

# Interspecies bacterial competition determines community assembly in the *C. elegans* intestine

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## 1 **Abstract**

2           From insects to mammals, a large variety of animals hold in their intestines complex  
3 bacterial communities that play an important role in health and disease. However, the  
4 complexity of these gut microbiomes and their hosts often constrains our ability to understand  
5 how these bacterial communities assemble and function. In order to elucidate basic principles of  
6 community assembly in a host intestine, we study the assembly of the microbiome of  
7 *Caenorhabditis elegans* with a bottom-up approach. We colonize the gut of the worm *C. elegans*  
8 with 11 bacterial species individually, in all possible pairs, and in selected trios, and we find an  
9 organized mixture of coexistence and competitive exclusion that indicates a hierarchical  
10 structure in the bacterial interactions. The capacity of a bacterial species fed in monoculture to  
11 colonize the *C. elegans* intestine correlates with its average fraction in co-culture experiments,  
12 yet fails to predict its abundance in many two- and three-species microbiomes. Hence, the  
13 bacterial fractional abundances in co-culture experiments—pairwise outcomes—are influenced  
14 by interspecies interactions. These pairwise outcomes accurately predict the trio outcomes in  
15 the worm intestine, further highlighting the importance of pairwise interactions in determining  
16 community composition. We also find that the *C. elegans* gut environment influences the  
17 outcome of co-culture experiments, and demonstrate that the low intestinal pH is one of the  
18 causes. These results highlight that a bottom-up approach to microbiome community assembly  
19 may provide valuable insight into the structure and composition of complex microbial  
20 communities.

## 21 **Introduction**

22           Bacterial communities are found almost everywhere in nature. Among the many  
23 ecosystems in which bacterial communities play a fundamental role, the animal digestive tract is  
24 one of remarkable importance. These large, complex, and highly organized consortia can

25 degrade food and deliver nutrients to their host, protect against invading pathogens, and even  
26 produce neurotransmitters that affect host behavior (1–7).

27         Despite considerable recent efforts toward elucidating the contents of these bacterial  
28 communities (8–11), the rules that govern their assembly are still poorly understood.  
29 Complicating this problem is the fact that these microbial communities are exceedingly complex  
30 and difficult to manipulate experimentally. The human gut microbiome, for instance, consists of  
31 approximately  $4 \times 10^{13}$  cells distributed across hundreds of species interacting in myriad ways  
32 across different regions of the gut (12). Moreover, these complex microbial communities vary  
33 between individuals based on many factors, including environmental conditions, diet, and the  
34 host genotype and immune system (13,14). This complexity and variability has stymied efforts  
35 to uncover general rules for microbial community assembly in the context of the host  
36 environment.

37         Current efforts have taken advantage of model systems to experimentally address the  
38 composition and assembly of simpler gut microbiomes (15,16). Animals such as zebrafish (17–  
39 19), honey bees (20–23), leeches (24–27), flies (28–31), and worms (32–35) can be raised and  
40 manipulated in the laboratory in order to assess their microbiomes. The bacterial communities  
41 associated with these animals contain a small number of taxa relative to humans, allowing  
42 bottom-up assembly of comprehensive, synthetic microbial communities. These experimentally  
43 tractable models provide an opportunity to observe population dynamics in host-associated  
44 microbiomes, by exposing a selected population of hosts to colonization under controlled  
45 conditions and monitoring the ensuing interactions among microbes and between microbes and  
46 host.

47         Here we use the nematode *Caenorhabditis elegans* (36) as a simple gut model to  
48 address issues of experimental tractability and to gain intuition about community assembly in

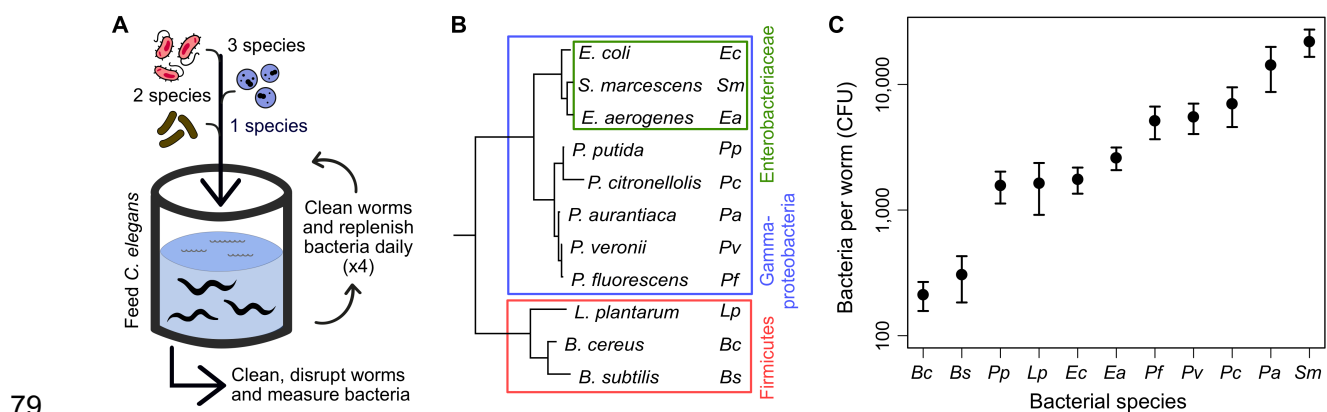
49 microbiomes. Despite comprising only 20 epithelial cells, the *C. elegans* intestinal tract shares  
50 many physiological features with the intestines of higher organisms, including microvilli, a mucin  
51 layer, epithelial junctions, cyclical movement via contraction of muscles, and extensive  
52 interaction with the host immune system (37–43). The gut of *C. elegans* is well known to host  
53 stable populations of bacteria, including important pathogens and probiotics of humans, at  
54 substantial population levels up to  $10^5$  bacterial cells per worm (34,44–46). Furthermore, recent  
55 reports have suggested that the gut of this millimetric organism is capable of filtering its bacterial  
56 environment and selecting a core microbiota (35).

57         In this study, we colonized the intestine of *C. elegans* with simple microbiomes to  
58 determine patterns in the assembly of microbial communities in a host intestine. We found that  
59 the inherent ability of a bacterial species to colonize the worm gut was an acceptable predictor  
60 of that strain’s average performance in two-species microbiomes, but these single-species data  
61 became inadequate at predicting the outcomes of specific two- and three-species communities.  
62 This suggests that interactions between bacterial species determine the shape and form of the  
63 microbial communities in this host. Importantly, we find that the outcome of two-species feeding  
64 experiments can be used to predict the composition of the three-species feeding experiments,  
65 indicating that “assembly rules” may provide insight into the composition of microbiome  
66 communities. Further, we identified a conserved bacterial competitive hierarchy between *in vivo*  
67 gut and *in vitro* liquid medium that is disabled in specific cases by the acidity of the *C. elegans*  
68 intestine. With this, we advance our understanding of the environmental filtering imposed by *C.*  
69 *elegans* and provide insight into bacterial community assembly.

## 70 **Results**

71         To investigate the colonization and growth dynamics of different bacterial species in the  
72 gut of *C. elegans*, we fed germ-free synchronized adult worms with eleven single bacterial

73 species over four days in a well-mixed liquid medium (Fig 1A, Methods). This length of feeding  
74 was long enough to ensure reliable colonization of the worms, yet short enough to avoid  
75 problems associated with pathogen-based killing by our strains (47). After this feeding and  
76 colonization period, we allowed worms to feed briefly on heat-killed bacteria to remove transient  
77 colonizers, cleaned the surface of the worms, and measured the intestinal bacterial densities by  
78 grinding batches of worms and counting colony forming units (CFU, hereafter called *cells*).

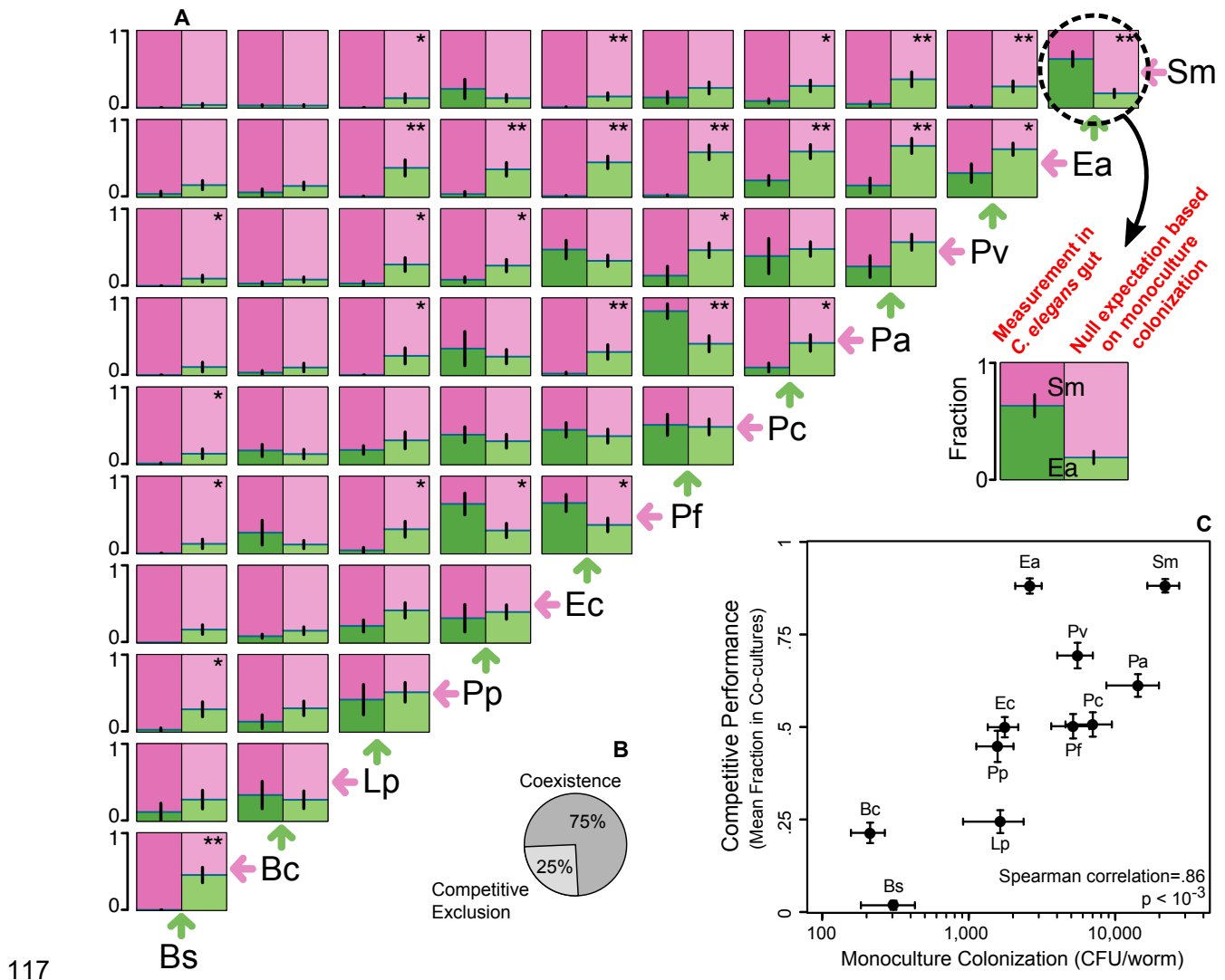


79  
80 **Figure 1. Eleven bacterial species colonize the gut of the worm in monoculture. (A)** One,  
81 two or three bacterial species are fed in liquid culture to a sterile population of *C. elegans* AU37.  
82 Equal bacterial concentrations are maintained during 4 days of colonization. Afterwards, worms  
83 are mechanically disrupted in batches of ~20 and CFU counts are used to determine bacterial  
84 population sizes. **(B)** Phylogeny of the 11 bacterial species used to colonize the gut of *C.*  
85 *elegans*. **(C)** Population sizes in monoculture colonization span two orders of magnitude and  
86 reflect the inherent capabilities of bacteria to colonize the worm intestine environment. Points  
87 represent the average of 8 or more biological replicates, and error bars as the standard error of  
88 the mean (SEM).

89 We utilized a set of eleven bacterial species representing the phyla Firmicutes (gram-  
90 positive) and Proteobacteria (gram-negative) (Fig 1B). From the latter phylum, we included soil  
91 isolates from the families *Enterobacteriaceae* and *Pseudomonadaceae*, abundant bacterial

92 clades found in the core microbiota of *C. elegans* grown on its native environment of decaying  
93 organic matter (46,48,49), but none of our strains was directly isolated from nematodes. These  
94 experiments can therefore be viewed as probing community assembly of a gut microbiome with  
95 bacterial species encountered in the natural environment and without a period of evolutionary  
96 adaptation. We found that all bacterial species successfully colonized the *C. elegans* intestine,  
97 with population sizes ranging from two hundred cells per worm in the case of *Bacillus cereus*  
98 (*Bc*), up to tens of thousands of cells in the case of *Serratia marcescens* (*Sm*) (Fig 1C). These  
99 differences in bacterial population sizes in monoculture colonization reflect differences in the  
100 ability of each bacterial species to not trigger an aversive olfactory response in *C. elegans* (50–  
101 52), survive passage through the *C. elegans* grinder (53–56), adhere to the intestinal lumen  
102 (32,57–59), proliferate within the worm, or a combination of these features (60).

103         Despite the importance of revealing which of these characteristics allows each bacterial  
104 species to surpass the environmental filtering of the worm gut and reach a given population  
105 size, in this study we aimed to understand the influence of monoculture colonization in the  
106 assembly of the *C. elegans* microbiome. Thus we constructed the simplest intestinal  
107 communities by performing all possible co-culture experiments with these eleven bacterial  
108 strains (55 pairs in total). We fed worms in a well-mixed liquid medium with pairs of bacteria  
109 present at equal concentrations (*cells/mL*) to ensure that strains had the same initial probability  
110 to be ingested (Methods). We found that a majority (41/55~75%) of pairs displayed coexistence  
111 of the two species (Fig 2A, left panels), whereas the remainder (14/55) led to competitive  
112 exclusion of a species (Fig 2B). Given that we are feeding the worms at equal concentrations,  
113 we cannot detect the possibility of bistability, in which the outcome of competition depends upon  
114 the starting ratio of the two species. Some bacterial species consistently exclude other species  
115 (*Sm*) or are excluded (*Bs*), but the large amount of coexistence (Fig S1) makes the simple  
116 winner/loser classification inappropriate for the observed bacterial competitive abilities.



117

118 **Figure 2. Monoculture colonization relates to, but does not predict pairwise outcomes in**

119 **the worm intestine. (A) Left:** Fractional abundances of 55 co-culture experiments inside the *C.*

120 *elegans* intestine. Error bars as the SEM of 2 to 8 biological replicates (Fig S1). Bacterial strains

121 are ordered by its mean fractional abundance. **Right:** Null expectation for the fractional

122 abundances based on a non-interacting model where each bacterial species reaches its

123 monoculture population size. Null expectation as the mean of all possible pairings of

124 monoculture information, and error bars as the SEM (Methods). \* and \*\* represent a statistically

125 significant difference between measurement and null expectation at *p*-values of .05 and .01,

126 respectively (Welch's T-test). **(B)** Coexistence is common and competitive exclusion at a

127 detection limit of 2% is rare. **(C)** Competitive performance, as the mean fractional abundance in  
128 co-culture experiments, correlates with monoculture population size. X-axis as in Fig 1B, and  
129 error bars on Y-axis as the propagated error from the SEM of each pairwise competition.

130 A better summary-metric of the pairwise outcomes is the mean fractional abundance of a  
131 species when competed against each of all the other species. Full dominance, extinction, and  
132 coexistence award 1 point, 0 points, and the fraction of point at which the species is present in  
133 each co-culture experiment, respectively, and the sum is then divided by the number of  
134 competitors. We found that this competitive performance score correlates to the population size  
135 reached in monoculture colonization (Fig 2C, Spearman correlation  $[r_s]=.86$ ,  $p<10^{-3}$ ). This  
136 positive relationship indicates that a bacterial species persists in two-species microbiomes due  
137 to similar properties to those favoring its monoculture colonization of the gut. Interestingly, the  
138 population sizes do not correlate to the mean relative yields (*cells/worm* of a species in  
139 competition divided by *cells/worm* of the same species in monoculture), indicating that a large  
140 population size does not protect a bacterial species from being harmed by competition in co-  
141 culture experiments (Fig S2).

142 The interactions we measured can be categorized as hierarchical, since a highly ranked  
143 competitor will frequently exclude or dominate a low-rank adversary (dominance of pink-color in  
144 left panels of Fig 2A). The hierarchy score of this network, 0.82, is significantly larger than the  
145 hierarchy score found in random matrices with the same distribution of pairwise outcomes ( $p$   
146  $<10^{-5}$ , Methods), suggesting that the hierarchy seen in our experiments is caused by bacterial  
147 features acting in a transitive manner. Consistent with this, we do not observe any cases of  
148 intransitive competition, in which the pairwise interactions of three bacterial species would be  
149 analogous to the rock-paper-scissors game and no absolute winner would exist (61,62). This  
150 intransitivity has been proposed as a major mechanism inducing coexistence in natural  
151 populations (63–67), but we do not observe it in any of our 165 possible trios. With a more



152 relaxed definition of non-transitivity, in which a species wins a competition by being more  
153 abundant than the competitor instead of needing to fully exclude it, we find two candidate trios  
154 with a rock-paper-scissors-like structure: *Ec-Pf-Pa* and *Pp-Pf-Pa* (although the dominance of  
155 some competitors is not statistically significant).

156 *Lp*, *Bs*, and *Bc*, the three species representing the Firmicutes phylum, were  
157 competitively excluded in most co-culture experiments, consistent with their poor colonization in  
158 monoculture. Likewise, the strongest single species colonizer, *Sm*, dominates the final  
159 population in most of its pairwise competitions. However, not all strains and interactions acted  
160 according to expectations from single species colonization (68–70). For example, even though  
161 its monoculture population size was just above average, *Enterobacter aerogenes* (*Ea*) was  
162 capable of outcompeting most of its adversaries, suggesting that direct interactions between  
163 microbial species can play a significant role in determining the outcome of pairwise colonization.

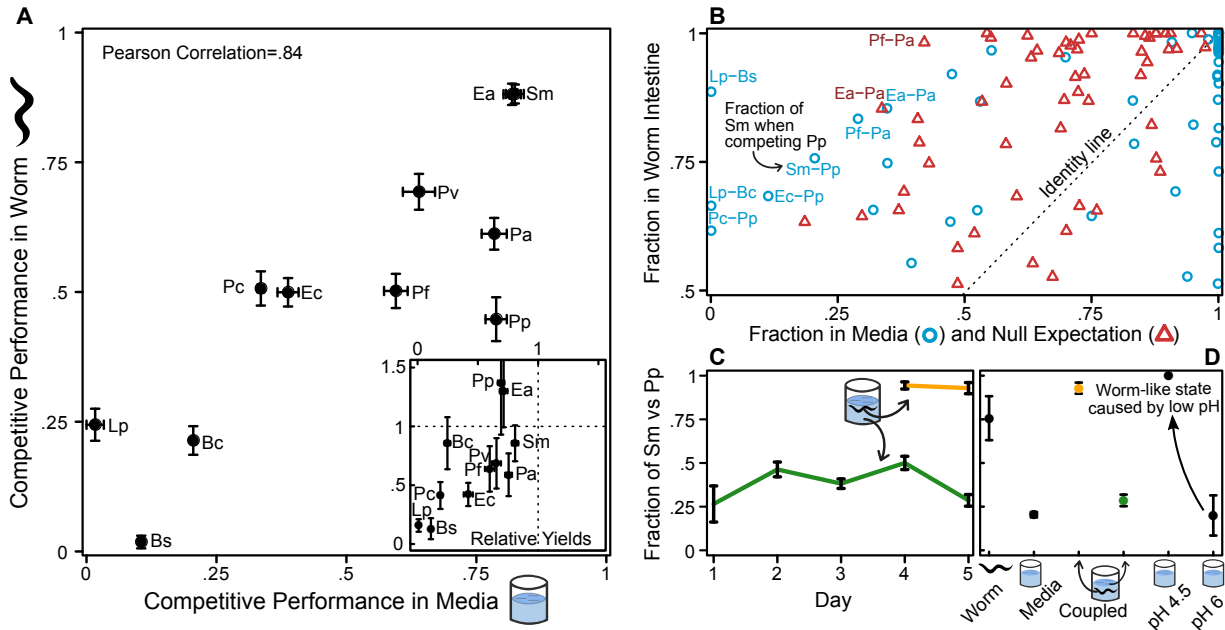
164 We therefore sought to determine whether interactions between microbes are an  
165 important determinant of community structure, or whether monoculture colonization ability is  
166 sufficient to predict the outcomes of pairwise competition in the host. We calculated a null  
167 expectation for the fractional abundances assuming that each species is able to reach the  
168 carrying capacity that was measured in monoculture colonization (Fig 2A, right panels). By  
169 comparing this null expectation with the experimentally measured fractions obtained from  
170 pairwise colonization (Fig 2A, left panels), we are able to identify the cases in which interspecies  
171 interactions play a dominant role in determining the composition of the gut microbiome. Across  
172 all pairs, the measured fractions deviate from the null expectations by a mean distance of .2,  
173 with a peak at low distance and a long tail corresponding to cases where the interspecies  
174 interactions are particularly important (Fig S3). In 28 cases this deviation is large enough to  
175 reject the null model ( $p < .05$ ), many more than the 2.75 cases expected by chance at this  
176 significance level and with these 55 pairwise combinations. This result indicates that a null

177 expectation, where each species' abundance in pairwise colonization is largely determined by  
178 the initial filtering event rather than by interactions between bacteria, is valid for some but not all  
179 pairs of species in this set.

180 The importance of interspecies interactions is further supported by an analysis of  
181 relative yields (Fig S4). Most species cannot reach their monoculture population size in co-  
182 culture experiments (relative yield < 1), suggesting that interactions between species are largely  
183 competitive. The 21 out of 110 cases (19%) where we measured a relative yield larger than one  
184 could be due to modest facilitation or due to variation in colonization and measurement between  
185 the monoculture and pairwise experiments. Collectively, these data suggest that the  
186 monoculture colonization ability of a bacteria relates to its abundance in pairwise experiments,  
187 but the mostly competitive interspecies interactions alter as many as half of the individual  
188 pairwise outcomes (Fig 2, S4).

### 189 ***Environmental filtering imposed by C. elegans***

190 Next, we sought to characterize the environmental filtering performed by *C. elegans*  
191 during microbial community assembly. Specifically, we sought to quantify the differences in  
192 bacterial relative abundances between the *in vivo* worm gut and the *in vitro* liquid medium used  
193 as feeding substrate (Fig 3). Thus, we performed all fifty five co-culture experiments in liquid  
194 medium and measured the equilibrated bacterial fractions after seven cycles of daily dilution  
195 (Methods, Fig S5). We found that competitive performance in the worm gut and liquid media are  
196 correlated (Fig 3A), which suggests that the environmental filtering that *C. elegans* provides is  
197 not strong enough to alter the hierarchical order of these eleven bacterial strains. This  
198 resemblance between the worm gut and liquid media with the coarse-grained mean fractional  
199 abundance is also observable when comparing the mean relative yields (Fig 3A, inset).



200

201 **Figure 3. Bacterial competitive hierarchy is conserved between *in vivo* gut and *in vitro***

202 **liquid media environments. (A)** Mean fractional abundance in co-culture experiments in the

203 worm and in the background feeding media correlate with each other, indicating that strong

204 competitors remain strong, and weak competitors vs remain weak in these two environments.

205 **(Inset)** Mean relative yields (*cells/worm* of a species in competition divided by *cells/worm* in

206 monoculture) in the worm and media also correlate. Error bars as the propagated error from the

207 SEM of co-culture experiments. **(B)** Fractional abundances in co-culture experiments in worm

208 intestine are as different from the background media as from the null expectation based on

209 monoculture colonization. Points at distance  $>.5$  from the identity line were labeled. **(C)** Fraction

210 of *Sm* in worm (yellow) and media (green) when environments are coupled by migration. Both

211 environments reach their different equilibrium points in the same test tube. **(D)** An acidic version

212 of the media resembling the average pH of the worm intestine (4.5) shifts back the pairwise

213 outcome of *Sm-Pp* to a worm-like state. Error bars in C and D as the SEM of at least 4

214 replicates.

215           Although competitive performance is similar between the worm gut and *in vitro* liquid  
216 media, we find that individual pairwise outcomes often display very different outcomes in these  
217 two environments (Fig 3B). We quantify the competitive performance of a bacterial species by  
218 averaging over all ten pairwise experiments; although these averages correlate between the  
219 worm intestine and the background media (Fig 3A), the underlying pairwise outcomes can be  
220 very different (Fig 3B, blue circles). The mean distance between the fractions in the media and  
221 the worm (0.22) is similar to the mean distance between the null expectation from monoculture  
222 colonization and the worm outcomes described earlier (Fig 3B, red triangles; Fig S3). If we  
223 wanted to predict the relative abundance of two-species communities in the worm intestine, both  
224 the monoculture colonization and the media pairwise outcomes would fail to a similar extent, but  
225 in dramatically different ways: while the null expectation overestimates the frequency of  
226 coexistence in the gut, the media competition displays less coexistence than observed in the  
227 worm gut. Our data therefore suggests that the *C. elegans* intestine doesn't alter the competitive  
228 hierarchy of its bacterial colonizers, but is capable of shifting specific pairwise outcomes.

229           In fact, several pairs of species displayed remarkably different outcomes in competition  
230 inside versus outside of the worm intestine, such as *Sm-Pp* and *Lp-Bs*. Since the two  
231 environments were capable of selecting a different microbial community composition, we  
232 wondered if migration between them would homogenize their microbial compositions. To  
233 answer this question, we fed again a population of worms with a mixture of *Sm-Pp*, but instead  
234 of keeping equal proportions of both species by adding fresh bacteria daily to the batch culture,  
235 we allowed the bacteria to be carried over during each dilution step. While the bacterial  
236 abundances in the media change and reach equilibrium, at the same time and in the same well,  
237 competition takes place within the intestine of worms. Migration between these environments  
238 takes place in the form of worms feeding from the media and defecating live bacteria into it. We  
239 found that despite the strong linkage between environments, *Sm* dominated in the gut yet was

240 present in a minority in the media outside the worm (Fig 3C). Similar differences in competition  
241 outcomes between the worm gut and outside media were observed for *Lp-Bs* (Fig S6). These  
242 results show that the environmental filtering imposed by the worm intestine can be strong  
243 enough to keep an internal bacterial community different from its surrounding environment.

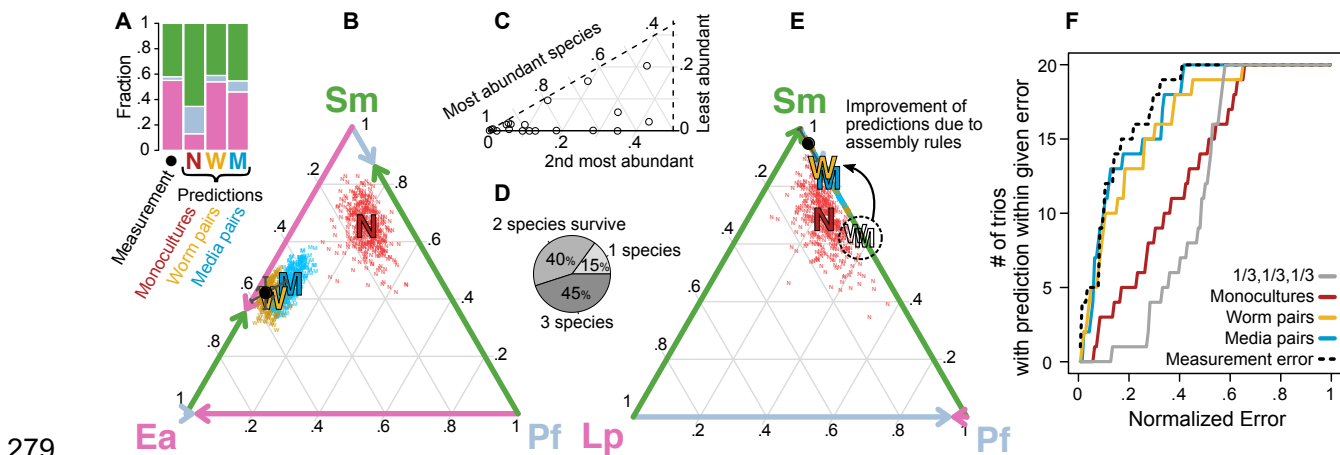
244         Given the importance of pH to microbial growth and competition (71–75), we tested  
245 whether the low pH of the worm gut could cause some of the observed differences between  
246 competitive outcomes in the intestine and the media. To this end, we repeated the *Sm-Pp*  
247 competition in liquid media at its normal pH 6, and at lower pH 4.5, where the latter  
248 approximates the conditions within the nematode intestine (76,77). For this pair of species,  
249 lowering the pH of the media was sufficient to alter the pairwise outcome, resulting in a  
250 community very similar to that observed in the worm intestine (Fig 3D). Similar results were  
251 observed in the pair *Lp-Bs* (Fig S6). We conclude that the acidic pH of the worm gut is an  
252 important component of the environmental filtering imposed by this host intestine during  
253 community assembly.

#### 254 ***Trio outcomes are predicted by pairwise outcomes***

255         Our results thus far indicate that monoculture colonization and pairwise interactions are  
256 both important for the outcomes of pairwise experiments in the *C. elegans* intestine (Fig 3B).  
257 However, it remains unclear how these two forces will impact the assembly of more diverse  
258 bacterial communities. We therefore constructed three-species communities to extend our  
259 analysis. From our eleven bacterial strains, we purposely selected a set of six (*Bc*, *Lp*, *Pf*, *Pv*,  
260 *Ea*, and *Sm*) that covers the different competitive abilities observed. We fed *C. elegans* with the  
261 20 possible three-species combinations of these six species, and measured the bacterial  
262 abundances after four days of colonization (Fig S7). To summarize these trio outcomes, we  
263 again utilized the mean fractional abundance of each species in all communities where it

264 participates. We found that the population size in monocultures correlates less strongly with this  
 265 mean fraction in trios (Fig S8, Spearman correlation  $[r_s]=.6$ ,  $p=.12$ ) than with the mean fraction  
 266 in pairwise experiments (Fig 2C). This result indicates that, as community diversity increases,  
 267 the ability to colonize in monoculture becomes less important.

268 The mean fractional abundance measured in our experiments agrees with previous  
 269 reports of the natural *C. elegans* microbiota (35), with our *Enterobacteriaceae*,  
 270 *Pseudomonadaceae*, and Firmicute isolates ranking first, second, and third in abundance,  
 271 respectively. However, more than confirming the potential that each bacterial species has when  
 272 competing in a natural setting, our bottom-up approach allows us to examine closely many  
 273 particular microbiomes. For instance, the trio outcome of *Ea-Pf-Sm* is shown in Figure 4A as a  
 274 stack of bars, and in Figure 4B as a point in a simplex, where the point moves closer to a vertex  
 275 when that given species increases in abundance. By plotting the measurements of all 20 trios  
 276 into one simplex (Fig 4C), we observe that most of the cases have one species as highly  
 277 abundant, yet full exclusion is rare and only accounts for 3 out of the 20 different communities  
 278 (Fig 4D).



280 **Figure 4. Fractional abundances in three-species communities are well predicted by**  
 281 **pairwise outcomes. (A) Outcome of trio *Ea-Pf-Sm* in *C. elegans* intestine, together with**

282 predictions based on monocultures (each bacterial species reaches its population size in  
283 monoculture) or pairwise outcomes (normalized arithmetic mean) in worm or media. **(B)** Simplex  
284 representation of trio outcome and predictions in (A), with the edges depicting the pairwise  
285 outcomes in the worm intestine. The error bars on measurement are the SEM of 4 biological  
286 replicates, and the clouds of small letters around predictions are 400 bootstrap replicates ('N's  
287 sampling the monoculture data, and 'W's and 'M's sampling the pairwise data). **(C)** 20 trio  
288 outcomes represented in one 6th of a simplex, with the highest, medium, and lowest abundant  
289 species taking the left, right and upper vertices, respectively. **(D)** 3, 8, and 9 out of the 20 trios  
290 show full competitive exclusion, two- and three-species coexistence, respectively. **(E)** Assembly  
291 rules help the quantitative prediction of the trio outcomes when one of the pairwise outcomes is  
292 competitive exclusion. **(F)** Cumulative distribution of error of predictions. Error calculated as the  
293 linear distance between prediction and measurement in the simplex. The distances are  
294 normalized by the maximal distance,  $\sqrt{2}$ . The dashed line represents the mean distance  
295 between the measured mean and the biological replicates of each trio.

296 As with the pairwise outcomes, the mean abundance of *Ea* is the least predictable based  
297 on its monoculture colonization (Fig S8), which suggests that the pairwise interactions favoring  
298 the abundance of *Ea* in co-cultures might favor *Ea* further in larger communities. To determine if  
299 pairwise interactions are the main drivers of trio outcomes, we made predictions of all trios  
300 based on either monocultures, or pairwise outcomes. To make quantitative predictions of trios  
301 based on monocultures, we extended the null expectation described earlier by assuming that all  
302 species will reach their monoculture population sizes ('N's in Fig 4 and S7). This null  
303 expectation based on monoculture colonization achieves poor results at predicting trio  
304 communities, for its mean error of 35.7% is just slightly better than the 43.8% mean error of an  
305 uninformed '1/3, 1/3, 1/3' prediction (Fig 4F). Hence, although monoculture colonization predicts  
306 pairwise outcomes with a lower mean error of 20% (Fig S3), monocultures become inadequate

307 at predicting three-species microbiomes, highlighting again the importance of interspecies  
308 interactions.

309         Alternatively, we can predict trios based on pairwise outcomes by taking the arithmetic  
310 mean of each species' abundance in the co-culture experiments against the other two species  
311 (here, a normalization factor of 2/3 is needed for the fractions of the three species to add to  
312 one). This *normalized arithmetic mean* prediction, applied to the pairwise outcomes obtained in  
313 the worm intestine or the *in vitro* liquid media ('W's and 'M's in Fig 4A and B, respectively),  
314 quantitatively predicts some trios with high accuracy, and reaches a lower mean error of 26%  
315 (Fig S9). However, this simple prediction is prone to error when one of the pairwise outcomes is  
316 competitive exclusion, such as *Sm-Lp* in the *Sm-Lp-Pf* trio (Fig 4E, empty letters). Fortunately, a  
317 recently proposed 'assembly rule' (69) is capable of adjusting these cases by simply removing a  
318 bacterial species from the trio prediction when it cannot survive all pairwise competitions (Fig 4E  
319 and S7). After the application of these assembly rules, the mean error of the predictions based  
320 on worm or media pairwise outcomes, 18.7 and 15.7%, comes close to the expected biological  
321 noise in trio outcomes, 13.3% (Fig 4F). The fact that pairwise outcomes can properly predict trio  
322 outcomes indicates that interactions between pairs of bacterial species are an important force in  
323 determining the outcome of multispecies communities and suggests that indirect interactions  
324 are uncommon or weak.

## 325 **Discussion**

326         Here we characterized the outcomes of all co-culture experiments among 11 bacterial  
327 species within the gut of the worm *C. elegans*. We find that monoculture population size in the  
328 worm intestine correlates well with competitive performance in this environment, suggesting that  
329 the ability to colonize and proliferate in the host is an important determinant of success.  
330 However, we find that the outcomes of pairwise competition, but not single-species colonization



331 ability alone, predict the outcomes of three-way competition, indicating that pairwise competitive  
332 interactions between microbes are an important driver of the assembly of more complex  
333 multispecies communities.

334 We found that most interactions among our set of species are competitive, and none of  
335 the 55 pairs that we measured displayed a strong mutualism in which both species benefited.  
336 Despite this prevalence of competitive interactions, we also found that coexistence between  
337 species was very common, indicating that the competition is relatively weak and only rarely  
338 leads to competitive exclusion. Interestingly, we do not observe any cases of strictly non-  
339 transitive interactions (rock-paper-scissors) (66) among any of the 165 possible trios,  
340 questioning once more the practical significance of this mechanism at stimulating coexistence  
341 and diversity in multispecies communities (63). However, the 11 species that we studied here  
342 are laboratory isolates rather than a naturally occurring community, which may have evolved to  
343 co-occupy their particular environment (78). Such communities could be enriched for mutualistic  
344 or rock-paper-scissors interactions; future work will be necessary to determine whether naturally  
345 occurring communities have significantly different network properties, although recent work  
346 suggests that non-transitive interactions are not common in natural microbial communities (79).

347 Although we observed a range of different outcomes, from coexistence to competitive  
348 exclusion, we have not extensively explored the mechanistic basis behind them. These  
349 outcomes could arise from simple resource competition, including competition for the limited  
350 space available within the *C. elegans* gut, or there could be more explicit forms of antagonism  
351 such as toxin production. In cases of coexistence, spatial partitioning within the host could play  
352 an important role; the role of spatial structure was recently found to be important in determining  
353 the competitive outcome of interspecies competition within the gut of the zebrafish (17). Further  
354 work will be required to elucidate these spatial dynamics within the worm.

355 Our results provide additional support for the importance of host-determined  
356 environmental factors, specifically pH, in shaping the microbiome. The low pH of the gut  
357 environment is thought to be a critical factor in host-microbe interactions, and recent work has  
358 explicitly demonstrated the importance of pH in modulating the interactions between microbes  
359 and determining the structure of synthetic and natural communities (71–73). Consistent with  
360 these results, we observed that reducing the pH of a liquid medium to simulate the host intestine  
361 could alter the outcomes of competition between species and substantially reduce the difference  
362 between *in vitro* media and *in vivo* gut.

363 The conserved competitive hierarchy among our set of microbial species inside and  
364 outside the worm suggest that similar traits are required for success in both environments.  
365 Notably, these experiments were carried out in an immune-deficient host strain (AU37) which is  
366 defective in recognition of and response to microbial colonization (80). It remains to be seen  
367 whether the more stringent environment of an immune-capable host would produce larger  
368 differences between *in vitro* and *in vivo* experiments. In future work, it will therefore be  
369 interesting to determine the extent to which modifying the properties of the host environment  
370 alters the filtration imposed by that environment and the interactions between colonizing  
371 microbial strains.

372 The work presented here focuses on population averages rather than the composition of  
373 bacterial communities within individual worms. Recent results from our group have  
374 demonstrated that variation between individuals can be informative - when colonization is very  
375 slow, there can be an extreme bottleneck during colonization of the worm gut that leads to  
376 marked heterogeneity between the gut communities in different worms (81). Similar results have  
377 recently been found in *Drosophila* (82), indicating that stochastic effects during colonization may  
378 be important in a wide range of host species. Under the feeding densities used in these  
379 experiments we expect more uniform colonization, but it will be important for future studies to

380 determine the role of stochasticity and priority effects during assembly of multispecies  
381 communities within the host.

382 Our results are based on a single time point after multiple days of colonization, at which  
383 point we expect any initial transients in population dynamics to have died out. This experimental  
384 choice allows us to focus on community composition after species have had time to interact, but  
385 also precludes the study of the early colonization dynamics. *C. elegans* in the wild is born sterile  
386 and is then colonized by the complex microbial communities present within the soil and rotting  
387 organic matter (83). This is similar to the microbial successions that have been observed in  
388 other contexts, such as in the digestive tract of human infants, *in situ* and *in vitro* cheese rind  
389 communities, enrichments of seawater in chitin particles, and *in vitro* enrichments of leaf  
390 communities (84–87). It would be fascinating to determine whether similar succession dynamics  
391 take place during early colonization of the worm intestine.

392 In this study, we have fed worms with monocultures, pairs, and trios of bacteria from a  
393 set of naive soil isolates to determine the role of interspecies interactions in the assembly of  
394 host-associated microbial communities. These results add to our understanding of how  
395 interactions between pairs of bacterial species can inform our understanding of more complex  
396 bacterial communities. Our results show the promise of experimental bottom-up microbial  
397 ecology as a tool for understanding the dynamics of bacterial gut communities in a simple model  
398 organism, providing insight into the forces that shape and control the structure of microbiomes.

## 399 **Methods**

### 400 ***Nematode culture***

401 Nematodes were grown, maintained, and manipulated with standard techniques (88).  
402 The *C. elegans* strain AU37 (*glp-4(bn2)* I; *sek-1(km4)* X) was used for all experiments. Due to

403 the *glp-4* mutation, AU37 does not develop gonads and is therefore sterile when raised at room  
404 temperature (23-25°C). Sterility ensured that all worms in a given experiment were of the same  
405 age and had the same life history. The mutation on the *sek-1* gene, part of the p38 MAPK  
406 signalling pathway (80), decreases immune function and makes AU37 more susceptible to  
407 bacterial colonization (41). The ensuing larger intestinal bacterial communities allowed better  
408 quantification of fractional abundances. Worm strains were provided by the Caenorhabditis  
409 Genetic Center, which is funded by NIH Office of Research Infrastructure Programs (P40  
410 OD010440).

411 L1 animals of the same age were obtained using the standard egg-extraction protocol  
412 (88). Starved larvae were then transferred to NGM plates with lawns of *E. coli* OP50, and after 2  
413 days of feeding at room temperature, a synchronous adult population of *C. elegans* was  
414 obtained. Worms were then transferred to S-medium (88) + 100 µg/mL gentamicin + 5X heat-  
415 killed OP50 for 24 hours to kill any bacteria inhabiting the intestine, resulting in germ-free  
416 worms. Heat-killed OP50 was used to trigger feeding behaviour in the worms. The adult worms  
417 were washed via sucrose flotation to remove debris before bacterial colonization.

#### 418 **Bacteria**

419 *Bacillus subtilis* (ATCC 23857), *Enterobacter aerogenes* (ATCC 13048), *Lactobacillus*  
420 *plantarum* (ATCC 8014), *Pseudomonas aurantiaca* (*Pseudomonas chlororaphis* subsp.  
421 *aurantiaca*) (ATCC 33663), *Pseudomonas citronellolis* (ATCC 13674), *Pseudomonas*  
422 *fluorescens* (ATCC 13525), *Pseudomonas putida* (ATCC 12633), *Pseudomonas veronii* (ATCC  
423 700474), *Serratia marcescens* (ATCC 13880) were obtained from ATCC. *Bacillus cereus* was  
424 obtained from Ward's Scientific Catalog. *Escherichia coli* MC4100 (CGSC #6152) was obtained  
425 from the *E. coli* Genetic Stock Center.

426 Bacterial strains were grown for 24hrs at 30°C in individual culture tubes with 2 mL of LB  
427 (Difco). To construct cultures to feed *C. elegans*, strains were centrifuged 1 minute at 7K RCF to  
428 pellet, washed once in S-medium, and then resuspended in 1% v/v Axenic Medium diluted in S-  
429 medium (1%AXN). 100 ml of 100% AXN were prepared by autoclaving 3g yeast extract and 3g  
430 soy peptone (Bacto) in 90 ml water, and subsequently adding 1g dextrose, 200µl of 5 mg/ml  
431 cholesterol in ethanol, and 10 ml of .5% w/v hemoglobin in 1 mM NaOH. To standardize the  
432 bacterial densities used to feed *C. elegans*, the bacterial cultures were diluted to  $\sim 10^8$  CFU/ml  
433 based on previously measured CFU counts.

#### 434 ***Bacterial colonization of C. elegans***

435 Germ-free adult worms were resuspended in 1%AXN to a concentration of  $\sim 1000$   
436 worms/mL. Aliquots of 120µL ( $\sim 100$  worms) were laid into 96-deep-well culture plates (1 mL  
437 well volume, Eppendorf). 15µL of each bacterial suspension were added to the corresponding  
438 wells in a matrix-like format (11 species x 11 species), leaving a final bacterial concentration of  
439  $\sim 2 \times 10^7$  CFU/ml, and a 150µl volume for monoculture and co-culture experiments. Extra  
440 replicates of monoculture colonization were done to complement the diagonals of the matrices  
441 containing the co-culture experiments. For trio competitions, 3 bacterial species were mixed  
442 evenly to reach a concentration of  $\sim 3 \times 10^7$  CFU/ml. Plates were covered with a Breathe-Easy  
443 sealing membrane and incubated with shaking at 400 RPM at 25°C. To maintain the bacterial  
444 concentrations constant throughout the 4-day feeding period, every day the worm samples were  
445 washed and the bacteria was replenished. Samples were washed with a VIAFLO 96 by adding  
446 500µl of M9 Worm Buffer (WB) + 0.1% v/v Triton X-100 (Tx), pipetting 10 times, and removing  
447 the supernatant after worms precipitated. The samples were then transferred to new 96-deep-  
448 well plates to leave behind possible biofilms, and then washed in the same way two more times  
449 with 1%AXN. Bacterial suspensions from new culture tubes were added as previously  
450 described.

## 451 ***Mechanical disruption of worms***

452           The worm samples were washed to remove most external bacteria with the previously  
453 described protocol. Samples were then incubated in 100 $\mu$ L S-medium + 2X heat-killed OP50 at  
454 25°C for one hour to allow the worms to evacuate any non-adhered bacterial cells from the  
455 intestine. Worms were then rinsed twice with WB + 0.1%Tx, cooled down 15 min at 4°C to stop  
456 peristalsis, and bleached for 6 minutes at 4°C with 100 $\mu$ L WB + .2% v/v commercial bleach.  
457 Worms were then rinsed three times with cold WB + 0.1%Tx to remove bleach.

458           For manual disruption, each worm sample was transferred to a small petri dish (6 cm)  
459 with 3mL of WB + 1%Tx (the high concentration of surfactant facilitates the upcoming grinding).  
460 To guarantee the background media was fully clean, 20 $\mu$ L of the supernatant in each petri dish  
461 were collected, serially diluted, and laid in agar plates (see below). Then, a batch of ~20 worms  
462 was collected by pipetting 200 $\mu$ L from each petri dish into a 0.5 mL microcentrifuge tube (Kimble  
463 Kontes), and the exact number of collected worms was recorded. The volume of each microtube  
464 was then reduced to 20 $\mu$ L and the worms were grinded for one minute with a motorized pestle  
465 (Kimble Kontes Pellet Pestle, Fisher Scientific). After disruption, tubes were centrifuged two  
466 minutes at 7K RCF to collect all material, and the resulting pellet was resuspended in 180 $\mu$ L WB  
467 (final volume 200 $\mu$ L) before transfer to 96-well plates for serial dilution in 1X phosphate-buffered  
468 saline (PBS).

469           For 96-deep-well plate disruption, rinsed worms were treated for 20 minutes with 100 $\mu$ L  
470 of a solution of SDS + DTT (WB + 0.25% v/v sodium dodecyl sulfate + 3% v/v dithiothreitol [1M,  
471 freshly mixed in water], chemicals from Sigma Aldrich) to partially disrupt the cuticle, and then  
472 washed twice in WB + 0.1%Tx. A deep-well plate (2 mL square well plate, Axygen) was  
473 prepared by adding ~0.2g of a sterile grit (36-mesh silicon carbide, Kramer Industries,  
474 autoclaved) to each well. Same as in the manual disruption, the worm samples were transferred

475 to a small petri dish and supernatant samples were taken. Then, batches of ~20 worms were  
476 pipetted into individual wells of the Axygen plate. The plate was covered with parafilm and kept  
477 at 4°C for one hour prior to disruption to reduce heat damage to bacteria. Parafilm plates  
478 were capped with square silicon sealing mats (AxyMat) and disrupted by shaking at high-speed  
479 (Retsch MM400, 30 hz for 3 minutes). Plates were then centrifuged at 3K RCF for 2 minutes to  
480 collect all material, resuspended by pipetting, and transferred to 96-well plates for serial dilution  
481 in PBS.

### 482 ***Measurement of bacterial fractional abundances***

483 The undiluted worm digests and supernatants, together with the serial dilutions  $10^{-1}$ ,  $10^{-2}$ ,  
484 and  $10^{-3}$  of each sample, were plated onto Nutrient Agar (3g yeast extract, 5g peptone, and 15g  
485 of agar [Bacto] in one liter of water). The samples were incubated at room temperature for two  
486 days to allow distinct colony morphologies to develop. Colonies in all distinguishable dilutions  
487 were counted afterwards.

488 Pairwise and trio outcomes were categorized as coexistence if the rare species was  
489 present at an average abundance of more than 2%. This threshold is just above our usual limit  
490 of detection of ~1%, which is inversely proportional to the number of colonies counted (~100).  
491 The pair *Pf-Ea* (1.7%-98.3%) was defined as coexisting since we could reassure the presence  
492 of *Pf* with more than one biological replicate.

### 493 ***Hierarchy scores and generation of random matrices***

494 Utilizing the 11x11 matrix with the fractional abundances in co-culture experiments, the  
495 hierarchy score is calculated by: 1) Ordering its rows and columns ascendingly based on the  
496 mean fractional abundance; and 2) Taking the mean value of the half-matrix under the diagonal.  
497 In a perfectly hierarchical matrix, each competitor will drive to extinction every other species with  
498 a lower rank, reaching a hierarchy score of 1. Random matrices were generated to calculate the

499 significance of the observed high hierarchy score. We conserved the distribution of fractional  
500 abundances by: 1) Sampling with replacement 55 values of the original matrix; 2) Assigning  
501 these random fractional abundances to the lower triangle of a new matrix; and 3) Assigning to  
502 the upper triangle of the matrix the values of 1-transpose. For each random matrix generated, a  
503 new hierarchy score is calculated as previously described.

#### 504 ***Null expectation of fractional abundances***

505 The null expectation based on monocultures is obtained by averaging the fractional  
506 abundances in all possible combinations of monoculture information. Since we have ~10  
507 replicates for each species colonizing *C. elegans* in monoculture, the null expectation for the  
508 pairwise and trio outcomes is the mean of ~100 and ~1000 different combinations of  
509 monoculture data, respectively. The SEM is calculated by dividing the standard deviation of all  
510 combinations by  $\sqrt{\text{least number of monoculture replicates}}$ . These mean and SEM can also  
511 be obtained by bootstrapping (sampling with replacement) the monoculture data.

#### 512 ***Co-culture experiments in vitro***

513 Utilizing the same S-medium + 1%AXN, 96-deep-well culture plates, 150 $\mu$ l volume per  
514 sample, and the same matrix format used to colonize *C. elegans*, pairs of bacterial species were  
515 mixed at a concentration of  $10^5$  CFU/ml each. We allowed the bacterial relative abundances' to  
516 equilibrate with seven growth-dilution cycles, where the bacteria are diluted 100-fold into fresh  
517 media each day. As previously described, we quantified the bacterial abundances by plating into  
518 agar.

#### 519 ***Phylogeny reconstruction***

520 Sequences of the full 16S rRNA gene were obtained from NCBI. *Sulfolobus solfataricus*,  
521 a thermophilic archaea, was used as an outgroup species to root the tree. Clustal X with default



522 parameters was used to align the sequences (89). PhyML-SMS with default parameters was  
523 used to select GTR+G+I as the best model and to infer the tree (90).

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