Complex third-party effects in the *Dictyostelium-Paraburkholderia* symbiosis: prey bacteria that are eaten, carried, or left behind

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Abstract

Symbiotic interactions may change depending on third parties like predators or prey. Third-party interactions with prey bacteria are central to the symbiosis between *Dictyostelium discoideum* social amoeba hosts and *Paraburkholderia* bacterial symbionts. Symbiosis with inedible *Paraburkholderia* allows host *D. discoideum* to carry prey bacteria through the dispersal stage where hosts aggregate and develop into fruiting bodies that disperse spores. Carrying prey bacteria benefits hosts when prey are scarce but harms hosts when prey bacteria are plentiful, possibly because hosts leave some prey bacteria behind while carrying. Thus, understanding benefits and costs in this symbiosis requires measuring how many prey bacteria are eaten, carried, and left behind by infected hosts. We found that *Paraburkholderia* infection makes hosts leave behind both symbionts and prey bacteria. However, the number of prey bacteria left uneaten was too small to explain why infected hosts produced fewer spores than uninfected hosts. Turning to carried bacteria, we found that hosts carry prey bacteria more often after developing in prey-poor environments than in prey-rich ones. This suggests that carriage is actively modified to ensure hosts have prey in the harshest conditions. Our results show that multifaceted interactions with third parties shape the evolution of symbioses in complex ways.

Introduction

The fitness effects of symbiotic interactions can change depending on the environment [1–5]. One crucial component of the environment that can affect selection on symbiotic interactions is a third species that interacts with hosts or symbionts [6–10]. For example, in the symbiosis between ants and *Acacia* plants, ants benefit *Acacia* by fending off herbivores. However, when herbivores were prevented from accessing *Acacias*, ordinarily mutualistic interactions between ants and *Acacias* shifted towards antagonism because ants no longer provided a benefit to host *Acacias* while still using plant resources [6]. Such shifts can influence whether host and symbiont fitness interests are aligned or in conflict [11,12]. Fitness alignment or conflict then shapes the evolution of these interactions [13,14]. However, the details of how third parties affect the fitness interests of symbiotic partners are not well understood for many kinds of symbioses [2].

An important model of microbial symbiosis is the social amoeba *Dictyostelium discoideum*, that is famous for its complex and well-studied multicellular life cycle [15,16]. *D. discoideum* is a soil amoeba that feeds on many bacteria [17–19]. Upon starvation, amoebae aggregate to form a multicellular fruiting body that disperses spores to new soil patches [15]. Development of the fruiting body progresses through well-studied stages starting with aggregation of individual amoebae which then become motile slugs [20]. Slugs further develop into a fruiting body. During fruiting body development, about 20% of the cells die and become stalk while the remaining cells develop into spores that sit atop the stalk in a structure called a sorus [21]. Spores are dispersed by other organisms [22,23] and germinate into new amoebae if the spores land in locations with prey bacteria to eat.

Ordinarily, *D. discoideum* cannot carry prey bacteria through its social development and must rely on spores being dispersed to soil with already available prey bacteria [17]. Wild clones that were exceptions to this pattern were discovered from soil isolates [24]. These wild clones could carry enough prey bacteria through development to allow multiple rounds of development on agar plates without any additional prey bacteria. The ability of hosts to carry prey was later shown to be due to infection by certain symbiotic *Paraburkholderia* species [25,26]. The mechanism that *Paraburkholderia* uses to allow carriage is currently unknown, but prey bacteria tend to be carried outside spores in the sorus while *Paraburkholderia* symbionts are carried inside spores [26].

Three species of symbiotic *Paraburkholderia* – *P. agricolaris*, *P. hayleyella*, and *P. bonniae* – have been identified that cause carriage with around 25% of wild-collected *D. discoideum* isolates being infected by these *Paraburkholderia* [27]. These Paraburkholderia symbionts are adapted for interacting with amoebae [28–30]. Symbionts move towards host amoebae [29] and make host phagosomes less acidic to evade host digestion [31]. *P. hayleyella* and *P. bonniea* also have reduced genomes relative to *P. agricolaris* and most other *Paraburkholderia* [30,32]. Such genomic reduction is often found in endosymbionts and pathogens that have been associated closely with their hosts during a long evolutionary history [33]. Some *D. discoideum* hosts may also be adapted to their symbionts. Hosts infected by *P. hayleyella* appear to suffer less from *P. hayleyella* toxicity than hosts that are naturally infected by *P. agricolaris* [28].

69 The fitness consequences of the symbiosis between *D. discoideum* and
70 *Paraburkholderia* depend crucially on the presence or absence of prey bacteria. When
71 host spores disperse to locations where prey bacteria are rare (prey-poor), they benefit
72 from *Paraburkholderia* infection because they are able to seed new prey bacteria
73 populations [4,24,25]. The ability of infected hosts to bring prey along contrasts sharply
74 with uninfected hosts that are unable to grow when food is scarce. On the other hand,
75 when hosts disperse to conditions with ample prey (prey-rich), infected hosts do not gain
76 from carrying prey bacteria and instead experience a cost. In this case, infected hosts
77 produce fewer spores relative to uninfected hosts [24,25]. Higher *Paraburkholderia*
78 densities in secondarily colonized locations impose higher costs on hosts but again this is
79 affected by third-party prey, with high *Paraburkholderia* densities being most harmful in
80 prey-rich conditions [4].
81 The third-party bacteria can also affect *Paraburkholderia* fitness due to
82 competition over resources [34]. Paraburkholderia symbionts may be freed from this
83 competition when they are carried to locations with fewer prey bacteria by the *D.*
84 *discoideum* host [4]. Thus prey-poor conditions for the host are low-competition
85 conditions for the symbiont (though for consistency, we will stick to calling them prey-
86 poor and prey-rich conditions rather than low-competition and high-competition
87 conditions).
88 While the importance of prey bacteria has been well established for this symbiosis
89 [4,25,26], there are still many questions about the effects of prey bacteria during
90 development of *D. discoideum*. In this study, it is the use of prey bacteria during host
91 development that is of interest – should they be eaten, carried, or left behind?

92 Leaving prey bacteria behind would not seem to be adaptive, but this appears to
93 happen when hosts are infected by *Paraburkholderia* [24,35]. Initially, before the
94 distinct roles of inedible symbionts and edible prey were appreciated, bacteria left
95 behind on plates was taken as a possible sign of "prudent predation" by host amoebae —
96 if some bacteria were going to be saved for carriage, hosts cannot eat all the available
97 prey and may stop feeding and start developing earlier [24]. It was hypothesized that
98 hosts leaving prey bacteria uneaten might explain why carriage is costly in some
99 conditions [24]. Hosts that were prudent predators left prey uneaten and missed out on
100 potential growth and proliferation. As a result of this missed opportunity, we expect hosts
101 that leave prey behind to starve and develop faster and produce fewer spores than those
102 that eat all available prey.
103 While infected hosts may leave some prey bacteria uneaten, they also gain the
104 ability to carry prey bacteria along with the dispersing host spores in sori [24–26]. The
105 number of carried prey bacteria has not been explored in the context of the prudent
106 predation hypothesis. It is unknown whether the number of carried prey bacteria changes
107 in different environments or if carriage is active or passive. If carriage is passive, we
108 expect hosts to carry more prey after developing in a prey-rich environment and fewer
109 prey bacteria after developing in a prey-poor environment.
110 Alternatively, the ability to carry prey bacteria could reflect the evolutionary
111 interests of hosts and symbionts. Since soil environments tend to be spatially and
112 temporally structured [36,37], developing in a prey-poor environment may be associated
113 with an increased probability that the next environment will also tend to be prey-poor. If
114 this is the case, it would be more adaptive for hosts if they carried more prey bacteria

115 after developing in a prey-poor environment than in a prey-rich environment. Of course,
116 hosts must not eat all their prey to have excess prey to carry. Host carrying more prey in
117 prey-poor conditions may also be adaptive for the *Paraburkholderia* symbionts since it
118 allows better survival of their hosts.

119 We investigate three questions about the complex role of prey bacteria in the
120 symbiosis between *D. discoideum* and two commonly studied *Paraburkholderia*
121 symbionts, *P. agricolaris* and *P. hayleyella* [4,28,34,38]. We first re-evaluate the main
122 observation behind the prudent predation hypothesis that infected hosts do leave behind
123 prey bacteria. Second, we ask whether leaving prey bacteria uneaten results in faster
124 development or reduced spore production because of hosts starving faster than if they
125 were to consume all available prey bacteria. Lastly, we turn to whether the number of
126 prey bacteria carried inside sori changes after hosts develop in prey-poor and prey-rich
127 environments.

128

129 **Methods**

130

131 *Clones and Culturing Methods*

132 In our experiments, we used several types of infected and uninfected hosts (Table
133 1). We used two types of infected host, naturally infected hosts (n=7 clones for each
134 symbiont species) and, to better control for infection levels, cured-and-reinfected hosts
135 (n=4 clones for each symbiont species). The naturally infected hosts were compared to
136 naturally uninfected controls (n=6 clones) and the cured-and-reinfected host were
137 compared to a different set of naturally uninfected controls (n=4 clones). To better

138 control for genetics, cured-and-reinfected hosts were also compared to their cured
139 clonemates (n=4 clones for each symbiont species). To generate cured-and-reinfected
140 clones, naturally infected clones were cleared of their natural infections with 30 μg/mL
141 tetracycline and then reinfected at 1:1000 ratios of *Paraburkholderia* to prey bacteria to
142 control infection densities [4]. Data reported here for cured-and-reinfected hosts and their
143 uninfected controls come from experiments performed in Scott et al. [4] but here we
144 focus on additional measures of prey bacteria not included in the original study. Naturally
145 uninfected controls in this comparison were also treated with antibiotics to control for
146 antibiotic treatment. We stored all prepared clones in a -80 C freezer for repeated use.
147 To remove any effects of being in the freezer and to ensure that infected amoebae
148 carried *K. pneumoniae* prey bacteria, we grew amoebae through one round of feeding and
149 fruiting body formation and then collected spores to initiate our experiments. This step
150 may allow infection densities time to equilibrate [39]. Amoebae were grown from frozen
151 spores at room temperature on 100 x 15mm plates filled with 30mL SM/5 agar (2 g
152 glucose (Fisher Scientific), 2 g Bacto Peptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g
153 $MgSO_4$ * $7H_2O$ (Fisher Scientific), 1.9 g KH_2PO_4 (Sigma-Aldrich), 1 g K_2HPO_4
154 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter). Plates were seeded with
155 200 μL of 1.5 optical density measured at 600 nm (OD_{600}) fluorescently labeled *K.*
156 *pneumoniae* suspended in KK2 buffer (2.25 g KH_2PO_4 (Sigma-Aldrich) and 0.67 g
157 K_2HPO_4 (Fisher Scientific) per liter) spread over the entirety of the agar. *K. pneumoniae*
158 used in this study expressed green-fluorescent protein (GFP) and were provided by
159 dictyBase (Fey et al. 2019). OD_{600} for plating bacteria was measured with a
160 BioPhotometer (Eppendorf, New York).

161

162 *Dispersal experiment setup*

163 To determine the consequences of hosts leaving behind bacteria, we performed a
164 dispersal experiment to seed new plates with sorus contents. To do this, we transferred
165 200 μL of $2x10^5$ spores/mL suspension obtained from the equilibration step to plates with
166 or without 200 μL of 1.5 OD_{600} GFP-expressing *K. pneumonia*e. Spore counts to make
167 spore suspensions were obtained using a hemocytometer. We let bacteria and amoebae on
168 plates proliferate at room temperature for six days, enough time for amoebae to form
169 fruiting bodies.

170 We replicated these dispersal experiments with cured-and-reinfected hosts along
171 with uninfected controls on two separate dates. To understand if our results held with
172 natural infections, we performed an additional dispersal experiment with naturally
173 infected clones and uninfected controls. To rule out that leaving uneaten prey bacteria
174 was a host trait, we also performed a separate set of dispersal experiments comparing
175 cured clones to their reinfected counterparts.

176 After dispersed amoebae and bacteria were allowed to proliferate for six days (to
177 allow hosts to complete development into fruiting bodies), we measured the density of
178 uneaten prey bacteria, *Paraburkholderia*, and host spore production. To measure the
179 density of uneaten prey bacteria, we first collected host spores and bacteria from after the
180 dispersal step. We collected host spores and bacteria by washing plates with 15 mL of
181 KK2 buffer after six days of growth. We counted host spores from this washed solution
182 using a hemocytometer. To measure bacterial density, we first removed host spores by
183 centrifuging the wash solution for three minutes at 360 rcf and reserving the supernatant.

184 We measured the optical density of the supernatant at 600 nm (OD_{600}) as well as
185 fluorescence with an excitation wavelength of 485 and emission wavelength of 515 nm in
186 a 96 well plate with a Tecan Infinite 200 Pro microplate reader. Removing host spores by
187 manually removing sori with a pipette tip resulted in similar densities of uneaten prey
188 bacteria consistent with earlier findings [26] that the number of prey bacteria inside
189 spores and sori is minimal relative to that left on the plate (data not shown).
190 To calculate the density of Paraburkholderia and prey bacteria in mixed solution,
191 we used the standard curve method in Scott et al. [4]. Since the supernatant from infected
192 clones contains GFP-expressing *K. pneumoniae* and unlabeled *Paraburkholderia*, the
193 total OD_{600} is due to both kinds of bacteria but fluorescence comes only from *K.*
194 *pneumoniae*. To calculate the amount of OD_{600} due to fluorescing *K. pneumoniae*, we
195 generated a standard curve relating fluorescence to OD_{600} using serial dilutions of GFP-
196 expressing *K. pneumoniae* in KK2. This standard curve predicted the OD_{600} of *K.*
197 *pneumoniae* in our samples. After subtracting the OD_{600} due to *K. pnuemoniae*, the
198 remaining OD_{600} is the amount due to *Paraburkholderia* symbionts. An OD_{600} of 0.1
199 translates to around 5×10^7 *K. pneumoniae* cells or 1×10^8 *Paraburkholderia* cells
200 according to the validation dataset in Scott et al. [4].
201
202 *Development assays for cured and reinfected hosts*
203 To determine how symbionts and their interactions with prey affected host
204 development time, we tracked host development by taking time-lapse images of cured
205 hosts and reinfected counterparts developing in six-well plates filled with 10 mL SM/5.
206 Each 6-well plate contained three wells with cured hosts and three wells with reinfected

hosts of either *P. hayleyella* clones (QS23, QS38, and QS45) or *P. agricolaris* clones (NC21, QS159, and QS161). We repeated development experiments on six-well plates three times for each *Paraburkholderia* species. We first grew clones from the freezer on SM/5 plates for six days in case there were freezer effects, as above. We then collected host spores and plated 30 μL of $2x10^5$ spores per mL and 30 μL of 1.5 OD_{600} *K. pneumoniae* in each well. Photos were taken every hour until fruiting bodies developed using a Canon EOS Mark IV. We inspected photos to determine time points for when aggregates, slugs, and fruiting bodies first appeared in each well.

Measurement of carried bacteria

To determine how prey context affected the number of prey bacteria carried in sori, we used cured-and-reinfected hosts that were grown on prey-poor and prey-rich plates after the dispersal step. Prey-rich plates were those from the dispersal experiment plated with 200 μL 1.5 OD_{600} fluorescently labeled *K. pneumoniae*. Prey-poor plates were plated with 200 μL KK2 without bacteria as a control. Prey-poor plates only harbor bacteria if they are carried in the sorus solution used in the dispersal experiments [4] We haphazardly sampled a single sorus from each of our experimental plates from reinfection experiments (replicated on two different dates resulting in 8 observations of 4 different clones for each species and condition). To count the number of carried prey, we suspended single-sorus contents in KK2 buffer and plated out serial dilutions from no dilution to 1:1000 dilutions. Since *K. pneumoniae* were labeled with GFP, we could differentiate colonies of *K. pneumoniae* and *Paraburkholderi*a. We counted colonies from the dilution plate that appeared closest to having 100 colonies and then back-

230 calculated to get the total count for a single sorus. To ensure that our results were robust
231 to sampling more fruiting bodies, we performed an additional experiment where we
232 sampled five fruiting bodies instead of one. For these additional experiments, we used
233 eight reinfected clones for each species (Table S1). We performed these additional
234 experiments in blocks of four clones with both prey-rich and prey-poor conditions
235 included in each block resulting in a total of 32 observations. Each block experiment was
236 performed on a separate day.
237
238 *Statistical Methods*
239 To understand how *Paraburkholderia* infection affected the density of *K.*
240 *pneumoniae* bacteria left uneaten, we fit linear models in R (version 3.6.3). Because
241 experiments with cured-and-reinfected (and uninfected control) hosts were performed on
242 two different dates, we used linear mixed models (LMM) with date as a random effect.
243 We fit LMMs using the nlme package [40] in R. For other comparisons that involved
244 only single measures, we used linear models fit by ordinary linear regression (LM) with
245 the lm function in R. We performed Tukey post-hoc tests for pairwise comparisons using
246 the *emmeans* package [41].
247 To determine whether the timing of development was affected by
248 *Paraburkholderia* infection, we used generalized linear mixed models (GLMMs) with
249 Poisson errors. We included the date that experiments were performed as a random
250 effect to capture variation within plates.
251 To test how prudent predation affects host spore production relative to other
252 explanations, we again used LMMs and LMs for cured-and-reinfected and naturally

253 infected infections, respectively, along with respective uninfected controls. If prudent
254 predation reduces host spore production, we expected a decrease in spore production with
255 increasing uneaten prey bacteria. Differences in host fitness could also be affected by the
256 density of *Paraburkholderia* left on plates [4] or by infection category (uninfected vs
257 infected) [24,25,27]. To account for these possibilities, we also fit models with these
258 variables along with a model that includes both uneaten prey density and
259 *Paraburkholderia* density. In total, we compared five models of spore production: (1)
260 uneaten prey density (Prey model), (2) *Paraburkholderia* density (Para model), (3)
261 uneaten prey density + *Paraburkholderia* density (Prey + Para model), (4) categorical
262 infection status (Infection model), and (5) a null model fit with only the intercept for each
263 *Paraburkholderia* species.
264 To compare effects more easily across the different models, we scaled all
265 variables by subtracting the mean and dividing by twice the standard deviation. This
266 scaling procedure is useful for comparing models with multiple continuous predictors
267 (density of prey bacteria and symbionts) and with categorical factors (infected and
268 uninfected) because all measures end up on roughly the same scale [42].
269 To compare the fit of the five models described above, we calculated AICc for
270 each model. AICc is a measure of model fit that accounts for model complexity and that
271 corrects for small sample sizes [43]. Models that fit the data best have lower AICc values.
272 For our model selection analysis we show results with 85% confidence intervals instead
273 of the standard 95% confidence intervals because 85% confidence intervals are more
274 consistent with model selection with AIC [44]. To measure potential collinearity in our
275 models that included prey and *Paraburkholderia* density, we calculated variance inflation

factors (VIF) using the check_collinearity function in the performance package [45]. VIFs lower than 5 indicate low collinearity, values between 5 and 10 indicate moderate collinearity, and values above 10 indicate high collinearity.

To compare the ability of hosts to carry *K. pneumoniae* inside fruiting bodies in prey-rich and prey-poor environments, we used a zero-inflated negative binomial model (ZINB) fit with the glmTMB package [46] in R. This zero-inflated model incorporated two processes: (1) zero-inflation for whether fruiting bodies contained or did not contain prey bacteria and (2) negative binomial for the counts of carried prey bacteria. Using this ZINB, we compared different hypotheses about how prey-rich and prey-poor environments affected the ability to carry and the number carried with AICc. We fit models to carriage data from both species and asked whether models including prey environment, *Paraburkholderia* species, or the interaction between prey environment and species had low AICc values. We did this model selection for both the zero-inflation part of the model and for the negative binomial part of the model. We also compared models with the random effects of clone and date included in either the zero-inflation or negative binomial parts of the model. For our additional experiment with five sampled fruiting bodies, we selected models as above but instead of random effects, we included the four-clone blocks as fixed effects to account for variation across blocks.

Results:

D. discoideum hosts infected with Paraburkholderia leave some prey bacteria uneaten

We investigated the prey bacteria left behind after hosts formed fruiting bodies by measuring the density of leftover *K. pneumoniae* from cured-and-reinfected hosts

299 compared to naturally uninfected controls. These prey densities are estimated from
300 fluorescence measurements since the *K. pneumoniae* used in this study expresses green
301 fluorescent protein (GFP; see methods). First, we wanted to confirm that some of the left-
302 behind bacteria are prey bacteria and not just *Paraburkholderia* symbionts. Leaving prey
303 bacteria uneaten so that hosts miss out on potential growth and proliferation was a key
304 component of the prudent predation hypothesis, but previous studies did not differentiate
305 what kind of bacteria were left behind (Brock et al. 2011, 2016*b*).
306 Cured-and-reinfected hosts with *P. agricolaris* left 4.4 times as much prey
307 bacteria as naturally uninfected hosts (LMM; estimate = 0.009, se = 0.003, df = 20, P <
308 0.005; Figure 1A). Cured-and-reinfected hosts that were infected with *P. hayleyella* left
309 7.4 times as much prey bacteria than naturally uninfected hosts (Figure 1A; LMM;
310 estimate = 0.017, se = 0.003, df = 20, P < 0.001).
311 This comparison supports the view that symbionts cause prey bacteria to be left
312 uneaten. To control for host clone genotypes, we next compared cured-and-reinfected
313 hosts to the same host genotypes that were cured of their native *Paraburkholderia*
314 symbionts but not reinfected. Cured-and-reinfected hosts left more prey bacteria than
315 their cured counterparts (*P. agricolaris*: LM; estimate = 0.004, se = 0.002, df = 6, P =
316 0.041, *P. hayleyella*: LM; estimate = 0.004, se = 0.001, df = 6, P = 0.019; Figure 1B).
317
318 *Host development time is not affected by Paraburkholderia infection*
319 We hypothesized that leaving prey bacteria uneaten would result in faster
320 starvation and faster development by infected hosts. To test this, we took time-lapse
321 photos of amoebae aggregating into fruiting bodies, comparing cured-and-reinfected

322 hosts to the same host genotypes that were cured of their *Paraburkholderia* symbionts.
323 We determined time courses for when hosts aggregated, formed slugs, and formed
324 fruiting bodies. We were unable to detect that development times were slower for cured-
325 and-reinfected hosts relative to cured hosts for *P. agricolaris* (GLMM; estimate across all
326 developmental timepoints = 0.015, se = 0.037, P = 0.682) or *P. hayleyella* (GLMM;
327 estimate across all developmental timepoints = 0.044, se = 0.037, P = 0.231; Figure
328 2A&B). Individual developmental stages also did not differ due to *Paraburkholderia*
329 infection (P > 0.05).
330
331 *Prey bacteria left on plates are not associated with reduced spore production in hosts*
332 Leaving more prey uneaten is suspected to lower host fitness because hosts are
333 leaving food on the table. This cost due to uneaten prey could explain the cost of
334 *Paraburkholderia* infection relative to uninfected hosts when in prey-rich environments
335 [24]. Alternately, the cost of infection may be more directly due to *Paraburkholderia*,
336 either infection itself or the density of *Paraburkholderia* symbionts. In addition to these
337 first three models (food bacteria density, *Paraburkholderia* infection status, and
338 *Paraburkholderia* density), we also fit a model that included both uneaten prey bacteria
339 density and *Paraburkholderia* density. To compare the relative support for these different
340 hypotheses, we fit a model for each hypothesis (see Methods) and compared fitted
341 models using AICc.
342 We first tested how host spore production is affected by uneaten prey bacteria and
343 *Paraburkholderia* left on plates using our cured-and-reinfected hosts and naturally
344 uninfected controls (Table 1). For infections with either species of *Paraburkholderia*,

345 null models fit to only the intercept fit the data best (Figure 3A & S1; the model that
346 includes uneaten prey bacteria and *Paraburkholderia* density for *P. agricolaris* cured-
347 and-reinfected hosts — Prey + Para — did show confidence intervals that did not overlap
348 zero, but this was the worst model in terms of AICc). We thus found no evidence that
349 leaving prey explained the cost of infection for cured-and-reinfected hosts relative to
350 uninfected controls.

351 Naturally infected hosts and their uninfected controls also showed no support for
352 a cost due to leaving prey bacteria behind. Models of host spore production that included
353 uneaten prey (Prey model in Figure 3B) were poor predictors of host spore production.
354 This poor performance of prey as a predictor was also true when *Paraburkholderia* was
355 included as a covariate (Prey + Para model). This lack of fit for the *P. hayleyella* Para +
356 Prey model may be due to correlations between *Paraburkholderia* density and uneaten
357 food bacteria (VIF = 7.62).

358 These results show no support for a cost due to leaving prey bacteria behind (even
359 when accounting for *Paraburkholderia* density). We next turned to the direct role of
360 *Paraburkholderia*. Because models fit to only the intercept were the best for cured-and-
361 reinfected hosts relative to their controls, there was no evidence that *Paraburkholderia*
362 was costly in these clones, the same result as previously shown [4]. In contrast, naturally
363 infected hosts were afflicted by a cost of *Paraburkholderia* infection (Figure 3B & S2).
364 The best models for both *P. agricolaris* and *P. hayleyella* showed that infected hosts had
365 lower spore production than uninfected hosts (*P. agricolaris*: LM; estimate = -0.508, se =
366 0.247, df = 11; *P. hayleyella*: LM; estimate = -0.804, se = 0.160, df = 11).
367 *Paraburkholderia* density predicted host spore production less well than infection status.

368 However, models that included *Paraburkholderia* density on its own were better than
369 models with prey bacteria density (Figure 3B & S2). These models thus show little
370 evidence for a cost of uneaten food bacteria and instead show that *Paraburkholderia*
371 infection is responsible for reduced host spore production in naturally infected hosts.
372
373 *Uneaten prey bacteria are a minority of left-behind bacteria*
374 Prey bacteria left behind did not have the predicted effects on either development
375 time or spore production costs. One reason may be that the amount of left behind prey is
376 not as great as formerly thought [24]. Hosts may leave too few prey bacteria to noticeably
377 affect spore production. In fact, uneaten prey bacteria make up a minority of left-behind
378 bacteria. Uneaten prey bacteria are about 9% and 14% of bacteria on plates for *P.*
379 *agricolaris* and *P. hayleyella* cured-and-reinfected hosts, respectively, and 21% and 19%
380 for *P. agricolaris* and *P. hayleyella* naturally infected hosts, respectively (Figure 4).
381
382 *More prey bacteria are carried after hosts develop in prey poor conditions*
383 To determine whether the number of prey bacteria carried in sori simply reflects
384 the number of prey bacteria in the previous environment or is modified according to the
385 interests of hosts or symbionts, we measured the number of fluorescent prey bacteria
386 inside sori after growth of cured-and-reinfected hosts on prey-rich and prey-poor plates
387 (Figure 5A). We found that for both *Paraburkholderia* species (Figure 5B&C), sori
388 produced in prey-rich environments were less likely to contain prey bacteria than those in
389 prey-poor environments (ZINB, zero-inflation estimate = 1.950, se = 0.987). We did not
390 detect a difference in the number of prey bacteria that were caried between prey-rich and

391 prey-poor environments (Figure 5D&E). However, using measurements from a single
392 sorus did not result in consistent carriage for some clones (Figure S3). This inconsistency
393 suggests that these estimates may be unreliable. To avoid this issue with single-sorus
394 measures, we repeated the experiment while sampling five fruiting bodies from each
395 plate and found a strikingly similar increase in the likelihood of carrying prey after host
396 developed in prey-poor conditions (ZINB, zero-inflation estimate = 1.710, se = 0.921;
397 Figure 6). This shows that the ability to carry prey bacteria is affected by prey context,
398 but in the opposite direction to that expected if prey was carried according to the density
399 of prey bacteria in the environment. Thus, the ability to carry prey bacteria may depend
400 on the fitness interests of hosts and symbionts that benefit from carrying prey bacteria
401 when harsh conditions are expected.
402
403 **Discussion**
404 Third parties that interact with symbiotic partners commonly affect the outcome
405 of symbiotic interactions [2,6,9,10] and alter selection for cooperation and conflict
406 between symbiotic partners [8,11]. Often the details of how third parties affect fitness
407 effects are unknown. In the symbiosis between *D. discoideum* social amoebae and
408 *Paraburkholderia* bacteria, the third-party prey bacteria are normally eaten by the host
409 but can also be carried or left behind. We studied how many prey bacteria are carried and
410 left behind, and their impacts, by tracking fluorescently labeled prey bacteria (*K.*
411 *pneumoniae*) during fruiting body formation by hosts.
412 Our most surprising finding was that infected hosts were more likely to carry prey
413 bacteria after growing in prey-poor environments (Figure 5 & 6). This result is surprising

414 because it means that hosts or symbionts actively change the tendency to carry prey
415 bacteria depending on environmental conditions. This result provides possible evidence
416 for mutual benefit between *D. discoideum* hosts and *Paraburkholderia* symbionts. Hosts
417 and symbionts are likely to benefit from modifying carriage in this way if future soil
418 environments tend to resemble past soil environments. Repeated prey-poor environments
419 are where hosts should most benefit from carrying and seeding out prey bacteria.
420 *Paraburkholderia* symbionts could also benefit from carriage of prey because keeping
421 hosts alive means there are hosts to further disperse *Paraburkholderia*. However, this
422 dispersal benefit to the symbionts must be balanced against the costs of increased
423 competition with the prey bacteria.

424 We speculate that carrying more prey in prey-poor contexts may represent
425 cooperation between hosts and symbionts that allows the symbiosis to persist over
426 repeated harsh environments. Such harsh conditions are potentially an important force
427 shaping cooperation in this [4,47] and other symbioses [48–50]. These studies in
428 symbiotic systems complement research on the role of harsh environments in the
429 evolution of cooperation that has been focused on interactions between members of the
430 same species [51], most commonly in cooperatively breeding birds [52–54].

431 Although seeding out prey bacteria has been shown to be important when we move
432 spores to a prey-poor environment in the lab, we have little idea how frequently this
433 occurs in nature. The finding that carriage of prey bacteria changes in an apparently
434 adaptive fashion suggests that it is indeed important in the field. The importance of third-
435 parties in this case may be an interesting exception to the relatively small effects of third
436 parties on fitness that were observed in a meta-analysis of other studies [2].

437 It is also interesting that the tendency to carry prey bacteria changed in prey-poor
438 and prey-rich conditions, but that when hosts carried prey bacteria, sori contained similar
439 numbers of prey bacteria across both conditions (Figure 5 & 6). The consistent number of
440 carried prey bacteria suggests that when prey are present, individual sori may be able to
441 support only a specific number of prey. The sorus consists of spores and a secreted matrix
442 of mucopolysaccharides and cellulose [15]. Prey bacteria may be able to use only some
443 of the available matrix material for their own growth and thus have limited populations in
444 sori.

445 Questions remain about how hosts and symbionts affect the carriage of prey
446 bacteria. *Paraburkholderia* are more often carried inside spores while prey bacteria are
447 more often carried outside spores [26]. Differences in carriage of bacteria could
448 potentially result from differences in lectins [55] or polyphosphates [56] that affect the
449 ability of bacteria to invade and survive inside hosts. Hosts in the absence of their
450 *Paraburkholderia* symbionts are able to modify the contents of fruiting bodies through
451 their immune cells that protect against toxins and bacteria by collecting potential threats
452 and dropping off during the slug stage [57]. However, the role of these immune cells in
453 the symbiosis [58,59] needs to be further explored. Manipulation of phagosomes could
454 also play a role in determining the contents of fruiting bodies. *Paraburkholderia*
455 symbionts increase the pH of phagosomes, presumably to prevent host digestion of
456 symbionts [31]. Similar modification of lysosomes is used by pathogens to evade human
457 immune cells during infection [60,61]. More work is needed to understand how
458 symbionts and food bacteria get into fruiting bodies and how both hosts and symbionts
459 contribute to bacterial carriage.

460 We also found that *Paraburkholderia* infection prevents hosts from eating all the
461 prey bacteria in an environment (Figure 1). A prior study suggested that leaving prey
462 bacteria uneaten because of prudent predation may explain the cost of infection relative
463 to uninfected hosts when in food-rich conditions [24]. Instead, we found that the quantity
464 of left-behind prey bacteria may be too small to noticeably affect host spore production
465 since we observed that only a minority of left-behind bacteria was food bacteria, with the
466 majority being indigestible *Paraburkholderia* (Figure 4).
467 To get a better sense of how important left-behind prey bacteria could be for
468 hosts, we can use the density vs bacteria count curve in Figure S1B of Scott et al. 2022
469 and the average left-behind prey bacteria in this study to estimate the number of
470 individual left-behind prey bacteria. Using this estimation, *P. agricolaris* infected hosts
471 left 7.060×10^6 individual prey bacteria and *P. hayleyella* infected hosts left 1.206×10^7
472 individual prey bacteria. Using Kessin's [15] rough estimate that an amoebae needs to eat
473 1,000 bacteria to divide, we calculated that the number of uneaten prey bacteria is only
474 enough to produce 0.002% and 0.004% more spores than what we recovered from plates
475 (collected spores were around 3×10^8) for *P. agricolaris* or *P. hayleyella* infected hosts,
476 respectively. These percentages are a rough and conservative approximation as they
477 assume that amoebae devote all consumed prey bacteria to proliferation. However, the
478 calculations show that it is unlikely that uneaten prey bacteria can affect host fitness in a
479 detectable manner.
480 Instead of hosts paying a cost in potential growth because they leave prey bacteria
481 uneaten, we suspect that *Paraburkholderia* infection causes both observations: that hosts
482 leave prey uneaten and that infected hosts pay a cost. Tentative support for this idea

483 comes from our findings that *Paraburkholderia* and uneaten prey bacteria densities were
484 correlated. This correlation could result from higher *Paraburkholderia* densities
485 interfering with the ability of hosts to eat prey bacteria. Additional support for
486 *Paraburkholderia* symbiont densities lowering host fitness comes from a recent study on
487 *P. bonniea* [62].

488 An interesting remaining question in the *D. discoideum-Paraburkholderia*
489 symbiosis and in other symbioses is the mechanisms that result in reduced fitness when
490 hosts are infected. One potential explanation is that *Paraburkholderia* symbionts directly
491 feed on host cells or otherwise extract nutrients from hosts. Measures of
492 *Paraburkholderia* density inside sori have so far not been found to be correlated with
493 host fitness within species [39], though the more toxic *P. hayleyella* does appear to infect
494 a higher percentage of spores than less toxic *P. agricolaris* [26,28]. Another promising
495 hypothesis that deserves further study is that hosts have lower spore production because
496 of "collateral damage" from competitive interactions between *Paraburkholderia* and prey
497 bacteria [4]. Competition between bacteria is often mediated by chemical warfare [63]
498 that could reduce host *D. discoideum* fitness as a side-effect.

499 The role of *Paraburkholderia* in reducing host spore production is more evidence
500 in support of there being some evolutionary conflict in this symbiosis [4,25]. However,
501 the magnitude of conflict may differ across *Paraburkholderia* symbiont species. This
502 conflict may be more pronounced between *P. hayleyella* and its hosts than between *P.*
503 *agricolaris* and its hosts. We found that *P. hayleyella* density could explain some of the
504 decrease in naturally infected host spore production but we did not find the same for *P.*
505 *agricolaris* density (Figure 3). This difference between *P. agricolaris* and *P. hayleyella* is

consistent with prior studies that found that *P. hayleyella* is more harmful than *P. agricolaris* [26–28]. In addition to being more harmful, *P. hayleyella* also has a reduced genome relative to *P. agricolaris* [30,32]. Reduced genomes are a common result of persistent host association in beneficial symbionts and pathogens [33]. While *P. hayleyella* has maintained its ability to harm hosts over this persistent association, it is important to remember that infection also comes with the ability to carry prey bacteria [25,26]. Thus, this symbiosis involves a balance between harm and benefits to hosts.

Whether a specific symbiosis involves fitness alignment or conflict may depend on a third party that affects the costs and benefits of symbiosis. Our results show that third parties can have complex effects on conflict and fitness alignment in symbioses; the symbiosis between *D. discoideum* and *Paraburkholderia* appears to involve elements of conflict and cooperation that depends on how third-party bacteria are eaten, carried, and left behind. Symbiosis may be worth the cost for *D. discoideum* hosts because they are better off with symbionts when prey are scarce. Third-party effects in other symbiotic interactions may similarly favor cooperation when times are tough.

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712 **Figure and Table Legends**

713 Table 1: Clones used in experiments.

Infection Type	Description	*Paraburkholderia* Treatment	Clones
Cured-and-reinfected	Clones that were cured of any native symbionts with tetracycline and reinfected in the lab with 0.1% of their native symbiont	*P. agricolaris* infected	QS159, QS161, QS606, NC21
		P. hayleyella infected	QS395, QS45, QS38, QS23
Naturally uninfected controls (for comparison with cured-and-reinfected)	Control clones that were treated with tetracycline.	Naturally uninfected control	QS6, QS138, QS472, QS527

Cured (for same-clone comparison with cured-and-reinfected)	Clones that were cured of any native symbionts with tetracycline, but not reinfected.	Cured of *P. agricolaris* infection	QS159, QS161, QS606, NC21
		Cured of *P. hayleyella* infection	QS395, QS45, QS38, QS23
Naturally infected hosts	Clones with natural infections that have not been treated with antibiotics.	*P. agricolaris* infected	QS494, QS756, QS788, QS113, QS453, QS70, QS606
		P. hayleyella infected	QS45, QS101, QS46, QS2, QS23, QS529, QS38
Naturally uninfected controls (for comparison with naturally infected)	Clones without natural infections that have not been treated with antibiotics.	Naturally uninfected control	QS4, QS6, QS14, QS18, QS9, QS8

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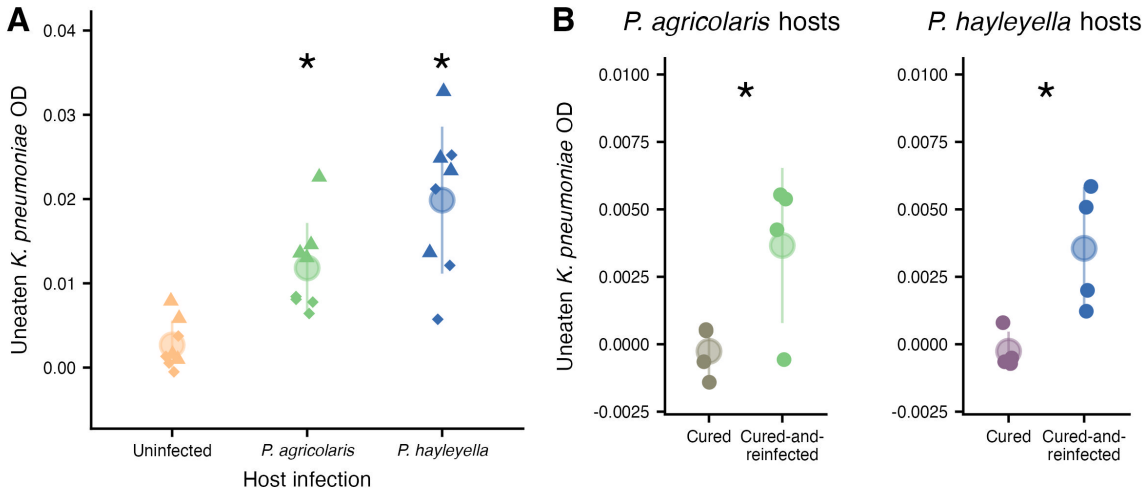
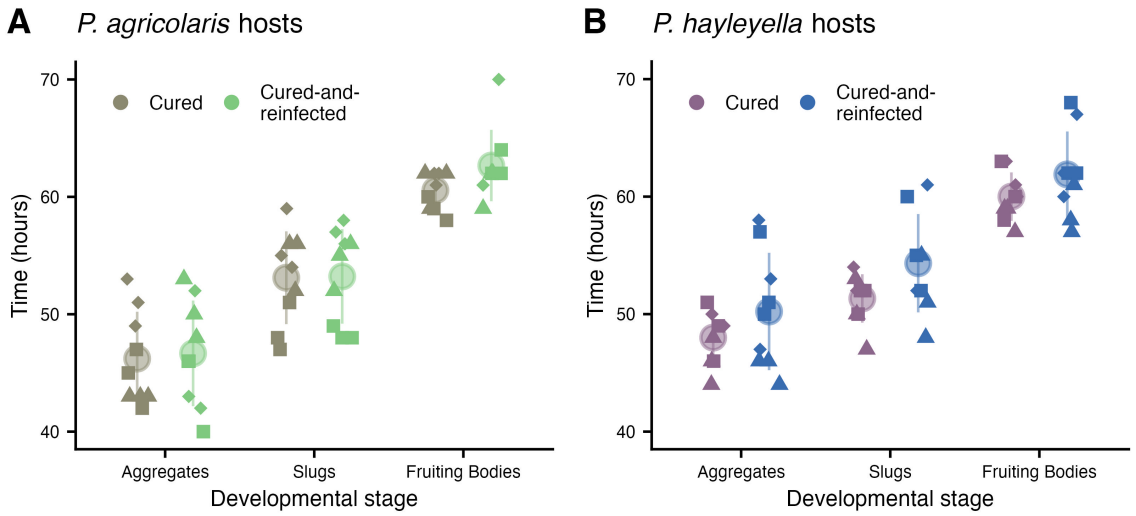


Figure 1: *Paraburkholderia* symbionts cause hosts to leave *K. pneumoniae* prey uneaten. (A) Density left on plate of uneaten *K. pneumoniae* prey bacteria (measured by OD_{600} after subtracting out OD_{600} from *Paraburkholderia*) for naturally uninfected hosts, *P. agricolaris* hosts that have been cured and then reinfected, and *P. hayleyella* hosts that have been cured and then reinfected. (B) Density left on plate of uneaten prey bacteria for cured *P. agricolaris* and *P. hayleyella* hosts compared to their cured-and-reinfected counterparts. Dot and lines show mean and standard deviation, respectively. Asterisks indicate significant differences (in panel A comparisons are between infected and uninfected). Point shapes of small points indicate different dates on which experiments were replicated.



729

730 **Figure 2**: *Paraburkholderia* symbionts do not affect developmental time. Developmental

731 time points at different stages of development for cured hosts and their counterparts that

732 have been cured and then reinfected with (A) *P. agricolaris* and (B) *P. hayleyella* hosts.

733 Dot and lines show mean and standard deviation, respectively. Point shapes of small

734 points indicate different dates on which experiments were replicated.

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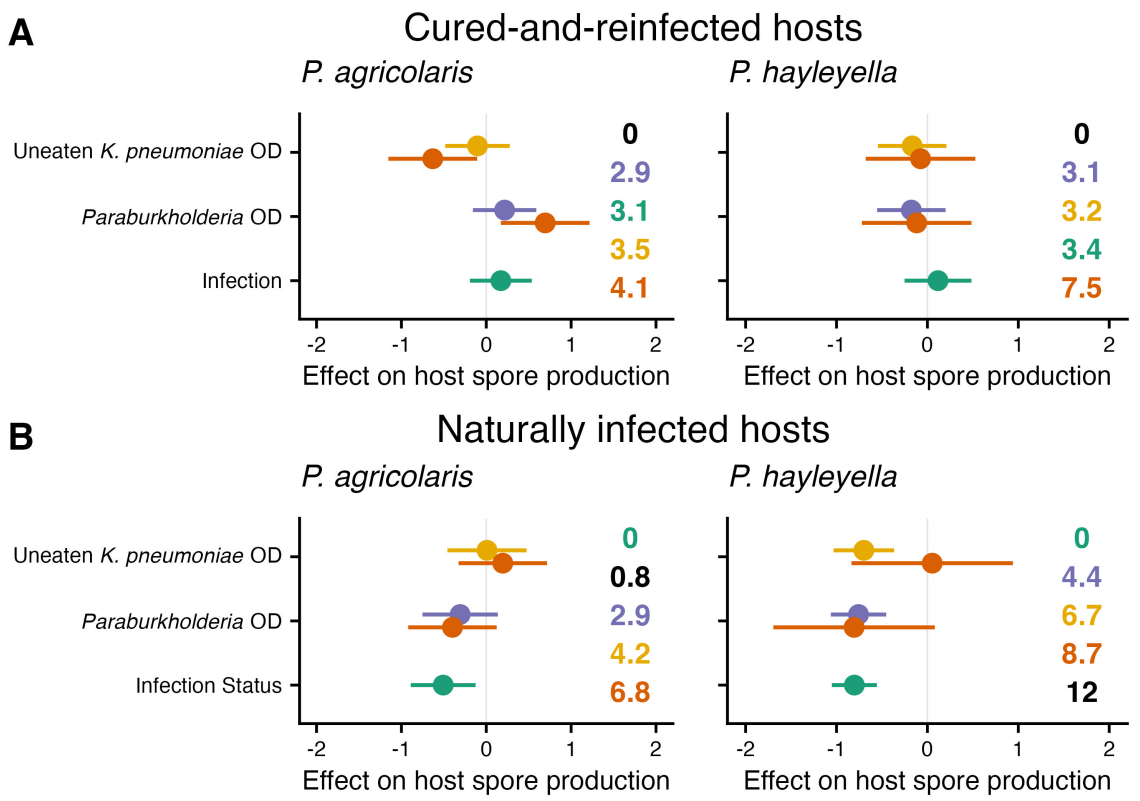

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740 **Figure 3:** Host spore production as a function of different bacteria densities on plates and
741 infection status in (A) cured-and-reinfected (B) and naturally infected hosts relative to
742 their respective uninfected counterparts (see Table 1). We compared models of host spore
743 production (shown in different colors), predicted by *Paraburkholderia* density only,
744 uneaten prey bacteria only, both *Paraburkholderia* and uneaten prey bacteria, or a
745 categorical variable for infection status (infected with *Paraburkholderia* or not).
746 Estimated effects are shown as points and 85% confidence intervals are shown as lines
747 (null models with only the intercept are not shown). A vertical line at 0 is included to
748 show whether estimated effects overlap 0. ΔAICc values are shown in increasing order
749 from top to bottom and colored according to the kind of model. Intercepts are not shown.

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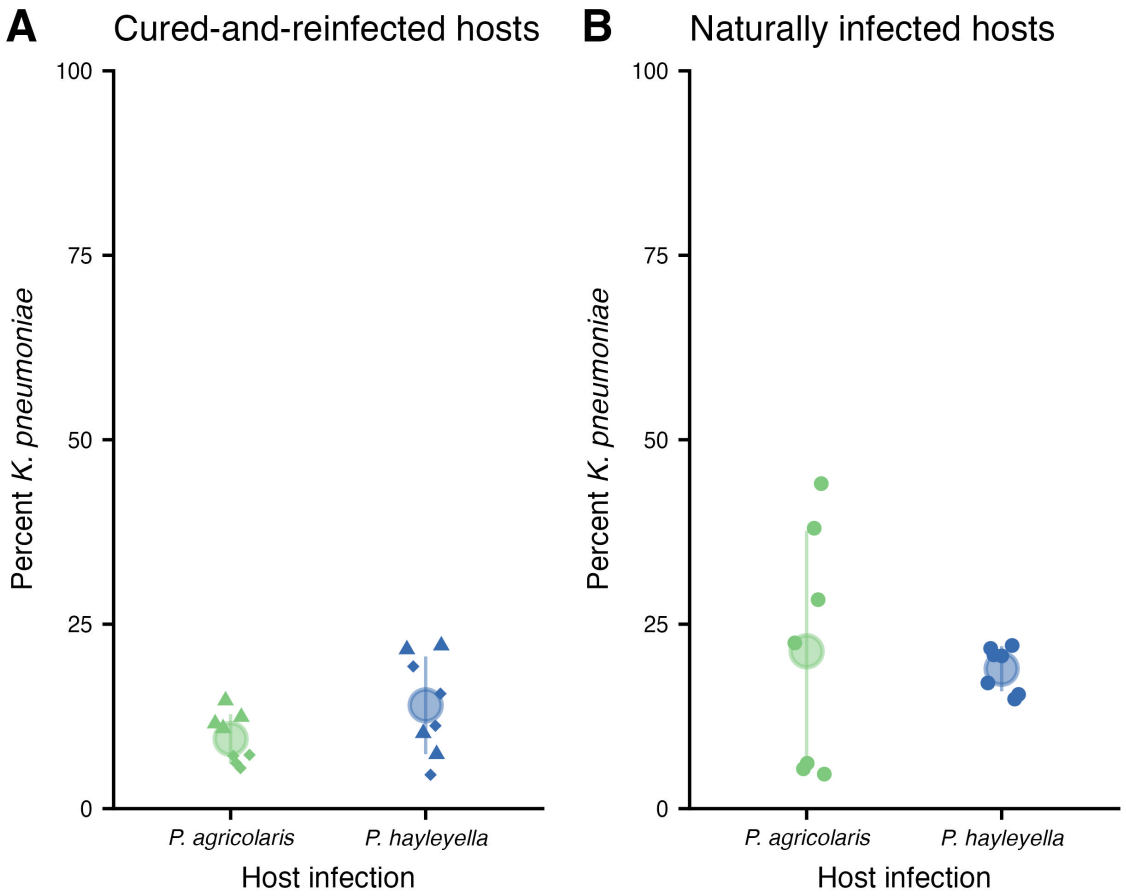

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752 **Figure 4:** Most of the bacteria left behind by infected hosts were not prey bacteria.

753 Percent of the total left-behind bacteria (includes prey bacteria and *Paraburkholderia*

754 symbionts) that was prey bacteria from cured-and-reinfected (A) and naturally infected

755 (B) hosts. Dot and lines show mean and standard deviation, respectively. Point shapes for

756 small points indicate different dates on which experiments were replicated. Data in this

757 figure come from the same experiments as in Figure 1A and in Figure 3.

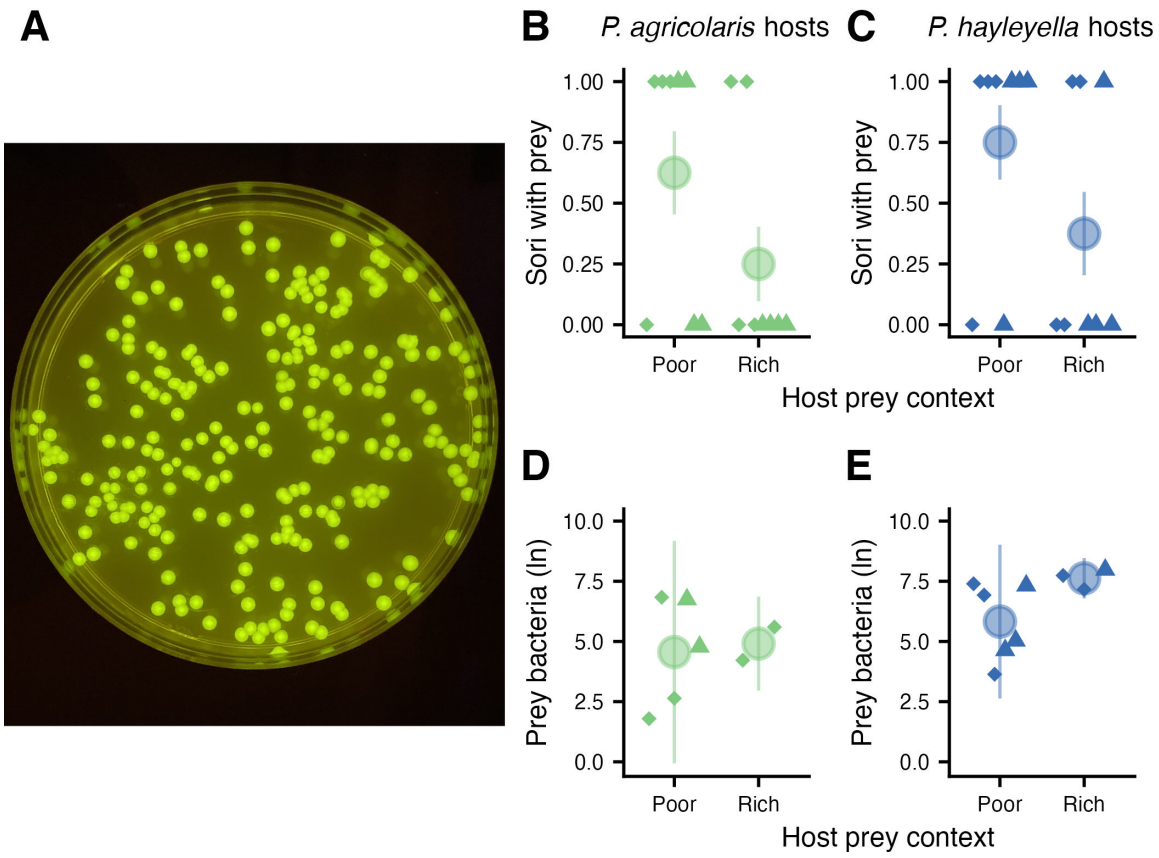

Figure 5: Hosts are less likely to carry prey bacteria in sori after developing in prey-rich environments. (A) Photo of fluorescent *K. pneumoniae* prey bacteria colonies plated from an individual sorus. (B&C) Sori with carried food bacteria for cured-and-reinfected *P. agricolaris* (B) or *P. hayleyella* (C) hosts from prey-poor and prey-rich contexts. Carriage outcomes for individual fruiting bodies are shown as small points that indicate carried prey (1) or did not carry prey (0). Large points and lines show the proportion of sori with prey and the standard deviation. (D&E) Number of prey bacteria of sori that contained prey (1s in B&C), for hosts carrying *P. agricolaris* (D) or *P. hayleyella* (E) from prey-poor and prey-rich contexts. Large points and lines in D&E show the average and standard deviation. Carried prey bacteria were quantified by plating serial dilutions (from

769 undiluted to 1:1000) of single fruiting bodies. Point shapes for individual fruiting bodies
770 (small points) indicate different dates on which experiments were replicated.
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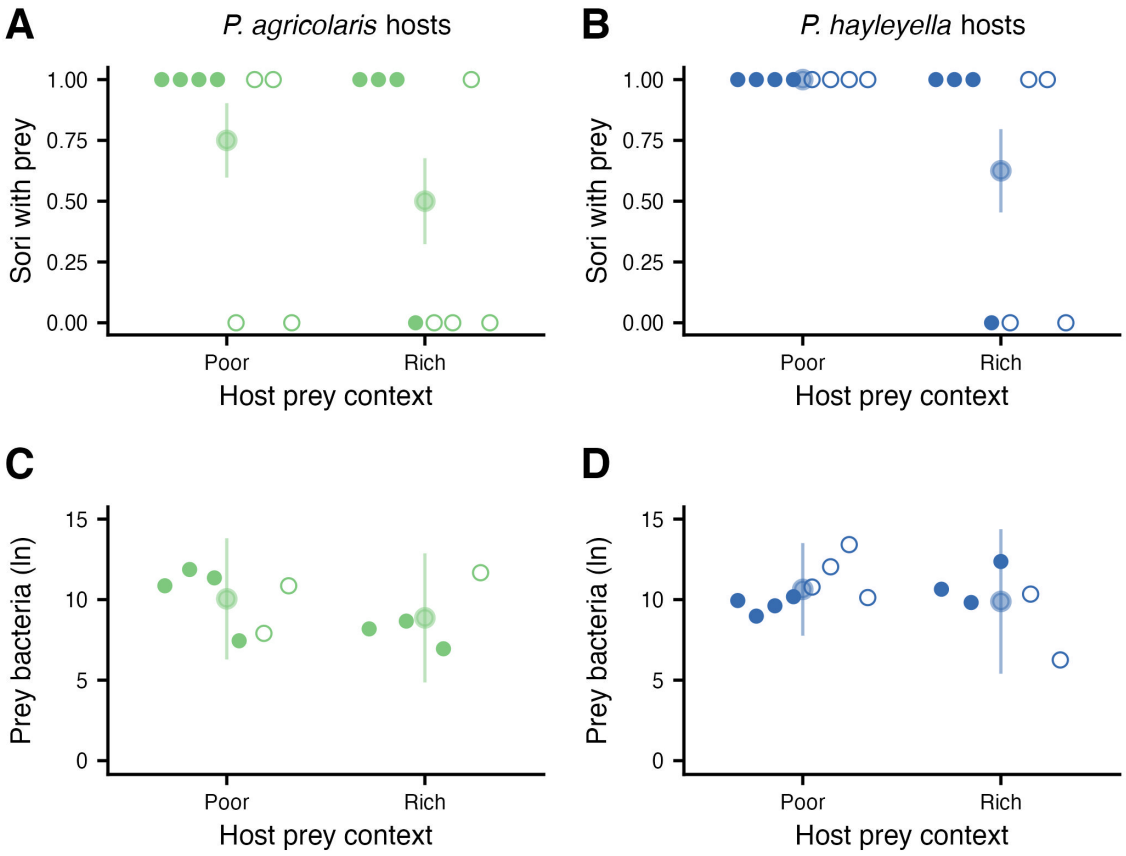

Figure 6: Hosts are less likely to carry prey bacteria in sori after developing in prey-rich environments when more sori are sampled. (A&B) Sori with carried food bacteria for cured-and-reinfected *P. agricolaris* (A) or *P. hayleyella* (B) hosts from prey-poor and prey-rich contexts. Carriage outcomes for individual fruiting bodies are shown as small points that indicate carried prey (1) or did not carry prey (0). Large points and lines show the proportion of sori with prey and the standard deviation. (C&D) Number of prey bacteria of sori that contained prey (1s in A&B), for hosts carrying *P. agricolaris* (C) or *P. hayleyella* (D) from prey-poor and prey-rich contexts. Large points and lines in C&D show the average and standard deviation. Carried prey bacteria were quantified by plating serial dilutions (from undiluted to 1:1000) of five fruiting bodies (in contrast to single

792 fruiting bodies shown in Figure 5). Point shapes for individual fruiting bodies (small
793 points) indicate different dates on which experiments were replicated.
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