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

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Conflict of interest

We have no conflicts of interest to declare.

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Abstract

Symbiotic interactions may change depending on the abundance of third parties like predators, prey, or pathogens. Third-party interactions with prey bacteria are central to the symbiosis between *Dictyostelium discoideum* social amoeba hosts and *Paraburkholderia* facultative bacterial symbionts. Symbiosis with inedible *Paraburkholderia* allows host *D. discoideum* to carry prey bacteria through the dispersal stage where host amoebae aggregate and develop into fruiting bodies that disperse spores. Carrying prey bacteria benefits hosts when prey bacteria are scarce but harms hosts when prey bacteria are plentiful. Symbiont-carrying hosts leave some bacteria behind; this could explain the harm to hosts if left-behind bacteria include uneaten prey bacteria. Thus, understanding both 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 small and did not explain why infected hosts produced fewer spores than uninfected hosts. Turning to the bacteria that are carried, we found that hosts carry more prey bacteria 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 (Bronstein, 1994; Chamberlain *et al.*, 2014; Horas *et al.*, 2022; Scott *et al.*, 2022). One crucial component of the environment that often selects for symbiotic interactions is a third species that interacts with hosts or symbionts (Palmer *et al.*, 2008; Wendling *et al.*, 2017; Wood *et al.*, 2018; Hafer-Hahmann & Vorburger, 2020; Cassidy *et al.*, 2022). 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* (Palmer *et al.*, 2008). Such shifts can influence whether host and symbiont fitness interests are aligned or in conflict (Keeling & McCutcheon, 2017; Iwai *et al.*, 2019). Fitness alignment or conflict then shapes the evolution of these interactions (Scott & Queller, 2019; O'Brien *et al.*, 2021). However, the details of how third parties affect the fitness interests of symbiotic partners are not well understood for many kinds of symbioses (Chamberlain *et al.*, 2014).

An important model of microbial symbiosis is the social amoeba *Dictyostelium discoideum*, that is famous for its complex and well-studied multicellular life cycle (Kessin, 2001; Jahan *et al.*, 2021). *D. discoideum* is a soil amoeba that feeds on many bacteria (Raper, 1937; Brock *et al.*, 2018). Upon starvation, amoebae aggregate to form a multicellular fruiting body that disperses spores to new soil patches (Kessin, 2001). Development of the fruiting body progresses through well-studied stages starting with aggregation of individual amoebae which then become motile slugs (Medina *et al.*,

47 2019). Slugs further develop into a fruiting body. During fruiting body development,
48 about 20% of the cells die and become stalk while the remaining cells develop into spores
49 that sit atop the stalk in a structure called a sorus (Strassmann & Queller, 2011).

50 *D. discoideum* has symbioses with certain facultatively intracellular
51 *Paraburkholderia* bacteria (DiSalvo *et al.*, 2015; Brock *et al.*, 2020). In natural isolates,
52 around 25% of wild-collected *D. discoideum* are infected (Haselkorn *et al.*, 2019). There
53 are three symbiont species – *P. agricolaris*, *P. hayleyella*, and *P. bonniae* – and we will
54 refer to them collectively in this paper as either symbionts or simply *Paraburkholderia*
55 (though readers should understand that there are many species of *Paraburkholderia* not
56 involved in this symbiosis).

57 These symbionts are adapted for interacting with amoebae (Shu *et al.*, 2018a; b;
58 Brock *et al.*, 2020). Symbionts move towards hosts (Shu *et al.*, 2018b) and make host
59 phagosomes less acidic so that the symbionts are not digested (Tian *et al.*, 2022). *P.*
60 *hayleyella* and *P. bonniea* also have reduced genomes relative to *P. agricolaris* and most
61 other *Paraburkholderia* (Brock *et al.*, 2020; Noh *et al.*, 2022) and such reduction is often
62 found in endosymbionts and pathogens that have been associated closely with their hosts
63 during a long evolutionary history (McCutcheon & Moran, 2012). Some *D. discoideum*
64 hosts may also be adapted to their symbionts. Hosts infected by *P. hayleyella* appear to
65 suffer less from *P. hayleyella* toxicity than hosts that are naturally infected by *P.*
66 *agricolaris* (Shu *et al.*, 2018a).

67 Fitness of *D. discoideum* hosts and *Paraburkholderia* symbionts is affected by
68 interactions with a third set of organisms, the various prey bacteria that are eaten by host

amoebae (Brock *et al.*, 2011, 2018; DiSalvo *et al.*, 2015; Scott *et al.*, 2022). *Paraburkholderia* symbionts are largely inedible by hosts but they cause host carriage of additional, edible bacteria (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Khojandi *et al.*, 2019). Edible prey bacteria are mostly carried extracellularly in the sorus while *Paraburkholderia* symbionts are mostly carried inside host spores (Khojandi *et al.*, 2019). When host spores disperse to locations where prey bacteria are rare, they benefit from *Paraburkholderia* infection because they are able to seed new prey bacteria populations (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Scott *et al.*, 2022). When hosts disperse to prey-rich conditions, infected hosts do not gain from carrying prey bacteria and instead experience a cost producing fewer spores relative to uninfected *D. discoideum* (Brock *et al.*, 2011; DiSalvo *et al.*, 2015). Higher *Paraburkholderia* densities impose higher costs on hosts but again this is affected by third-party prey, with the effect being stronger in pre-rich conditions (Scott *et al.*, 2022).

The third-party bacteria can also affect *Paraburkholderia* fitness due to competition (Medina *et al.*, 2023). One consequence is that symbionts reach higher densities when they are carried to locations with fewer prey bacteria (Scott *et al.*, 2022). Thus prey-poor conditions for the host are low-competition conditions for the symbiont (though for consistency, we will stick to calling them prey-poor and prey-rich conditions rather than low-competition and high-competition conditions).

While the importance of prey bacteria has been well established for this symbiosis (DiSalvo *et al.*, 2015; Khojandi *et al.*, 2019; Scott *et al.*, 2022), there are still many questions about the effects of prey bacteria during development of *D. discoideum*. In this

study, it is the disposition of prey bacteria during host development that is of interest – should they be eaten, carried, or left behind?

Leaving prey bacteria behind would not seem to be adaptive, but this appears to happen when hosts are infected by *Paraburkholderia* (Brock *et al.*, 2011, 2016b). Initially, before the distinct roles of inedible symbionts and edible prey were appreciated, bacteria left behind on plates was taken as a possible sign of "prudent predation" by host amoebae — if some bacteria were going to be saved for carriage, hosts cannot eat all the available prey and may stop feeding and start developing earlier (Brock *et al.*, 2011). It was hypothesized that hosts leaving prey bacteria uneaten might explain why carriage is costly in some conditions (Brock *et al.*, 2011). Hosts that were prudent predators left prey uneaten and missed out on potential growth and proliferation.

The essential role of *Paraburkholderia* in the symbiosis, including causing hosts to leave behind bacteria is now known (DiSalvo *et al.*, 2015; Brock *et al.*, 2016b). At least some of the bacteria left behind are not prey bacteria but inedible *Paraburkholderia* symbionts (Scott *et al.*, 2022). Because of this new information, the prudent predation hypothesis should be re-evaluated by determining the relative abundances of left behind prey bacteria and *Paraburkholderia* symbionts. The fitness consequences of leaving prey behind, in terms of spore production and development time, should also be evaluated.

While infected hosts may leave some prey bacteria uneaten, they also gain the ability to carry prey bacteria along with the dispersing host spores in sori (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Khojandi *et al.*, 2019). It is unknown whether the number of carried prey bacteria changes in different environments. If carriage is passive, we expect

113 hosts to carry more prey after developing in a prey-rich environment and fewer prey
114 bacteria after developing in a prey-poor environment.

115 Alternatively, the ability to carry prey bacteria could reflect the evolutionary
116 interests of hosts and symbionts. Since soil environments tend to be spatially and
117 temporally structured (Sun *et al.*, 2003; Vos *et al.*, 2013), developing in a prey-poor
118 environment may be associated with an increased probability that the next environment
119 will also tend to be prey-poor. If this is the case, it would be more adaptive for hosts if
120 they carried more prey bacteria after developing in a prey-poor environment than in a
121 prey-rich environment. This may also be adaptive for the *Paraburkholderia* symbionts
122 since it allows better survival of their hosts.

123 We investigate three questions about the complex role of prey bacteria in the
124 symbiosis between *D. discoideum* and two commonly studied *Paraburkholderia*
125 symbionts, *P. agricolaris* and *P. hayleyella* (Shu *et al.*, 2018a; Garcia *et al.*, 2019; Scott
126 *et al.*, 2022; Medina *et al.*, 2023). We first re-evaluate some of the ideas behind the
127 prudent predation hypothesis. Second, we ask whether any prey bacteria that are left
128 uneaten results in costs due to delayed development or reduced spore production. Lastly,
129 we turn to whether the number of prey bacteria carried inside sori changes between prey-
130 poor and prey-rich environments.

131

132 **Methods**

133

134 *Clones and Culturing Methods*

135 In our experiments, we used several types of infected and uninfected hosts (Table
136 1). We used two types of infected host, naturally infected hosts (n=7 for each symbiont
137 species) and, to better control for infection levels, cured-and-reinfected hosts (n=4 for
138 each symbiont species). The naturally infected hosts were compared to naturally
139 uninfected controls (n=6) and the cured-and-reinfected host were compared to a different
140 set of naturally uninfected controls (n=4). To better control for genetics, cured-and-
141 reinfected hosts were also compared to their cured clonemates (n=4 for each symbiont
142 species). To generate cured-and-reinfected clones, naturally infected clones were cleared
143 of their natural infections with antibiotics and then reinfected at 1:1000 ratios of
144 *Paraburkholderia* to prey bacteria to control infection densities (Scott *et al.*, 2022). Data
145 reported here for cured-and-reinfected hosts and their uninfected controls come from
146 experiments performed in Scott et al. (2022) but here we include additional measures not
147 included in the original study. Naturally uninfected controls in this comparison were also
148 treated with antibiotics to control for antibiotic treatment. We stored all prepared clones
149 in a -80 C freezer for repeated use.

150 To remove any effects of being in the freezer and to ensure that infected amoebae
151 carried *K. pneumoniae* prey bacteria, we grew amoebae through one round of feeding and
152 fruiting body formation and then collected spores to initiate our experiments. This step
153 may allow infection densities time to equilibrate (Miller *et al.*, 2020). Amoebae were
154 grown from frozen spores at room temperature on 100 x 15mm plates filled with 30mL
155 SM/5 agar (2 g glucose (Fisher Scientific), 2 g Bacto Peptone (Oxoid), 2 g yeast extract
156 (Oxoid), 0.2 g $MgSO_4$ * $7H_2O$ (Fisher Scientific), 1.9 g KH_2PO_4 (Sigma-Aldrich), 1 g
157 K_2HPO_4 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter) with 200 µL of

158 1.5 OD_{600} fluorescently labeled *K. pneumoniae* suspended in KK2 buffer (2.25 g
159 KH_2PO_4 (Sigma-Aldrich) and 0.67 g K_2HPO_4 (Fisher Scientific) per liter) spread over
160 the entirety of the agar. *K. pneumoniae* used in this study expressed green-fluorescent
161 protein (GFP) and were provided by dictyBase (Fey et al. 2019).
162
163 *Dispersal experiment setup*
164 To determine the consequences of hosts leaving behind bacteria, we performed a
165 dispersal experiment to seed new plates with sorus contents. To do this, we transferred
166 200 μL of $2x10^5$ spores/mL suspension obtained from the equilibration step to plates with
167 or without 200 μL of 1.5 OD_{600} GFP-expressing *K. pneumonia*e. Spore counts to make
168 spore suspensions were obtained using a hemocytometer. OD_{600} for plating bacteria was
169 measured with a BioPhotometer (Eppendorf, New York). We let bacteria and amoebae on
170 plates proliferate at room temperature for six days, enough time for amoebae to form
171 fruiting bodies.
172 We replicated these dispersal experiments with cured-and-reinfected hosts along
173 with uninfected controls on two separate dates. To understand if our results held with
174 natural infections, we performed an additional dispersal experiment with naturally
175 infected clones and uninfected controls. To rule out that leaving uneaten prey bacteria
176 was a host trait, we also performed a separate set of dispersal experiments comparing
177 cured clones to their reinfected counterparts.
178
179 *Measurement of bacteria and host spore production*

180 After dispersed amoebae and bacteria were allowed to proliferate for six days (to
181 allow hosts to complete development into fruiting bodies), we measured the density of
182 uneaten prey bacteria, *Paraburkholderia*, and host spore production. To measure the
183 density of uneaten prey bacteria, we first collected host spores and bacteria from after the
184 dispersal step. We collected host spores and bacteria by washing plates with 15 mL of
185 KK2 buffer after six days of growth. We counted host spores from this washed solution
186 using a hemocytometer. To measure bacterial density, we first removed host spores by
187 centrifuging the wash solution for three minutes at 360 rcf and reserving the supernatant.
188 We measured the optical density of the supernatant at 600 nm (OD_{600}) as well as
189 fluorescence with an excitation wavelength of 485 and emission wavelength of 515 nm in
190 a 96 well plate with a Tecan Infinite 200 Pro microplate reader. Removing host spores by
191 manually removing sori with a pipette tip resulted in similar densities of uneaten prey
192 bacteria consistent earlier findings (Khojandi *et al.*, 2019) that the number of prey
193 bacteria inside spores and sori is minimal relative to that left on the plate (data not
194 shown).

195 Since the supernatant from infected clones contains GFP-expressing *K.*
196 *pneumoniae* and unlabeled *Paraburkholderia*, the total OD_{600} is due to both kinds of
197 bacteria but fluorescence comes only from *K. pneumoniae*. To calculate the amount of
198 OD_{600} due to fluorescing *K. pneumoniae*, we generated a standard curve relating
199 fluorescence to OD_{600} using serial dilutions of GFP-expressing *K. pneumoniae* in KK2
200 and used this curve to predict the OD_{600} of *K. pneumoniae* in our samples. The remaining
201 OD_{600} is the amount due to *Paraburkholderia* symbionts, after also subtracting off the
202 background OD_{600} of the KK2 buffer. An OD_{600} of 0.1 translates to around 5×10^7 *K.*

203 *pneumoniae* cells or 1 x 10^8 *Paraburkholderia* cells according to the validation dataset in
204 Scott et al. (2022).
205
206 *Development assays for cured and reinfected hosts*
207 To determine how symbionts and their interactions with prey affected host
208 development time, we took time-lapse images of cured hosts and reinfected counterparts
209 developing in six-well plates filled with 10 mL SM/5. Each 6-well plate contained three
210 wells with cured hosts and three wells with reinfected hosts of either *P. hayleyella* clones
211 (QS23, QS38, and QS45) or *P. agricolaris* clones (NC21, QS159, and QS161). We
212 repeated development experiments on six-well plates three times for each
213 *Paraburkholderia* species. We first grew clones from the freezer on SM/5 plates for six
214 days in case there were freezer effects, as above. We then collected host spores and
215 plated 30 μL of 2x10^5 spores per mL and 30 μL of 1.5 OD_{600} *K. pneumoniae* in each
216 well. Photos were taken every hour until fruiting bodies developed using a Canon EOS
217 Mark IV. We inspected photos to determine time points for when aggregates, slugs, and
218 fruiting bodies first appeared in each well.
219
220 *Measurement of carried bacteria*
221 To determine how prey context affected the number of prey bacteria carried in
222 sori, we used cured-and-reinfected hosts that were grown on prey-poor and prey-rich
223 plates after the dispersal step. We haphazardly sampled a single sorus from each of our
224 experimental plates from reinfection experiments (replicated on two different dates). To
225 count the number of carried prey, we suspended single-sorus contents in KK2 buffer and

226 plated out serial dilutions from no dilution to 1:1000 dilutions. Since *K. pneumoniae* were
227 labeled with GFP, we could differentiate colonies of *K. pneumoniae* and
228 *Paraburkholderi*a. We counted colonies from the dilution plate that appeared closest to
229 having 100 colonies and then back-calculated to get the total count for a single sorus.
230 This experiment was completely replicated on two separate dates.
231
232 *Statistical Methods*
233 To understand how *Paraburkholderia* infection affected the density of *K.*
234 *pneumoniae* bacteria left uneaten, we fit linear models in R (version 3.6.3). Because
235 experiments with cured-and-reinfected (and uninfected control) hosts were performed on
236 two different dates, we used linear mixed models (LMM) with date as a random effect.
237 We fit LMMs using the nlme4 package (Pinheiro & Bates, 2006) in R. For other
238 comparisons that involved only single measures, we used linear models fit by ordinary
239 linear regression (LM) with the lm function in R. We performed Tukey post-hoc tests for
240 pairwise comparisons using the *emmeans* package (Lenth *et al.*, 2019).
241 To determine whether the timing of development was affected by
242 *Paraburkholderia* infection, we used generalized linear mixed models (GLMMs) with
243 Poisson errors. We included the date that experiments were performed as a random
244 effect to capture variation within plates.
245 To test how prudent predation affects host spore production relative to other
246 explanations, we again used LMMs and LMs for cured-and-reinfected and naturally
247 infected infections, respectively, along with respective uninfected controls. If prudent
248 predation reduces host spore production, we expected a decrease in spore production with

249 increasing uneaten prey bacteria. Differences in host fitness could also be affected by the
250 density of *Paraburkholderia* left on plates (Scott *et al.*, 2022) or by infection category
251 (uninfected vs infected) (Brock *et al.*, 2011; DiSalvo *et al.*, 2015; Haselkorn *et al.*, 2019).
252 To account for these possibilities, we also fit models with these variables along with a
253 model that includes both uneaten prey density and *Paraburkholderia* density. In total, we
254 compared five models of spore production: (1) uneaten prey density (Prey model), (2)
255 *Paraburkholderia* density (Para model), (3) uneaten prey density + *Paraburkholderia*
256 density (Prey + Para model), (4) categorical infection status (Infection model), and (5) a
257 null model fit with only the intercept for each *Paraburkholderia* species.
258 To compare effects more easily across the different models, we scaled all
259 variables by subtracting the mean and dividing by twice the standard deviation. This
260 scaling procedure is useful for comparing models with multiple continuous predictors
261 (density of prey bacteria and symbionts) and with categorical factors (infected and
262 uninfected) because all measures end up on roughly the same scale (Gelman *et al.*, 2020).
263 To compare the fit of the five models described above, we calculated AICc for
264 each model. AICc is a measure of model fit that accounts for model complexity and that
265 corrects for small sample sizes (Burnham & Anderson, 2004). Models that fit the data
266 best have lower AICc values. For our model selection analysis we show results with 85%
267 confidence intervals instead of the standard 95% confidence intervals because 85%
268 confidence intervals are more consistent with model selection with AIC (Arnold, 2010).
269 To measure potential collinearity in our models that included prey and *Paraburkholderia*
270 density, we calculated variance inflation factors (VIF) using the check_collinearity
271 function in the performance package (Lüdecke *et al.*, 2021). VIFs lower than 5 indicate

272 low collinearity, values between 5 and 10 indicate moderate collinearity, and values
273 above 10 indicate high collinearity.
274 To compare the ability of hosts to carry *K. pneumoniae* inside fruiting bodies in
275 prey-rich and prey-poor environments, we used a zero-inflated negative binomial model
276 (ZINB) fit with the glmTMB package (Brooks *et al.*, 2017) in R. This zero-inflated
277 model incorporated two processes: (1) zero-inflation for whether fruiting bodies
278 contained or did not contain prey bacteria and (2) negative binomial for the counts of
279 carried prey bacteria. The zero-inflation parameter captures whether hosts are more likely
280 to carry while the negative binomial parameter captures whether hosts carry more prey
281 bacteria when they carry. Using this ZINB, we compared different hypotheses about how
282 prey-rich and prey-poor environments affected the ability to carry and the number carried
283 with AICc. We fit models to carriage data from both species and asked whether models
284 including prey environment, *Paraburkholderia* species, or the interaction between prey
285 environment and species had low AICc values. We did this model selection for both the
286 zero-inflation part of the model and for the negative binomial part of the model. We also
287 compared models with the random effects of clone and date included in either the zero-
288 inflation or negative binomial parts of the model.
289
290 **Results:**
291 *D. discoideum hosts infected with Paraburkholderia leave some prey bacteria uneaten*
292 We investigated the prey bacteria left behind after hosts formed fruiting bodies by
293 measuring the density of leftover *K. pneumoniae* from cured-and-reinfected hosts
294 compared to naturally uninfected controls. These prey densities are estimated from

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

318 We determined time courses for when hosts aggregated, formed slugs, and formed
319 fruiting bodies. We were unable to detect that development times were slower for cured-
320 and-reinfected hosts relative to cured hosts for *P. agricolaris* (GLMM; estimate across all
321 developmental timepoints = 0.015, se = 0.037, P = 0.682) or *P. hayleyella* (GLMM;
322 estimate across all developmental timepoints = 0.044, se = 0.037, P = 0.231; Figure
323 2A&B). Individual developmental stages also did not differ due to *Paraburkholderia*
324 infection (P > 0.05).
325
326 *Prey bacteria left on plates are not associated with reduced spore production in hosts*
327 Leaving more prey uneaten is suspected to lower host fitness and could therefore
328 explain the cost of *Paraburkholderia* infection relative to uninfected hosts (Brock *et al.*,
329 2011). Alternately, the cost of infection may be more directly due to *Paraburkholderia*,
330 either infection itself or the density of *Paraburkholderia* symbionts. In addition to these
331 first three models (food bacteria density, *Paraburkholderia* infection status, and
332 *Paraburkholderia* density), we also fit a model that included both uneaten prey bacteria
333 density and *Paraburkholderia* density. To compare the relative support for these different
334 hypotheses, we fit a model for each hypothesis (see Methods) and compared fitted
335 models using AICc.
336 We first tested the role of uneaten prey bacteria and *Paraburkholderia* left on
337 plates on host spore production using our cured-and-reinfected hosts and naturally
338 uninfected controls (Table 1). For infections with either species of *Paraburkholderia*,
339 null models fit to only the intercept fit the data best (Figure 3A & S1; the model that
340 includes uneaten prey bacteria and *Paraburkholderia* density for *P. agricolaris* cured-

341 and-reinfected hosts — Prey + Para — did show confidence intervals that did not overlap
342 zero, but this was the worst model in terms of AICc). We thus found no evidence that
343 leaving prey explained the cost of infection for cured-and-reinfected hosts relative to
344 uninfected controls.

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

352 These results show no support for a cost due to leaving prey bacteria behind (even
353 when accounting for *Paraburkholderia* density). We next turned to the direct role of
354 *Paraburkholderia*. Because models fit to only the intercept were the best for cured-and-
355 reinfected hosts relative to their controls, there was no evidence that *Paraburkholderia*
356 was costly in these clones, the same result as previously shown (Scott *et al.*, 2022). In
357 contrast, naturally infected hosts were afflicted by a cost of *Paraburkholderia* infection
358 (Figure 3B & S2). The best models for both *P. agricolaris* and *P. hayleyella* showed that
359 infected hosts had lower spore production than uninfected hosts (*P. agricolaris*: LM;
360 estimate = -0.508, se = 0.247, df = 11; *P. hayleyella*: LM; estimate = -0.804, se = 0.160,
361 df = 11). *Paraburkholderia* density predicted host spore production less well than
362 infection status. However, models that included *Paraburkholderia* density on its own
363 were better than models with prey bacteria density (Figure 3B & S2). These models thus

364 show little evidence for a cost of uneaten food bacteria and instead show that
365 *Paraburkholderia* infection is responsible for reduced host spore production in naturally
366 infected hosts.
367
368 *Uneaten prey bacteria are a minority of left-behind bacteria*
369 Prey bacteria left behind did not have the predicted effects on either development
370 time or spore production costs. One reason may be that the amount of left behind prey is
371 not as great as formerly thought (Brock *et al.*, 2011). Hosts may leave too few prey
372 bacteria to noticeably affect spore production. In fact, uneaten prey bacteria make up a
373 minority of left-behind bacteria. Uneaten prey bacteria are about 9% and 14% of bacteria
374 on plates for *P. agricolaris* and *P. hayleyella* cured-and-reinfected hosts, respectively,
375 and 21% and 19% for *P. agricolaris* and *P. hayleyella* naturally infected hosts,
376 respectively (Figure 4).
377
378 *More prey bacteria are carried after hosts develop in prey poor conditions*
379 To determine whether the number of prey bacteria carried in sori simply reflects
380 the number of prey bacteria in the previous environment or is modified according to the
381 interests of hosts or symbionts, we measured the number of fluorescent prey bacteria
382 inside sori after growth of cured-and-reinfected hosts on prey-rich and prey-poor plates
383 (Figure 5A). We found that for both *Paraburkholderia* species (Figure 5B&C), sori
384 produced in prey-rich environments were less likely to contain prey bacteria than those in
385 prey-poor environments (ZINB , zero-inflation estimate = 1.950, se = 0.987). This shows
386 that the ability to carry prey bacteria is affected by prey context, but in the opposite

387 direction to that expected if prey was carried according to the density of prey bacteria in
388 the environment. We did not detect a difference in the number of prey bacteria that were
389 caried between prey-rich and prey-poor environments (Figure 5D&E). Thus, the ability to
390 carry prey bacteria may depend on the fitness interests of hosts and symbionts that benefit
391 from carrying prey bacteria when harsh conditions are expected.
392
393 **Discussion**
394 Third parties that interact with symbiotic partners commonly affect the outcome
395 of symbiotic interactions (Palmer *et al.*, 2008; Chamberlain *et al.*, 2014; Hafer-Hahmann
396 & Vorburger, 2020; Cassidy *et al.*, 2022) and alter selection for cooperation and conflict
397 between symbiotic partners (Keeling & McCutcheon, 2017; Wood *et al.*, 2018). Often the
398 details of how third parties affect fitness effects are unknown. In the symbiosis between
399 *D. discoideum* social amoebae and *Paraburkholderia* bacteria, the third-party prey
400 bacteria are normally eaten by the host but can also be carried or left behind. We studied
401 how many prey bacteria are carried and left behind, and their impacts, by tracking
402 fluorescently labeled prey bacteria (*K. pneumoniae*) during fruiting body formation by
403 hosts.
404 Our most surprising finding was that infected hosts were more likely to carry prey
405 bacteria after growing in prey-poor environments (Figure 5). This result is surprising
406 because it means that hosts or symbionts actively change the number of carried prey
407 bacteria depending on environmental conditions. This result provides possible evidence
408 for mutual benefit between *D. discoideum* hosts and *Paraburkholderia* symbionts. Hosts
409 and symbionts are likely to benefit from modifying carriage in this way if future soil

410 environments tend to resemble past soil environments. Repeated prey-poor environments
411 are where hosts should most benefit from carrying and seeding out prey bacteria.
412 *Paraburkholderia* symbionts could also benefit from carriage of prey because keeping
413 hosts alive means there are hosts to further disperse *Paraburkholderia*. However, this
414 dispersal benefit to the symbionts must be balanced against the costs of increased
415 competition with the prey bacteria.

416 We speculate that carrying more prey in prey-poor contexts may represent
417 cooperation between hosts and symbionts that allows the symbiosis to persist over
418 repeated harsh environments. Such harsh conditions are potentially an important force
419 shaping cooperation in this (Scott *et al.*, 2022, 2023b) and other symbioses (Henry *et al.*,
420 2021; Veresoglou *et al.*, 2021; Cornwallis *et al.*, 2023). These studies in symbiotic
421 systems complement research on the role of harsh environments in the evolution of
422 cooperation that has been focused on interactions between members of the same species
423 (Kennedy *et al.*, 2018), most commonly in cooperatively breeding birds (Cornwallis *et*
424 *al.*, 2017; Griesser *et al.*, 2017; Capilla-Lasheras *et al.*, 2021).

425 Although seeding out prey bacteria has been shown to be important when we move
426 spores to a prey-poor environment in the lab, we have little idea how frequently this
427 occurs in nature. The finding that carriage of prey bacteria changes in an apparently
428 adaptive fashion suggests that it is indeed important in the field. The importance of third-
429 parties in this case may be an interesting exception to the relatively small effects from
430 third-parties that have been observed across other studies (Chamberlain *et al.*, 2014).

431 Questions remain about how hosts and symbionts affect the carriage of prey
432 bacteria. *Paraburkholderia* are more often carried inside spores while prey bacteria are

433 more often carried outside spores (Khojandi *et al.*, 2019). Differences in carriage of
434 bacteria could potentially result from differences in lectins (Dinh *et al.*, 2018) or
435 polyphosphates (Rijal *et al.*, 2020) that affect the ability of bacteria to invade and survive
436 inside hosts. Hosts in the absence of their *Paraburkholderia* symbionts are able to modify
437 the contents of fruiting bodies through their immune cells that protect against toxins and
438 bacteria by collecting potential threats and dropping off during the slug stage (Chen *et al.*,
439 2007). However, the role of these immune cells in the symbiosis (Brock *et al.*, 2016a;
440 Scott *et al.*, 2023a) needs to be further explored. Manipulation of phagosomes could also
441 play a role in determining the contents of fruiting bodies. *Paraburkholderia* symbionts
442 increase the pH of phagosomes, presumably to prevent host digestion of symbionts (Tian
443 *et al.*, 2022). Similar modification of lysosomes is used by pathogens to evade human
444 immune cells during infection (Isberg *et al.*, 2009; Leseigneur *et al.*, 2020). More work is
445 needed to understand how symbionts and food bacteria get into fruiting bodies and how
446 both hosts and symbionts contribute to bacterial carriage.

447 We also found that *Paraburkholderia* infection prevents hosts from eating all the
448 prey bacteria in an environment (Figure 1). A prior study suggested that leaving prey
449 bacteria uneaten because of prudent predation may explain the cost of infection relative
450 to uninfected hosts (Brock *et al.*, 2011). Instead, we found that the quantity of left-behind
451 prey bacteria may be too small to noticeably affect host spore production since we
452 observed that only a minority of left-behind bacteria was food bacteria, with the majority
453 being indigestible *Paraburkholderia* (Figure 4).

454 To get a better sense of how important left-behind prey bacteria could be for
455 hosts, we can use the density vs bacteria count curve in Figure S1B of Scott et al. 2022

456 and the average left-behind prey bacteria in this study to estimate the number of
457 individual left-behind prey bacteria. Using this estimation, *P. agricolaris* infected hosts
458 left 7.060×10^6 individual prey bacteria and *P. hayleyella* infected hosts left 1.206×10^7
459 individual prey bacteria. Using Kessin's (2001, p. 21) rough estimate that an amoebae
460 needs to eat 1,000 bacteria to divide, we calculated that the number of uneaten prey
461 bacteria is only enough to produce 0.002% and 0.004% more spores than what we
462 recovered from plates (collected spores were around 3×10^8) for *P. agricolaris* or *P.*
463 *hayleyella* infected hosts, respectively. These percentages are a rough and conservative
464 approximation as they assume that amoebae devote all consumed prey bacteria to
465 proliferation. However, the calculations show that it is unlikely that uneaten prey bacteria
466 can affect host fitness in a detectable manner.

467 Instead of hosts paying a cost in potential growth because they leave prey bacteria
468 uneaten, we suspect that *Paraburkholderia* infection causes both observations: that hosts
469 leave prey uneaten and that infected hosts pay a cost. Tentative support for this idea
470 comes from our findings that *Paraburkholderia* and uneaten prey bacteria densities were
471 correlated. This correlation could result from higher *Paraburkholderia* densities
472 interfering with the ability of hosts to eat prey bacteria.

473 An interesting remaining question in the *D. discoideum-Paraburkholderia*
474 symbiosis and in other symbioses is the mechanisms that result in reduced fitness when
475 hosts are infected. One potential explanation is that *Paraburkholderia* symbionts directly
476 feed on host cells or otherwise extract nutrients from hosts. Measures of
477 *Paraburkholderia* density inside sori have so far not been found to be correlated with
478 host fitness within species (Miller *et al.*, 2020), though the more toxic *P. hayleyella* does

479 appear to infect a higher percentage of spores than less toxic *P. agricolaris* (Shu *et al.*,
480 2018a; Khojandi *et al.*, 2019). Another promising hypothesis that deserves further study
481 is that hosts have lower spore production because of "collateral damage" from
482 competitive interactions between *Paraburkholderia* and prey bacteria (Scott *et al.*, 2022).
483 Competition between bacteria is often mediated by chemical warfare (Granato *et al.*,
484 2019) that could reduce host *D. discoideum* fitness as a side-effect.

485 The role of *Paraburkholderia* in reducing host spore production is more evidence
486 in support of there being some evolutionary conflict in this symbiosis (DiSalvo *et al.*,
487 2015; Scott *et al.*, 2022). However, the magnitude of conflict may differ across
488 *Paraburkholderia* symbiont species. This conflict may be more pronounced between *P.*
489 *hayleyella* and its hosts than between *P. agricolaris* and its hosts. We found that *P.*
490 *hayleyella* density could explain some of the decrease in naturally infected host spore
491 production but we did not find the same for *P. agricolaris* density (Figure 3). This
492 difference between *P. agricolaris* and *P. hayleyella* is consistent with prior studies that
493 found that *P. hayleyella* is more harmful than *P. agricolaris* (Shu *et al.*, 2018a;
494 Haselkorn *et al.*, 2019; Khojandi *et al.*, 2019). In addition to being more harmful, *P.*
495 *hayleyella* also has a reduced genome relative to *P. agricolaris* (Brock *et al.*, 2020; Noh
496 *et al.*, 2022). Reduced genomes are a common result of persistent host association in
497 beneficial symbionts and pathogens (McCutcheon & Moran, 2012). While *P. hayleyella*
498 has maintained its ability to harm hosts over this persistent association, it is important to
499 remember that infection also comes with the ability to carry prey bacteria (DiSalvo *et al.*,
500 2015; Khojandi *et al.*, 2019). Thus, this symbiosis involves a balance between harm and
501 benefits to hosts.

502 Whether a specific symbiosis involves fitness alignment or conflict may depend
503 on a third party that affects the costs and benefits of symbiosis. Our results show that
504 third parties can have complex effects on conflict and fitness alignment in symbioses; the
505 symbiosis between *D. discoideum* and *Paraburkholderia* appears to involve elements of
506 conflict and cooperation that depends on how third-party bacteria are eaten, carried, and
507 left behind.

508

509

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693 **Figure and Table Legends**

694 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	Clones that were cured of any native symbionts with	Cured of *P. agricolaris* infection	QS159, QS161, QS606, NC21

with cured-and-reinfected)	tetracycline, but not reinfected.	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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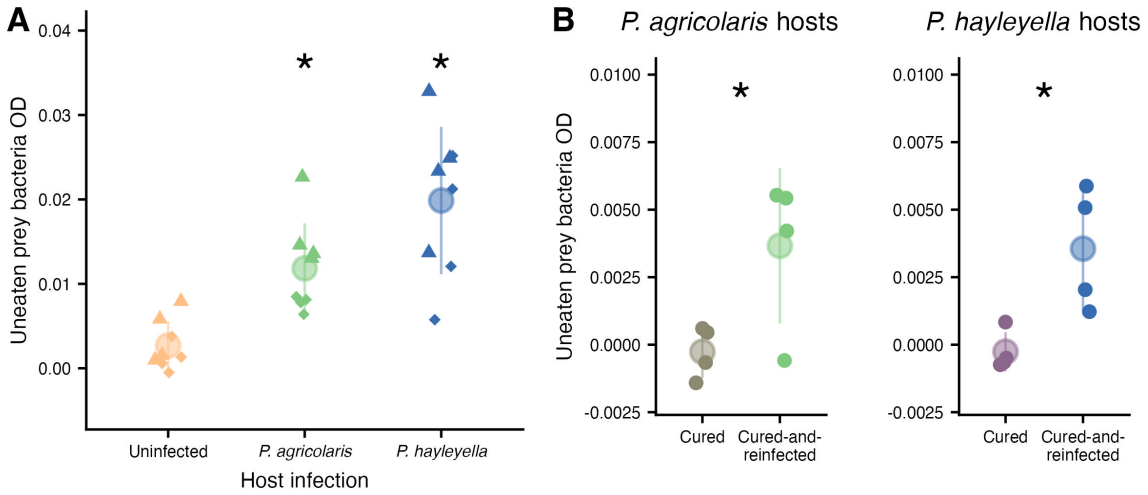


Figure 1: Symbionts cause hosts to leave prey uneaten. (A) Density left on plate of uneaten *K. pneumoniae* prey bacteria (measured by OD_{600}) for naturally uninfected hosts, *P. agricolaris* hosts that have been cured and then reinfected, and *P. hayleyella* hosts that have been cured and then reinfected. (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.

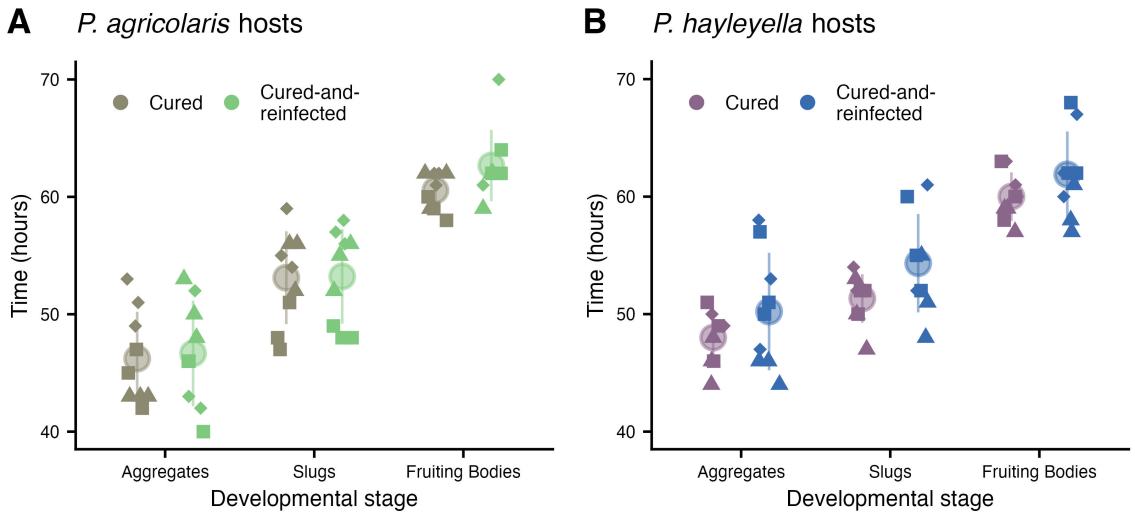


Figure 2: Symbionts do not affect developmental time. Developmental time points at different stages of development for cured hosts and their counterparts that have been cured and then reinfected with (A) *P. agricolaris* and (B) *P. hayleyella* hosts. Dot and lines show mean and standard deviation, respectively. Point shapes of small points indicate different dates on which experiments were replicated.

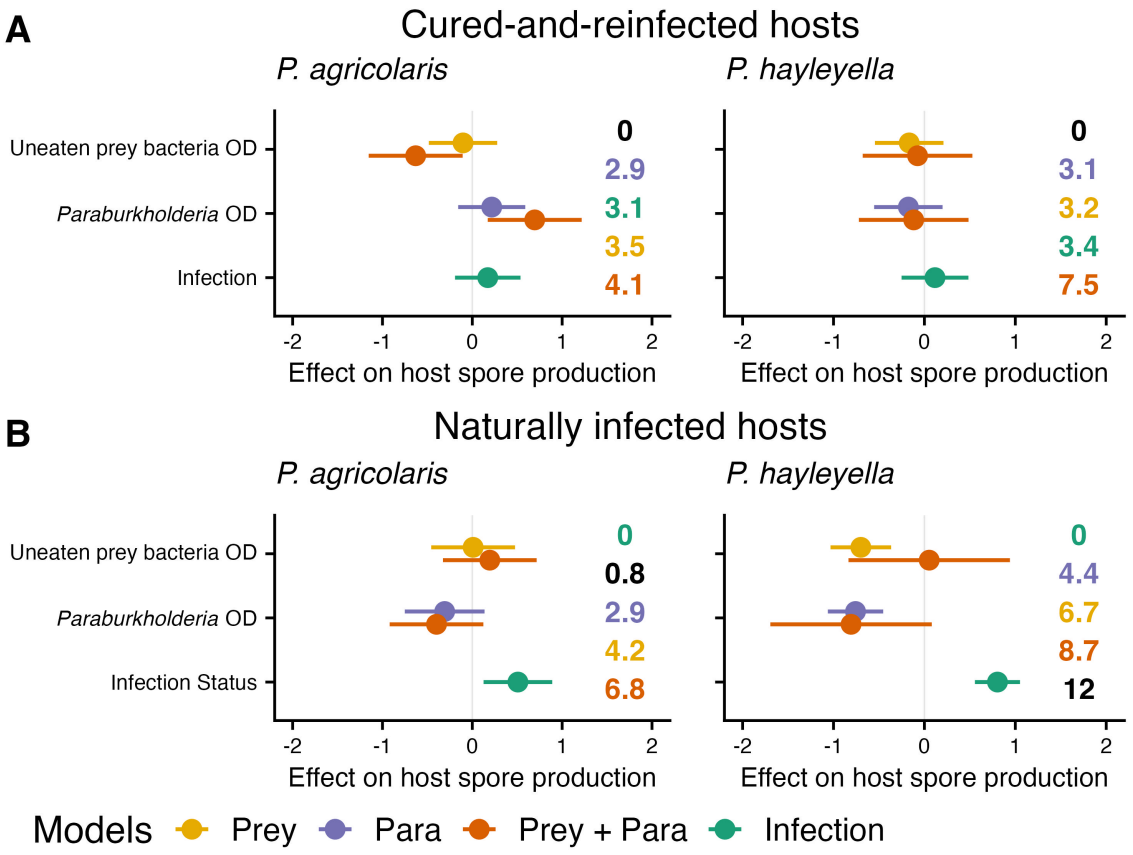
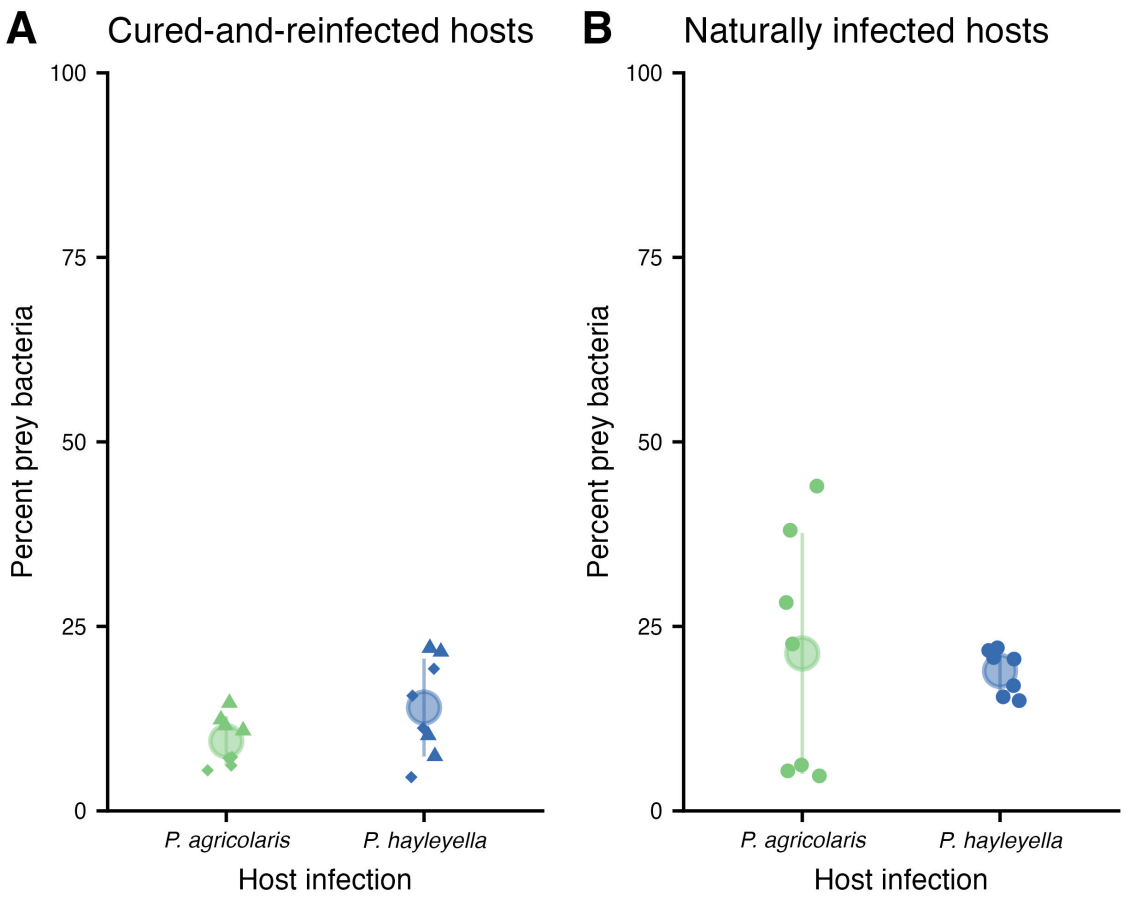


Figure 3: Host spore production as a function of different bacteria densities on plates and infection status in (A) cured-and-reinfected (B) and naturally infected hosts relative to their respective uninfected counterparts (see Table 1). We compared models of host spore production (shown in different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



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

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

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

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

735 small points indicate different dates on which experiments were replicated

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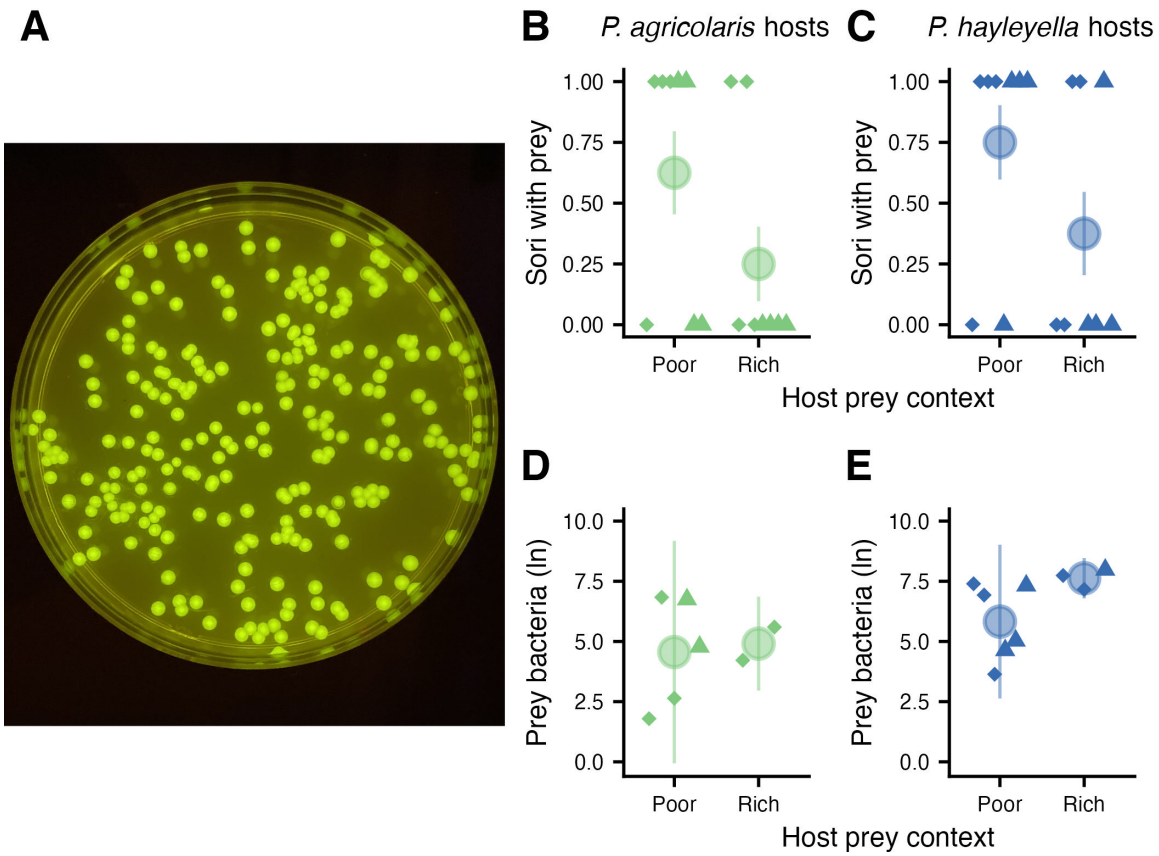

Figure 5: Hosts are less likely to carry prey bacteria in sori after developing in prey-rich environments. (A) Photo of fluorescent *K. pneumoniae* prey bacteria colonies plated from an individual sorus. (B&C) Sori with carried food bacteria for cured-and-reinfected *P. agricolaris* (B) or *P. hayleyella* (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

748 undiluted to 1:1000) of single fruiting bodies. Point shapes for individual fruiting bodies

749 (small points) indicate different dates on which experiments were replicated.

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