Interaction variability shapes succession of synthetic microbial ecosystems

Feng Liu¹,²,³,⁴,₁², Junwen Mao¹,²,⁵,₁², Wentao Kong¹,², Qiang Hua³,⁴, Youjun Feng⁶, Rashid Bashir¹,⁷,⁸ & Ting Lu¹,²,⁹,₁⁰,₁¹∗

Cellular interactions are a major driver for the assembly and functioning of microbial communities. Their strengths are shown to be highly variable in nature; however, it is unclear how such variations regulate community behaviors. Here we construct synthetic Lactococcus lactis consortia and mathematical models to elucidate the role of interaction variability in ecosystem succession and to further determine if casting variability into modeling empowers bottom-up predictions. For a consortium of bacteriocin-mediated cooperation and competition, we find increasing the variations of cooperation, from either altered labor partition or random sampling, drives the community into distinct structures. When the cooperation and competition are additionally modulated by pH, ecosystem succession becomes jointly controlled by the variations of both interactions and yields more diversified dynamics. Mathematical models incorporating variability successfully capture all of these experimental observations. Our study demonstrates interaction variability as a key regulator of community dynamics, providing insights into bottom-up predictions of microbial ecosystems.
Microbial communities are assemblages of multi-species microorganisms that live and interact with each other. They regulate the biogeochemical cycling of the planet, fertilization of crops, and metabolism of our human body, thereby affecting profoundly the environment, agriculture and human health. As the dynamics of a community often underlies its emergent properties, being able to predict ecosystem succession is central to the elucidation of community organization and function, rational design of artificial ecosystems and introduction of intervention. To that end, rapidly developed is the toxicity of organic acids in the culture of Escherichia coli, the antagonism from Pseudomonas aeruginosa, nutrient and stress, microbial interactions are highly variable, rather than static, in nature. Indeed, microbial interactions often change with environmental cues such as pH, nutrient and stress. The mutualism between Escherichia coli and Rhodopseudomonas palustris is moderated by the toxicity of organic acids in the culture; the antagonism from Pseudomonas aeruginosa to Staphylococcus aureus increases with ion depletion. Microbial interactions are also subjective to the population of microorganisms generating the interactions as well as the presence of other species. For instance, Lactococcus lactis produces nisin to suppress pathogens such as Staphylococcus aureus through quorum sensing of its own population while Enterococcus faecalis secrets cytolysin when sensing the presence of target cells. Additionally, as cellular interactions are typically fulfilled through the production of metabolites and proteins—biochemical processes that are fundamentally stochastic, there are intrinsic fluctuations for all microbial interactions including those ‘constant’. Recognizing this characteristic of microbial interactions, a handful of mathematical frameworks have been proposed to consider interaction variations. In contrast, there is a lack of systematic experimental investigations that quantify the degree of variability for given microbial interactions. It also remains unclear to what extent such variations drive ecosystem succession and alter community structures and characteristics. Accordingly, it is unknown how the incorporation of variability into modeling shapes the predictive power of bottom-up mathematical modeling.

Here we hypothesize that variations of microbial interactions are a key modulator of community behaviors and characterizing and incorporating the variability empowers predictive understanding of ecosystem succession from the bottom up. To test the hypotheses, we design and build a set of synthetic three-strain microbial consortia, which involve both cooperation and competition, and use them as our experimental model systems. Compared to native ecologies, such synthetic communities possess a significantly reduced degree of complexity while offering the feasibility for mechanistic dissection and quantitative measurement. In parallel, we develop mathematical models with an explicit incorporation of interaction variability to analyze ecosystem succession. For the consortium containing a variable cooperation, we quantify the variability of cooperation, elucidate the alteration of ecosystem dynamics arising from the variations, and demonstrate the power of variability-incorporated modeling in capturing community development. For the ecosystem whose cooperation and competition both fluctuate, more complex ecosystem dynamics arises but characterizing the variabilities again lead to successful succession predictions. Together, our results elucidate the role of interaction variability in regulating community dynamics, providing fundamental insights into bottom-up understanding of microbial ecosystem succession.

Results
Creation of a cooperation between synthetic populations. We start by engineering a cooperation in synthetic populations because it is ubiquitous among microorganisms and critical to their organization. Specifically, the interaction involves two engineered Lactococcus lactis strains, Ca and Cb, both of which harness the biosynthetic pathway of LcnG, a Class II two-subunit lactococcus bacteriocin (Fig. 1a and Methods). Here, Ca constitutively expresses lagA, lagD, and lagE which encode the peptide α precursor, ABC transporter and accessory protein of the pathway respectively, allowing the strain to synthesize and secrete the α subunit of LcnG. Similarly, Cb constitutively expresses the pathway’s β precursor gene lagB, transporter gene lagD and accessory protein gene lagE, enabling the synthesis and secretion of the β peptide, the other subunit of LcnG. In the extracellular milieu, the two subunits α and β self-assemble into a bioactive antimicrobial which inhibits the growth of L. lactis strains. Through this fashion of division of labor, Ca and Cb achieve a cooperation for successful LcnG production. As LcnG inhibits all L. lactis strains, the immunity gene lagC was introduced into both Ca and Cb to confer them an immunity. Additionally, two reporter genes, yemGFP and mCherry, were loaded into Ca and Cb respectively to enable the quantification of ecosystem dynamics.

To validate the cooperation, we conducted inhibition zone assays using the supernatants of Ca and Cb monocultures (Methods). Four supernatant combinations, including blank culture (GM17 media supplemented with chloramphenicol, −/−), supernatant of Ca monoculture (+/−), supernatant of Cb monoculture (−/+), and mix of the two supernatants (+/+), were loaded into single wells in the solid agar plated with a lawn of LcnG-sensitive cells (L. lactis NZ9000 loaded with a chloramphenicol resistant gene). Upon 8 hours (h) of incubation at 30°C (Methods), only the well loaded with the supernatant mix (+/+ ) produced an inhibition zone (Fig. 1b). In addition, the four supernatant combinations were loaded into separate wells in agar plates covered with LcnG sensitive cells. We found that a clear inhibition zone formed between adjacent wells only when they were loaded with the Ca and Cb supernatants respectively (Fig. 1c). Together, the results confirmed two pieces of information. First, combination of Ca and Cb produced the active LcnG but individual strains alone did not. Second, peptides α and β were able to autonomously assemble into an active bacteriocin without the need for any assistance.

Characterizing the variations of cooperation. Driven by diverse biotic and abiotic factors, microbial interactions are highly variable. Here we aimed to experimentally determine the variability of the cooperation originating from its division of labor nature. Specifically, to quantitate how labor partition, reflected by the cooperator ratio, affects the strength of cooperation, we grew the
monocultures of Cα0 and Cβ0, (the reporter-free version of Cα and Cβ), mixed their supernatants with varied ratios while fixing the total volume (30 μL), and further used the mixes to perform inhibition zone experiments (Methods). Our results (Fig. 2a, top row) showed that, across the ratios from 30:1 to 1:30, the size of the inhibition zones varies from small to large and back to small with the largest occurring at the 1:1 ratio. As zone size correlates positively to a mix’s lcnG level and hence the strength of cooperation, the results suggested that initial labor partition can induce significant variations of the cooperation. Using the supernatants of Cα0, Cβ0 co-cultures with varied initial relative population abundance, we also observed the same dependence of the lcnG level on the initial population partition (Fig. 2a, bottom row). To quantitatively determine the variations, we further measured the relative lcnG level of each sample by normalizing its lcnG level to the largest occurring at the 1:1 ratio (Supplementary Fig. 2a, c) because, in monocultures, Cα and Cβ had the same amount of nutrient and, thereby, produced a comparable level of subunits. To further confirm that the maximal cooperation is characterized by the stoichiometric ratio of the lcnG subunits, we derived new strains with altered peptide productivities, including Cα0v1/3, Cβ0v1/3, Cα0v1/5, and Cβ0v1/5 whose β productivities are reduced to 1/3 and 1/5 of that of Cβ0 (Supplementary Fig. 4). In theory, the combinations of Cα0 and Cβ0v1/3, Cα0 and Cβ0v1/5, Cα0v1/3 and Cβ0v1/5, and Cα0v2 and Cβ0v1/5 would shift the optimal ratios from 1:1 to 1:3, 1:5, 1:6, and 1:10 respectively, which were subsequently confirmed by the experiments (Fig. 2c).

For any microbial ecosystem, there are intrinsic random fluctuations of cellular populations arising from various stochastic processes, which motivated us to quantify the variability of cooperation from intrinsic stochasticity by specifically examining the effects of sampling of initial populations (i.e., genetic drift). We first inoculated the consortium from an initial culture (1:1 Cα-to-Cβ ratio, 1.0 optical density at 600 nm (OD)) into fresh media through serial 1:10 dilution to generate samples with different initial ODs (10−2, 10−4, 10−6 and 10−8) (Fig. 2d and Methods). Here, 10−8 was selected as the minimal initial OD since it is the minimal density for both Cα and Cβ to stably grow in monocultures (Supplementary Fig. 5). Next, we grew the cultures for the defined incubation time, collected their supernatants, and measured the lcnG levels (Methods). Figure 2e...
shows the lcnG levels of the co-cultures normalized by the productivity of Caβ, a single-strain lcnG producer we created. We found that, with the reduction of initial OD, the mean lcnG productivity of the consortium decreased monotonically but, in the meanwhile, the sample-to-sample variation increased. Such a trend was also clearly observed in the inhibition zones formed by the co-cultures (Fig. 2f and Supplementary Fig. 6). By conducting the same experiments for the reporter-free version of the consortium, the Caα-Cβ ecosystem (Supplementary Fig. 7), we confirmed that random sampling continued to serve as a key inducing factor of the variation.

Notably, partition alteration and random sampling are two independent sources of cooperation variations; however, they are intrinsically connected. The both alter cooperation strength by varying the division of labor among the cooperators, but the former is a controlled, deterministic alteration of the partition while the latter is unintended, stochastic alteration.

Ecosystem successions driven by cooperation variations. To elucidate the consequences of interaction variations on community behaviors, we designed a three-strain consortium composed of the two cooperators (Ca and Cβ) and a competitive third strain (Ks). Here, Ks is an engineered strain capable of constitutively secreting lacticoccin A (lcnA)62, a bacteriocin that effectively kills all L. lactis strains including Ca and Cβ unless immunized. Experimentally, Ks was built by introducing the lcnA pathway into the L. lactis MG1363 (Fig. 3a). To efficiently count Ks in the three-strain ecosystem, it was inserted with constitutively expressed gusA3, a beta-glucuronidase gene that enables colorimetric quantification upon the supplementation of X-Gluc63. As Ks does not contain the lcnG immunity gene lagC, it is sensitive to lcnG cooperatively produced by Ca and Cβ. Therefore, the community involves a cooperation between Ca and Cβ and a competition of Ks with Ca and Cβ (Fig. 3b).

Meanwhile, we hypothesized that predicting ecosystem behaviors requires mathematical models that take in account the variability of interaction. To test the hypothesis, we devised a dynamic ecosystem model using a systematic, bottom-up fashion (Methods, Supplementary Information 1). Briefly, we first constructed models of Ca and Cβ monocultures (Supplementary Equations 1-2, Supplementary Fig. 3b), and used them as modules to derive a model of Ca-Cβ co-culture incorporating variability (Supplementary Equation 4) with their parameters specified with experiments (Supplementary Fig. 8). We then constructed a model of lcnA-producing Ks monoculture (Supplementary Equation 5) and determined its parameters experimentally (Supplementary Fig. 9a, b). Finally, by merging the modules of the Ca-Cβ co-culture model with the Ks monoculture and characterizing the inhibitions between the modules (Supplementary Equations 6-7, Supplementary Fig. 9c-f), we obtained a model for the Ca-Cβ-Ks consortium (Supplementary Equation 9). For batch fermentations starting from a fixed (Ca + Cβ):Ks ratio (2:1), the model predicted that the ecosystem evolves temporally into Ks dominance at imbalanced cooperator partitions and Ks subordinate at close partitions (Fig. 3c), suggesting that the variations of cooperation can modulate the succession of the consortium dramatically.

To test the predictions, we experimentally assembled the consortium by mixing Ca, Cβ and Ks with altered initial ratios but a fixed total OD (10^{-2}) as our model construction (Methods). Consistent with the predictions, our fermentations showed that
Fig. 3 Succession of a three-strain ecosystem driven by cooperation variations from labor partition. a Design of a competitive third strain Ks. The lcnA biosynthesis pathway, including the genes lcnA, lceA, lciA, and lcnA, is placed under constitutive promoters for constant lcnA secretion. The gene gusA3 is also introduced for colorimetric quantification. b A three-strain consortium composed of Ca, Cβ, and Ks. Ca and Cβ cooperate to produce lcnG that inhibits Ks; in turn, Ks secretes lcnA to oppose Ca and Cβ. c, d Model-predicted (c) and experimentally measured (d) temporal dynamics of the Ks abundance in the Ca-Cβ-Ks ecosystem. The initial Ca:Cβ partition was varied across 30:1 to 1:30, but the total (Ca + Cβ): Ks ratio was fixed as 2:1. e Design of a control strain Kr. L. lactis MG1363 is loaded with the constitutively expressed lagC, the lcnG immunity gene, to confer resistance to lcnG and gusA3 for colorimetric quantification. f A three-strain ecosystem composed of Ca, Cβ and Kr. As Kr is deficient in lcnA production but resistant to lcnG, the consortium does not have active bacteriocin-mediated interactions. g, h Model predictions (g) and experimental measures (h) of temporal dynamics of the Kr abundance in the Ca-Cβ-Kr ecosystem. The initial Ca:Cβ partition was varied across 30:1 to 1:30, but the total (Ca + Cβ): Kr ratio was fixed as 2:1. Source Data available in the source data file.

The Ks percentage indeed increased over time and eventually became dominant when the Ca:Cβ ratio was extremely imbalanced (30:1 and 1:30); in contrast, when their abundances were close (3:1, 1:1, and 1:3), the Ks percentage declined monotonically and diminished eventually (Fig. 3d). Opposite with the Ks percentage, the relative abundances of Ca and Cβ both decreased at imbalanced Ca:Cβ ratios (30:1 and 1:30) but increased when the ratio is close (3:1, 1:1, and 1:3) (Supplementary Fig. 10a, b). Meanwhile, although the total OD of the ecosystem remained largely consistent regardless of the Ca:Cβ ratios (Supplementary Fig. 8, circles), the lcnG level changed significantly (Supplementary Fig. 8, bars): it remained limited throughout the fermentations in imbalanced scenarios but accumulated rapidly at the balanced cases, consistent with our previous characterization (Fig. 2a–c). The correspondence between higher lcnG production (Supplementary Fig. 8) and lower Ks abundance (Fig. 3c, d) suggested a strong correlation between cooperation variations and diversified ecosystem succession.

To further confirm that it is a causal relationship between interaction variations and diversified succession, we designed a Ks variant, named Kr, which is resistant to lcnG co-produced by Ca and Cβ and deficient in producing lcnA. Experimentally, Kr was established by removing the lcnA biosynthetic pathway from Ks while introducing the lcnG immunity gene lagC (Fig. 3e, Supplementary Fig. 11). The mixture of Ca, Cβ and Kr formed a control consortium where the cooperation and competition are both abolished (Fig. 3f). Our mathematical model (Methods, Supplementary Equation 11) predicted that, upon the same alterations of the initial Ca:Cβ ratio, the structure of the ecosystem remained largely consistent regardless of the Cα-Cβ ratio.
In parallel, we experimentally mixed the strains C\textit{m}eters (Supplementary Tables 7 and 8) and combining the lacking Ks remains the original culture color (yellow). Each test tube contains only the supernatants of the co-culture after X-Gluc treatment. Source Data the experiment in at 1:1:1 ratio but different total ODs; for each initial OD, 10 experimental replicates were performed.

To examine if the modulation of ecosystem succession by interaction variability is specific to the origin of variation, we investigated the dynamics of the Ca-Cβ-Ks consortium upon fluctuations in sampling, another source of variation we characterized. Mathematically, we used a bottom-up strategy to create a corresponding dynamic model by introducing sampling-induced variations into the previous Ca-Cβ co-culture module (Supplementary Equation 4) and Ks monoculture module (Supplementary Equation 5) with experimentally derived parameters (Supplementary Tables 7 and 8) and combining the resulting modules into a single model (Methods). Using the model that encapsulates variations from sampling, we conducted multiple repeats of computational batch fermentations for the consortium starting with the 1:1:1 ratio but different initial ODs. In parallel, we experimentally mixed the strains Ca, Cβ and Ks in 1:1:1 ratio to form the co-culture, inoculated them into ten replicates at specific initial total ODs (10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}) and measured their population dynamics over time (Methods). Notably, due to the difference in initial conditions, the culturing time and sampling time were altered accordingly to enable a consistent and proper comparison (Supplementary Fig. 12).

Our model predictions and subsequent experiments showed that the Ks fraction consistently declined over time at high initial ODs (e.g., 10^{-2} and 10^{-4}) across all replicates (Fig. 4a–f), suggesting that Ca and Cβ robustly outperformed Ks. Conversely, when the initial OD was low (10^{-6} and 10^{-8}), the consortium exhibited two divergent modes of succession (Fig. 4c–d, g–h): the Ks fraction declined monotonically as in the high initial OD cases; alternatively, it increased over time and dominated the population. Furthermore, comparison of all four cases suggested that the chance of Ks dominance increased with reducing initial OD. To directly visualize such succession outcomes, we further collected the co-cultures at the end of fermentations and performed colorimetric assays by adding X-Gluc to the co-cultures (Methods). Because Ks encodes beta-glucuronidase which can produce a clear blue green color, the colors of the treated supernatants (Fig. 4i) reflected the Ks dominance in individual experiments. Linking to the characterization of sampling-induced variations (Fig. 4e, Supplementary Tables 7, 8), these results confirmed that increasing sampling-induced variations equally drives the consortium into divergent outcomes. Supporting the statement, we computationally turned off the sampling-induced variations in our mathematical model and found that, without the variations, Ks persistently declined regardless of initial ODs (Fig. 4a–d, bold lines). The theory-experiment consistency suggested that incorporating variability into ecosystem modeling provides a predictive capacity over community behaviors.

**Construction of pH-dependent competition and cooperation.** To examine if variability-modulated ecosystem succession is general to different cellular interactions, we designed a new cooperation-competition consortium composed of the cooperators—Ca and Cβ—and Kp, a strain that opposes Ca and Cβ and resists their killing in a pH-dependent manner. Using \textit{L. lactis} MG1363 as the host, the pH-dependent Kp-to-Ca/Cβ inhibition was created by applying a pH-inducible promoter P_{774} to control \textit{lcnG}, the precursor gene of the \textit{lcnA} pathway and the constitutive promoters P_{1} and P_{2} to drive the rest genes (\textit{lceA}, \textit{lcmA}, and \textit{kicA}) in the pathway (Fig. 5a). Similarly, the pH-dependent resistance to Ca/Cβ-to-Kp inhibition was enabled by using the promoter P_{774} to drive \textit{lagC}, the immunity gene of \textit{lcnG} co-produced by Ca and Cβ. Additionally, \textit{gusA} was constitutively expressed to enable colorimetric quantification of the strain.

Previous studies showed that the promoter P_{774} is active when the environmental pH is below 6.5 but switched to be inactive when above 7.6. Thus, Kp’s \textit{lcnA} production (i.e., inhibition over Ca and Cβ) and \textit{lagC} production (i.e., resistance to killing by Ca and Cβ) are no longer constant but, instead, vary with the environment. Importantly, these strains are all derived from \textit{L.\textit{lactis}}.
lactis which naturally produces a large amount of lactic acid and, thus, can lower the pH of culture in fermentation, which suggests that both the Kp-to-Cα/Cβ and Cα/Cβ-to-Kp inhibitions can be highly dynamic even in simple batch fermentation.

To validate the interactions, we grew Kp monoculture under three settings: pH ≥ 7, pH ≤ 6, and no pH control (Methods) (Fig. 5b and Supplementary Fig. 13). Our results (Fig. 5c and Supplementary Fig. 14) showed that the size of the lcnA inhibition zones (Methods) remains undetectable during the pH ≥ 7 fermentation, suggesting no lcnA production. By contrast, in the pH ≤ 6 fermentation, lcnA was detected as early as 3 h after fermentation and the culture yielded the highest lcnA level. For the case of no pH control, lcnA was detected after 4 h of fermentation and the culture yielded the highest lcnA level. These results confirmed that lcnA production (Kp-to-Cα/Cβ inhibition) is highly correlated with the environmental pH. To confirm the pH-dependence of the Ca/Cβ-to-Kp inhibition, we cultured Kp and Ks in pH-defined media mixed with the supernatant of Ca-Cβ co-culture normalized by those growing in the media mixed with the Ca-Cβ’ supernatant in three pH-defined conditions. Source Data available in the source data file.

Dynamics jointly regulated by multiple interaction variations. To illustrate how the Ca-Cβ-Kp consortium evolves upon both cooperation and competition variations, we assembled a dynamic community model (Supplementary Equation 17) from the bottom up (Methods). Then we used the model to explore the succession of the consortium when its interaction strengths vary due to simultaneous pH and cooperator partition alterations. In parallel, we performed Ca-Cβ-Kp co-culture experiments under the conditions identical to the computational test (Methods).

For the consortium starting from a fixed initial (Ca + Cβ):Kp ratio (2:1), the model predicted and subsequent experiments confirmed that, when pH was controlled above 7, Kp abundance declined gradually over fermentation for different initial Ca-Cβ ratios but reached to the lowest at 1:1 (Fig. 6d, g). In contrast, when pH was below 6, Kp became increasingly dominant over time for all Ca-Cβ ratios but augmented the most at unbalanced cases (30:1 and 1:30) (Fig. 6c, h). When there was no pH control, the consortium succession displayed two distinct patterns: At unbalanced ratios, Kp evolved to be dominant as the case of pH ≤ 6 but, at close ratios, Kp abundance declined over time as the pH ≥ 7 case (Fig. 6f, i).

Although seemingly diversified, these successions can be elucidated by considering the multiple interaction variations caused by pH modulation and labor partition. First, the systematic shift of the Kp abundance from consistent decrease (Fig. 6d, g) to consistent increase (Fig. 6e, h) and divergent
development (Fig. 6f, i) originated from the pH-induced variation of the interactions: At pH ≥ 7, Kp-to-Ca/Cβ inhibition was abolished but Ca/Cβ-to-Kp inhibition remained potent; at pH ≤ 6, Kp-to-Ca/Cβ inhibition became effective (Supplementary Fig. 18) but Ca/Cβ-to-Kp inhibition was significantly reduced; when there was no pH control, Ca and Cβ inhibited Kp at beginning but were later suppressed by Kp (Fig. 6a–c). Second, within a single pH setting, the final Kp abundance was lower at close Ca:Cβ partitions (e.g. 1:1) than at imbalanced (e.g. 30:1 and 1:30) because Ca and Cβ had a stronger lcnG productivity when their partitions are close (Supplementary Fig. 17). These results showed that for ecosystems containing multiple variable interactions, at least for those we tested, their succession is determined jointly by all of the variations but not by any one of them. Of note, the divergent dynamics in the absence of pH control (Fig. 6f, i) exemplified the superposition of pH and labor partition effects: At imbalanced Ca:Cβ partitions, Ca and Cβ had the potential to kill Kp at beginning but their lcnG yield was too low; later, Kp gained the lcnG resistance and further secreted lcnA to kill Ca and Cβ, leading to the monotonous increase of Kp abundance. In contrast, at the close partitions, Ca and Cβ produced significant lcnG to efficiently inhibit Kp during the initial fermentation and the lcnG remained in the culture continued to suppress Kp even though the interaction topology was later altered.

To further demonstrate this finding, we conducted additional assays for the consortium by varying pH and initial total OD. Here, the model and the experimental setups were the same as previous except for the initial conditions (Methods). Accordingly, the sources of variation became pH modulation and random sampling. Our results showed that, at pH ≥ 7, the Kp abundance consistently reduced at high initial ODs (Fig. 7a–f), owing to the cooperative inhibition of Ca and Cβ to Kp; However, at low initial ODs, it could also remain largely invariant in some replicates (Fig. 7c–d, g–h) since increasing variations at random sampling abolished the cooperation (Fig. 2e–f). By contrast, in the absence of sampling-induced variations, the Kp abundance always declined regardless of initial ODs (Fig. 7a–d, bold lines). At pH ≤ 6, Kp became increasingly dominant regardless of initial ODs (Fig. 7i–l, m–p), because Kp constitutively suppressed Ca and Cβ and such a suppression was not affected by the fluctuations of Ca/Cβ ratios. These results are consistent with the ecosystem succession when sampling-induced variations were eliminated (Fig. 7i–l, bold lines). When there was no pH control, Kp declined minorly to a plateau at high initial ODs (Fig. 7q–r, u–v), attributed to the factors that Ca and Cβ collaborated to suppress Kp initially but were later suppressed by Kp due to pH reduction. At low initial ODs, it declined as the high initial OD case or diverged to be dominant (Fig. 7s–t, w–x) because increasing randomness diminished the Ca/Cβ-to-Kp inhibition at
**Fig. 7** Model-predicted and experimentally measured succession of the Cα-Cβ-Kp ecosystem for varied pH conditions and initial densities. a–h Predicted (a–d) and experimentally measured (e–h) time courses of Kp abundance in the Cα-Cβ-Kp consortium when pH ≥ 7. i–p Predicted (i–l) and measured (m–p) time courses of Kp abundance in the Cα-Cβ-Kp consortium when pH ≤ 6. q–x Predicted (q–t) and measured (u–x) time courses of Kp abundance in the Cα-Cβ-Kp consortium when there is no pH control. For both model predictions and experimental measurements, the co-culture was inoculated at 1:1:1 ratio but the initial OD was varied from 10⁻² (first column) to 10⁻⁴ (second column), 10⁻⁶ (third column) and 10⁻⁸ (fourth column). For each condition, a total of 100 simulation replicates and 10 experimental replicates were performed. For comparison, the deterministic dynamics of Kp without sampling-induced variability were displayed with bold lines in a–d, i–l, and q–t. Source Data available in the source data file.
the beginning but did not affect Kp-to-Ca/Cβ suppression later. For comparison, in the absence of Kp, there was no Ca dominance under all initial conditions (Fig. 7q–t, bold lines). These results demonstrated again that it is the joint regulation from multiple interaction variations that determines the dynamics of the ecosystems.

To quantitatively evaluate the capacity of the variability-incorporated modeling scheme, we calculated relative errors, defined as the differences between simulation and experimental results divided by experimental measures, for all of the simulations and experimental data above. The results (Supplementary Fig. 19) showed that, for most of the comparisons, the simulations agree quantitatively with experimental measures with the mean absolute relative errors (MARE) falling within the range of (0, 0.2) (panels a–d, g). For a subset of the cases, the models have larger relative errors but yet qualitatively agree well with the experimental findings (panels e, f, h). The encouraging agreements between the simulations and experiments demonstrated that incorporating interaction variability into ecosystem modeling is a promising strategy for quantitative and predictive understanding of complex community behaviors. Meanwhile, the discrepancies in certain cases suggest that the current models may need to consider additional processes involved in the experimental ecosystems, such as the nonlinearity observed during parameter fitting in Supplementary Fig. 8, in order to achieve a better modeling-experiment agreement.

Discussion

Microbial interactions are often modeled invariant, however, in nature, they constantly fluctuate over time and such fluctuations in strength are shown to be profound to ecosystem behaviors. Using synthetic microbial consortia as simple and reliable platforms, we showed that variations of interaction diversifies an ecosystem’s succession into distinct outcomes. We also showed that, when there are multiple variable interactions, these variations collectively, but not a single one, regulate the behaviors of a community. Together, our results established interaction variability as a critical modulator of ecosystem behaviors.

Our synthetic ecosystems are relatively simple as they contain multiple microbial and environmental factors that modulate cellular interactions. We thus speculate that interaction variability is not specific to our synthetic systems and potentially a universal determinant for microbial ecosystem succession.

Searching for assembly rules has been invaluable to our understanding of community organization. Using microbial consortia as simple and reliable platforms, we showed that increasing variations of interaction diversifies a community; instead, the discrepancies in certain cases suggest that the current models may need to consider additional processes involved in the experimental ecosystems, such as the nonlinearity observed during parameter fitting in Supplementary Fig. 8, in order to achieve a better modeling-experiment agreement.

Engineering of microbial consortia for various biotechnological applications.

Methods

Strain and growth conditions. All strains are derived from L. lactis MG1363 and grown at 30 °C in M17 broth supplemented with 0.5% (w/v) glucose and 5 μM β-lactam (GM17/Cm). Tween 80 was added at a final concentration of 0.1% (w/v) when necessary. Cells cultures are adjusted to 2 M NaOH solution every hour to maintain pH above 7. To achieve pH 6, cell cultures are adjusted by 1 M HCl every hour. Strains used in this study are described in Supplementary Table 1.

Plasmid construction. All plasmids used in this study were developed from a L. lactis-E. coli shuttle vector, pleiss-Nuc68, and described in Supplementary Table 1. Oligos for plasmid construction are listed in Supplementary Table 2. To generate the plasmid pleiss-lcnG for lactococcin G production, a 5-kb fragment of lcnG gene cluster including lagA, lagB, lagC, lagD, and lagE was amplified from the genome of L. lactis LGM 2081 using primers of lcnG-F and lcnG-R69, and subsequently assembled with a fragment of pleiss-Nuc amplified with primers Pβ-F and Pβ-R using Gibson assembly. The plasmid pleiss-lcnC was then transformed into L. lactis MG1363 to obtain the lcnG producing strain Ca. To construct lcnC subunit expression plasmids pleiss-Cα and pleiss-Cβ, the α and β coding gene was deleted from the plasmid pleiss-lcnG by reverse PCR and Gibson assembly using two pairs of primers: Caα-F/Caβ-R and Cβα-F/Cββ-R. The resulting plasmids were transformed into L. lactis MG1363 to obtain Ca and Cβ. To enable screening and counting of cells with different subunits, a gfp or rfp reporter gene, yem65GFP or mCherry was introduced using primers Pα-F/Pβ-R and Cα-F/Cβ-R, generating the plasmids pleiss-Cα and pleiss-Cβ. These plasmids were subsequently transformed into L. lactis MG1363 to construct the reporter version of α and β peptide producer Ca and Cβ. As a control, plasmid pleiss-Cβ was generated by deleting the β precursor gene lagC from the plasmid pleiss-Cβ with primers Pβ-F, Pβ-R; Cβ-F and Cβ-R. The resulting plasmid was then transformed into L. lactis MG1363 to get a β-free variant Cβ. To increase the productivity of a peptide in Cαα, an additional copy of expression cassette of a under the control of Pα promoter was inserted to pleiss-Cαα using primers Pαα-F/Pαα-R and Cαα-F/Cαα-R and Cαα-F/Cαα-R69, generating the variant Caαα, and Cβαα, with approximately 1/3 and 1/5 of the productivity of the wild-type, were constructed by inserting simple short repeat sequence (ATA) into the spacer region of ribosome binding site of β peptide to weaken the translational initiation rate with primers Pβαα-F/Pβαα-R, Cβαα-F/Cβαα-R, Cβαα-F/Cβαα-R, Cβαα-F/Cβαα-R, Cβαα-F/Cβαα-R69. The lactococcin A producing plasmid pleiss-lcnA was constructed by assembling the lcnA gene cluster from the plasmids pFI2396 and pFI2148 with pleiss-Nuc vector68. To simplify the detection of the lcnA-producing strain, a reporter gene gusA3 was amplified from the plasmid pTRK89265 and then inserted into pleiss-lcnA using primers Pαα-F/Pαα-R and lcnA-F/lcnA-R and gusA3-F/gusA3-R. The resulting plasmid pleiss-lcnA-gusA3 was transformed into L. lactis MG1363 to generate the lcnA producing strain Kp, as a control strain for tractability of lcnG and a gusA3 reporter, was created by assembling the immunity gene lagC of lcnG and gusA3 using the primers of Pβαα-F/Pβαα-R and lcnA-F/lcnA-R and gusA3-F/gusA3-R. To create the strain with pH-dependent lcnA production and lcnG resistance, plasmid pleiss-Pαβ-lcnA-gusA3 was firstly created by replacing the lcnA’s native promoter Pαα by the plasmid pleiss-lcnA with a pH inducible promoter Pβα. Subsequently, pleiss-Pαβ-lcnA-gusA3 was assembled with the fragment of Pβα-F/lagC using two pairs of primers: Pβα-F/lagC-R and Pβα-F/lagC-R69, generating the variant Cβαβ, generating the final plasmid pleiss-Pβαβ-lcnA-Pβαβ-lagC-gusA3. The final plasmid was then transformed into L. lactis MG1363 to generate the strain Kp.

Measurement of lcnG productivity. The agar diffusion assay was performed using a protocol adapted from a previous study66. Specifically, cultures of lcnG producing strains were grown in GM17/Cm/Tween broth at 30 °C overnight under corresponding culture conditions. The overnight cultures were inoculated in fresh media at 1:50 dilution and grown to the early stationary phase. Supernatants were obtained by centrifuging at 10,000 × g for 10 min. Then, 30 μL samples were added into the wells in a double-layer agar (15 mL of GM17/Cm/Tween with 0.75% agar for each layer) in which the bottom layer was seeded with 50 μL of overnight culture of inducer strain L. lactis NZ9000/pleiss-Nuc. After incubation at 30 °C for 8 h, the inhibition zones were characterized by the blank circles around wells. To establish a standard curve of relative lcnG concentration for quantitatively evaluating samples, the concentration of lcnG in the supernatant of Caβ culture at the early stationary phase is defined as 100%. And then the cell-free supernatant of Caβ culture was diluted with fresh GM17/Cm/Tween media to the relative lcnG concentrations of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2%, and 1%. Next, 30 μL of samples were added into the wells in the top layer agar. After incubation at 30 °C for 8 h, the inhibition zones emerged and a standard curve was established. By measuring the diameter of inhibition zones produced by different relative concentrations of lcnG. Using this curve, the relative concentrations of lcnG from tested samples were estimated.

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Characterization of variations from labor partition. Monocultures of Ca and Cβ (Ca0 and Cβ0) were inoculated in GM17/Cm/Tween liquid medium at 30 °C overnight. The optical density at 600 nm (OD600) of Kp (Kp0) monocultures were measured and the co-cultures of Ca and Cβ (Ca0 and Cβ0) were mixed at a variety of ratios (30:1, 10:1, 3:1, 1:1, 1:3, 1:10, 1:30) with a start total OD600 of 10^{-2}. After 8 h, the co-culture were centrifuged at 10,000 g for 10 min to obtain the co-culture supernatants. To test the cooperative strength in supernatant mixtures, overnight monocultures were firstly transformed to fresh GM17/Cm/Tween broth at the initial OD600 of 10^{-2} individually. Then supernatants were extracted after growth for 8 h by centrifuging and mixed at different ratios as mentioned above. Finally, 30 μL of samples were used to determine the activity of lcnG by agar diffusion assay in the presence of the variability of co-culture supernatants and monoculture supernatant mixtures. Additionally, to evaluate the role of subunit stoichiometric ratio in cooperation variation, monoculture supernatants were also prepared from Ca0, Cβ0, Cα0, Cβ1, Cβ2, and Cβ0v2 as described above and mixed at ratios of 30:1, 10:1, 3:1, 1:1, 1:3, 1:5, 1:6, 1:10 to 1:30. Among them, ratios of 1:5 and 1:6 were used for determining the optimal cooperativity between Ca0 and Cβ0 or Cα0; Cβ0v2. Then, activities of lcnG in these combinations were determined by agar diffusion assay. Similarly, to measure the cooperative inhibition from Ca and Cβ to Ks (Kp), three steps were involved. First, starting media were prepared by mixing 2-fold concentrated GM17/Cm/Tween medium with equal volume of supernatants from Ca-Cβ co-culture at the early stationary phase that were filtered with sterile 0.22 μm filter and adjusted to necessary pH. Second, overnight Ks (Kp) monoculture was inoculated with an initial OD of 10^{-2} to the starting media. Third, the relative abundances of Ks were measured with fluorescence microscope from the starting to 8 h of incubation, during which pH was controlled properly if needed. For comparison, starting media were also prepared for the diluted co-culture samples and Ks (Kp) monoculture supernatants. To test the cooperative strength in supernatant mixtures, monoculture supernatants were inoculated with an initial OD of 10^{-2} to the starting media. The initial total OD600 of three strains Cα, Cβ, and Ks were serially diluted to an OD600 of 10^{-2} to 10^{-13} at 1:10 dilution and the survival ratio of Ca-Cβ cooperation was calculated by counting the growing cultures in twenty replicates of each OD600. After incubation at 30 °C for 24 h, the tubes with cell growth were counted to calculate the survival rate of different initial OD600. Our experiment (Supplementary Fig. 5) showed that 10^{-8} is the minimum OD600 with a 100% survival rate for almost all strains. An inoculation with an initial OD600 of 10^{-8} would result in failure in growth and would then disturb the studies of small number fluctuations in the community. Therefore, the initial OD600 after dilution in the serial dilution experiments should be higher than 10^{-8}.

Characterization of cooperation variations from sampling. To examine the effect of sampling on the variability of Ca-Cβ cooperation, overnight cultures of Ca and Cβ were washed twice with sterile PBS buffer (pH = 7) and re-suspended in PBS buffer. Then Cβ suspensions were diluted to an OD600 of 1.0 with PBS buffer, and mixed together at 1:1 ratio. The resulting suspension was used as a start culture and diluted to the OD600 of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} through serial 1:10 dilutions with PBS buffer. The total volume of a start culture was set at 5 mL. After being prepared, all samples were centrifuged at 10,000 g for 15 min to remove supernatants, and 5 mL of fresh GM17/Cm/Tween media were subsequently added for cell growth. At the end of incubation, the supernatants were obtained. Subsequently, inhibition zone assays were conducted to determine the strength of cooperation. As cultures with different initial ODs require different incubation times, for each initial condition we chose sampling time based on the corresponding growth profile so that the ODs at each time point are comparable across the samples (Supplementary Fig. 12). Such samplings enable a consistent and proper comparison. Ten trials were performed for each initial condition.

Three-strain cooperator-variying experiments. The initial total OD600 of three strains Ca, Cβ and Ks (Kr or Kp) was set at 10^{-2}. The start abundance of Ks (Kr or Kp) was fixed at 33.3% in the population but the ratios of Ca and Cβ were set at 30:1, 10:1, 3:1, 1:1, 1:3, 1:10, and 1:30. During incubation, samples were taken every two hours for measuring their ODs and the relative numbers of green (Ca), red (Cβ), and non-fluorescent cells were counted under an AMG EVOS FL fluorescence microscope using green, red and bright field channels. Notably, for the pH-controlled three-strain experiments, the overnight culture of Kp was washed twice with sterilePBS buffer (pH = 7) and then inoculated to an OD600 of 10^{-2} into fresh medium with proper different pH controls (pH ≥ 7, pH ≤ 6 and no pH control). During the course of fermentation, culture samples were collected every hour to determine environmental pH using pH meter and Kp’s lcnA productivity using the GusA3 enzyme assay. To directly visualize the Ks in three-strain system, GusA3 protein was used as a reporter to produce a blue green color. At the end of growth in three-strain consortium, a final concentration of 2 mM of 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (X-Gluc) was added into the cultures and the blue green color formation was monitored. After incubation at 37 °C for 1 h, the supernatants were obtained by centrifuging the cultures at 10,000 g for 10 min and images were taken.

Mathematical modeling. In concert with the experimental ecosystem assembly, a bottom-up strategy was utilized to construct the dynamic models of the synthetic ecosystems. Briefly, we first created and characterized growth models of monocultures (Ca, Cβ, Ks, Kr, Kp), then models for the cooperative Ca-Cβ species as well as their interactions, and finally assembled individual modules into integrated models that represent complete ecosystems (e.g., Ca-Cβ-Ks, Ca-Cβ-Kr and Ca-Cβ-Kp consortia). During model construction, ordinary differential equations were used to quantitatively describe the kinetics of three major classes of variables: nutrient availability, cell populations and bacteriocins mediating cellular interactions. Reaction parameters were determined using data in the literature or by fitting the models to our experiments. MATLAB software was used to simulate the models, produce plots, and fit data for the models. A detailed description of the models is available in Supplementary Information.

Statistical analysis. All of the experiments were performed for multiple times. Replicate numbers of the experiments (n) are indicated in the figure legends. Sample sizes were chosen based on standard experimental requirement in molecular biology. Data are presented as mean ± s.d.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Strains and plasmids constructed in this study are available from the corresponding author upon request. Data supporting the results in this paper are available within the paper and its supplementary information files. The source data of Figs. 2bc, e, 3acg, h, 4a, 5b, 6c, 7 and Supplementary Figs. 1a, 2c, 3, 5, 7b and 8a are provided as a Source Data file. All other material and data are available from the author upon reasonable request.

Code availability. Custom MATLAB codes developed in this study are available from the corresponding author upon request.
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Author contributions
T.L. conceived the project; T.L., Q.H., Y.F. and R.B. designed research; F.L. performed experiments; J.M. developed mathematical models; W.K. contributed new reagents/analytic tools; F.L., J.M. and T.L. analyzed data; F.L., J.M. and T.L. wrote the paper with input from all other authors.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to T.L.

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