when co-cultured with Lp in buffered media, suggesting that Lp-mediated inhibition
223 was linked to its ability to shift the pH of the surrounding environment. To test this
224 hypothesis *in vivo*, we performed bacterial load analysis on axenic and gnotobiotic flies
225 infected with Ecc15 on the standard fly diet and on a fly diet buffered to pH 6.0 with 2-
226 (*N*-morpholino)ethanesulfonic acid (MES) (**Figures 4E, S5**). Interestingly, axenic and
227 Lp-colonized flies feeding on the buffered diet had significantly higher levels of Ecc15 in
228 their intestines 48 hours-post infection. This demonstrates that acidification of the gut is
229 crucial to the protective effect of Lp during infection. To assess gut pH, we fed axenic
230 and gnotobiotic flies bromophenol blue for 24 hours, dissected their guts, and imaged
231 them to assess intestinal pH (**Figure 4F**). We found that flies colonized with either Lp or

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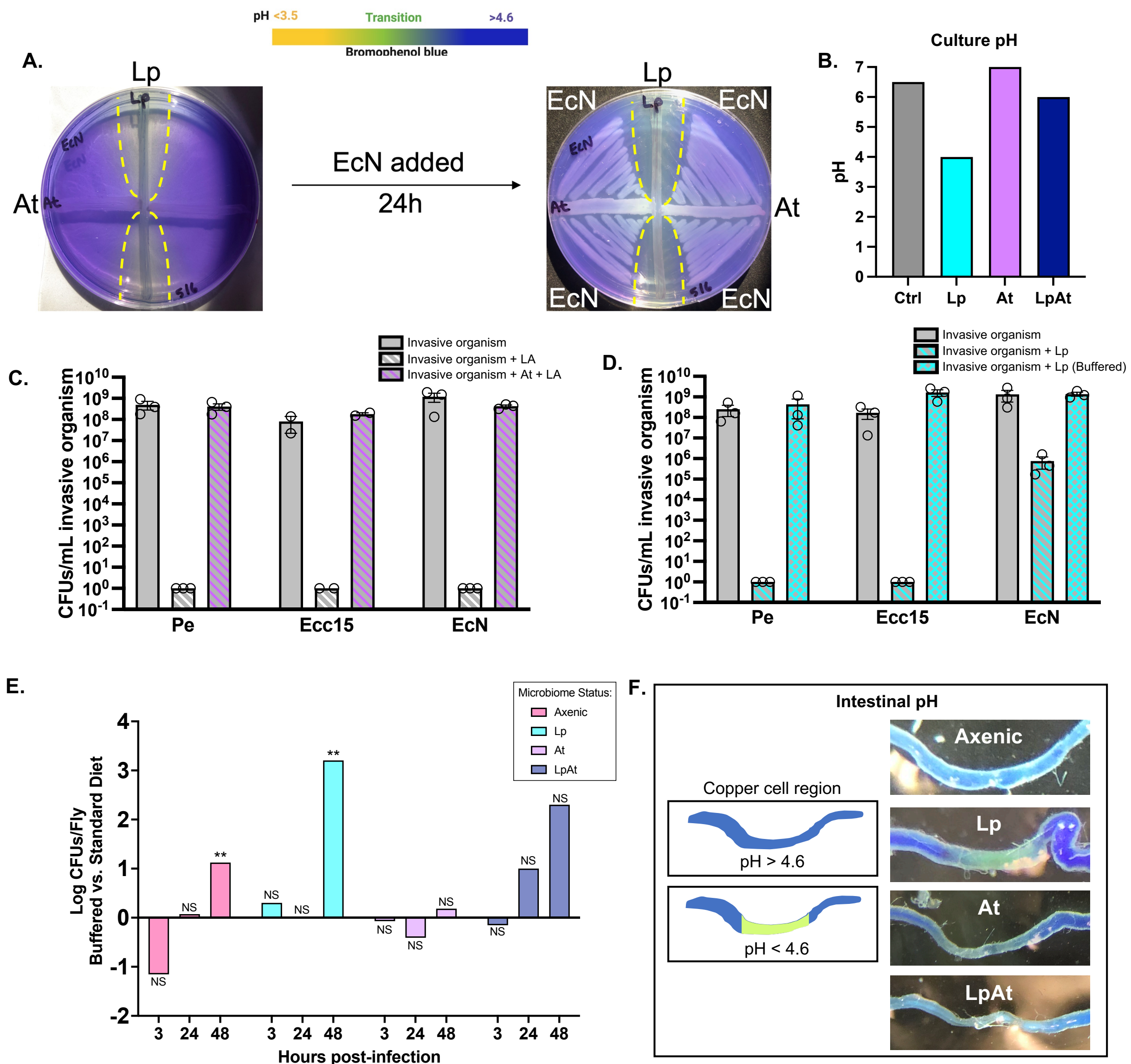
232 At had distinct acidic copper cell regions (indicated by a green-yellow color) while the
233 guts of axenic flies or flies colonized with Lp and At lacked such distinct regions,
234 suggesting that microbiome composition alters the pH of the intestine.

235




236

237 **Figure 4. Microbiome-derived shifts in pH contribute to inhibition by Lp. A)** Multi-
238 organism interaction assay showing the effects of Lp and At on EcN growth on media
239 containing the pH indicator bromophenol blue. After three days of growth, a yellow

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240 acidic zone appears around Lp, but it tapers off near the At interaction point. When EcN
241 is added, the zone of inhibition overlaps with the acidic region. **B)** pH measurement of
242 microbiome mono-cultures and co-cultures reveals a sharp acidification of culture media
243 by Lp. **C)** Density of invasive organisms in media adjusted to pH 4.0 with lactic acid (LA)
244 with or without 24 hours of prior growth with At. Each bar represents mean CFUs/mL
245 ±SEM of 3 biological replicates. **D)** Microbial concentration of invasive organisms in
246 media buffered to pH 6.0 with phosphate buffer with or without 24 hours of prior growth
247 with Lp. Each bar represents mean CFUs/mL ±SEM for 3 biological replicates. **E)** Ratio
248 of Ecc15 microbial load in flies infected on buffered vs. standard fly diets, n=15 flies per
249 treatment per time point over three biological replicates. Statistical significance was
250 determined for flies of each microbiome status between standard and buffered diets
251 using the Kruskal-Wallis method with Dunn's multiple comparisons analysis. P-values
252 are represented as follows: NS, $P>0.05$; **, $P \leq 0.01$. **F)** The pH of the copper cell region
253 of the intestine was approximated by feeding axenic and gnotobiotic flies food soaked
254 with 2% bromophenol blue. Guts were dissected and imaged immediately. A
255 yellow/green color in the copper cell region indicates an acidified environment (pH <
256 4.6).
257

258 **Microbiome composition alters the chemical environment of fly food**

259 The observation that Lp rapidly acidifies bacterial culture media raised a question about
260 the effect of the microbiome members on the chemistry of fly food. Fly food is a rich
261 source of microbes as flies feed upon and defecate microbes continuously, and as
262 such, it has been described as a "reservoir" for the microbiome[27]. We tested the effects

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263 of gut microbes on the pH of fly food in two different ways: by applying bacterial cultures
264 directly to fly food (**Figure 5A**) and by introducing axenic and gnotobiotic flies to vials of
265 fly food (**Figure 5B**). In both experiments, the control fly food was ~pH 6.0. Fly food
266 colonized by Lp was reduced to ~pH 4.0. *A. tropicalis* had more minimal impacts on
267 food pH, with slightly higher pH (6.5) when culture-inoculated and slightly lower pH (5.5)
268 when fly-inoculated. Interestingly, the pH of fly food co-inoculated with Lp and At was
269 ~pH 5.0 in both experiments, indicating a level of acidity intermediate between Lp and
270 At alone. These data demonstrate that microbiome composition has a critical impact on
271 the pH of fly food, as fly food containing Lp alone was found to be ~100x more acidic
272 than uninoculated controls, while At had a less dramatic effect on pH. Given the
273 different levels of acidity observed in the presence of different gut microbes, we asked
274 whether the invasive organisms experience similar interactions with gut microbes on fly
275 food. To test this, we added gut microbes to the surface of fly food, allowed them to
276 grow for three days, and applied Pe, Ecc15, or EcN to the same vial and cultured them
277 from the fly food 24 hours later to determine the levels of invasive microbes and
278 microbiome members (**Figure 5C, Figure S6).** As observed in previous analyses, At
279 had little effect on the growth of the invasive microbes on fly food. Lp completely
280 prevented the growth of Pe and Ecc15, while greatly reducing the level of EcN growth
281 compared to the control. Levels of invasive microbes were partially rescued when Lp
282 and At were grown together on fly food, suggesting that At alters the inhibitory capacity
283 of Lp on fly food. These experiments support the idea that fly food acts as a reservoir for
284 not only the microbiome community members, but also the metabolic products they
285 release into the environment. These include acidic products such as lactic acid, which

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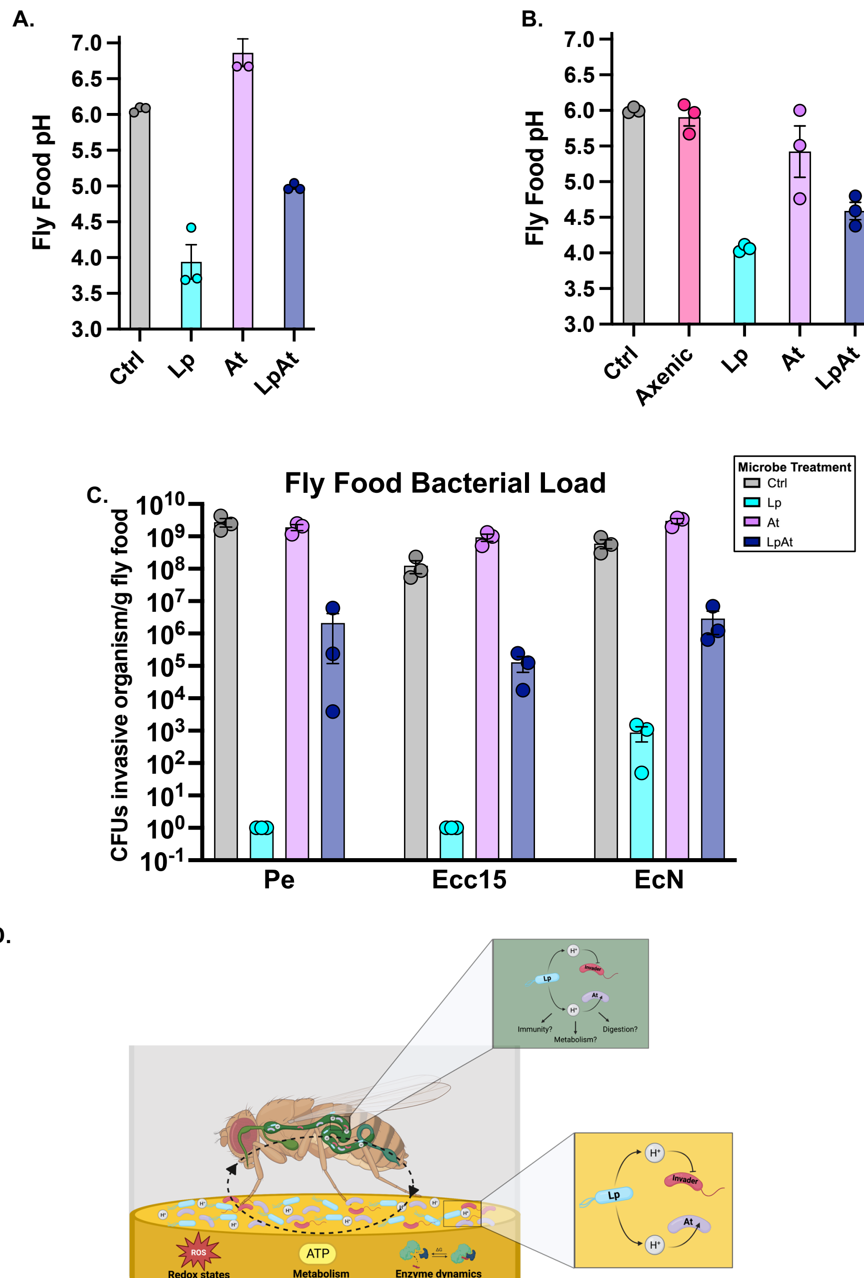
286 have the potential to play an important role in modulating the environment and affecting
287 both host and microbial processes **(Figure 5D)**.
288




289

290 **Figure 5. Microbiome composition alters the chemical environment of fly**
291 **food. A)** pH analysis of fly food after three days of growth of Lp, At, or Lp/At, with

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292 culture media added as a control. Bars represent the mean pH of three biological
293 replicates ±SEM. **B)** pH analysis of fly food three days after the addition of 40 male flies
294 of different microbiome statuses (Axenic, Lp, At, Lp/At). Bars represent the mean pH
295 ±SEM of three biological replicates. **C)** Bacterial load of Pe, Ecc15, and EcN present on
296 fly food initially inoculated with microbiome members Lp, At, or Lp/At, with culture media
297 as a control. Each bar represents the mean CFUs/g fly food ±SEM for three biological
298 replicates. **D)** Pictorial representation of microbe-microbe interactions between
299 microbiome members and Gram-negative invasive bacteria on fly food and during
300 consumption by the host.
301
302 **Discussion**
303 The microbiome protects the host during infection with invasive organisms
304 We examined microbe-microbe interactions in a *D. melanogaster* model, which has the
305 advantage of possessing a simple microbial community that can be removed and
306 manipulated to provide a high level of control over the system. Previous work showed
307 that axenic flies had reduced survival during infection with *Pseudomonas aeruginosa*
308 and *Serratia marcescens* compared to flies raised with their microbiomes intact[27]. Our
309 study expanded upon this finding by assessing survival and microbial burden in
310 gnotobiotic flies with defined microbial communities. We found that flies harboring gut
311 microbes *Lactiplantibacillus plantarum* (Lp), *Acetobacter tropicalis* (At), or a consortium
312 of the two had an improved probability of survival compared to axenic flies when
313 infected with the gut pathogen *Pseudomonas entomophila* (Pe) and that this was
314 accompanied by a reduction in microbial burden of Pe, most starkly in the Lp

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315 gnotobiotes (**Figure 1**). We questioned whether the apparent protective effect of the
316 microbiome was specific to lethal pathogens or whether a more generalized mechanism
317 of protection was at play. To test this, we screened two other microbiome-invasive but
318 non-lethal microbes *Pectobacterium carotovora* (Ecc15) and *Escherichia coli Nissle*
319 *1917* (EcN) in our model and observed a similar reduction in microbial burden of Ecc15
320 in Lp gnotobiotes, but no such reduction of EcN, suggesting that EcN is a better invader
321 into the microbiome (**Figure 2**).
322

323 Weak organic acids act as gatekeepers for invasion of microbial communities

324 Microbial invasion has been studied extensively and has implications on a variety of
325 biological processes from pathogenesis to agriculture. It involves the introduction and
326 establishment of a microorganism, initially foreign, into a new environment[1]. Multiple
327 biotic and abiotic barriers must be overcome before a microbe can successfully invade
328 an existing microbial community[1]. In this study, we characterized microbiome-derived
329 acidity as a chemical barrier to microbial invasion. *In vitro* analyses of microbe-microbe
330 interactions revealed that Lp inhibited the three invasive organisms we tested, and the
331 observation that At lessened this inhibition when grown in close proximity to Lp was a
332 key finding as it suggested that At modulates the environment to make it more favorable
333 for invasive organisms (**Figure 3**). We found a link between the microbial inhibition
334 phenotype and the pH of the culture media, as evidenced by the acidification of the
335 surrounding environment by Lp and neutralization of acidity by At (**Figure 4**). These
336 findings suggest that the composition and invasion resiliency of the microbiome is tightly
337 linked to the biochemical environment created by the resident microbes. We

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338 hypothesize that acidity from the microbiome acts as a chemical barrier and gatekeeper
339 to the establishment of an invader. While this harsh environment eliminates many
340 potential invaders, microbes that overcome this hurdle can become established and
341 eventually displace the resident community. Another interesting finding from our study
342 was that not all acids have the same inhibitory capacities, even when set at an identical
343 pH. For example, when we adjusted culture media to pH 4.0, lactic acid completely
344 inhibited EcN, while hydrochloric acid had little effect on its growth (**Figures 4C, S4**). A
345 similar finding was made with a human isolate of *E. coli* O157:H7[31]. This is likely
346 explained by the ability of the acid to enter the microbial cytoplasm. Strong acids such
347 as HCl ($pK_a$ -5.1) fully dissociate in aqueous environments, and the positively charged
348 $H^+$ ions cannot readily permeate the plasma membrane[32,33]. This contrasts with weak
349 acids such as lactic acid ($pK_a$ 3.86), which do not fully dissociate and can diffuse more
350 easily into the microbial cell where they can dump their protons and acidify the
351 cytoplasm. This causes issues with protein stability, redox balance, and the proton
352 motive force[32] (**Figure S4**). Considering the types of acids present and their abundance,
353 and how different microbiome compositions shape these attributes will be of interest for
354 future study. For example, in our system the amount of lactic acid needed to match the
355 Lp culture pH (~4.0) was 3.5 times higher than the amount of lactate we measured in
356 these cultures, suggesting additional acids are contributing to the final pH and inhibition
357 by Lp (**Figure S4**).
358
359 Establishing common rules for microbial invasion across hosts

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360 Taken together, our results add to the growing body of evidence describing the rules for
361 the control of microbial invasion. The deployment of acids acts as an initial sieve to
362 block the establishment of foreign invaders, with later, more specific interventions by the
363 host immune response and direct competition by the microbiome also acting as
364 barriers[1,6,13,16,34,35]. The finding that Lp protects the host by acidifying the environment
365 parallels interactions observed in the human vaginal microbiome. In particular,
366 *Lactobacillus crispatus* plays an important role in maintaining homeostatic conditions by
367 producing weak organic acids that maintain the pH of the lower reproductive tracts of
368 reproductive-aged women at ~ 4.5[22,36]. If an invasive organism perturbs the vaginal
369 microbial community and neutralizes the acidity (similarly to At in our model), the host
370 becomes more susceptible to opportunistic pathogens such as *Candida albicans*[25,37].
371 This is consistent with our findings and bolsters the utility of the *D. melanogaster* gut as
372 a model, as it serves as a vacuum to study inter-species microbial interactions on
373 epithelial surfaces in granular detail.
374

375 Fly food as a reservoir for the microbiome and its metabolites

376 When considering how the *D. melanogaster* microbiome interacts with external
377 microbes encountered by the host, the microenvironment associated with fly food is an
378 important consideration. Fly food has previously been suggested to behave as a
379 "reservoir" for the microbiome, as frequent transfer of flies to sterile fly food diminishes
380 the microbiome load observed[27]. We previously established that fly microbiome
381 community members alter the nutritional content of fly food, leading to an increased
382 protein-to-carbohydrate ratio in the diet[38,39]. In the present study, we have expanded our

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383 understanding of how the microbiome affects the chemical makeup of fly food by
384 demonstrating strong acidification of the diet by Lp, which is absent when At alone is
385 added to the food. Interestingly, when Lp and At are both added to fly food, the level of
386 acidity is intermediate, suggesting that At quenches some of the acidic compounds
387 produced by Lp, which is unsurprising considering that many *Acetobacter* species utilize
388 lactate as a carbon source[40,41]. This is an important observation because quite often,
389 the fly food environment is overlooked as a contributor to host physiology. We argue
390 that under laboratory conditions, as in wild *D. melanogaster* populations, the food
391 substrate is a critical component of the host-microbe relationship in that it acts as a
392 source and a sink for the microbiome, as well as its metabolites. Moreover, these
393 metabolites and biochemical impacts likely alter food even prior to fly ingestion (**Figure**
394 **5D**). Acidic fly diets were previously shown to increase fly gustatory responses and food
395 intake while also extending lifespan[42]. Another study found that interactions among
396 microbiome members leading to the production of acetic acid protected developing
397 larvae against pathogenic fungi and influenced host behavior[43]. These interactions and
398 their effects on the chemical environment could potentially impact invasive microbes by
399 altering redox states, metabolites, and enzyme kinetics. Inside the host gut
400 environment, the microbiome and its acidic products may play direct roles in modulating
401 immunity, metabolism, and digestion[34,44,45]. Going forward, this will be an important
402 consideration when designing host-microbe studies in *D. melanogaster* as the
403 microbiome's relationship with the fly diet comes further into focus.
404

405 **Materials and Methods**

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406 Fly stocks and rearing

407 Oregon-R flies obtained from the Bloomington *Drosophila* Stock Center (BDSC #5)
408 were initially treated with a final concentration of 0.05% tetracycline in food to eliminate
409 *Wolbachia* and were maintained for at least an additional three generations prior to
410 starting experiments. Stocks were maintained at 25°C with 12 hour light:dark cycling on
411 the Broderick Standard diet[46] containing, per liter: 50 grams inactive dry yeast, 70
412 grams yellow cornmeal, 40 grams sucrose, 6 grams *Drosophila* agar, and 1.25 grams
413 methyl paraben (dissolved first in 5 mL of 100% ethanol). Fly food was autoclaved
414 before use and newly emerged adults were passaged into new tubes every 3-4 days.

415

416 Bacterial culturing methods

417 Unless otherwise specified, bacterial cultures were prepared as follows:

| **Organism** | **Media** | **T˚C** | **Reference** |
|---|---|---|---|
| *Lactiplantibacillus plantarum* (Lp) | MRS | 29 | Broderick et al., 2014 [26] |
| *Acetobacter tropicalis* (At) | MRS | 29 | Judd et al., 2018 [47] |
| *Pseudomonas entomophila* (Pe) | LB | 29 | Vodovar et al., 2005 [14] |
| *Pectobacterium carotovora* (Ecc15) | LB | 29 | Basset et al., 2003 [15] |
| *Escherichia coli Nissle 1917* (EcN) | LB | 37 | Mutaflor ® |

418

419 *Pseudomonas entomophila* (Pe). GFP-expressing *Pseudomonas entomophila* was
420 grown from -80°C on Lysogeny broth (LB- 10g tryptone, 5g yeast extract, 5g NaCl) agar
421 containing 1% milk and incubated at 29°C for two days. Colonies expressing GFP and
422 protease activity (visualized as clearing in milk plates) were grown shaking in liquid LB
423 containing 3% NaCl for 20 hours at 29°C.

424

425 Generation of axenic and gnotobiotic flies

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426 Adult flies were kept in an embryo collection chamber with grape juice agar smeared
427 with yeast paste overnight. Embryos were rinsed with ethanol, dissociated from the
428 grape juice agar using PBS and sterile swabs, and collected in a cell strainer. The
429 chorion was removed using 5% bleach and dechorionated embryos were rinsed with
430 sterile water and ethanol before being transferred via pipette to sterile medium. Vials of
431 food were exposed to ultra-violet light for 15 minutes before use for axenic flies, and all
432 flipping of axenic stocks was done in a biosafety cabinet or near a flame.
433 To generate gnotobiotic flies, *L. plantarum* and *A. tropicalis* were grown, overnight
434 cultures were set to $OD_{600nm}$ = 0.5, and 150 µL of the diluted cultures (or a 1:1 mix of
435 both) were fed to axenic flies. Stocks of gnotobiotes were maintained for at least six
436 generations prior to being used in infection experiments.
437

438 <u>Preparing invasive organisms for oral infection</u>
439 Liquid cultures of Pe, Ecc15, and EcN were centrifuged for 20 minutes at 3700 rpm at
440 4°C, the supernatant was removed, and the pellets were resuspended by light
441 vortexing. Cultures were adjusted using media to $OD_{600nm}$=200 (OD200) for infections.
442 Bacterial pellets (or media for controls) were mixed in a 1:1 ratio with 2.5% sucrose, and
443 140 µL of this infection medium was used to saturate paper filters on top of fly food.
444 Adult female flies (4-7 days old) were starved for 2 hours at 29°C before being
445 transferred to infection tubes and kept at 29°C for the duration of the experiment.
446 Pathogen-independent deaths were recorded at t=2 hours. Experimental vials were
447 passaged at 24 hours, then every two days (for Pe).
448

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449 Bacterial load analysis

450 For *P. entomophila* infection analysis, two sets of flies were infected as described above
451 per replicate (N=3 replicate infections); one set per replicate infection was monitored for
452 survival while individuals were taken from the other set for assessment of bacterial load
453 of Pe, Lp, and At at 24 hours, 72 hours, and 7 days post-infection start. Flies infected
454 with Ecc15 and EcN were sacrificed at 3 hours, 24 hours, and 48 hours post-infection
455 start and assessed for bacterial load. Each infection vial was subjected to $CO_2$
456 anesthetization, and a sub-sample of individuals was removed, surface sterilized in 70%
457 ethanol, rinsed in sterile PBS, and homogenized in sterile screw-top vials containing
458 glass beads and 0.6 mL of PBS. 3-5 flies were taken at each time point from LB control
459 infection tubes, and five flies were taken from invasive organism-infected tubes (or as
460 many living flies were left, up to five). Following homogenization, samples were diluted
461 to $10^{-5}$ and spot-plated as 3 µL spots. Pe-containing samples were plated on LB+milk
462 agar. Ecc15- and EcN-containing samples were plated on LB agar. All samples were
463 also plated on MRS agar to capture Lp and At. Samples containing EcN were also
464 plated on MRS agar supplemented with 50 µg/ml ampicillin. All plates were incubated at
465 29°C. Counts were recorded 1-2 days later.

466

467 Visualization of *in vitro* microbe-microbe interactions

468 *Multi-organism interaction assays.* To visualize the combined effects of Lp-At
469 interactions on the growth of invasive organisms, Lp and At were grown in
470 perpendicular lines on a Petri dish containing mannitol agar for two days. Each invasive
471 organism was streaked in the adjacent quadrants and grown for an additional day.

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472 Plates were photographed, and inhibition of the test organism was measured in
473 millimeters proximal and distal from the point of interaction between Lp and At.
474
475 *In vitro pH approximation.* pH of interactions on plates was approximated by
476 supplementing mannitol agar with 0.02% bromophenol blue, which turns yellow at ~pH
477 3.5. The pH of bacterial cultures was determined using universal pH indicator solution
478 (Sigma).
479
480 *Co-culture assays.* Lp and At were grown in MRS broth for 18 hours, shaking at 200
481 rpm at 29˚C. Both cultures were adjusted to $OD_{600}$=0.2 (OD0.2). These starter cultures
482 were used to inoculate experimental co-cultures by spiking 100 µl of Lp, At, or a 1:1 mix
483 into test tubes containing 5 mL of mannitol broth. Mono-cultures of Pe, Ecc15, and EcN
484 were prepared in 5 mL of LB. Mono-cultures and co-cultures were grown for 18 hours,
485 shaking at 200 rpm at 29˚C. Mono-cultures of invasive organisms were adjusted to
486 OD0.2, and 100 µl of the appropriate organism was spiked into each co-culture. Mono-
487 cultures of the invasive organisms were also prepared in 5 mL of mannitol broth from
488 the OD0.2 starter cultures for comparison. All cultures were grown for an additional 18
489 hours, shaking at 200 rpm at 29˚C. Cultures were serially diluted and plated on both LB
490 agar and MRS agar and grown for 1-2 days at 29˚C.
491
492 *Lactic acid determination.* Lactic acid concentrations of overnight cultures of Lp, At, and
493 LpAt co-cultures were determined using the Lactate Assay Kit II (Sigma) according to
494 the manufacturers' instructions.

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495

496 *Acid co-cultures.* To mimic the lactic acid content of an Lp culture, mannitol media was
497 supplemented with 4 mM lactic acid. To mimic the pH of an Lp culture, mannitol media
498 was titrated with either lactic acid or hydrochloric acid to pH 4.0. *A. tropicalis* was grown
499 for 18 hours in MRS broth, shaking at 200 rpm at 29˚C and adjusted to $OD_{600}$=0.2. Two
500 sets of cultures were set up and grown overnight in test tubes containing 5 mL of acid-
501 supplemented media: one set spiked with 100μl of At and one set lacking At. Pe, Ecc15,
502 and EcN were grown overnight in LB, adjusted to $OD_{600}$=0.2, and 100 μl was spiked into
503 the appropriate acid-supplemented media tube. The co-cultures were grown for an
504 additional 18 hours. Cultures were serially diluted and plated on both LB agar and MRS
505 agar and grown for 1-2 days at 29˚C.

506

507 *Buffered co-cultures.* Mannitol media was supplemented with 60 mM phosphate buffer
508 ($Na(HPO_4)_2$ + $Na_2HPO_4$). The media was titrated with HCl to pH 6.0. Lp was grown
509 overnight and adjusted to $OD_{600}$=0.2. Two sets of Lp cultures were set up and grown
510 overnight in test tubes containing 5 mL of either buffered or non-buffered mannitol
511 media. Pe, Ecc15, and EcN were grown overnight in LB, adjusted to $OD_{600}$=0.2, and
512 100 μl was spiked into the appropriate media tube. The co-cultures were grown for an
513 additional 18 hours. Cultures were then serially diluted and plated to determine bacterial
514 density.

515

516 *Gut pH measurement.* To approximate pH regionality of the gut, axenic and gnotobiotic
517 flies were placed in fly food vials supplemented with 2% bromophenol blue indicator

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518 (Sigma). Flies were allowed to feed for 24 hours before being anesthetized and
519 dissected. Intestines were rapidly photographed as gut contents quickly escape
520 following dissection.
521
522 *Buffered fly food infections.* Fly food was prepared using the standard Broderick Lab
523 recipe (see above) with the liquid portion supplemented with 60 mM 2-(*N*-
524 morpholino)ethanesulfonic acid (MES). The resulting slurry was adjusted to pH 6.0 with
525 NaOH, distributed into vials, and autoclaved. Ecc15 infections were then carried out as
526 described above.
527
528 *Fly food pH measurement*. For culture-inoculated fly food analysis, Lp and At were
529 grown overnight in MRS media and adjusted to $OD_{600}$=0.5. 150 µl of Lp, At, or a 1:1
530 mixture of Lp and At was applied to the surface of fly food and allowed to soak in for 3
531 days at 25˚C. For gnotobiotic fly-inoculated fly food analysis, 40 axenic/gnotobiotic male
532 flies were sorted aseptically, transferred to sterile fly food, and incubated for 3 days at
533 25˚C. Fly food was removed from the vial and weighed, vortexed vigorously with
534 deionized water, and the pH was measured using an Orion Star pH meter
535 (ThermoFisher).
536
537 *Fly food bacterial load determination*
538 Lp and At were grown overnight and adjusted to $OD_{600}$=0.5 with MRS. 150 µl of Lp, At,
539 or an LpAt mix (~$10^7$ cells total) were deposited on the surface of fly food vials, with
540 MRS added as a control. After three days at 25˚C, 150 µl of OD0.5 Pe, Ecc15, or EcN

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541 was added to the appropriate tubes, with LB as a control. 24 hours later, 1 g of fly food
542 was aseptically collected, serially diluted, and plated on LB and MRS agar to determine
543 the density of gut microbes and invasive organisms.
544

545 **Acknowledgements**

546 The authors thank Dr. Arne Schon (Johns Hopkins Department of Biology) for his
547 helpful discussions about the project. Figures were created with GraphPad Prism and
548 BioRender.
549

550 This project was supported by NIH grant R35GM128871.
551

552 The authors have no conflicts of interest to declare.
553

554 A.J.B, D.N.A.L., J.D., and S.C. performed research, and A.J.B., D.N.A.L., and N.A.B.
555 contributed to designing research, analyzing data, and writing the paper.
556

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