The effect of symbiosis on symbiont fitness – interactions within a simple metaorganism

Peter Deines[a]*, Katrin Hammerschmidt[b] and Thomas CG Bosch[a]

[a]Zoological Institute, Christian Albrechts University Kiel, 24118 Kiel, Germany

[b]Institute of General Microbiology, Christian Albrechts University Kiel, 24118 Kiel, Germany

*Correspondence:

Peter Deines, Zoological Institute, Christian Albrechts University Kiel, Am Botanischen Garten 1-9, 24118 Kiel, Germany

Tel: +49-431-880-4171

Email: pdeines@zoologie.uni-kiel.de

Running title: Interactions drive microbiome species composition

Abstract

Organisms and their resident microbial communities form a complex and mostly stable ecosystem. It is known that the specific composition and abundance of certain bacterial species affect host health and *Darwinian fitness,* but the processes that lead to these microbial patterns are unknown. We investigate this by deconstructing the simple microbiome of the freshwater polyp *Hydra*. We contrast the performance of its two main bacterial associates, *Curvibacter* and *Duganella*, on germ free hosts with two *in vitro* environments over time. We show that interactions within the microbiome but also host modulation lead to the observed species frequencies and abundances. More specifically, we find that rare microbiome members are essential for achieving the observed community composition, which ultimately sets the maximum carrying capacity. Bacterial fitness strongly depends on the environment: while *Duganella* performs better than *Curvibacter* in a non-host habitat, *Curvibacter* benefits through the host association. This is of particular interest because *Curvibacter* and its host show a history of co-evolution, as inferred from phylogenies, whereas the colonization with *Duganella* seems to be a recent event. Our findings oppose the assumption that bacteria always benefit through the association with the host and poses questions regarding the long-term maintenance of such relationships.

42 **Introduction**

43 Eukaryotes form a distinct habitat for microbial communities (microbiomes) and these
44 microbial associations are integral to life. The host with its associated microbial
45 community, often dominated by bacteria but co-habited by fungi, protozoa, archaea, and
46 viruses, is termed metaorganism. Microbiomes can contain from few up to thousands of
47 microbial species - the human microbiome, for example, is estimated to be comprised of
48 about 5000 bacterial species (1-3). These host-associated microbial communities have
49 been shown to enhance host function and contribute to host fitness and health (4).
50 Changes in microbiome diversity, function, and density have been linked to a variety of
51 disorders in many organisms (5-8).

52 A major goal in host-microbe ecology is to unravel the ecological and
53 evolutionary dynamics of microorganisms within their communities. Of particular
54 relevance are the factors that shape the stability and resilience of such communities,
55 despite different fitness trajectories of the microbiome members. The microbial
56 response to stress or perturbations, e.g. exposure to a new substrate, provides a
57 selective advantage to certain members of the community. If the system cannot tolerate
58 the change, the microbial community dramatically shifts until a different equilibrium
59 state is reached (9). Frequency-dependent selection forces the host to adapt to these
60 changes and select for or against the most frequent genotypes of their associated
61 microbiota (10). There is, for example, strong evidence that species-specific
62 antimicrobial peptides (AMPs) shape, control, and confine host-species specific bacterial
63 associations (11, 12). In addition, microbial communities are not evenly distributed, e.g.
64 along the gastrointestinal tract or between the lumen and the epithelial surfaces (2, 13,
65 14). These significant differences in niches or micro-habitats and their occupancy is
66 known as spatial heterogeneity and will affect community assembly rules and dynamics
67 (15, 16). Interspecies metabolic exchange is another key biotic force acting as a major
68 driver of species co-occurrence in diverse microbial communities (17).

69 One aspect that has been so far neglected in metaorganism research is why a
70 stable microbiome can persist in a specific host lineage for many host generations.
71 Central to this is the question whether microbes benefit from the association with the
72 host. This could either happen through direct interaction with the host or indirectly,
73 through interactions with another microbial strain within the microbiome. Host-
74 dependent advantages for the symbiont range from the availability of host-derived
75 nutrients, a competition-free environment to reduced predation risk (18). When
76 members of the microbiome benefit from the association with a particular host, one
77 would expect their capacity to reproduce, i.e. their *Darwinian fitness*, to be higher in the
78 host relative to an alternative habitat, as demonstrated for the squid-*Vibrio* system (19).
79 Surprisingly, this aspect has been tested less frequently than the effect of microbes on
80 host fitness, which has been quantified in many systems.

81 Methods for quantifying microbial fitness range from estimating absolute
82 fitness, where population growth is measured from which maximum growth rates can
83 be calculated, to estimates of relative fitness. The latter is of particular relevance as the
84 fitness of a focal microbial lineage is measured *relative* to another microbial lineage.
85 This provides a better representation of the turnover of strains in microbial populations
86 and also closely corresponds to the meaning of fitness in evolutionary theory (20). The
87 basic design of such an experiment involves mixing populations of the two strains
88 together in a particular initial ratio in the test environment and measuring their relative
89 contributions to future generations. Under certain ecological conditions the relative
90 fitness of two strains is dependent upon their relative frequencies (frequency-
91 dependent selection) (21). These methods of measuring microbial fitness can easily be
92 applied to host-microbe systems and help us in understanding the underlying dynamics.
93 This will yield insights into the role that microbes played in the evolution of eukaryotes
94 and into the ecology and evolution of host-microbe associations in general (22). Here, it
95 is important to unravel the extent to which the observed pattern simply result from

96 intrinsic properties, such as microbial growth rates, interactions of the microbes, which
97 could be recapitulated *in vitro* with co-culture experiments, or whether it is an emergent
98 property of that particular host-microbe system (23).

99 We here test whether microbial associates benefit from their association with
100 the host and their fellow community members and follow their ecological interactions
101 within the microbiome. We use the freshwater polyp *Hydra vulgaris* and its microbiome,
102 which has become a valuable experimental model in metaorganism research as it
103 provides an excellent bridge between the simplicity of synthetic communities and the
104 mouse model (24). *Hydra's* ectodermal epithelial cells are covered with a multi-layered
105 glycocalyx that provides a habitat for a species-specific and core microbiome of low
106 complexity (11, 25, 26), from which most microbes can be cultured *in vitro* (26, 27). This
107 allows for an integrated approach based on constructing synthetic communities of
108 various complexities and contrasting the host (*in vivo)* to *in vitro* habitats (24). We focus
109 on the two most abundant members of the microbiome that together constitute about
110 85% of *Hydra*'s simple microbiome, *Curvibacter* sp. AEP1.3 and *Duganella* sp. C1.2,
111 (hereafter called *Curvibacter* and *Duganella*) where abundances of *Curvibacter* are
112 several magnitudes higher as compared to *Duganella* (27). Previous work on species
113 interactions suggested that continued coexistence depends heavily on relative growth
114 rates. Using invasion-from-rare experiments we explore the potential for coexistence
115 under different environmental conditions (host and non-host habitat) and contribute to
116 a better understanding whether the host alters microbial interactions that potentially
117 lead to coexistence in simple microbiomes.

118

119 **Results**

120 **Carrying capacity of the *Hydra* host**

121 The carrying capacity of the *Hydra* habitat is highly stable among single *Hydra* wild-type
122 polyps with $1.7*10^5$ CFUs/polyp and at its maximum with the native microbiome.

123 Manipulations of the microbial community lead to significant changes in bacterial
124 numbers (Welch ANOVA, F_3=7.054; P<0.005; Fig. 1A). The addition of single bacterial
125 species (*Curvibacter* or *Duganella*) to wild-type polys does not increase the carrying
126 capacity but, in the case of *Curvibacter*, leads to a significant reduction in microbial
127 population size. In contrast does the addition of *Duganella* not significantly shift the
128 bacterial numbers (Fig. 1A). Conventionalised animals (germ-free polyps incubated with
129 tissue homogenates of wild-type animals) show a carrying capacity, which is not
130 significantly different compared to wild-type polyps (Fig. 1A), indicating the usability of
131 the germ-free polyps for the manipulation and construction of synthetic bacterial
132 communities on the host/ *in vivo*.
133
134 **Tracking microbial mono-colonisations in *Hydra* over time**
135 In mono-associations *Curvibacter* and *Duganella* populations display typical bacterial
136 growth dynamics. Following a lag and exponential phase, both strains reach a stationary
137 phase after roughly 72 h with a stable population size/carrying capacity per host (Fig.
138 1B). After 72 h we find significant differences in the carrying capacity between both
139 strains, *Curvibacter* being higher than *Duganella* (estimated by post hoc contrasts;
140 Generalized linear model: Full model: χ^2=54.360, d.f.=9, P<0.0001; bacterial species x
141 days post exposure: χ^2=18.326, d.f.=4, P=0.0011). These significant differences last until
142 the end of the experiment. Once carrying capacity of mono-associations is reached, we
143 find a population size of about 10^4 CFUs per host for *Curvibacter*, whereas for *Duganella*
144 the population size reaches on average only $1.5*10^3$ CFUs per host. Further, both mono-
145 associations do not reach the carrying capacity of wild-type polyps. The variation in
146 bacterial density between hosts is significantly higher in *Curvibacter* than in *Duganella*
147 (Levene: F_1=21.496, P<0.0001). No extinction events are observed in either strain after
148 successful establishment during the mono-colonisation experiments.
149

150 **Microbial growth kinetics of mono-associations *in vivo* and *in vitro***

151 Growth rates of *Curvibacter* did not significantly differ between the host and the
152 microcosm environments. This is in marked contrast to *Duganella*, where significantly
153 higher growth rates were observed in the non-host as compared to the host
154 environment. In all environments, except for the host, *Duganella* achieved a significantly
155 higher growth rate than *Curvibacter* (determined by post hoc t-tests; ANOVA: R^2=0.827;
156 Full model: $F_{5,15}$ = 14.333; P<0.0001; bacterial species x environment: F_2 = 15.592;
157 P=0.0002; Fig. 2).

158

159 **Microbial di-association experiments *in vivo* and *in vitro***

160 *Carrying capacity*

161 Results of host and microcosm experiments (static and mixed) show that during di-
162 associations the carrying capacity in all habitats is reached at about 72 h after
163 inoculation. Both microcosm environments are characterized by a carrying capacity of
164 10^7-10^8 CFUs/ml, and so exceeding the *in vivo* carrying capacity by a factor of 10^4 (Fig.
165 3). Nevertheless, di-associations on the host also fail to reach the carrying capacity of
166 wild-type polyps and reach a comparable carrying capacity as in the mono-colonisations
167 of *Curvibacter*. Both bacterial species do not match the carrying capacities as measured
168 in mono-colonisations on the host: whereas *Curvibacter* fails by a power of 10 to reach
169 its density in the mono-colonisations, *Duganella* outgrows it by a power of 10.

170

171 *Changes in fractions in microbial di-association experiments*

172 When competing *Curvibacter* and *Duganella* in three different starting frequencies in the
173 host, and the non-host environments, several interesting patterns can be observed (Fig.
174 4). In both non-host environments, *Duganella* outcompetes *Curvibacter* within 48 h post
175 exposure. From then onwards, frequencies of *Curvibacter* are low, reaching a maximum
176 of about 10%. This pattern does not depend on the initial frequency at the start of the

experiment. The host shows a different pattern. Here, a decrease in *Curvibacter* fraction can be observed in all three initial frequencies but never to a point where it cannot be detected in the population. From 72 h post exposure onwards the population on the host has reached a stable state, with *Curvibacter* making up 20% of the total bacterial population.

Microbial growth kinetics of di-associations

Overall *Curvibacter* growth rate in di-associations are lower or not different from the mono-associations (Fig. 5; as estimated by post hoc contrasts; Generalized linear model: Full model: χ^2=45.790, d.f.=11, P<0.0001; environment x initial frequency: χ^2=33.685, d.f.=6, P<0.0001). *Curvibacter* grows significantly differently when inoculated in equal densities as compared to the rare and dominant starting frequencies across the different environments. Whereas in the host, *Curvibacter* grows better when in equal density with *Duganella*, the opposite is true for both *in vitro* environments.

As observed for the growth of *Duganella* in mono-colonisations, growth rates are always higher in the non-host environments irrespective of initial frequency (Generalized linear model: Full model: χ^2=130.278, d.f.=11, P<0.0001; environment x initial frequency: χ^2=59.723, d.f.=6, P<0.0001). Whereas, in di-associations, negative growth rates can be detected only once for *Duganella*, it happens more frequently in the *Curvibacter*, indicating a direct or indirect negative effect of *Duganella*.

Relative performance of Curvibacter

As estimation for the relative performance of *Curvibacter* in competition with *Duganella* we determined the selection rate constant (r) for all di-association experiments (28, 29). We observed r < 0 for all environments and frequencies, indicating that *Curvibacter* performs worse than *Duganella* when they are in direct competition (Fig. 6). There is no overall effect of the initial frequency - only in the mixed environment differences can be

204 detected with the lowest performance of *Curvibacter* at equal frequencies of the two
205 competitors (Generalized linear model: Full model: χ^2=51.168, d.f.=8, P<0.0001;
206 environment x initial frequency: χ^2=19.147, d.f.=4, P=0.0007). In general, *Curvibacter*
207 performs significantly better in the host environment irrespective of initial frequency.
208

209 **Discussion**

210 One of the major challenges in microbiome research is to understand the factors that
211 influence the dynamics and stability of host-associated microbial communities. Of
212 particular relevance for this are the processes governing assembly (30, 31) and
213 resilience (32). Insights into such processes in bacterial populations within their native
214 host environments can be gained through a number of ways. Relationships between
215 different community members in complex microbiomes, such as the human gut, can, for
216 example, be inferred by analysing co-occurrence data and correlation patterns from
217 sequencing-based metagenomic time-series experiments (33, 34). Another approach,
218 where ecological interactions can experimentally be dissected, allows for hypothesis
219 testing. For this, a relatively simple system, such as *Hydra* and its microbiome is ideal for
220 'deconstructing' a metaorganism and its interactions (24). This strategy is novel in
221 meta-organism research but has yielded exciting results regarding rules that determine
222 community assembly and stability in non-host associated microbial communities (35,
223 36). In microbiome research, the added advantage of including a host provides the
224 opportunity to study the performance of the individual microbiome members in their
225 natural environment. In addition, the comparison of the *in vivo* to *in vitro* environments
226 provides information on host effects in regulating its microbiome.

227 Carrying capacity is defined as the largest population size that an ecosystem can
228 sustainably support without degrading the ecosystem. Therefore population size is
229 constrained by the carrying capacity of the environment. This is also true for bacteria
230 populating a particular environment, including hosts. In microbiome research, the

231 significance of host carrying capacity has been largely overlooked until very recently
232 where a link between host health and microbiome density has been reported (8).
233 Bacterial levels have been quantified for only a few model organisms such as in the gut
234 of larval zebrafish (37) or the gut of *Drosophila melanogaster* (38). The host's carrying
235 capacity ultimately sets the upper boundary for bacterial fitness relative to any
236 competitors, and provides a reference for investigating the interactions within the host
237 microbiota at spatial and temporal scales.

238 We here show that wild-type *Hydra* is characterized by a specific carrying
239 capacity of about 10^5 bacteria per polyp that is stable in adult polyps and can be
240 artificially assembled through the re-population of germ-free animals with the natural
241 bacterial community. This is an important prerequisite for conducting the *in vivo*
242 experiments, where colonization patterns of single species from *Hydra's* microbiome are
243 individually followed. When *Curvibacter* and *Duganella* are introduced separately to the
244 host (in mono-associations), each bacterial species is capable to robustly colonize the
245 host to high abundances, which are maintained throughout the experimental period,
246 indicating the ability to independently utilize the resources of the *Hydra* habitat. This
247 confirms earlier findings from Wein *et al.* (39) that *Curvibacter* is able to robustly
248 colonize its host. A similar observation has been made for *Aeromonas* and *Vibrio*
249 colonizing patterns of the gut of larval zebrafish (23). While both, *Curvibacter* and
250 *Duganella*, fail to reach their respective carrying capacities as compared to wild type
251 *Hydra* by a factor of 10, their respective proportions of about 75% and 11% (27) are
252 nevertheless maintained. Results from the di-association experiments clearly show that
253 the abundances and relative frequencies of *Curvibacter* and *Duganella* as measured in
254 wild-type *Hydra* cannot be explained by their (positive) interactions in the host context
255 alone as this led to a frequency reversal, making *Duganella* more abundant than
256 *Curvibacter*. *Duganella* however reaches comparable carrying capacities as measured in
257 wild-type *Hydra* polyps. This implies that the presence of *Curvibacter* has a positive

258 effect on the performance of *Duganella* but *Duganella* negatively affects *Curvibacter* in
259 the host habitat. These observations indicate that the less frequent community members
260 (each 2% and less) are of central importance for reaching the full carrying capacity of
261 the *Hydra* microbiome. We hypothesise that two aspects are of importance here – (i) the
262 low abundant microbes might be able to utilize different resources as compared to
263 *Curvibacter* and *Duganella* and so inhabit different ecological niches within the
264 microbiome, which the two main colonizers cannot fill and (ii) they likely interact in a
265 positive way (either directly or indirectly) with (at least) *Curvibacter*, enabling it to
266 reach higher carrying capacities. It is thus important to note that *Hydra*'s carrying
267 capacity is not solely determined by the host (resources) alone but also by the
268 interactions within the microbiome.

269 To determine the relative importance of the host, interactions of *Curvibacter* and
270 *Duganella*, and intrinsic properties of the bacteria, such as ability to grow, in shaping the
271 observed patterns in the host environment, we performed the same mono- and di-
272 association experiments in two i*n vitro* environments. We chose to contrast static, which
273 closely resembles the host habitat in that it provides spatial heterogeneity facilitating
274 bacterial interactions and mixed, where direct interactions between individual bacteria
275 cannot be established but where individual bacteria have (unlimited) access to
276 resources and oxygen. Because community structure can be influenced by initial species
277 abundances (40), we performed all di-association experiments using various initial
278 starting frequencies. In general, both bacterial species reached much higher carrying
279 capacities in both *in vitro* environments as compared to the *in vivo* environment, with
280 *Duganella* reaching up to 10^8 CFUs/ml and *Curvibacter* up to 10^5 CFUs/ml. Interestingly
281 we were not able to replicate the *in vivo* dynamics between *Curvibacter* and *Duganella in*
282 *vitro*, suggesting that the host mediates the interaction between the two bacterial
283 species. Previous work has suggested that continued coexistence between bacterial
284 species is dependent on their relative growth. Coexistence is maintained if the slower-

285 growing species is released from its dependence on the other (41), which in our case
286 could be compensated by the host. This aspect will be subject of further investigation.
287 Wright and Vetsigian (42) recently demonstrated in pairwise competitions between
288 bacteria of the genus *Streptomyces* that the winner is often the species that starts at high
289 initial abundance. We find that 'survival of the common' does not apply to *Curvibacter* in
290 a non-host environment. Whereas pairwise competitions in the di-association
291 experiments resulted in coexistence of *Curvibacter* and *Duganella* in the host habitat, it
292 led to an overgrowth of *Duganella* in both *in vitro* habitats. More specifically, we found
293 that in the *Hydra* habitat *Curvibacter*, independent of its inoculation frequency, and after
294 the initial establishment period of 72 h, reached a constant fraction of about 20%,
295 whereas it was only present at very low frequencies in both *in vitro* habitats due to the
296 high continuous growth rates of *Duganella* (see also (43) for homogeneous
297 environment). This pattern seems to be more pronounced in the static environment,
298 fitting to the general assumption that extinctions are more likely to occur in spatially
299 structure environments. In terms of cross-feeding interactions one could reason that the
300 faster growing-species is released from its dependence on the other, resulting in the
301 slower-growing species being lost (a hypothesis that is termed 'feed the faster grower')
302 (41).

303 Many host-associated microbial communities live in highly competitive
304 surroundings, in which they struggle to persist. According to Ghoul and Mitri (44), a
305 strain is competitive if it shows phenotypes that cause a fitness decrease in a competitor
306 strain. Competition can be either passive, where strains compete for the same resources
307 or active, where strains directly harm one another (44). Li *et al.* (43) showed that during
308 co-culture experiments in a homogenous environment the presence and frequency of
309 *Curvibacter* greatly influenced the growth rate of *Duganella* (quadratic function), which
310 we also found. Interestingly we find the same pattern on the host, although growth rates

311 are drastically reduced. In the static environment we find the inverse of the quadratic
312 function.

313 When comparing the bacterial growth rates in mono-associations we observe
314 that *Curvibacter* performs equally well in all habitats, whereas *Duganella* shows a
315 reduced growth rate compared to *Curvibacter* on the host and performs best in the non-
316 host habitat. This suggests that *Duganella* is not particularly adapted to the host niche.
317 Interestingly the two species also differ in their phylogenetic association patterns with
318 different *Hydra* species (45). Whereas different lineages of *Curvibacter* have been shown
319 to be associated with the majority (N=6) of *Hydra* species analysed (N=7), *Duganella*
320 was detected in less than half (N=3). This indicates that *Duganella* is a more recent
321 member of the *Hydra* microbiome and that it is not as co-evolved as it has been
322 suggested for one of *the* keystone species of *Hydra's* microbiome, *Curvibacter* (43).
323 Nevertheless, the specific microbiome of *Hydra vulgaris*, which is studied here, has been
324 shown to be stable as animals living in the wild were colonized by a similar microbiome
325 as compared to the ones that had been maintained in the laboratory for a relatively long
326 time (25). One of the few metaorganisms, where symbiont performance within the host
327 relative to a non-host environment has been contrasted, is the squid-*Vibrio* system. For
328 this obligate association Wollenberg and Ruby (19) demonstrated that symbionts have
329 an increased reproductive capacity and higher fitness when associated with the host. In
330 combination with the results from this study, this indicates that more specifically
331 associated symbionts indeed benefit from growing in the host habitat.

332 Oscillatory dynamics, chaotic fluctuations and stochastic perturbations influence
333 species abundances and allow for the coexistence of many species and have therefore a
334 significant impact on species survival even in simple communities containing only a few
335 interacting species (46-48). Interestingly we observed such sporadic collapses in the
336 *Curvibacter* population during di-association experiments in the non-host environment
337 but not on the host. An earlier study following the bacterial colonization pattern of

338 *Hydra* hatchlings showed sporadic collapses of the *Curvibacter* population during the
339 first 15 weeks post hatching (49). In adult polyps, which were used in the experiments,
340 we did not observe such dynamics, which points to the fact that these sporadic collapses
341 might be buffered in adult polyps or are caused by the other species of the *Hydra*
342 microbiome. For the zebrafish host model it has been shown that *Aeromonas* during
343 mono-association is not resistant to disturbances related to intestinal motility and that
344 in the presence of *Vibrio* the magnitude of collapses are even greater (23). *Hydra* is also
345 characterized by spontaneous contractile activity (50) but our data suggest that this
346 does not have such far-reaching effects on the microbiota as found in the zebrafish gut.

347 Whereas in other host-microbe systems such as the zebrafish, *in vivo* dynamics
348 between two bacterial species can be replicated *in vitro* (51), in our system this is not
349 the case. We find the fitness of *Curvibacter* and *Duganella* to be highly dependent on the
350 specific environments tested. Whereas *Duganella* performs best *in vitro*, in terms of
351 carrying capacities reached, absolute fitness (growth rates) but also relative fitness
352 (fractions in population, selection rate constant), the opposite is true for all parameters
353 in *Curvibacter* which generally performs best in the host habitat, except for its carrying
354 capacity that is higher *in vitro*. Our experiments reveal that not only direct bacterial
355 interactions are important but also highlight the fact that the host plays an important
356 role in mediating these interactions (Fig. 7). Further, we were able to show that carrying
357 capacity is a fundamental property of a host-microbe system and that it is not only
358 determined by the physiological features of the host but also by the interactions within
359 the microbiome. Detecting a selection rate constant of $r < 0$ for the main *Hydra*
360 colonizer, *Curvibacter*, in the host environment and a reduced carrying capacity in both,
361 mono- and di-associations, suggests that low-abundance species of the *Hydra*
362 microbiome are likely playing a major role in maintaining the density of *Curvibacter* on
363 the host. Evidence for the importance of rare-species comes from the human intestinal
364 microbiota, which contains many low-abundance species (52) with some of them having

365 a large impact on inducing dysbiosis in the microbiome and on guaranteeing host health
366 (53, 54).
367
368 **Materials and Methods**
369 **Animals used, culture conditions and generation of germ-free animals**
370 *Hydra vulgaris* (strain AEP) was used for carrying out experiments and cultured
371 according to standard procedures at 18°C in standardized culture medium (Hydra
372 medium (HM)) (55). Animals were fed three times a week with 1st instar larvae of
373 *Artemia salina*. Germ-free (GF) polyps were obtained by treating wild-type (WT)
374 animals with an antibiotic cocktail solution containing 50 μg/ml ampicillin, neomycin,
375 streptomycin, rifampicin and 60 μg/ml spectinomycin as previously described (45, 50).
376 The antibiotic cocktail solution was exchanged every 48 h and the antibiotic treatment
377 lasted for two weeks, after which polyps were transferred into antibiotic-free sterile HM
378 for recovery (four days). The germ-free status of polyps was confirmed as previously
379 described (45). During antibiotic treatment and re-colonization experiments, polyps
380 were not fed.
381
382 **Bacterial strains and media**
383 The bacterial strains used in this study are *Curvibacter* sp. AEP1.3 and *Duganella* sp.
384 C1.2., which have been isolated from *Hydra vulgaris* (strain AEP) (27). These bacteria
385 were cultured from existing isolate stocks in R2A medium at 18°C, shaken at 250 r.p.m
386 for 72 h before use in the different experiments.
387
388 **Carrying capacity of the host**
389 To determine the carrying capacity of the *Hydra* habitat the microbial load of individual
390 *Hydra* polyps (N=16) was determined. In addition to wild-type polyps the carrying
391 capacity of conventionalized polys (N=12), obtained by incubating germ-free polyps

392 with tissue homogenates of wild-type animals (per germ-free polyp one wild-type polyp
393 was used) for 24 h was also determined. To test whether the carrying capacity can
394 artificially be increased or destabilises upon self-challenge we added either *Curvibacter*
395 or *Duganella* to wild-type polyps (N=6) (approximately $5x10^3$ cells for 24 h). After
396 incubation all polyps were washed with and transferred to sterile HM and further
397 incubated at 18°C and sampled after 120 h.
398
399 **Tracking microbial mono-associations in *Hydra* over time**
400 Germ-free polys were inoculated in their aquatic environment with single bacterial
401 strains (mono-associations). Individual germ-free polyps were incubated with $5x10^3$
402 cells of *Curvibacter* or *Duganella* in 1.5 ml Eppendorf tubes containing 1 ml of sterile
403 HM. After 24 h of incubation all polyps were washed with and transferred to sterile HM,
404 incubated at 18°C and followed over a period of 216 h. For each treatment 6 polys per
405 time point were independently analysed. Every 48 h individual polyps were
406 homogenized in an Eppendorf tube using a sterile pestle and serial dilutions of the
407 homogenate were plated on R2A agar plates to determine colony-forming units (CFUs)
408 per polyp.
409
410 **Microbial growth kinetics of mono- and di-associations *in vivo* and *in vitro***
411 To study the initial phase of colonization, i.e. 96 h post inoculation (see Fig. 1B) in more
412 detail microbial growth of *Curvibacter* and *Duganella* was determined in different
413 habitats; the host habitat (*in vivo*) and two different microcosm environments (*in vitro*).
414 The static incubation provided a spatially structured habitat (heterogeneous), whereas
415 shaking of the microcosms (mixed treatment) eliminated the spatial structure
416 (homogenous).
417 *Mono-associations:* All germ-free polyps and microcosms were inoculated from
418 the same bacterial inoculation culture with approximately $5x10^3$ cells of *Curvibacter* or

419 *Duganella* for 24 h, washed with and transferred to sterile HM. Samples were taken
420 every 12 h for 96 h. For *Hydra* six polyps were sacrificed at each time point and colony-
421 forming units (CFUs) were determined as described above. As microcosms 24-well
422 plates were used. Wells were filled with 2 ml of R2A medium, inoculated and incubated
423 at 18°C either under static or shaken (200 r.p.m) conditions. Each time point was
424 replicated four times and serial dilutions were plated on R2A agar plates to determine
425 CFUs. Growth rates of each strain (A and B) were determined for the exponential growth
426 phase (36-48 h) and were calculated as $g = \ln (A_f/A_0)$ and $g = \ln (B_f/B_0)$, where A_0, B_0 is
427 the starting density at time 0 and A_f, B_f is the final density at time *f*.

428 *Di-associations:* Density dependent competiveness fitness assays of the two most
429 dominant colonizers *Curvibacter* and *Duganella* were performed *in vivo* and *in vitro.* The
430 same host and microcosm experiments as described above were performed except for
431 using microbial di-associations of *Curvibacter* and *Duganella* with the frequency of
432 *Curvibacter* being rare (10:90), equal (50:50), or dominant (90:10). As *Curvibacter* and
433 *Duganella* form distinct colonies on R2A agar plates, their frequency can be determined
434 by plating serial dilutions (43). Six polyps and four microcosm replicates were assayed
435 per treatment (static and mixed) and time point. Also this data allowed determining the
436 different carrying capacities of the *in vivo* and *in vitro* habitats used. Malthusian
437 parameters were calculated for both bacterial strains (A and B), $m = \ln (N_f/N_0)$/hour,
438 where N_0 and N_f were the initial and final densities. The relative performance of the two
439 strains was expressed in terms of the selection rate constant (*r*). Note that this measure
440 of performance should be preferentially used over the more commonly used relative
441 fitness (*W*), when one competitor is much less fit than the other (28, 29), as was the case
442 in our study. The selection rate constant was calculated as $r = m_B - m_A$. Accordingly, $r = 0$
443 implies that there is no difference in growth rates of the two strains, $r > 0$ indicates that
444 strain B is advantageous in population growth ($r = 1$ indicates a 10-fold increase in the

445 ratio of B vs. A abundances), while r < 0 suggests that strain B fails in invading
446 population of strain A.
447
448 **Statistical analysis**
449 A Welch ANOVA (and subsequent Dunnett's posthoc test) was used to test for
450 differences in bacterial abundance patterns ('bacteria per *Hydra*') in wild-type versus
451 manipulated hosts as variances between the different groups were not equally
452 distributed.
453 Differences during mono-colonizations of *Curvibacter* and *Duganella* over time
454 were assessed using a Generalized linear model (error structure: normal; link function:
455 identity). The response variable was 'bacteria per *Hydra*', and explanatory variables
456 were 'bacterial species', 'time' and 'bacterial species' x 'time'. Differences between the
457 two bacterial species on each day were detected with post hoc contrasts.
458 Analysis of variance (ANOVA) and subsequent post hoc t-tests were used to test
459 for differences in growth rates of the two competitors when grown singly in the
460 different environments. The response variable was 'growth rate', and explanatory
461 variables were 'bacterial species', 'environment' and 'bacterial species' x 'environment'.
462 Differences in the growth rates in the di-associations of *Curvibacter* and
463 *Duganella* in the different environments and dependence on initial frequency were
464 assessed using a Generalized linear model (error structure: normal; link function:
465 identity) and post hoc contrasts. For each bacterial species, a separate model was
466 calculated with the response variables being either 'growth rate *Curvibacter*' or 'growth
467 rate *Duganella*', and the explanatory variables were 'environment', 'starting density' and
468 'environment' x 'starting density'.
469 Differences in the selection rate constant of *Curvibacter* in the different
470 environments and dependent on initial frequency were assessed using a Generalized
471 linear model (error structure: normal; link function: identity) and post hoc contrasts.

472 The response variable was 'selection rate constant *Curvibacter*' and the explanatory
473 variables were 'environment', 'starting density' and 'environment' x 'starting density'.
474 Sample size was chosen to maximise statistical power and ensure sufficient
475 replication. Assumptions of the tests, that is, normality and equal distribution of
476 variances, were visually evaluated. Non-significant interactions were removed from the
477 models. All tests were two-tailed. Effects were considered significant at the level of $P <$
478 0.05. All statistical analyses were performed with JMP 9. Graphs were produced with
479 GraphPad Prism 5.0, and Adobe Illustrator CS5.1.
480

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Conflict of Interests

The authors declare no conflict of interest.

497 **References**

498 1. **Turnbaugh PJ**, **Ley RE**, **Hamady M**, **Fraser-Liggett CM**, **Knight R**, **Gordon**
499 **JI**. 2007. The human microbiome project. Nature **449**:804–810.

500 2. **Huttenhower C**, **Gevers D**, **Knight R**, **Abubucker S**, **Badger JH**, **Chinwalla**
501 **AT**, ***et al.***. 2012. Structure, function and diversity of the healthy human
502 microbiome. Nature **486**:207–214.

503 3. **Pasolli E**, **Asnicar F**, **Manara S**, **Zolfo M**, **Karcher N**, **Armanini F**, ***et al.***.
504 2019. Extensive unexplored human microbiome diversity revealed by over
505 150,000 genomes from metagenomes spanning age, geography, and
506 lifestyle. Cell **176**:649–662.e20.

507 4. **Mueller UG**, **Sachs JL**. 2015. Engineering microbiomes to improve plant
508 and animal health. Trends Microbiol **23**:606–617.

509 5. **McKenney PT**, **Pamer EG**. 2015. From hype to hope: The gut microbiota in
510 enteric infectious disease. Cell **163**:1326–1332.

511 6. **Gilbert JA**, **Blaser MJ**, **Caporaso JG**, **Jansson JK**, **Lynch SV**, **Knight R**. 2018.
512 Current understanding of the human microbiome. Nat Med **24**:392–400.

513 7. **McKenzie VJ**, **Kueneman JG**, **Harris RN**. 2018. Probiotics as a tool for
514 disease mitigation in wildlife: insights from food production and medicine.
515 Ann N Y Acad Sci **287**:443.

516 8. **Contijoch EJ**, **Britton GJ**, **Yang C**, **Mogno I**, **Li Z**, **Ng R**, ***et al.***. 2019. Gut
517 microbiota density influences host physiology and is shaped by host and
518 microbial factors. eLife **8**:337.

519 9. **Lozupone CA**, **Stombaugh JI**, **Gordon JI**, **Jansson JK**, **Knight R**. 2012.
520 Diversity, stability and resilience of the human gut microbiota. Nature
521 **489**:220–230.

522 10. **Sachs JL**, **Essenberg CJ**, **Turcotte MM**. 2011. New paradigms for the
523 evolution of beneficial infections. Trends Ecol Evol **26**:202–209.

524 11. **Deines P**, **Lachnit T**, **Bosch TCG**. 2017. Competing forces maintain the
525 Hydra metaorganism. Immunol Rev **279**:123–136.

526 12. **Macke E**, **Tasiemski A**, **Massol F**, **Callens M**, **Decaestecker E**. 2017. Life
527 history and eco-evolutionary dynamics in light of the gut microbiota. Oikos
528 **126**:508–531.

529 13. **Eckburg PB**, **Bik EM**, **Bernstein CN**, **Purdom E**, **Dethlefsen Les**, **Sargent**
530 **M**, **Gill SR**, **Nelson KE**, **Relman DA**. 2005. Diversity of the human intestinal
531 microbial flora. Science **308**:1635–1638.

532 14. **Zhang Z**, **Geng J**, **Tang X**, **Fan H**, **Xu J**, **Wen X**, ***et al.***. 2014. Spatial
533 heterogeneity and co-occurrence patterns of human mucosal-associated
534 intestinal microbiota. ISME J **8**:881–893.

535 15. **Britton RA**, **Young VB**. 2012. Interaction between the intestinal microbiota
536 and host in Clostridium difficile colonization resistance. Trends Microbiol

537 **20**:313–319.

538 16. **Levy R**, **Borenstein E**. 2014. Metagenomic systems biology and metabolic
539 modeling of the human microbiome. Gut Microbes **5**:265–270.

540 17. **Zelezniak A**, **Andrejev S**, **Ponomarova O**, **Mende DR**, **Bork P**, **Patil KR**.
541 2015. Metabolic dependencies drive species co-occurrence in diverse
542 microbial communities. Proc Natl Acad Sci USA **112**:6449–6454.

543 18. **Garcia JR**, **Gerardo NM**. 2014. The symbiont side of symbiosis: do
544 microbes really benefit? Front Microbiol **5**:1103.

545 19. **Wollenberg MS**, **Ruby EG**. 2012. Phylogeny and fitness of *Vibrio fischeri*
546 from the light organs of *Euprymna scolopes* in two Oahu, Hawaii
547 populations. ISME J **6**:352–362.

548 20. **Wiser MJ**, **Lenski RE**. 2015. A comparison of methods to measure fitness in
549 *Escherichia coli*. PLoS ONE **10**:e0126210.

550 21. **Lenski RE**. 1992. Relative fitness: its estimation and its significance for
551 environmental applications of microorganisms, pp. 183–198. *In* Levin, M,
552 Seidler, R, Rogul, M (eds.), Microbial Ecology: Principles, Applications and
553 Methods. myxo.css.msu.edu, New York.

554 22. **Mushegian AA**, **Ebert D**. 2015. Rethinking "mutualism" in diverse host-
555 symbiont communities. Bioessays **38**:100–108.

556 23. **Wiles TJ**, **Jemielita M**, **Baker RP**, **Schlomann BH**, **Logan SL**, **Ganz J**, ***et al.***.
557 2016. Host gut motility promotes competitive exclusion within a model
558 intestinal microbiota. PLoS Biol **14**:e1002517.

559 24. **Deines P**, **Bosch TCG**. 2016. Transitioning from microbiome composition
560 to microbial community interactions: The potential of the metaorganism
561 *Hydra* as an experimental model. Front Microbiol **7**:1610.

562 25. **Fraune S**, **Bosch TCG**. 2007. Long-term maintenance of species-specific
563 bacterial microbiota in the basal metazoan *Hydra*. Proc Natl Acad Sci USA.

564 26. **Bosch TCG**. 2013. Cnidarian-microbe interactions and the origin of innate
565 immunity in metazoans. Annu Rev Microbiol **67**:499–518.

566 27. **Fraune S**, **Anton-Erxleben F**, **Augustin R**, **Franzenburg S**, **Knop M**,
567 **Schröder K**, ***et al.***. 2015. Bacteria-bacteria interactions within the
568 microbiota of the ancestral metazoan *Hydra* contribute to fungal resistance.
569 ISME J **9**:1543–1556.

570 28. **Lenski RE**, **Rose MR**, **Simpson SC**, **Tadler SC**. 1991. Long-term
571 experimental evolution in *Escherichia coli*. I. Adaptation and divergence
572 during 2,000 generations. Am Nat **138**:1315–1341.

573 29. **Travisano M**, **Lenski RE**. 1996. Long-term experimental evolution in
574 *Escherichia coli*. IV. Targets of selection and the specificity of adaptation.
575 Genetics **143**:15–26.

576 30. **Sonnenburg JL**, **Angenent LT**, **Gordon JI**. 2004. Getting a grip on things:

how do communities of bacterial symbionts become established in our intestine? Nat Immunol **5**:569–573.

31. **Shafquat A**, **Joice R**, **Simmons SL**, **Huttenhower C**. 2014. Functional and phylogenetic assembly of microbial communities in the human microbiome. Trends Microbiol **22**:261–266.

32. **Allison SD**, **Martiny JBH**. 2008. Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci USA **105**:11512–11519.

33. **Faust K**, **Raes J**. 2012. Microbial interactions: from networks to models. Nat Rev Microbiol **10**:538–550.

34. **Trosvik P**, **de Muinck EJ**, **Stenseth NC**. 2015. Biotic interactions and temporal dynamics of the human gastrointestinal microbiota. ISME J **9**:533–541.

35. **Friedman J**, **Higgins LM**, **Gore J**. 2017. Community structure follows simple assembly rules in microbial microcosms. Nat Ecol Evol **1**:109.

36. **Higgins LM**, **Friedman J**, **Shen H**, **Gore J**. 2017. Co-occurring soil bacteria exhibit a robust competitive hierarchy and lack of non-transitive interactions. bioRxiv 175737.

37. **Jemielita M**, **Taormina MJ**, **Burns AR**, **Hampton JS**, **Rolig AS**, **Guillemin K**, ***et al.***. 2014. Spatial and temporal features of the growth of a bacterial species colonizing the zebrafish gut. mBio, **5**:e01751-14.

38. **Pais IS**, **Valente RS**, **Sporniak M**, **Teixeira L**. 2018. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. PLoS Biol **16**:e2005710.

39. **Wein T**, **Dagan T**, **Fraune S**, **Bosch TCG**, **Reusch TBH**, **Hülter NF**. 2018. Carrying capacity and colonization dynamics of *Curvibacter* in the *Hydra* host habitat. Front Microbiol **9**:185.

40. **Fukami T**. 2015. Historical contingency in community assembly: Integrating niches, species pools, and priority effects. Annu Rev Ecol Evol Syst **46**: 1-23.

41. **Hammarlund SP**, **Chacón JM**, **Harcombe WR**. 2019. A shared limiting resource leads to competitive exclusion in a cross-feeding system. Environ Microbiol **21**:759–771.

42. **Wright ES**, **Vetsigian KH**. 2016. Inhibitory interactions promote frequent bistability among competing bacteria. Nat Commun **7**:11274.

43. **Li X-Y**, **Pietschke C**, **Fraune S**, **Altrock PM**, **Bosch TCG**, **Traulsen A**. 2015. Which games are growing bacterial populations playing? J R Soc Interface **12**:20150121–10.

44. **Ghoul M**, **Mitri S**. 2016. The ecology and evolution of microbial competition. Trends Microbiol **24**:833–845.

45. **Franzenburg S**, **Walter J**, **Künzel S**, **Wang J**, **Baines JF**, **Bosch TCG**, ***et al.***.

617 2013. Distinct antimicrobial peptide expression determines host species-
618 specific bacterial associations. Proc Natl Acad Sci USA **110**:E3730–8.

619 46. **Armstrong RA**, **McGehee R**. 2015. Competitive exclusion. Am Nat
620 **115**:151–170.

621 47. **Sousa WP**. 1984. The role of disturbance in natural communities. Annu Rev
622 Ecol Syst 353–391.

623 48. **Huisman J**, **Weissing FJ**. 1999. Biodiversity of plankton by species
624 oscillations and chaos. Nature **402**:407–410.

625 49. **Franzenburg S**, **Fraune S**, **Altrock PM**, **Künzel S**, **Baines JF**, **Traulsen A**,
626 ***et al.***. 2013. Bacterial colonization of Hydra hatchlings follows a robust
627 temporal pattern. ISME J **7**:781–790.

628 50. **Murillo-Rincon AP**, **Klimovich A**, **Pemöller E**, **Taubenheim J**, **Mortzfeld**
629 **B**, **Augustin R**, ***et al.***. 2017. Spontaneous body contractions are modulated
630 by the microbiome of *Hydra*. Sci Rep **7**:15937.

631 51. **Rolig AS**, **Parthasarathy R**, **Burns AR**, **Bohannan BJM**, **Guillemin K**. 2015.
632 Individual members of the microbiota disproportionately modulate host
633 innate immune responses. Cell Host Microbe **18**:613–620.

634 52. **Arumugam M**, **Raes J**, **Pelletier E**, **Le Paslier D**, **Yamada T**, **Mende DR**, ***et***
635 ***al.***. 2011. Enterotypes of the human gut microbiome. Nature **473**:174–180.

636 53. **Hajishengallis G**, **Darveau RP**, **Curtis MA**. 2012. The keystone-pathogen
637 hypothesis. Nat Rev Microbiol **10**:717–725.

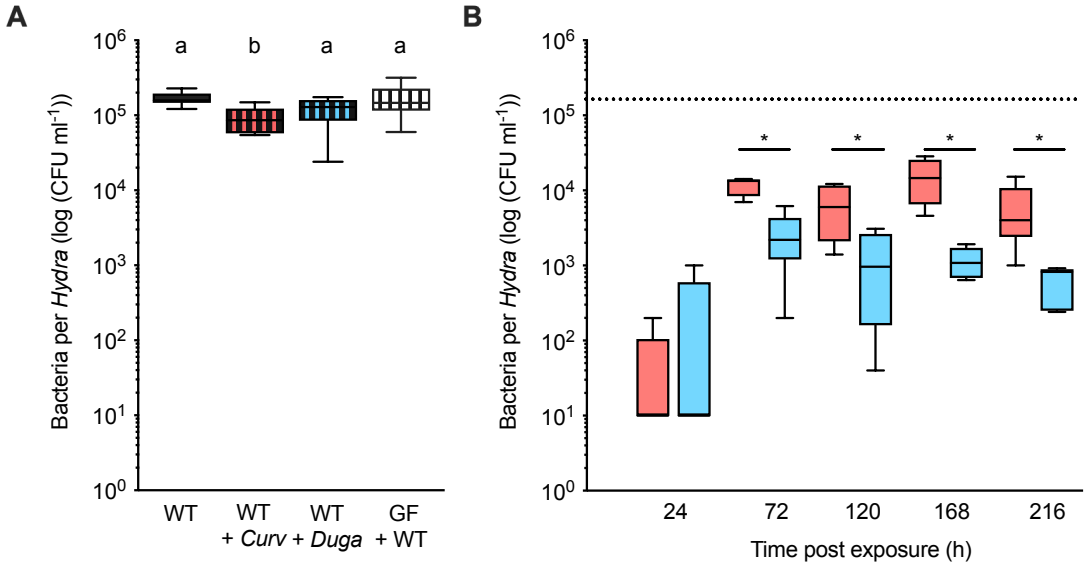
638 54. **Sokol H**, **Pigneur B**, **Watterlot L**, **Lakhdari O**, **Bermúdez-Humarán LG**,
639 **Gratadoux J-J**, ***et al.***. 2008. F*aecalibacterium prausnitzii* is an anti-
640 inflammatory commensal bacterium identified by gut microbiota analysis
641 of Crohn disease patients. Proc Natl Acad Sci USA **105**:16731–16736.

642 55. **Lenhoff HM**, **Brown RD**. 1970. Mass culture of *Hydra*: an improved method
643 and its application to other aquatic invertebrates. Lab Anim **4**:139–154.

644

645 **Figures**

646



647

648 Fig 1. (A) Carrying capacity of the *Hydra* habitat in wild-type (WT) polyps, wild-type
649 polyps and the addition of either the focal species *Curvibacter* (*Curv*) or *Duganella*
650 (*Duga*) and germ-free (GF) animals incubated with native *Hydra* microbiota
651 (conventionalised polyps) (each boxplot N≥6≤16). (B) Time course analysis of microbial
652 abundances in mono-associations of germ-free polyps with either *Curvibacter* (red) or
653 *Duganella* (blue) (each boxplot N=6). The dashed line indicates the carrying capacity of
654 wild-type polys.

655

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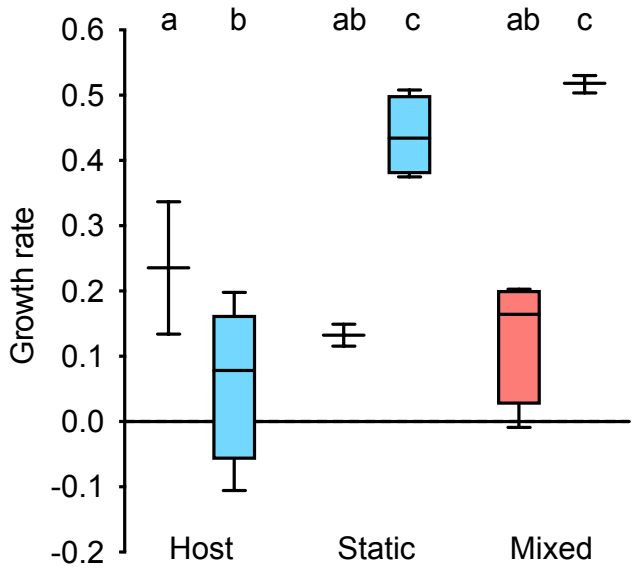

657

658 Fig 2. Bacterial growth rates of *Curvibacter* (red; each boxplot N≥2≤4) and *Duganella*
659 (blue; each boxplot N≥3≤6) in mono-associations are habitat dependent. Compared are
660 the host habitat (*in vivo*), and two *in vitro* environments: heterogeneous (static
661 microcosms), and homogenous (mixed microcosms).

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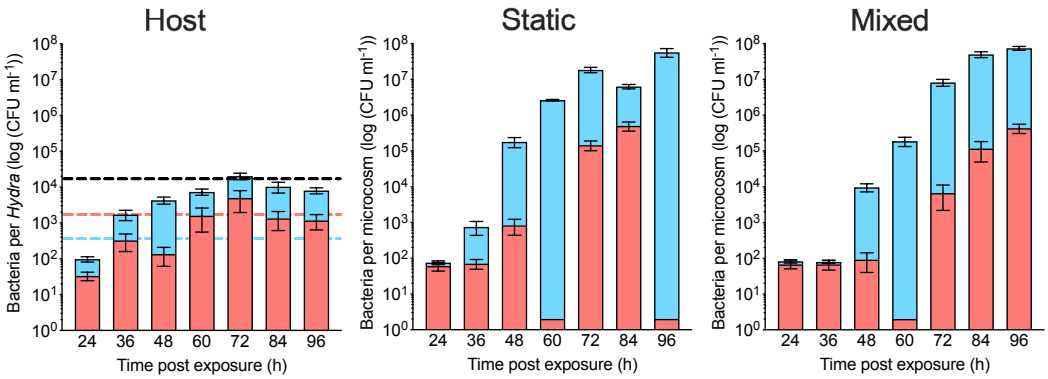
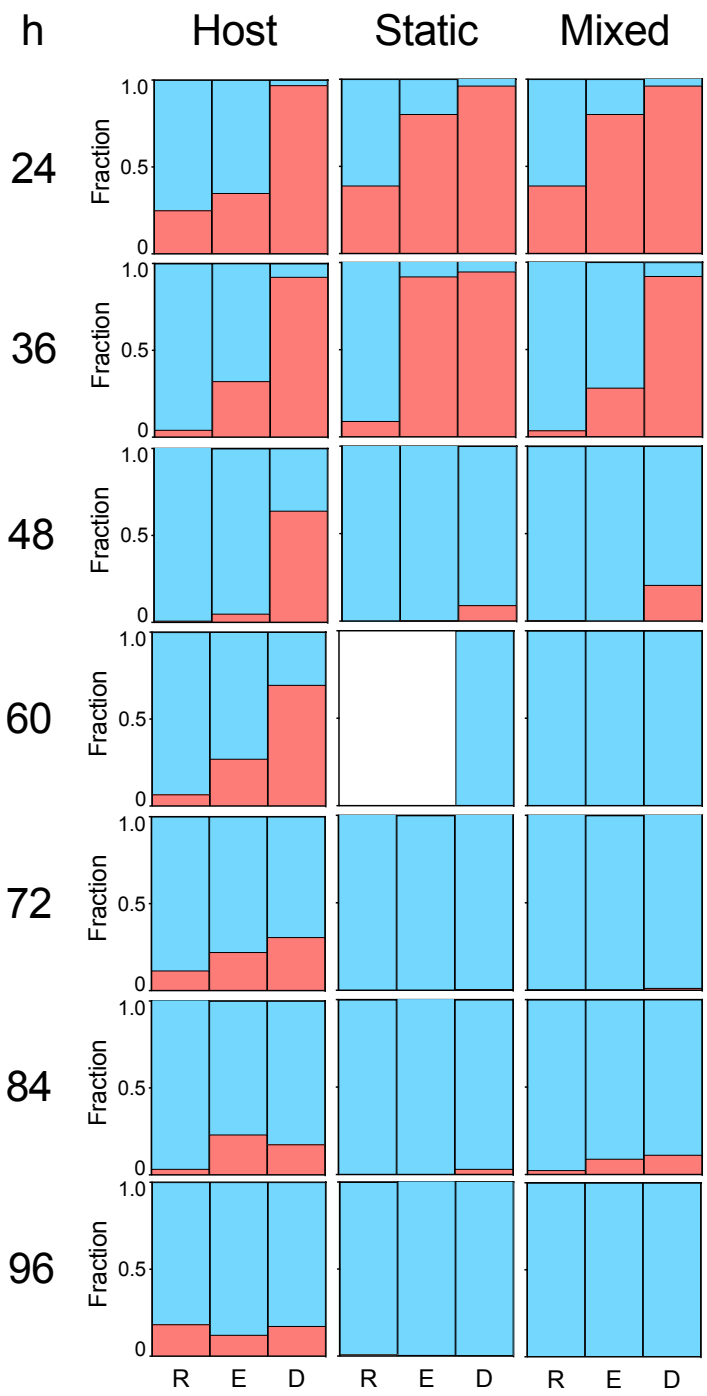


664

665 Fig 3. Carrying capacity of the *in vivo* and *in vitro* habitats used in this study. Shown are
666 pooled total numbers of colony forming units (CFUs) from all di-association
667 experiments with *Curvibacter* (red) and *Duganella* (blue) (shown are s.e.m. based on
668 N≥11≤18 for the host, N≥9≤12 for static, and N≥4≤12 for mixed). The dashed black line
669 indicates the carrying capacity of WT polyps, the light grey line the carrying capacity of
670 polyps during *Curvibacter* mono-associations and the dark grey line the carrying
671 capacity of polyps during *Duganella* mono-associations.

672



673

674 Fig 4. Time course of fractions of *Curvibacter* (red) and *Duganella* (blue) in the three
675 different habitats obtained from di-association experiments. The initial inoculation
676 frequency of *Curvibacter* varied from being rare (R), to equal (E), to dominant (D) in
677 comparison to *Duganella* (each bar N≥3≤6; except for: Host, D, 24 h, where N=2, and for
678 Static, R and E 60 h, where N=0 due to contamination of plates).

679

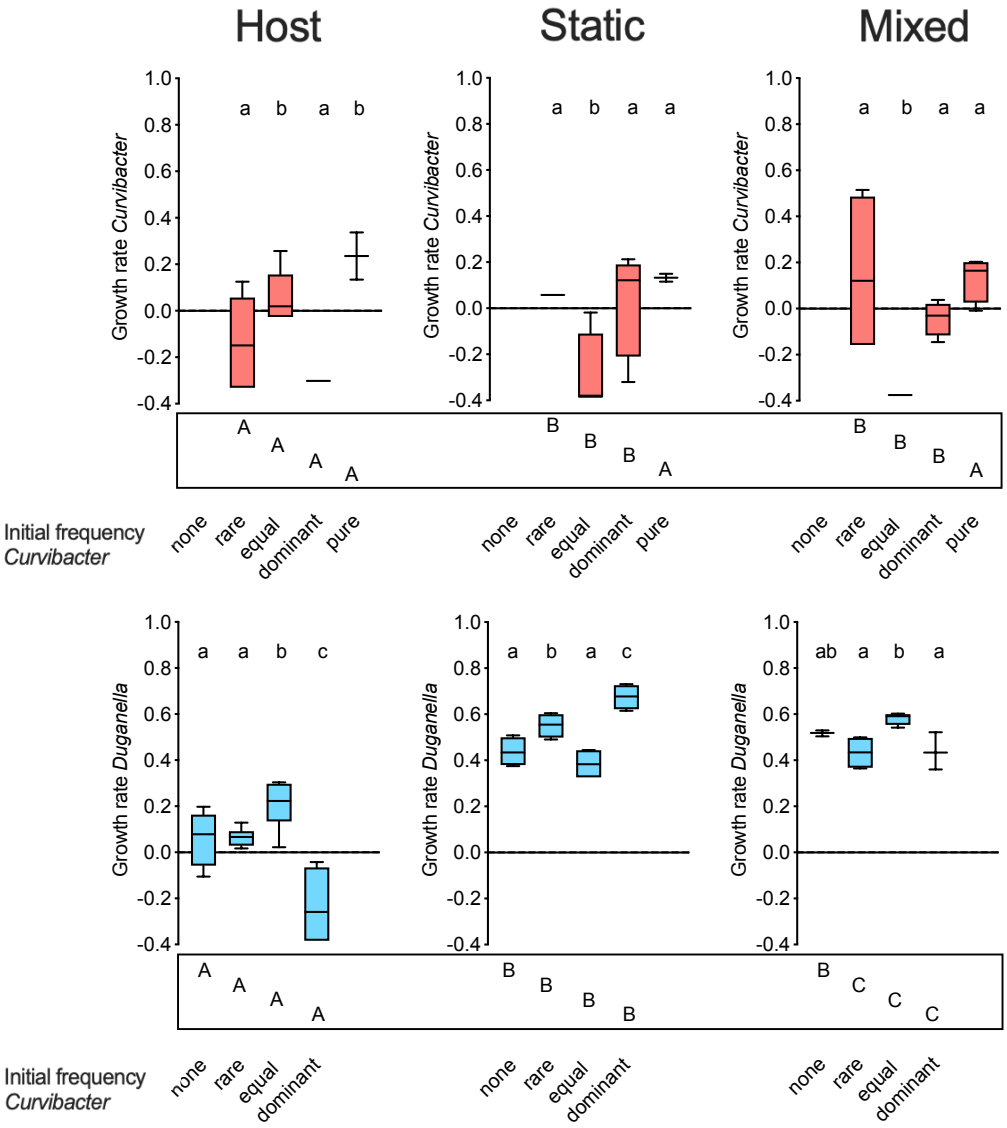


Fig 5. Bacterial growth rates of *Curvibacter* (red) and *Duganella* (blue) from mono- and di-association experiments across the different habitats and initial frequencies tested (each boxplot N≥3≤4).

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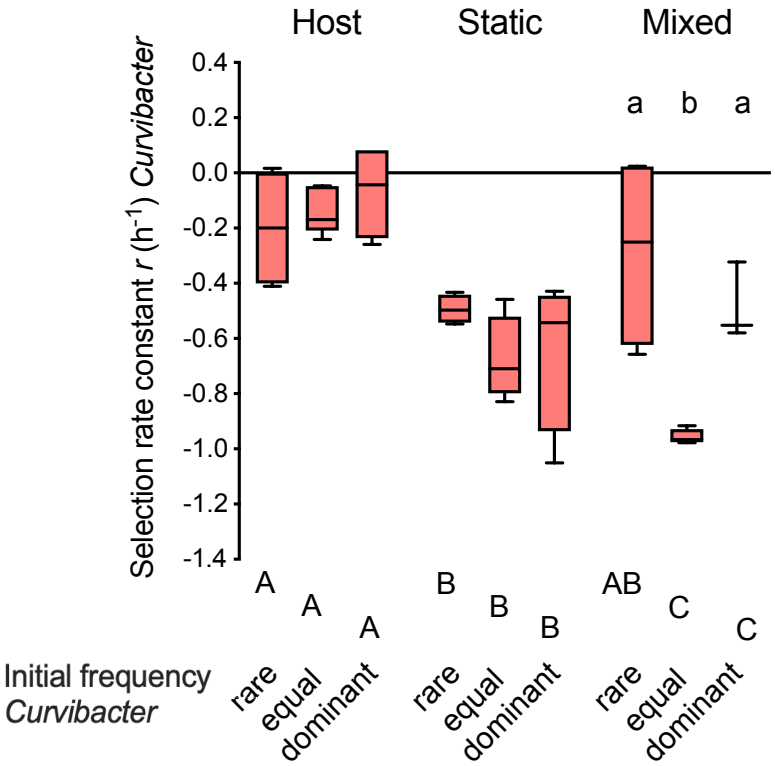

687

688 Fig. 6. Selection rate constants of *Curvibacter* based on competition experiments with

689 *Duganella* for the different starting frequencies, (each boxplot N≥3≤6).

690

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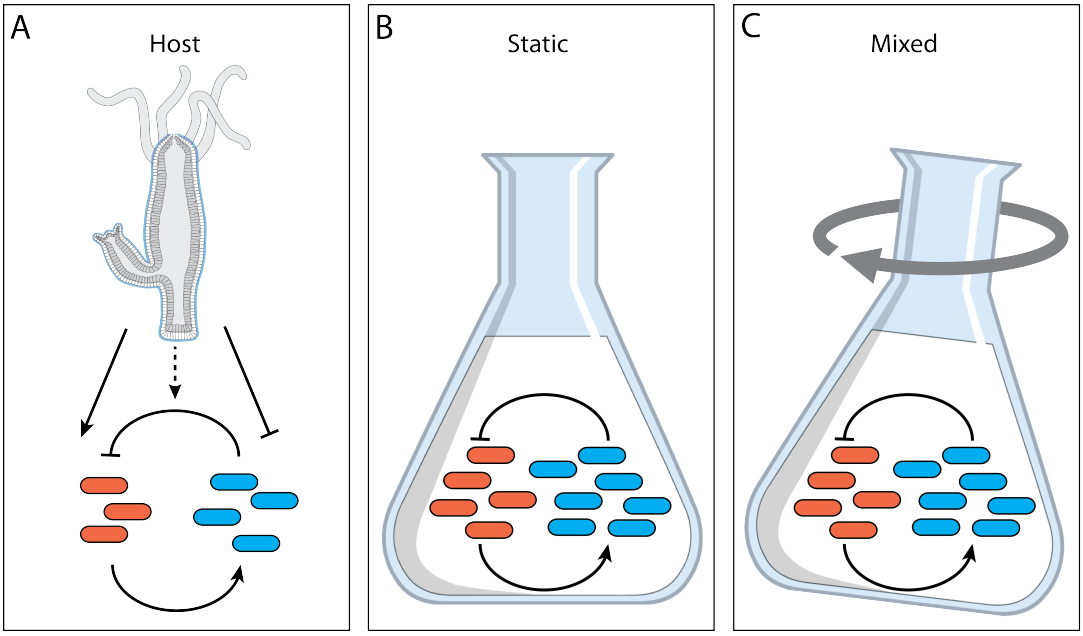

692

693 Fig. 7. Model of the microbe-microbe and host-microbe interactions occurring in
694 different habitats (host (A) and non-host (B and C)) that influence bacterial abundances,
695 which are indicated by the number of bacterial cells (*Curvibacter*: red, *Duganella*: blue).
696 Whereas (A) allows to study the effect of the host environment on the two microbial
697 species and their interactions, microcosm experiments allow to study species
698 interaction in a heterogeneous (B) and a homogeneous environment (C). Arrow-headed
699 lines indicate promotion, whereas bar-headed lines indicate inhibition.