Emergent coexistence in multispecies microbial communities

Chang-Yu Chang[1,2], Djordje Bajic[1,2], Jean Vila[1,2], Sylvie Estrela[1,2], Alvaro Sanchez[1,2,*,#]

[1]*Department of Ecology & Evolutionary Biology and* [2]*Microbial Sciences Institute, Yale University, New Haven, CT, USA.*

*New Address: *Department of Microbial Biotechnology. Centro Nacional de Biotecnología - CSIC. Campus de Cantoblanco, Madrid, Spain.*

#Correspondence: A.S. alvaro.sanchez@cnb.csic.es

Abstract

Microbial communities are highly diverse, and understanding the factors that promote and modulate this diversity is a major area of research in microbial ecology. Recent work has proposed a reductionist perspective to microbial coexistence, where pairwise coexistence between strains in isolation is required for their coexistence in a more complex community. In this view, species exclusion in pairwise co-culture would preclude their coexistence in a more complex community too. An alternative view is that coexistence is a more complex property of the entire community, requiring the presence of additional community members. If this view were correct, competitive exclusion in pairwise co-culture would not necessarily preclude species coexistence in more complex community contexts. Empirically testing these alternative hypotheses is complicated by the intractably high microbial diversity of most natural communities, and the challenges of reconstituting every pair of coexisting species under the exact same habitat where their community of origin was assembled. To address this challenge, we have experimentally reconstituted all possible pairwise co-cultures between stably coexisting species from 13 different, low diversity microbial enrichment communities, which had previously been assembled in a well-controlled synthetic habitat. We find that, when isolated from the rest of their community members, most species pairs fail to coexist. This result highlights the importance of community context for microbial coexistence, and indicates that pairwise exclusion may not reflect the ability of species to coexist in more complex, multispecies ecosystems.

INTRODUCTION

Explaining species coexistence and the bewildering diversity of ecological communities has been one of the major goals of Ecology[1]. Historically, research on the mechanisms that make coexistence possible has focused on pairs of competing species[2]. This work has been instrumental to develop our current understanding of the fundamental mechanisms that modulate biodiversity, and it occupies a central place in ecological theory. Yet, species are found in nature forming complex ecological communities. It is thus plausible that their coexistence may be mediated by interactions that require the presence of more than two species, including higher-than-pairwise interactions, but also complex networks of pairwise effects[3–7].

Is species coexistence primarily a reductionist pairwise phenomenon, or is it an emergent property of the whole community[8]? (Fig. 1A). In other words, does coexistence within a complex community require that two microbial species also coexist in isolation? To unambiguously answer this question, one would have to break down a stable multispecies community into its simplest units (species pairs), and determine whether all of those pairs coexist too. If we found that all (or, more generously, most) of them did, this would lend support to the reductionist hypothesis. If, on the other hand, we found that few of those species coexist as a pair when removed from the ecological context provided by the other community members[9], we would have to conclude that multispecies coexistence is an emergent, systems-level property of the community as a whole. An extreme scenario is the maintenance of biodiversity through nontransitive (non-hierarchical) competition, which may allow multiple species to coexist even when none of them do as a pair in isolation[4,5,10–13].

Resolving this question is particularly urgent in microbial ecology, given the enormous and still largely unexplained diversity of microbial ecosystems[14–21]. The answer is likely going to be system-specific. Recent empirical research has provided support to the reductionist paradigm, finding that the composition of synthetic bacterial communities can be predicted from a simple pairwise assembly rule: the species that

coexist together in a complex community are those that coexist with one another as pairs in isolation[22,23]. However, synthetic bacterial communities have also exemplified nontransitive loops (i.e., rock-paper-scissors) where a trio coexists despite all pairwise co-cultures ending up in exclusion[24,25]. Testing these two scenarios in natural communities is generally not feasible, given their diversity: even if we managed to isolate most community members from a natural habitat, the number of replicate natural environments we would need in order to incubate every single pair would scale quadratically with the community richness.

Given the technical, financial, and even ethical difficulty of doing such an experiment in environmental or medical settings, previous studies took an inference-based approach where they estimated the pairwise interaction coefficients by fitting models of population dynamics to the temporal behaviors of the community and/or a subset of sub-communities[26,27]. From these models, one may infer the outcome of pairwise co-cultures. Despite its mathematical elegance, generating predictions from this approach requires a large collection of data and may be prone to artifacts and fail predictions, as the models generally assume that high-order interactions are absent and impose specific functional forms on the density-dependence of pairwise interactions. As an alternative approach, recent studies have attempted to directly determine pairwise coexistence by reconstituting species pairs from natural communities in well-controlled laboratory environments[22,28,29]. A limitation, however, was that only a very small subset of all of the coexisting species in those natural communities was included in the study, typically those which could grow on their own under laboratory conditions. Perhaps most importantly, the pairs were reconstituted in a laboratory environment that is very different from the original environment experienced by the community where those species pairs were coexisting. Thus, while pairwise assembly rules were generally successful at predicting the final composition of small ($N<5$) synthetic consortia in novel environments, they did not permit one to determine whether or not multispecies coexistence in the communities these isolates came from was a pairwise phenomenon.

In past work, we have assembled a collection of hundreds of microbial enrichment communities that stably coexist in well-controlled synthetic environments containing one single supplied limiting nutrient[16,30–33]. These communities were formed in a manner that is very similar to the "random zoo" model in theoretical ecology[34]: we added a large number of species to a new habitat (which none of them had encountered before) without giving them much time to adapt and allowed these species to spontaneously assemble into a new stable community (Fig. 1B). These newly assembled communities reach compositional stability after ~50-80 generations of serial passaging. Once stabilized, they have a modest diversity (N<25, generally[30,31,33,35]), and most of the species can be isolated without much work, including those that cannot grow on their own[30,31]. Because the communities were assembled in liquid, synthetic media under laboratory conditions that can be easily and faithfully recreated in high throughput, it is possible to break each community apart and reconstitute every pairwise species combination in exactly the same habitat where the multispecies community from which they came from was assembled in the first place. This is critically important, as it allows us to evaluate whether a pair coexists in isolation under identical abiotic conditions where they coexist as a part of a multispecies community. All of these advantages overcome the limitations of past laboratory studies, providing a middle ground between natural and purely synthetic communities[32].

Motivated by this realization, we set out to test whether coexistence is a pairwise or an emergent phenomenon in this empirical system. To that end, we empirically characterized the competition outcomes in all 186 within-community pairwise combinations among 68 microbial species from 13 representative multispecies enrichment communities, comprising ~90% of all the Exact Sequence Variants (ESVs) in those communities[31]. As shown below, we find that most species pairs fail to coexist in isolation under the same ecological conditions where they do coexist as members of the same multispecies community. Among those pairs that do coexist, stable rather than neutral coexistence is the norm. Among

non-coexisting pairs, competitive exclusion is strongly hierarchical, a point that is consistent with previous findings[29].

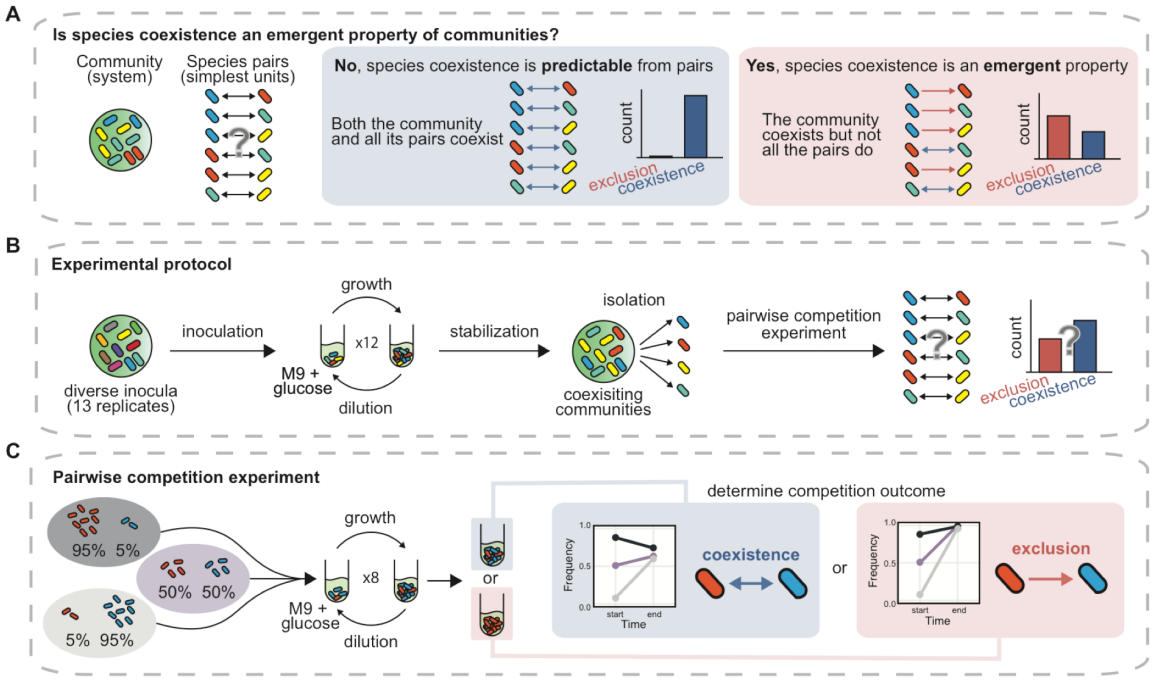

Figure 1. Overview of the question and our experimental design. (**A**) Testing whether species coexistence is an emergent property requires whether the property occurs at the levels of both the system (community) and its elemental units (species pairs). If all species isolated from a community coexist with one another in pairs, the coexistence at the community level is predictable from the knowledge of pairs, therefore can be reducible to the additive condition of all pairs. Whereas when some pairs do not coexist, the species coexistence should be an emergent property of the diverse communities that requires the presence of more than two species. (**B**) 13 representative microbial enrichment communities were chosen from a collection of 96 communities that had been stabilized in a simple medium containing a single limiting carbon source (M9 + glucose)[30]. After a stabilization phase of 12 growth cycles, bacterial strains were isolated from the communities by culture-dependent methods, and their pairwise competition relationships were further determined. **(C)** We show a hypothetical example of two possible outcomes from the pairwise competition experiments. For a pair of strains, the competition outcome was determined by competing them in three varied initial proportions to test whether they are capable of invading each other from rare. The competition outcome was determined by a mutual invasion criterion that has been used in previous studies[22,28]: a pair stably coexists when one strain can invade the other from rare and vice versa; whereas if one strain always excludes another regardless of its initial frequencies, this pair was deemed as a competitive exclusion pair.

RESULTS

Multi-replicated enrichment microbial communities are an ideal model system to test whether multispecies coexistence is a pairwise or emergent property. Over the past few years, we have gathered a collection of hundreds of self-assembled communities in well-controlled synthetic habitats[30]. Starting from natural bacterial communities that were serially passaged every 48 hr in glucose minimal medium (Methods), these enrichment communities adopted stable compositions containing N<25 Exact Sequence Variants (ESVs) after ~50 generations. The simple synthetic medium and incubation conditions make it straightforward to reconstruct the abiotic environment, allowing us to characterize the pairwise competition outcomes under the exact abiotic conditions where the original community had been assembled.

To empirically test whether multispecies coexistence is a pairwise phenomenon in our system, we chose 13 representative communities containing 3-12 isolated strains (Fig. 1B). We were able to isolate 68 bacterial strains whose full-length 16S rDNA matched the majority of the relative abundances obtained from the exact sequencing variants (89.5% of relative abundance) (Fig. S1). Pairwise competition experiments were then carried out by mixing pairs of the isolates' inocula with standardized optical density (OD=0.1 at 620 nm) at three sets of given volumes (the cell proportion then approximately 5:95, 50:50, and 95:5), and passaging them for eight growth-dilution cycles in the same glucose minimal medium at the same 30°C (Fig. 1C). During each growth cycle, the cells were incubated for 48 hr, after which the resulting culture was diluted by 1/125 into fresh media as we did in the original community assembly experiment. At the end of the last dilution cycle, the composition of our pairwise communities was measured by CASEU, a Sanger sequencing-based approach identifying the fraction of amplicon mixture[36] (Methods). The pair is identified as "stable coexistence" if both species could invade each other when rare[22,28]. By contrast, competitive exclusion is deemed to be the outcome when one of the species drops in abundance below the detection limit of our experiments regardless of its initial frequency.

Besides stable coexistence and competitive exclusion, other more rare scenarios (frequency-dependent coexistence, mutual exclusion, and neutrality) comprising <9% of the pairs were also detected (Methods).

Species coexistence is an emergent property in our enrichment microbial communities. To recapitulate the experiment described in Fig. 1C, we reconstituted every possible pair of species from each of the 13 communities, in the exact same environment where the community had been assembled. Thus, all of these pairs consisted of two species that coexist together in the context of a more complex community. After characterizing the outcome of every one of these pairwise co-cultures, we found that competitive exclusion was the most common outcome, and only 34.9% of the species pairs were able to coexist in pairwise co-culture when isolated from the rest of the species in the community. All of the 13 communities contained at least one pair (but generally many more) that did not coexist (Fig. 2A) and the fraction of pairs exhibiting exclusion was remarkably consistent and comparable for all of the 13 enrichment communities, regardless of their richness (Fig. 2B).

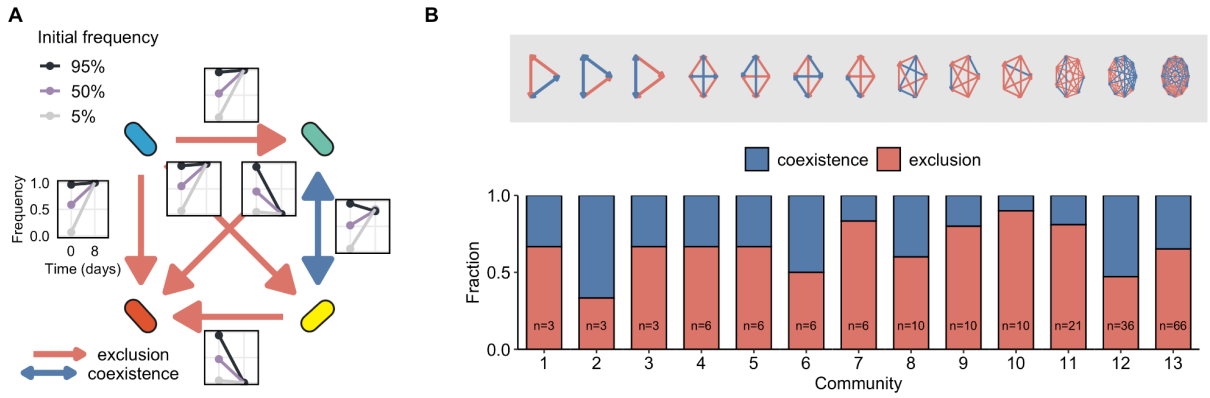

Figure 2. Multispecies coexistence is an emergent property as most species pairs fail to coexist on their own. (**A**) Analysis of the pairwise competition outcomes of a representative community (from community 7) reveals a combination of both pairwise exclusion and pairwise coexistence among the four constituent taxa. Each inset line chart shows the frequency changes between the start (day 0) and the end (day 8) of the competition experiments in a pair. The color intensity in the insets represents the set of initial frequencies used in the pairwise competition: 95% (darkest purple), 50% (medium purple), to 5% (lightest purple). The pairwise competition outcomes of all 13 enrichment communities are shown as the network in (**B**), ordered by the number of strains in each community from the smallest (3 taxa) to the largest (12 taxa). The sample size in each bar shows the number of pairs per network.

Our results indicate that the simple pairwise assembly rule proposed in ref. [22] would have failed when applied to our consortia, as it would have predicted a much less diverse set of communities composed only of those taxa that coexist as an isolated pair. This result suggests that, at least under our experimental conditions, multispecies coexistence is a complex, emergent property of the entire community that cannot be trivially reduced to the outcome of pairwise co-cultures in isolation. This suggests that the outcome of competition experiments between pairs of isolates in the absence of additional community members has a more limited value than previously thought, at least when we seek to predict community composition.

Among the coexisting pairs, the majority were found to coexist stably (Methods), as evidenced by negative frequency-dependent selection: both species were able to invade each other when rare (Fig. 3 and S2). A smaller number of pairs (5.9% of the total) exhibited frequency-dependent coexistence (Fig. 3). Interestingly, neutral coexistence among isolated pairs was very rare, with only one pair of species exhibiting dynamics that did not rule out neutrality (Methods).

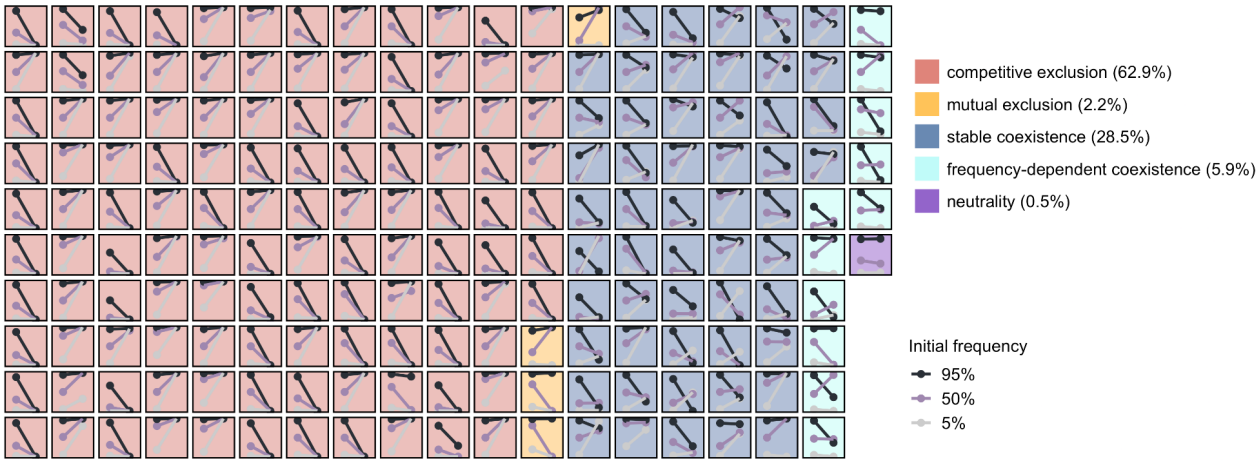

Figure 3. Species pairs from coexisting communities exhibit mutual exclusion, frequency-dependent coexistence, and neutral coexistence. Competition outcomes of a total of 186 pairs across the 13 enrichment communities. Mutual exclusion occurs when the abundance of a species starting rare eventually drops to the detection limit and vice versa (Methods); frequency-dependent coexistence happens when two frequency treatments converge to equilibrium but the third one shows history-dependent exclusion; a pair is deemed as neutrality when the initial frequencies remain over the course of the experiment. Each of the inset grids indicates the dynamic change from day 0 to 8 of one species pair. The background color represents the identified competition outcomes whereas the line color indicates the treatments of three initial frequencies as described above.

Competitive exclusion among species pairs in isolation is transitive and strongly hierarchical. An extreme case of emergent coexistence may occur when coexistence networks are nontransitive. An example is the "rock-paper-scissors" scenario, where species A outcompetes species B, species B outcompetes species C, and species C outcompetes species A. To determine whether this scenario might play a role in stabilizing coexistence in our communities, we ordered all strains in each community by their competitive ranking, which is calculated as how many times an isolate won in a pairwise competition minus how many times it lost (Fig. 4A). Regardless of the community size, we observed a consistent hierarchical structure in all 13 communities when these strains are ordered in their rankings (Fig. 4B). To quantify the hierarchies of our communities, we introduce a simple metric (h) which captures whether the direction of pairwise exclusion (from winner to loser) conforms to the strains' competitive ranking:

$$h = \frac{N_f}{N_f + N_v}.$$

Here, N_f represents the number of exclusion pairs that follow the strains' competitive rankings (e.g. where the higher-rank species excludes the lower-rank competitor), and N_v represents the number of rank-violating pairs (e.g. where the lower-rank species excludes the higher-rank competitor). Note that this measure only considers exclusion pairs. If exclusion were perfectly hierarchical, we would expect to see h=1, as none of the lower-ranked species would exclude a higher-ranked community member. All of the 13 communities in our experiments as well as those in our simulations exhibit a very high degree of hierarchy (h~1) (Fig. 4C). Overall, we see no evidence of nontransitivity in our coexistence/exclusion networks.

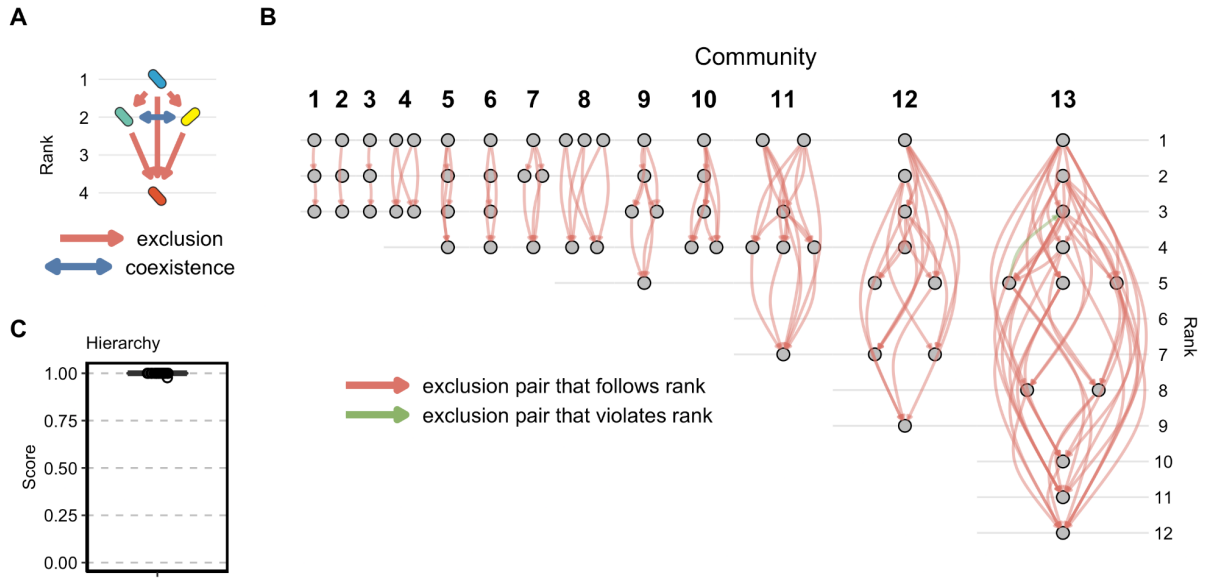

Figure 4. Competitive hierarchy prevails among species pairs in stably coexisting communities. (A) Ordering strains by competitive ranking reveals a competitive hierarchy of a representative community (from community 7). The direction of the exclusion arrow (red) connects a winner toward the loser, whereas the pairwise coexistence links (blue) are mutual. (**B**) The competitive rankings of strains are shown for 13 experimental communities. For the sake of representation, only the exclusion pairs are shown and coexistence pairs are removed from the networks. The gray nodes in the networks denote the microbial strains. (**C**) The hierarchy scores. Each point represents the hierarchy score for an enrichment community.

DISCUSSION

At the outset of this paper, we set out to empirically address a fundamental question in microbial community ecology, using an empirical system that is uniquely suited for that purpose: Is species coexistence primarily a reductionist pairwise phenomenon, or is it an emergent property of the whole community? To address this question, we reconstituted every possible pairwise co-culture out of the (N=3-12) coexisting members of 13 different enrichment communities, in the same environment where the communities had been assembled, and determined whether the pairs coexisted on their own in the absence of the other members of their communities. We found that they do generally not: pairwise competitive exclusion prevails across these communities. This was true in all of our communities, which exhibit highly similar fractions of coexisting pairs regardless of their diversity. These findings indicate

that the community context is essential to explain pairwise coexistence within multispecies communities. Our results also suggest that coexistence cannot be trivially reduced to pairwise coexistence rules, and indicate that pairwise competition experiments where two species are grown in isolation may not be very informative about the ability of those species to coexist, even when done under identical abiotic conditions as those experienced by the community. Our results do not allow us to determine whether the complex nature of multispecies coexistence derives from higher-order interactions, or whether it can be explained by a complex network of pairwise interactions. Additional experiments will have to be done in future work to settle this question.

Several theoretical studies have suggested that nontransitivity stabilizes the coexistence of a large number of competing species when the competitors have differential competitive abilities on multiple limiting resources[4,5], or in the presence of spatial heterogeneity[10]. Despite the well-established theoretical basis of this idea, empirical studies on the prevalence of interspecific nontransitivity are still sparse. Taking the advantage of the mutual invasion experiments of 186 species pairs from each of the 13 replicate communities, we found strongly hierarchical competition among our species, and found no evidence of nontransitivity, suggesting that the role of nontransitive competition in promoting species coexistence may be negligible in our communities. The discrepancy between the theory and our findings may arise from the underlying ecological interactions among competing species. In our communities, nutrient exploitation on the single limiting nutrient and cross-feeding are the dominant ecological interactions in determining the community structure[30,31], whereas laboratory microbial experiments have suggested that nontransitivity may emerge through mechanisms such as changes in species' competitiveness across resources[37] and the balance among toxin-producing, resistant, and sensitive strains[24].

Future work should address to what extent the topology of the resource competition and cross-feeding networks may affect the answer to the question raised above. While this is beyond the scope of this paper,

the empirical system developed here could be extended to environments containing a larger number of resources, as done elsewhere[32,35,38]. This could be a way to manipulate these networks empirically. In sum, we hope that the methodology developed here will help us determine under what conditions one should expect simple pairwise assembly rules to predict the assembly of microbial communities. Our findings highlight the challenges of predicting species coexistence from a bottom-up perspective and emphasize the importance of developing models that take into account the full community context.

METHODS

Isolation of microbial strains. Communities used in this study were assembled from various foliage- and soil-derived microbiomes in a minimal medium with M9 salts and 0.2% glucose as the single supplied carbon source. These communities were allowed to equilibrate for 12 transfers (~84 generations) in batch culture. Detailed assembly processes and community properties were described in our previous work[30]. To isolate the microbial strains for pairwise competition experiments, we streaked out the frozen stocks of 13 representative communities on nutrient agar plates (trypticase soy agar; TSA), picked morphologically distinguishable colonies, and re-streaked them on selective agar plates of M9 salts with glucose or citrate as carbon sources. We then sequenced the 16S ribosomal RNA gene of each isolated strain and compared the sequencing results to the bacterial 16S rRNA database[39] to determine their taxonomic identities. For storing the stocks of the isolated strains, we dissolved the colonies with phosphate buffer saline (PBS) and mixed them with an equal volume of 80% glycerol, and saved the stocks at -80℃.

Pairwise competition experiments. We streaked out the frozen stocks of 68 bacterial strains on the nutrient TSA agar plates and grew them for 48 hours at 30℃. The agar plates were then saved at 4℃ for up to two weeks before being used in the competition experiments. On the first day of the experiment, we prepared the strain inocula by suspending the colonies of each strain in PBS, which were standardized to

an optical density (OD) of 0.1 at 620 nm. To initiate the competition with three initial mixing proportions, we mixed the inocula by volume to the desired ratios of 5%:95%, 50%:50%, and 95%:5% in a master 96-well 200 μL nunc plate. We initiated the pairwise competition experiments by inoculating 4 μL of the inoculum mixtures to 500 μL of M9 glucose liquid medium that was contained in 2 mL 96-well deep-well plates sealed with porous film. For each growth cycle, we incubated the cultures at 30℃ without shaking for 48 hours and diluted the mature cultures by a factor of 1/125 into a fresh M9 glucose medium to start the next cycle. We carried out the competition experiments for eight growth cycles and made an experimental duplicate for each of the three mixing proportions. The duplicate was included in the experimental design as a sanity check and only one replicate was eventually sequenced. At the end of the competition experiment, we mixed 300 μL of the mature media with an equal volume of 80% glycerol and saved the mixture stocks at -80 ℃ for later sequencing.

Sanger sequencing preparation. We determined the final proportion of pairs in the competition experiments using an approach based on Sanger sequencing called CASEU[36], which takes the amplicons mixture of synthetic communities and amplicons of its known members as inputs. We followed the experimental protocol suggested in ref. [36] on preparing the sequencing samples. Briefly, we centrifuged and pelleted 400 μL of the frozen stocks of both the pairwise cultures and the monocultures. The DNA pellets were then extracted using DNeasy Blood and Tissue 96-well Kit (QIAGEN 69504). We then PCR-amplified 10 μL of the extracted DNA with the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) in duplicate PCR, following the incubation condition listed in ref. [36]. After obtaining the PCR products, we mixed the PCR duplicate outcomes before cleaning and standardizing them into a concentration of 1.25 ng/μL using SequalPrep Normalization 96-well Plate Kit (ThermoFisher A1051001). To meet the sample concentrations required for Sanger sequencing by Genewiz, we added 5 μL of 27F primer at 5 μM to 10

μL of template DNA of concentration 1.25 ng/μL. The resulting ABIF files from Sanger sequencing were analyzed using the R package associated with the CASEU protocol[36].

Bootstrapping the frequency changes. Once the final relative frequencies in the competition experiments were measured, we implemented bootstrapping to determine whether the change in the relative frequencies was statistically significant. The statistical significance of frequency changes from initial inocula to final culture was produced by bootstrapping the frequencies based on a presumed normal distribution that has a mean and variance from empirical measurements. The uncertainty of frequencies was obtained from the fitting errors reported in the CASEU outputs. In each bootstrap, the value of initial frequency was compared to a random value drawn from the normal distribution, where the mean was the measured frequency in the final culture and the standard deviation was the estimated uncertainty. We repeated the bootstrapping 1000 times. The changes were considered statistically significant if more than 950 of the 1000 permutations (p=0.05) have the same sign of change. For example, if a strain starting at an equal proportion with its competitors consistently increases in frequency for more than 95% of the 1000 bootstraps, the result for this frequency treatment was scored as "increase". Whereas if neither the results of increase nor decrease exceeds 95% of the bootstraps, this frequency treatment was statistically insignificant. We conducted the same bootstrapping approach for all three frequency treatments and obtained a combination of "increase", "decrease", and "non-significance" for each species pair.

Determining the outcomes of pairwise competition. We followed a mutual invasion criterion to determine whether the pairwise competition outcomes are stable coexistence[2,22,28]. Each of the three frequency treatments (5%, 50%, or 95%) has three possible signs of change (increase, decrease, or non-significance), giving rise to 27 possible combinations of frequency changes, where only 22 of the 27 possibilities were realized (Table S1). The two main outcomes are competitive exclusion and stable coexistence. A pair was considered as competitive exclusion when the frequency of one species always

decreased regardless of its initial frequencies, and we determined the competitive dominance by connecting the competitively superior to the inferior species. Stable coexistence occurs whenever one species always increases in frequency from rare and vice versa, converging to a stable equilibrium. This included the cases where two frequency treatments converged to an equilibrium and the third one showed history-dependent exclusion (abundant strain excluding rare strain) (11/186=5.9%). Besides competitive exclusion and stable coexistence, we observed rare cases of mutual exclusion (4/186 = 2.2%) and neutral coexistence (1/186 = 0.5%). Mutual exclusion occurred whenever a strain starting from abundant always excluded its rare competitor and vice versa. Neutrality occurred when all the frequency treatments of a species pair remained constant over pairwise experiments. For simplicity in the analysis, these rare cases were classified into exclusion and coexistence: the winner of mutual exclusion was determined by which species won twice among the three frequency treatments; neutral coexistence was classified into the coexistence pairs.

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