Costless metabolic secretions as drivers of interspecies interactions in microbial ecosystems

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Metabolic exchange mediates interactions among microbes, helping explain diversity in microbial communities. As these interactions often involve a fitness cost, it is unclear how stable cooperation can emerge. Here we use genome-scale metabolic models to investigate whether the release of “costless” metabolites (i.e. those that cause no fitness cost to the producer), can be a prominent driver of intermicrobial interactions. By performing over 2 million pairwise growth simulations of 24 species in a combinatorial assortment of environments, we identify a large space of metabolites that can be secreted without cost, thus generating ample cross-feeding opportunities. In addition to providing an atlas of putative interactions, we show that anoxic conditions can promote mutualisms by providing more opportunities for exchange of costless metabolites, resulting in an overrepresentation of stable ecological network motifs. These results may help identify interaction patterns in natural communities and inform the design of synthetic microbial consortia.
The astonishing number of microbial species observed in nature\textsuperscript{1–3} seems to contradict classical ecological theory, which predicts far less biodiversity in many nutrient-poor environments\textsuperscript{4,5}. A variety of explanations have been proposed as possible solutions to this discrepancy\textsuperscript{6–10}, including metabolic cross-feeding\textsuperscript{11–13}. This phenomenon, in which one species produces metabolites that are then consumed by another, has been shown to enhance the capacity of microbes to survive in resource-poor environments\textsuperscript{14–16}. However, it is not clear how these cooperative phenotypes emerge, as they often involve the exchange of metabolites that are costly for the producer. This apparent altruism introduces the potential for the rise of cheating behaviors\textsuperscript{14,16,25}

It is known that microbes often secrete waste products (e.g., Escherichia coli secreting acetate under limited oxygen) that can support other species\textsuperscript{15}. This phenomenon may allow community benefits to emerge as a product of otherwise selfish acts by individual organisms. It is not obvious, however, whether such behavior extends beyond a few fermentation byproducts or how widely it varies across microbial species and environmental contexts\textsuperscript{22}. Moreover, it is not clear whether these byproducts have the potential to enable or enhance growth of other species in complex communities.

Here, we use computational metabolic modeling to explore the environmental modifications brought about by “costless” metabolite secretion, as well as the interspecies interactions enabled by this type of exchange. For our purposes, we define a metabolite as costless if the predicted growth rate of an organism secreting that metabolite is not less than its growth rate when the metabolite is not secreted. As we will illustrate in detail, the costless nature of a given metabolic secretion is strongly dependent on environmental conditions and may hinge on the techniques used to generate predictions. Our computational framework is based on flux balance analysis (FBA)\textsuperscript{24}, which we use to predict the growth phenotypes and beneficial interactions mediated by costless metabolites for 24 microbial species under a large set of carbon source combinations. Through this method, we obtain a global view of cross-feeding opportunities that can mediate the emergence of beneficial interactions and the maintenance of biodiversity in natural communities.

Previous computational models of microbial community metabolism have yielded useful predictions on mutually beneficial and competitive behaviors\textsuperscript{14,16,25–29}. In this study, we evaluate the impact of costless metabolic secretions by applying FBA to a large space of microbial species and environmental conditions. Moreover, we quantify the degree to which environmental variables, such as oxygen and carbon source, contribute to costless metabolic secretions, interspecies interactions, and community stability. Though the present work focuses on secretions and interactions predicted computationally, we restricted our analysis to microbes associated with high-quality and experimentally verified in silico models, which have allowed us to make predictions consistent with previously established empirical knowledge. However, the current analysis should be viewed as an exploration of a large space of stoichiometrically possible costless interactions (inscrutable to such an extent at the experimental level), whose global patterns can motivate and inform future experimental and theoretical endeavors.

Results

Metabolite secretion cost depends on environmental context. Understanding whether the secretion of a metabolite by an organism is associated with a decrease in fitness (interpreted here as growth rate) is difficult to assess experimentally, but can be readily calculated using genome-scale models of metabolism (see Methods). For example, one can impose the secretion of a given compound at a given rate \(v_s\), and then ask whether this constraint is expected to cause the organism’s growth rate \(v_g\) to be less than its growth rate without this constraint \(v_{g,0}\). A small set of simulations of this kind for a single organism (Supplementary Fig. 1) exemplifies the spectrum of possible outcomes: depending on the carbon sources provided, different metabolites can be produced either (i) at the expense of growth capacity, (ii) with no apparent effect, or (iii) even to its benefit. For cases (ii) and (iii), since fitness is not reduced by the metabolite secretion, \(v_{g,0} \geq v_{g,s}\). The existence of solutions that satisfy this equation (i.e., flux states that have higher growth in presence of a metabolic secretion) forms the basis of our subsequent calculations. The above equation can thus be viewed as the defining characteristic of a costless metabolic secretion.

We note that while FBA provides mechanistic insight into the tradeoff between metabolic reaction costs and benefits, current predictions (including those on the costless nature of a metabolic secretion) may depend on factors not captured by our method, including temperature\textsuperscript{30}, signaling and gene regulation\textsuperscript{31–33}, pH changes\textsuperscript{34}, and explicit pathway-dependent cost of enzyme production\textsuperscript{35}. Our definition of “costless” may therefore be interpreted as a heuristic that captures expected spontaneous metabolite secretions, in contrast to secretion that would be associated with a growth or fitness reduction.

Costless secretions promote environmental enrichment. Having illustrated in an individual case how metabolite secretion costs can strongly depend on carbon sources, we sought to map the prevalence of costless secretions across a broad set of organisms and environments. As an initial core analysis, we carried out a total of 1,051,596 unique simulations, each with two organisms from a set of 14 genome-scale models of facultative anaerobes and two carbon sources from a set of 108 compounds (Fig. 1, Supplementary Data 1, Supplementary Table 1). We chose facultative anaerobes in order to enable a direct comparison of secretion profiles in oxic vs. anoxic conditions. Each simulation was conducted as an iterative process that emulates a coculture experiment, uniquely defined by the organisms involved, the carbon sources provided, and the availability of oxygen. At each iteration, we used FBA to determine the ability of each organism to grow on the provided medium, in addition to the set of metabolites predicted to be spontaneously (i.e., costlessly) secreted by each microbe. We incorporated several measures into these simulations to minimize false-positive reporting of secreted metabolites (see Methods).

If at the first iteration \(c = 1\), Fig. 1) at least one organism was able to grow on the carbon sources provided, all newly secreted costless metabolites were added to the medium for the next iteration. This process was repeated until no new metabolites were produced (defining a final iteration \(c = c_f\)). Upon running such simulations for all combinations of species and environments, we obtained distributions for the value of \(c_f\) (Fig. 2a). A majority of cases reached a steady state after only one iteration, possibly due to organisms secreting multiple byproducts that only contributed weakly to subsequent secretions.

In aggregate, our simulations showed a rightward shift in the diversity of metabolites secreted under anoxic conditions when compared to the number secreted when oxygen was available.
A growth medium ($M_c$) containing two carbon sources (α, β) with or without oxygen ($Ω$) is provided to genome-scale metabolic models of two microbial organisms (i, j). If at least one organism grows, any costlessly secreted metabolites ($σ_c$) are added to the medium, which is fed back to the organisms. This process is repeated for a series of iterations $c$, and terminates at iteration $c_σ$, defined as the last iteration in which any new metabolites were secreted into the medium.

(Fig. 2b), as well as a shift in the quantity of metabolites secreted between the first and last iteration of each simulation (Fig. 2c). This latter effect reflects organisms taking up metabolites secreted by themselves or their partner, and secreting different metabolites as a response. Based on these results, we hypothesized that oxygen availability would be among the best indicators of the metabolites secreted in a simulation. To quantify this effect, we applied a machine learning approach to a modified simulation set consisting of all 14 organisms individually feeding on a single carbon source (see Methods). Using this method, we found that sets of secreted metabolites could be used to yield varying degrees of information on simulation starting conditions. Specifically, oxygen availability, species identity, and carbon source type could be predicted with cross-validation accuracies of 93.4%, 58.0%, and 85.3%, respectively. Notably, organism identity appeared to be not strongly associated with specific costless secretions compared to carbon source and oxygen. This may be due to the fact that, while an organism may have a pathway to secrete a particular byproduct, utilization of that pathway would be strongly contingent on the presence of the necessary substrates. The observed associations of secretions with the carbon source mirrored previous experimental observations, which identified carbon sources as the main drivers of community composition through metabolic cross-feeding. While the specific concentration of environmental substrates could in principle affect predicted secretions, we found through dynamic FBA (dFBA) simulations that substrate concentration had a negligible effect on the identity of secreted metabolites (see Methods and Supplementary Data 2). Nonetheless, one should consider the possibility that this result may be due to limitations of constraint-based modeling, which may be overcome in future studies.

**Useful costlessly secreted byproducts are abundant.** Our analysis revealed that most organisms secreted a broad distribution of metabolically useful compounds without cost in a variety of environmental conditions (Fig. 3, Supplementary Fig. 4a). Though inorganic compounds such as water and carbon dioxide were, as expected, the most commonly secreted molecules across
all simulations, nitrogen-containing compounds such as nitrite, ammonium, and urea were secreted in 73.5% of the analyzed cases, suggesting maintenance of an appropriate carbon-to-nitrogen ratio in the cell. We note specifically that nitrite is secreted in fewer than 100 simulations with oxygen, but its secretion is prevalent in anoxic simulations—a phenomenon previously observed in anaerobic enteric bacteria. Moreover, ~10% of anoxic simulations resulted in at least one organism fully reducing nitrate into nitrogen gas, suggesting that anaerobic respiration was a preferred strategy in some environments. Organic acids made up the second most abundant category of costless secretions, followed by nucleotides, peptides, and carbohydrates. Altogether, this space of secreted metabolites points to a large variety of molecules that can be freely produced in resource-poor environments.

Despite our careful design of the simulation process, it remains difficult to quantify the degree to which these secretions will be observed experimentally. For this reason, we have relied exclusively on genome-scale metabolic models that have undergone experimental validation under conditions that in many cases mirror those that we have simulated, in addition to imposing our own set of constraints (see Methods). Moreover, though empirical testing of every simulation we performed is inaccessible, we note that experimental data from previously published work supports key portions of our predictions (Supplementary Table 4). In an additional effort to ensure the accuracy of the set of secreted metabolites, we also carried out all simulations using alternative objective functions. In particular, though optimization of growth reflects the possibility of organisms “selfishly” growing as rapidly as possible and “unintentionally” secreting useful metabolites, alternative objective functions may best capture metabolic regimes relevant across different conditions. We therefore compared metabolite secretion profiles inferred by maximizing growth to those obtained through minimization of biomass production, as well as maximization and minimization of ATP production. All objectives gave rise to secretion profiles highly similar to each other, with an increase of only 0.18% of all predicted metabolic secretions in the growth maximization condition relative to the others (see details and results in Supplementary Fig. 5, and Supplementary Table 5).

Given the abundance of secretions from different organisms, we asked whether specific metabolite secretions were highly correlated. We thus used a Spearman correlation analysis to identify secretion patterns that appeared with high frequency (Supplementary Fig. 6). In the presence of oxygen, we observed a strong co-occurrence of glycerol, lactate, succinate, malate, and acetate, which may reflect the high frequency of secretion of these carbon-containing compounds. We also observed positive, but weaker correlations between these metabolites and other central carbon compounds such as fumarate, citrate, and 2-oxoglutarate. Our analysis also pointed to the simultaneous release of multiple nitrogen-containing compounds, chiefly urea, ammonium, and nitrate. Without oxygen, we observed stronger correlations between secretion of nitrogen-containing compounds and fermentation byproducts. Amino acids also co-occurred with high frequency without oxygen in patterns consistent with examples of previously studied exometabolomic profiles, including those showing co-secretion of central carbon intermediates in E. coli and of amino acids in yeast41,42, as well as time-dependent patterns of metabolites released simultaneously in soil communities. These co-secretion profiles suggest that environments modified by metabolic activities of existing organisms may be simultaneously enriched by specific combinations of molecules.

Having mapped the space of metabolites secreted at no fitness cost to the producer, we sought to understand which metabolites could be subsequently taken up by other organisms. We found that the organic metabolites most commonly exchanged across species were central carbon intermediates, secreted mainly in anoxic conditions (Supplementary Fig. 4b). These secretion patterns mirrored those of anoxic gut bacteria43. These co-secretion profiles suggest that environments modified by metabolic activities of existing organisms may be simultaneously enriched by specific combinations of molecules. Costless metabolite exchange enhances growth capabilities. We next assessed how often the exchange of costlessly produced molecules could directly enable growth of other organisms that would otherwise not grow on the initial environmental nutrients. Before taking into account the costless secretions, 18.2% and 11.9% of simulations predicted growth of both organisms with and without oxygen, respectively (Fig. 4a). After the organism pairs were allowed to exchange costlessly secreted metabolites, our algorithm predicted a substantial increase in growth-supporting environments (72.7% with oxygen and 82.5% without oxygen relative to minimal medium), suggesting that exchange of costlessly secreted metabolites can enable growth of additional organisms in resource-poor environments.

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Both organisms grew in minimal medium
Both organisms grew only after cross-feeding
One or no organisms grew

In addition to a global increase in growth capabilities due to costless metabolite secretion, we observed species-specific growth patterns that varied widely across our dataset (Fig. 4b). Lactococcus lactis and Porphyromonas gingivalis, for example, are host-associated microbes that are auxotrophic for a wide range of metabolites and that often depend on metabolic products from the host or other commensal microbes50,51. In our study, these organisms failed to grow in all simulations even after costless metabolites were made available by a partner. This failure to sustain growth of highly dependent organisms suggests that there is an upper limit to the degree to which costless metabolite production can enable species growth, especially in the minimal environments that were tested. Nonetheless, most of the metabolites that these organisms require to grow were producible separately by multiple species, suggesting a possible important role of multi-partner cross-feeding interactions in complex communities. Aside from these extreme cases, our analysis shed light on the performance of generalist organisms, such as E. coli, K. pneumoniae, S. cerevisiae, and S. enterica. These organisms grew in at least half of all tested environmental conditions, in contrast with organisms such as Methylobacterium extorquens or Zymomonas mobilis, which exhibited much more limited pairwise growth capabilities. The growth patterns of these latter organisms suggest a greater dependence on the metabolic byproducts of their partners, particularly in anoxic conditions.

As our study relied on a limited set of curated metabolic models, we wondered how sensitive these results were to the organisms being assessed. In order to explore possible bias, we conducted additional simulations in which we binned organisms by environmental habitat. These simulations were separated into three sets: the first with 13 aquatic microbes grown aerobically, the second with 12 soil microbes grown aerobically, and the third with 12 human gut-associated microbes grown anaerobically. These simulations employed additional genome-scale models...
(including obligate aerobes or obligate anaerobes, see Supplementary Data 1) that were not used in our core analysis of 14 facultative anaerobes. By analyzing the expanded set of organisms in a habitat-specific manner, we found that exchange of costless metabolites substantially improved the ability of minimal environments to support pairwise growth in all three habitats (Supplementary Fig. 7a). Notably, metabolite secretion and exchange for aquatic and soil microbes resembled the profiles found for the core organisms grown with oxygen (Supplementary Fig. 7b, c, Supplementary Table 6). Conversely, the distribution of secreted metabolites for the core organisms grown anoxically (Supplementary Fig. 7d, Supplementary Table 6) yielded pairwise growth.

Competition and non-competition for oxic (out of 164,939 simulations that yielded pairwise growth) and anoxic conditions (out of 115,463 simulations that yielded pairwise growth). Overall distributions of competitive and non-competitive interactions for oxic and anoxic conditions. These interactions at the level of secreted metabolites exist simultaneously with competition or no competition for a primary carbon source.

Carbon sources exhibit cooperation in promoting growth. In addition to characterizing the global space of in silico growth phenotypes, we examined how cooperativity of carbon sources could enhance growth capabilities in organism pairs. Drawing from techniques used to quantify epistatic interactions52, we defined the cooperativity index $C$ of two carbon sources $a$ and $b$ as the difference between the number of simulations that resulted in growth from both carbon sources ($g_{ab}$) and the product of the number of simulations that resulted from single carbon sources ($g_{a}g_{b}$). These counts were normalized by the total number of simulations involving the specific pairing of carbon sources being analyzed (represented here by the combinatorial formula $\binom{N}{2}$), as follows:

$$C_{a,b} = \frac{g_{a,b}}{\binom{N}{2}} - \left( \frac{g_{a}}{\binom{N}{2}} \times \frac{g_{b}}{\binom{N}{2}} \right). \quad (1)$$

This metric reflects the cooperative potential of each carbon source pair relative to that of each carbon source in isolation. Upon averaging a single carbon source over its cooperativity index, we obtain a relative degree to which a carbon source “depends” on another to sustain growth. By framing cooperativity in this context, we observed that simple sugars such as glucose...
and sucrose had relatively low cooperativity indices, that is, they were able to sustain growth efficiently on their own. In contrast, more complex molecules and dipeptides had higher average cooperativity indices, indicating they performed better in the presence of another carbon source. We grouped these average cooperativity indices through hierarchical clustering (Supplementary Fig. 8, Supplementary Table 7) and observed general clustering by carbon source type—especially with sugars and amino acids appearing in distinct groups. This analysis illustrates the nonlinear effects of adding additional nutrients to a minimal medium, underscoring the observed complex metabolite usage patterns in organism pairs.

Costless crossfeeding can offset competition for nutrients. Our analysis so far has examined the contexts in which a metabolite can be secreted costlessly, as well as the potential for these metabolites to promote growth. Additional insight about the relevance of these interactions can be obtained by comparing them to ecological expectations of cooperation and competition. Towards this goal, we defined six types of possible interactions: non-interaction, commensalism (unidirectional exchange), and mutualism (bidirectional exchange), each with or without competition for a primary carbon source (Fig. 5a). We chose to decouple competition for nutrients from exchange of secreted metabolites in order to more fully understand the degree to which the latter can promote organism coexistence despite resource scarcity. When analyzing our dataset under this framework, we found that competition for one or both carbon sources constituted the majority of all interactions (Fig. 5b), as previously observed experimentally. However, these predicted competitive phenotypes were observed to frequently occur simultaneously with potentially beneficial interactions mediated by metabolic byproducts.

Our modeling predicted bidirectional interactions to be far more common without oxygen than with oxygen (Fig. 5c). We obtained a more fine-grained perspective on costless metabolic interactions by considering the distributions of interaction types by species pairs (Fig. 5d). For example, the majority of pairings of M. extorquens with B. subtilis, E. coli, and K. pneumoniae exhibited commensal interactions (chiefly with M. extorquens receiving). In contrast, the distribution of interactions shifted toward mutualism when oxygen was made unavailable. These patterns were also mirrored in a majority of individual species pairings. As with the positive shift observed in the distributions of secreted metabolites (Fig. 2b, c), we attributed the increased prevalence of mutualistic interactions without oxygen to a greater availability of metabolic byproducts that contributed to reciprocity. To test this hypothesis, we performed a small subset of “hybrid” in silico experiments, where we analyzed the interactions that arose from one species being grown with oxygen and the other without oxygen. We studied the examples of E. coli with B. subtilis and S. enterica, whose pairwise simulations showed greater amounts of mutualistic interactions without oxygen (Fig. 6). These hybrid simulations demonstrated how an organism grown anoxically can provide a higher number of useful byproducts to its aerobic partner, leading to bidirectional interactions when both are grown without oxygen.

Costless secretions can produce stable interaction motifs. Lastly, we combined data generated by our algorithm with ecological network simulations to understand how the simultaneous competition for common nutrients and cooperation through costless metabolite exchange could jointly affect the stability of pairwise consortia. Using the general interaction types outlined...
Fig. 7 Interaction motif analysis and dynamical modeling of motif stability. **a** Schematic representation of specific motif types. Motifs are named according to three features: the interaction type (non-interacting, N; commensal, C; mutualistic, M), the number of carbon sources consumed by the pair (1–2), and competition for a primary carbon source (no competition, a; competition, b). Orange circles denote organisms, black dots denote primary carbon sources, and violet dots indicate any arbitrary number of costlessly shared metabolites. Arrows indicate direction of metabolite flow. **b** Frequency of specific motif types. Height of empty white bars indicate the total number of simulations that exhibited the general motif type (non-interacting, commensal, mutualistic). Colored bars within indicate the number of the specific motif type (N1a, N1b, and so on). **c** Stability space of motifs from dynamical chemostat modeling, as a function of the specific growth rates of the two organisms involved (GR1, GR2). Red indicates area of stable coculture previously (non-interaction), commensalism, and mutualism with and without competition), we first enumerated all possible interaction network motifs (Fig. 7a) and calculated the frequency with which each motif was observed in our simulation set (Fig. 7b). For non-interacting motifs, our simulations predicted an almost exclusive representation of relationships involving competition for a primary carbon source. The distribution between competitive and non-competitive motifs was more balanced for commensal and mutualistic interactions, showing a slight preference for interactions involving competition.

In order to simulate how these interactions could contribute to stable symbioses, we created a dynamical chemostat model of two arbitrary species consuming carbon sources and exchanging costless metabolites according to each motif type (see Methods, Supplementary Fig. 9). By varying the maximum specific growth rates (μmax) of each species from 0 to 1 h⁻¹, we simulated the growth of the pair under each motif type for 500 h. If both species survived at the end of the simulation, we marked the motif type as stable at that combination of specific growth rates. We mapped the space of stable species pairs under each motif type, observing that competitive interactions generally had a reduced parameter space for enabling stability (Fig. 7c). Notably, though motif N1b was highly prevalent in the costless FBA simulation set, this motif represents competitive exclusion and cannot result in long-term stability. In contrast, though complete nutrient–organism orthogonality can yield stability over the whole space of parameters (N2a), this motif was not predicted to occur in the FBA simulations. An intermediate case between these two extremes (N2b) represents a balance between competition and independence with respect to external carbon source utilization: in this case, which frequently occurs in our dataset, stability is achievable only for a narrow set of specific growth rates.

Our models predicted a marked increase in stability when costless metabolite exchange was enabled. In motif C1a, for example, the rate of costless metabolite secretion from organism 1 is enough to sustain organism 2, even when the specific growth rate of organism 2 is greater. This nonintuitive space of stable solutions is the result of the effective growth rate of organism 2 being reduced such that its rate of byproduct consumption does not exceed the rate of secretion by organism 1. Nonetheless, competition for primary carbon sources leads to decreases in the space of possible stable solutions, as observed in motifs C1b and C2b. In motif C2b, both organisms are competing for a carbon source and organism 1 is providing one or more costless metabolites to organism 2. Our dynamical modeling showed that the specific growth rate of organism 1 must usually be greater than that of organism 2 in order for both species to be stable. When feedback is allowed to occur (mutualism), the potential for stability greatly increases across our parameter space, even in the presence of competition for carbon sources. These motifs, with their associated prevalence data and dynamical properties, can overall serve as an atlas for guiding the engineering of stable synthetic consortia built off of costless metabolic relationships.

**Discussion**

We have investigated the pairwise growth phenotypes and interactions of 24 diverse microbial species in over 2 million computational experiments. We found that resource-poor environments can provide the basis for the release of a wide variety of useful metabolic products secreted without cost by their producing
organism; these costless metabolic products provide, in a manner chiefly dependent on oxygen availability and environmental composition, valuable environmental enrichment, nearly doubling the potential of minimal environments to sustain growth. We further found that exchange of costless metabolites established beneficial uni- and bidirectional interspecies interactions, associated with different chances of stability of the ensuing consortia.

Our iterative medium expansion method allowed us to observe which metabolites were secreted in response to others in a mechanistic fashion, highlighting the capability of costless metabolites to enrich minimal environments and sustain biodiversity, even when organisms were competing for the same primary nutrients. Though spatial segregation of competing organisms can also allow for stable communities, purely competitive phenotypes can lead to community collapse in homogenous environments, which can limit taxonomic diversity in nature. By studying the prevalence of mutually beneficial interactions in the presence of competition, our study can help understand metabolic dynamics in resource-poor environments, such as the oligotrophic communities found in the open ocean. Costless secretions, some of which resemble the metabolic laggages that underlie the Black Queen Hypothesis, may also contribute to the maintenance of small genomes in resource-poor environments, as the metabolic needs of some organisms can be fulfilled by others. Interestingly, the prediction that resource-poor environments can lead to diverse secretions and thus to the emergence and maintenance of beneficial interspecies interactions is consistent with prior suggestions that resource abundance or lack of stress may reduce the reliance of communities on metabolic exchange.

Our interaction analysis provides deeper mechanistic insight into the increased prevalence of mutualistic interactions without oxygen, a phenomenon that has been previously predicted computationally and that provides a window into metabolic relationships in environments harboring steep oxygen gradients, such as the human gut. By carrying out a set of hybridoxic–anoxic in silico experiments, we observed that the additional metabolites secreted anoxically by a facultative anaerobe could provide valuable nutrients for aerobically growing organisms. This phenomenon has been suggested to play an important role in maintaining equilibrium in communities at oxic–anoxic interfaces in the mammalian gut and could be the subject of further mechanistic studies.

Although our modeling method considered a wide space of mechanistic constraints in predicting costless metabolic exchange, we acknowledge that secretion patterns and exchange potential are also defined by a variety of other biological factors that fall outside the scope of our modeling framework, such as temperature, signaling and regulatory-based decisions, pH changes and metabolite toxicity, and concentration-dependent thermodynamic gradients. In addition, the cost of metabolite secretion and the cooperative or competitive nature of an interaction may change when interpreted across different timescales. A quality that is not fully captured by the framework described here. In spite of these limitations, however, our analysis is able to demonstrate the plausibility of widespread costless cross-feeding in nature. Our results can also serve as a basis for prioritizing future specific experiments, for which model predictions could be thought of as a null hypothesis against which to compare empirical measurements. Dynamical modeling coupled with these metabolic analyses could then be used to obtain the parameter space most likely to yield desired stable partnerships in vivo. Because this approach relies on screening, in a scalable way, synergy-inducing environments, as opposed to engineering individual strains, it can simplify the process of assembling symbiotic communities and enhance our understanding of microbiomes.

Methods
Selection and modification of genome-scale metabolic models. A genome-scale metabolic reconstruction was obtained for each of the 14 facultative anaerobic organisms used in the analysis, as well as for the organisms used in our habitat-specific simulations (Supplementary Data 1). Genome-scale metabolic models are mathematical representations of an organism’s known metabolic network, which are used to generate mechanistic predictions of growth and resource allocation in a variety of environmental conditions. The process of generating a genome-scale metabolic model has been outlined conceptually and described procedurally by various groups, and generally comprises an automatic generation of a model based on pathway and genome data followed by manual curation by integrating phenotyping, metabolic, or transcriptomic data. We note that although an automatically generated draft metabolic model can be constructed for virtually any organism for which a genome annotation exists, the space of high-quality, experimentally verified metabolic models that have undergone the manual curation process summarized above is comparatively very small. This is due to the time and resources needed to complete the curation process, which can span from 6 months to >10 years for the iteratively refined model of E. coli K-12. We nonetheless consider this process to be essential in producing models that can generate the mechanistic cross-feeding predictions detailed here, which rely on verified metabolic capabilities in monoculture.

The models used in this analysis span five taxonomic phyla, as well as a variety of primary metabolic strategies (Supplementary Data 1). In addition, these models describe several organisms that are commonly used for in vivo studies (E. coli K-12, S. enterica LT2, and so on), making the resulting costless cross-feeding predictions particularly useful for synthetic ecology experiments and microbial community assembly. Importantly, each metabolic model includes reactions that account for the energy requirements of organism growth, as well as those of metabolic production and secretion. These requirements are often incorporated as two key reactions: (1) NPGM, or non-growth-associated ATP maintenance, which comprises an ATP hydrolysis step that simulates ATP usage for processes that are not needed for growth; and (2) GAM, or growth-associated ATP maintenance, which accounts for energy usage in growth-associated processes such as macromolecule and protein synthesis. A minimum level of flux (typically determined experimentally) must flow through each of these reactions in order for the models to grow in silico. In this way, energetic costs of growth and metabolism production and transport are accounted for in the models.

Each model was imported into MATLAB (The MathWorks, Inc., Natick, MA) using the constraint-based reconstruction and analysis (COBRA) Toolbox, a software platform for constraint-based modeling of metabolic networks. In order to enable in silico cross-feeding to be correctly classified, the namespace of all of the metabolic compounds in each of the models was standardized to be internally consistent. This was performed via a computational pipeline with additional manual curation for irregularly annotated metabolites.

Computational methodology description and inputs. Our computational method comprises a set of programs written in MATLAB that use FBA to mechanistically define the growth status and metabolic exchange of microbes through costlessly secreted byproducts. Briefly, FBA is a mathematical method that determines an optimal distribution of metabolic flux through a biochemical network that will maximize a given objective, usually biomass. An FBA problem is framed in the context of several constraints, namely: (i) S, the stoichiometric matrix of dimensions m × n where m is the number of metabolites and n is the number of reactions in the model; (ii) v, the vector of all reaction fluxes, and (iii) v_{min} and v_{max} flux constraints placed on v, defined by enzymatic capacity and experimentally measured uptake rates.

We employ FBA to determine if an organism is able to grow on the in silico growth media conditions we define, in addition to which metabolites are taken up and costlessly secreted. We first apply FBA by maximizing for growth and obtaining an optimal growth rate for an organism, v_{max}. To determine which metabolites are costlessly secreted, we set this growth rate as a minimum for the biomass flux and apply FBA again, recording any metabolites that were secreted and the new growth rate, v_{max}. We also apply the additional constraint of minimizing all reaction fluxes across the network to more closely simulate efficient use of the proteome and minimize cycling of metabolites through the network. Our linear program therefore becomes:

\[
\min \|v\|
\]

s.t.:

\[
S \times v = 0,
\]

\[
v_{\min} \leq v \leq v_{\max},
\]

\[
v_{\max} \geq v_{\min},
\]
This optimization aims to encompass any enzymatic cost incurred by the organism in synthesizing and exporting any metabolite we deem to be costless. During each step in which growth or metabolism absorption and secretion are computed, FBA optimizations are performed separately for each in silico organism $i$ and $j$, with biomass production set as the objective function while minimizing the sum of the absolute value of $v$. Because we focus on the emergence of potential metabolic exchange through the availability of costlessly secreted metabolites, our modeling framework purposefully keeps FBA optimizations separate for each model without accounting for spatial or temporal community structure. It is also for this reason that we establish the biomass fluxes of each in silico organism as the objective functions to be optimized, as we are concerned with secretion of potentially useful metabolic byproducts that arise out of “selfish” optimal growth. This assumption of maximum growth with proteome optimality is also key for translating these organisms and predictions to in vivo synthetic ecologies, where biomass optimization more closely describes the behavior of organisms in batch or continuous culture\cite{Note35}. In simulations where we employed alternative objective functions, we primed either minimization of biomass production, maximization of ATP production, or minimization of ATP production (all with a lower bound for biomass flux set at 0.01 per hour) to compare secretion profiles to those observed under biomass maximization. For ATP maximization and minimization functions, we set the ATP maintenance reaction in each of the models as the objective.

Our algorithm requires six inputs: (1) a data structure containing the genome-scale metabolic models to be used, (2) a list of carbon sources, (3) the number $N_M$ of in silico organisms to be simulated together (for pairwise simulations $N_M = 2$), (4) the number $N_C$ of carbon sources to be provided to each simulation, (5) a Boolean variable $Ω$ that specifies if oxygen will be made available to the in silico organisms, and (6) a list of metabolites that becomes up a simulated base growth medium, $M_{base}$. This base medium contains various nitrogen, sulfur, and phosphorus sources, as well as vitamins, ions, and metals needed for growth of the organisms (Supplementary Table 2).

We focused on pairwise species growth with two carbon sources ($N_M, N_C = 2$). Although each genome-scale metabolic model we used has been manually curated to reflect in vivo metabolic capabilities, very few experiments have been performed to verify FBA-generated predictions for more than a single species\cite{Note35,Note36}. We therefore limit the number of in silico species to two, in order to interpret the growth and cross-feeding predictions with greater confidence. This limit alsoconstantly the combinatorial space of the simulations, which grows exponentially and becomes numerically intractable with more models and carbon sources. In addition, limiting simulations to $N_M = 2$ allows for greater experimental accessibility for assembling synthetic ecologies based on costless metabolic exchange. Our algorithm can nonetheless be applied to any $N_M, N_C > 2$.

To simulate possible carbon sources secreted primarily from the carbon sources contained in the BIOLOG Phenotyping MicroArray 1 (PM1) plate, which is used for phenotyping and curation of genome-scale metabolic models\cite{Note37,Note38,Note39,Note40,Note41}. The carbon sources we selected are common mono-, di-, and polysaccharides, all 20 amino acids, dipeptides, and organic acids contained in the PM1 plate. We also supplemented the list with additional carbon sources known to be consumed by the in silico organisms, for a total of 108 (Supplementary Table 1).

To permit uptake of the metabolites in the medium, the constraint on the uptake flux bound $v_{max}$ for each exchange reaction pertaining to a medium metabolite was removed in each of the models $i$ and $j$. This bound was fully removed ($v_{max} = 1000 \text{mmol} \times \text{gDw}^{-1} \times \text{h}^{-1}$) for non-limiting medium components, and was set to ($v_{max} = 10 \text{mmol} \times \text{gDw}^{-1} \times \text{h}^{-1}$) for the growth-limiting carbon sources $α$ and $β$. This latter value is drawn from experimentally estimated uptake rates of sugars by $E. coli$ in exponential growth conditions\cite{Note42,Note43,Note44,Note45,Note46}, and is applied equally to all other species to simulate general availability of the carbon sources in the environment. In all aerobic simulations, the maximum uptake rate for oxygen was also unconstrained ($v_{max} = 1000 \text{mmol} \times \text{gDw}^{-1} \times \text{h}^{-1}$), so as not to explicitly model conditions similar to those of overflow metabolism. All other exchange reaction $v_{max}$ values are set to zero to block uptake of metabolites not in the medium.

Computing growth, secretion, and cross-feeding. We describe the FBA operations at the core of our algorithm as a function $F$ that, given a medium condition $M$ and organisms $i$ and $j$, outputs the binary growth status $g$ of the organisms, as well as the set of metabolites $σ$ secreted costlessly by the organisms:

$$F([M, i, j]) = [g, σ].$$  

(2)

Each in silico experiment $E$ for a given organism pair with a pair of carbon sources is made up of an initialization step, an expansion step consisting of series of applications of $F$, and a completion step (Supplementary Fig. 2). In the initialization step, two organisms $i$ and $j$ are selected, and a medium $M_i$ is defined. $M_i$ contains the minimal medium $M_{base}$, two carbon sources $α$ and $β$, and the variable $Ω$, which denotes the presence or absence of oxygen.

In the expansion step, the function $F$ is applied for a series of iterations $c$. In each iteration, $F$ simulates the growth of both organisms in the current medium condition and returns the Boolean growth status $g$ (where $g_{ij} = [0; 1]$) of both organisms and the set of any costlessly secreted metabolites, $σ_i$. To avoid recording metabolites reported to be secreted only as a result of numerical uncertainty in FBA, a minimal lower flux bound of 0.01 mmol x gDw$^{-1}$ x h$^{-1}$ was applied as a cutoff for determining secretion. If at least one organism in the pair grows, the medium is supplemented with $σ_i$:

$$M_i+1 = M_i + σ_i.$$  

(3)

As long as new metabolites continue to be secreted into the medium, that is,

$$M_i > M_{i-1},$$  

(4)

$F$ continues to be applied. This stepwise expansion simulates the organisms responding to the costlessly secreted metabolites being secreted and generating a richer medium. The completion step occurs when no new metabolites are secreted,

$$M_i = M_{i-1}$$  

(5)

and the final iteration before this stabilization occurs is defined as $c_0$. Our algorithm therefore carries out individual in silico experiments $E_{i,j}^{c_0}$, defined as the output resulting from $c_0$ applications of $F$ given organisms $i$ and $j$, carbon sources $α$ and $β$, and the presence or absence $Ω$ of oxygen:

$$E_{i,j}^{c_0} = ([M, i, j])_{c_0-1}.$$  

(6)

Dynamical modeling of interaction motifs. We designed a dynamical modeling method to simulate the long-term stability of each pairwise interaction type observed in our in silico experiments. We first established a graph theory framework to map each simulation to a specific interaction motif, each of which accounted for the general interaction type (non-interacting, commensal, or parasitic). To verify FBA-generated predictions for more than a single species\cite{Note35}, we selected one representative topology from these motifs to simplify the space of dynamical modeling simulations. These equations were modeled off Monod dynamics\cite{Note47} and are intended to simulate growth of species in a chemostat, with constant replenishment of medium components. The abundance of each organism $s_i$ in g/L is modeled as follows:

$$\frac{ds_i}{dt} = \mu_{max} \left( \frac{m_i}{k_{i,m_i} + m_i} \right) - D s_i$$  

(7)

where $\mu_{max}$ is the maximum specific growth rate of organism $i$ in h$^{-1}$, $m_i$ is the concentration of carbon source $α$ in g × L$^{-1}$, $k_{i,m_i}$ is the concentration of $α$ at which organism $i$ reaches half its maximal growth rate in g × L$^{-1}$, and $D$ is the chemostat dilution rate in h$^{-1}$. If two carbon sources are present and the organism is determined to take up both by the motif definition, the equation is modified to include a carbon source $β$ as follows:

$$\frac{ds_i}{dt} = \mu_{max} \left( \frac{m_i}{k_{i,m_i} + m_i} \right) - D s_i$$  

(8)

The concentrations of each carbon source are defined as follows:

$$\frac{dm_i}{dt} = I_{s_i} - \mu_{max} \left( \frac{s_i}{k_{s_i,m_i} + s_i} \right) - D m_i$$  

(9)

where $I_{s_i}$ is the nutrient stock concentration for $m_i$ in g × L$^{-1}$, and $K_{s_i,m_i}$ is the ratio of nutrient consumed by the organism in g $\text{g}^{-1}$. This equation is modified with an additional term (organism $j$) to simulate competition for $m_i$:

$$\frac{dm_i}{dt} = k_{s_i,j} \times s_j - \mu_{max} \left( \frac{m_i}{k_{s_i,j} + m_i} \right) - D m_i$$  

(10)

Here, metabolite $m_i$ is produced by organism $j$ and consumed by organism $i$. $k_{s_i,j}$ is the synthesis rate of the metabolite in h$^{-1}$, $K_{s_i,j}$ is the ratio of metabolite consumed by the population $s_j$ in $\text{g}^{-1}$, and $k_{s_i,j}$ is the concentration of metabolite needed for the population $s_j$ to reach half of its maximum growth rate in g × L$^{-1}$.

We then combine Eqs. (7, 9, and 10) to fit the particular motif being modeled (Supplementary Fig. 9). The values of the parameter values are described in Supplementary Table 3 and are based on values reported by Smith\cite{Note48}, Balagadde et al\cite{Note49}, and those based on reasonable estimates for resource consumption. For
each motif, we vary the maximum specific growth rate of both organisms from 0 to 1 per hour and run the simulation for 500 h. If both organism abundances are above 0.05 g × L−1 at the end of the simulation, we determine the motif to be stable at the prescribed growth rates.

Support vector machine classification. We trained three separate support vector machine (SVM) classifiers to quantify the degree to which oxygen availability, species identity, and carbon source type contribute to the variability in secretion profiles. SVMs were constructed using the MATLAB function “fitcsvm” for the two-class oxygen availability vector, and using the MATLAB function “fitcecoc” for the multi-class species and carbon source category vectors. Secretion profiles were represented as a binary matrix, with each row representing a simulation and each column denoting a metabolite. Cross-validation was performed by: (1) randomly

Dynamic flux balance analysis simulations. We carried out a set of simulations to determine the effect of substrate concentration on metabolism secretion patterns. For these simulations, we used the COMETS (computation of microbial ecosystems in time and space) software package39, which uses dFBA38 to integrate metabolic fluxes over sequential time intervals. This method enables specification of substrate concentrations as well as measurement of biomass growth and byproduct concentrations. As dFBA is more time- and computationally intensive than our primary FBA-based algorithm, we limited the number of COMETS simulations to analyzing single organisms growing on one carbon source. For each organism–carbon source pair, we ran COMETS with three different carbon source concentrations (0.01, 20, and 200 mM) for a simulated 2 h to capture differences in secreted metabolites. These combinations yielded a total of 9072 distinct simulations.

Code availability. Code for running pairwise cross-feeding simulations is available at github.com/segrelab/CostlessExchange.

Data availability
All supplementary tables are contained in the Supplementary Information file. Additional data are provided in Supplementary Data files 1 and 2. Raw results data for all pairwise cross-feeding simulations are available at github.com/segrelab/ CostlessExchange. A Reporting Summary for this Article is available as a Supplementary Information file.

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