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 $1.7 \times 10^5$ CFUs/polyp and at its maximum with the native microbiome.
Manipulations of the microbial community lead to significant changes in bacterial numbers (Welch ANOVA, $F_s=7.054; P<0.005$; Fig. 1A). The addition of single bacterial species (*Curvibacter* or *Duganella*) to wild-type polyps does not increase the carrying capacity but, in the case of *Curvibacter*, leads to a significant reduction in microbial population size. In contrast, does the addition of *Duganella* not significantly shift the bacterial numbers (Fig. 1A). Conventionalised animals (germ-free polyps incubated with tissue homogenates of wild-type animals) show a carrying capacity, which is not significantly different compared to wild-type polyps (Fig. 1A), indicating the usability of the germ-free polyps for the manipulation and construction of synthetic bacterial communities on the host/ *in vivo*.

**Tracking microbial mono-colonisations in Hydra over time**

In mono-associations *Curvibacter* and *Duganella* populations display typical bacterial growth dynamics. Following a lag and exponential phase, both strains reach a stationary phase after roughly 72 h with a stable population size/carrying capacity per host (Fig. 1B). After 72 h we find significant differences in the carrying capacity between both strains, *Curvibacter* being higher than *Duganella* (estimated by post hoc contrasts; Generalized linear model: Full model: $\chi^2=54.360, d.f.=9, P<0.0001$; bacterial species x days post exposure: $\chi^2=18.326, d.f.=4, P=0.0011$). These significant differences last until the end of the experiment. Once carrying capacity of mono-associations is reached, we find a population size of about $10^4$ CFUs per host for *Curvibacter*, whereas for *Duganella* the population size reaches on average only $1.5*10^3$ CFUs per host. Further, both mono-associations do not reach the carrying capacity of wild-type polyps. The variation in bacterial density between hosts is significantly higher in *Curvibacter* than in *Duganella* (Levene: $F_1=21.496, P<0.0001$). No extinction events are observed in either strain after successful establishment during the mono-colonisation experiments.
Microbial growth kinetics of mono-associations *in vivo* and *in vitro*

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

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

Carrying capacity

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

Changes in fractions in microbial di-association experiments

When competing *Curvibacter* and *Duganella* in three different starting frequencies in the host, and the non-host environments, several interesting patterns can be observed (Fig. 4). In both non-host environments, *Duganella* outcompetes *Curvibacter* within 48 h post exposure. From then onwards, frequencies of *Curvibacter* are low, reaching a maximum of about 10%. This pattern does not depend on the initial frequency at the start of the
experiment. The host shows a different pattern. Here, a decrease in *Curvibacter* fraction can be observed in all three initial frequencies but never to a point where it cannot be detected in the population. From 72 h post exposure onwards the population on the host has reached a stable state, with *Curvibacter* making up 20% of the total bacterial population.

**Microbial growth kinetics of di-associations**

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

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

**Relative performance of Curvibacter**

As estimation for the relative performance of *Curvibacter* in competition with *Duganella* we determined the selection rate constant (r) for all di-association experiments (28, 29). We observed \( r < 0 \) for all environments and frequencies, indicating that *Curvibacter* performs worse than *Duganella* when they are in direct competition (Fig. 6). There is no overall effect of the initial frequency - only in the mixed environment differences can be
detected with the lowest performance of *Curvibacter* at equal frequencies of the two competitors (Generalized linear model: Full model: $\chi^2=51.168$, d.f.=8, $P<0.0001$; environment x initial frequency: $\chi^2=19.147$, d.f.=4, $P=0.0007$). In general, *Curvibacter* performs significantly better in the host environment irrespective of initial frequency.

**Discussion**

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

Carrying capacity is defined as the largest population size that an ecosystem can sustainably support without degrading the ecosystem. Therefore population size is constrained by the carrying capacity of the environment. This is also true for bacteria populating a particular environment, including hosts. In microbiome research, the
significance of host carrying capacity has been largely overlooked until very recently where a link between host health and microbiome density has been reported (8). Bacterial levels have been quantified for only a few model organisms such as in the gut of larval zebrafish (37) or the gut of Drosophila melanogaster (38). The host’s carrying capacity ultimately sets the upper boundary for bacterial fitness relative to any competitors, and provides a reference for investigating the interactions within the host microbiota at spatial and temporal scales.

We here show that wild-type Hydra is characterized by a specific carrying capacity of about $10^5$ bacteria per polyp that is stable in adult polyps and can be artificially assembled through the re-population of germ-free animals with the natural bacterial community. This is an important prerequisite for conducting the in vivo experiments, where colonization patterns of single species from Hydra’s microbiome are individually followed. When Curvibacter and Duganella are introduced separately to the host (in mono-associations), each bacterial species is capable to robustly colonize the host to high abundances, which are maintained throughout the experimental period, indicating the ability to independently utilize the resources of the Hydra habitat. This confirms earlier findings from Wein et al. (39) that Curvibacter is able to robustly colonize its host. A similar observation has been made for Aeromonas and Vibrio colonizing patterns of the gut of larval zebrafish (23). While both, Curvibacter and Duganella, fail to reach their respective carrying capacities as compared to wild type Hydra by a factor of 10, their respective proportions of about 75% and 11% (27) are nevertheless maintained. Results from the di-association experiments clearly show that the abundances and relative frequencies of Curvibacter and Duganella as measured in wild-type Hydra cannot be explained by their (positive) interactions in the host context alone as this led to a frequency reversal, making Duganella more abundant than Curvibacter. Duganella however reaches comparable carrying capacities as measured in 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 in 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-
growing species is released from its dependence on the other (41), which in our case could be compensated by the host. This aspect will be subject of further investigation. Wright and Vetsigian (42) recently demonstrated in pairwise competitions between bacteria of the genus *Streptomyces* that the winner is often the species that starts at high initial abundance. We find that ‘survival of the common’ does not apply to *Curvibacter* in a non-host environment. Whereas pairwise competitions in the di-association experiments resulted in coexistence of *Curvibacter* and *Duganella* in the host habitat, it led to an overgrowth of *Duganella* in both *in vitro* habitats. More specifically, we found that in the *Hydra* habitat *Curvibacter*, independent of its inoculation frequency, and after the initial establishment period of 72 h, reached a constant fraction of about 20%, whereas it was only present at very low frequencies in both *in vitro* habitats due to the high continuous growth rates of *Duganella* (see also (43) for homogeneous environment). This pattern seems to be more pronounced in the static environment, fitting to the general assumption that extinctions are more likely to occur in spatially structure environments. In terms of cross-feeding interactions one could reason that the faster growing-species is released from its dependence on the other, resulting in the slower-growing species being lost (a hypothesis that is termed ‘feed the faster grower’) (41).

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

When comparing the bacterial growth rates in mono-associations we observe that Curvibacter performs equally well in all habitats, whereas Duganella shows a reduced growth rate compared to Curvibacter on the host and performs best in the non-host habitat. This suggests that Duganella is not particularly adapted to the host niche. Interestingly the two species also differ in their phylogenetic association patterns with different Hydra species (45). Whereas different lineages of Curvibacter have been shown to be associated with the majority (N=6) of Hydra species analysed (N=7), Duganella was detected in less than half (N=3). This indicates that Duganella is a more recent member of the Hydra microbiome and that it is not as co-evolved as it has been suggested for one of the keystone species of Hydra’s microbiome, Curvibacter (43).

Nevertheless, the specific microbiome of Hydra vulgaris, which is studied here, has been shown to be stable as animals living in the wild were colonized by a similar microbiome as compared to the ones that had been maintained in the laboratory for a relatively long time (25). One of the few metaorganisms, where symbiont performance within the host relative to a non-host environment has been contrasted, is the squid-Vibrio system. For this obligate association Wollenberg and Ruby (19) demonstrated that symbionts have an increased reproductive capacity and higher fitness when associated with the host. In combination with the results from this study, this indicates that more specifically associated symbionts indeed benefit from growing in the host habitat.

Oscillatory dynamics, chaotic fluctuations and stochastic perturbations influence species abundances and allow for the coexistence of many species and have therefore a significant impact on species survival even in simple communities containing only a few interacting species (46-48). Interestingly we observed such sporadic collapses in the Curvibacter population during di-association experiments in the non-host environment 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
a large impact on inducing dysbiosis in the microbiome and on guaranteeing host health (53, 54).

Materials and Methods

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

_Hydra vulgaris_ (strain AEP) was used for carrying out experiments and cultured according to standard procedures at 18°C in standardized culture medium (Hydra medium (HM)) (55). Animals were fed three times a week with 1st instar larvae of _Artemia salina_. Germ-free (GF) polyps were obtained by treating wild-type (WT) animals with an antibiotic cocktail solution containing 50 μg/ml ampicillin, neomycin, streptomycin, rifampicin and 60 μg/ml spectinomycin as previously described (45, 50).

The antibiotic cocktail solution was exchanged every 48 h and the antibiotic treatment lasted for two weeks, after which polyps were transferred into antibiotic-free sterile HM for recovery (four days). The germ-free status of polyps was confirmed as previously described (45). During antibiotic treatment and re-colonization experiments, polyps were not fed.

Bacterial strains and media

The bacterial strains used in this study are _Curvibacter_ sp. AEP1.3 and _Duganella_ sp. C1.2., which have been isolated from _Hydra vulgaris_ (strain AEP) (27). These bacteria were cultured from existing isolate stocks in R2A medium at 18°C, shaken at 250 r.p.m for 72 h before use in the different experiments.

Carrying capacity of the host

To determine the carrying capacity of the _Hydra_ habitat the microbial load of individual _Hydra_ polyps (N=16) was determined. In addition to wild-type polyps the carrying 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 $5 \times 10^3$ 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 $5 \times 10^3$ 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 $5 \times 10^3$ 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 \( g = \ln \left( \frac{A_f}{A_0} \right) \) and \( g = \ln \left( \frac{B_f}{B_0} \right) \), 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), \( m = \ln \left( \frac{N_f}{N_0} \right) \)/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
ratio of B vs. A abundances), while $r < 0$ suggests that strain B fails in invading population of strain A.

**Statistical analysis**

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

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

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

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

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

Sample size was chosen to maximise statistical power and ensure sufficient replication. Assumptions of the tests, that is, normality and equal distribution of variances, were visually evaluated. Non-significant interactions were removed from the 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 GraphPad Prism 5.0, and Adobe Illustrator CS5.1.
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Conflict of Interests

The authors declare no conflict of interest.
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Figures

Fig 1. (A) Carrying capacity of the *Hydra* habitat in wild-type (WT) polyps, wild-type polyps and the addition of either the focal species *Curvibacter* (*Curv*) or *Duganella* (*Duga*) and germ-free (GF) animals incubated with native *Hydra* microbiota (conventionalised polyps) (each boxplot N≥6≤16). (B) Time course analysis of microbial abundances in mono-associations of germ-free polyps with either *Curvibacter* (red) or *Duganella* (blue) (each boxplot N=6). The dashed line indicates the carrying capacity of wild-type polys.
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).
Fig 3. Carrying capacity of the *in vivo* and *in vitro* habitats used in this study. Shown are pooled total numbers of colony forming units (CFUs) from all di-association experiments with *Curvibacter* (red) and *Duganella* (blue) (shown are s.e.m. based on N≥11≤18 for the host, N≥9≤12 for static, and N≥4≤12 for mixed). The dashed black line indicates the carrying capacity of WT polyps, the light grey line the carrying capacity of polyps during *Curvibacter* mono-associations and the dark grey line the carrying capacity of polyps during *Duganella* mono-associations.
Fig 4. Time course of fractions of *Curvibacter* (red) and *Duganella* (blue) 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).
Fig 5. Bacterial growth rates of *Curvibacter* (red) and *Duganella* (blue) from mono- and di-association experiments across the different habitats and initial frequencies tested (each boxplot N≥3≤4).
Fig. 6. Selection rate constants of *Curvibacter* based on competition experiments with *Duganella* for the different starting frequencies, (each boxplot \(N\geq3\leq6\).
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: red, Duganella: blue).

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.