Intracellular *Burkholderia* Symbionts Induce Extracellular Secondary Infections; Driving Diverse Host Outcomes that Vary by Genotype and Environment

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Abstract

Symbiotic associations impact and are impacted by their surrounding ecosystem. The association between *Burkholderia* bacteria and the soil amoeba *Dictyostelium discoideum* is a tractable model to unravel the biology underlying symbiont-endowed phenotypes and their impacts. Several *Burkholderia* species stably associate with *D. discoideum* and typically reduce host fitness in food-rich environments while increasing fitness in food-scarce environments. *Burkholderia* symbionts are themselves inedible to their hosts but induce co-infections with secondary bacteria that can serve as a food source. Thus, *Burkholderia* hosts are “farmers” that carry food bacteria to new environments, providing a benefit when food is scarce. We examined the ability of specific *Burkholderia* genotypes to induce secondary co-infections and assessed host fitness under a range of co-infection conditions and environmental contexts. Although all *Burkholderia* symbionts intracellularly infected *Dictyostelium*, we found that co-infections are predominantly extracellular, suggesting that farming benefits are derived from extracellular infection of host structures. Furthermore, levels of secondary infection are linked to conditional host fitness; *B. agricolaris* infected hosts have the highest level of co-infection and have the highest fitness in food scarce environments. This study illuminates the phenomenon of co-infection induction across *Dictyostelium* associated *Burkholderia* species and exemplifies the contextual complexity of these associations.

36 **Introduction**

37 Symbiotic interactions can alter the fitness and evolutionary trajectory of both partners
38 (1–4). Clearly detrimental or mutualistic associations have been investigated for obvious
39 reasons: to eliminate infectious disease, boost health, and restore ecosystems.
40 However, many symbiotic associations evade simple characterization and related
41 mechanisms can underlie opposing outcomes (5,6). Invasion and replication strategies
42 employed by mutualists and pathogens often resemble each other, while genotypes and
43 external factors modify subsequent outcomes (7). Genotype pairing determines the
44 outcome of plant-mycorrhizae interactions (8) and amplification of a genomic region in a
45 normally beneficial *Wolbachia* symbiont leads to over-replication at the hosts expense
46 (9). Light mediates pathogenicity of a fungal plant endosymbiont (10), temperature
47 affects reproductive fitness of aphids hosting *Buchnera* (11), and parasitoid pressure
48 determines whether *Hamiltonella defensa* is beneficial to host aphids (12). These
49 examples demonstrate that even canonically beneficial or detrimental associations may
50 produce alternative effects in alternative contexts (4,13–17).

51 Eukaryotic microbes, such as amoebae, are attractive models for exploring eukaryote-
52 prokaryote interactions. Amoebae are ubiquitous and efficient phagocytic predators of
53 bacterial prey, making them important shapers of the microbial community (18). This
54 pressures prey microbes to evolve virulence strategies that enable evasion of
55 phagocytosis or subsequent digestion (19). Amoebae are thereby potential training
56 grounds and environmental reservoirs for bacterial pathogens. Amoebae phagocytosis
57 also enables bacteria to gain easy access to an attractive intracellular niche, bypassing
58 the requirement for evolving specialized cell-entry mechanisms. After invasion, bacteria

59 can be retained in an environmentally resistant cyst or spore (20). A number of bacterial
60 pathogens, such as *Francisella tularenis* and *Legionellae pneumophila* (21), are
61 harbored in different species of amoebae and there is a growing list of recently identified
62 amoebae symbionts (22,23).

63 The social amoebae *Dictyostelium discoideum* has been appreciated as a model host
64 for studying bacterial pathogens for some time (24,25). Recently, work with wild isolates
65 has emphasized its power for exploring naturally occurring microbial symbioses (26,27).
66 As a social amoeba *Dictyostelium* exhibits a unique life cycle, transitioning between
67 single- and multi-cellular forms. Under favorable conditions, it lives as a unicellular
68 amoeba, consuming bacteria and dividing by binary fission. When bacterial food is
69 depleted, amoebae secrete cAMP, which triggers the transition to multi-cellularity.
70 During this phase, amoebae aggregate to form a multicellular slug that seeks out a
71 location for fruiting body formation (such as the soil surface). Fruiting bodies are
72 comprised of a spherical sorus containing hardy spore cells resting atop a long stalk of
73 dead cells. This positioning of spore cells likely aids in their dispersal (28). Once
74 dispersed, spores germinate, and the cycle continues.

75 *D. discoideum* grown with a variety of bacterial food traditionally form germ-free sori,
76 clearing residual bacteria from the multicellular state during development. Microbial
77 clearance is aided by immune-like sentinel cells which engulf debris and slough off the
78 migrating slug (29,30). However, approximately one third of wild *D. discoideum* isolates
79 are naturally and stably colonized by *Burkholderia* bacteria (31,32). *Burkholderia* can be
80 easily eliminated from host populations with antibiotic treatment and new associations
81 can be readily initiated through co-culture. These *Burkholderia* symbionts establish

82 intracellular infections which persist through host development, resulting in sori
83 containing both extracellular and intracellular bacteria (32). *Burkholderia* symbionts
84 thereby remain associated with host populations during spore dispersal and can be
85 acquired through vertical and horizontal transmission routes. This mixed mode of
86 transmission has interesting implications for the fitness consequences and evolutionary
87 trajectory of the symbiosis.

88 *Burkholderia* symbionts of *D. discoideum* are members of the plant beneficial
89 environmental group within the *Burkholderia* genus (33). Symbiont strains are
90 genetically diverse, belonging to three species arising from two independent lineages:
91 *B. agricolaris, B. hayleyella,* and *B. bonniea* (34). *Burkholderia* differentially impacts
92 host fitness according to host-symbiont genotype combinations and environmental
93 context (31,32,35). Symbionts generally reduce host fitness in food rich conditions but
94 enhance fitness in food scarce environments (31,32,35). Fitness benefits are attributed
95 to retention of bacteria within host spores, allowing them to reseed new environments
96 with bacterial food. This trait is called farming and *Burkholderia* infected hosts are thus
97 referred to as “farmers”. *Burkholderia* symbionts themselves are poor food sources for
98 their hosts (31,32). However, *Burkholderia* infection appears to increase host
99 susceptibility to secondary bacterial infection, promoting the formation of a mini-
100 microbiome. It is these secondary bacteria that can serve as an amoebae food source
101 and thereby provide the farming benefit.

102 Given the importance of secondary infection in farming, we sought to explore the
103 underlying dynamics of this interaction. While a commonly used lab food strain, *K.*
104 *pneumoniae,* can be identified as an occasional co-infecting partner, co-infection

105 dynamics might vary depending on particular bacterial pairings (32). Thus, food bacteria
106 identity is an important environmental context that may affect outcomes. Furthermore,
107 the three different *Burkholderia* symbiont species have divergent evolutionary histories
108 of association with *D. discoideum*. While they have converged on the farming
109 phenotype, the effects and underlying mechanisms of infection may differ across
110 *Burkholderia* species (Haselkorn et al. in review).

111 Here, we reveal the density and location of secondary co-infections induced by each
112 *Burkholderia* species with a collection of secondary bacteria. Next, we clarified the
113 downstream benefits of *Burkholderia* infection in varied food availability contexts and
114 link these with host outcomes according to co-infection induction. Specifically, we
115 analyzed co-infection patterns and host outcomes with a variety of secondary bacteria
116 including: laboratory food *Klebsiella pneumoniae, Rhizobium* and *Serratia* isolates that
117 naturally co-occur with *D. discoideum*, and *Agrobacterium tumefaciens* and
118 *Pseudomonas aeruginosa* as pathogens that *D. discoideum* may encounter in nature.
119 We found that all *Burkholderia* symbionts induce some degree of secondary infection in
120 host sori but the density and location of secondary infections is dependent on
121 *Burkholderia* genotype and secondary bacterial identity. Contrary to previous inference,
122 secondary infections are predominantly extracellular with intracellular co-infections only
123 readily visualized in *B. agricolaris* infected spores. Overall, *B. agricolaris* induces the
124 highest density of combined co-infection resulting in a higher fitness benefit hosts in
125 food scarce environments. *B. bonniea* and *B. hayleyella* induce lower levels of
126 secondary co-infection but only *B. bonniea* provides significant host benefits under
127 specific dispersal conditions. This work illuminates the interplay between symbiont

128 genotypes and environmental context in mediating the expression and consequences of
129 novel symbiont endowed phenotypes.

130 **Materials and Methods**

131 Bacterial Strains and Culturing

132 *Burkholderia* strains were isolated in the Queller Strassmann lab from *D. discoideum*
133 hosts. *Klebsiella pneumoniae*, *Rhizobium* and *Serratia* originate from the Queller
134 Strassmann lab with *Rhizobium* and *Serratia* isolated from wild *Dictyostelium* fruiting
135 bodies grown directly from Virginia Mountain Lake Biological Station soil and 16s rRNA
136 gene sequenced with universal primers for putative identification (genbank accession
137 numbers MH997560 and MH997561). *Pseudomonas aeruginosa* PAO1-GFP (derived
138 from ATCC15692) was provided by R. Fred Inglis (36). *Agrobacterium tumefaciens*
139 (A136) was provided by Daniel Gage (reclassified as *Rhizobium radiobacter* 51350 in
140 ATCC). We used GFP labeled secondary bacteria for all experiments, with the
141 exception of *K. pneumoniae*, which is unlabeled when mixed with other bacteria. We
142 grew all bacteria on SM/5 medium (Formedium: 2g Peptone, 0.2g yeast extract, 2g
143 glucose, 1.9g KH_2PO_4, 1.3g $K_2HPO_4.3H_20$, 0.49g MgO_4.anhydrous, 17g agar/liter) at
144 room temperature. To prepare bacteria for culturing *Dictyostelium*, we suspended
145 bacterial colonies from SM/5 medium into KK2 (2.2g KH2PO4 monobasic and 0.7g
146 K2HPO4 dibasic/liter) and set to an OD_{600nm} of 1.5. For *K. pneumoniae*/secondary
147 bacterial mixtures, we combined bacterial suspensions equally by volume. For
148 *Burkholderia* infections, we added 5% by volume *Burkholderia*-RFP to indicated
149 bacterial mixtures.

150 Construction of Fluorescent Bacterial Strains

151 We generated RFP labeled *Burkholderia* by triparental mating with *E. coli* helper strain
152 E1354 (pTNS3-asdEc) and *E. coli* donor strain E2072 (pmini-Tn7-gat-P1-rfp) and
153 confirmed identity of RFP conjugants using a *Burkholderia* specific PCR as previously
154 described (32,37,38). We GFP labeled *Rhizobium, Serratia*, *A. tumefaciens,* and *K.*
155 *pneumoniae* through triparental mating with *E. coli* donor WM3064 (pmini-Tn7-KS-GFP)
156 and *E. coli* helper E1354 (pUXBF13) as previously described (39) and confirmed
157 identity of GFP positive conjugants through 16s rRNA gene sequencing. *P. aeruginosa*-
158 GFP was described previously (36).

159 *Dictyostelium* Culture Conditions

160 We used *D. discoideum* clone QS864 (naturally symbiont free) for all experiments.
161 Cultures were initiated by plating spores on SM/5 medium with *K. pneumoniae* and
162 incubating under lights at room temperature until fruiting bodies developed (4-7 days).
163 For experiments, 10^5 spores were harvested from developed sori and plated with 200µL
164 of the appropriate bacterial mixtures. For all experiments (unless otherwise indicated)
165 we analyzed sori 5 days after plating.

166 For co-infection assays we plated uninfected spores on bacterial mixtures with
167 *Burkholderia,* uninfected controls were plated without *Burkholderia.* To compare spore
168 productivity under food variable conditions, we harvested sori from indicated co-
169 infection conditions and plated 10^5 spores onto SM/5 with *K. pneumoniae* at an OD_{600} of
170 1.5 for food-rich conditions or with heat-killed (30 min at 80°C) *K. pneumoniae* at an
171 OD_{600} of 6 for food scarce conditions.

172 Spore Production Assays

173 To harvest total spores, we flooded each plate with 5–10 mL KK2 + 0.1% Nonidet P-40
174 alternative and collected the entire surface contents into 15-mL Falcon tubes. We then
175 diluted samples in KK2 and counted spores on a hemocytometer. At least five replicates
176 were analyzed for each treatment.

177 Confocal Microscopy

178 We imaged spores by staining with 1% calcofluor in KK2, placing on glass bottom
179 culture dishes (Electron Microscopy Sciences) and overlaying with 2% agarose. We
180 imaged samples on an Olympus Fluoview FV1000 confocal microscope using Plan Apo
181 Oil 1.4NA 60X objective. Z-sections were taken every 0.5 microns at 1024 resolution.
182 Calcofluor was visualized with DAPI, GFP with FITC, and RFP with Cy3 then
183 pseudocolored grey, green, and red respectively. We imaged at least three individual
184 replicates and counted more than 30 spores for each.

185 Colony forming unit quantification

186 To quantify secondary bacteria, we harvested sori grown from the indicated co-culture
187 conditions from 6- or 14-day incubations. We suspended individual sori in KK2+0.05%
188 Nonidet P-40 alternative, counted spores on a hemocytometer, plated serial dilutions on
189 SM/5 medium and incubated plates at room temperature until colony formation (~2
190 days), and counted GFP colonies using a safe-light imaging system. We performed 3 or
191 more independent replicates for each treatment.

192 Streak test

193 Our streak test assay was initiated from the indicated co-culture conditions by touching
194 individual sori with sterile pipette tips and transferring them to SM/5 plates along a
195 ~1inch streak. We incubated plates face up under lights at room temperature and
196 examined them 5 days (or 2 weeks) after streaking. We determined the percentage of
197 streaks with bacterial growth, percentage of bacterial positive streaks with fruiting
198 bodies, and number of fruiting bodies in positive streaks. Streaks were photographed on
199 a Cannon Eos7D with a macro-lens. Six sori were streaked for each replicate for at
200 least 4 individual replicates per condition.

201 Statistical analysis

202 We analyzed all data using R (version 3.3.1). For normally distributed data we
203 determined significance using a standard one-way analysis of variance (ANOVA) and a
204 *post hoc* Tukey HSD test. For non-normally distributed data we performed a Kruskall-
205 wallis test and *post hoc* analysis with a Dunn test using the dunnTest function in the
206 FSA package. We used *Burkholderia* status as fixed effects for all conditions.

207 **Results**

208 *Burkholderia* and Secondary Bacterial Combinations

209 To investigate the induction of secondary infection by *Burkholderia* symbionts, we
210 cultured an uninfected natural isolate of *D. discoideum* with different *Burkholderia*-RFP
211 and secondary bacteria-GFP combinations. We began with three *Burkholderia* strains:
212 Ba.70, Bh.11, and Bb.859, each representing one *D. discoideum* symbiont species *B.*
213 *agricolaris, B. hayleyella*, and *B. bonniea* (34). Secondary bacteria consisted of a

214 *Klebsiella pneumoniae* strain, soil isolated *Rhizobium* and *Serratia*, and lab
215 *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* strains. We chose these
216 representatives because: 1) *K. pneumoniae* is used widely as a lab food source for *D.*
217 *discoideum* and serves as a good starting point for experimental conditions. 2) The
218 *Rhizobium* and *Serratia* strains where co-isolated with *D. discoideum* and are thereby
219 ecologically relevant potential co-associates. 3) *A. tumefaciens,* in addition to its use in
220 plant molecular biology, is an important soil dwelling plant pathogen. As such, amoebae
221 may interact with *A. tumefaciens* in the environment and this could subsequently impact
222 the surrounding ecosystem. 4) *P. aeruginosa* is an important opportunistic human
223 pathogen whose association with other bacterial species in biofilms (such as pathogenic
224 *Burkholderia cenocepacia*) influences infection outcomes (40,41). Adding
225 *Pseudomonas* to the *Burkholderia-Dictyostelium* system provides a novel approach to
226 explore microbial interactions and virulence.

227 Host Outcomes Differ According to *Burkholderia* and Secondary Bacteria Conditions.

228 First, we examined host fitness when amoebae were co-cultured with *Burkholderia* and
229 secondary bacteria. We determined total spore productivity of host amoebae after one
230 social cycle on each labeled secondary bacterium, either alone or in a 50% mixture with
231 *K. pneumoniae.* Five percent by volume of *Burkholderia*-RFP was included to establish
232 infections (Figure 1). *D. discoideum* was unable to develop on any conditions where *P.*
233 *aeruginosa* was the only food source suggesting that this strain was toxic and/or
234 inedible for amoebae. All other conditions supported fruiting body development, but
235 spore productivity varied across conditions (Figure 1). In line with previous studies,
236 *Burkholderia* species differentially impact spore productivity on *K. pneumoniae*.

237 Typically, *B. hayleyella* was the most detrimental for host fitness with *B. agricolaris* and
238 *B. bonniea* being neutral or moderately detrimental. However, these patterns and the
239 degree by which symbiont altered host fitness varied across culture conditions (Figure 1
240 and Table 1). These results highlight the variability of fitness outcomes caused by
241 distinct *Burkholderia* symbionts and suggest that surrounding bacterial communities
242 also impact fitness outcomes.

243 Intracellular Co-infection is Rare and Depends on *Burkholderia* and Secondary Bacterial
244 Combinations.

245 To investigate the induction of secondary infections we imaged *D. discoideum* sori
246 contents after development on *Burkholderia* and secondary bacteria. We used 50/50 *K.*
247 *pneumoniae/*secondary bacteria-GFP conditions as they resulted in better amoebae
248 development than secondary bacteria-only conditions. We also imaged sori grown from
249 *K. pneumoniae*-GFP. Importantly, we do not detect any secondary bacteria in sori in the
250 absence of *Burkholderia* (Figure 2). Thus, these bacteria are not capable of infecting *D.*
251 *discoideum* on their own. In contrast, we can detect secondary-GFP cells in sori from
252 amoebae co-exposed to *Burkholderia* (Figure 3). To determine their prevalence in host
253 spore populations, we quantified the percent of spores intracellularly infected with
254 *Burkholderia*-RFP and with secondary bacteria-GFP. First, the percent of spores
255 infected with each *Burkholderia* species significantly differs (χ^2=44.02, df=2, p<0.001).
256 On average across conditions, *B. hayleyella* infects the most (89.2%), *B. bonniea*
257 infects an intermediate (68.6%), and *B. agricolaris* infects the fewest (33%) percent of
258 spores. However, we only readily observe intracellular secondary co-infections in *B.*
259 *agricolaris* host spores (average of 5.5% across conditions). Only rarely, if ever, do we

260 observe intracellular GFP in *B. hayleyella* and *B. bonniea* hosts (0.01% and 0.05%
261 respectively) (Figure 3). We did not observe intracellular GFP in the absence of
262 intracellular RFP, suggesting that secondary bacteria are only retained in *Burkholderia*
263 co-infected spores.

264 The secondary bacterium also plays a role in the prevalence of intracellular infections
265 for both *Burkholderia* and secondary bacteria. For instance, slightly fewer spores are
266 infected with *B. bonniea* when cultured with *P. aeruginosa*-GFP (29%) than with all
267 other bacteria (85.9-75.6%) (χ^2=10.843, df=4, p=0.028). On the flip side, significantly
268 more spores are infected with *B. hayleyella* when cultured with *P. aeruginosa*-GFP
269 (98.9%) than with all other bacteria (81.2-91%) (χ^2=10.217, df=4, p=0.036). For *B.*
270 *agricolaris* hosts, the degree of secondary co-infections significantly varied by
271 secondary bacteria (χ^2=15.019, df=4, p=0.004). *K. pneumoniae*-GFP is localized in only
272 0.2% of total spores while *Rhizobium*-GFP was localized in 14.9%. When secondary
273 bacterial infections are considered as a percentage of spores co-infected with *B.*
274 *agricolaris*, *Rhizobium*-GFP is co-localized in almost half of total infected spores. This
275 suggests that should *B. agricolaris* infection levels increase due to a higher infectious
276 dose or conditions that promote higher infection titers, secondary infections may
277 correspondingly increase.

278 <u>*Burkholderia* Symbionts Induce Extracellular Secondary Infections</u>

279 Although we found minimal intracellular co-infections in most conditions, the farming
280 phenotype may instead by explained by extracellular secondary infections. To get an
281 initial indication of extracellular co-infections, we determined the percent of confocal

282 images in which any extracellular GFP could be visualized (Figure 4a). We found that all
283 *Burkholderia* symbionts induced at least some level of extracellular co-infections, as we
284 could visualize external GFP in confocal images for each condition. Similar to our
285 observations for intracellular co-infections, extracellular secondary bacteria appeared
286 most frequently in *B. agricolaris* host sori (Figure 4a).

287 To quantify overall secondary co-infection, we counted GFP colony-forming units per
288 sori for *K. pneumoniae*-GFP$^{100\%}$ and *Rhizobium*-GFP$^{50\%}$ conditions. After six days of co-
289 culturing amoebae we counted the number of GFP positive colonies that developed
290 from individual sori (Figure 4b). No bacterial colonies were recovered when we plated
291 sori from fruiting bodies grown without *Burkholderia*, again indicating that these bacteria
292 do not by themselves infect *D. discoideum*. We found that *B. agricolaris* induces the
293 highest level of secondary infection, with *B. agricolaris* host sori colonized by an
294 average of 1.29×10^5 *K. pneumoniae*-GFP cfu's and 1.17×10^6 *Rhizobium*-GFP cfu's
295 (Figure 4b). We recover notable, albeit far fewer, secondary bacterial colonies from *B.*
296 *hayleyella* and *B. bonniea* hosts. *B. hayleyella* sori host an average of 206 and 169, and
297 *B. bonniea* an average of 153 and 276, *K. pneumoniae*-GFP and *Rhizobium*-GFP cfu's,
298 respectively.

299 To explore whether secondary bacteria could further amplify within fruiting bodies over
300 time, we also quantified R*hizobium*-GFP colony-forming units 14 days after plating. We
301 found that cfu's did not increase for *B. agricolaris* hosts, but dramatically increased for
302 *B. hayleyella* and *B. bonniea* hosts, which produced 2.87×10^5 and 8.37×10^5 GFP cfu's,
303 respectively. This brought the number of cfu's in all *Burkholderia* infected sori up to fairly
304 similar levels, perhaps representing a peak carrying capacity. However, we noticed that

305 the number of spores per sori for *B. hayleyella* and *B. bonniea* infected hosts appeared
306 to decrease over time (not shown). Thus, replication of secondary bacteria within these
307 sori could be damaging to spores, counter-acting potential benefits of hosting more food
308 bacteria.

309 <u>*Burkholderia* Symbionts Benefit Hosts in Food Scarce Conditions in Relation to Co-</u>
310 <u>infection Induction</u>.

311 Farmers have been shown to have an advantage when dispersed to food scarce
312 environments (31,32). This is attributed to the induction of secondary bacterial food
313 carriage, enabling host spores to reseed new environments with edible bacteria.
314 However, this benefit has previously only been measured as an average fitness
315 outcome across hosts infected with genotypically diverse *Burkholderia* symbionts (32).
316 Whether or how specific *Burkholderia* genotypes correspond with this beneficial
317 outcome remained unknown. Given our results demonstrating that *B. agricolaris*
318 induces the highest level of secondary co-infections, we speculated that *B. agricolaris*
319 hosts would have the highest reproductive fitness after dispersal to food scarce
320 environments.

321 To compare host fitness under different food availability conditions, we first plated
322 uninfected spores with *Burkholderia* and secondary bacteria (*K. pneumoniae*-GFP$^{100\%}$
323 and *Rhizobium*-GFP$^{50\%}$) under the same conditions employed previously. After five
324 days of incubation we harvested developed sori and transferred 10^5 spores with rich
325 (live *K. pneumoniae*-GFP) or scarce (heat killed *K. pneumoniae*) food on nutrient
326 medium. After five days of incubation in these conditions, we measured total spore

327 productivity (Figure 5a). For food rich conditions, we again found significant differences
328 according to amoebae infection status (F=7.41, df=3, p 0.0015 and χ^2=13.95, df=3,
329 p=0.0029 for *K. pneumoniae*-GFP$^{100\%}$ and *Rhizobium*-GFP$^{50\%}$ conditions respectively).
330 However, in this experiment only *B. hayleyella* hosts produced significantly fewer spores
331 than the uninfected control (Figure 5a). For food scarce conditions, spore productivity
332 was also significantly different according to *Burkholderia* infection status (χ^2=11.87 and
333 20.616, df=3, p=0.0078 and <0.001 for *K. pneumoniae*-GFP$^{100\%}$ and *Rhizobium*-GFP$^{50\%}$
334 conditions, respectively). Here, *B. agricolaris* hosts had the highest spore productivity
335 for both secondary conditions (Figure 5a). *B. bonniea* also resulted in slightly higher, but
336 not significantly different, spore productivity compared to the uninfected control (Figure
337 5a). Thus, *B. agricolaris* infections endow a benefit for their amoeba host when
338 dispersed to food scarce environments.

339 For the above assay, we evenly distributed spores on plates. If secondary bacteria are
340 less numerous in host sori (as for *B. hayleyella* and *B. bonniea*), they might be spread
341 too far from germinating spores to access and thus benefit from. Further, this assay
342 might not best simulate spore dispersal in nature, where spores might be deposited in
343 smaller denser patches by passing soil inhabitants. Therefore, we examined host fitness
344 in food scarce conditions using a “streak” dispersal strategy. Here, we deposited
345 individual sori from fruiting bodes grown on *K. pneumoniae*-GFP$^{100\%}$ or *Rhizobium*-
346 GFP$^{50\%}$ in small patches (~1-inch streaks) on nutrient medium. After a week of
347 incubation, we measured the percent of fruiting body positive streaks and the number of
348 fruiting bodies per each streak (Figure 5b-d).

349 Streaks from uninfected control sori did not produce bacterial colonies nor fruiting
350 bodies. Over 95% of *Burkholderia* infected sori produced streaks with bacteria,
351 however, the percent of streaks with fruiting bodies growing from these bacterial
352 colonies significantly varies across *Burkholderia* species (χ^2=13.728 and 12.127, df=2,
353 p=0.001 and 0.0023 for *K. pneumoniae*-GFP$^{100\%}$ and *Rhizobium*-GFP$^{50\%}$ conditions
354 respectively). *Burkholderia* species also significantly influences the number of fruiting
355 bodies per streak (χ^2=14 and 11.24, df=2, p<0.001 and 0.0036 for *K. pneumoniae*-
356 GFP$^{100\%}$ and *Rhizobium*-GFP$^{50\%}$ conditions respectively). *B. agricolaris* infected sori
357 generated significantly more fruiting bodies than *B. hayleyella* hosts from both
358 conditions and *B. bonniea* hosts from *K. pneumoniae*-GFP$^{100\%}$ conditions. The number
359 of fruiting bodies also increased the longer streak plates were left to incubate (Figure 6).
360 Since fruiting bodies often developed from *B. bonniea* host sori, this suggests they gain
361 better access to food under this dispersal strategy.

362 We rarely witnessed fruiting bodies from *B. hayleyella* host sori streaks, providing an
363 interesting counterpart to *B. bonniea*. Both symbionts induce similar densities of co-
364 infection (Figure 4) yet differ in downstream benefits (Figure 5). This may be explained
365 by the relative detriment each species exerts on its host. *B. hayleyella* reduces host
366 fitness compared to *B. bonniea*, whereas *B. bonniea* hosts are often indistinguishable
367 from uninfected counterparts (Figure 1 and 5). Thus, *B. hayleyella* toxicity may inhibit
368 host development despite food availability.

369 <u>Co-infections and Conditional Benefits are Consistent Across *Burkholderia* Species</u>
370 <u>Members.</u>

371 Our representative *Burkholderia* symbiont species significantly differed in their induction
372 of co-infection and host impacts. We next asked whether these phenotypes were similar
373 across strains of the same *Burkholderia* species. We imaged (Figure 7) and streak
374 tested (Figure 8) host sori for additional *Burkholderia*-RFP strains after growth on
375 *Rhizobium*-GFP$^{50\%}$. We again found noticeable, but low, levels of intracellular
376 *Rhizobium* in *B. agricolaris* infected spores, with co-infection rare or absent in *B.*
377 *hayleyella* and *B. bonniea* infected spores (Figure 5). The percent of spores infected by
378 *Burkholderia* was significantly different depending on genotype (χ^2=22.65, df=5, and
379 P<0.001), with *B. agricolaris* strains infecting fewer spores than *B. hayleyella* and *B.*
380 *bonniea* (Figure 5b). Despite low levels of intracellular *Rhizobium* co-infection, we
381 again frequently observed extracellular GFP in sori (Figure 7c).

382 Next, we investigated the benefits of infection by these strains in food scarce
383 environments (Figure 8). We found that *Burkholderia* genotype significantly influences
384 both the percentage of sori that generate fruiting bodies and the number of fruiting
385 bodies in positive streaks (χ^2=12.127, df=2, p=0.002 and χ^2=11.24, df=2, p=0.003,
386 respectively). In accordance with our previous pattern *B. agricolaris* infections result in
387 high, *B. bonniea* intermediate, and *B. hayleyella* low, levels of fruiting body production
388 (Figure 8). These results demonstrate that species members similarly induce co-
389 infections and result in similar subsequent host benefits.

390 **Discussion:**

391 Since elucidating the link between farming and *Burkholderia,* it has been assumed that
392 all *Burkholderia* symbionts allow for secondary co-infections through an intracellular co-

393 infection event. Intracellular co-infections of *B. agricolaris* with *K. pneumoniae* had
394 previously been identified (32). Co-phagocytosis with *Burkholderia* and subsequent
395 inhibition of phagocytic digestion was posited as a parsimonious mechanistic
396 explanation for the phenomenon. *Burkholderia* can be visualized within intracellular
397 vacuoles that appear similar to phagosomes (35) but how *Burkholderia* invades and
398 survive within amoebae is not currently resolved. Nonetheless, in this scenario
399 secondary bacteria must first be liberated from infected amoebae or spores (via
400 regurgitation or host cell lysis) so that surrounding amoebae may reap the benefits of
401 farming. Here, we found that co-intracellular infection is actually quite rare, occurring
402 with some frequency for only *B. agricolaris* strains, and occurring differentially across
403 secondary bacterial species. In contrast, all *Burkholderia* symbiont species generate
404 extracellular secondary bacterial infection that can be visualized for all secondary
405 species. The induction of overall secondary bacterial infection and its corresponding
406 downstream benefits is significantly different across symbionts. *B. agricolaris* strains
407 induce the highest levels of secondary infection (both intracellularly and overall) with *B.*
408 *hayleyella* and *B. bonniea* strains generating exclusively extracellular infections at
409 similarly low densities. This suggests that *Burkholderia* symbionts could induce
410 secondary infections by multiple mechanisms that qualitatively and quantitatively differ
411 between genotypes.

412 Overall, these results indicate that the predominant route by which different
413 *Burkholderia* symbionts induce farming leans more towards extracellular bacterial
414 carriage than intracellular co-infections. Susceptibility to secondary infections could be
415 due to *Burkholderia* symbionts compromising the primitive immune system of their

416 multicellular hosts. Sentinel cells serve as immune-like cells in multicellular slugs by
417 trapping unwanted cargo through direct phagocytosis and/or neutralization by DNA net
418 formation (29,30). When sentinel cells have accumulated cargo, they drop out the slug,
419 thereby cleansing it of potential toxic entities (29). A gene deletion that reduces sentinel
420 cells leads to retention of secondary bacteria through the slug stage and into the sorus
421 (29). *Burkholderia* host slugs have fewer sentinel cells than uninfected counterparts and
422 this defect goes away when hosts are cured of their *Burkholderia* symbiont via antibiotic
423 treatment (42). Thus, the induction of secondary infections could be an indirect
424 consequence of *Burkholderia* symbiosis resulting in sentinel cell reduction. This
425 scenario may be comparable to the phenomenon of secondary infections in mammalian
426 systems whereby primary infectious agents compromise the immune system of their
427 hosts. Despite this, we cannot rule out the possibility that extracellular secondary
428 infection originates from intracellular co-infection. It is possible that co-infected cells are
429 more susceptible to lysis, rupturing and spewing their secondary bacterial passengers
430 into the extracellular matrix. In either situation, secondary bacteria might then amplify
431 within sori.

432 Intracellular co-infections are most frequent with *B. agricolaris* and the soil *Rhizobium*
433 strain. This might reflect an ecologically relevant association between these species in
434 nature. *Burkholderia* and *Rhizobium* are both ubiquitous in soil and contain several
435 important symbiont species which have been found co-colonizing the same hosts (43–
436 46). Predation by amoebae in soil and aquatic systems shapes microbial community
437 assembly and overall food webs (47). Given the likely co-occurrence of soil amoebae
438 with *Burkholderia* and other soil microbes it's tempting to speculate on how these

439 multipartite interactions influence overall microbial communities and higher trophic
440 levels. Here, we show that amoebae co-disperse *Burkholderia* symbionts and
441 secondary bacterial hitchhikers to new environments. Thus, the impact of amoebae on
442 their surrounding microbial network can go well beyond predator-prey dynamics. Finally,
443 our observation of *Burkholderia* and *P. aeruginosa* co-infection amplifies the concern
444 that soil amoebae can serve as reservoirs for bacterial pathogens. These results
445 suggest that *Burkholderia* symbionts can increase the suite of potential pathogenic
446 partners hosted by amoebae.

447 *Burkholderia*-fungal associations have been well recognized for their importance in the
448 soil ecosystem and for their bio-restoration potential (48–50). There are compelling
449 parallels between *Burkholderia-Dictyostelium* and *Burkholderia-fungal* associations.
450 Some *Burkholderia* (notably *B. terrae*) are capable of adhering to and migrating with
451 growing fungal hyphae through soil (51). Similar to our system, some of these fungal
452 associates assist in the co-migration of other (non-migrating) bacteria (52). Several
453 mechanisms have been proposed to underlie these interactions, such as direct receptor
454 binding and indirect biofilm co-aggregation (53). *B. terrae* extracellularly colonizes
455 fungal hyphae but many other *Burkholderia* symbionts of diverse hots persist
456 intracellularly (13,54). A particularly interesting example is the *Rhizopus microsporus*
457 endosymbiont *B. rhizoxinica* fungi, which produces the rice seedling blight toxin (55).
458 Recently, secretion systems have been shown to be important for the active invasion of
459 *B. rhizoxinica* across the fungal cell wall and into the host cytoplasm (54). Secretion
460 systems have also been implicated in *B. psuedomallei* infections (56,57). However
461 many plant mutualistic *Burkholderia* species, which are closer relatives to *Burkholderia*

462 symbionts of *Dictyostelium,* appear to lack some of these systems (56–58). The
463 hypothesized portal of entry into *Dictyostelium* is via phagocytosis, which could
464 circumvent the need for invasion specific mechanisms. Overall, *Burkholderia* symbionts
465 of other hosts can help inform our understanding of the *Burkholderia*-amoebae
466 symbiosis and vice versa.

467 Biofilm formation is particularly intriguing to consider as a mechanistic explanation of
468 secondary infection. *Burkholderia* adherence to secondary bacteria would increase the
469 likelihood of co-phagocytosis or extracellular co-colonization. Different adhesive
470 capacities of *Burkholderia* and secondary species could explain differences in the extent
471 of secondary infections across bacterial combinations. Indeed, co-aggregation could be
472 a simple explanation for the observation of high levels of co-infection with *B. agricolaris*
473 and *Rhizobium*. Interestingly, recent work implicates *Dictyostelium* lectins in the farming
474 phenomenon, higher lectin expression was detected in farmer *D. discoideum* clones
475 and addition of endogenous lectins induced bacterial carriage (59). Although this work
476 did not consider the presence or impact of *Burkholderia*, we think *Burkholderia*
477 symbionts play a key role. *Burkholderia* could induce farming via induction of lectin
478 expression in amoebae or more simplistically, *Burkholderia* lectins may mediate
479 adherence of secondary bacteria. Indeed, lectin expression by *B. cenocepacia* is an
480 important component of biofilm formation and lectin aids in adherence of *B. cepacia* to
481 host tissues (40,60). Future exploration into lectin expression and adhesion
482 mechanisms will be helpful for clarifying these themes.

483 In addition to elucidating the phenomenon of secondary infections our results exemplify
484 the context dependency of symbiotic outcomes in this system. We found that the costs

485 and benefits of this symbiosis can be modified by different bacterial conditions and
486 spore dispersal processes. The nature and extent of farming induction by *Burkholderia*
487 symbionts differs across symbiont species and so do their corresponding contextual
488 fitness outcomes. Ultimately, further research into the mechanisms, consequences, and
489 ecological framework of the *Burkholderia-Dictyostelium* symbiosis will help illuminate
490 microbial interaction dynamics relevant to infection biology and microbial ecology.

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495 **Conflict of interest** We declare no conflict of interests.

496 **Author Contributions Statement**

497 NK and SD designed the study. NK and SD performed experiments with assistance
498 from ME, RN, and TH. SD and TH wrote the manuscript.

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501 **Data Availability Statement**

502 The raw data supporting the conclusions of this manuscript will be made available by
503 the authors, without undue reservation, upon request.

504 **Table 1. *Burkholderia* infections significantly alter spore productivity in most (but**
505 **not all) bacterial culture conditions.** Statistical Analysis of Spore Fitness from Figure
506 1. All DF values=3.

Condition	χ^2	p value
K. pneumoniae-GFP$^{100\%}$	23.537	>0.001
Rhizobium-GFP$^{100\%}$	20.81	>0.001
Rhizobium-GFP$^{50\%}$	18.759	>0.001
Serratia-GFP$^{100\%}$	6.411	0.09
Serratia-GFP$^{50\%}$	11.292	0.01
Agrobacterium-GFP$^{100\%}$	18.76	>0.001
Agrobacterium-GFP$^{50\%}$	16.267	>0.001
Pseudomonas-GFP$^{50\%}$	23.36	>0.001

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682 **FIGURES**
683
684 **Figure 1. Culture Conditions Modify the Impact of *Burkholderia* Infections on Host**
685 **Fitness.** Total spore productivity for *D. discoideum* after one round of development on
686 the indicated GFP labeled bacterial species (Kleb= *K. pneumoniae*, Rhizo=*Rhizobium*,
687 Serr=*Serratia*, Agro=*A, tumefaciens,* and Pseu=*P. aeruginosa*. 50% cultures are mixed
688 with 50% unlabeled *K. pneumoniae* by volume). Dark grey, light grey, and medium grey
689 boxes indicate cultures wherein *Burkholderia* infections are initiated by inclusion of 5%
690 *B. agricolaris.*70-RFP, *B. hayleyella.*11-RFP, and *B. bonniea.*859-RFP, respectively.
691 Points within boxes indicate individual replicates. Letters indicate post-hoc significance
692 within panels.
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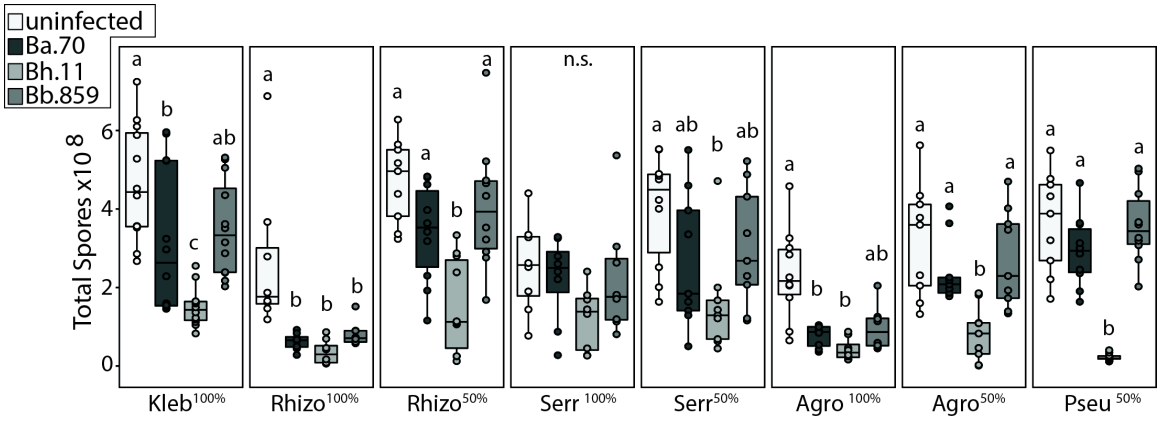

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699 **Figure 2. Secondary Bacteria do not Infect Symbiont Free Spores.** Representative
700 confocal micrographs of sori contents after growth on the indicated GFP labeled
701 bacterial cultures. Scale bar=10um.
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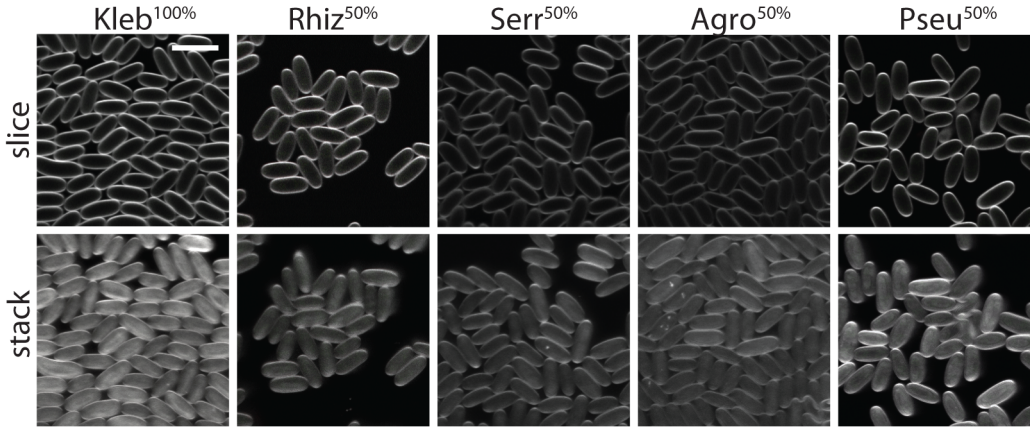

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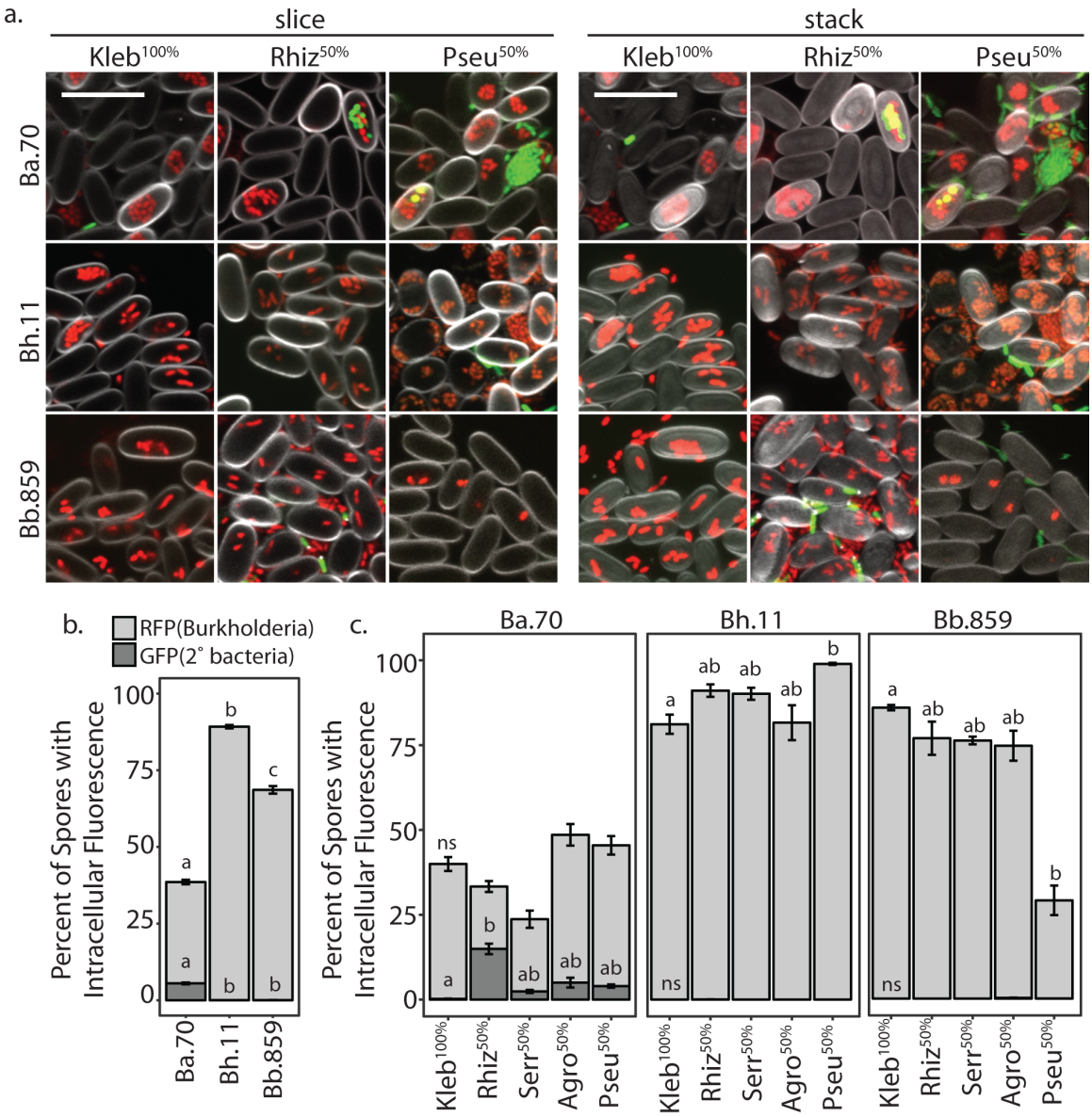
Figure 3. *Burkholderia* Differentially Induce Intracellular Co-Infections. a) Representative confocal micrographs of *Burkholderia*-RFP infected sori contents after development on the indicated GFP labeled secondary bacteria. Scale bar= 10um. b) Percent of spores infected with *Burkholderia*-RFP (light grey) and co-infected with secondary bacteria-GFP (dark grey) averaged across all replicates of all secondary bacterial conditions. c) Average percent of spores infected (as in b) for each secondary culture condition. Error bars ±SE.



723 **Figure 4. *Burkholderia* Induce Extracellular Co-infections.** a) Percent of confocal
724 micrographs wherein secondary bacterial-GFP cells were visualized extracellularly
725 within sori contents. Numbers nested within bars indicate the number of images with
726 visible GFP over the number of total images collected per condition. b) Number of GFP
727 colony forming units (log 10) from *Burkholderia* infected sori contents when grown on *K.*
728 *pneumoniae*-GFP and *Rhizobium*-GFP. Individual sori contents were harvested either 6
729 or 14 days after plating on the *Burkholderia*/Secondary bacterial culture conditions.
730 These numbers may represent both extracellular and intracellularly derived secondary
731 bacteria. Points represent the GFP-CFU count for individual replicates.
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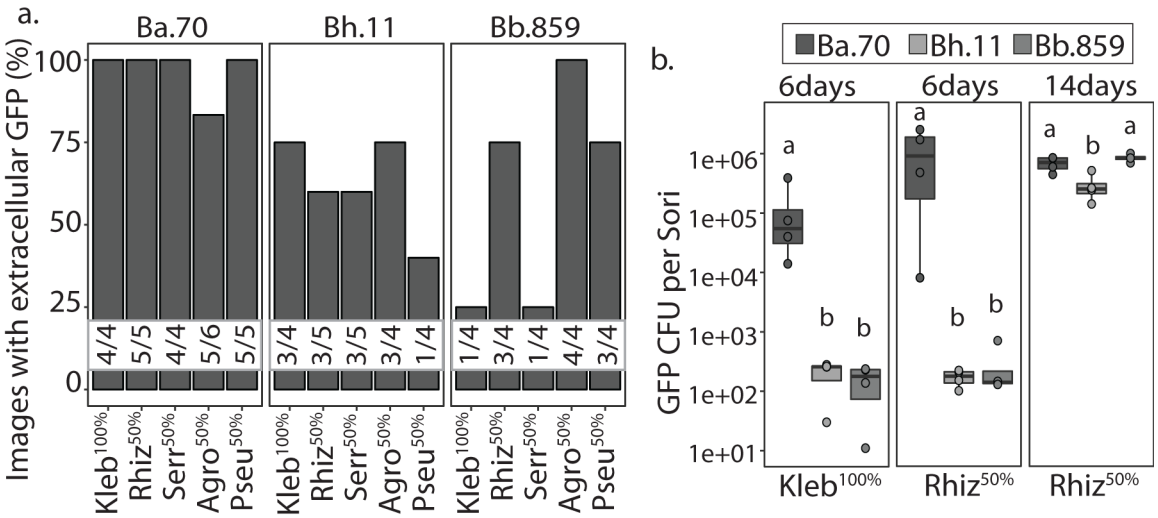

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Figure 5. Farming Benefits Vary by Symbiont and Dispersal Strategy. For all figures, top panels represent data from sori pre-grown on *K. pneumoniae*-GFP$^{100\%}$ and bottom panels represent sori pre-grown on *Rhizobium*-GFP$^{50\%}$, both with (greys) and without (white) supplementation of 5% *Burkholderia*. a) Quantification of total spores harvested from food rich and food scarce plating conditions after transfer from *K. pneumoniae*-GFP$^{100\%}$ (top) or *Rhizobium*-GFP$^{50\%}$ (bottom) plating conditions. Points represent data from each individual replicate. b) Representative images of individual sori streaks from fruiting bodies developed on *K. pneumoniae*-GFP$^{100\%}$ (top) or *Rhizobium*-GFP$^{50\%}$ (bottom) plates. Top panels are magnified sections of bottom panels. c) Percentage of bacterial positive sori streaks with observable fruiting bodies. Error bars ±SE. d) Number of fruiting bodies per fruiting body positive streaks for each individual replicate. All letters indicated post-hoc significance within panels. For streak tests, all sori where streaked five days after plating on *K. pneumoniae*-GFP$^{100\%}$ (top panels) or *Rhizobium*-GFP$^{50\%}$ (bottom panels) and all data was collected 5 days after streaking.

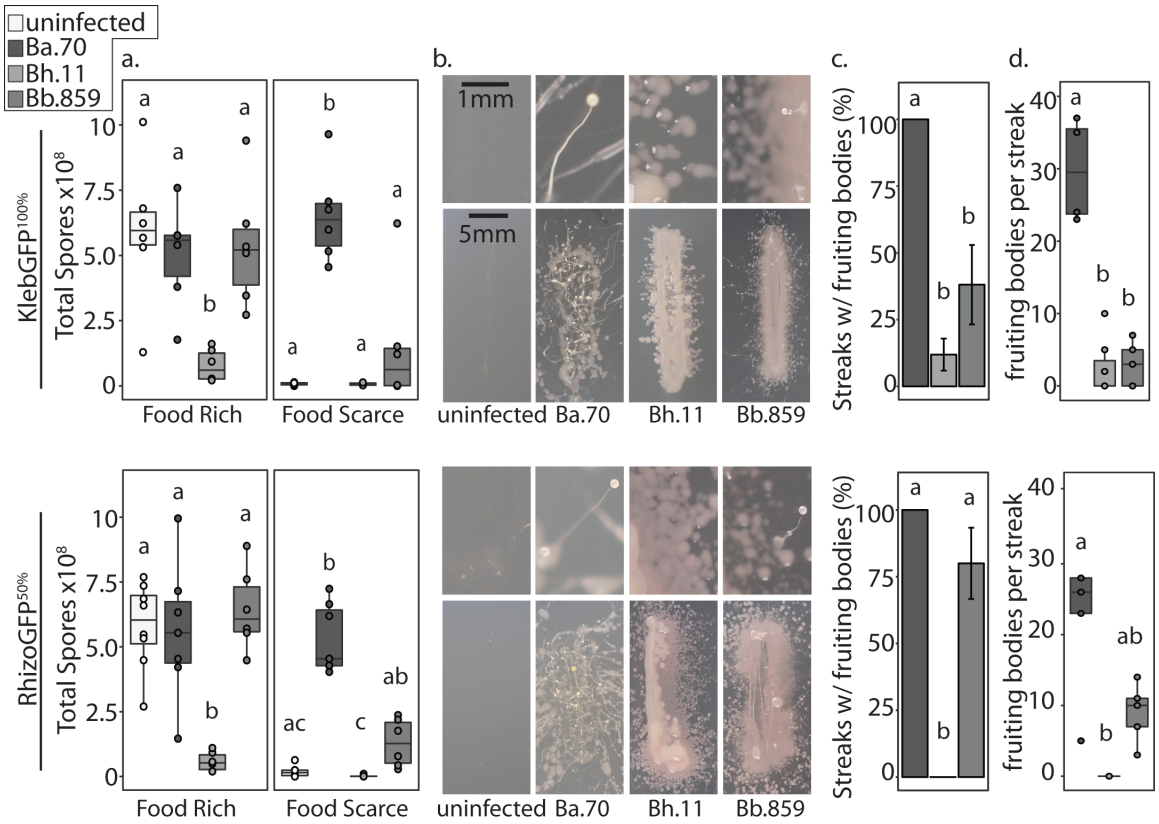

Figure 6. Fruiting Bodies Amplify in *Burkholderia* Infected Sori Streaks. Images of sori streaks two weeks after streak-testing. Sori were harvested from individual fruiting bodies that developed five days after plating spores on *Rhizobium*-GFP$^{50\%}$ culture conditions with 5% of the indicated *Burkholderia*-RFP strain.

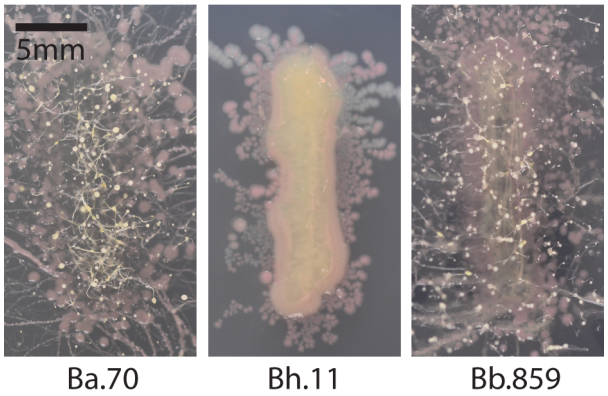

Figure 7. Co-infection Patterns Correspond to *Burkholderia* Species. a) Representative confocal micrographs of sori after five days post plating with 5% of the indicated *Burkholderia*-RFP strain and *Rhizobium*-GFP$^{50\%}$. b) Average percent of spores visualized with intracellular *Burkholderia*-RFP (light grey) and intracellular *Rhizobium*-GFP (dark grey) for each of the indicated *Burkholderia* species. Letters indicate post-hoc significance. c) Percent of images in which external GFP was visualized out of the total number of independent images acquired. Numbers above bars represent the raw number of external GFP positive images over the number of images acquired for each *Burkholderia* infection condition.

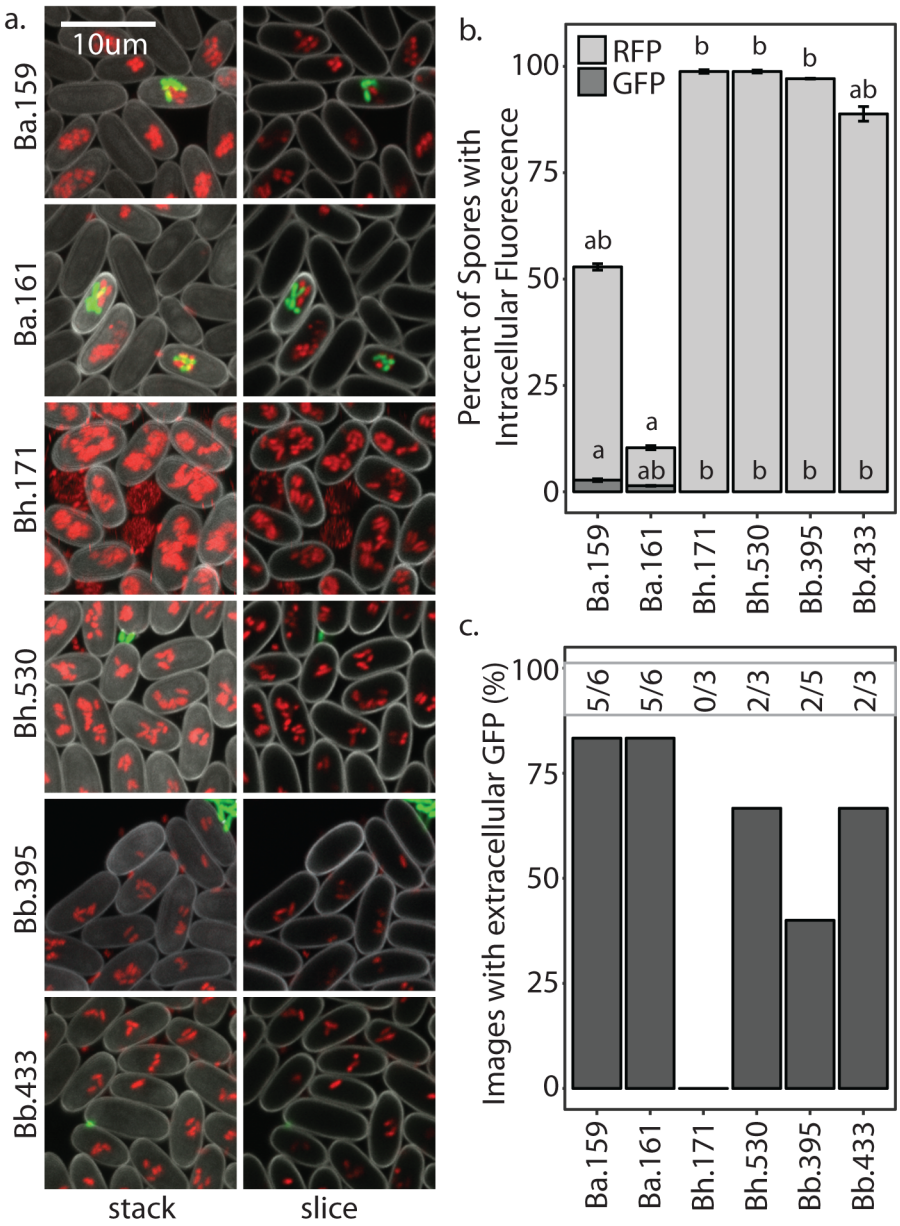
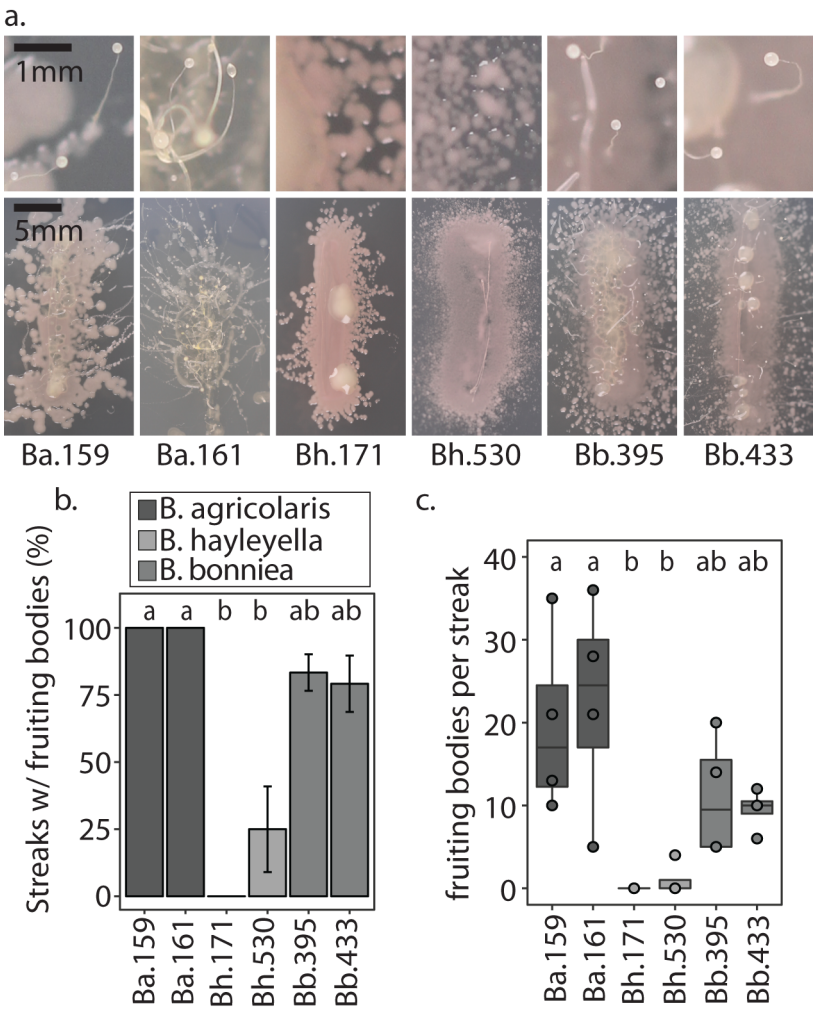
Figure 8. Host Benefits Correspond to *Burkholderia* Species and their Induction of Co-infections. a) Images of representative sori streaks from fruiting bodies grown on *Rhizobium*-GFP$^{50\%}$ with 5% of the indicated *Burkholderia* strain. Top panels are magnified sections of bottom panels. b) Percentage of bacterial positive sori streaks with observable fruiting bodies. Error bars ±SE. c) Number of fruiting bodies per fruiting body positive streaks for each individual. All letters indicated post-hoc significance within panels. All sori where streaked five days after plating on *Rhizibium*-GFP$^{50\%}$ and all data was collected 5 days after streaking.