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# Modelling of microbial interactions in anaerobic digestion: from black to glass box☆

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Anaerobic and microaerophilic environments are pervasive in nature, providing essential contributions to the maintenance of human health, biogeochemical cycles and the Earth's climate. These ecological niches are characterised by low free oxygen and oxidants, or lack thereof. Under these conditions, interactions between species are essential for supporting the growth of syntrophic species and maintaining thermodynamic feasibility of anaerobic fermentation. Kinetic models provide a simplified view of complex metabolic networks, while genome-scale metabolic models and flux-balance analysis (FBA) aim to unravel these systems as a whole. The target of this review is to outline the main similarities, differences and challenges associated with kinetic and metabolic modelling, and describe state-of-the-art modelling practices for studying syntrophies in the anaerobic digestion (AD) case study.

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## Introduction

Anaerophilic organisms are microbial species, including both Archaea and Bacteria, adapted to live in anaerobic environments. These environments may seem 'extreme' or 'hostile' from an anthropogenic point of view, but no environment is too unwelcoming when considering the whole tree of life [1]. Anaerobes derive their energy from transfer of key molecules participating in the maintenance of global biogeochemical cycles [2], with crucial influence on the Earth climate [3], but also in influencing human health and serving as cell factories.

While developing mathematical models is important for hypothesis testing and characterisation of biological functions, it faces significant challenges due to the limited genomic and physiological characterisation of anaerobic organisms. Their cultivation is more difficult and the use of culture-independent approaches, such as metagenomics, is needed to investigate their activity. These challenges thus limit data availability and collection for model assembly, which requires appropriate strategies.

Here, we describe the different modelling approaches used to study metabolic activity and interactions in the context of the anaerobic digestion (AD) process. During AD, complex organic compounds, such as carbohydrates, proteins and lipids, are enzymatically converted into simpler molecules. AD represents a controlled and tractable, yet complex and complete system that can mirror other similar anaerobic environments (e.g. the human gut). In this context, mathematical models provide a structured framework for integrating biological knowledge and experimental data, helping us to understand intracellular activity and interspecies interactions.

## Mathematical models of anaerobic environments

Mathematical models have long been developed for the description of anaerobic bacterial and archaeal processes. These models are designed to enhance our understanding of bioconversion abilities, growth requirements and susceptibility of anaerobic microorganisms to inhibitors.

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Modelling of anaerobic environments has come a long way since their early representatives, becoming standard tools in process design, operation planning and optimisation [4]. Simple models of the past, focused entirely on macroscopic community and species interactions, are gradually becoming more performing, taking advantage of rapidly evolving sequencing and computational techniques [5]. This trend is also reflected in the evolution of modelling approaches, traversing from simplified ecological and primarily mechanistic (kinetic) models to constraint-based (metabolic) models. In the following, these approaches are discussed highlighting their primary features and limitations.

### Kinetic models of anaerobic environments

Kinetic modelling (KM), with 'kinesis' representing the dynamic response of a biological system to stimuli, is an approach for unravelling the biochemical mechanisms in anaerobic environments. With the spread of KM, more and more AD subprocesses could be described mathematically, providing structured and reproducible explanations of the system layers previously uncharted.

Based on their level of complexity, KM of AD can be divided into descriptive (or heuristic) and mechanistic. Descriptive models comprise basic process calculators used in applied industrial biotechnology, while mechanistic kinetic equations pertain to well-defined subprocesses of anaerobic metabolism. Mechanistic kinetic equations provide a reduced, yet biologically more accurate glimpse into the workings of the organisms involved, typically representing individual steps of the AD funnel [6]. KM might be used by plant operators for a first overview about material flows, energy production and other macrokinetic aspects of designing or operating AD plants.

A description of first-order models and logistic models, as well as a comprehensive collection of models specific to AD can be found in Supplementary Figure 1, with the associated list of references reported in Supplementary Table 1. Here, we report 'Monod-type models' that were fundamental for the genesis of more complex models such as the Anaerobic Digestion Model No. 1 (ADM1) and more complex, mechanistic models, introduced here.

### Monod-type models

In 1949, Jacques Monod published his acclaimed study on 'the growth of bacterial cultures' [7]. His work focused on nutrient-limited bacterial growth in general, and used a simple empirical formula to correlate growth with substrate availability (Equation 1).

$$r = \mu_{\max} * \frac{S}{S + K_S} \quad (1)$$

The new growth-modulating term is dependent on the substrate concentration $S$, and a substrate affinity (or half-saturation) constant $K_S$.

Monod-type multiterm expressions have been widely used for modelling anaerobic bioconversion. Examples for rate equation and biomass calculations are shown in Supplementary Figure 2.

### The Anaerobic Digestion Model No. 1

Owing to an ever-growing interest in the field, fruitful decades of progress eventually culminated in the creation of the detailed ADM1 [8]: a major milestone in the standardisation of anaerobic metabolism modelling. ADM1 is an advanced model describing all four layers of the cascade bioconversion process considered in AD (i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis). As a testament to its relevance, in the past 20 years, the ADM1 has been applied, extended and improved through the work of hundreds of researchers, opening a new era for multidisciplinarity in AD modelling [9]. The importance of this aspect, which also propelled the model-based study of syntrophies in anaerobic microbiomes, will further be highlighted in a dedicated section. Overall, this reductionist philosophy (as it will be demonstrated in the next paragraphs) is in stark contrast to the holistic approach employed by genome-scale metabolic models (GSMMs).

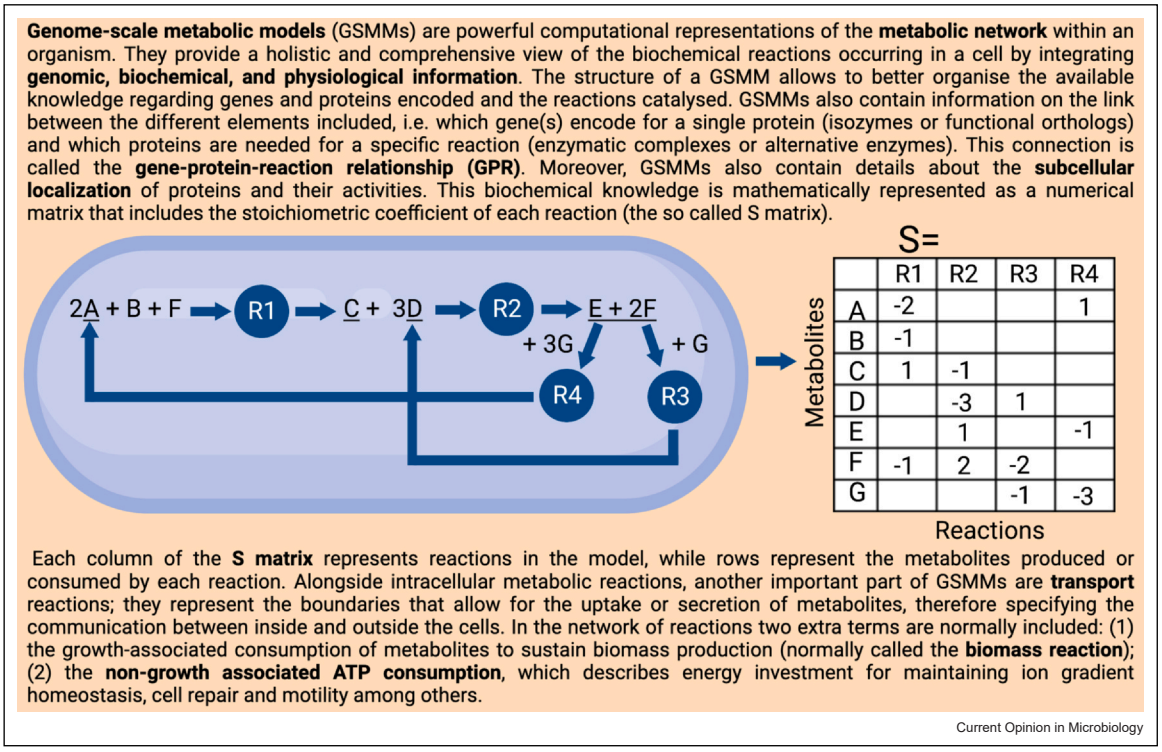
### Large-scale kinetic metabolic models

The gap between metabolic network knowledge and experimental data in conditions far from steady state is progressively narrowing as KM expands in scale, from elementary reaction mass action to the more comprehensive Monod–Wyman–Changeux model [10]. Large-scale KM captures time-dependent changes in metabolite concentrations, reaction rates and metabolic fluxes on a systems level [11], thus having increasing potential for understanding, predicting and optimising living organism behaviour. Clearly, the main obstacle for their development is the sheer number of reaction kinetic expressions and associated parameters required, which often limit their size to a set of key pathways. In this context, complementary approaches able to account for model uncertainty are actively being developed. For instance, REKINDLE (Reconstruction of Kinetic Models using Deep Learning) [12] is a framework based on deep learning that allows for the generation of KM capable of matching the observed dynamic properties of cells. This approach thus offers the ability to explore various physiological states of metabolism using a limited amount of data, and reducing computational requirements.

### Genome-scale metabolic modelling of anaerobic environments

GSMMs are composed of a whole set of stoichiometrically balanced reactions, which collectively describe

**Figure 1**




| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| A | -2 | | | 1 |
| B | -1 | | | |
| C | 1 | -1 | | |
| D | | -3 | 1 | |
| E | | 1 | | -1 |
| F | -1 | 2 | -2 | |
| G | | | -1 | -3 |

Description of GSMMs and of their mathematical representation.

the gene-protein-reaction associations of the entire metabolic repertoire for one or more organisms [13] (more details about GSMMs are in Figure 1).

They are commonly used to simulate metabolic network activity by various constraint-based techniques such as flux-balance analysis (FBA) and quantifying steady-state fluxes of biochemical reactions [14] (Figure 2). These models are often referred to as knowledge bases, and their ability to accurately describe the metabolic behaviour particularly suffers from limited characterisation of the organisms of interest. As such, one of the most important constraints to improve accuracy of GSMM predictions are measured uptake and release fluxes. The integration of transcriptomic data constitutes another improvement of the reliability of the models, allowing to integrate enzyme-specific constraints [15]. Even so, GSMMs are heavily underdetermined, and FBA always results in one of thousands of plausible optimal solutions. This is caused by the presence of multiple combinations of reaction fluxes that can satisfy the constraints imposed. As a consequence, multiple issues must be taken into account when approaching modelling of anaerobic systems. However, some '*escamotages*' have been adopted for some particular examples that will be presented below.

## Genome annotation and pathways characterisation

GSMMs are based on genomic annotation and experimentally obtained information [16]. With the advent of next-generation sequencing, the number of taxa characterised only as a means of genome-centric metagenomics is exponentially increasing [17].

Gap-filling is a method employed to incorporate reactions into metabolic models based on established growth needs. The lack of biosynthetic capacity of certain compounds in many anaerobes makes them challenging to cultivate and hampers automated gap-filling processes, resulting in incomplete or erroneous metabolic networks. With reference to this particular aspect, particularly outstanding is the work of Huang and colleagues [18] charting the integration of $H_2$-producing pathways in the metabolic reconstructions. The models enabled a correct description of glucose fermentation revealing conversions of a range of metabolites (i.e. ethanol, propionic acid and butyric acid) to acetate.

GSMM reconstruction of methanogenic Euryarchaeota faces additional challenges due to alternative pathways (i.e. aceticlastic, hydrogenotrophic and methylotrophic methanogenesis [19]). Furthermore, methanogens could be classified in species with and without cytochromes

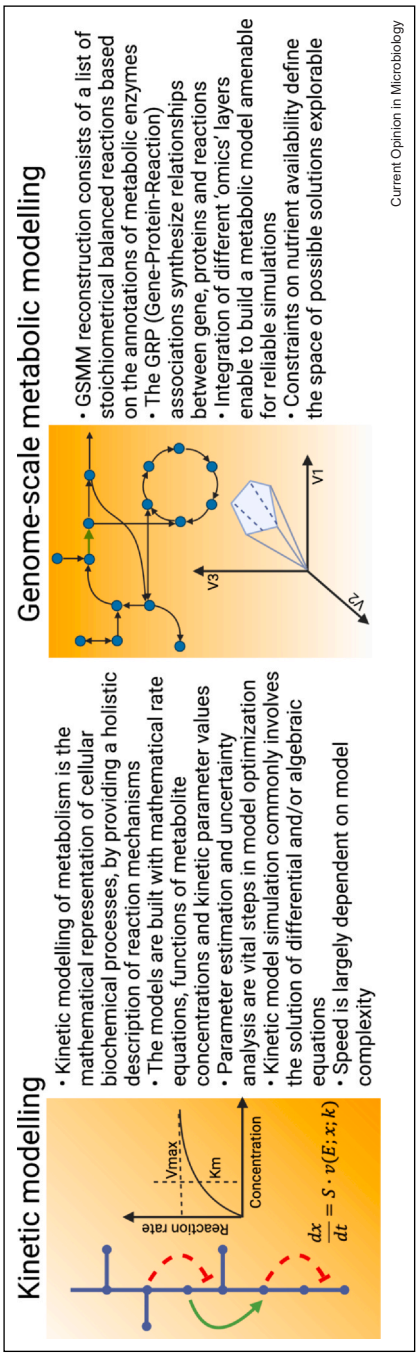



**Figure 2**

Schematic representation of the main steps involved in kinetic and metabolic modelling approaches.

[20]. Nonetheless, the underlying energy-conserving process is dependent on the presence or absence of cytochromes. *Methanosarcina barkeri* is a methanogen with cytochromes and, therefore, reactions using methanophenazine are active in the model [21]. The model of *Methanothermobacter thermautotrophicus* uses an electron bifurcating reaction, coupling energetic unfavourable electron transfer from $H_2$ to ferredoxin, with the energetic favourable reduction of CoM–S–S–CoB. Machado et al. implemented a curated model template containing metabolic pathways and compartments specific for archaea; GSMMs are then constructed as a subset of these reactions [22]. The recently developed tool Gapseq [23] includes a workflow targeting the presence or absence of cytochromes to fetch archaeal metabolic reconstructions.

### Biomass composition

Along with gene annotation, the definition of an organism biomass composition is fundamental to reliably simulate its metabolism. In general, such a task is based on direct measurement, on the extension of related species biomass or on the propagation of template compositions for classes of organisms [24]. Moreover, it must be considered that in vivo macromolecular cell composition constantly changes also based on the growth environment. In this regard, metabolism and expression models were shown to capture such dependency in *Escherichia coli* by explicitly accounting for cofactors and amino acid synthesis [25]. Yet, difficulties may arise when implementing these approaches on uncultivated organisms.

### Redox homoeostasis and thermodynamics integration

Despite the knowledge gaps, GSMMs are particularly suited to elucidating peculiarities of anaerobic metabolism. Perhaps most importantly, redox homoeostasis is controlled by overflowing metabolism and a complex balance of cofactor availability and specificity [26]. Therefore, large-scale redox equilibria can influence pathway utilisation in a non-intuitive manner, and require detailed stoichiometric models to be captured. Kinetic parameters are not considered in the standard computation of the optimal solution, as in FBA, it is assumed that the cells are in a steady-state growth phase where there is no net metabolite accumulation [27]. In this regard, particularly standing is the work carried out by Hatzimanikatis and colleagues [28] targeting the integration of thermodynamic information in FBA. The metabolic reconstruction is complemented by thermodynamics with a three-step approach where Gibbs energy of compound biosynthesis, Gibbs energies of reaction and transported metabolites and pH, ionic strength and membrane potentials are integrated [29]. Another option is the use of enzyme-constrained models (ECMs). ECMs combine GSMMs with enzyme kinetic data (i.e. enzyme turnover number [30]). These models

accurately simulate maximum growth abilities, metabolic shifts and proteome allocations by constraining the whole-cell metabolic network with enzyme-catalytic capacities [31]. Furthermore, although unequivocally proving its occurrence requires special attention, direct interspecies electron transfer (DIET) has a relevant yet scarcely studied role in this context [32].

### Supra-organism approaches

A possible way to overcome difficulties related to single-species reconstruction, is the so-called 'enzyme-soup' or 'supra-organism' approach. In this method, microbial genes, metabolites and reactions are inferred directly from metagenomic data generating a pan-microbiome reconstruction [33]. As a disadvantage, this method ignores species–species boundaries and transport mechanisms, which greatly influence the metabolic fluxes in the community. Following this approach, a model of fermentative bacteria, named iFermGuilds789 [34], has been developed. iFermGuilds789 considers metabolic functions of mixed microbial communities to be shared among a maximum of six functional guilds. The free exchange of reducing equivalents (e.g. the reduced form of nicotinamide adenine dinucleotide, ferredoxin) among members of the microbial consortia is reduced by simulated role stratification. Conclusively, the redox balance of individual microorganisms is constrained, enhancing adenosine triphosphate and product yields.

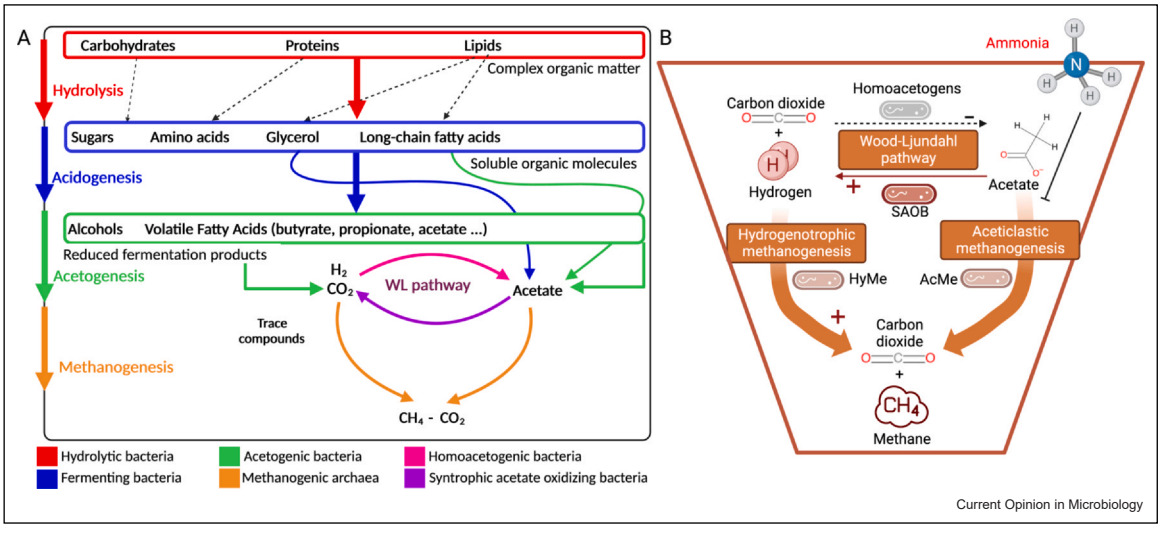
## Case study: anaerobic digestion

AD is a complex and biologically mediated process occurring in anaerobic environments and involving the degradation of organic matter into simple compounds such as methane and carbon dioxide [38]. This process can take place in wetlands [35], landfills [36], and it can mirror similar processes occurring in the intestinal tract of animals [37]. Nevertheless, it has substantial biotechnological potential and can be harnessed as a renewable energy source [38]. The four main steps of the anaerobic degradation process are hydrolysis, acidogenesis, acetogenesis and methanogenesis [39], each of which is performed by highly specialised members of the associated microbial community, including *Bacteria* and *Archaea* (Figure 3**a**). Campanaro and colleagues identified as 3.7 the percentage of metagenome-assembled genomes not assignable at the species taxonomic level [6]. These organisms are commonly termed 'microbial dark matter', and are involved in yet-to-be identified relationships [40,41] (Supplementary Information). As an important aspect of modelling AD, microbial interactions have thus been studied extensively in the past decades.

### Kinetic models of syntrophies in anaerobic environments

Capson-Tojo and colleagues [42] first implemented syntrophic acetate oxidation (SAO) in the ADM1 [8]. Unlike most other anaerobic model implementations, where distinct microbial groups catalyse independent bioconversion processes, their model extension considered a direct competition for acetate utilisation between SAO bacteria and acetoclastic methanogens (Figure 3**b**). More recently, the addition of homoacetogenic bacteria as a third group of interest [43]

**Figure 3**




Microbial cohorts in the Anaerobic Digestion process. **(a)** Schematic representation of the four trophic layers of AD. The arrow colours are related to the functional guild performing the conversion between different compounds, as indicated in the bottom part of the figure. **(b)** Graphical representation of the coexistence balance among homoacetogens, syntrophic acetate-oxidising bacteria (SAOB), hydrogenotrophic archaea and aceticlastic archaea without ammonia and after its injection or production during protein degradation.

further increased the level of detail in simulating the methanogenic step in AD. The implementation was achieved through modelling the triangle of metabolic exchange between acetoclastic and hydrogenotrophic methanogens, together with homoacetogens. At the same time, hydrogen can also be seen as a thermodynamic regulator of biochemical conversion [44]. Jin used $H_2$ partial pressure as a key variable in available Gibbs free energy calculations, and determined the rate of butyrate fermentation through the combination of kinetic and thermodynamic process information. It was therefore shown that kinetic fermentation modelling (albeit not restricted to butyrate fermentation) can benefit from the inclusion of thermodynamics during model conceptualisation and design.

Other studies from the last decade targeted exhaustive mathematical analyses to investigate the effect of substrate concentrations, process inhibition and cell mortality on the kinetics of microbial growth and syntrophies in AD. The importance of accounting for inhibitions while ensuring balanced archaeal growth for model stability was advocated by the study of Weederman et al. [45] In this study, a model community of four microbial groups was designed, with acetoclastic and hydrogenotrophic methanogens co-producing biogas. Meanwhile, the focus of Fekih-Salem et al. [46] was on modelling the interaction between acetogenic bacteria and hydrogenotrophic methanogens. The simulation was extended with a review of more than 10 earlier mathematical AD models, considering varying number of process steps and microbial interactions.

With regard to alternative forms of kinetic AD models, Zakaria and co-workers implemented a microbial electrolysis cell-assisted AD system, and demonstrated the importance of propionate-to-acetate ratio (HPr:HAc) for microbial syntrophy [47]. Their model was based on a modified version of ADM1 and assumed a synthetic microbial trio of electroactive bacteria, hydrogenotrophic methanogenic archaea and homoacetogenic bacteria. The authors found that at low HPr:HAc ratios (around 0.5–1.5), a balanced syntrophy could be maintained between the members of the community, while higher HPr:HAc ratios partially inhibited electroactive bacteria responsible for DIET, overall resulting in methanogenesis destabilisation.

The additional level of model complexity brings about significant technical difficulties that must be evaluated critically. Nevertheless, by transferring the findings of microbial analyses focused on mixed cultures to kinetic anaerobic models, and considering the relevant intra- and intercellular processes, the quality of model prognoses can be significantly improved.

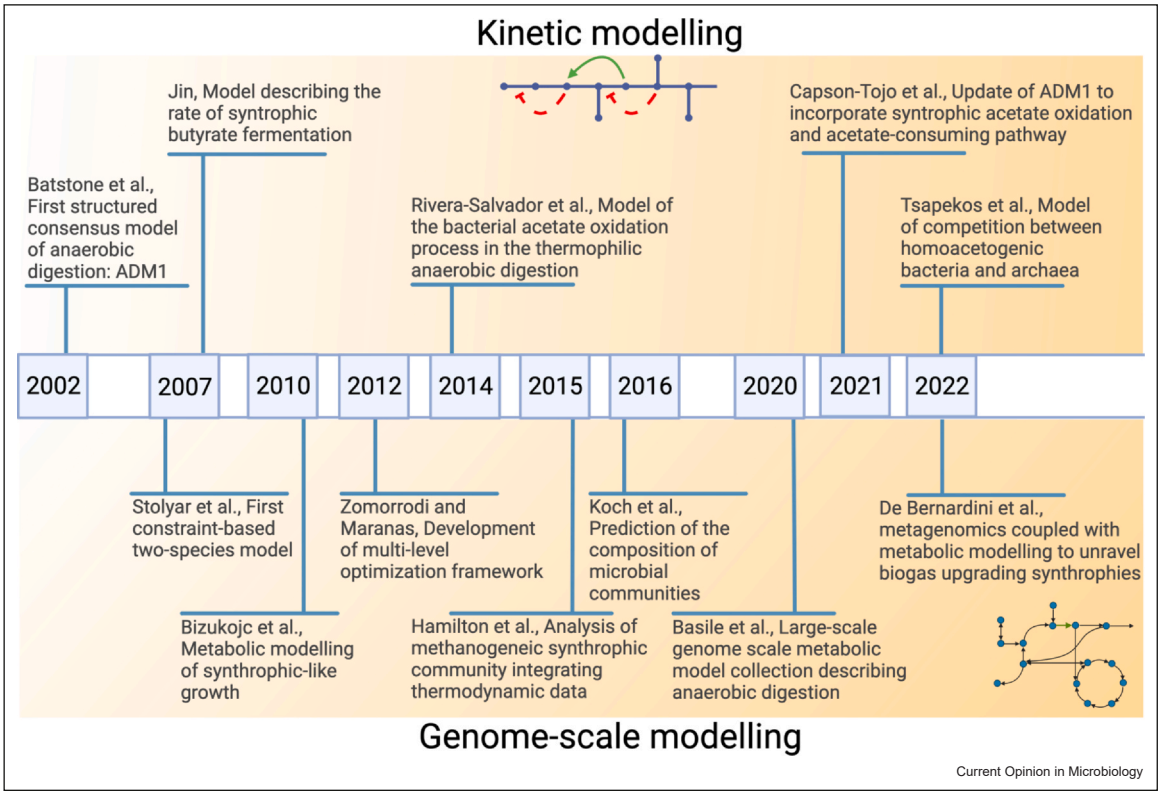
## Genome-scale metabolic modelling of syntrophies in anaerobic environments

From the early 2000s, FBA has been used as a powerful tool to shed light on the behaviour of different microbial guilds with recent emphasis on the uncultivable majority, focusing on the syntrophies between the bacterial and archaeal guilds.

To this aim, the first pioneering work was published in 2007 by Stolyar and colleagues. In particular, they analysed the syntrophic association between *Desulfovibrio vulgaris* and *Methanococcus maripaludis* [48]. The simulations published by Stolyar et al. indicated formate interspecies electron transfer as facultative, while hydrogen exchange as mandatory for syntrophic growth. In another study [49], the conditions leading to a complete conversion of $H_2$ and formate produced by *Syntrophobacter fumaroxidans* by *Methanospirillum hungatei* were identified combining the metabolic reconstructions of the two species named *i*Sfu648 and *i*Mhu428. The models were combined taking into account thermodynamic information, and the main drivers of their interaction were identified in the simultaneous exchange of $H_2$ and formate. The interaction between *D. vulgaris* and *M. maripaludis* was further investigated taking into account a third player, *Methanosarcina barkeri* [50]. The simulations consisted of three different scenarios, including a competitive one, with both methanogens using the hydrogenotrophic methanogenesis. The second scenario assumes that *M. barkeri* can use only the acetoclastic pathway for methane production, and the third that *M. barkeri* has both hydrogenotrophic and acetotrophic pathways. The maximum theoretical methane yield was obtained in the second scenario, where all the acetate was consumed.

Embree and colleagues [51] analysed the interaction network of seven different species: *Smithella* sp. ME-1, *Syntrophomonas wolfei*, *Desulfovibrio magneticus*, *Methanoculleus marisnigri*, *Methanosaeta concilii*, *Methanocorpusculum labreanum* and *Melioribacter roseus*. The species were binned from metagenomic experiments inspecting the hexadecane degradation process. Species-specific gene expression profiles were recovered from metatranscriptomic experiments, and it was underlined that amino acid auxotrophies reinforce interdependence and collaboration within the community. Only recently, Basile et al. [52] targeted the description of the whole AD community, by automatically reconstructing more than 800 metabolic models from MAGs, describing both archaeal and bacterial species. Basile et al. used a mixed approach involving pairwise interactions and community modelling. The analysis revealed that auxotrophs are driven mainly by exchanges of glucogenic amino acids, thereby completing the tricarboxylic acid cycle. Another

**Figure 4**




Timeline of the studies modelling AD syntrophies with kinetic (upper part) or genome-scale (lower part) simulations. Realised with Biorender.

application coupling genome-centric metagenomics and FBA targeted a biofilm community formed in a reactor during biogas upgrading, which included five dominant species [53]. The analysis revealed that formate and amino acid exchanges among species were crucial to support the stability of the consortium. This strategy can pave the way to the prediction of most favourable growth conditions and to the optimisation of methane production in biotechnological applications.

## Future trends and conclusions

Model-based research focused on the microbiology of anaerobic environments has gained increasing interest in recent years, and was mainly focused on the human metabolism, biotechnological applications and data-driven environment. In particular, interspecies interactions are key to the structure and function of the microbial community under investigation, with macroscopic features revealed as emerging properties derived from microscopic synergies [54].

There is a long-lasting debate over idealised process-level models, or kinetic biochemical models that provide clearer insights into the true dynamics of anaerobic environments (Figure 4). According to the authors, the solution might be somewhere halfway, with the possibility of integrating the two approaches. Such integration can enable the transfer of microbe–microbe interactions not accounted for in FBA, for example, inhibitory effects of metabolites on microbe growth. Another plausible compromise is to use genomic data and create detailed metabolic models, based on the fundamental principles of stoichiometry and mass conservation [55]. This is the principle used in whole-cell models, foreseeing the full characterisation of the dynamics of all molecules within a living organism. However, so far, this approach has only been applied to the model organism *Escherichia coli* [56].

As computational tools are becoming more powerful and numerical solvers widely accessible, the development of new tools for modelling anaerobic systems can accelerate significantly, mostly at the intersection of biotechnology, molecular biology, microbiology and bioinformatics.

## CRediT authorship contribution statement

**Arianna Basile**: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Guido Zampieri**: Writing – original draft, Writing – review & editing. **Adam Kovalovszki**: Writing – original draft, Writing – review &

editing, Visualization. **Behzad Karkaria**: Writing – original draft. **Laura Treu**: Funding acquisition, Writing – review & editing. **Kiran Raosaheb Patil**: Funding acquisition, Writing – review & editing. **Stefano Campanaro**: Conceptualization, Project administration, Supervision, Writing – review & editing.

## Data Availability

No data were used for the research described in the article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mib.2023.102363.

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