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Metabolic modelling approaches for describing and engineering microbial communities

Beatriz García-Jiménez[1,2], Jesús Torres[1], Juan Nogales[1,3,*]

[1] Department of Systems Biology, Centro Nacional de Biotecnología (CSIC), 28049 Madrid, Spain
[2] Centro de Biotecnología y Genómica de Plantas (CBGP, UPM-INIA), Universidad Politécnica de Madrid (UPM) - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Campus de Montegancedo-UPM 28223-Pozuelo de Alarcón (Madrid) Spain
[3]Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain.

* Correspondence to: J. Nogales, j.nogales@csic.es

Abstract

Microbes do not live in isolation but in microbial communities. The relevance of microbial communities is increasing due to the awareness about their biotechnological influences in a huge number of environmental, health and industrial processes. Hence, being able to control and engineer the output of both natural and synthetic communities would be of great interest. However, most of the available methods and biotechnological applications (both *in vivo* and *in silico*) have been developed in the context of isolated microbes. *In vivo* microbial consortia development, i.e. to reproduce the community life conditions in the wet-lab, is extremely difficult and expensive requiring of computational approaches to advance knowledge about microbial communities, mainly with descriptive modelling, and further with engineering modelling. In this review we provide a detailed compilation of available examples of engineered microbial communities as a launch pad for an exhaustive and historical revision of those computational methods devoted so far toward the better understanding, and rational engineering of natural and synthetic microbial communities.

Keywords:

Genome-scale metabolic modelling, microbial community, optimization, design, engineering, computational methods, synthetic microbial consortia.

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1. Introduction

Microbes play a pivotal role in fields as diverse as human health, environmental science and biotechnology. In the latter, microbial production of chemicals has become increasingly attractive across industry due to its consistency with the development of sustainable strategies. Microbial biotechnology platforms, integrating tools from systems and synthetic biology has successfully enabled the production of a large portfolio of chemical compounds [1–6]. Early applications of such system metabolic engineering to maximize metabolite production were focused on the engineering of single competitive strains, which has brought obstacles such as metabolic burden and heterogeneity [7,8]. As a direct consequence, production of the target chemical is not always cost-effective and great efforts to improve yield and productivity are required. In response, the use of microbial consortia has been promoted as an alternative to overcome these limitations [9,10]. This is because cooperation among several strains allows microbial communities to accomplish complex functions that are out of reach for individual cells cannot. Pathway modularization allows distribution of metabolic reactions between highly specialized strains, requiring less genetic load per individual. The increase in bioproduction performance and efficiency in source transformation would be achieved with distinct substrates, and/or synthesizing parallel products, and/or avoiding intermediate metabolite accumulation. In addition, robustness provided by microbial communities avoids environmental stresses [11–13].

Following these advantages, significant progress has been made in recent years in analysing, understanding, designing and developing both natural and synthetic microbial communities. Such progress has been applied to improving health, food and chemical production and to dealing with environmental challenges. Therefore, microbial biotechnology will probably tend towards engineering whole communities rather than single strains, using species selection, manipulation of strain ratios and/or genetic engineering of community members. However, there are unresolved challenges to metabolically engineering microbial communities [14] e.g., identifying the co-culture conditions, growth compatibility, and selecting the cross-feeding metabolites among different strains in the consortium, among others. Microbial community modelling emerged with

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need to improve the knowledge and understanding of interactions among heterogeneous cells. The way of modelling these interactions is by metabolite exchange, where the actual metabolites and the extent to which they are exchanged need to be defined. Community interactions include cross-feeding, nutrients competition, symbiotic relations (such as plant-microorganism) or parasitism (such as human-pathogen) or multi-tissues [19,20]. Therefore, these pioneer efforts have highlighted the need for novel computational-system approaches in order to facilitate more rational designs. Here we review pioneer achievements on microbial consortia-based bioprocess and available computational tools developed to provide a better understanding and rational engineering of such microbial communities.

2.- Learning from nature: functional and stability-based design of synthetic microbial consortia.

In nature, microorganisms are involved in a large array of complex interactions with other organisms and their environment, contributing to stability and functionality. Among others, such complex relationships traditionally include commensalism, amensalism, mutualism, predation and competition. These natural relationships have been profusely used as mechanisms for the establishment of synthetic metabolic interactions when designing synthetic microbial communities (SMC) [12,15,16] (Figure 1A). Therefore, the two main questions emerging when designing a SMC are: i) how will the microbial community structure be established to ensure the consortium's stability? and ii) how will the relationships within the SMC driving the final community output be established?

In terms of stability, relationships can be grouped into two main categories, unidirectional and bidirectional. Unidirectional interactions are those in which the population of one of the components in the consortium is regulated (either positively or negatively) by another component. In contrast, bidirectional interactions are those in which all the microbial components of the community actively interact with each other to maintain the stability of the entire consortium by sharing positive or negative goods (Figure 1B). Similarly, relationships within the community also determine, to a great

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extent, the SMC's functionality and its grade of complexity. Overall, as a function of the complexity level, functionality can be categorized as non-distributed or distributed. Non-distributed functionality implies low complexity level and often only one member of the consortium is responsible for the final community output. Contrary, higher complexity SMC outputs require the involvement of several community partners in the consortium. Labor is split in a distributed process, thus increasing the consortium's efficiency. Therefore, SMC can be classified into four main categories according to the relationships contributing to design stability and functionality: Unidirectional Non-Distributed, Bidirectional Non-distributed, Unidirectional Distributed and Bidirectional Distributed (Figure 1B). In order to define the field's state-of-the-art, in the following sections we have categorized and contextualized current efforts on synthetic microbial consortia engineering based on the above classification. A detailed review of outstanding examples is summarized in Table 1.

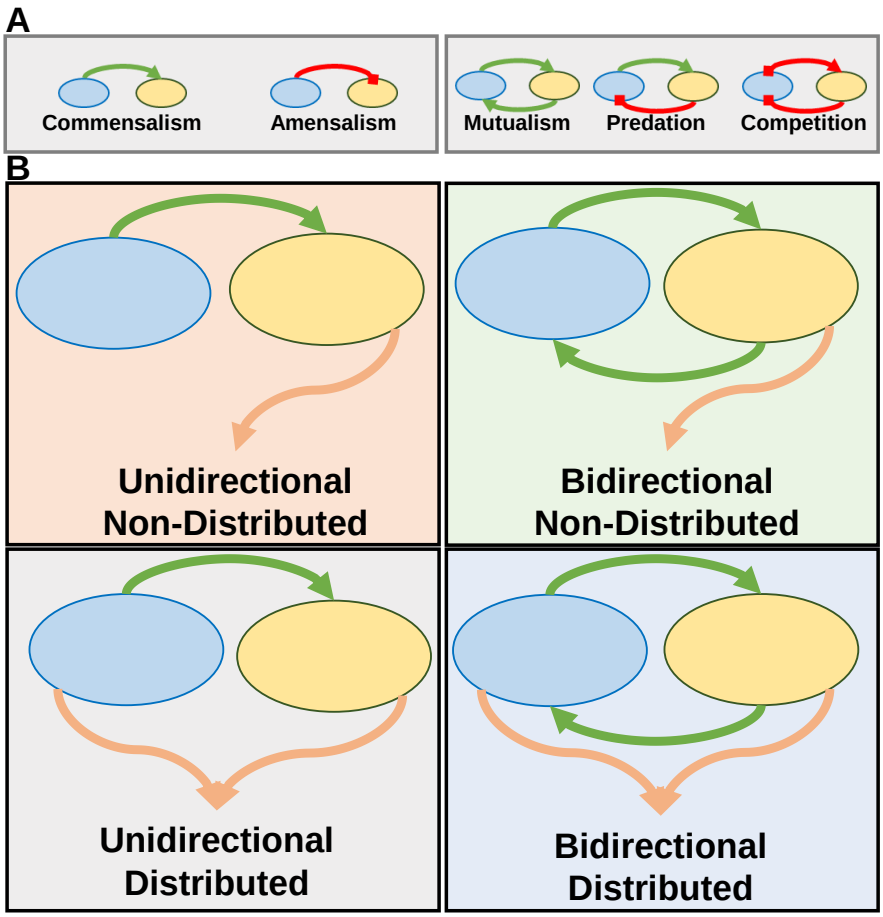

Figure 1. A, Schematic representation of the basic ecological interactions between the microbial strains in co-culture, Green positive and red negative interactions. B, Schematic representation of the SMCs categories. Green stability interactions and orange functionality interactions.

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2.1 Unidirectional Non-Distributed

This category includes the simplest community in which the stability is provided by a single unidirectional relationship (e.g., one microbial component is responsible for feeding the other, either directly or by feedstock processing) while the second strain is in charge of the whole consortium's functionality. Unidirectional Non-Distributed SMCs designs provide significant advantage over single cultures by joining complementary metabolic traits of the cognate microbial partners. Therefore, SMCs expand the scope of the target bioprocess in terms of either access to new feedstock and/or providing additional biosynthetic properties. Under this scenario, a single member will address the catabolism of a complex feedstock (e.g., xylan, cellulose, syrup, etc.) to release low-complexity carbon sources. These easy-to-uptake carbon sources will be subsequently used by the other consortium component which is in charge of delivering the non-distributed biotechnological output. Many consortia fitting these criteria have been already constructed, mainly guided to the production of biofuels. For instance, the fungi *Trichoderma reesei* was used to hydrolyse cellulose in a co-culture with *Escherichia coli*, which was in charge of synthetizing isobutanol [17]. Similarly, the co-culture of two clostridium species (*C. thermocellum* and *C. saccharoperbutylacetonicum*) were used for the production of butanol [16], while a consortium made up of two specialized strains of *E. coli* was used to produce ethanol from xylan [25]. Within this category, an interesting group of consortia are those in which one of the members is the producer of the primary carbon source. For instance, the cyanobacteria *Synechococcus elongatus* has been profusely used as sucrose producer in co-cultures with heterotrophic organisms such as *Pseudomonas putida* and *E. coli* for the production of polyhydroxyalkanoates and 3-hydroxypropinoic acid, respectively [19,20].

2.2 Bidirectional Non-Distributed

These represent an additional layer of complexity. In these consortia, the stability of the community is achieved through the establishment of co-dependency relationships between community microorganisms, while only one consortium member is in charge of the labor. The co-

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dependency relationships allow better control of the stability of microbial populations. An example of co-dependency interaction could be metabolite cross-feeding relationships in which all members of a consortium are responsible for feeding each other. For example, Sgobba *et al* [21] developed a bidirectional non-distributed consortium for cadaverine production in which a lysine auxotroph *E. coli* strain released glucose from starch, feeding *C. glutamicum* that in turn produced lysine for *E. coli*. Bidirectional relationships can also be established by competition mechanisms, e.g. in a co-culture established for *Bacillus subtilis* and *Streptomyces* sp. Mg1, the growth of *B. subtilis* stimulated the production of chalcomycin A by *Streptomyces* sp Mg1, an inhibitor of *B subtilis* growth [22].

2.3 Unidirectional Distributed

This category adds complexity to the unidirectional complexes because all members of the SMC participate jointly in the functionality of the system. Engineering of Unidirectional Distributed SMCs relies mainly on splitting complex biosynthetic pathways among two or more microbial strains. The division of the microbial labor between more than one strain allows for resource optimization, thus reducing the metabolic burdens and increasing the efficiency of the processes. For instance, *E. coli* and yeast *S. cerevisiae* were used for the production of oxygenated taxanes. Both strains were metabolically engineered so that *E. coli* produced taxadiene, which in turn was used by *S. cerevisiae* to produce oxygenate taxane [23]. In this SMC, *E. coli* utilized xylose as carbon source, producing acetate which was used by *S. cerevisiae*. Unidirectional distributed consortia have been extensively used for phenylpropanoids production. Several examples are shown in Table 1. Thus, for example, the production of naringenin was implemented in a SMC using two engineered *E. coli* strains, one for the production of the p-coumarate intermediate and the other to produce naringenin from p-coumarate [24]. A similar strategy was used to produce resveratrol and resveratrol glucosides using *E. coli* synthetic consortia [25,26]. The functional specialization in these kinds of consortia can be addressed by more than two microbial strains. For example, Li *et al.* [27] used three genetically modified strains of *E. coli* for the production of

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rosmarinic acid, and four different engineered *E. coli* strains were used for synthetizing anthocyanins.[28] (see Table 1 for details).

2.4 Bidirectional Distributed

In this category, all members of the SMC participate in maintaining both the stability of the system and its functionality. Bidirectional Distributed SMCs represent a significant increase in the complexity of SMCs. To accommodate the microbial strains for their cooperativeness in the stability and functionality of the consortia, these SMCs require important metabolic engineering processes of the involved strains. One example is a consortium made up of two *E. coli* strains for the production of salidroside [29]. Both strains were engineered to establish cooperative metabolite cross-feeding so that each strain complemented the other's auxotrophy. In parallel, one strain produced tyrosol, which was used by the other to synthetize salidroside. To avoid competition over the carbon source, both strains were also engineered to use different sources of carbon, i.e. the tyrosol producer used xylose and the salidroside producer used glucose.

Overall, it is evident that by increasing the complexity of SMCs in terms of both stability and functionality, the revalorization of complex feedstocks and the cost-effective production of increasingly complex metabolites become more accessible. However, scaling up the design from simple Unidirectional Non-Distributed to Bidirectional Distributed consortia is not always straightforward, but is instead a trial and error process. Thus, more holistic approaches will be required. In this sense, biotechnological applications using monocultures have benefits largely on the construction and application of metabolic models. Given the clear advantages that modelling contributes to the development of more precise and complex SMCs, in the following sections we provide a comprehensive review of classical and recent computational modelling methods developed for the description and engineering of natural and synthetic microbial consortia.

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3. The long journey of community-based modelling: from ecological to genome-scale models.

Multiple community-level modelling approaches have been developed to gain insights in the understanding of complex ecosystems [30,31]. Overall, they have been classified as ecological, individual-based and metabolic models [32,33]. Ecological models describe the community with ecological parameters such as pairwise interactions, growth rates, etc., where interactions depend mainly on correlations. Individual-based models focus on the individual rather than the population level. Finally, metabolic models predict interactions, as ecological models do. However, they are based on a metabolic context while providing a community dynamics description. The lack of knowledge of kinetic parameters is promoting the wide use of stoichiometric, constrained-based rather than kinetic models [34]. These metabolic models are sub-categorized into topological and constrained, as detailed below.

3.1 Ecological Models.

Ecological models are focused on representing and/or discovering potential interactions among different species [35,36]. They include mainly evolutionary game theory and non-linear dynamics, that could be evolved with stochastic processes [37]. Evolutionary game emerges as an adaptation of the classical game theory to biological systems, given that the rationality assumptions to define strategies where the success of one individual depends on the choices of others does not apply in biology [38]. Thus, in evolutionary game theory, natural selection and mutation are what guide changes in the biological community. This theory has been applied to explain the behaviour of microbial communities in terms of interactions such as cooperation [39,40] or competition [41]. A new application of game theory combined with metabolic models has been proposed recently in community modelling to infer evolutionary stable interactions by analysing the behaviour of a pair of microbes with complementary autotrophies and cross-feeding relationships [42].

Lotka-Volterra (LV) are the first non-linear dynamic systems describing biological populations with a mathematical model based on ordinary or partial differential equations. LVs are

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deterministic, not representing randomness present in nature. From a static point of view, these methods infer models as similarity- or regression- or rule-based networks [43]; while from a dynamic point of view, generalized Lotka-Volterra (gLV) is the main approach [44,45]. gLV requires knowledge of the growth rates and interaction strengths of the different community members. gLV equations have been applied to model a variety of different microbial communities including cheese fermentation [46], marine phage communities [47] and human microbiome [48–50]. In this later context, gLV was extended to take external perturbations over time into account [48], and successfully applied to predict species abundances in the community [49]. Finally, it has been possible to qualitatively infer interaction types without a dynamic model, or quantitatively interaction strengths and growth rates when gLV was considered [50].

3.2 Individual-based modelling.

In individual-based modelling (IBM) (or agent-based modelling) [51,52] microbes are individually simulated as concentration state variables rather than at population-level. Each cell evolves over time, following predefined probabilistic rules that introduce the randomness required to model the dynamics. This approach includes genes, transcripts, proteins and metabolites, although usually just a representative subset of them is selected to reduce the complexity. Its main advantage is that they take intra-population heterogeneity into account. A detailed review of IBM models is included in [53].

3.3 Genome-scale metabolic models (GEMs)

GEMs are structured representations of a target organism based on existing genetic, biochemical and physiological information. Therefore, GEMs represent the metabolic capabilities of a particular organism and can be used in combination with algorithms such as Flux Balance Analysis (FBA) to predict phenotype from genotype [54–56]. An important advantage of metabolic models is their accuracy without requiring kinetic information [56]. Therefore, it is not surprising that this approach has started to be applied for modelling not only individual organisms but entire microbial communities, thus resulting in numerous successful stories. Therefore, GEMs are seen by many as optimal computational tools for optimizing SMC-based biotechnological endeavours.

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Community models using GEMs have not been used only at microbial scale, but also to create multi-tissue models such as the liver in humans and to predict the effect of kinetically-modelled drugs [57]. Interestingly this approach has been also applied to the construction of whole-plant models including leaf, stem, seed and root of barley [58]. The combination of several genome-scale models in a community, however, entails several challenges [59]. For instance, defining a community objective function is a tricky point in both biological and mathematical terms. On the other hand, determining exchange rates and metabolic fluxes to constrain the models in a community context is a new topic because current techniques measure the *in vivo* data in an isolated organism and are thus not applicable to the community, where the contribution of the distinct organisms must be measured. An additional challenge is to define the medium composition for the combined culture or simulation of the microbial consortium, taking interchanged metabolites into account.

Due to the increasing interest and applicability of GEMs, we focus here on the different approaches available so far to model microbial communities using these metabolic models. Two different stages can be distinguished in microbial community modelling with GEMs: The first stage includes descriptive methods to understand and describe the communities. The second level of development, which took off recently, advances in building methods that lead not just to the description but also the engineering of these microbial communities.

4. Dynamics-based classification of descriptive metabolic modelling approaches

Descriptive methods for metabolic modelling of microbial communities are useful to describe how consortia work, to understand them and to identify relationships among the strains taking part in the community. However, they do not allow to engineer consortia, as is explained below. Several attempts to classify and categorize the different approaches aimed at modelling microbial communities have been made. The first classification was based on available knowledge about the community and its complexity [60,61]. An alternative categorization was based on the scope of the community, as defined by Bosi *et al* [62]. A most recent classification based on the definition of the objective function have been proposed (simplified linear, multi-level or non-linear

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function) [63]. In order to complement current approaches, we propose a classification from a point of view of the microbial community dynamics (see Table 2). Therefore, we classified available descriptive methods as static/unified, static/multi-part and dynamic.

4.1 Static/Unified methods

This approach considers all strains unified in a common metabolic model, with only one copy of the shared reactions and metabolites. The model is completed by adding strain-specific metabolic content and a combined community-based biomass objective function. This approach, also called 'lumped network' or 'enzyme soup', is the simplest and, although only useful to have a general perspective about how the community works, it allows high scalability (Table 3). In network-based models, the unified approach would be the closest as it considers all reactions in a unique common graph, irrespective of stoichiometry, which is ignored in favour of topology. Network-based models use metabolic reactions of the different strains in the community to build a graph where metabolites are the nodes connected in a directed way, following the reactions' direction from substrates to products, being reactions represented by edges. This approach could be applied to poor-quality GEM reconstructions because the main data source are the reaction chains. Some tools or algorithms designed following this unified approach are:

- Borestein's group uses a graph or network-based community model representation that is regardless of stoichiometry. With this unified static approach, they mainly study relationships among different microbes [64].
- Kbase is a community data-driven network reconstruction [65]. It builds a unique community model rather than aggregating individual models and is focused on predicting interactions between species in a community. In the absence of data for single species due to lack of individual cultivability, this approach uses relevant community-level data as input. Single and community modelling is carried out within the Kbase software platform, including automatic gap-filling analysis by providing a particular community-based growth condition (www.kbase.us) [66].

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- MO-FBA and MO-FVA, multi-objective FBA and FVA (Flux Variability Analysis) algorithms extensions to community level [67]. These methods model microbial consortia by grouping several constrained based individual models in a combined big stoichiometric matrix. The multi-objective is referred to allow a weighted combination of the individual objective of each strain.

4.2 Static/Multi-part methods

This category of models preserves the individual metabolic matrices and has a pool of metabolites, which could be defined by pre-fixed reactions (compartment per guild in other classifications) or with new stoichiometric reactions after a first optimization step (bi-level optimization). The single strain models are directly connected by exchange reactions, assuming no change in the concentrations of extracellular metabolites and no accumulation in the medium. This approach has been profusely applied to describe microbial communities (Table 3). Several algorithms that fit this category are summarized below:

- The method described by Stolyar *et al* [68] provided the first metabolic modelling of a microbial consortium and the distribution of its associated metabolic fluxes. This method has since been applied with different objectives, such as to categorize interactions [69], to estimate medium composition [70], to predict relative biomass abundances [71], and to define a host-pathogen interaction between the human alveolar macrophage and *M. tuberculosis* in a multi-tissue model [72].
- OptCom [73] and d-OptCom [74]: are two closely related methods mainly focused on engineering microbial communities (see a longer description in section 5), although they also have a descriptive version.
- cFBA [71]: this method assumes a balanced and fixed growth rate for all microbes in the consortium. Subsequently, cFBA (community FBA) maximizes this growth rate using a non-linear multi-objective function. This approach implies constant species abundance

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ratios in the community and it's applicable to cells grown in chemostat or in waste-water microbial communities scenarios [59].

- MMinte [75]: this method allows to explore the pairwise microbial metabolic interactions that occur in a community model limited to two strains. Metabolic models are automatically reconstructed using ModelSEED, using metagenomics data as input (16S rRNA sequences).
- SteadyCom [76]: this system maximizes community stability, i.e. constant growth rate across all microbes in the community, with an iterative linear programming approach. Additionally, it applies FVA to predict microbial abundances under changes in uptake rates.
- CarveMe [77]: it is mainly focused on the automatic reconstruction of single strains. In addition, it allows to automatically merge several single-species models into a unique community model, with a common or individual extracellular compartment.
- Microbiome modelling toolbox [78]: a COBRA Toolbox extension to analyse microbial communities, studying interactions (intra- or with the host).
- MICOM [79]: this static approach predicts growth rates and fluxes from *in-vivo* data such as abundances of species in a microbiome sample, consequently it infers metabolic interactions in the microbiota.

4.3 Dynamic methods

Static approaches ignore temporal events. The dynamic or hybrid approaches are based on dynamic Flux Balance Analysis (dFBA) [80], which allows representations of the temporal evolution of the community, including changes on metabolite concentrations and cell densities over time. This approach is preferred to represent microbial interactions because shared metabolites vary dynamically. It is limited by the fact that dynamic approaches entail kinetic parameter configuration and require higher computational resources, running FBA multiple time points per strain in the consortium and thus limiting the analysis to smaller size communities.

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- DMMM [81]: this was the first method using dFBA at community level. In DMMM each strain optimizes its growth rate.
- COMETS [82]: this algorithm includes the spatial distribution of the cells, in addition to the dynamic simulation of communities using dFBA. The biomasses and fluxes per time points reported as output could be visualized using VisANT tool [83].
- MCM [84]: this framework simulates dynamic community models and additionally presents statistical evaluation and parameter calibration based on experimental data. Initially, it was tested in a homogeneous *E. coli* community, and later it combined different species in nitrifying and methanogenic bioreactors [85,86].
- BacArena [87]: this combines metabolic modelling with individual-based modelling, in contrast to population-based modelling (one model per strain with a certain amount of biomass). Therefore, BacArena allows the modelling of metabolically heterogeneous populations in which each individual cell is represented by a unique metabolic model depending on its spatial resource allocation. In BacArena, metabolite diffusion (implemented with partial differential equations) produces gradient concentrations resulting in spatial niches where different metabolic pathways are activated. It predicted novel cross-feeding interactions through fermentation products. COMETS and BacArena allow spatial resolution, taking diffusion parameters into account.
- Daphne [88]: Daphne combines two different modelling strategies: GEM (metabolism) and ODE (Ordinary Differential Equations). ODE allows to model the kinetics of the strain's growth and the medium metabolite consumption and production dynamically, through a set of equations that can be mathematically solved.
- MMODES [89]: this also integrates GEMs and ODEs to simulate the dynamics of the biomass and metabolites over time. In addition, MMODES allows perturbing the community with external longitudinal interventions such as changes in the medium and/or strains composition (e.g. increasing a metabolite concentration and/or biomass of a species).

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4.4 Practical applications of descriptive modelling methods

Overall, each of the three categories of descriptive methods described above are suitable for modelling different microbial scenarios and their interactions. The unified approach is appropriate for systems with limited knowledge and/or many strains, where details about individual assignations of reactions and metabolites could be unknown, such as in metagenomics. Quantifying metabolic fluxes or representing inter-species interactions requires more complex approaches such as the multi-part or dynamic models. However, the dynamic approach is the only one suitable for medium composition and metabolite concentrations prediction because multi-part transfers metabolites from one model strain to another. Therefore, the dynamic approach involves the highest capabilities to represent complex situations in microbial communities. Dynamic approaches take all time-dependent elements into account, although they only can be applied to small communities because they require many details about individual strains, and are more time consuming than the other approaches. Nevertheless, they are recommended for engineered or synthetic microbial communities for biotechnology applications, i.e. scenarios that usually have low species richness.

Static/Unified, Static/Multi-part and Dynamic approaches have been applied to model a large number of microbial communities (see 'modelled species' column in Table 3), in the context of a variety of scenarios; such as food biotechnology [90], human health (including GEMs for microbes, tissues and organs) [91], and marine microbiome [92]. Table 3 collects applications of microbial consortia from a descriptive point of view, grouped by categories defined in Table 2. The most extended approach is the static/multi-part one, with around twice the number of applications of static/unified or dynamic approaches. In some cases, the study defines a new computational method, while in other ones those methods are re-used. Sometimes the corresponding *in vivo* consortium has been deployed, although mostly not. The microbial consortia applications are assorted in terms of: i) species richness, some are monoclonal populations, others are consortia with less than 10 strains or hundreds of heterogeneous strains, such as those present in the gut

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microbiota; ii) species diversity, going from only one cell per strain to big consortia with hundreds of cells per organism; and iii) environment: industrial bio-transformations, human health and plants.

The application of these methods for modelling human gut microbiome has received a special attention [63]. Knowledge about the components of the gut microbial community has increased recently thanks to the improvement in sequencing. However, the relationships among them and between them and the human cells remain mainly unknown. Hence, microbial community modelling techniques contribute to understanding the complex behaviour of the gut microbiome and its associations with human diseases. In many cases, only a small (less than 10) and simplified subset of representative species from the microbiome have been taken into account [93,94]. However, in recent studies the size of the modelled microbiomes has been expanded to tens or even hundreds of species. The static/multi-part applications are often focused on this scenario. Noteworthy, Thiele's group has modelled the metabolism of the whole human gut microbiota communities using constraint-based models [95]. This modelling has been applied to study the interactions between gut microbes and human intestinal enterocytes, under anoxic and normoxic conditions [96]; to predict short chain fatty acids levels to use as treatment for Crohn's disease [97]; or to determine if metformin treatment increases agmatine production by gut microbiota, explaining changes in host lifespan [98]. More recently, microbiome modelling has been contextualized within human organ models [99]. Finally, in multi-omics modelling, metabolome data have begun to be combined with microbiome data [100–102].

5. Engineering metabolic modelling: design and optimization

All the descriptive approaches explained in the previous section refers to the group of non-optimizing modelling methods. In the context of modelling of individual cells, immediately after the development of descriptive methods, new tools targeted on the design and engineering of high performance strains were profusely developed [103], including strain designing algorithms such as OptKnock [104], OptStrain [105], OptGene [106] and GDLS [107], among others. Unfortunately, progress at community level has not caught up, i.e. most of the current approaches do not allow

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design and even less optimization of natural and/or synthetic microbial communities. However, pioneer efforts toward this aim pave the way for future development of fully community-based design and optimization methods. In the design and optimization of SMC, there are assorted goals (and even combined) that can be optimized as a function of the final application. Key parameters/goals to be optimized include, among others: final bioproduction yield, pathway distribution, community stability, medium composition, spatial cells organization and a combination of goals, i.e. a flexible objective. Therefore, beyond methodological classifications [55], the applications of microbial communities engineering can be also grouped according to their target optimization goal (see Figure 2). In this context, there are a few tools that can be considered as generic, i.e. that could be applied in the optimization of several applications (see Table 4). However, multiple applications have been developed just as an *ad hoc* system for optimizing a very particular task (Table 4). In the following sections we describe and categorize in detail the first attempts of GEM-based methods to design microbial communities toward biotechnological applications.

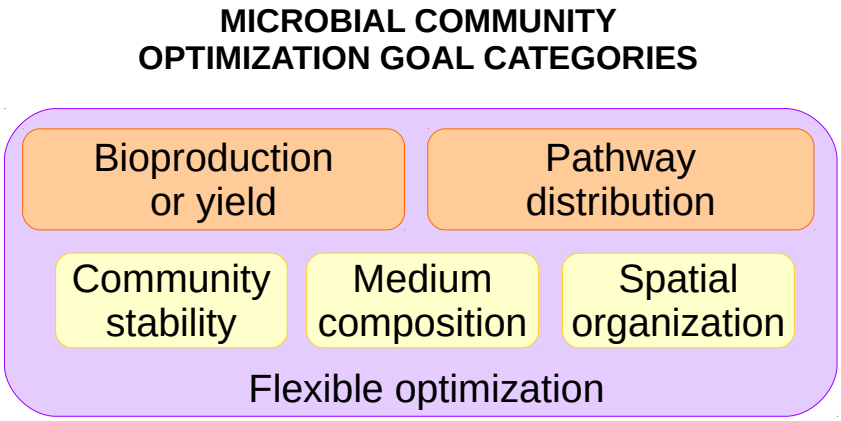

Figure 2: engineering microbial community optimization/design goal categories. Table 4 shows detailed applications and methods of these different categories. Square size according to the number of applications. The flexible optimization category covers all the optimization goals.

5.1 Bioproduction or yield.

The optimization goal in this group of applications is often the maximization of the bioproduction/yield of a specific metabolite of industrial, health or environmental interest. The

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outputs of these methods are parameters linked to the design of the community that fulfils this aim, e.g., strains ratio, C-source ratio, metabolites uptakes, initial biomasses, cross-feeding rates, etc.

The main generic method in this group has been developed by the optimization capabilities of the OptCom system [73]. OptCom implemented a bi-level optimization, at single strain and community levels. By default, OptCom optimizes the community biomass by assuming fixed single strain growth, and returns strains ratio and substrate uptakes and secretion rates. OptCom has been applied to different microbial communities: a syntrophic association through hydrogen between *D. vulgaris* and *M. maripaludis* [73]; a phototrophic microbial community based on *Synechococcus spp* as initial feeder in daylight metabolism [73]; a sub-surface anaerobic environments with electron accepting interactions [73]; and to maximize uranium reduction [74].

Apart from OptCom, some *ad-hoc* methods have been developed to optimize bioproduction: to maximize ethanol production with *S. cerevisiae* and *E.coli* [108]; to maximize flavonoids production with *E.coli* strains using a scaled-Gaussian model [109]; or to maximize yield with three *E.coli* mutants in a chemostat model of competition for a simple sugar (glucose limited conditions) [110].

5.2 Pathway distribution.

Methods and applications in this category allow engineering consortia focused on the fragmentation and distribution of a given complex metabolic pathway between strains within the consortium, thus allowing division of labor through metabolite exchange. They are mainly graph-based methods [36] because they allow easily identifying which species produces a metabolite and which metabolites are exchanged among strains. For example, CoMiDA [111] identifies putative sub-pathways to synthetize a target product for a series of given substrates while minimizing the number of species to combine in a community. Regardless-stoichiometry methods are the most usual within graph-based methods even though they are not independent and require a post-processing step (taking stoichiometry into account) to verify whether their designs are plausible. Some approaches count on stoichiometry, based on MILP (Mixed Integer Linear Programming) rather than FBA, to allocate reactions among the metabolic models of the single strains in the community, in order to optimize a particular community goal (growth rate or uptake of one

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compound) [112]. Contrary to fragment the network, other approaches have been designed to expand the network with an agglomerative algorithm that adds reactions iteratively [113].

Generic methods optimizing pathway distributions have been also developed, including: MultiPlus (static/unified), DOLMN (static/multi-part) and BioLEGO 2 (static/multi-part). MultiPlus [114] has two fixed objectives: minimizing the number of reactions and minimizing the exchanged metabolites, in a *de novo* synthesis pathway; starting from an hypergraph that integrates several GEM models. Following a MILP optimization approach, DOLMN [115] identifies communities able to survive under constraints (e.g. limited number of reactions) that are difficult to identify manually. BioLEGO 2 [116] allows large-scale simulations of several knockouts (KO) simultaneously, running an exhaustive search to identify the KOs maximizing ethanol yield.

5.3 Community stability.

These applications predict optimal individual growth parameters resulting in a stable community over time. Two generic methods have been developed to optimize this goal, d-OptCom [74] and SteadyCom [76].

d-OptCom includes both descriptive (dynamic) and engineering approaches, similar to its static version, OptCom. d-OptCom is a highly complex method, e.g. it requires bilinear FBA solver, optimizing with a global search approach (BARON), kinetics parameters and additional LP constraints need to be defined in order to configure a MILP problem with new reactions for new interactions between strains. In addition, d-OptCom is defined as a 'comprehensive computational framework', not providing any software that allows reproducibility or new applications. It was used to predict the optimal strains ratio in several auxotrophic pairs of *E.coli* consortia [74]. SteadyCom is focused on predicting a common growth rate for all members in the community and then expecting a stable community. Contrary to other multi-objective methods such as d-OptCom and the flexible methods, it entails a fixed objective. SteadyCom's technical features include linear FBA solver complexity and optimizing with iterative LP. Apart from FBA, SteadyCom is compatible with FVA. The method was applied in a multi *E.coli* community with amino acid auxotrophy as proof of

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concept and in a reduced human gut microbiome community represented by 9 species in order to analyse the influence of fibre [76].

On the other hand, the *ad-hoc* CASINO toolbox [94] is a computational platform focused on the human gut microbiome. It is designed to study metabolic interactions among microbial species and the host metabolism. From a static point of view, CASINO predicted alterations of amino-acids metabolism due to diet interventions. This method follows a bi-level optimization approach, like d-OptCom. First, it maximizes growth rate at individual species level to determine uptakes, and, in a second step, the growth rate and resources distribution are optimized at community level. Contrary to other previous methods, CASINO requires experimental data as input including strains abundances to configure their models.

5.4 Medium composition

Applications under this category aim to predict the optimal concentration of metabolites in a given medium providing maximal community performance in terms of growth, production, decontamination, etc. The unique application of this optimization goal is the study of Zampieri and Sauer [117] that described a model-based medium selection to minimize the cost of metabolic cooperation in microbial ecosystems. It maximized metabolite concentration to add to the medium, minimizing the cost of shared essential metabolites while guaranteeing growth only inside a consortium, not individually. It is a comprehensive optimization approach that solves a bi-level MILP problem with high computational complexity. A descriptive static/multi-part approach has also been used to optimize the medium composition [70]. For this, a minimum medium with all metabolites required to sustain growth was initially defined using a pair of metabolic models. Subsequently, they iteratively removed a carbon source (thus hampering growth) and iteratively added a new metabolite to recover growth. It was concluded that medium composition makes symbiotic relationships possible between binary pairs of 7 different strains.

5.5 Spatial organization

The methods developed for this objective predict the physical distribution of the strains belonging to the community along a 2D/3D space. The unique generic method of this group is

IndiMeSH [118], which dynamically modelled bacterial dispersion and nutrient diffusion in a 2D pore network, depending on pore size and nutrient gradients. Modelling space implies simplifications of other issues, such as using a reduced version of the GEMs (reducing reactions and metabolites from thousands to hundreds of them) or integrating all bacterial biomass per spatial unit in a single reaction without intra-species variation. IndiMeSH was applied to the soil habitat with two different consortia: a syntrophic community of *E. coli* with *S. enterica*, and a multistrain community comprising the obligate aerobic *P. putida* and the facultative anaerobic *P. stutzeri*. BacArena and MMODES, listed above in the descriptive methods, include some spatial features, although optimizing spatial organization is not their principal goal. In an *ad hoc* application, GEMs were combined with partial differential equations (allowing metabolite diffusion), which resulted in a dynamic model that was able to predict the biofilm thickness [119]. Spatially Linked Microbial Consortia (SLMC) is a conceptual design to engineer consortia. Spatial distribution is optimized through isolated modules with distinct growth medium to increase the control and facilitate new strains combination. SLMC is reviewed by Sala [120] by including GEMs in the design of compatible synthetic communities.

5.6 Flexible optimization

This section describes applications of methods accounting for a goal-agnostic optimization, i.e. the optimization goal and consortium parameters to be predicted can be independently defined and be different in each case. They allow designing and engineering microbial communities by selecting the best consortium configuration to optimize a given goal. To the extent of our knowledge, FLYCOP [55] is the only framework capable of doing this. The goal can be defined in a flexible way depending on the consortium's functionality and the particular interest, including community growth rate, stability, medium composition, etc. For example, FLYCOP can be configured to optimize the medium composition with a finite list of metabolites from which to select the final compounds and their initial concentrations. A flexible approach could contribute to the metabolic modelling of microbial communities, apart from different and multiple optimization goals not limited to maximizing growth rate. One example of this are applications where we would seek

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to maximize yields of a product of interest and its synthesis pathway has been split among different strains. Another example would be the comparison of the optimization of different products as is done experimentally in [23] with different fitness functions. Another advantage of FLYCOP's flexible approach is that multiple parameters can be optimized at once rather than one independent optimization process for each parameter [108]. Besides, the flexible approach lends itself to applications with obligatorily mutualistic communities, while other engineering approaches do not, e.g. d-OptCom [74]. However, at the individual level, bioproduction optimization methods can maximize yield, while FLYCOP can optimize the flux of a reaction within the metabolic model. FLYCOP could manage applications including GEMs with thousands of reactions versus other methods limited to small models. The flexibility is also applied to single-strain models where each has a different growth rate, thus not requiring a single growth rate for all strains in the model as other applications do [71,121]. Additionally, the FLYCOP approach allows an automatic search optimization versus a systematic exploration of different configurations. Regarding technical features, FLYCOP has linear FBA solver complexity and optimizes with a local search approach (SMAC).

Among the different categories of applications when engineering microbial communities with GEMs (see Figure 2), the widespread optimization goals are bioproduction yield followed by pathway distribution. There are fewer examples focused on the optimization of the other parameters e.g., stability, medium composition, spatial organization, or even a flexible goal. The most common applications are to two-strain consortia (see 'strains' column in Table 4), although there are also some with a higher number of strains, both homogeneous and heterogeneous.

6.- Summary and Outlook

As we realize that microbes rarely act alone but in the context of complex communities, the need for computational tools able to provide mechanistic knowledge about how these communities work and evolve over time becomes critically important. Microbial communities are already recognized as critical players in human health and they have begun to be seen as promising biocatalysts in biotechnology. Following the development of modelling approaches towards

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individual cells and pioneer efforts on community-level modelling, it is largely expected that methods for the efficient analysis and engineering of such communities will appear in the coming years.

In this context, there is a long debate about the real objective function at community level [122]. The general assumption is that the microbial community's goal is to maximize growth under a natural selection scenario. However, optimizing biomass might not be the right microbial goal with genetically engineered organisms or when the environment is different from that where its evolution occurs [84]. Thus, alternative community configurations implying alternative goals are ignored by most of the available methods. Thus, it would be interesting to have methods that allow the optimization of different community goals.

Current dynamic methods rely on the analysis of a few species and only static/unified methods can be used to analyse complex communities, thus preventing the deeper understanding of such communities. An important challenge to address in the near future is the development of tools allowing dynamic analyses of large microbial communities in the context of high-quality GEMs [63]. This will require not only an increase in the current portfolio of GEMs but collecting large sets of kinetic parameters while developing novel computational methods to reduce the very time-consuming solving step using dFBA.

In the long term, model-guided microbial community engineering should trend towards the development of technologies capable of determining potential genetic modifications, at community-level (similar to individual-level OptKnock or GDLS algorithms). The same applies to all the useful and comprehensive COBRA-related algorithms applied to design individual engineered strains.

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Table 1. Recent examples of engineering Synthetic Microbial consortia.

Microorganism	Interaction	Goal to optimize	C-source	Yield	Ref.
Unidirectional Non-Distributed					
Synechoccocus elongatus *Pseudomonas putida*	*S. elongatus* produces sucrose from CO_2 and light. It was used for *P. putida,* growing and cleaning 2,4-DNT while produces polyhydroxyalcanoates (PHA)	− Sucrose production in presence of 2,4-DNT − 2,4-DNT cleaning − PHA production	CO_2	− 1.2 g/L sucrose at 120h. − 250 mM 2,4-DNT cleaning at 24 h. − 5.1 mg/L day PHA	[20]
S. elongatus *Escherichia coli*	*S. elongatus* produces sucrose from CO_2 and light. The sucrose is used as C-source for *E. coli,* producing 3-hydroxypropinoic acid (3-HP)	− 3-HP production − Sucrose production	CO_2	− Up to 68.29 mg/L 3-HP at 7 days − 600 mg/L sucrose at 144 h	[19]
Klebsiella pneumoniae *Shewanella oneidensis*	*K. pneumoniae* uses glycerol as C-source, producing lactate. *S. oneidensis* uses the lactate producing electrons.	− Lactate production − Flavin production (S. oneidensis) − Inoculum ratio − Electric power	Glycerol	− 2.1-times increase lactate production − 7.9-time increase flavin production − Inoculum ratio 1:10 − 19.9 mW/m^2 power density	[123]
Ralstonia eutropha *Bacillus subtilis*	*B. subtilis* hydrolyses sucrose in fructose and glucose, producing propionic acid. They are used by *R. eutropha,* producing PHA or poly (3-hydroxybutyrate-co-3hydroxyvalerate) [P(3HB-co-3HV].	− Biomass − PHA production − P(3HB-co-3HV) production	Sucrose	− Biomass 3.79 g dcw/L − PHA 63% w/w − P(3HB-co-3HV) 66% w/w	[124]
Citrobacter amalonaticus *Sporomusa ovata*	*C. amalonaticus* uses CO as carbon source, producing CO_2 and H_2 which are used by *S. ovata* producing acetate	− Acetate production	CO	− 0.157 mM acetate from 0.439 mM CO	[125]
Trichoderma reesei *Rhizopus delemar* *or* *T. ressei* *R. orizae*	*T. reesei* hydrolyses cellulose into monomeric sugars. *R. delemar* uses these sugars producing fumaric acid and *R. oizae* producing lactic acid.	− Organic acids production	Corn stove	− 6.87 g/L fumaric acid − 4.4 g/L lactic acid	[126]
Clostridium thermocellum *C. saccharoperbutylace*	*C. thermocellum* hydrolyses cellulose releasing the C-source for butanol production by *C. saccharoperbutylacetonicum*.	− Butanol production	Rice straw	− 6.5 g/L butanol from 40 g/L rice straw	[16]

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tonicum					
E. coli *Acinetobacter baylyi*	*E. coli* utilizes glucose as C-source producing acetate. The acetate is used by *A. baylyi*	− *E. coli* biomass accumulation − Acetate removal	Glucose	− Increase of *E. coli* biomass from 2.1 g/l in monoculture to 5.1 g/l in co-culture − Acetate reduction from 13 mM to 3mM	[127]
T. reesei *E. coli*	*T. reesei* hydrolyses cellulose into monomeric sugars. *E. coli* uses these sugars producing isobutanol.	− Isobutanol production	Cellulose	− 1.88 g/L from 20g/L cellulose	[17]
E. coli *E. coli*	*E. coli* E609Y produces xylanase extracellularly, hydrolysing xylan to xylooligosaccharides. they are used by *E. coli* KO11 producing ethanol.	− Xylane hydrolysis − Ethanol production	Xylan	− 38.6% hydrolysis − 3.71 g/L ethanol	[18]
Rhodotorula glutinis *Dwbaryomyces castellii*	*D. castelli* hydrolyses corn syrup into sugars, which are used by *R. glutinis*, producing carotenoids.	− Carotenoids production	Corn syrup	− 8.2 mg/L carotenoids	[128]
Bidirectional Non-Distributed					
E. coli Corynebacterium glutamicum	*E. coli* (Lys auxotroph) produces amylase extracellularly, hydrolysing starch into glucose, which is used by *C. glutamicum*, producing cadaverine or L-pipecolic acid (L-PA) and Lys, necessary for *E. coli* growth.	− Production of Lys and cadaverine or L-PA	Starch	− 12.3 mM Lys − 6.8 mM cadaverine or 3.4 mM L-PA	[21]
Sacharomyces cerevisiae -Bacillus. Amyloliquefacien or *S. cerevisiae - Lactobacillus fermentum*	*B. amyloliquefaciens* / *L. fermentum* produces amylase, hydrolysing starch into glucose and oligosaccharides. they are used by *S. cerevisiae*. Its growth stimulates the production of more amylase for *B. amyloliquefaciens* / *L. fermentum*.	− α-amylase production − Co-culture conditions	Starch	− 1.8-times increase α-amylase production − Bacterial;yeast ratio of 1:125; T^a of 33.5ºC and pH of 5.5	[129]
Streptomyces sp. Mg1 *B. subtilis*	In co-culture *B. subtilis* stimulates *Streptomyces* sp Mg1 to produce chalcomycin A (macrolide antibiotic). Chalcomycin A inhibits *B subtilis* growth.	− Chalcomycin A	Maltose	− n.d.	[22]
Pseudomonas aeruginosa *Enterobacter aerogenes*	*E. aerogenes* use glucose, producing 2,3-butanediol which is used by *P. aeruginosa* producing phenazines, They are used for *E. aerogenes* as electron acceptor.	− Electric density	Glucose	− 14-times increase of the electric density	[130]
Unidirectional Distributed					

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E. coli *S. cerevisiae*	Hydrogel compartmentalized *E. coli* and *S. cerevisiae* were co-cultured, using glucose as C-source, *E. coli* produces L-DOPA, that is used by *S. cerevisiae* to produce betaxhantins	− Stability of the compartmentalized consortium − Inoculum ratio − Betaxhanthins production	Glucose	− Up to 10 times reutilization of the compartmentalized consortium − Inoculum *S. cerevisiae*:*E. coli* ratio of 6:1 − Optimized betaxhantin production	[131]
Three *E. coli* strains	The rosmarinic acid biosynthetic pathway was divided in three *E. coli* strains, one producing caffeic acid, other salvinic acid, and a third strains that use those intermediaries to produce rosmarinic acid. All of them use glucose as carbon source	− Rosmarinic acid	Glucose	− 172 mg/L rosmarinic acid	[27]
E. coli *E.coli*	The glutarate biosynthetic pathway from Lys was splitted in two *E. coli* strains. The first one use Lys, producing 5-aminovaleric acid, that is used by the second *E. coli* strain producing glutarate	− Glutarate production	Lysine	− 43.8 g/L glutarate	[132]
E. coli *E.coli*	*E. coli* RES produces resveratrol from p-coumarate. The resveratrol is glycosylated by *E. coli* RGL. Both strains use glucose as carbon source.	− Resveratrol glucosides	Glucose	− 92 mg/L resveratrol glucosides	[25]
Halomonas sp. HL-48 *Marinobacter sp. HL-58*	When both strains are growing using glucose as carbon source they compete for it. When xylose is used instead of glucose, *Halomonas* consumes xylose, producing metabolites that are used for *Marinobacter* growth.	− Growth	Xylose	− Changed from competitive to cooperative interaction the growth was improved in co-culture	[133]
E. coli *E. coli*	*E. coli* P2C produces Tyr and p-coumarate from glucose. Both are used for *E. coli* BLNA to produce naringenin using glucose as carbon source	− Inoculation ratio − Naringenin production	Glucose	− P2C:BLNA ratio 1:5 − 41.5 mg/L naringenin at 36 h	[24]
Four strains of *E. coli*	The synthetic plants pathway to produce Anthocyanins was divided and inserted in four different *E. coli* strains. The first produces phenylpropanoic acid, that is used for the second, producing flavonones. A third strain produces flavan-3-ols from flavonones. Finally, the last *E. coli* strain produces anthocyanins from flavan-3-ols.	− Anthocyanins production	Glucose	− 9 mg/L anthocyanidin-3-O-glucosides	[28]
E. coli *E. coli*	The resveratrol biosynthetic pathway is divided in two E. *coli* strains. Both strains use glycerol as carbon source. One of them produces P-coumarate, which is used for the other to produce resveratrol.	− Resveratrol production	Glycerol	− 22.6 mg/L resveratrol in 30 hours	[26]

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E. coli *S. cerevisiae*	*E. coli* utilizes xylose as C-source, producing acetate which is the C-source for *S. cerevisiae*. In parallel, *E. coli* is producing taxadiene, that is oxygenated by *S. cerevisiae*.	− Co-culture stability − Oxygenated taxanes	Xylose	− 33 mg/L oxygenated taxanes	[23]
E. coli *E. coli*	One *E. coli* strain uses xylose as C-source, producing 3-dehydroshikimic acid (DHS), uses for the other strain to produce muconic acid or 4-hydroxybenzoic acid, using glucose as C-source.	− Muconic acid − 4-hydroxybenzoic acid	Glucose Xylose	− 4.7 g/L of muconic acid − 2.3 g/L of 4-hydroxybenzoic acid	[134]
Four strains of *S. cerevisiae*	The enzymatic pathway to produce ethanol from cellulose was divided in four *S. cerevisiae* strains.	− Ethanol production	Cellulose	− 1.25 g/L of ethanol	[15]
Bidirectional Distributed					
Dietzia sp strain DQ1245-1b *Pseudomonas stutzeri SLG510A3-8*	*Dietzia* uses hexadecane as C-source, producing hexadecanoid acid, α-ketoglutaric acid and R-3-hydroxybutanoic acid, that are used by *P. stutzeri,* that in turn produces glutamate and acetate for *Dietzia*. The consortium increase the diesel degradation	− Diesel biodegradation	Hexadecane	− 85.54 % diesel removal	[135]
E. coli *E. coli*	One *E. coli* strain uses xylose, producing tyrosol. The other consumes glucose and produces salidroside (from tyrosol). The relationship between both strains had been stablished by cross-feeding. The xylose consuming strain is Phe auxotroph, while the glucose consuming is Tyr auxotroph.	− Salidroside production − C-source ratio − Inoculum ratio	Xylose Glucose	− 6.03 g/L at 120 h fermentation − Glucose:xylose ratio 4:1 − Inoculum ratio tyrosol producer:salidroside producer 1:2	[29]
E. coli *E. coli*	One *E. coli* strain uses glucose as C-source, producing lysine. The other *E. coli* strain intakes the lysine producing cadaverine. This strain use glycerol as carbon source	− Cadaverine production − C-source ratio − Inoculum ratio − C:N ratio − Fermentation conditions	Glucose Glycerol	− Up to 28.5 g/L with constant feeding at 40 h − Glucose:glycerol ratio 8:1 − Strains ratio 10:1 − C:N ratio 3:2 − others	[136]
E. coli *B. subtilis* *S. oneidensis*	*E. coli* utilizes glucose as C-source, producing lactate and an electron donor; *B. subtilis* uses also glucose producing riboflavin as an electron shuttle. *S. oneidensis* uses the electron donor and the shuttle generating electricity and oxidizing lactate to acetate, which is used by *E. coli* and *B. subtilis* as C-source	− Electricity production	Glucose	− 15 days production with an efficiency of 55.7%	[137]
S. oneidensis *E. coli*	*E. coli* ferments glucose producing formate, which is used by *S. oneidensis*, producing flavins, uses by	− Current density	Glucose	− Increase of the current density to 2.0 μA/cm^2.	[138]

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	E. coli. Their activity increase the electric current from cathode to anode in a MFC				
E. coli *E. coli*	*E. coli* L is Leu auxotroph and *E. coli* K is Lys. They co-culture provide each other with the necessary amino acids, increasing the growth rate and the biomass.	− Growth	Glucose	− 3-fold growth rate increase	[139]

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Table 2: descriptive microbial community modelling methods classification. The 'In-vivo consortia categories' defines the most complex category from those defined in Figure 1 that could be modelled with the descriptive computational approach (both unidirectional and bidirectional could be modelled in all computational categories).

	In-vivo consortia categories	**Properties**
Static / Unified	Uni/Bidirectional Non-Distributed	- Unique GEM - Combined biomass objective function - No metabolite exchanges - High number of strains
Static / Multi-part	Uni/Bidirectional Distributed	- Individual GEMs - Pool of metabolites - Models connected by direct exchange reactions - No metabolite accumulation in the medium
Dynamic	Uni/Bidirectional Distributed	- Allowing community evolution over time - Metabolite concentration in the medium - Low number of strains

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Table 3: applications descriptive microbial community modelling approaches. There are three blocks corresponding to the descriptive modelling approach category described in Table 2. The 'tool' column includes the name of the algorithm or method defined in that application to describe the communities, and link to the software if it is available. '*In vivo* validation' column indicates if the application has been validated with *in vivo* data or they are *in silico*-based results.

Modelled Species	Application	*In vivo* validation	Tool	Ref.
Static / unified				
Several anaerobic fermentative strains	Description of product formation in fermentative conditions, from glucose depending on pH and substrate concentration.	No	*ad hoc*	[140]
- 478 species *- 154 human microbiome species*	Large-scale studies based on integration of metabolic capabilities in a common network with multiple species, with nodes representing metabolites and edges connecting substrates to products. Phylogenetic analysis and prediction of interactions based on that metabolic network.	No	*ad hoc*	[141,142]
Assorted 113 bacterial species	Study of metabolic variability and cohabitation categorize interactions versus growth rate.	No	*ad hoc*	[143]
Synechococcus spp, Chloroflexus spp, and sulfate reducing bacteria	The first microbial consortia modelling classification, representing the consortium with different approaches. Description of relative abundances, biomass productivity and generation of toxic by-products.	No	*ad hoc*	[60]
Clostridium cellulolyticum, *Desulfovibrio vulgaris* Hildenborough, and *Geobacter sulfurreducens*	Study of trophic and electron accepting interactions of subsurface anaerobic environments.	Yes	*ad hoc*	[144]
2 naphthalene-contaminated soil communities; with 13 and 12 species, including: Achromobacter, Azospirillum, Comamonas, Achromobacter and Pseudoxanthomonas [145].	Description of common metabolic network of naphthalene-degrading bacterial communities based on metaproteomic and taxonomic data.	Yes	*ad hoc*	[146]
261 assorted species of diverse habitats, such as soil, water and the human gut.	Study the extent of resource competition and metabolic exchanges in over 800 microbial communities.	No	*ad hoc*	[147]
Microbialites and microbial mats (structures similar to corals and stromatolites)	Study of autotrophic capabilities (identification of pathways for C and N assimilation) with a metabolic network based on metagenomics data.	No	*ad hoc*	[148]

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Thermosynechococcus elongatus BP-1 and Meiothermus ruber Strain A	Study of photoautotrophic cyanobacterium-heterotroph consortium.	No	KBase	[65]
The same as Taffs et al., 2009 [60] *(see above)*	Description of ecosystem of hot spring microbial mats, with different behaviour between day and night.	No	MO-FBA/ FVA	[67]
Static / multi-part				
D. vulgaris and Methanococcus maripaludis	Study of mutualistic interactions between sulphate-reducing bacteria and methanogens, predicting fluxes (intracellular and exchange between species).	Yes	*ad hoc*	[68]
Clostridium butyricum and Methanosarcina mazei	Studying a syntrophic interaction to increase methane production in anaerobic conditions, with an efficient consumption of by-products.	No	*ad hoc*	[149]
- Hepatocyte (liver), adipocyte (fat) and myocyte (skeletal muscle) human cells *- leaf, stem and root of Arabidopsis thaliana cells*	Defining multi-tissue models, to study diabetes in human (including gene expression data) or analysing how *Arabidopsis* minimizes energy usage for plant growth.	No	*ad hoc*	[72,150]
Plasmodium falciparum and the host red blood cell (erythrocyte)	Study of the metabolism of malaria infection, over different life cycle stages of the pathogen.	No	*ad hoc*	[151]
E. coli, Bacillus subtilis, Helicobacter pylori, Salmonella typhimurium, Methanosarcina barkeri, S. oneidensis and Methylobacterium extorquens	Estimation of medium composition to allow symbiosis between binary pairs of species.	No	*ad hoc*	[70]
46 pairs of auxotroph E. coli	Description of synthetic mutualism interactions in auxotrophic *E.coli*.	Yes	*ad hoc*	[152]
The same as Stolyar,2007, Taffs *et al.*, 2009 and *Miller et al.,2010* [60,68,144] *(see above)*	Quantifying a syntrophic association; assessing the level of sub-optimal growth in phototrophic microbial mats depending on community composition; and evaluating the direction of inter-species metabolite and electron transfer.	No	OptCom	[73]
- Two imaginary species 'i' consuming glucose and ammonium and producing succinate and species 'j' consuming succinate, fixing nitrogen gas and excreting ammonium. *- E. coli polymorphism in Long Term Experimental Evolution experiment* [153].	Analysis community parameters (relative biomass abundances, etc) at balanced growth.	No	cFBA	[71]
Geobacter metallireducens and G. sulfurreducens	Study of interspecies electron transfer mechanisms in syntrophic associations, in genomic and transcriptomics.	Yes	*ad hoc*	[154]

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Bacteroides thetaiotamicron, Eubacterium rectale and Methanobrevibacter smithii	Prediction of interactions between 3 key representative bacteria in the human gut, and analysing their individual contributions to secrete SCFA.	Yes	*ad hoc*	[93]
Bifidobacterium adolescentis L2-32 and Faecalibacterium prausnitzii A2-165	Predicting demand for acetate and production of butyrate, in 2 gut strains related to Chron's disease, using OptCom tool.	No	*ad hoc*	[155]
Ketogulonicigenium vulgare and Bacillus megaterium	Understanding of vitamin C production by an artificial consortium, study of subsystems and other possible metabolites to secrete.	No	*ad hoc*	[156]
11 representative gut microbes (E. coli, H. pylori, Salmonella enterica, S. thermophilus, etc.)	Study of interactions between gut microbes and human small intestinal enterocytes, under anoxic and normoxic conditions.	No	*ad hoc*	[96]
Leptospirillum ferriphilum and Ferroplasma acidiphilum	Study of bioleaching (oxidizing iron) in a bacteria-archaea consortium presents in natural environment, with chemo-mixotrophic growth.	No	*ad hoc*	[157]
AOB, ammonia oxidizing bacteria: Nitrosomonas europaea, Nitrosomonas eutropha, Nitrosospira multiformis, and Nitrosococcus oceani. And NOB, nitrite oxidizing bacteria: Candidatus Nitrospira defluvii, Nitrobacter winogradskyi, Nitrobacter hamburgensis, Nitrospina gracilis.	Assessment of NO redox reactions contributes to N2O formation during nitrification, in 9 different consortia with variable composition selected among 4 AOB and 4 NOB.	Yes	*ad hoc*	[158]
- Human Microbiome Project data *- Desulfovibrio piger, B. thetaiotaomicron, Bacteroides caccae, Bacteroides ovatus, E. rectale, Marvinbryantia formatexigens, Collinsella aerofaciens, E. coli and Clostridium symbiosium*	Exploring pairwise microbial metabolic interactions, using 16S data from microbiome studies. Evaluating a sulphate-reducing bacteria growth in gut microbiome with different diets with data from [159]..	No	MMinte	[75]
- 4 *E. coli* auxotrophic for amino acids - Gut microbiome	Maximizing community stability (common growth).	No	SteadyCom	[76]
- 74 human gut bacterial strains from AGORA collection *- 5587 species from NCBI RefSeq in groups of 20 strains per community*	Automatic reconstruction of single strain models (from 238 to 2472 reactions per model) with the possibility to merge in a community one, analysing the number of compounds that can be exchanged.	No	CarveMe	[77]
Human gut strains from AGORA collection [95] *and human cells (Brunk et al., 2018)* [160]	Analysis of pairwise interactions (microbe-microbe and host-microbe) of different types (competition, parasitism, etc.) with a join matrix of GEMs, and modelling of microbial communities given the relative abundances, used to	No	Microbiome modelling toolbox	[78]

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	personalize community biomass reaction and simulating under different diets.			
Human gut strains from AGORA collection	Predicting growth rates and metabolic fluxes from microbe abundances as input. Using an heuristic optimization approach based on L2 regularization to allow different growth rates per strain.	No	MICOM	[79]
Dynamic				
E. coli	Exploring the metabolic variability among bacterial strains and identifying interactions, across different single-carbon-source conditions. They use a combination of a graph-theoretic approach together with a metabolic model.	No	*ad hoc*	[161]
Clostridium acetobutylicum and Clostridium cellulolyticum	Improving bioprocessing of cellulose with a clostridial consortia, with DMMM.	No	*ad hoc*	[162]
G. sulfurreducens and Rhodoferax ferrireducens	Designing of uranium bioremediation scenarios with two competing heterogeneous species	No	DMMM	[81,163]
- E. coli auxotrophs *- G. sulfurreducens, R. ferrireducens, and S. oneidensis*	Study of impact of lactate vs acetate addition on the composition of uranium-reducing community. In-vivo validation of *E.coli* auxotrophs with Wintermute and Silver, 2010 [164] results.	Yes	d-OptCom	[74]
E. coli, S. enterica and Methylobacterium extorquens	Simulation of spatiotemporal dynamics of microbial communities, predicting species ratios and investigating the influence of spatial structure on competition in mutualistic systems, and with a competitor between the cross-feeding pair.	Yes	COMETS	[82]
Homogeneous E.coli consortia	Combining metabolic model with statistical analysis and calibration to experimental data, in this case related to Lenski's experiment LTEE.	Yes	MCM	[84]
- E. coli and S. enterica [87] *- B. fragilis, B. longum, C. difficile, E. coli, H. pylori and L. acidophilus*	Visualization of metabolic interaction networks between microbes in a community.	No	VisANT	[83]
E. coli (*E. coli* B, not K12)	Analysis of evolution. LTEE: divergence in glucose-limited conditions, with daily transfers.	No	evoFBA	[165]
- Clostridium beijerinckii and M. barkeri *- Anaerostipes caccae, B. thetaiotaomicron, Bifidobacterium longum, Blautia producta, Clostridium ramosum, E. coli and Lactobacillus plantarum*	Analysis of interactions and spatial and temporal distributions of microbes in communities using individual-based metabolic modelling.	No	BacArena	[87]

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L. plantarum	Study of cross-feeding with short-chain fatty acids from glucose in the human gut microbiome, using DMMM with spatial addition. The *L. plantarum* GEM is converted in a 'supra-model' increased by pathways crucial in carbohydrate fermentation in the colon.	No	*ad hoc*	[166]
N.s europaea and N. winogradskyi	Study of the dynamics of nitrification-derived N oxide production, with aerobic ammonia- and nitrite-oxidizing bacteria, using DMMM.	Yes	*ad hoc*	[167]
E.coli	Analysis of diauxic shift in two homogeneous subpopulations, combining ordinary differential equations (ODE) with GEMs.	No	Daphne	[88]
- F. prausnitzii and B. adolescentis *- P. aurescens, H. stevensii, Halobacillus sp.*	Simulation of heterogeneous microbial communities behaviour over time with ODE and GEMs under perturbations, i.e. changes in availability of metabolites and biomass of different strains.	No	MMODES	[89]

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Table 4: engineering modelling applications. Grouped by the optimization community goal. Focus on optimization/engineering topics. 'Bioproduction' group includes to optimize different community parameters (strains ratio, carbon source ratio, initial biomass, etc). GR=Growth Rate. Output means the configuration parameters that are predicted. If there is a software available, it is referred to and linked in the column 'references' too.

Specific goal of optimization	Output	Strains	Results and additional details	Ref.
Bioproduction				
Maximizing ethanol production	- carbon source ratio (glucose/xylose) - mutant initial biomasses	- *S. cerevisiae (or S. stipitis)* - *E. coli*	- ethanol productivity of ~1.08 gr/L/h - *In vivo* experiments to determine kinetics parameters	[108,168,169]
Maximizing flavonoids production	- carbon sources ratio (glucose/glycerol) - strains ratio	*E. coli strains* (flavonoid pathway fragmented in 2 strains)	- Using a scaled-Gaussian model: carbon source ratio of 0:1 (glucose:glycerol), strains ratio of 7:3 (upstream:downstream) - Production of flavonoids to 40.7±0.1 mg/L, i.e. a 970-fold improvement - Also *in vivo* experiments to validate the results	[109]
2 maximization goals: - methane production (high community GR) - methane yield (low community GR)	- initial biomasses (strains ratio) - flux rates (input and output metabolites)	- *D. vulgaris* - *M. maripaludis* - *M. barkeri*	- Predicted (max. methane, ATP and biomass yield) and some *in vivo* data (biomass yield and ATP maintenance) - Low biomass yield per strain, vs community goal - 2 first strains consortium: 0.45 mol. methane/mol. ethanol - *In vivo* validation with literature data from [170]	[121]
Maximizing yield	- initial glucose concentration for stable consortia - strains ratio - uptake glucose and glycerol	*E. coli:* -glucose specialist CV103-'respirer' - acetate specialist CV101-'fermenter' - glycerol specialist CV116	- *In vivo* data from [153]. Originally growing in tryptone - 3 mutants after evolution in-vivo, with different GRs - Glucose limited conditions (LTEE) - Chemostat model of competition for a simple sugar - *In silico* model predictions for different glucose concentrations - >0.0033% of acetate specialist to allow a viable consortium - Strain rations: CV101:CV103:CV116 ~= 0.10:0.65:0.025 - CV103 best takes up the limiting resource glucose, but excretes acetate and glycerol (and/or a closely-related compound, glycerol 3-phosphate)	[110,171]
Maximizing (together): - community biomass - yield per single	- strains ratio - substrate uptakes	- *D. vulgaris* - *M. maripaludis*	- *In vivo* data from [68] - *In silico* model with OptCom - Strain ratio: 2:1 *in vivo* and 2.28:1 *in silico* - lactate uptake= 48 μM/h	OptCom [73]

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strain (OptCom fixed goal)			- formate and hydrogen accumulation =0 - Additional *in silico* predictions: concentration of acetate, methane, CO2 and total biomass	
Maximizing (together): - community biomass - yield per single strain (OptCom fixed goal)	- strains ratio - O_2/CO_2 ratio	- *Synechococcus* spp (SYN) - filamentous anoxygenic phototrophs (FAP) related to *Chloroflexus* and *Roseiflexus* spp - sulphate-reducing bacteria (SRB)	- *In vivo* data from [60]. - *In silico* model with OptCom - Fluxes ratio O2/CO2 reactions: 0.03-0.07 - Strain ratio: 1:6:1 experimentally, and from 1:5:1 to 3:5:1 with metagenomics data - SYN/FAP strain ratio: 1.5-3.5 *in vivo* and from 7.94 (with O2/CO2=0.07) to 20.26 (0.03) *in silico*	OptCom [73]
Maximizing (together): - community biomass - yield per single strain (OptCom fixed goal)	- strains ratio - substrate uptakes	- *C. cellulolyticum* - *D. vulgaris* - *G. sulfurreducens*	- *In vivo* data from [144]. - *In silico* model with OptCom - Biomasses: 0.8:0.1:0.13 *in vivo* and 0.036:0.0045:0.0059 in *silico* - acetate: 2.7 *in vivo* and 2.48 *in silico* - CO2: 3.3 *in vivo* and 3.2 *in silico* - Several metabolite fluxes details in Fig.5	OptCom [73]

Maximizing uranium reduction	- strains ratio
	- acetate and Fe(III) uptakes
		- *S. oneidensis* (acetate producer)
		- *G. sulfurreducens*
		- *R. ferrireducens*
		Two first ones are uranium reducers
			- *In vivo* data from [163].
			- *In silico* model with OptCom
			- Carbon source: lactate= 5mM
			- In ammonium excess condition ([NH4] = 400 µM)
			- Decrease in the biomass of the uranium-reducing species (SO, GS):
			  - Strain ratio max.community biomass: 0.056:0.051:0.055
			  - Strain ratio max.uranium reduction: 0.039:0.041:0.056
			- Acetate (GS/RF): 14.9/1.49 when max.uranium reduction
			- Fe(III) (SO/GS/RF): 28.3/110/2.06 when max.uranium reduction
			- Alternative optimization objective in the manuscript
				OptCom [74]

2 cases of study: - maximizing butyrate production - maximizing atrazine degradation	Interventions in medium composition or biomass of strains	- *F. prausnitzii and B. adolescentis* - *P. aurescens, H. stevensii, Halobacillus sp.*	- *In silico* model combining GEMs with a Markov Decision Process - Predict how to modify the community over time to reach a state of maximum performance - Intervention for max. butyrate: inulin increase - Intervention for max. atrazine degradation: depending on the microbiome state, increase of the biomass of *H.stevensii* is often	MDPbiomeGEM [89]
Pathway distribution				

Optimizing metabolite secretion Secondary goal: medium composition	- medium composition - 2 selected strains - secreted metabolite	122 strains (6 from [70]) and 116 from [69] combined in >6500 different consortia of 2 members	- *In silico* framework to design synthetic communities, evaluating which new metabolites could be secreted - secreted emergent metabolites (highlighting the most common ones), with their associated two-strain consortium and medium composition - *E. coli/B. subtilis* emergent secretion of both succinate and urea (see Figure S4 and F6 from the original study for more pairs and metabolites)	[172]
Maximizing growth or compound yield	Allocated reactions per strain	2 generic bacteria with reduced central carbon metabolism	- *In silico* model following a MILP optimization approach (higher computational cost than LP (FBA)), with a Static/Multi-part method - Given metabolic reactions to distribute - Strains can only survive through cross-feeding	[112]
Minimizing number of species	Selected species to combine in the community	Human gut microbiome	- *In silico* model with CoMiDA - Graph-based approach (not GEM) combined with Integer Linear Programming (ILP) - Given selected substrates and products, and a set of available species - Identify putative metabolic pathways from substrates to product - Glycolysis pathway, glucose → pyruvate, 284 species: minimal solution with one species was found. Also, they forced for multi-species solution - With 10000 random pairs of substrate-product metabolites, 1-3 species are selected among 2051 species	CoMiDA [111]
2 cases of study: - maximizing antibiotics production, - maximizing 1,3-propanediol and methane yield Secondary goal: bioproduction	All reactions to include and their distribution among strains	*- Streptomyces cattleya and M. barkeri (selected from 4 strains)* *- K. pneumoniae and M. mazei*	- *In silico* model with MultiPlus, following static/Unified approach - *De novo* synthesis of bioactive metabolites - Results: - Case study 1 (antibiotics): 4 solutions with 528 reactions (2 transports, 3 insertions, and 28 endogenous reactions) - Case study 2 (industrial): 6 solutions with 110 reactions (1 transition and 10 endogenous reactions)	MultiPlus [114]
Optimizing metabolic exchange rates	- carbon/nitrogen exchange and uptake rates - kinetic parameters	*- C. acetobutylicum* *- Wolinella succinogenes*	- *In silico* model with DMMM, following a dynamic approach - Model parameters adjusted to *in vivo* data (kinetic ones, biomass, carbon and nitrogen sources ratio) - Anaerobic species with hydrogen and nitrogen cross-feeding - Co-cultures with uni- and bidirectional metabolic interactions - The metabolic models can simulate their experimental data, in 4 different cultivation conditions (with/out NH_4 and/or NO_3), with distinct metabolic capabilities	[173]
Surviving under	Cross-feeding	*E. coli* (2-3 strains)	- *In silico* model with DOLMN, following a MILP optimization approach, with a	DOLMN

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constraints	partnerships and division of labor		Static/Multi-part method - Results: - core: 91 combinations of 2 strains. Split the TCA cycle into two halves - full with reduced functionalities: 2207 combinations for 2 strains, and 2402 for 3 strains. At least 215 and 203 internal reactions to grow, respectively for 2 and 3 strain consortia. Loss one reaction is not compensated with adding one metabolite in the medium (nonlinear boundary)	[115]
Maximizing ethanol yield	KO in strains	*S. cerevisiae* *E. coli*	- *In silico* model with BioLEGO 2. Based on Microsoft Azure Cloud. - Analysis of two-step fermentation pathway of *Ulva sp.* biomass into ethanol with KOs in each strain from the consortium - 6,649,115 possible single KO analysed scenarios - Ethanol yield increased at 170% of WT (for 867 KO candidate pairs)	BioLEGO 2 [116]
Stability				
Maximizing (together): - biomass per single strain - community biomass concentration (cells/L)	strains ratio	Auxotrophic *E. coli pairs*: (argH-lysA) (lysA, trpC) (metA, ilvE)	- *In vivo* data from [152]. - *In silico* model with dOptCom - Biomass ratios (approx. values from Fig.2): argH-lysA: 0.8:0.2 *in vivo* and 0.97:0.03 *in silico* lysA-trpC: 0.9:0.1 *in vivo* and 0.98:0.02 *in silico* metA-ilvE: 0.15:0.85 *in vivo* and 0.15:0.85 *in silico*	dOptCom [74]
GR in auxotroph evolution	strains ratio	*E. coli* lysine and leucine KOs long-term	- *In vivo* data to constrains the model - Glucose minimal medium, with uptake rate 10 mmol/gDW/hour - Increased GR by 3 folds, while decreased growth in mono-culture - Strain ratio depending on the aa uptake rate	[139]
Common growth Secondary goal: spatial distribution	- strains ratio - cross-feeding rate - spatial distribution	- *E. coli* (KO metE) in lactate - *S. enterica* (secretes methionine)	- *In vivo* data from [174] and itself - *In silico* model with COMETS - Strain ratios: *E. coli*:*S. enterica*=75-80:25-20% - Spatial distribution: presence of a strain competitor between cross-feeding species reduces the growth of those strains	COMETS [82]
GR with optimum distribution of resources	- metabolites (amino-acids) consumption	*E. rectale or F. prausnitzii, B. thetaiotaomicron, B. adolescentis and R. bromii*	- *In vivo* data to constrains the model - *In silico* model with CASINO - Quantifying diet-induced metabolic changes of the human gut microbiome, using metabolomics data	CASINO [94]
Common growth	- strains ratio	- 4 *E. coli* auxotrophic	- *In silico* model with SteadyCom	SteadyCom

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	- community GR	for amino acids - Gut microbiome (9 species)	- 4 *E. coli* case of study: - GR: 0.736 $gDWh^{-1}$ - Strains ratio: Ec1-Ec2=50%, Ec3-Ec4=50%. Direct competition Ec1-Ec4 and Ec2-Ec3 - Gut microbiome case of study: values depending on fibre uptake from *B. thetaiotaomicron*: - GR: ~0.06-0.08 $gDWh^{-1}$, variable depending on fibre uptake	[76]
Medium composition				
Minimizing the cost of metabolic cooperation	Combination of nutrients allowing synergistic growth	*E. coli* arginine and leucine KOs	- *In silico* model following a static/Multi-part approach - Selected nutrients: supplementation of nucleotide precursors (maltose, xanthine and inosine) to the medium - *In vivo* experimental validation: the predicted medium allows growth	[117]
Spatial organization				
Spatial Partitioning	-spatial distribution - biofilm thickness - growth with by-products	*P. aeruginosa* *S. aureus* (chronic wound biofilm)	- *In silico* dynamic model combining GEM with partial differential equations - Results: - Tendency of the two bacteria to spatially partition, as observed experimentally. Nutrient gradients influence (oxygen-top-aerobic, glucose-bottom-anaerobic) - Different biofilm thickness than isolated	[119]
Spatial Partitioning	-spatial distribution - strain ratio - shift due to perturbations	2 case of study (reduced models): - *E. coli, S. enterica* - *P. putida, P. stutzeri*	- *In silico* model with IndiMeSH, following a dynamic approach - Study of soil habitat - Compared to COMETS and experimental data	IndiMeSH [118]
Flexible				
Optimizing PHA accumulated Secondary goal: bioproduction	- initial biomasses - NH_4 concentration - sucrose secretion rate	- *S. elongatus* - *P. putida*	- *In silico* model with FLYCOP - biomasses: 2, 0.2 gr/L - NH_4: 0.5 mM - sucrose secretion rate: 40% - PHA production: 22.43 mM/100h	FLYCOP [55]
Stability maximization (common growth)	- strains ratio - amino acid secretion rate	4 *E. coli* auxotrophic for amino acids	- *In silico* model with FLYCOP - strains ratio: Ec1=35%, Ec2=10%, Ec3=15%, Ec4=40% - aa secretion rate (in terms of %GR): Arg=1.5, Lys=2, Met=1.6, Phe=1	FLYCOP [55]

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Several optimization goals: maximizing yield or biomass or GR, and minimizing time	Uptake rates per strain (glucose, acetate, oxygen)	2 *E. coli* polymorphism: - glucose specialist - acetate specialist	- *In silico* model with FLYCOP - *In vivo* data from Lenski's experiment (LTEE) - Different configurations are predicted depending on the optimization goal. A polymorphism with 2 strains growing is the best configuration under limited oxygen conditions; else only one strain growing	FLYCOP [55]