Microbial species coexistence depends on the host environment

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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 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 the host environment lead to the observed species frequencies and abundances. More specifically, we find that both microbial species can only stably coexist in the host environment, whereas *Duganella* outcompetes *Curvibacter* in both *in vitro* environments irrespective of initial starting frequencies. While *Duganella* seems to benefit through secretions of *Curvibacter*, its competitive effect on *Curvibacter* depends upon direct contact. The competition might potentially be mitigated through the spatial structure of the two microbial species on the host, which would explain why both species stably coexist on the host. Interestingly, the fractions of both species on the host do not match the fractions reported previously nor the overall microbiome carrying capacity as reported in this study. Both observations indicate that the rare microbial community members might be relevant for achieving the native community composition and carrying capacity. Our study highlights that for dissecting microbial interactions the specific environmental conditions need to be replicated, a goal difficult to achieve with *in vitro* systems.

Importance

This work studies microbial interactions within the microbiome of the simple cnidarian, *Hydra*, and investigates whether microbial species coexistence and community stability depends on the host environment. We find that the outcome of the interaction between the two most dominant bacterial species in *Hydra*'s microbiome differs depending on the environment and only results in a stable coexistence in the host context. The interactive ecology between the host, the two most dominant microbes, but also the less abundant members of the microbiome, are critically important for achieving the native community composition. This indicates that the metaorganism environment needs to be taken into account when studying microbial interactions.

53 **Introduction**

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

63 A major goal in host-microbe ecology is to unravel the ecological and
64 evolutionary dynamics of microorganisms within their communities. Of particular
65 relevance are the factors that shape the stability and resilience of such communities,
66 despite different fitness trajectories of the microbiome members. The microbial
67 response to stress or perturbations, e.g. exposure to a new substrate, provides a
68 selective advantage to certain members of the community. If the system cannot tolerate
69 the change, the microbial community dramatically shifts until a different equilibrium
70 state is reached (9). Frequency-dependent selection forces the host to adapt to these
71 changes and select for or against the most frequent genotypes of their associated
72 microbiota (10). There is, for example, strong evidence that species-specific
73 antimicrobial peptides (AMPs) shape, control, and confine host-species specific bacterial
74 associations (11, 12). In addition, microbial communities are not evenly distributed, e.g.
75 along the gastrointestinal tract or between the lumen and the epithelial surfaces (2, 13,
76 14). These significant differences in niches or micro-habitats and their occupancy is
77 known as spatial heterogeneity and will affect community assembly rules and dynamics
78 (15, 16). Interspecies metabolic exchange is another key biotic force acting as a major
79 driver of species co-occurrence in diverse microbial communities (17).

80 To experimentally address the composition and assembly of animal
81 microbiomes current efforts have taken advantage not only of the traditional models
82 such as the zebrafish, the fruit fly, and the nematode worm but also of other systems
83 such as the honeybee, and crustacean species belonging to the genus *Daphnia* (18). All of
84 these simple animal models can be raised and manipulated in the laboratory allowing
85 for the discovery of fundamental principles of animal-microbiome interactions. As most
86 of these models contain only a small number of taxa, a bottom-up approach can help to
87 better understand these host-associated microbiomes using synthetic microbial
88 communities (19, 20).

89 We here apply a reductionist approach to disentangle the inherent complexity of
90 interactions in host-microbiomes. We use the freshwater polyp *Hydra vulgaris* and its
91 microbiome, which has become a valuable experimental model in metaorganism
92 research (21). *Hydra's* ectodermal epithelial cells are covered with a multi-layered
93 glycocalyx that provides a habitat for a species-specific and core microbiome of low
94 complexity (11, 22, 23), from which most microbes can be cultured *in vitro* (23, 24). This
95 allows the construction of synthetic communities of various complexities and
96 contrasting the host (*in vivo)* to *in vitro* habitats (21). We focus on the two most
97 abundant members of the microbiome that together constitute about 85% of *Hydra*'s
98 simple microbiome, *Curvibacter* sp. AEP1.3 and *Duganella* sp. C1.2, (hereafter called
99 *Curvibacter* and *Duganella*), where abundances of *Curvibacter* are several magnitudes
100 higher as compared to *Duganella* (24). In this study, we want to understand the factors
101 leading to this pattern of species coexistence and hypothesize that the respective
102 environmental conditions are key for the outcome of microbial species interactions.
103 After a characterization of the population dynamics of the two main colonizers when
104 grown singly, we perform experiments where we combine host and non-host
105 environments with invasion-from-rare set-ups to elucidate the effect of the host

106 environment on microbial interactions. More specifically we test whether microbial
107 species coexistence and community stability depends on the host environment.
108
109 **Results**
110 **The *Hydra* ecosystem is characterized by an overall carrying capacity**
111 Carrying capacity is defined as the maximum population size that an ecosystem can
112 sustainably support without being degraded. This concept from macro-ecology can also
113 be applied to host-microbe ecosystems. We here determine whether the *Hydra*
114 ecosystem is characterized by a specific carrying capacity, and whether it can be
115 reached again after the incubation of germ-free polyps with tissue homogenates of wild-
116 type animals (conventionalized animals). We find that the carrying capacity of *Hydra* is
117 highly stable among single *Hydra* wild-type polyps with $1.7*10^5$CFUs per individual
118 (standard deviation of $\pm 0.3*10^5$). This carrying capacity cannot be exceeded through the
119 artificial addition of either *Curvibacter* or *Duganella* to wild-type polys. In the contrary,
120 the addition of *Curvibacter*, leads to a significant reduction in overall microbial
121 population size (Welch ANOVA, $F_3=7.054$; $P<0.005$; Fig. 1A). Most importantly, the
122 carrying capacity of wild-type and conventionalized polyps does not differ. These
123 findings indicate the usability of germ-free polyps for the manipulation and construction
124 of *in vivo* synthetic bacterial communities
125
126 **Species-specific carrying capacities for *Hydra*'s two main colonizers**
127 In any habitat, the carrying capacity for each species is different and depends on several
128 factors, including availability of nutrients, spatial distribution, and inter- and intra-
129 species interactions. To assess the capabilities to colonize *Hydra*, we assess the
130 population growth of *Curvibacter* and *Duganella* in mono-associations until they reach
131 their individual carrying capacities. After about 72 h of growth on the host, both
132 microbial species reach a stable population size (Fig. 1B). This carrying capacity, when

133 grown singly on the host, differs between both strains, with *Curvibacter* reaching a
134 higher population size than *Duganella* (estimated by post hoc contrasts; Generalized
135 linear model: Full model: χ^2=54.360, d.f.=9, P<0.0001; bacterial species x days post
136 exposure: χ^2=18.326, d.f.=4, P=0.0011). These significant differences last until the end of
137 the experiment. The carrying capacity of mono-associations is about 10^4 CFUs per
138 individual for *Curvibacter*, whereas for *Duganella* the population size reaches on average
139 only $1.5*10^3$ CFUs per individual. Further, both mono-associations do not reach the
140 overall carrying capacity of wild-type polyps. The variation in bacterial density between
141 hosts is significantly higher in *Curvibacter* than in *Duganella* (Levene: F_1=21.496,
142 P<0.0001).
143
144 **Deconstructing the metaorganism: role of the host environment and of the most**
145 **dominant co-colonizing microbial species**
146 Here we perform an experiment to deconstruct some of the interactions within the
147 *Hydra* metaorganism by (i) exploring the role of the host as a microbial habitat through
148 the comparison of microbial population dynamics *in vivo* with *in vitro* environments,
149 and (ii) determining the role of the second most-dominant co-colonizer within the
150 *Hydra* microbiome through performing di-association experiments with different
151 starting frequencies of both microbial species. The previous experiment showed that the
152 carrying capacity for each species was reached after 72 h post inoculation and stably
153 maintained thereafter. Here we adjust our sampling intervals accordingly: we focus on
154 the critical period that determines the outcome of the colonization process, i.e. the first
155 96 h post inoculation, and shorten the intervals in-between sampling to 12 h. This
156 should allow for a detailed monitoring of the microbial population dynamics until the
157 respective carrying capacities are reached.
158
159 *The effect of the environment on microbial growth kinetics in mono-associations*

160 To determine the relative importance of the host for microbial population dynamics and
161 community stability, we chose two *in vitro* environments to contrast to the host: static,
162 which closely resembles the host habitat in that it provides spatial heterogeneity
163 facilitating bacterial interactions and mixed, where direct interactions between
164 individual bacteria cannot be established but where individual bacteria have (unlimited)
165 access to resources and oxygen.

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

173

174 *The effect of microbial interactions on microbial performance varies depending on the*
175 *environment*

176 Here we test whether microbial performance is affected by the presence of the other,
177 most dominant co-colonizer from the *Hydra* microbiome. Again, we contrast the *in vivo*
178 environment with the two *in vitro* environments to be able to test whether the
179 environment, the fellow microbes or an interaction of both determines microbial
180 population dynamics and community stability.

181 The overall population dynamics in di-associations resembles the one observed
182 in mono-associations, namely that irrespective of the environment, the carrying capacity
183 in all habitats is reached at about 72 h after inoculation. Both microcosm environments
184 are characterized by a carrying capacity of 10^7-10^8 CFUs/ml, and so exceed the *in vivo*
185 carrying capacity by a factor of 10^4 (Fig. 3). Nevertheless, di-associations on the host
186 also fail to reach the overall carrying capacity of wild-type polyps (Welch ANOVA,

187 F_1=441.929; P<0.001) and reach a comparable carrying capacity as in the mono-
188 colonisations of *Curvibacter* and *Duganella* (ANOVA: $F_{2,44}$ = 2.011, P=0.146). Both
189 bacterial species do not match the species-specific carrying capacities as measured in
190 mono-colonisations on the host: whereas *Curvibacter* fails by a power of 10 to reach its
191 density in the mono-colonisations, *Duganella* outgrows it by a power of 10.

192 To control for frequency-dependent microbial population dynamics, we
193 competed *Curvibacter* and *Duganella* in three different starting frequencies (Fig. 4). In
194 both non-host environments, *Duganella* outcompetes *Curvibacter* within 48 h post
195 exposure. From then onwards, frequencies of *Curvibacter* are low, reaching a maximum
196 of about 10%. This pattern does not depend on the initial frequency at the start of the
197 experiment. Most interestingly, this pattern is not observed in the host environment:
198 here, a decrease in the fraction of *Curvibacter* can be observed in all three initial
199 frequencies but never to a point where it cannot be detected in the population. From 72
200 h post exposure onwards the population on the host has reached a stable state, with
201 *Curvibacter* making up 20% of the total bacterial population.
202

203 **Zooming in on the interaction between the two most dominant microbes in the**
204 ***Hydra* microbiome**

205 In every environment, *Duganella* had a negative impact on *Curvibacter*, while the
206 presence of *Curvibacter* led to an increased *Duganella* carrying capacity in both, host and
207 microcosm environments. We here performed growth assays of both microbial species
208 in spent medium (cell-free supernatant) of the other microbial species to test whether
209 contact dependent or contact independent interactions determined the observed
210 population dynamics. Interestingly, we did not observe reduced growth of *Curvibacter* in
211 the supernatant of *Duganella*, but the opposite, with *Curvibacter* growing to higher
212 abundances in cell-free supernatant as compared to abundances in *Hydra* medium
213 (ANOVA: R^2=0.924, $F_{1,8}$ = 97.312, P<0.0001; Fig. 5A). Also, *Curvibacter* supernatant led to

214 higher *Duganella* abundances as compared to *Hydra* medium (ANOVA: R^2=0.946, $F_{1,8}$ =
215 140.628, P<0.0001) – but only after a significantly longer lag time (ANOVA: R^2=0.996,
216 $F_{1,8}$ = 1876.566, P<0.0001; Fig. 5B).
217
218 **Discussion**
219 One of the major challenges in microbiome research is to understand the factors that
220 influence the dynamics and stability of host-associated microbial communities. Of
221 particular relevance for this are the processes governing assembly (25, 26) and
222 resilience (27). Insights into such processes in bacterial populations within their native
223 host environments can be gained through a number of ways. A bottom-up approach, for
224 example, allows to elucidate the basic principles of community assembly as has been
225 shown in non-host associated microbial communities (28, 29). This strategy is becoming
226 increasingly popular in metaorganism research and has produced informative results,
227 such as in the zebrafish (30) or the nematode *Caenorhabditis elegans* (19). Here we use
228 *Hydra* and its microbiome for 'deconstructing' a metaorganism and its interactions (21).
229 To determine the relative importance of the host on the interactions of *Curvibacter* and
230 *Duganella*, we performed mono- and di-association experiments *in vivo* and in two i*n*
231 *vitro* environments (Fig. 6). As community structure can be influenced by initial species
232 abundances (31), we also performed all di-association experiments using various initial
233 starting frequencies. Interestingly we found that in the *Hydra* habitat *Curvibacter*,
234 independent of its inoculation frequency, and after the initial establishment period of 72
235 h, reached a constant fraction of about 20%, whereas it was only present at very low
236 frequencies or went extinct in both *in vitro* habitats (see also (32) for similar patterns in
237 a homogeneous *in vitro* environment). Wright and Vetsigian (33) recently demonstrated
238 in pairwise competitions between bacteria of the genus *Streptomyces* that the winner is
239 often the species that starts at high initial abundance. We find that 'survival of the
240 common' does not apply to *Curvibacter* in a non-host environment, whereas pairwise

241 competitions in the host habitat show signs of stabilisation between *Curvibacter* and
242 *Duganella*. This suggests that within the host environment both strains can stably
243 coexist, which is in contrast to the *in vitro* environment, where we find competitive
244 exclusion (*Duganella* excludes *Curvibacter*). Nevertheless, the resulting fractions in the
245 *in vivo* di-association experiments (*Curvibacter* 20% and *Duganella* 80%) do not
246 represent the species fractions found in wild-type polyps. Here *Curvibacter* represents
247 75% and *Duganella* 11% of the whole community (24), which indicates that the rare
248 microbiome members might be relevant for achieving the native community
249 composition. When *Curvibacter* and *Duganella* are introduced separately to the host (in
250 mono-associations), each bacterial species is capable to robustly colonize the host to
251 high abundances. This confirms earlier findings from Wein *et al.* (34) that *Curvibacter* is
252 able to reach stable abundances on the host. A similar observation has been made for
253 *Aeromonas* and *Vibrio* colonizing patterns of the gut of larval zebrafish (35) and of
254 microbes colonizing the gut of *C. elegans* (19). While in mono-associations *Curvibacter*
255 reaches higher abundances than *Duganella*, the opposite is true for the di-associations.
256 Here, *Curvibacter* fails to reach its species-specific carrying capacities by a factor of 10
257 (as compared to the mono-association), *Duganella* outgrows it by a factor of 10.

258 Results from the di-association experiments clearly show that the abundances
259 and relative frequencies of *Curvibacter* and *Duganella* as measured in wild-type *Hydra*
260 cannot be explained by their interactions in the host context alone as this led to a
261 frequency reversal, making *Duganella* more abundant than *Curvibacter*. *Duganella*
262 however reaches comparable carrying capacities as measured in wild-type *Hydra*
263 polyps. These observations indicate that the less frequent community members (each
264 2% and less) are important for achieving the overall carrying capacity of the *Hydra*
265 microbiome. We hypothesise that two aspects are of importance here – (i) the low
266 abundant microbes might be able to utilize different resources as compared to
267 *Curvibacter* and *Duganella* and so inhabit different ecological niches within the

268 microbiome, which the two main colonizers cannot fill and (ii) they likely interact in a
269 positive way (either directly or indirectly) with (at least) *Curvibacter*, enabling it to
270 reach higher carrying capacities. Evidence for the importance of rare-species comes
271 from the human intestinal microbiota, which contains many low-abundance species (36)
272 with some of them having a large impact on inducing dysbiosis in the microbiome and
273 on guaranteeing host health (37, 38). It is thus important to note that *Hydra*'s carrying
274 capacity is not solely determined by the host (resources) alone but also by the
275 interactions within the microbiome. In microbiome research, the significance of an
276 overall host carrying capacity has been largely overlooked until very recently where a
277 link between host health and microbiome density has been reported (8). Bacterial levels
278 have been quantified for only a few model organisms such as in the gut of larval
279 zebrafish (39) or the gut of *Drosophila melanogaster* (40). We here show that also wild-
280 type *Hydra* is characterized by an overall carrying capacity of about 10^5 bacteria per
281 polyp that is stable in adult polyps and can be artificially assembled through the re-
282 population of germ-free animals with the native microbial community. This is an
283 important prerequisite for conducting the *in vivo* experiments, where colonization
284 patterns of single species from *Hydra's* microbiome are individually followed.

285 To investigate the interaction between *Curvibacter* and *Duganella* in more detail,
286 we tested whether the interactions between the two species are contact dependent by
287 performing spent medium assays. Our results indicate that the effect of *Duganella* on
288 *Curvibacter* might depend on direct contact, as *Duganella* supernatant did not negatively
289 affect the growth of *Curvibacter*. In contrast, *Curvibacter* supernatant led to an initial
290 time lag in *Duganella* growth, which was followed by an exponential growth phase after
291 about 35 h (note that such a time lag is not visible in the other media). The same pattern
292 can be observed in the fractions in the di-association experiments – also here, *Duganella*
293 started to outcompete *Curvibacter* only after an initial delay of 36 h (Fig. 4). It is
294 interesting to speculate what might lead to this pattern. The fact that this also becomes

295 apparent in the supernatant experiments suggests that it is mediated by products in the
296 supernatant of *Curvibacter*, which might metabolically be not directly assessable, so
297 *Duganella* needs to adjust its physiology accordingly.

298 The observation that *Curvibacter* is not negatively affected by the *Duganella*'s
299 supernatant suggests that direct contact is needed between the two species for
300 *Duganella* to outcompete *Curvibacter*. The competition can be either passive, where
301 strains compete for the same resources or active, where strains directly harm one
302 another (41). Thus, one explanation for the stable coexistence of the two species on
303 *Hydra* could be that the host environment leads to a (partial) spatial segregation of
304 *Hydra*'s most dominant colonizers, reducing between-species contact, as has been
305 shown for biofilms (42). For *Hydra* it is known that it shapes its microbiota through the
306 secretion of antimicrobial peptides (43) and neuropeptides (44), which influences the
307 microbial spatial structure. In addition, other host mechanisms have been predicted,
308 such as the provisioning of carbon sources via epithelial feeding or releasing of specific
309 adhesive molecules from epithelial surfaces targeted at specific microbes (45). Thus, it is
310 important to conclude that while stability of microbial communities depend on
311 interactions between different bacterial strains and species, these interactions need to
312 occur in their native environment, the host.
313

314 **Materials and Methods**

315 **Animals used, culture conditions and generation of germ-free animals**

316 *Hydra vulgaris* (strain AEP) was used for carrying out experiments and cultured
317 according to standard procedures at 18°C in standardized culture medium (*Hydra*
318 medium (HM)) (46). Animals were fed three times a week with 1st instar larvae of
319 *Artemia salina*. Germ-free (GF) polyps were obtained by treating wild-type (WT)
320 animals with an antibiotic cocktail solution containing 50 μg/ml ampicillin, neomycin,
321 streptomycin, rifampicin and 60 μg/ml spectinomycin as previously described (43, 47).
322 The antibiotic cocktail solution was exchanged every 48 h and the antibiotic treatment
323 lasted for two weeks, after which polyps were transferred into antibiotic-free sterile HM
324 for recovery (four days). The germ-free status of polyps was confirmed as previously
325 described (43). During antibiotic treatment and re-colonization experiments, polyps
326 were not fed.

327

328 **Bacterial strains and media**

329 The bacterial strains used in this study are *Curvibacter* sp. AEP1.3 and *Duganella* sp.
330 C1.2., which have been isolated from *Hydra vulgaris* (strain AEP) (24). These bacteria
331 were cultured from existing isolate stocks in R2A medium at 18°C, shaken at 250 r.p.m
332 for 72 h before use in the different experiments. R2A was chosen as medium as it was
333 used to isolate bacterial strains and to be able to compare results to previous published
334 *Hydra* papers (24, 32).

335

336 **Carrying capacity of the host**

337 To determine the carrying capacity of the *Hydra* habitat the microbial load of individual
338 *Hydra* polyps (N=16) was determined. In addition to wild-type polyps the carrying
339 capacity of conventionalized polys (N=12), obtained by incubating germ-free polyps
340 with tissue homogenates of wild-type animals (per germ-free polyp one wild-type polyp

341 was used) for 24 h was also determined. To test whether the carrying capacity can
342 artificially be increased or destabilises upon self-challenge we added either *Curvibacter*
343 or *Duganella* to wild-type polyps (N=6) (approximately $5x10^3$ cells for 24 h). After
344 incubation all polyps were washed with and transferred to sterile HM and further
345 incubated at 18°C and sampled after 120 h. Polyps were first washed three times with
346 sterile HM to remove non-associated bacteria and then transferred to an Eppendorf tube
347 containing sterile HM. After homogenization using a sterile pestle serial dilutions of the
348 homogenate were plated on R2A agar plates to determine colony-forming units (CFUs)
349 per individual.
350
351 **Tracking microbial mono-associations in *Hydra* over time**
352 Germ-free polys were inoculated in their aquatic environment with single bacterial
353 strains (mono-associations). Individual germ-free polyps were incubated with $5x10^3$
354 cells of *Curvibacter* or *Duganella* in 1.5 ml Eppendorf tubes containing 1 ml of sterile
355 HM. After 24 h of incubation all polyps were washed with and transferred to sterile HM,
356 incubated at 18°C and followed over a period of 216 h. For each treatment 6 polys per
357 time point were independently analysed. Every 48 h individual polyps were collected to
358 determine CFUs a described above.
359
360 **Microbial growth kinetics of mono- and di-associations *in vivo* and *in vitro***
361 To study the initial phase of colonization, i.e. 96 h post inoculation (see Fig. 1B) in more
362 detail microbial growth of *Curvibacter* and *Duganella* was determined in different
363 habitats; the host habitat (*in vivo*) and two different microcosm environments (*in vitro*).
364 The static incubation provided a spatially structured habitat (heterogeneous), whereas
365 shaking of the microcosms (mixed treatment) eliminated the spatial structure
366 (homogenous).

367 *Mono-associations:* All germ-free polyps and microcosms were inoculated from
368 the same bacterial inoculation culture with approximately $5x10^3$ cells of *Curvibacter* or
369 *Duganella* for 24 h, washed with and transferred to sterile HM. Samples were taken
370 every 12 h for 96 h. For *Hydra* six polyps were sacrificed at each time point and colony-
371 forming units (CFUs) were determined as described above. As microcosms 24-well
372 plates were used. Wells were filled with 2 ml of R2A medium, inoculated and incubated
373 at 18°C either under static or shaken (200 r.p.m) conditions. Each time point was
374 replicated four times and serial dilutions were plated on R2A agar plates to determine
375 CFUs. Growth rates of each strain (A and B) were determined for the exponential growth
376 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
377 the starting density at time 0 and A_f, B_f is the final density at time *f*.
378 *Di-associations:* Density dependent competiveness fitness assays of the two most
379 dominant colonizers *Curvibacter* and *Duganella* were performed *in vivo* and *in vitro.* The
380 same host and microcosm experiments as described above were performed except for
381 using microbial di-associations of *Curvibacter* and *Duganella* with the frequency of
382 *Curvibacter* being rare (10:90), equal (50:50), or dominant (90:10). As *Curvibacter* and
383 *Duganella* form distinct colonies on R2A agar plates, their frequency can be determined
384 by plating serial dilutions (32). Six polyps and four microcosm replicates were assayed
385 per treatment (static and mixed) and time point. Also this data allowed determining the
386 different carrying capacities of the *in vivo* and *in vitro* habitats used. Growth rates of
387 each strain were calculated as above (Fig. S1).
388
389 **Spent medium assay to measure interaction activity between microbial strains**
390 Spent medium (cell-free supernatant (CFS)) of both microbial strains *Curvibacter* and
391 *Duganella* was prepared by growing them for 72 h in R2A medium at 18°C (shaken at
392 250 r.p.m) until stationary phase was reached. Cultures were then centrifuged at 1000 g
393 for 20 min and the supernatant was passed through a 0.22 µm filter. Growth assays

394 consisted of mixing 100 µl of a culture of *Curvibacter* or *Duganella* (adjusted to an OD_{600}
395 of 0.025) with the corresponding supernatant (100 µl) of the other strain. As control the
396 same volume of HM was added to the strains instead of the supernatant. Growth kinetics
397 were examined in a 96-well plate using a Tecan Spark 10M microplate reader. The plate
398 was incubated at 18°C and moderately shaken for 10 sec prior to each read. Absorbance
399 at 600 nm was measured every 30 min over a period of 48 h, and data from 5 h intervals
400 are plotted for clarity. Each treatment was replicated 5 times.
401
402 **Statistical analysis**
403 A Welch ANOVA (and subsequent Dunnett's posthoc test) was used to test for
404 differences in bacterial abundance patterns ('bacteria per *Hydra*') in wild-type versus
405 manipulated hosts as variances between the different groups were not equally
406 distributed.
407 Differences during mono-colonizations of *Curvibacter* and *Duganella* over time
408 were assessed using a Generalized linear model (error structure: normal; link function:
409 identity). The response variable was 'bacteria per *Hydra*', and explanatory variables
410 were 'bacterial species', 'time' and 'bacterial species' x 'time'. Differences between the
411 two bacterial species on each day were detected with post hoc contrasts.
412 Carrying capacities were compared between wild-type *Hydra* and di-
413 associations using a Welch ANOVA, and between mono- and di-associations using an
414 ANOVA.
415 Analysis of variance (ANOVA) and subsequent post hoc t-tests were used to test
416 for differences in growth rates of the two competitors when grown singly in the
417 different environments. The response variable was 'growth rate', and explanatory
418 variables were 'bacterial species', 'environment' and 'bacterial species' x 'environment'.
419 Differences in the growth rates in the di-associations of *Curvibacter* and
420 *Duganella* in the different environments and dependence on initial frequency were

421 assessed using a Generalized linear model (error structure: normal; link function:
422 identity) and post hoc contrasts. For each bacterial species, a separate model was
423 calculated with the response variables being either 'growth rate *Curvibacter*' or 'growth
424 rate *Duganella*', and the explanatory variables were 'environment', 'starting density' and
425 'environment' x 'starting density'.

426 Differences between the maximal bacterial abundances of both species and lag
427 time of *Duganella* when grown in the supernatant of the respective other species as
428 compared to growth in *Hydra* medium were assessed using ANOVA.

429 Sample size was chosen to maximise statistical power and ensure sufficient
430 replication. Assumptions of the tests, that is, normality and equal distribution of
431 variances, were visually evaluated. Non-significant interactions were removed from the
432 models. All tests were two-tailed. Effects were considered significant at the level of $P <$
433 0.05. All statistical analyses were performed with JMP 9. Graphs were produced with
434 GraphPad Prism 5.0, and Adobe Illustrator CS5.1.

435

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Conflict of Interests

The authors declare no conflict of interest.

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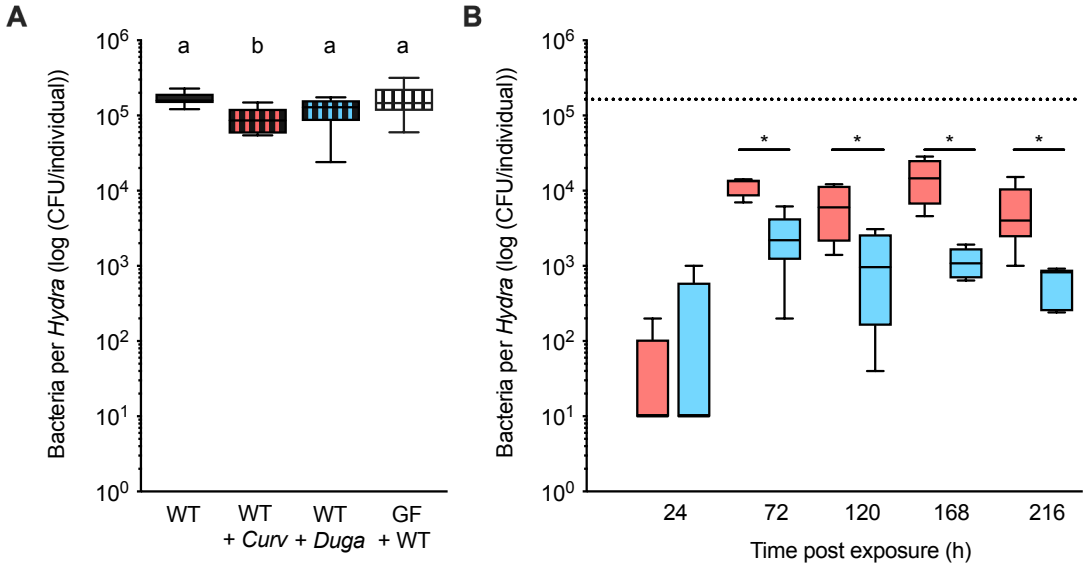
637

638 **Figures**

639



640

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

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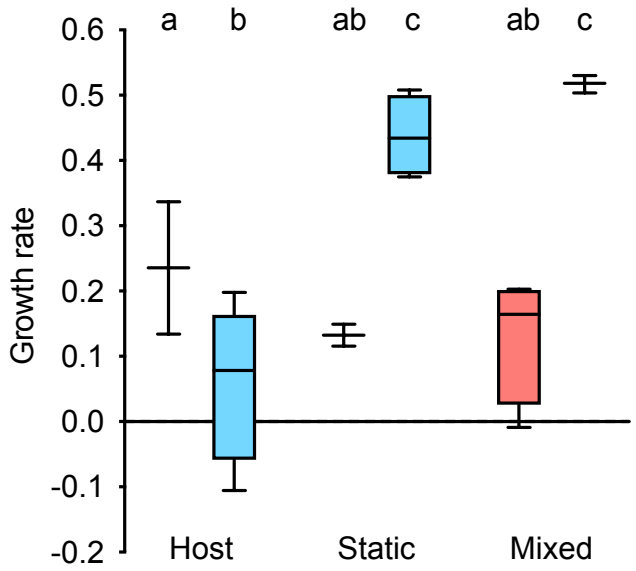

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651 Fig. 2 Bacterial growth rates of *Curvibacter* (red; each boxplot 4≥N≥2) and *Duganella*
652 (blue; each boxplot 6≥N≥3) in mono-associations are habitat dependent. Compared are
653 the host habitat (*in vivo*), and two *in vitro* environments: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

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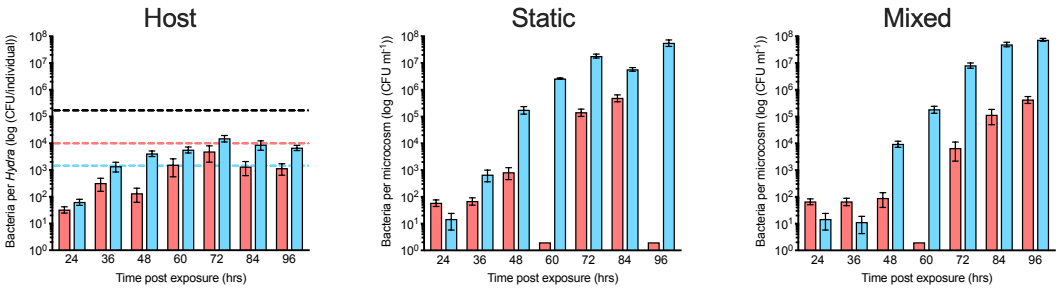
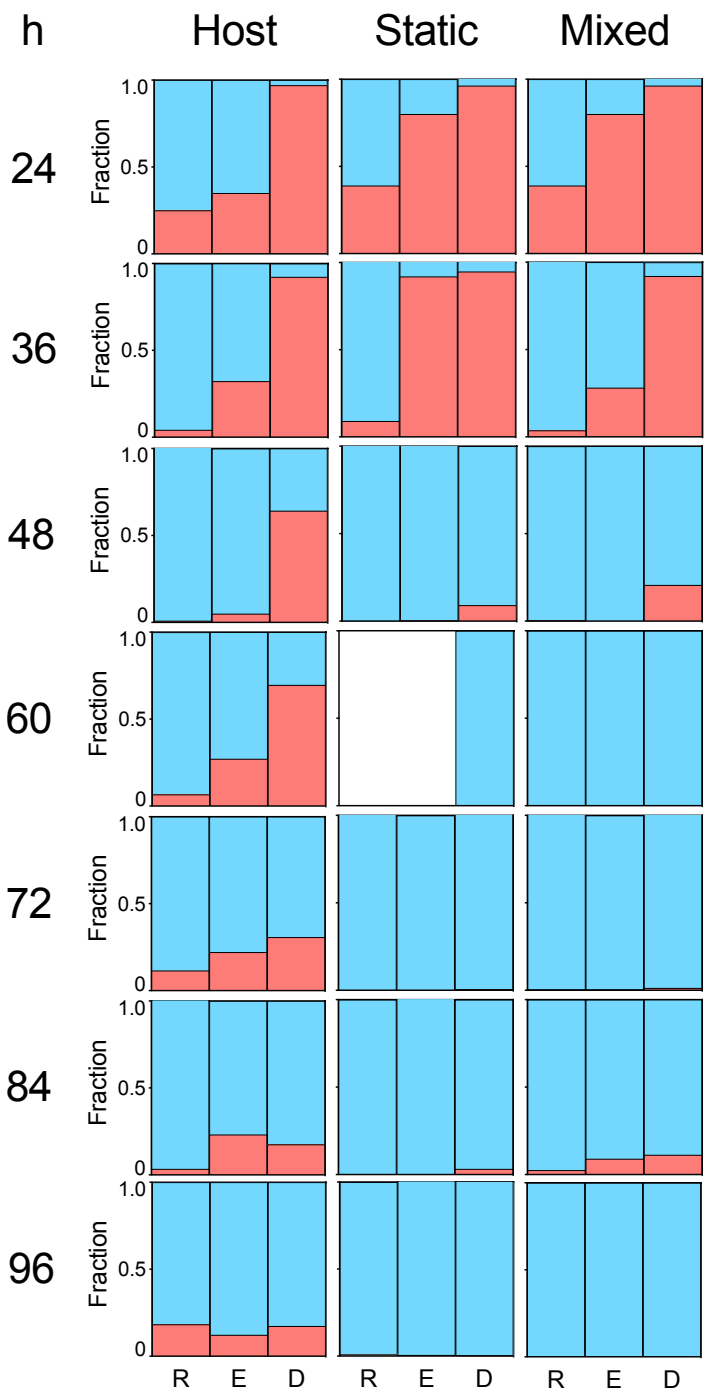


657

658 Fig. 3 Carrying capacity of the *in vivo* and *in vitro* habitats used in this study. Shown are
659 pooled total numbers of colony forming units (CFUs) from all di-association
660 experiments with *Curvibacter* (red) and *Duganella* (blue) (shown are s.e.m. based on
661 18≥N≥11 for the host, 12≥N≥9 for static, and 12≥N≥4 for mixed). The dashed black line
662 indicates the carrying capacity of WT polyps, the dashed red line the species-specific
663 carrying capacity of polyps during *Curvibacter* mono-associations and the dashed blue
664 line the species-specific carrying capacity of polyps during *Duganella* mono-
665 associations.

666

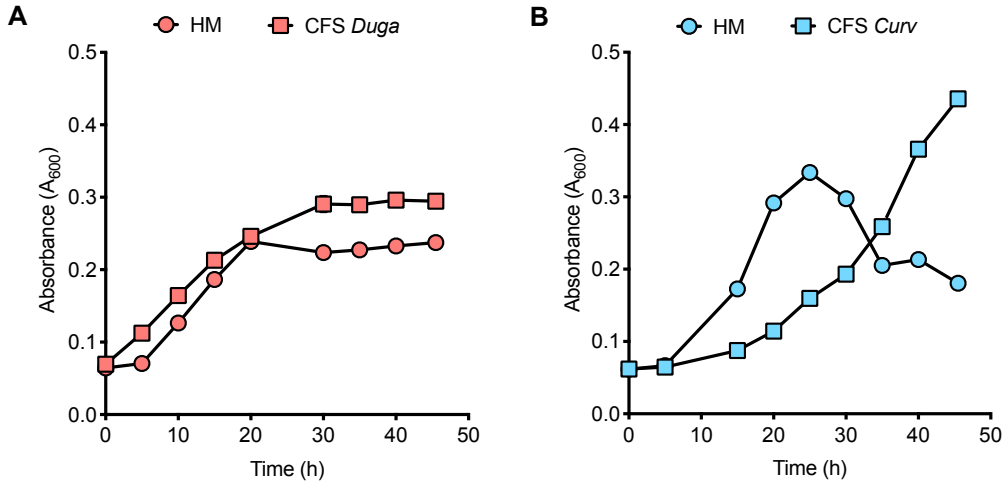


667

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

673

674



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676 Fig. 5 Bacterial growth dynamics of *Curvibacter* (red; A) and *Duganella* (blue; B) in

677 *Hydra* medium (HM) and in the presence of *Duganella* (CFS *Duga*) or *Curvibacter* (CFS

678 *Curv*) cell-free supernatant (CFS). Plotted are means (± s.e.m. based on N=5).

679

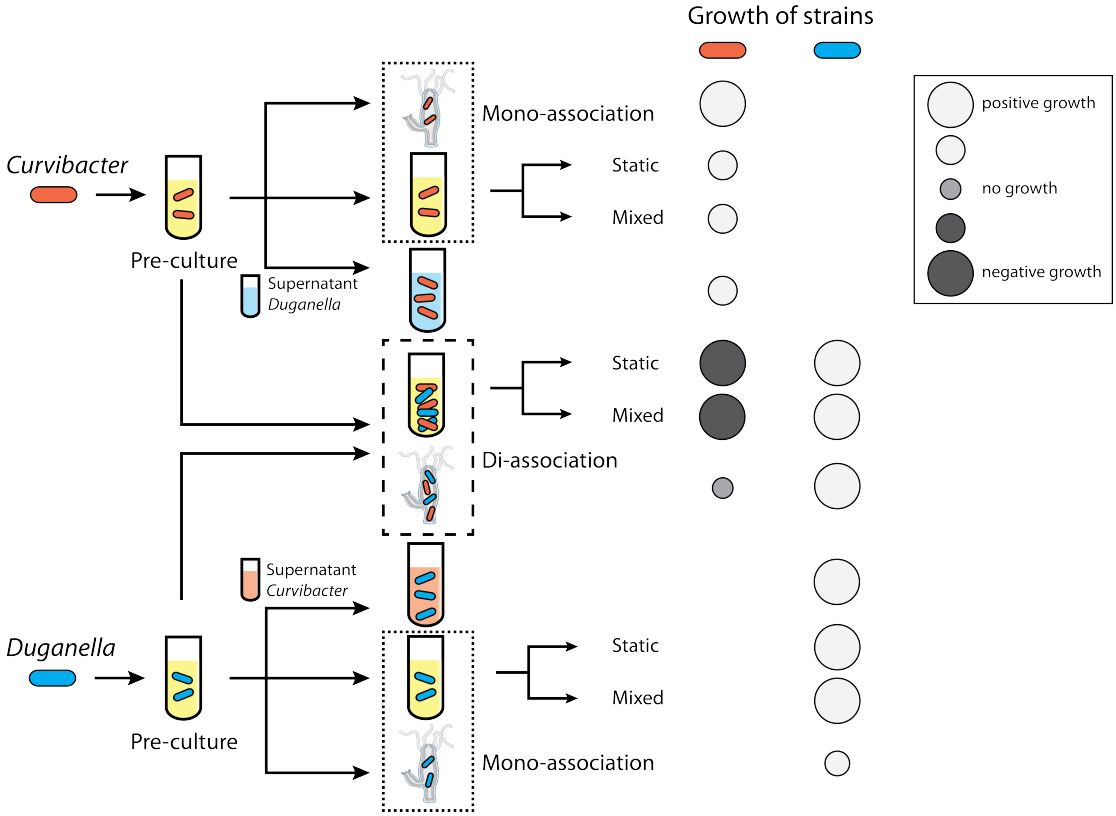

Fig. 6 Overview of performed experiments and effects on growth of *Curvibacter* (red) and *Duganella* (blue).

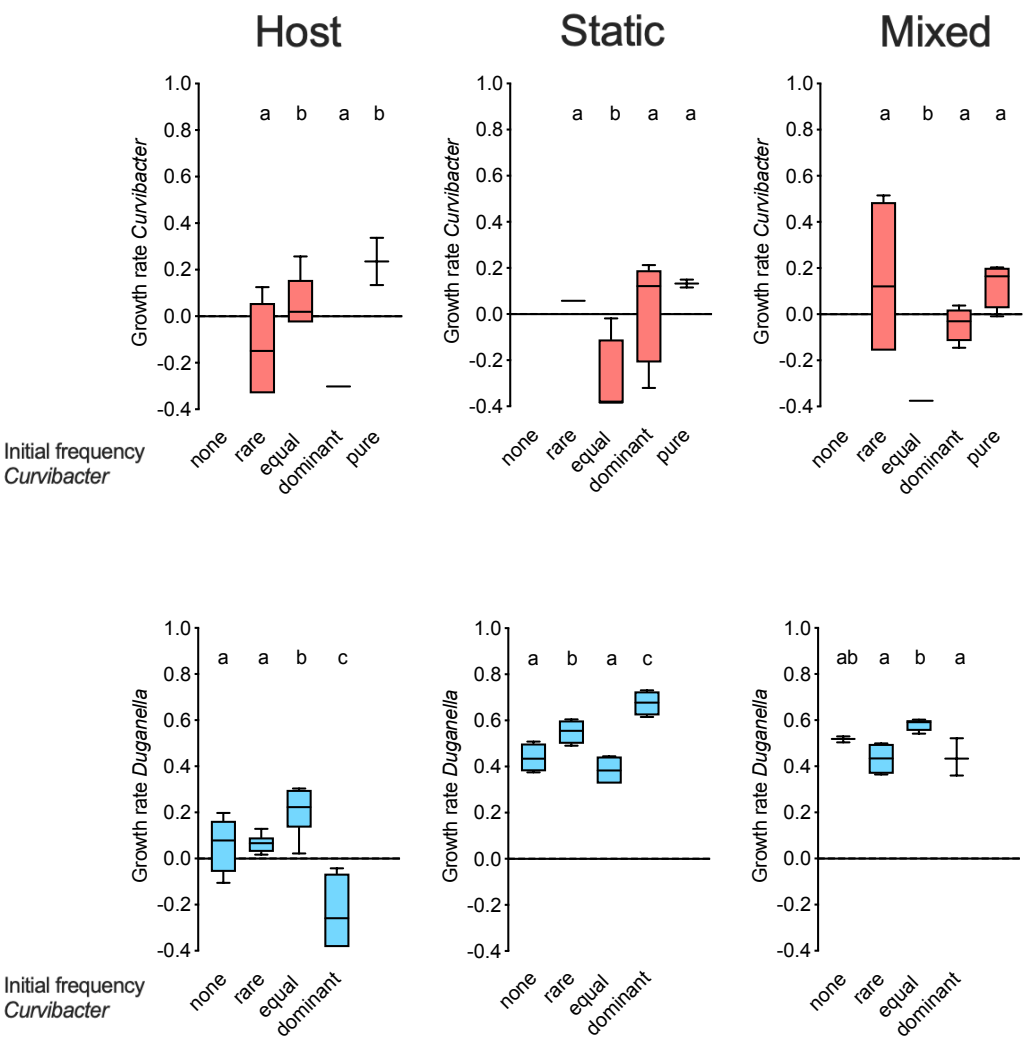

Fig. S1 Bacterial growth rates of *Curvibacter* (red) and *Duganella* (blue) from mono- and di-association experiments across the different habitats and initial frequencies tested (each boxplot 4≥N≥3). Overall *Curvibacter* growth rate in di-associations are lower or not different from the mono-associations (as estimated by post hoc contrasts; Generalized linear model: Full model: $\chi2=45.790$, d.f.=11, $P<0.0001$; environment x initial frequency: $\chi2=33.685$, 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

697 (Generalized linear model: Full model: $\chi 2=130.278$, d.f.=11, $P<0.0001$; environment x
698 initial frequency: $\chi 2=59.723$, d.f.=6, $P<0.0001$). Whereas, in di-associations, negative
699 growth rates can be detected only once for *Duganella*, it happens more frequently in the
700 *Curvibacter*, indicating a direct or indirect negative effect of *Duganella*.
701