Fitness effects of host-bacterial interactions – the microbial perspective

Peter Deines[1]*, Katrin Hammerschmidt[2] and Thomas CG Bosch[1]

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

[2]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

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Conflict of Interests

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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 *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 explore the ecological and evolutionary dynamics of microorganisms within their communities. Of particular relevance is to improve our understanding of factors that shape the stability and resilience of these 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 and the microbial community shifts dramatically, a different equilibrium state will be 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 [for example 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]. If 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.

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 [19]. 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) [20]. 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 [21]. Here, it is important to unravel the extent to which the observed pattern simply result from

100 intrinsic properties, such as microbial growth rates, interactions of the microbes, which
101 could be recapitulated *in vitro* with co-culture experiments, or whether it is an emergent
102 property of that particular host-microbe system [22].

103 We here test whether microbial associates benefit from their association with
104 the host and their fellow community members and follow their ecological interactions
105 within the microbiome. We use the freshwater polyp *Hydra vulgaris* and its microbiome,
106 which has become a valuable experimental model in metaorganism research as it
107 provides an excellent bridge between the simplicity of synthetic communities and the
108 mouse model [23]. *Hydra's* ectodermal epithelial cells are covered with a multi-layered
109 glycocalyx that provides a habitat for a species-specific and core microbiome of low
110 complexity [11, 24, 25], from which most microbes can be cultured *in vitro* [25, 26]. This
111 allows for an integrated approach based on constructing synthetic communities of
112 various complexities and contrasting the host (*in vivo)* to *in vitro* habitats [23]. We focus
113 on the two most abundant members of the microbiome that together constitute about
114 85% of *Hydra*'s simple microbiome, *Curvibacter* sp. AEP1.3 and *Duganella* sp. C1.2,
115 (hereafter called *Curvibacter* and *Duganella*) where abundances of *Curvibacter* are
116 several magnitudes higher as compared to *Duganella* [26]. Previous work on species
117 interactions suggested that continued coexistence depends heavily on relative growth
118 rates. Using invasion-from-rare experiments we explore the potential for coexistence
119 under different environmental conditions (host and non-host habitat) and contribute to
120 a better understanding whether the host alters microbial interactions that potentially
121 lead to coexistence in simple microbiomes.
122

123 **Materials and Methods**

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

125 *Hydra vulgaris* (strain AEP) was used for carrying out experiments and cultured
126 according to standard procedures at 18°C in standardized culture medium (Hydra
127 medium (HM)) [27]. Animals were fed three times a week with 1st instar larvae of
128 *Artemia salina*. Germ-free (GF) polyps were obtained by treating wild-type (WT)
129 animals with an antibiotic cocktail solution containing 50 μg/ml ampicillin, neomycin,
130 streptomycin, rifampicin and 60 μg/ml spectinomycin as previously described [28, 29].
131 The antibiotic cocktail solution was exchanged every 48 h and the antibiotic treatment
132 lasted for two weeks, after which polyps were transferred into antibiotic-free sterile HM
133 for recovery (four days). The germ-free status of polyps was confirmed as previously
134 described [28]. During antibiotic treatment and re-colonization experiments, polyps
135 were not fed.

136

137 **Bacterial strains and media**

138 The bacterial strains used in this study are *Curvibacter* sp. AEP1.3 and *Duganella* sp.
139 C1.2., which have been isolated from *Hydra vulgaris* (strain AEP) [26]. These bacteria
140 were cultured from existing isolate stocks in R2A medium at 18°C, shaken at 250 r.p.m
141 for 72 h before use in the different experiments.

142

143 **Carrying capacity of the host**

144 To determine the carrying capacity of the *Hydra* habitat the microbial load of individual
145 *Hydra* polyps (N=16) was determined. In addition to wild-type polyps the carrying
146 capacity of conventionalized polys (N=12), obtained by incubating germ-free polyps
147 with tissue homogenates of wild-type animals (per germ-free polyp one wild-type polyp
148 was used) for 24 h was also determined. To test whether the carrying capacity can
149 artificially be increased or destabilises upon self-challenge we added either *Curvibacter*

150 or *Duganella* to wild-type polyps (N=6) (approximately $5x10^3$ cells for 24 h). After
151 incubation all polyps were washed with and transferred to sterile HM and further
152 incubated at 18°C and sampled after 120 h.
153
154 **Tracking microbial mono-associations in *Hydra* over time**
155 Germ-free polys were inoculated in their aquatic environment with single bacterial
156 strains (mono-associations). Individual germ-free polyps were incubated with $5x10^3$
157 cells of *Curvibacter* or *Duganella* in 1.5 ml Eppendorf tubes containing 1 ml of sterile
158 HM. After 24 h of incubation all polyps were washed with and transferred to sterile HM,
159 incubated at 18°C and followed over a period of 216 h. For each treatment 6 polys per
160 time point were independently analysed. Every 48 h individual polyps were
161 homogenized in an Eppendorf tube using a sterile pestle and serial dilutions of the
162 homogenate were plated on R2A agar plates to determine colony-forming units (CFUs)
163 per polyp.
164
165 **Microbial growth kinetics of mono- and di-associations *in vivo* and *in vitro***
166 To study the initial phase of colonization, i.e. 96 h post inoculation (see Fig. 1B) in more
167 detail microbial growth of *Curvibacter* and *Duganella* was determined in different
168 habitats; the host habitat (*in vivo*) and two different microcosm environments (*in vitro*).
169 The static incubation provided a spatially structured habitat (heterogeneous), whereas
170 shaking of the microcosms (mixed treatment) eliminated the spatial structure
171 (homogenous).
172 *Mono-associations:* All germ-free polyps and microcosms were inoculated from
173 the same bacterial inoculation culture with approximately $5x10^3$ cells of *Curvibacter* or
174 *Duganella* for 24 h, washed with and transferred to sterile HM. Samples were taken
175 every 12 h for 96 h. For *Hydra* six polyps were sacrificed at each time point and colony-
176 forming units (CFUs) were determined as described above. As microcosms 24-well

177 plates were used. Wells were filled with 2 ml of R2A medium, inoculated and incubated
178 at 18°C either under static or shaken (200 r.p.m) conditions. Each time point was
179 replicated four times and serial dilutions were plated on R2A agar plates to determine
180 CFUs. Growth rates of each strain (A and B) were determined for the exponential growth
181 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
182 the starting density at time 0 and A_f, B_f is the final density at time *f*.

183 *Di-associations:* Density dependent competiveness fitness assays of the two most
184 dominant colonizers *Curvibacter* and *Duganella* were performed *in vivo* and *in vitro.* The
185 same host and microcosm experiments as described above were performed except for
186 using microbial di-associations of *Curvibacter* and *Duganella* with the frequency of
187 *Curvibacter* being rare (10:90), equal (50:50), or dominant (90:10). As *Curvibacter* and
188 *Duganella* form distinct colonies on R2A agar plates, their frequency can be determined
189 by plating serial dilutions [30]. Six polyps and four microcosm replicates were assayed
190 per treatment (static and mixed) and time point. Also this data allowed determining the
191 different carrying capacities of the *in vivo* and *in vitro* habitats used. Malthusian
192 parameters were calculated for both bacterial strains (A and B), $m = \ln (N_f/N_0)$/hour,
193 where N_0 and N_f were the initial and final densities. The relative performance of the two
194 strains was expressed in terms of the selection rate constant (*r*). Note that this measure
195 of performance should be preferentially used over the more commonly used relative
196 fitness (*W*), when one competitor is much less fit than the other [31, 32], as was the case
197 in our study. The selection rate constant was calculated as $r = m_B - m_A$. Accordingly, $r = 0$
198 implies that there is no difference in growth rates of the two strains, $r > 0$ indicates that
199 strain B is advantageous in population growth ($r = 1$ indicates a 10-fold increase in the
200 ratio of B vs. A abundances), while $r < 0$ suggests that strain B fails in invading
201 population of strain A.

202 **Statistical analysis**

203 A Welch ANOVA (and subsequent Dunnett's posthoc test) was used to test for
204 differences in bacterial abundance patterns ('bacteria per *Hydra*') in wild-type versus
205 manipulated hosts as variances between the different groups were not equally
206 distributed.

207 Differences during mono-colonizations of *Curvibacter* and *Duganella* over time
208 were assessed using a Generalized linear model (error structure: normal; link function:
209 identity). The response variable was 'bacteria per *Hydra*', and explanatory variables
210 were 'bacterial species', 'time' and 'bacterial species' x 'time'. Differences between the
211 two bacterial species on each day were detected with post hoc contrasts.

212 Analysis of variance (ANOVA) and subsequent post hoc t-tests were used to test
213 for differences in growth rates of the two competitors when grown singly in the
214 different environments. The response variable was 'growth rate', and explanatory
215 variables were 'bacterial species', 'environment' and 'bacterial species' x 'environment'.

216 Differences in the growth rates in the di-associations of *Curvibacter* and
217 *Duganella* in the different environments and dependence on initial frequency were
218 assessed using a Generalized linear model (error structure: normal; link function:
219 identity) and post hoc contrasts. For each bacterial species, a separate model was
220 calculated with the response variables being either 'growth rate *Curvibacter*' or 'growth
221 rate *Duganella*', and the explanatory variables were 'environment', 'starting density' and
222 'environment' x 'starting density'.

223 Differences in the selection rate constant of *Curvibacter* in the different
224 environments and dependent on initial frequency were assessed using a Generalized
225 linear model (error structure: normal; link function: identity) and post hoc contrasts.
226 The response variable was 'selection rate constant *Curvibacter*' and the explanatory
227 variables were 'environment', 'starting density' and 'environment' x 'starting density'.

228 Sample size was chosen to maximise statistical power and ensure sufficient
229 replication. Assumptions of the tests, that is, normality and equal distribution of
230 variances, were visually evaluated. Non-significant interactions were removed from the
231 models. All tests were two-tailed. Effects were considered significant at the level of $P <$
232 0.05. All statistical analyses were performed with JMP 9. Graphs were produced with
233 GraphPad Prism 5.0, and Adobe Illustrator CS5.1.
234
235 **Results**
236 **Carrying capacity of the *Hydra* host**
237 The carrying capacity of the *Hydra* habitat is highly stable among single *Hydra* wild-type
238 polyps with $1.7*10^5$ CFUs/polyp and at its maximum with the native microbiome.
239 Manipulations of the microbial community lead to significant changes in bacterial
240 numbers (Welch ANOVA, $F_3=7.054$; $P<0.005$; Fig. 1A). The addition of single bacterial
241 species (*Curvibacter* or *Duganella*) to wild-type polys does not increase the carrying
242 capacity but, in the case of *Curvibacter*, leads to a significant reduction in microbial
243 population size. In contrast does the addition of *Duganella* not significantly shift the
244 bacterial numbers (Fig. 1A). Conventionalised animals (germ-free polyps incubated with
245 tissue homogenates of wild-type animals) show a carrying capacity, which is not
246 significantly different compared to wild-type polyps (Fig. 1A), indicating the usability of
247 the germ-free polyps for the manipulation and construction of synthetic bacterial
248 communities on the host/ *in vivo*.
249
250 **Tracking microbial mono-colonisations in *Hydra* over time**
251 In mono-associations *Curvibacter* and *Duganella* populations display typical bacterial
252 growth dynamics. Following a lag and exponential phase, both strains reach a stationary
253 phase after roughly 72 h with a stable population size/carrying capacity per host (Fig.
254 1B). After 72 h we find significant differences in the carrying capacity between both

255 strains, *Curvibacter* being higher than *Duganella* (estimated by post hoc contrasts;
256 Generalized linear model: Full model: χ^2=54.360, d.f.=9, P<0.0001; bacterial species x
257 days post exposure: χ^2=18.326, d.f.=4, P=0.0011). These significant differences last until
258 the end of the experiment. Once carrying capacity of mono-associations is reached, we
259 find a population size of about 10^4 CFUs per host for *Curvibacter*, whereas for *Duganella*
260 the population size reaches on average only $1.5*10^3$ CFUs per host. Further, both mono-
261 associations do not reach the carrying capacity of wild-type polyps. The variation in
262 bacterial density between hosts is significantly higher in *Curvibacter* than in *Duganella*
263 (Levene: F_1=21.496, P<0.0001). No extinction events are observed in either strain after
264 successful establishment during the mono-colonisation experiments.
265
266 **Microbial growth kinetics of mono-associations *in vivo* and *in vitro***
267 Growth rates of *Curvibacter* did not significantly differ between the host and the
268 microcosm environments. This is in marked contrast to *Duganella*, where significantly
269 higher growth rates were observed in the non-host as compared to the host
270 environment. In all environments, except for the host, *Duganella* achieved a significantly
271 higher growth rate than *Curvibacter* (determined by post hoc t-tests; ANOVA: R^2=0.827;
272 Full model: $F_{5,15}$ = 14.333; P<0.0001; bacterial species x environment: F_2 = 15.592;
273 P=0.0002).
274
275 **Microbial di-association experiments *in vivo* and *in vitro***
276 *Carrying capacity*
277 Results of host and microcosm experiments (static and mixed) show that during di-
278 associations the carrying capacity in all habitats is reached at about 72 h after
279 inoculation. Both microcosm environments are characterized by a carrying capacity of
280 10^7-10^8 CFUs/ml, and so exceeding the *in vivo* carrying capacity by a factor of 10^4 (Fig.
281 3). Nevertheless, di-associations on the host also fail to reach the carrying capacity of

282 wild-type polyps and reach a comparable carrying capacity as in the mono-colonisations
283 of *Curvibacter*. Both bacterial species do not match the carrying capacities as measured
284 in mono-colonisations on the host: whereas *Curvibacter* fails by a power of 10 to reach
285 its density in the mono-colonisations, *Duganella* outgrows it by a power of 10.
286
287 *Changes in fractions in microbial di-association experiments*
288 When competing *Curvibacter* and *Duganella* in three different starting frequencies in the
289 host, and the non-host environments, several interesting patterns can be observed (Fig.
290 4). In both non-host environments, *Duganella* outcompetes *Curvibacter* within 48 h post
291 exposure. From then onwards, frequencies of *Curvibacter* are low, reaching a maximum
292 of about 10%. This pattern does not depend on the initial frequency at the start of the
293 experiment. The host shows a different pattern. Here, a decrease in *Curvibacter* fraction
294 can be observed in all three initial frequencies but never to a point where it cannot be
295 detected in the population. From 72 h post exposure onwards the population on the host
296 has reached a stable state, with *Curvibacter* making up 20% of the total bacterial
297 population.
298
299 *Microbial growth kinetics of di-associations*
300 Overall *Curvibacter* growth rate in di-associations are lower or not different from the
301 mono-associations (Fig. 5; as estimated by post hoc contrasts; Generalized linear model:
302 Full model: χ^2=45.790, d.f.=11, P<0.0001; environment x initial frequency: χ^2=33.685,
303 d.f.=6, P<0.0001). *Curvibacter* grows significantly differently when inoculated in equal
304 densities as compared to the rare and dominant starting frequencies across the different
305 environments. Whereas in the host, *Curvibacter* grows better when in equal density with
306 *Duganella*, the opposite is true for both *in vitro* environments.
307 As observed for the growth of *Duganella* in mono-colonisations, growth rates are always
308 higher in the non-host environments irrespective of initial frequency (Generalized linear

309 model: Full model: χ^2=130.278, d.f.=11, P<0.0001; environment x initial frequency:
310 χ^2=59.723, d.f.=6, P<0.0001). Whereas, in di-associations, negative growth rates can be
311 detected only once for *Duganella*, it happens more frequently in the *Curvibacter*,
312 indicating a direct or indirect negative effect of *Duganella*.
313
314 *Relative performance of Curvibacter*
315 As estimation for the relative performance of *Curvibacter* in competition with *Duganella*
316 we determined the selection rate constant (r) for all di-association experiments [31, 32].
317 We observed r < 0 for all environments and frequencies, indicating that *Curvibacter*
318 performs worse than *Duganella* when they are in direct competition (Fig. 6). There is no
319 overall effect of the initial frequency - only in the mixed environment differences can be
320 detected with the lowest performance of *Curvibacter* at equal frequencies of the two
321 competitors (Generalized linear model: Full model: χ^2=51.168, d.f.=8, P<0.0001;
322 environment x initial frequency: χ^2=19.147, d.f.=4, P=0.0007). In general, *Curvibacter*
323 performs significantly better in the host environment irrespective of initial frequency.
324
325 **Discussion**
326 One of the major challenges in microbiome research is to understand the factors that
327 influence the dynamics and stability of host-associated microbial communities. Of
328 particular relevance for this are the processes governing assembly [33, 34] and
329 resilience [35]. Insights into such processes in bacterial populations within their native
330 host environments can be gained through a number of ways. Relationships between
331 different community members in complex microbiomes, such as the human gut, can, for
332 example, be inferred by analysing co-occurrence data and correlation patterns from
333 sequencing-based metagenomic time-series experiments [36, 37]. Another approach,
334 where ecological interactions can experimentally be dissected, allows for hypothesis
335 testing. For this, a relatively simple system, such as *Hydra* and its microbiome is ideal for

336 'deconstructing' a metaorganism and its interactions [23]. This strategy is novel in meta-
337 organism research but has yielded exciting results regarding rules that determine
338 community assembly and stability in non-host associated microbial communities [38,
339 39]. In microbiome research, the added advantage of including a host provides the
340 opportunity to study the performance of the individual microbiome members in their
341 natural environment. In addition, the comparison of the *in vivo* to *in vitro* environments
342 provides information on host effects in regulating its microbiome.

343 Carrying capacity is defined as the largest population size that an ecosystem can
344 sustainably support without degrading the ecosystem. Therefore population size is
345 constrained by the carrying capacity of the environment. This is also true for bacteria
346 populating a particular environment, including hosts. In microbiome research, the
347 significance of host carrying capacity has been largely overlooked until very recently
348 where a link between host health and microbiome density has been reported [8]. The
349 host's carrying capacity ultimately sets the upper boundary for bacterial fitness relative
350 to any competitors, and provides a reference for investigating the interactions within
351 the host microbiota at spatial and temporal scales.

352 We here show that wild-type *Hydra* is characterized by a specific carrying
353 capacity of about 10^5 bacteria per polyp that is stable in adult polyps and can be
354 artificially assembled through the re-population of germ-free animals with the natural
355 bacterial community. This is an important prerequisite for conducting the *in vivo*
356 experiments, where colonization patterns of single species from *Hydra's* microbiome are
357 individually followed. When *Curvibacter* and *Duganella* are introduced separately to the
358 host (in mono-associations), each bacterial species is capable to robustly colonize the
359 host to high abundances, which are maintained throughout the experimental period,
360 indicating the ability to independently utilize the resources of the *Hydra* habitat. While
361 both species fail to reach their respective carrying capacities as compared to wild type
362 *Hydra* by a factor of 10, their respective proportions of about 75% and 11% [26] are

363 nevertheless maintained. Results from the di-association experiments clearly show that
364 the abundances and relative frequencies of *Curvibacter* and *Duganella* as measured in
365 wild-type *Hydra* cannot be explained by their (positive) interactions in the host context
366 alone as this led to a frequency reversal, making *Duganella* more abundant than
367 *Curvibacter*. *Duganella* however reaches comparable carrying capacities as measured in
368 wild-type *Hydra* polyps. This implies that the presence of *Curvibacter* has a positive
369 effect on the performance of *Duganella* but *Duganella* negatively affects *Curvibacter* in
370 the host habitat. These observations indicate that the less frequent community members
371 (each 2% and less) are of central importance for reaching the full carrying capacity of
372 the *Hydra* microbiome. We hypothesise that two aspects are of importance here – (i) the
373 low abundant microbes might be able to utilize different resources as compared to
374 *Curvibacter* and *Duganella* and so inhabit different ecological niches within the
375 microbiome, which the two main colonizers cannot fill and (ii) they likely interact in a
376 positive way (either directly or indirectly) with (at least) *Curvibacter*, enabling it to
377 reach higher carrying capacities. It is thus important to note that *Hydra*'s carrying
378 capacity is not solely determined by the host (resources) alone but also by the
379 interactions within the microbiome.

380 To determine the relative importance of the host, interactions of *Curvibacter* and
381 *Duganella*, and intrinsic properties of the bacteria, such as ability to grow, in shaping the
382 observed patterns in the host environment, we performed the same mono- and di-
383 association experiments in two i*n vitro* environments. We chose to contrast static, which
384 closely resembles the host habitat in that it provides spatial heterogeneity facilitating
385 bacterial interactions and mixed, where direct interactions between individual bacteria
386 cannot be established but where individual bacteria have (unlimited) access to
387 resources and oxygen. Because community structure can be influenced by initial species
388 abundances [40], we performed all di-association experiments using various initial
389 starting frequencies. In general, both bacterial species reached much higher carrying

390 capacities in both *in vitro* environments as compared to the *in vivo* environment, with
391 *Duganella* reaching up to 10^8 CFUs/ml and *Curvibacter* up to 10^5 CFUs/ml. Interestingly
392 we were not able to replicate the *in vivo* dynamics between *Curvibacter* and *Duganella in*
393 *vitro*, suggesting that the host mediates the interaction between the two bacterial
394 species. Whereas pairwise competitions in the di-association experiments resulted in
395 coexistence of *Curvibacter* and *Duganella* in the host habitat, it led to an overgrowth of
396 *Duganella* in both *in vitro* habitats. More specifically, we found that in the *Hydra* habitat
397 *Curvibacter*, independent of its inoculation frequency, and after the initial establishment
398 period of 72 h, reached a constant fraction of about 20%, whereas it was only present at
399 very low frequencies in both *in vitro* habitats due to the high continuous growth rates of
400 *Duganella* (see also [30] for homogeneous environment).

401 Many host-associated microbial communities live in highly competitive
402 surroundings, in which they struggle to persist. According to Ghoul and Mitri [41], a
403 strain is competitive if it shows phenotypes that cause a fitness decrease in a competitor
404 strain. Competition can be either passive, where strains compete for the same resources
405 or active, where strains directly harm one another [41]. Li *et al.* [30] showed that during
406 co-culture experiments in a homogenous environment the presence and frequency of
407 *Curvibacter* greatly influenced the growth rate of *Duganella* (quadratic function), which
408 we also found. Interestingly we find the same pattern on the host, although growth rates
409 are drastically reduced. In the static environment we find the inverse of the quadratic
410 function.

411 When comparing the bacterial growth rates in mono-associations we observe
412 that *Curvibacter* performs equally well in all habitats, whereas *Duganella* shows a
413 reduced growth rate compared to *Curvibacter* on the host and performs best in the non-
414 host habitat. This suggests that *Duganella* is not particularly adapted to the host niche.
415 Interestingly the two species also differ in their phylogenetic association patterns with
416 different *Hydra* species [28]. Whereas different lineages of *Curvibacter* have been shown

417 to be associated with the majority (N=6) of *Hydra* species analysed (N=7), *Duganella*
418 was detected in less than half (N=3). This indicates that *Duganella* is a more recent
419 member of the *Hydra* microbiome and that it is not as co-evolved as it has been
420 suggested for one of *the* keystone species of *Hydra's* microbiome, *Curvibacter* [30]. The
421 specific microbiome of *Hydra vulgaris*, which is studied here, has shown to be stable as
422 animals living in the wild were colonized by a similar microbiome as compared to the
423 ones that had been maintained in the laboratory for a relatively long time [24].

424 Oscillatory dynamics, chaotic fluctuations and stochastic perturbations influence
425 species abundances and allow for the coexistence of many species and have therefore a
426 significant impact on species survival even in simple communities containing only a few
427 interacting species [42-44]. Interestingly we observed such sporadic collapses in the
428 *Curvibacter* population during di-association experiments in the non-host environment
429 but not on the host. An earlier study following the bacterial colonization pattern of
430 *Hydra* hatchlings showed sporadic collapses of the *Curvibacter* population during the
431 first 15 weeks post hatching [45]. In adult polyps, which were used in the experiments,
432 we did not observe such dynamics, which points to the fact that these sporadic collapses
433 might be buffered in adult polyps or are caused by the other species of the *Hydra*
434 microbiome. For the zebrafish host model it has been shown that *Aeromonas* during
435 mono-association is not resistant to disturbances related to intestinal motility and that
436 in the presence of *Vibrio* the magnitude of collapses are even greater [22]. *Hydra* is also
437 characterized by spontaneous contractile activity [29] but our data suggest that this
438 does not have such far-reaching effects on the microbiota as found in the zebrafish gut.

439 Whereas in other host-microbe systems such as the zebrafish, *in vivo* dynamics
440 between two bacterial species can be replicated *in vitro* [46], in our system this is not
441 the case. We find the fitness of *Curvibacter* and *Duganella* to be highly dependent on the
442 specific environments tested. Whereas *Duganella* performs best *in vitro*, in terms of
443 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. 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 [47] with some of them having a large impact on inducing dysbiosis in the microbiome and on guaranteeing host health [48, 49].

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471 **References**

472 1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The
473 human microbiome project. *Nature* 2007;**449**: 804–810.
474 2. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, *et al.*
475 Structure, function and diversity of the healthy human microbiome. *Nature* 2012;
476 **486**: 207–214.
477 3. Pasolli E, Asnicar F, Manara S, Zolfo M, Karcher N, Armanini F, *et al.* Extensive
478 unexplored human microbiome diversity revealed by over 150,000 genomes from
479 metagenomes spanning age, geography, and lifestyle. *Cell* 2019; **176**: 649–662.
480 4. Mueller UG, Sachs JL. Engineering microbiomes to improve plant and animal
481 health. *Trends Microbiol* 2015; **23**: 606–617.
482 5. McKenney PT, Pamer EG. From hype to hope: The gut microbiota in enteric
483 infectious disease. *Cell* 2015; **163**: 1326–1332.
484 6. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current
485 understanding of the human microbiome. *Nat Med* 2018; **24**: 392–400.
486 7. McKenzie VJ, Kueneman JG, Harris RN. Probiotics as a tool for disease mitigation in
487 wildlife: insights from food production and medicine. *Ann N Y Acad Sci* 2018;
488 **1429**: 18–30.
489 8. Contijoch EJ, Britton GJ, Yang C, Mogno I, Li Z, Ng R, *et al.* Gut microbiota density
490 influences host physiology and is shaped by host and microbial factors. *eLife* 2019;
491 **8**: e40553.
492 9. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability
493 and resilience of the human gut microbiota. *Nature* 2012; **489**: 220–230.
494 10. Sachs JL, Essenberg CJ, Turcotte MM. New paradigms for the evolution of
495 beneficial infections. *Trends Ecol Evol* 2011; **26**: 202–209.
496 11. Deines P, Lachnit T, Bosch TCG. Competing forces maintain the *Hydra*
497 metaorganism. *Immunol Rev* 2017; **279**: 123–136.

498 12. Macke E, Tasiemski A, Massol F, Callens M, Decaestecker E. Life history and eco-
499 evolutionary dynamics in light of the gut microbiota. *Oikos* 2017; **126**: 508–531.
500 13. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen Les, Sargent M, *et al.*
501 Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635–1638.
502 14. Zhang Z, Geng J, Tang X, Fan H, Xu J, Wen X, *et al.* Spatial heterogeneity and co-
503 occurrence patterns of human mucosal-associated intestinal microbiota. *ISME J*
504 2014; **8**: 881–893.
505 15. Britton RA, Young VB. Interaction between the intestinal microbiota and host in
506 Clostridium difficile colonization resistance. *Trends Microbiol* 2012; **20**: 313–319.
507 16. Levy R, Borenstein E. Metagenomic systems biology and metabolic modeling of the
508 human microbiome. *Gut Microbes* 2014; **5**: 265–270.
509 17. Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, Patil KR. Metabolic
510 dependencies drive species co-occurrence in diverse microbial communities. *Proc*
511 *Natl Acad Sci USA* 2015; **112**: 6449–6454.
512 18. Garcia JR, Gerardo NM. The symbiont side of symbiosis: do microbes really
513 benefit? *Front Microbiol* 2014; **5**: 510.
514 19. Wiser MJ, Lenski RE. A comparison of methods to measure fitness in *Escherichia*
515 *coli*. *PLoS ONE* 2015; **10**: e0126210.
516 20. Lenski RE. Relative fitness: its estimation and its significance for environmental
517 applications of microorganisms. In: Levin M, Seidler R, Rogul M (eds). *Microbial*
518 *Ecology: Principles, Applications and Methods*. McGraw-Hill Book Co: New York, NY,
519 USA, 1992, pp 183–198.
520 21. Mushegian AA, Ebert D. Rethinking "mutualism" in diverse host-symbiont
521 communities. *Bioessays* 2015; **38**: 100–108.
522 22. Wiles TJ, Jemielita M, Baker RP, Schlomann BH, Logan SL, Ganz J, *et al.* Host gut
523 motility promotes competitive exclusion within a model intestinal microbiota.
524 *PLoS Biol* 2016; **14**: e1002517.

525 23. Deines P, Bosch TCG. Transitioning from microbiomecomposition to microbial
526 community interactions: The potential of the metaorganism *Hydra* as an
527 experimental model. *Front Microbiol* 2016; **7**: 1610.
528 24. Fraune S, Bosch TCG. Long-term maintenance of species-specific bacterial
529 microbiota in the basal metazoan *Hydra*. *Proc Natl Acad Sci USA* 2007; **104**:
530 13146–13151.
531 25. Bosch TCG. Cnidarian-microbe interactions and the origin of innate immunity in
532 metazoans. *Annu Rev Microbiol* 2013; **67**:499–518.
533 26. Fraune S, Anton-Erxleben F, Augustin R, Franzenburg S, Knop M, Schröder K, *et al.*
534 Bacteria-bacteria interactions within the microbiota of the ancestral metazoan
535 *Hydra* contribute to fungal resistance. *ISME J* 2015; **9**: 1543–1556.
536 27. Lenhoff HM, Brown RD. Mass culture of *Hydra*: an improved method and its
537 application to other aquatic invertebrates. *Lab Anim* 1970; **4**: 139–154.
538 28. Franzenburg S, Walter J, Künzel S, Wang J, Baines JF, Bosch TCG, *et al.* Distinct
539 antimicrobial peptide expression determines host species-specific bacterial
540 associations. *Proc Natl Acad Sci USA* 2013; **110**: E3730–E3738.
541 29. Murillo-Rincon AP, Klimovich A, Pemöller E, Taubenheim J, Mortzfeld B, Augustin
542 R, *et al.* Spontaneous body contractions are modulated by the microbiome of
543 *Hydra*. *Sci Rep* 2017; **7**:15937.
544 30. Li X-Y, Pietschke C, Fraune S, Altrock PM, Bosch TCG, Traulsen A. Which games are
545 growing bacterial populations playing? *J R Soc Interface* 2015; **12**: 20150121.
546 31. Lenski RE, Rose MR, Simpson SC, Tadler SC. Long-term experimental evolution in
547 *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *Am Nat*
548 1991; **138**: 1315–1341.
549 32. Travisano M, Lenski RE. Long-term experimental evolution in *Escherichia coli*. IV.
550 Targets of selection and the specificity of adaptation. *Genetics* 1996; **143**: 15–26.
551 33. Sonnenburg JL, Angenent LT, Gordon JI. Getting a grip on things: how do

552 communities of bacterial symbionts become established in our intestine? *Nat*
553 *Immunol* 2004; **5**:569–573.
554 34. Shafquat A, Joice R, Simmons SL, Huttenhower C. Functional and phylogenetic
555 assembly of microbial communities in the human microbiome. *Trends Microbiol*
556 2014; **22**: 261–266.
557 35. Allison SD, Martiny JBH. Resistance, resilience, and redundancy in microbial
558 communities. *Proc Natl Acad Sci USA* 2008; **105**: 11512–11519.
559 36. Faust K, Raes J. Microbial interactions: from networks to models. *Nat Rev*
560 *Microbiol* 2012; **10**: 538–550.
561 37. Trosvik P, de Muinck EJ, Stenseth NC. Biotic interactions and temporal dynamics
562 of the human gastrointestinal microbiota. *ISME J* 2015; **9**: 533–541.
563 38. Friedman J, Higgins LM, Gore J. Community structure follows simple assembly
564 rules in microbial microcosms. *Nat Ecol Evol* 2017; **1**: 0109.
565 39. Higgins LM, Friedman J, Shen H, Gore J. Co-occurring soil bacteria exhibit a robust
566 competitive hierarchy and lack of non-transitive interactions. *bioRxiv* 2017:
567 175737. doi:10.1101/175737.
568 40. Fukami T. Historical contingency in community assembly: Integrating niches,
569 species pools, and priority effects. *Annu Rev Ecol Evol Syst* 2015; **46**: 1–23.
570 41. Ghoul M, Mitri S. The ecology and evolution of microbial competition. *Trends*
571 *Microbiol* 2016; **24**: 833–845.
572 42. Armstrong RA, McGehee R. Competitive exclusion. *Am Nat* 2015; **115**:151–170.
573 43. Sousa WP. The role of disturbance in natural communities. *Annu Rev Ecol Syst*
574 1984; **15**: 353–391.
575 44. Huisman J, Weissing FJ. Biodiversity of plankton by species oscillations and chaos.
576 *Nature* 1999; **402**: 407–410.
577 45. Franzenburg S, Fraune S, Altrock PM, Künzel S, Baines JF, Traulsen A, *et al.*
578 Bacterial colonization of *Hydra* hatchlings follows a robust temporal pattern. *ISME*

579 *J* 2013; **7**: 781–790.

580 46. Rolig AS, Parthasarathy R, Burns AR, Bohannan BJM, Guillemin K. Individual
581 members of the microbiota disproportionately modulate host innate immune
582 responses. *Cell Host Microbe* 2015; **18**: 613–620.

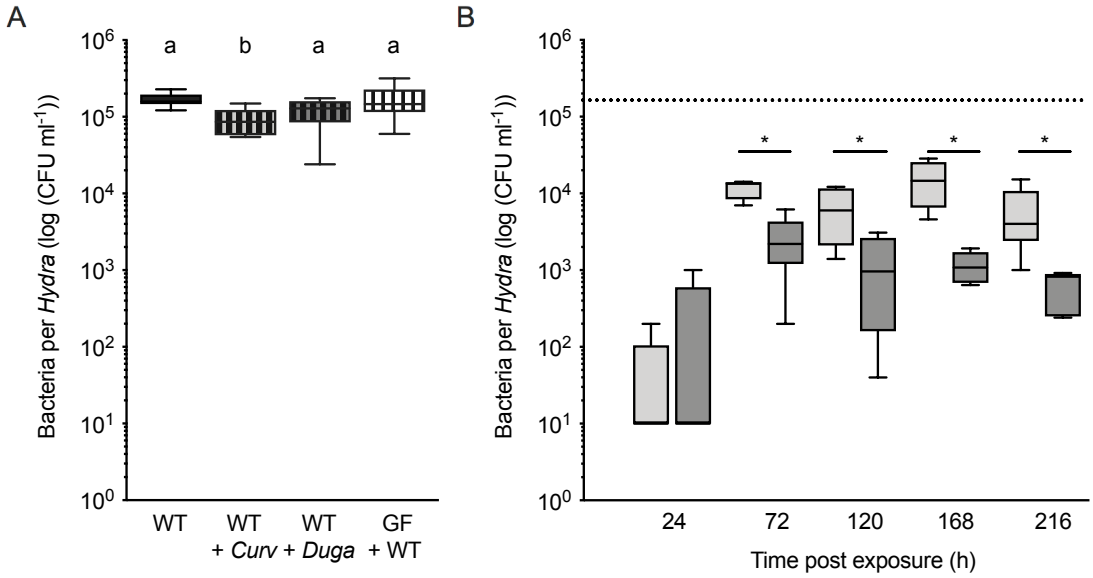
583 47. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, *et al.*
584 Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174–180.

585 48. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. *Nat*
586 *Rev Microbiol* 2012; **10**: 717–725.

587 49. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J,
588 *et al. Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium
589 identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci*
590 *USA* 2008; **105**: 16731–16736.

591

592 **Figures**



593

594

595 Fig 1. (A) Carrying capacity of the *Hydra* habitat in wild-type (WT) polyps, wild-type
596 polyps and the addition of either the focal species *Curvibacter* (*Curv*) or *Duganella*
597 (*Duga*) and germ-free (GF) animals incubated with native *Hydra* microbiota
598 (conventionalised polyps) (each boxplot N≥6≤16). (B) Time course analysis of microbial
599 abundances in mono-associations of germ-free polyps with either *Curvibacter* (light
600 grey) or *Duganella* (dark grey) (each boxplot N=6). The dashed line indicates the
601 carrying capacity of wild-type polys.

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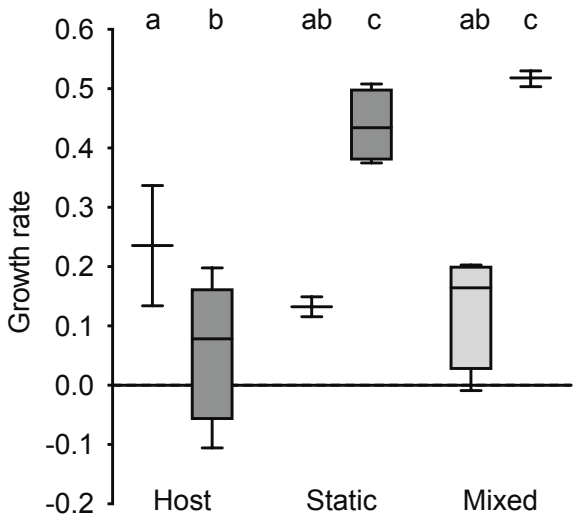

Fig 2. Bacterial growth rates of *Curvibacter* (light grey; each boxplot N≥2≤4) and *Duganella* (dark grey; each boxplot N≥3≤6) in mono-associations are habitat dependent. Compared are the host habitat (*in vivo*), and two *in vitro* environments: heterogeneous (static microcosms), and homogenous (mixed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

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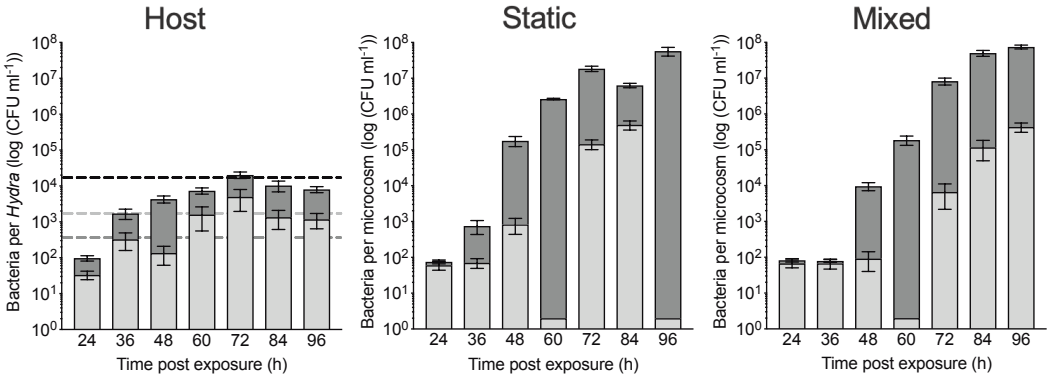

611

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

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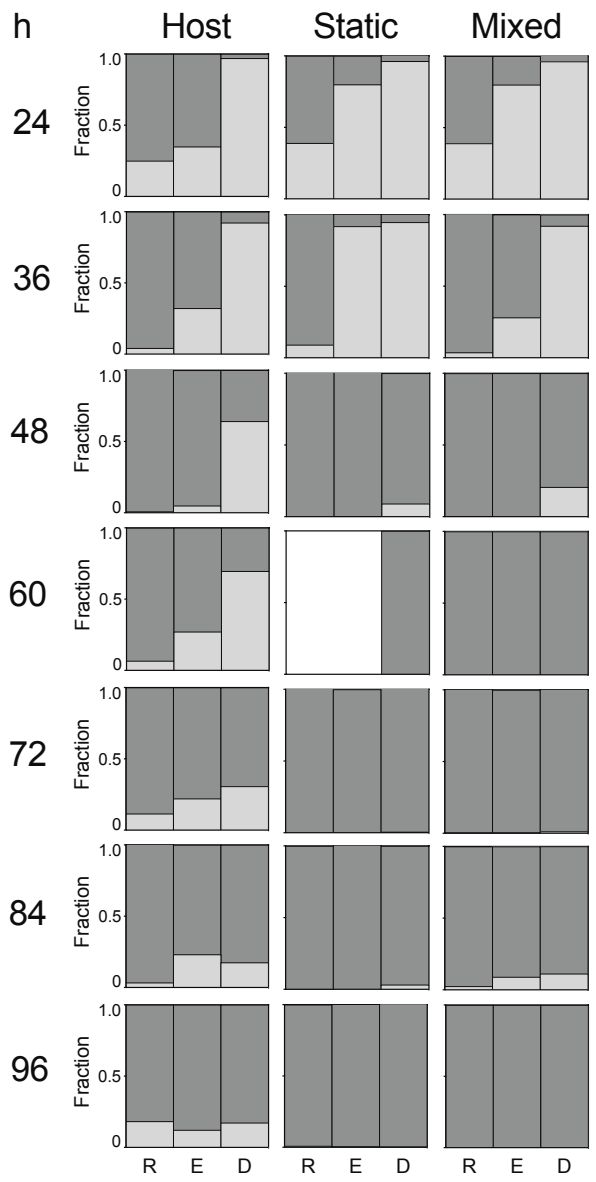
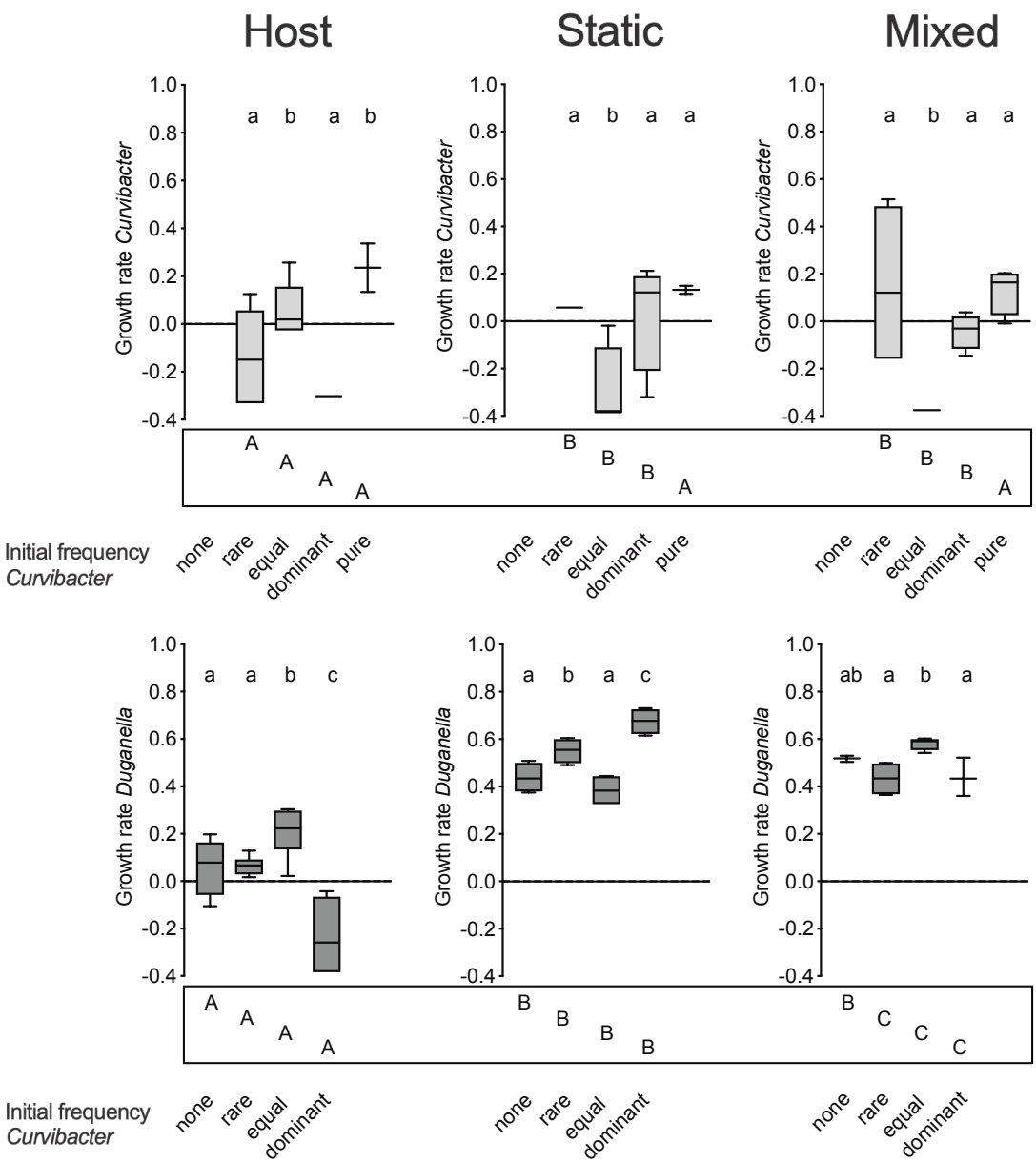


Fig 4. Time course of fractions of *Curvibacter* (light grey) and *Duganella* (dark grey) in the three different habitats obtained from di-association experiments. The initial inoculation frequency of *Curvibacter* varied from being rare (R), to equal (E), to dominant (D) in comparison to *Duganella*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



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629

630 Fig 5. Bacterial growth rates of *Curvibacter* (light grey) and *Duganella* (dark grey) from

631 mono- and di-association experiments across the different habitats and initial

632 frequencies tested (each boxplot N≥3≤4).

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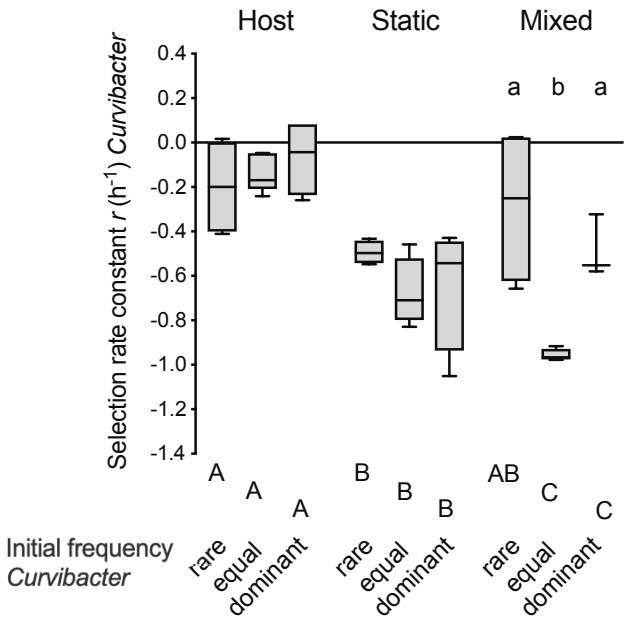

Fig. 6. Selection rate constants of *Curvibacter* based on competition experiments with *Duganella* for the different starting frequencies, (each boxplot N≥3≤6).

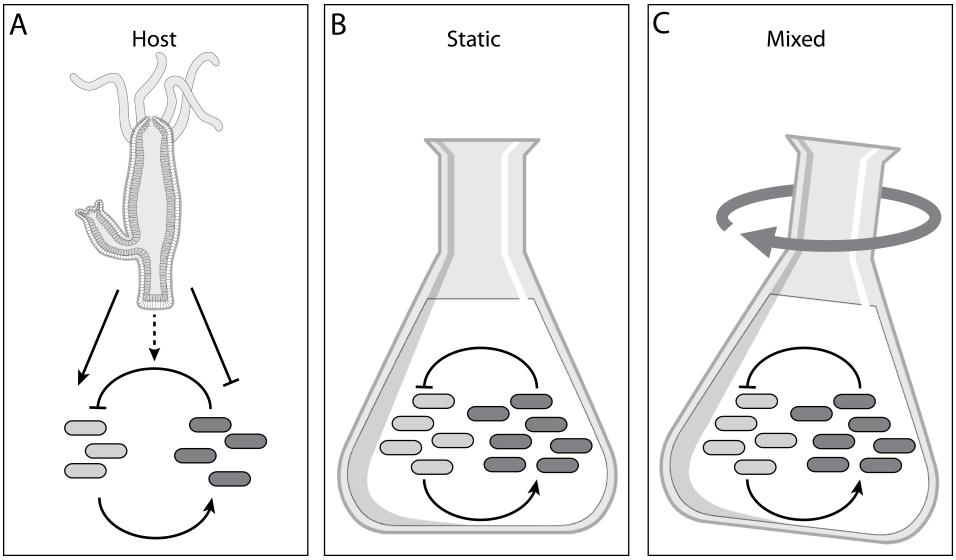

Fig. 7. Model of the microbe-microbe and host-microbe interactions occurring in different habitats (host (A) and non-host (B and C)) that influence bacterial abundances, which are indicated by the number of bacterial cells (*Curvibacter*: light grey, *Duganella*: dark grey). Whereas (A) allows to study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