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Model-aided targeted volatile fatty acid production from food waste using a defined co-culture microbial community

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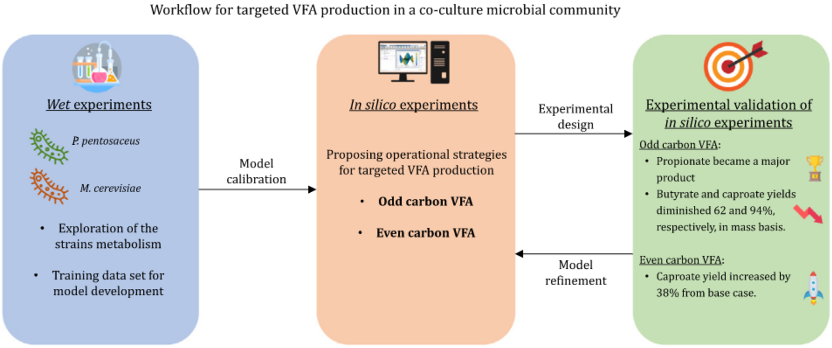
HIGHLIGHTS

- A co-culture of *P. pentosaceus* and *M. cerevisiae* converts food waste into VFA.
- The selective production of odd and even carbon VFA is targeted.
- A workflow combining mathematical models and wet experimentation is proposed.
- Butyrate and caproate dominate the spectrum in the even carbon VFA strategy.
- Propionate became dominant and butyrate yield halved when targeting odd carbon VFA.

GRAPHICAL ABSTRACT



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ABSTRACT

The production of volatile fatty acids (VFA) is gaining momentum due to their central role in the emerging carboxylate platform. Particularly, the production of the longest VFA (from butyrate to caproate) is desired due to their increased economic value and easier downstream processing. While the use of undefined microbial cultures is usually preferred with organic waste streams, the use of defined microbial co-culture processes could tackle some of their drawbacks such as poor control over the process outcome, which often leads to low selectivity for the desired products. However, the extensive experimentation needed to design a co-culture system hinders the use of this technology. In this work, a workflow based on the combined use of mathematical models and wet experimentation is proposed to accelerate the design of novel bioprocesses. In particular, a co-culture consisting of *Pediococcus pentosaceus* and *Megaphaera cerevisiae* is used to target the production of high-value odd- and even-carbon VFA. An unstructured kinetic model was developed, calibrated and used to design experiments with the goal of increasing the selectivity for the desired VFA, which were experimentally validated. In the case of even-carbon VFA, the experimental validation showed an increase of 38 % in caproate yield and, in the case of enhanced odd-carbon VFA experiments, the yield of butyrate and caproate diminished by 62 % and 94 %, respectively, while propionate became one of the main end products and valerate yield value increased from 0.007 to 0.085 $g_{valearte}$ per $g_{consumed\ sugar}$. The workflow followed in this work proved to be a sound tool for bioprocess design due to its capacity to explore and design new experiments in silico in a fast way and ability to quickly adapt to new scenarios.

Abbreviations: VFA, volatile fatty acid(s); FW, food waste; DBTL, design-build-test-learn; NRSMD, normalized root square mean deviation; k_I, inhibition constant; K_S, affinity constant; Y, biomass yield; q, specific substrate uptake rate; q_{max}, maximum specific substrate uptake rate.

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1. Introduction

Volatile fatty acids (VFA) are carboxylic acids comprising of 2 to 6 carbon atoms that can be produced biologically in fermentation processes using organic wastes as substrate (Atasoy et al., 2018; Jankowska et al., 2017; Shanthi Sravan et al., 2018). They are gaining attention in research and industrial contexts for their central role in the carboxylate platform, one of the emerging biorefinery paradigms, as an alternative to the current oil dependency of the chemical and energy industry (Agler et al., 2011; Dahiya and Mohan, 2019; Moscoviz et al., 2018). In particular, the production of the longest VFA (i.e. from butyrate to caproate) is desired due to their higher economic value, their easier recovery from the fermentation broth and their wider range of applications, such as animal feed additive for its antimicrobial properties, bulk fuels or solvents and bioplastics precursors (Atasoy et al., 2018; Carvajal-Arroyo et al., 2019; De Groof et al., 2019).

Among different organic waste sources, food waste (FW) represents an optimal candidate for the biorefinery concept due to its high and well-distributed availability. FW generation is predicted to increase from 2005 to 2025 by more than 40 % in developing countries and was predicted to increase by 40 % in Europe from 2006 to 2020 (Capson-Tojo et al., 2016). Furthermore, FW usually has a high concentration of volatile solids and particularly easily degradable carbohydrates, making it a good substrate for the carboxylate platform (Capson-Tojo et al., 2016; Strazzera et al., 2018; Zhang et al., 2020). To produce VFA from FW and organic wastes in general, a top-down approach is usually followed in which a specific metabolic function, or set of metabolic functions, are enriched from an undefined rich community. These open mixed-culture processes (i.e. undefined and varying mixtures of microorganisms that are present in the substrate) are usually preferred due to economic reasons (e.g. substrate does not need to be sterilized) (Regueira et al., 2020). However, relying on undefined microbiomes also poses severe disadvantages as the process outcome is intrinsically variable, which makes it difficult to control and steer reliably towards the production of the desired compounds (Arslan et al., 2016; Carballa et al., 2015). In consequence, the titers and productivities of specific targeted compounds are usually low. In this sense, the use of defined microbial communities can tackle some of the mentioned issues and provide an alternative way for waste valorization in the framework of the carboxylate platform.

Defined co-culture systems (i.e. processes using a microbial community formed by two or more pure species) follow a bottom-up approach, as they assemble selected isolated species based on their desired metabolic traits. In comparison with single pure culture systems, co-culture systems may present a higher metabolic flexibility, as the members of the community can cooperate to metabolize the substrate(s), as well as giving place to collaborative behaviors such as commensalism (e.g. cross-feeding or division of labor) or mutualism (Jagmann and Philipp, 2014). These characteristics increase the robustness and stability of the process against possible environmental perturbations. Additionally, a co-culture might be more flexible than a single pure community and their outcome might be modulated by modifying some process parameters. This flexibility opens the possibility of targeting the production of different VFA with the same co-culture system. However, to understand and design a co-culture system targeting the production of specific products (i.e. a particular VFA mixture) is not a straightforward task, as it is difficult to anticipate the behavior of the microbial species (Song et al., 2014). For example, in the case of substrate competition or cross feeding, the two species will interact via common compounds (e.g. substrate or intermediate compounds) or, in the case of the system showing product inhibition, the different species might be inhibited to a different extent. Additionally, design parameters, such as substrate (s) concentration or the inoculum concentration of each strain, might affect the result of the process. Therefore, to find the optimal operational conditions for a desired outcome, a lot of experimental trials are needed (Hellweger et al., 2016). In this sense, the use of mathematical modeling (i.e. replacing wet experiments for *in silico* experiments) can help tremendously in reducing the cost and time needed for optimizing the selection of operational conditions (Hellweger et al., 2016; Ibrahim et al., 2021; Lopatkin and Collins, 2020).

Unstructured kinetic models are simple mathematical models that treat cells as black boxes and do not describe intracellular processes (i.e. they are just biocatalyzers of chemical reactions). Even though they are simpler than more elaborated approaches (e.g. structured or metabolic models), they are capable of capturing the dynamics of the systems to describe and they are relatively quick to develop. Kinetic models usually rely on experimental data of particular systems for parameter calibration, but the experimentation needed to adapt a kinetic model to a different system (i.e. a change of the selected strains for the co-culture system) is low, unlike metabolic models, which require substantial modifications due to their higher complexity. For their simplicity to (re)adapt, unstructured kinetic models can be easily integrated in a Design-Build-Test-Learn (DBTL) approach, which, in brief, consists of designing a system, testing it and, from the results, extracting the causal relationships between design parameters and the process outcome in a iterative way until the process goals are achieved (Kim et al., 2021). In this scheme, an unstructured kinetic model would constitute an excellent tool with which to provide a preliminary process design. In literature, unstructured kinetic models for describing VFA or methane production from FW are available (Coelho et al., 2020; Karki et al., 2022; Li et al., 2018; Ma et al., 2019; Morais et al., 2020; Wang et al., 2017). However, their main focus is usually to propose a process description (i.e. validate a conversion mechanism) or to correlate some of the main operational variables (e.g. pH) with variations on estimated kinetics parameters and they are not intended to be used as design tools.

The aim of this work was to develop a workflow based on the combined use of mathematical models and wet lab experimentation for the design of novel bioprocesses. In particular, this approach was applied for designing a co-culture system to produce higher value VFA (i.e. with a chain length of 4 or more carbon atoms) using food waste as substrate. The selected strains are *Pediococcus pentosaceus* and *Megasphaera cerevisiae*. Both of these robust strains are classified as biosafety level 1 (BSL1) organisms, which puts them in a low-risk group and thus their handling in industrial processes is simpler than higher biosafety level organisms. *P. pentosaceus* is a lactate producing bacterium, and is widely used as a starter culture in different types of food fermentation, with the ability of utilizing a variety of carbon sources and tolerate acidic environments (Jiang et al., 2021; Xu et al., 2021). *M. cerevisiae* is known as a beer-spoilage bacterium and has the potential of converting lactate into more valuable VFAs, such as butyrate and valerate (Jeon et al., 2017). With this purpose, a mathematical model of the selected co-culture system was developed using experimental data to gain understanding of the process sensitivity to different operational parameters and subsequently applied to design a process with high selectivity for the desired products.

2. Materials and methods

2.1. Selected strains for VFA production and food waste characteristics

The VFA production process developed in this research is based on a bacterial co-culture of a VFA producing *M. cerevisiae* and lactate producing *P. pentosaceus*. Both of these strains were obtained from VTT's culture collection (culturecollection.vtt.fi). They were stored as frozen glycerol stocks (at −80 °C) and activated by inoculating a loopful of stock into 5 mL of Reinforced Clostridial Medium broth (RCMb, Thermo Scientific™ Oxoid™). *M. cerevisiae* was incubated under anaerobic conditions at 30 °C and *P. pentosaceus* was incubated under aerobic conditions at 37 °C for 72 h. For the seed train, 3 % (*v*/*v*) of culture was transferred into fresh RCM broth (data not shown). For the bioreactor experiments, an initial OD_{630} of 0.1 was targeted.

Food industry waste was used as a carbon source in VFA production. Cabbage residue was selected after an initial screening process (section A of the Supplementary Materials). Vaissi Oy (Haapamäki, Finland) provided the cabbage residue, which contained slightly steamed cabbage leaves rejected as unsuitable for food processing. Two pretreatment methods were applied to homogenize the cabbage leaves, either mechanical pressing or enzyme hydrolysis. In the mechanical pressing, the pulp was discarded and only the juice was used for the media. In the enzymatic hydrolysis, all parts of the cabbage were utilized. Hydrolysis was performed as previously described by Selder et al. (2021). The composition for cabbage hydrolysate (Cab1 medium) is 17.6 ± 0.1 g L^{-1} of fructose and 27.8 ± 0.1 g L^{-1} of glucose with a dry matter content of 6.6 ± 0.03 %. The composition of cabbage juice (Cab2 medium) is 24.6 ± 0.3 g L^{-1} of fructose and 27.9 ± 0.4 g L^{-1} of glucose with a dry matter content of 6.4 ± 0.01 %.

2.2. Kinetic model

2.2.1. Kinetic model development

The developed model describes the VFA production (i.e. acetate, propionate, butyrate, valerate and caproate) from glucose, fructose and lactate in a bioreactor operated in batch mode. The consumption of all substrates is modelled following a Monod equation (Eq. (1)) and assuming that all processes are inhibited by the global VFA accumulation. End-product inhibition in acidogenic fermentation processes is a well-documented phenomenon (Jones et al., 2015) and different models simulating VFA production in pure or mixed cultures include VFA inhibition terms (Infantes et al., 2012; Nandasana and Kumar, 2008; Rivera et al., 2007; Song et al., 2008). In this work, VFA inhibition is mathematically described as a non-competitive inhibition of the sum of VFA concentrations (Eq. (2)), as also featured in other models (Batstone et al., 2002).

$$q = q_{max} \cdot \frac{S}{K_S + S} \cdot X \cdot I_{VFA} \qquad (1)$$

$$I_{VFA} = \frac{1}{1 + \frac{\sum_i S_{VFA,i}}{K_I}} \qquad (2)$$

where q is the specific consumption rate ($g_{SUBS}\, g_{BM}^{-1}\, h^{-1}$), q_{max} is the maximum specific consumption rate ($g_{SUBS}\, g_{BM}^{-1}\, h^{-1}$), S is the substrate concentration ($g_{SUBS}\, L^{-1}$), K_S is the half-saturation constant ($g_{SUBS}\, L^{-1}$), X is the biomass concentration ($g_{BM}\, L^{-1}$), I_{VFA} is the inhibition term, $S_{VFA,I}$ ($g_{VFA}\, L^{-1}$) is the concentration of the ith VFA compounds and K_I is the inhibition constant ($g_{VFA}\, L^{-1}$).

The modelled system comprises two microbial species that perform the metabolism described in Table 1. Biomass decay is not considered in the model. The model is built on the mass balances of the different compounds (states) in a batch reactor and implemented in MATLAB (R2016a) as an ordinary differential equation (ODE) problem. The model considers 10 compounds (glucose, fructose, lactate, acetate, propionate, butyrate, valerate, caproate, and *P. pentosaceus* and *M. cerevisiae* biomass) and 9 possible reactions (Table 1), leading to a 10 × 9 stoichiometry matrix.

To correctly describe the functionality of each strain and build the stoichiometry matrix of the model, the information provided by the individual strain screening experiments was used (Figs. S1 and S2). These experiments showed that *P. pentosaceus* is able to consume both glucose and fructose and only produces lactate as end product (Table 1). The catabolic capacity of *M. cerevisiae* is broader in terms of possible end products as it yielded acetate, propionate, butyrate, valerate and caproate in these preliminary experiments, while its ability to consume sugars is more limited, as it did not consume any glucose.

Table 1
Proposed molar stoichiometry for the reactions occurring the co-culture system.

Strain	No.	Reaction
Pediococcus pentosaceus	1	1 glucose → 2 lactate
	2	1 fructose → 2 lactate
Megasphaera cerevisiae	3	1 fructose → 1 butyrate + $2CO_2$
	4	1 fructose → 0.67 butyrate + 0.67 acetate + $2CO_2$
	5	1 fructose + 2 butyrate → 2 caproate + $2CO_2$
	6	1 lactate → 0.67 propionate + 0.33 acetate + 0.33 CO_2
	7	1 lactate → 0.5 butyrate + CO_2
	8	1 lactate + acetate → 1 butyrate + CO_2
	9	1 lactate + propionate → 1 valerate + CO_2

To disentangle the stoichiometry of VFA production by *M. cerevisiae*, an additional experiment using fructose as carbon source was performed (Fig. S4). Additionally, to clarify the role of lactate in its catabolism, a lactate spike was done in the middle of the experiment. The results show that before the addition of lactate, mostly butyrate and caproate were yielded, meaning that only even carbon VFA are produced from fructose. After the lactate spike, the production of odd carbon VFA (i.e. propionic and valerate) starts and the production of even carbon VFA increases its rate, indicating a clear link with lactate consumption. Therefore, lactate is used in two ways: i) to yield propionate and acetate following the well-established 2:1 ratio (Seeliger et al., 2002); and ii) as electron donor in chain elongation reactions, as in other members of the same genus (Rombouts et al., 2020). In this way, the dependency of odd chain VFA production on lactate addition is explained as propionate is only produced from lactate and its elongation to valerate is also linked to lactate presence. A more in-depth analysis of the results is available in section A of the Supplementary Materials.

Therefore, the proposed stoichiometry of *M. cerevisiae* catabolism (Table 1) comprises fructose conversion to acetate, butyrate and caproate, being the last yielded via chain elongation from butyrate and using fructose as electron donor. Butyrate is described in the model to be yielded together with acetate in a 1:1 molar ratio assuming the stoichiometry proposed by Regueira et al. (2018) featuring the electron bifurcation biochemical mechanism. Additionally, fructose full conversion to butyrate is also considered in the metabolic network of *M. cerevisiae* (Rombouts et al., 2020), as the ability to perform the electron bifurcation mechanism is not ubiquitous among fermenting bacteria. For lactate, the assumed catabolism includes its conversion to acetate and propionate (as propionate and its chain elongation products, valerate, are not detected until lactate addition) and chain elongation reactions using it as electron donor that yield butyrate and valerate, with acetate and propionate as starting VFA. Also, direct production of butyrate from lactate without acetate consumption is included as proposed elsewhere (Rombouts et al., 2020). Caproate production is modelled with butyrate and fructose as only substrates, as it seems not to be affected by lactate addition (Fig. S4).

This versatile catabolism is described in the stoichiometric matrix of the model (Supplementary Materials Table S1 and Table S2) by assigning to each catabolic transformation a process (i.e. a row in the stoichiometric matrix), with its independent kinetic parameters (i.e. maximum specific consumption rate and half-saturation constant). In this way, the complex stoichiometry of the co-culture experimental system can be easily described by combination of the different individual processes and, besides, the different kinetic characteristics of these individual transformation processes can be captured effectively in the model.

2.2.2. Parameter estimation

Most kinetic parameters of the model are estimated from experimental data of the co-culture system. Some parameters were not estimated from the experimental data and instead fixed values were used in all simulations (Table S3). Biomass yields (Y_{SUGAR} and Y_{LAC}) were not estimated as the microbial biomass concentration was not followed during the experiments. An accurate biomass concentration measurement was infeasible due to the turbidity of the fermentation medium caused by the cabbage particles. Instead, biomass yields were set to similar values reported previously for strains producing and consuming lactate (Rombouts et al., 2020). Additionally, it was decided not to include the inhibition constant (K_I) and the half-saturation constant of the different processes ($K_{S,SUGAR}$, $K_{S,LAC}$, $K_{S,VFA}$) in the estimation procedure as the experimental data did not provide enough information to estimate such a high number of parameters without avoiding identifiability issues, which would result in an overparametrized model (i.e. a given modification of one parameter can be compensated by linear combination of the other parameters of the set without changes in the model output) (Brun et al., 2001). Therefore, the half-saturation constants and the inhibition constant were set fixed to the reported values in preliminary parameter estimations and those values were used for the final and reported parameter estimation (Table S3). The parameters to identify are the maximum uptake rate values of all the 9 processes included in the stoichiometric matrix (Table S2).

Parameters were estimated following the methodology described elsewhere (Regueira et al., 2021). The procedure starts with minimising the normalized root squared mean deviation (NRSMD) between the experimental data at different time points and the data obtained by the model when mimicking those experiments (Eq. (3)). NRSMD minimisation is performed with the command *lsqnonlin* using the trust-region-reflective algorithm.

$$NRMSD = \frac{1}{n \cdot m} \cdot \sqrt{\sum_{j=1}^{n} \sum_{i=1}^{m} \left(\frac{\widehat{y}_{j,i}(\theta) - y_{j,i}}{\sigma_j} \right)^2} \tag{3}$$

where m is the number of measurement times throughout the batch experiment (6), n is the number of experimentally measured compounds (8), $\widehat{y}$ is the simulated concentration value, y is the experimental concentration value, θ is the vector of parameters being estimated and σ is a normalisation factor meant to scale the residuals to comparable magnitudes (σ is the range of the experimental values for all measurements). The subscript i refers to a measurement over time of the compound j.

To ensure a robust estimation of the parameters and avoid the model getting stuck at local minima, the parameter estimation follows a bootstrap procedure to determine the value and uncertainty interval of the estimated parameters. Finally, to assess the propagation of parameter uncertainty on the model outputs (substrates and VFA concentrations over time), the estimation methodology features a Monte Carlo procedure, as described in Saltelli et al. (2008). A number of 500 iterations were performed for this using Latin Hypercube Sampling to ensure a maximum coverage of the value space of the estimated kinetic parameters (Helton and Davis, 2003).

2.3. Wet experiments description

2.3.1. Cultivation media characteristics

The experiments were conducted with minimal media with pretreated cabbage residue. Minimal media contained yeast extract (0.6 g L^{-1}), L-cysteine hydrochloride (0.5 g L^{-1}), sodium acetate (13.7 g L^{-1}), trace elements (80 mL L^{-1}) and vitamin solution (1 mL L^{-1}). Trace elements contained $CaCl_2$ (0.125 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.125 g L^{-1}), K_2HPO_4 (1 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $NaHCO_3$ (5 g L^{-1}), NaCl (2 g L^{-1}), $(NH_4)_2SO_4$ (2.5 g L^{-1}), $MnSO_4{\cdot}H_2O$ (0.05 g L^{-1}), $FeSO_4{\cdot}7H_2O$ (0.05 g L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.05 g L^{-1}) and $CoSO_4{\cdot}6H_2O$ (0.005 g L^{-1}). Vitamin solution contained biotin (0.5 mg L^{-1}), pyridoxine (20 mg L^{-1}) and calcium pantothenate (20 mg L^{-1}). Minimal media was enriched with either hydrolyzed cabbage residue (Cab1) or pressed cabbage juice (Cab2). The concentration of pretreated cabbage in the medium varied between experiments to optimize the production of targeted volatile fatty acids. Therefore, the concentrations of the substrates, glucose and fructose, are presented separately for each experiment in the *Results* section.

2.3.2. Bioreactor setups

Bench-top bioreactors with a working volume of 400 mL (Multifors2, Infors AG, Bottmingen, Switzerland) were used for process development (section A of the Supplementary Materials). BioFlo 510 bioreactors with a working volume of 10 L (New Brunswick Scientific Co., New Jersey, USA) were operated for the model calibration and validation experiments. Bioreactors were operated under anaerobic conditions (flushed with N_2) at 30 °C. The bioreactor pH was set to 6.5 and it was controlled by online addition of NaOH (4 M) or H_3PO_4 (15 %). Slow agitation (100–150 rpm) was used during the experiments. Daily samples from 8 to 10 mL were harvested to measure sugar consumption and production of VFAs.

2.3.3. Analytical methods

VFAs including caproate, valerate, butyrate, propionate and acetate were detected by Gas Chromatography and the concentrations of glucose, fructose and lactate were determined by high-performance liquid chromatography, as described previously by Selder et al. (2021). The optical density (OD) was measured with a spectrophotometer (Shimadzu UV-1201 & UV–Vis, Australia) at wavelength 630 nm.

3. Results and discussion

3.1. Robust estimation of kinetic parameters

Kinetic parameter estimation was performed using a co-culture experimental data set where a minimal media with cabbage residue (Cab1) is the sole carbon source (Fig. S5). The model reproduces the observed evolution over time of the different substrates (i.e. glucose and fructose), final products (i.e. VFA) and lactate, which behaves both as a product and substrate, acting as a cross-fed compound (Fig. 1). For this reason, the uncertainty of lactate concentration prediction is higher at intermediate reaction times as the prediction of this compound bears the uncertainty of the parameters related to its production and consumption.

The experimental data allowed to identify robust kinetic parameters with a low collinearity factor of 9.6 (section D of the Supplementary Materials) (Table 2), indicating that the model reproduces satisfactorily the experimental data and that the stoichiometric matrix and the process description are valid for this co-culture system. The NRMSD (Eq. (3)) value between the calibrated model and experimental results remained low (0.0128) and the estimated parameter uncertainty is quite narrow for most of the cases. The estimated q_{max} values show a relatively broad range from 0.825 $g_{SUBS}\ g_{BM}^{-1}\ h^{-1}$, for fructose uptake, to 4.725 $g_{SUBS}\ g_{BM}^{-1}\ h^{-1}$, for glucose uptake in *P. pentosaceus*, and from 0.070 $g_{SUBS}\ g_{BM}^{-1}\ h^{-1}$, for caproate production, to 8.529 $g_{SUBS}\ g_{BM}^{-1}\ h^{-1}$, for butyrate production, in the case of *M. cerevisiae*, which indicates the dominance of some pathways over others. These results indicate that each strain performs the considered processes at a very distinct rate showing preferences for one or the other process. For example, *P. pentosaceus* consumes preferably glucose rather than fructose and that *M. cerevisiae* is specialized in consuming lactate rather than fructose, with a preference for producing butyrate (Table 2).

3.2. Design of in silico experiments

The information provided by the kinetic parameters can help to understand how selectivity works in the co-culture system and gives insight on how to steer the process to yield a desired product spectrum. Regarding sugars consumption, *P. pentosaceus* has a much higher capacity for consuming glucose and fructose than *M. cerevisiae*, with a clear preference for glucose (Table 2). In line with the expected metabolism of *M. cerevisiae*, processes consuming lactate present a much higher maximum rate than those consuming fructose and yielding even carbon VFA (i.e. butyrate and caproate). Among lactate-consuming processes, the one producing butyrate is the fastest followed by lactate conversion to acetate and propionate. On the contrary, the process yielding valerate from lactate and propionate is the slowest, meaning that the expected production of even carbon VFA should be higher than odd carbon VFA production, as confirmed by the experimental results (Figs. 2 and S3). Regarding valerate production, it should be considered that propionate, unlike acetate, is not present at the beginning of the experiment and therefore it needs to be produced first by lactate oxidation before it can be elongated to valerate.

Therefore, if the objective is to favor even carbon VFAs production (i.e. butyrate and caproate), sugar conversion to lactate should be avoided as much as possible to favor its direct conversion to even-carbon VFA. To achieve this, the conversion of fructose to lactate by *P. pentosaceus* should be avoided to force fructose consumption by *M. cerevisiae* to yield more butyrate and caproate. To make this strategy possible, the two strains of the co-culture should be inoculated separately in the system as *M. cerevisiae*

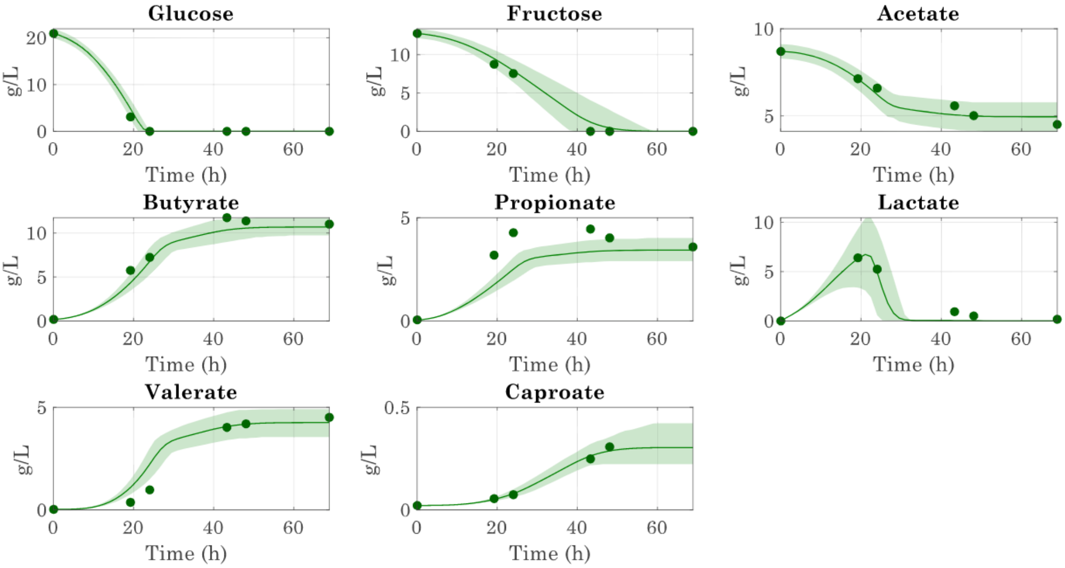

Fig. 1. Fit between the experimental data (filled circles) and the model results (continuous lines) with the estimated kinetics parameters (Table 2). The shaded areas represent the 95 % interval confidence and were estimated by error propagation of the parameter uncertainties following a Monte Carlo procedure.

consumed fructose slower than *P. pentosaceus*. Then, *M. cerevisiae* should be inoculated in the first place to convert fructose to butyrate and caproate, and when fructose is exhausted, *P. pentosaceus* can be inoculated to convert the glucose still present and complete the production of VFA. An additional measure to increment the yield of butyrate and of even-carbon VFA would be to increase the proportion of fructose in the initial mix of sugars by supplementing a waste stream with high fructose concentration such as fruit pressing residue to the waste stream.

On the contrary, if the intention is to favor the production of odd-carbon VFAs (i.e. propionate and especially valerate), the strategy should be the opposite: maximize the conversion of sugars to lactate, as propionate and valerate production directly depends on processes consuming it. Therefore, the inoculation of *M. cerevisiae* should be delayed allowing *P. pentosaceus* to convert all present sugars to lactate. Once sugars are fully converted to lactate, *M. cerevisiae* is inoculated to convert lactate to the final odd-carbon VFA compounds. To further increase the share of valerate, the target product, in the final product spectrum, the proportion of glucose in the initial sugar mixture could be increased as it is the preferred substrate for lactate production.

Based on the aforementioned information, in silico experiments were designed with the objective of validating the two envisioned strategies and predicting quantitatively the changes in the yield of the desired products (i.e. butyrate and valerate since caproate production is very limited) in each of the envisioned strategies (Table 3). Additionally, a standard co-cultivation referred as the base case experiment was included to be used as control experiment. In the sequential inoculations, the second inoculation was simulated to take place 100 h after the start of the experiment, to allow for full consumption of the substrate(s).

The model results (Table 3) indicate that both strategies fulfil their purpose of directing the production towards valerate or butyrate but with different success. While the odd-carbon VFA strategy can increase more than 30 % the yield value of valerate, the even-carbon VFA strategy is only able to raise the yield of butyrate marginally. This might indicate that the system could already be quite optimised towards the yield of even-carbon VFA and butyrate and that a further increased in even-carbon VFA yield is not possible with only changing operational parameters.

3.3. Experimental validation of in silico experiments

This section contains the results of the experiments performed to test the envisioned strategies in Section 3.2 to favor the production of even and odd-carbon VFA, including the base case. All experiments used cabbage hydrolysate medium (Cab 1). The base case (Fig. S6) behaved as expected with low lactate accumulation (2 g L^{-1}) and with butyrate as main product, as predicted by the model (Table 3). When compared to the model

Table 2
Estimated maximum specific consumption rates (mean value [estimated confidence interval with α = 0.05]) for the different processes considered in the co-culture system.

Reaction number	q_{max} (g_{SUBS} g_{BM}^{-1} h^{-1})
1 (Glu → 2 Lac)	4.725 [4.356, 4.958]
2 (Fruc → 2 Lac)	0.825 [0.633, 1.024]
3 (Fruc → But)	0.474 [0.021, 0.974]
4 (Fruc → 0.5 Ac + 0.5 But)	[a]
5 (Fruc + 2 But → 2 Cap)	0.070 [0.061, 0.082]
6 (Lac → 0.67 Pro + 0.33 Ac)	6.527 [5.833, 7.191]
7 (Lac → 0.5 But)	[a]
8 (Lac + Ac → But)	8.529 [7.743, 9.208]
9 (Lac + Pro → Val)	4.795 [4.079, 5.579]

[a] Preliminary estimations did not select these reactions and therefore, they were excluded from the complete parameter estimation procedure.

Table 3
Details of the first round of designed in silico experiments and modeled yields results.

	Base case	Odd-carbon VFA strategy	Even-carbon VFA strategy
Inoculation details			
First strain inoculated	Both	*P. pentosaceus*	*M. cerevisiae*
Second strain inoculated (at 100 h)		*M. cerevisiae*	*P. pentosaceus*
Initial substrate concentration			
Initial glucose concentration (g L^{-1})	25	40	25
Initial fructose concentration (g L^{-1})	15	15	30
Yield results (g_{VFA}/g_{SUGAR})			
Acetate	0.176	0.094	0.113
Propionate	0.179	0.177	0.154
Butyrate	0.442	0.424	0.448
Valerate	0.158	0.210	0.157
Caproate	0.010	0.000	0.022
Increase in yield of the targeted VFA			
Valerate		33.2 %	
Butyrate			1.4 %

predictions, the yield of odd-chain VFA is inferior and the yield for butyrate slightly increased.

However, the experiments for validating the envisioned strategies did not provide the expected results. In the experiment for enhanced odd-carbon VFA, *M. cerevisiae* was not able to consume lactate after its generation by *P. pentosaceus* from glucose and fructose, suggesting a severe inhibition of its activity due to the high lactate concentration (higher than 50 g L^{-1}) in the broth (Fig. 2A). This effect is not exclusive of *M. cerevisiae* since the growth of other bacteria, of the same or other genus, is reported to be severely impaired at moderate and high lactate concentration. For example, a strain of *Megasphaera elsdenii* was reported to be severely inhibited in the presence lactate concentrations of 23 g L^{-1} (Weimer and Moen, 2013) and a *Clostridium* genus isolate, a butyrate-producer from lactate, stopped converting lactate when its initial concentration was higher than 12 g L^{-1} (Tao et al., 2016). In the case of the even-carbon VFA strategy, while butyrate was the only product until *P. pentosaceus* inoculation, in line with the strategy, *M. cerevisiae* could only consume fructose slowly, which limited the extent to which even-carbon VFA production could be enhanced. Other *Megasphaera* strains proved to have a similar behaviour as experiments with *M. elsdenii* at increasing initial sugar concentrations showed that values above 54 g L^{-1} significantly decreased substrate consumption and hence VFA production (Weimer and Moen, 2013). Moreover, and most importantly, *M. cerevisiae* was not able to consume the lactate generated when *P. pentosaceus* was inoculated, limiting significantly the overall VFA yield. These results were interpreted as suggesting that *M. cerevisiae* was inhibited by high sugars and lactate concentrations and that lower sugar concentration should be used in the design of the co-culture process. The issue of lactate inhibition was not detected before because in the previous experiments where both strains were present from the beginning, lactate only accumulated up to a maximum of 6.4 g L^{-1} (Fig. S5).

As the model experimental design could not be validated due to the inhibition of *M. cerevisiae* at high sugars and lactate concentrations, the metabolic limits of this strain should be investigated to make the next round of in silico experiments more accurate. With that aim, experiments using *M. cerevisiae* were done to assess the limits of lactate tolerance (a detailed description is available in section E of the Supplementary Materials). The results indicate that a lactate concentration of 34 g L^{-1} is already quite inhibitory for *M. cerevisiae* as little lactate was consumed throughout the experiments (Table S4). A second and lower concentration of 24 g L^{-1} was tested and while lactate was consumed at a much faster rate, the consumption was not complete. Therefore, these results indicate that lactate concentration should be kelp below 20 g L^{-1} to avoid *M. cerevisiae* inhibition.

Table 4
Details of the designed in silico experiments and modelled yields results.

	Base case	Odd-carbon VFA strategy	Even-carbon VFA strategy
Inoculation details			
First strain inoculated	Both	*P. pentosaceus*	*M. cerevisiae*
Second strain inoculated (at 70 h)		*M. cerevisiae*	*P. pentosaceus*
Initial substrate concentration			
Initial glucose concentration (g L^{-1})	10	13.3	6.7
Initial fructose concentration (g L^{-1})	10	6.7	13.3
Yield results (g_{VFA}/g_{SUGAR})			
Acetate	0.370	0.357	0.393
Propionate	0.158	0.173	0.134
Butyrate	0.358	0.354	0.371
Valerate	0.080	0.095	0.056
Caproate	0.008	0.000	0.015
Increase in yield of the targeted VFA			
Valerate		19.3 %	
Butyrate			3.5 %

3.4. Targeting the production of odd- and even carbon VFAs in in silico experiments

A second round of in silico experiments was done with the kinetic model. In this case, the initial total sugar concentration was lowered to 20 g L^{-1} in all strategies, following the results from experiments testing the sensitivity of *M. cerevisiae* to different initial sugar and lactate concentrations (Table 4). Instead of adding extra glucose or fructose to increase their concentration in the waste stream, the total sugar concentration was kept constant while the glucose to fructose ratio was modified. The medium was changed from Cab1 to Cab2 (Section 2.1), since in parallel with the development of the model, the production process itself was optimized by Selder et al. (2021). Due to the change in glucose and fructose concentrations, the ratio between glucose and fructose initial concentration was assumed to be 1.0 in all in silico experiments. An initial acetate concentration of 10 g L^{-1} was set in the simulations as in the previous sets of experiments. Due to the lower total sugars concentration, the second

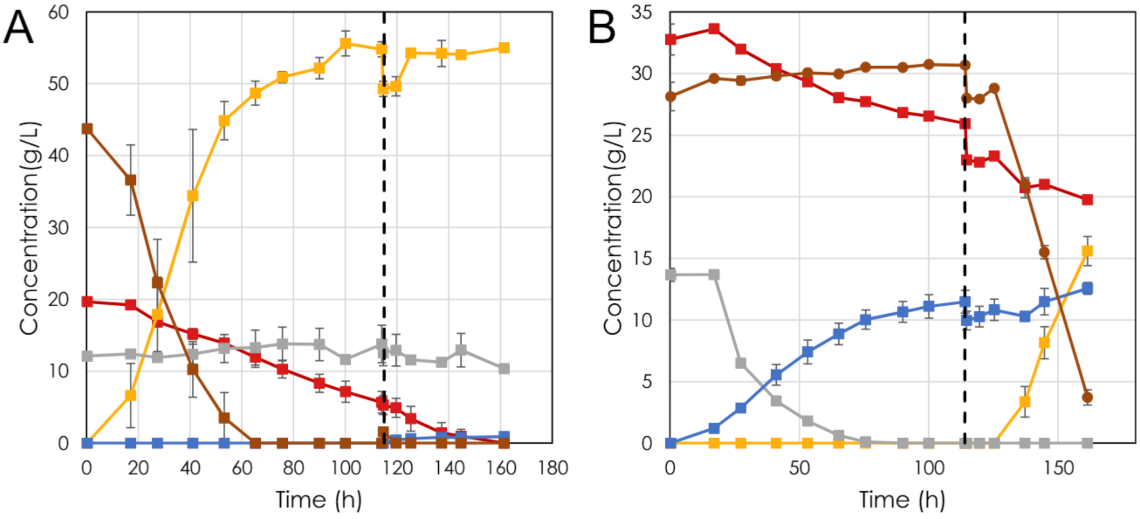

Fig. 2. Fructose, glucose, lactate and VFA concentration over time in the experiments for validation of the first round of in silico experiments. A: odd-carbon VFA strategy with delayed *M. cerevisiae* inoculation (at $t = 115$ h) and extra glucose supplementation. B: even-carbon VFA strategy with delayed *P. pentosaceus* inoculation (at $t = 114$ h) and additional fructose initial concentration. The data points show the average value of biological duplicate experiments and the error bars indicate the standard deviation. 🟥 fructose, 🟫 glucose, 🟨 lactate, ⬜ acetate, 🟦 butyrate.

inoculation was simulated to take place 70 h after the start of the experiment, in line with the time needed for substrate(s) consumption in the first validation experiment set.

The second-round model results (Table 4) present some differences when compared to the results of the first round of in silico experiments (Table 3). Acetate yield has now increased while, on the contrary, the yield values for odd-carbon VFA diminished. Also, the potential for enhancing the yield of valerate in the first strategy has decreased from 33.2 % to 19.3 %, which could be explained by the lower lactate concentration attained in the process. The potential of the even-carbon VFA strategy to improve butyrate yield remains very low, indicating again the process is already likely optimal to produce even-carbon VFA.

3.5. Experimental validation of second-round in silico experiments

The in silico experiments of the previous section were validated in wet experiments using the Cab2 medium (Table 1). Fig. 3 shows the yield for the products of interest at the end of the three experiments performed: i) the base case, ii) the even-carbon strategy case targeting butyrate production, and iii) the odd-carbon strategy case targeting valerate production. The detailed results of these three experiments are available in section F of the Supplementary Materials.

The base case presented a clear selectivity for butyrate, as predicted by the model. However, it must be pointed out that the yield for butyrate in this base case cultivation (Fig. 3) was significantly higher than in the previous equivalent experiments (Figs. S5 and S6). While in this base case the yield achieved a value of 0.64 ± 0.01 $g_{butyrate}$ per $g_{consumed\ sugar}$, in the experiments used for model calibration (Fig. S5), it achieved a yield of 0.33 $g_{butyrate}$ per $g_{consumed\ sugar}$, and of 0.36 ± 0.03 $g_{butyrate}$ per $g_{consumed\ sugar}$, in the base case of the first in silico experiments validation (Section 3.3 and Fig. S6). The process yield for caproate was also higher than in previous experiments, attaining now values of 0.14 ± 0.001 $g_{caproate}$ per $g_{consumed\ sugar}$, in contrast with the very low value of 0.013 $g_{caproate}$ per $g_{consumed\ sugar}$ achieved in the base case of the first in silico experiments validation (Fig. S6). This shift towards even-carbon VFA could be justified by the lower lactate concentrations attained, which drives the process towards the yield of odd-carbon VFA, due to the lower initial sugar concentration or to the higher proportion of fructose in the feeding due to the change in the real waste stream used. As discussed in Section 3.2, a higher fructose proportion in the feeding favors the generation of even-carbon VFA. Additionally, in this base case (Fig. S8), fructose consumption was faster than glucose consumption, which was not case in previous co-culture experiments (Figs. S5 and S6). This anomaly could indicate that in this experiment *M. cerevisiae* could capture a higher share of sugars, which will be exclusively used for even-carbon VFA production according to the metabolic network.

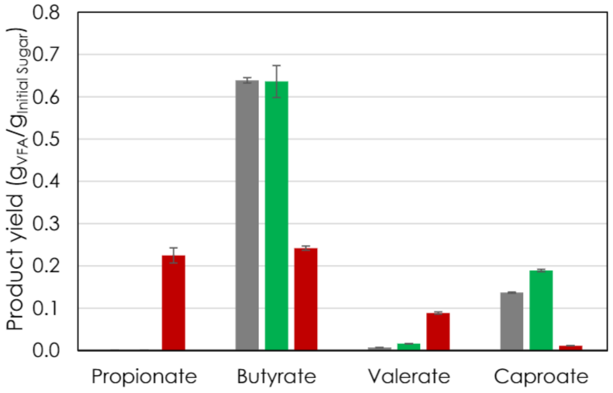

Fig. 3. Main product yields of the validation experiments for a targeted VFA production. The yield is determined with the batch end concentration. Bars represent the average yield and error bar indicate the standard deviation between the biological duplicates. ■ base case 🟩 even-carbon VFA strategy 🟥 odd-carbon VFA strategy.

The even-carbon strategy did not provide any significant change in comparison with the base case when it comes to butyrate yield, which is line with the model predictions (Fig. 3 and Table 4). Yet, the proposed strategy proved to be effective and provided a significant increase in the yield of the system for caproate up to a value of 0.19 ± 0.002 $g_{caproate}$ per $g_{consumed\ sugar}$, representing a 38 % increase. The final titer attained was of 3.24 ± 0.035 $g_{caproate}\ L^{-1}$, which is in the same range as caproate concentration achieved in the enrichment experiments producing selectively this compound from sugar-rich substrates (Allaart et al., 2021; Carvajal-Arroyo et al., 2019; Mariën et al., 2022). We believe that caproate yield is limited because its production by *M. cerevisiae* seems to be strongly linked to the use of fructose as substrate rather than lactate (Engelmann and Weiss, 1985), as also observed in other species of the *Megasphaera* genus, such as *M. elsdenii* (Marounek et al., 1989; Tao et al., 2017). In the co-culture system of this work, there is a strong competition for fructose consumption (e.g. lactate and butyrate production) as shown by the model parameters (Table 2), thus limiting the potential for caproate production.

The odd-carbon VFA strategy proved to be effective (red bars in Fig. 3). In comparison with the base case, the even-carbon VFA yield values (i.e. butyrate and caproate) diminished importantly (62 % and 94 %, respectively) and, at the same time, propionate became one of the major end products (0.225 $g_{propionate}$ per $g_{consumed\ sugar}$), while it was not detected in the base case. Also, valerate yield increased from a mere 0.007 to 0.085 $g_{valearte}$ per $g_{consumed\ sugar}$, which represents over a 10-fold increase. Both yield values are close to the predicted values (Table 4), demonstrating that the model is a suitable tool to explore experimental designs in process development. The higher selectivity for odd-carbon VFA strategy can be of use in applications as, for example, the production of bioplastics as polyhydroxyalkanoates (PHA). PHA are bio-sourced biodegradable polyesters that can be produced biologically using VFA as substrate and that require a balanced mixture of odd- and even-carbon VFA to have properties that allow them to effectively substitute current oil-based polymers (Duque et al., 2014; Melendez-Rodriguez et al., 2021). If the VFA mixture obtained in the base case would be used for the synthesis, the obtained PHA would instead have much poorer mechanical properties, limiting the use for common applications such as packaging films (Cunha et al., 2016).

The validation experimental set showed the goodness of the predictions made by the model in the in silico experiments. First, the prediction that the system was already significantly optimised for even-carbon VFA production was corroborated by the experimental results. In second place, the experiments validated successfully the odd-carbon VFA production strategy and the model could predict satisfactorily the achieved propionate and valerate yield values. The strategies envisioned with the developed model in Section 3.2 remained valid, even when the experimental conditions changed, which underlines the robustness of the methodology for capturing the complex dynamics of the system. If a more accurate quantitative predictions were needed for the development of a process, it would be recommendable to perform a model recalibration to update the kinetic parameters to the new experimental scenario (i.e. new substrate concentration and modified substrate).

4. Discussion: the combined use of experimental and modeling work in bioprocess development

Based on the potential showed by the results of this work and following the rules of the DBTL approach, we propose a design pipeline for bioprocess development that comprises two pieces: experimental data acquisition in expressly designed experiments and the development and/or use of unstructured kinetic models. The use of unstructured kinetic models is interesting in this scheme as they are easy and quick to adapt to a new scenario (i.e. selection of different strain or substrate). In general, they only require one experimental test to extract the kinetic parameters.

The design workflow envisioned in this study, combines the first-hand information provided by controlled experiments and the power of

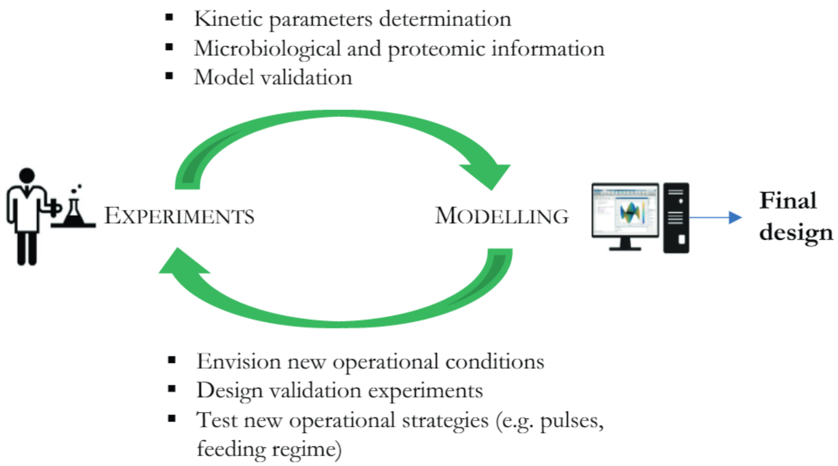

Fig. 4. Envisioned workflow combining the use of wet lab experimentation and modeling for bioprocess design.

mathematical models of extracting intrinsic information contained in the experimental data that is not obvious at first glance and of assisting in the design of future experiments. These two pieces exchange information with each other in an iterative way as depicted in Fig. 4. In the first cycle of the workflow, the kinetic model uses experimental information for its calibration (Section 3.1) and provides an experimental design proposal (i.e. the experimental conditions of the process) based on the intrinsic information provided by the kinetic parameters and simulations results (Section 3.2), which is experimentally validated (Section 3.3). If the in silico experiments are not satisfactorily validated, as it was the case of this work, a new round of in silico experiments should be performed once the needed adjustments are done (as shown in Section 3.4 to adjust for lactate and sugar inhibition). Again, these predictions are experimentally validated, as in Section 3.5. In the case differences were still detected, this routine of model-based designs and subsequent experimental validation would be repeated. For example, a model recalibration could be advised as suggested in Section 3.5. This need comes from the fact that, in some cases, kinetic models present problems to predict behaviors outside of the area of environmental conditions in which they were calibrated. To be efficient, this process should be done following an Optimal Experimental Design procedure, which aims at formulating experiments providing the maximum and best statistical quality information for model calibration and refinement using mathematical optimisation algorithms (Banga and Balsa-Canto, 2008). However, as pointed out earlier, the time required to readapt the kinetic model to these new environmental conditions is very limited. Other information coming from the wet experiments, such as data on microbial community composition or on proteomics, can also be used for model refinement as it could help identifying which metabolic functions are being truly expressed by the microorganisms involved in the process.

5. Conclusions

This work shows how the combination of mathematical modeling and wet lab experiments is a sound avenue for the design of novel bioprocesses. Specifically, this workflow was applied to develop a process to produce volatile fatty acids in a targeted way from food waste using a defined co-culture (*Pediococcus pentosaceus* and *Megasphaera cerevisiae*). The developed workflow proved to being able of capturing the complex dynamics of the co-culture system and of designing experiments with enhanced selectivity towards even-carbon chain products (e.g. butyrate or caproate) or odd-carbon chain products (e.g. propionate or valerate), which were experimentally validated. In the case of even-carbon VFA, the model detected that the base experiment was already quite optimised for butyrate production, although the proposed enhanced experiments yielded 38 % more caproate. For the enhanced odd-carbon VFA experiments, the yield of butyrate and caproate diminished 62 and 94 %, respectively, while propionate, which was absent in the base case, became a major product and the valerate yield increased from 0.007 to 0.085 $g_{valearte}$ per $g_{consumed\ sugar}$. Overall, the workflow proposed in this study integrates advantages from its two main pillars (i.e. modeling and experimentation) and offers a good compromise between understanding the main sensitivity of the process, flexibility and predictability, making it attractive and sound for developing processes based on the use of defined microbial communities for VFA production.

CRediT authorship contribution statement

Alberte Regueira: Methodology, Investigation, Data analysis, Software, Writing – original draft, Writing – review & editing. **Rosaliina Turunen**: Methodology, Investigation, Sample processing, Data analysis, Writing – original draft, Writing – review & editing. **Kiira S. Vuoristo**: Methodology, Investigation, Supervision, Data analysis, Writing – original draft, Writing – review & editing. **Marta Carballa**: Methodology, Conceptualization, Funding acquisition; Supervision, Data Analysis, Writing – review & editing. **Juan M. Lema**: Methodology, Conceptualization, Supervision; Writing – review & editing. **Jaana Uusitalo**: Methodology, Conceptualization, Funding acquisition; Supervision, Data Analysis, Writing – review & editing. **Miguel Mauricio-Iglesias**: Methodology, Conceptualization, Funding acquisition, Supervision, Data Analysis, Writing – review & editing.

Data availability

Data will be made available on request.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159521.

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