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

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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.

Introduction

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

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

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

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

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

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

Results

Carrying capacity of the *Hydra* host

The carrying capacity of the *Hydra* habitat is highly stable among single *Hydra* wild-type 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

177 experiment. The host shows a different pattern. Here, a decrease in *Curvibacter* fraction
178 can be observed in all three initial frequencies but never to a point where it cannot be
179 detected in the population. From 72 h post exposure onwards the population on the host
180 has reached a stable state, with *Curvibacter* making up 20% of the total bacterial
181 population.
182
183 *Microbial growth kinetics of di-associations*
184 Overall *Curvibacter* growth rate in di-associations are lower or not different from the
185 mono-associations (Fig. 5; as estimated by post hoc contrasts; Generalized linear model:
186 Full model: χ^2=45.790, d.f.=11, P<0.0001; environment x initial frequency: χ^2=33.685,
187 d.f.=6, P<0.0001). *Curvibacter* grows significantly differently when inoculated in equal
188 densities as compared to the rare and dominant starting frequencies across the different
189 environments. Whereas in the host, *Curvibacter* grows better when in equal density with
190 *Duganella*, the opposite is true for both *in vitro* environments.
191 As observed for the growth of *Duganella* in mono-colonisations, growth rates are always
192 higher in the non-host environments irrespective of initial frequency (Generalized linear
193 model: Full model: χ^2=130.278, d.f.=11, P<0.0001; environment x initial frequency:
194 χ^2=59.723, d.f.=6, P<0.0001). Whereas, in di-associations, negative growth rates can be
195 detected only once for *Duganella*, it happens more frequently in the *Curvibacter*,
196 indicating a direct or indirect negative effect of *Duganella*.
197
198 *Relative performance of Curvibacter*
199 As estimation for the relative performance of *Curvibacter* in competition with *Duganella*
200 we determined the selection rate constant (r) for all di-association experiments (28, 29).
201 We observed r < 0 for all environments and frequencies, indicating that *Curvibacter*
202 performs worse than *Duganella* when they are in direct competition (Fig. 6). There is no
203 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

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

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

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

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

with tissue homogenates of wild-type animals (per germ-free polyp one wild-type polyp was used) for 24 h was also determined. To test whether the carrying capacity can artificially be increased or destabilises upon self-challenge we added either *Curvibacter* or *Duganella* to wild-type polyps (N=6) (approximately $5x10^3$ cells for 24 h). After incubation all polyps were washed with and transferred to sterile HM and further incubated at 18°C and sampled after 120 h.

Tracking microbial mono-associations in *Hydra* over time

Germ-free polys were inoculated in their aquatic environment with single bacterial strains (mono-associations). Individual germ-free polyps were incubated with $5x10^3$ cells of *Curvibacter* or *Duganella* in 1.5 ml Eppendorf tubes containing 1 ml of sterile HM. After 24 h of incubation all polyps were washed with and transferred to sterile HM, incubated at 18°C and followed over a period of 216 h. For each treatment 6 polys per time point were independently analysed. Every 48 h individual polyps were homogenized in an Eppendorf tube using a sterile pestle and serial dilutions of the homogenate were plated on R2A agar plates to determine colony-forming units (CFUs) per polyp.

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

To study the initial phase of colonization, i.e. 96 h post inoculation (see Fig. 1B) in more detail microbial growth of *Curvibacter* and *Duganella* was determined in different habitats; the host habitat (*in vivo*) and two different microcosm environments (*in vitro*). The static incubation provided a spatially structured habitat (heterogeneous), whereas shaking of the microcosms (mixed treatment) eliminated the spatial structure (homogenous).

Mono-associations: All germ-free polyps and microcosms were inoculated from the same bacterial inoculation culture with approximately $5x10^3$ cells of *Curvibacter* or

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

Di-associations: Density dependent competiveness fitness assays of the two most dominant colonizers *Curvibacter* and *Duganella* were performed *in vivo* and *in vitro.* The same host and microcosm experiments as described above were performed except for using microbial di-associations of *Curvibacter* and *Duganella* with the frequency of *Curvibacter* being rare (10:90), equal (50:50), or dominant (90:10). As *Curvibacter* and *Duganella* form distinct colonies on R2A agar plates, their frequency can be determined by plating serial dilutions (43). Six polyps and four microcosm replicates were assayed per treatment (static and mixed) and time point. Also this data allowed determining the different carrying capacities of the *in vivo* and *in vitro* habitats used. Malthusian parameters were calculated for both bacterial strains (A and B), $m = \ln (N_f/N_0)$/hour, where N_0 and N_f were the initial and final densities. The relative performance of the two strains was expressed in terms of the selection rate constant (*r*). Note that this measure of performance should be preferentially used over the more commonly used relative fitness (*W*), when one competitor is much less fit than the other (28, 29), as was the case in our study. The selection rate constant was calculated as $r = m_B - m_A$. Accordingly, $r = 0$ implies that there is no difference in growth rates of the two strains, $r > 0$ indicates that 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 $P < 0.05$. 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.

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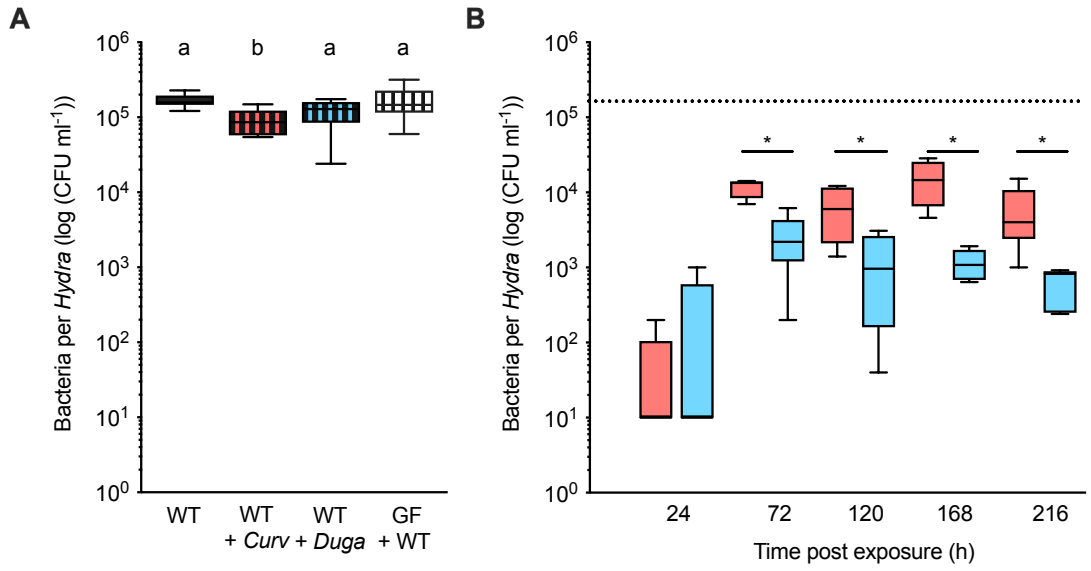
645 **Figures**

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.

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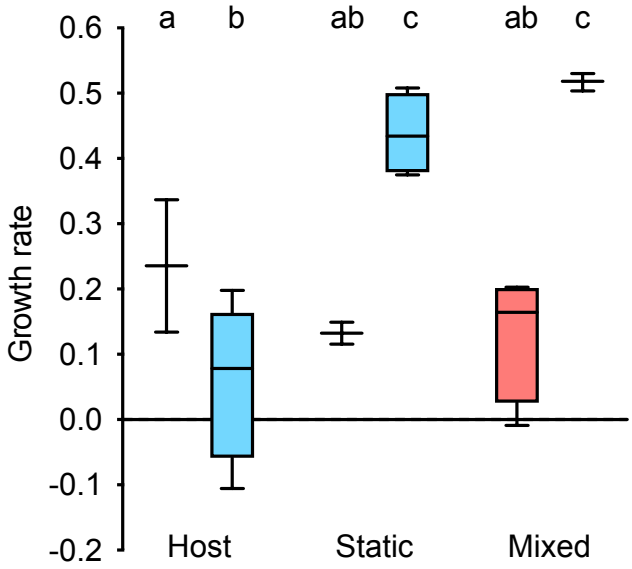

Fig 2. Bacterial growth rates of *Curvibacter* (red; each boxplot N≥2≤4) and *Duganella* (blue; each boxplot N≥3≤6) in mono-associations are habitat dependent. Compared are the host habitat (*in vivo*), and two *in vitro* environments: heterogeneous (static microcosms), and homogenous (mixed microcosms).

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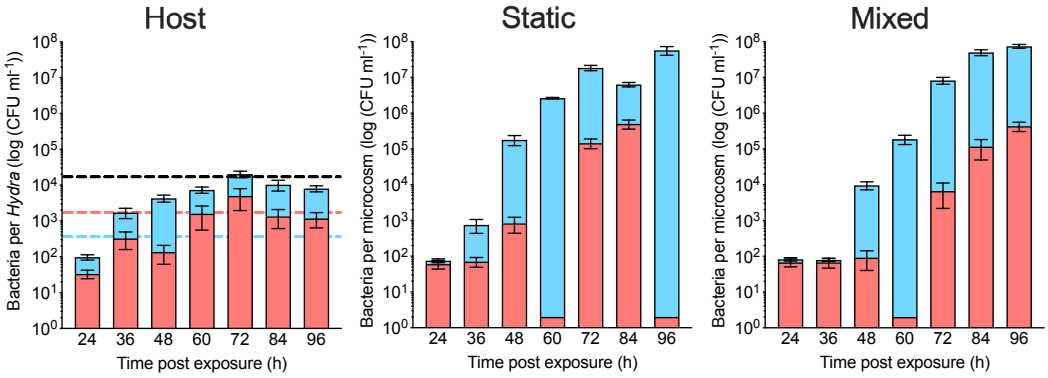
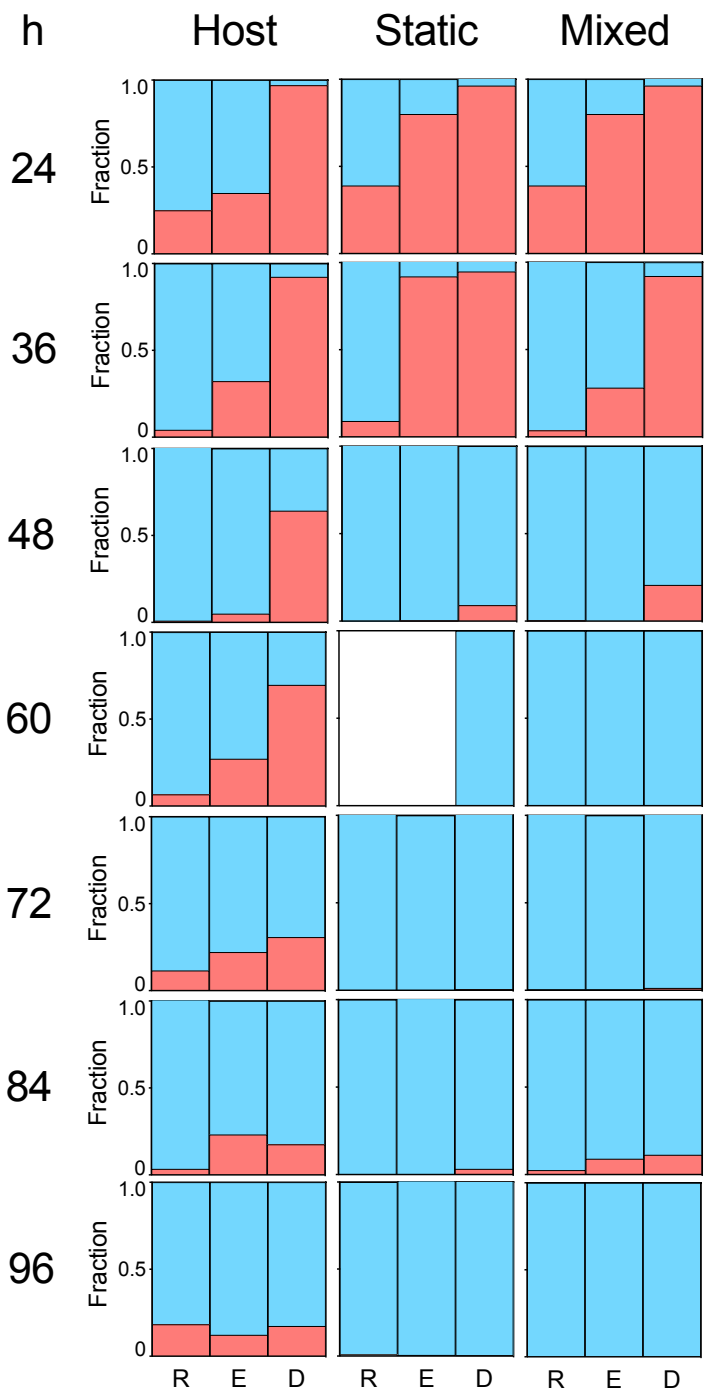


664

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

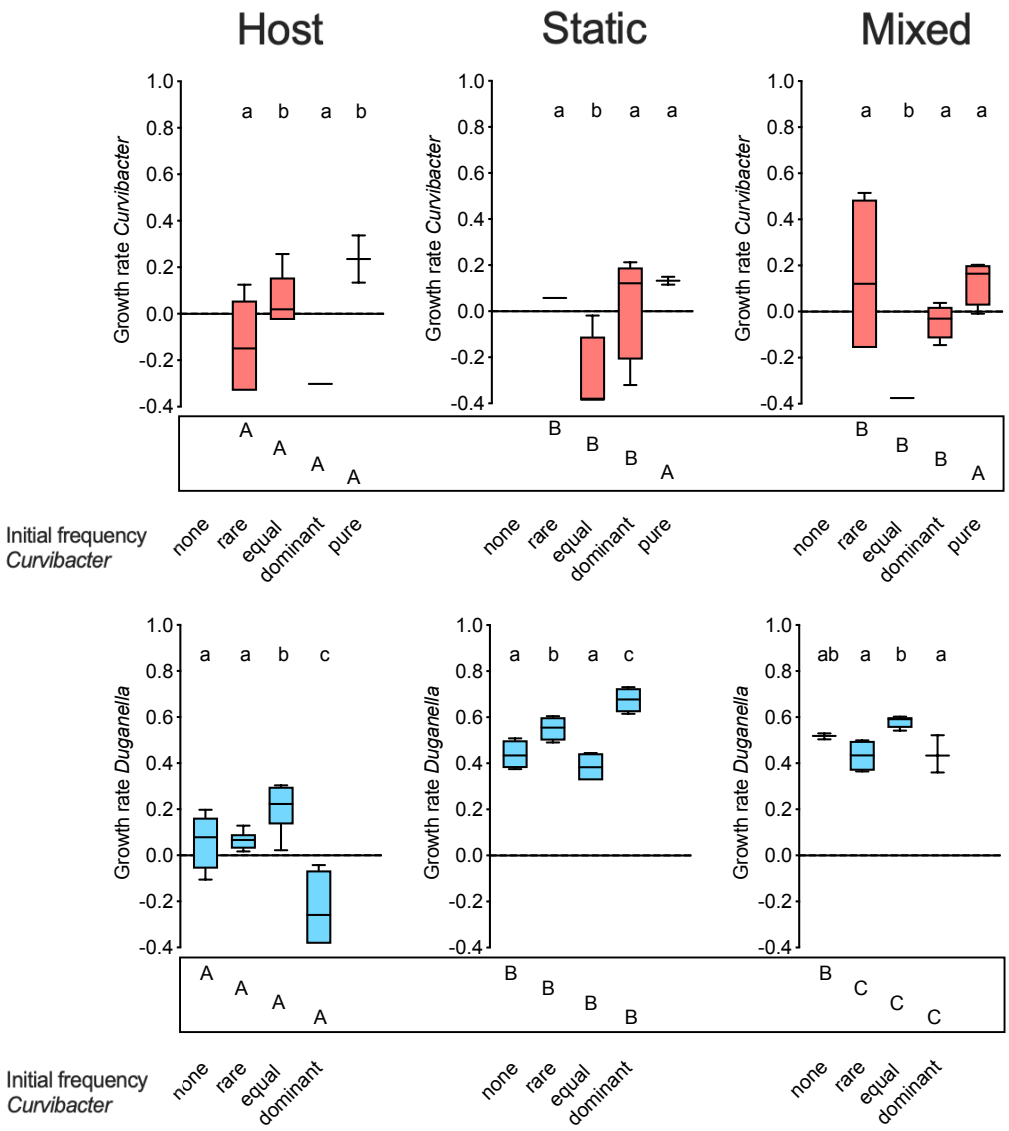


673

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

680



681

682 Fig 5. Bacterial growth rates of *Curvibacter* (red) and *Duganella* (blue) from mono- and

683 di-association experiments across the different habitats and initial frequencies tested

684 (each boxplot N≥3≤4).

685

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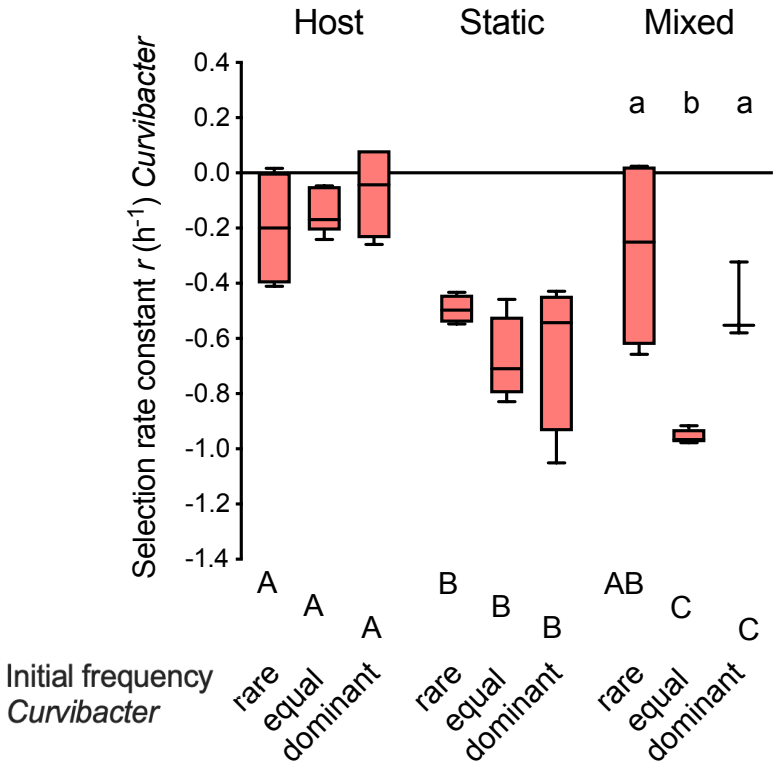

687

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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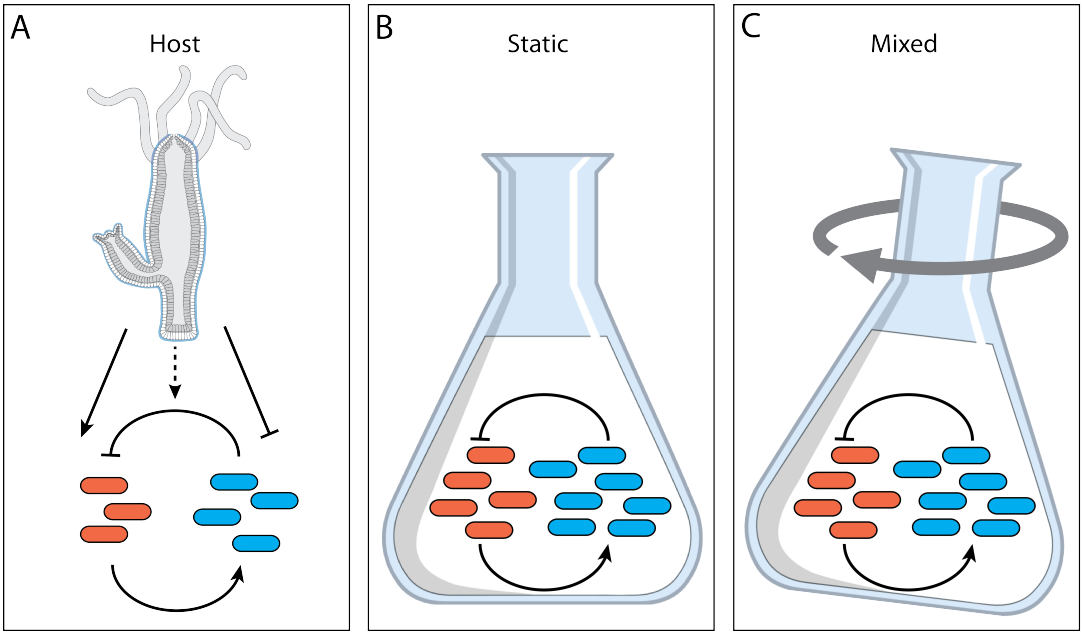

692

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.