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 4×10^{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.

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*).

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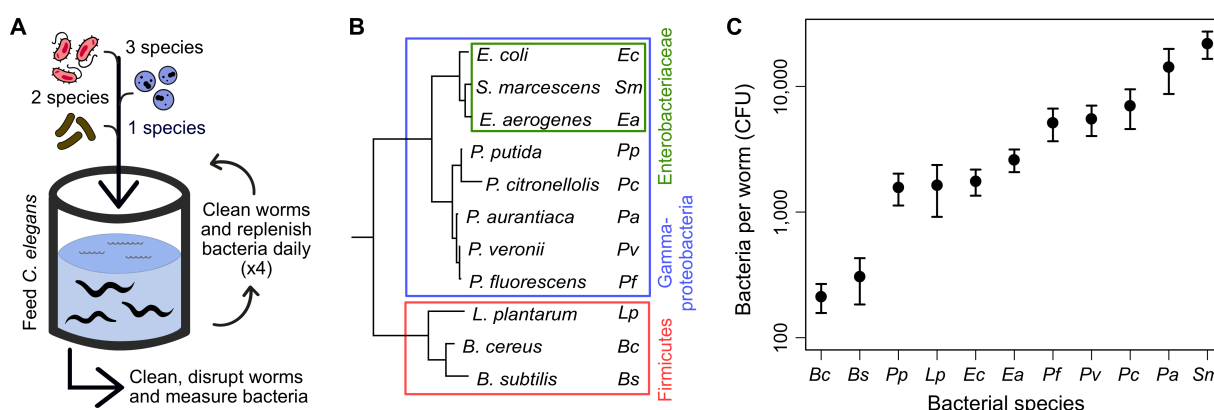
80 **Figure 1. Eleven bacterial species colonize the gut of the worm in monoculture. (A)** One,

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

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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.
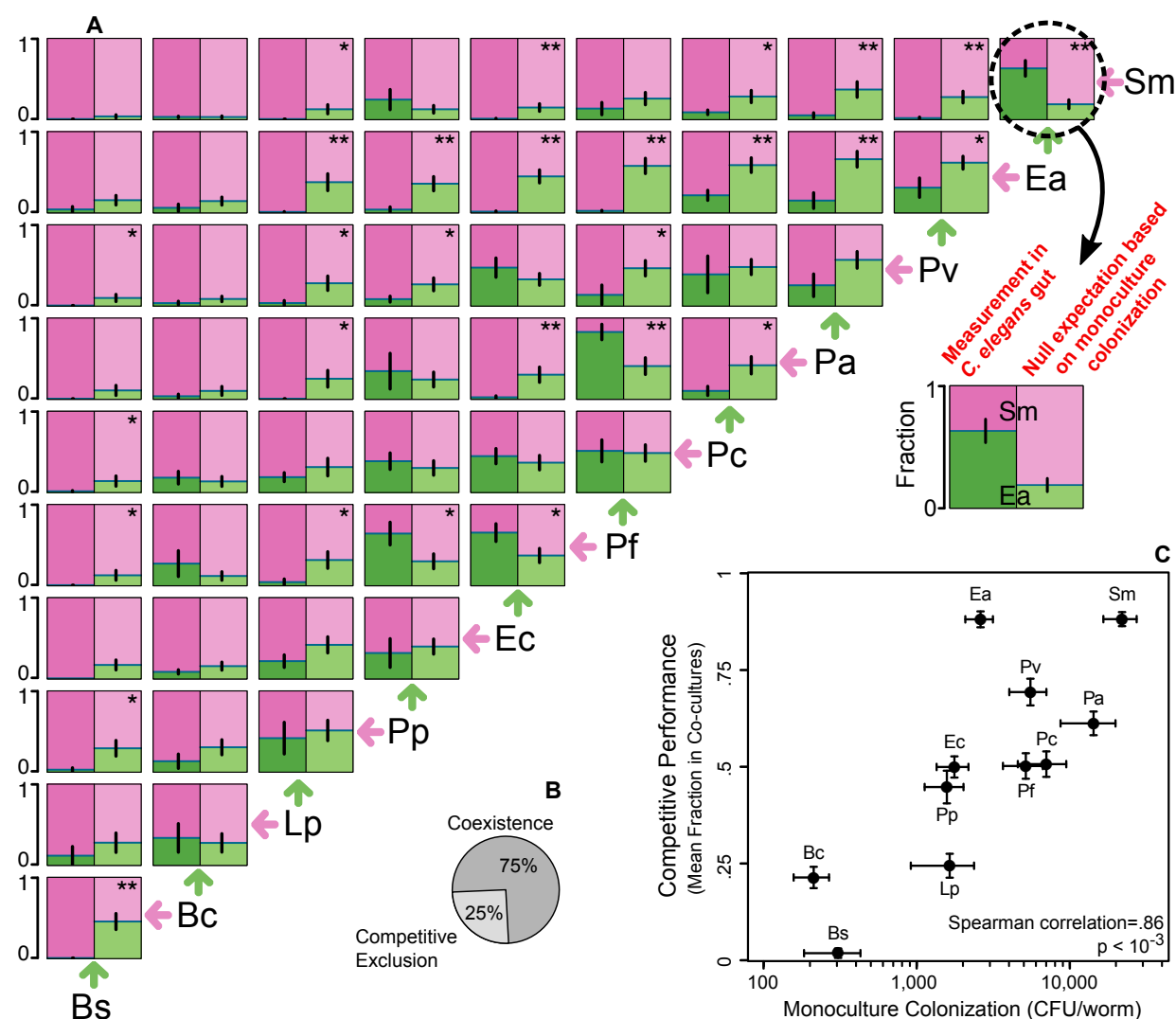
Figure 2. Monoculture colonization relates to, but does not predict pairwise outcomes in the worm intestine. (A) Left: Fractional abundances of 55 co-culture experiments inside the *C. elegans* intestine. Error bars as the SEM of 2 to 8 biological replicates (Fig S1). Bacterial strains are ordered by its mean fractional abundance. **Right:** Null expectation for the fractional abundances based on a non-interacting model where each bacterial species reaches its monoculture population size. Null expectation as the mean of all possible pairings of monoculture information, and error bars as the SEM (Methods). * and ** represent a statistically significant difference between measurement and null expectation at *p*-values of .05 and .01, respectively (Welch's T-test). **(B)** Coexistence is common and competitive exclusion at a

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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).

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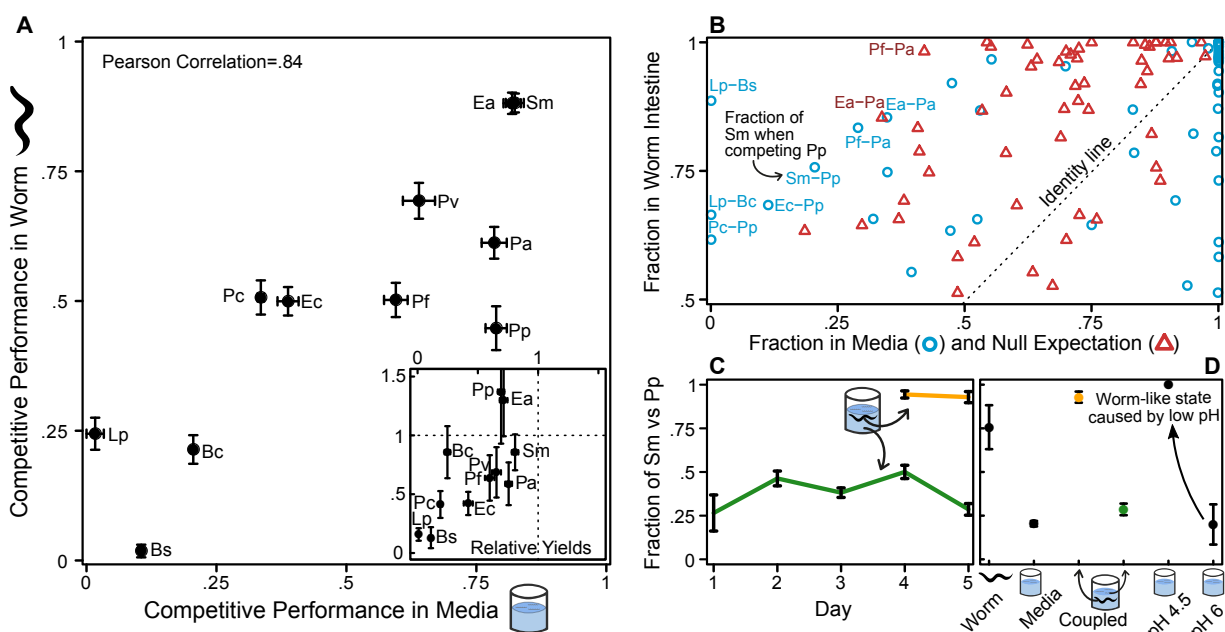
Figure 3. Bacterial competitive hierarchy is conserved between *in vivo* gut and *in vitro* liquid media environments. (A) Mean fractional abundance in co-culture experiments in the worm and in the background feeding media correlate with each other, indicating that strong competitors remain strong, and weak competitors remain weak in these two environments. **(Inset)** Mean relative yields (*cells*/worm of a species in competition divided by *cells*/worm in monoculture) in the worm and media also correlate. Error bars as the propagated error from the SEM of co-culture experiments. **(B)** Fractional abundances in co-culture experiments in worm intestine are as different from the background media as from the null expectation based on monoculture colonization. Points at distance >.5 from the identity line were labeled. **(C)** Fraction of *Sm* in worm (yellow) and media (green) when environments are coupled by migration. Both environments reach their different equilibrium points in the same test tube. **(D)** An acidic version of the media resembling the average pH of the worm intestine (4.5) shifts back the pairwise outcome of *Sm-Pp* to a worm-like state. Error bars in C and D as the SEM of at least 4 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

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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

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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).
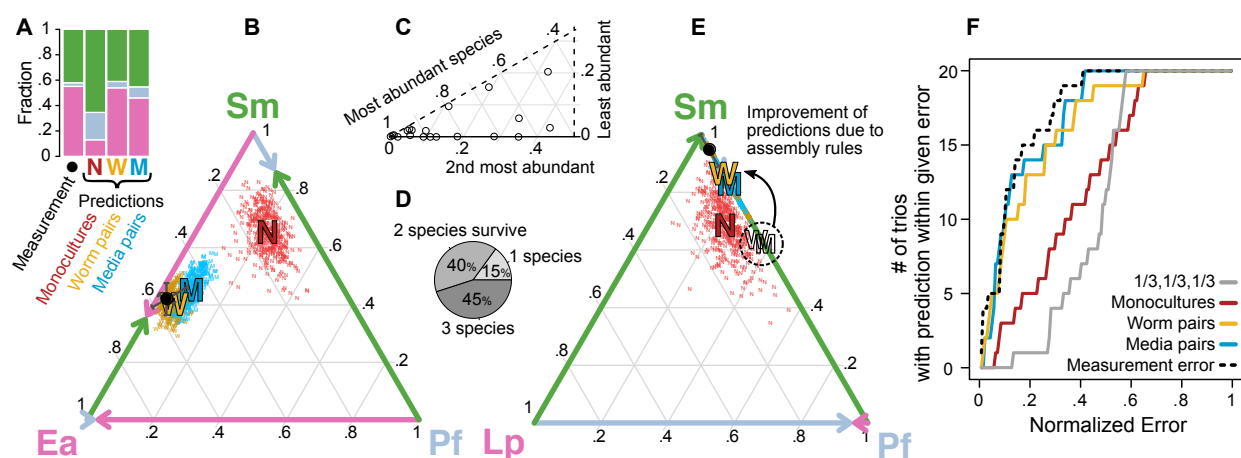
280   **Figure 4. Fractional abundances in three-species communities are well predicted by**

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 '⅓, ⅓, ⅓' prediction (Fig 4F). Hence, although monoculture colonization predicts

306   pairwise outcomes with a lower mean error of 20% (Fig S3), monocultures become inadequate

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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

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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

18

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 (23-25ºC). 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.

20

426   Bacterial strains were grown for 24hrs at 30ºC 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 ~10^8 CFU/ml

433   based on previously measured CFU counts.

Bacterial colonization of C. elegans

435   Germ-free adult worms were resuspended in 1%AXN to a concentration of ~1000

436   worms/mL. Aliquots of 120µL (~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   ~$2x10^7$ 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 ~$3x10^7$ CFU/ml. Plates were covered with a Breathe-Easy

443   sealing membrane and incubated with shaking at 400 RPM at 25ºC. 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 100µL S-medium + 2X heat-killed OP50 at

454 25ºC 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 4ºC to stop

456 peristalsis, and bleached for 6 minutes at 4ºC with 100µL 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, 20µl 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 200µl 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 20µl 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 180µL WB

467 (final volume 200µL) 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 100µL

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. Parafilmed 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.

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.

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

23

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(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, 150µl 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

24

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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