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Research article

The driving role of proteins in enhancing lactate and propionate production during food waste fermentation: metabolic constraints and kinetic modeling

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A B S T R A C T

This study aimed to quantify the fraction of lactate, derived from the fermentation of Food Waste (FW) attributable to proteins. Infact, recent studies based on cellular resource allocation suggest that proteins can promote the growth of lactic acid bacteria and enhance lactate production.

The experiments were conducted in a chemostat, at a HRT of 20 h and a pH = 5 and 5.5, using a synthetic substrate composed of glucose and albumin in a 4:1 COD ratio, mimicking household FW composition. A pure glucose solution was used for comparison.

Although lactate was detected at low concentrations, the glucose-albumin substrate produced a considerable amount of propionate, even when albumin was not consumed, in contrast to the pure glucose solution.

It was hypothesized that most propionate was generated via the acrylate pathway, rather than glycolysis, thus confirming the significance of the lactate metabolic pathway. A mathematical model based on the variable stoichiometry approach was adopted as a tool to investigate this hypothesis. The model accounted for the metabolic constraint imposed by the conservation of electron carriers (NADH) throughout the fermentation process.

The metabolic constraint made it possible to estimate that propionate production occurred with high probability via the acrylate pathway, thereby revealing that proteins were crucial in driving the metabolic shift of glucose fermentation toward lactate; model calibration estimated that lactate accounted for 35 – 65 % of the total electron flux.

Furthermore, lactate was separately supplemented to the substrate in additional experiments, inducing a metabolic shift from acrylate pathway to chain elongation.

1. Introduction

Anaerobic Digestion (AD) is a widely used technology for the treatment of organic substrates (Chen et al., 2026). AD has been traditionally employed for biogas production as a source of renewable energy; biogas is comprised of methane, carbon dioxide and hydrogen.

The methanogenic process involves the oxidation of the Volatile Fatty Acids (VFAs) produced along direct fermentation reactions, such as glycolysis. The oxidative process takes place through the progressive shortening of the VFAs molecular chains, which are subsequently converted into acetic acid and hydrogen (Yeghiazaryan et al., 2026); the latter is generated via protons reduction, since protons represent the only available extracellular electron carriers in the anaerobic environment. Both compounds are then converted into methane, via acetoclastic and hydrogenorophic methanogenesis, respectively. Methane is then combusted to produce renewable energy which, in most cases, requires subsidies to make the process economically viable.

More recently it was proposed to stop the digestion process at the fermentative stage, without proceeding to methane production (Kleerebezem et al., 2015). This can be achieved by increasing hydrogen partial pressure, to suppress VFAs oxydation to acetate, and at the same time by lowering the pH, to inhibit acetoclastic methanogenesis. Both these goals can be achieved by increasing the organic loading rate (Zhang et al., 2025a).

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However, the VFAs dispersed in the fermentation broth are difficult to extract, due to their low molecular weight and high solubility (Suo et al., 2025); to address this limitation, fermentation can be effectively integrated with Chain Elogation (CE), an anaerobic process enabling the production of longer-chain compounds, namely Medium Chained Fatty Acids (MCFAs), which are more hydrophobic and therefore more readily recovered from the aqueous phase.

CE encompasses a wide range of reactions in which an electron donor (ED) is first converted into acetyl-Coa and subsequently metabolized through the reverse β-oxydation cycle pathway (Zhang et al., 2026). Within this patway, acetyl-Coa reacts with an electron acceptor (EA) thereby promoting the elongation of the molecular chain and the production of MCFAs (Angenent et al., 2016; Atasoy et al., 2026; Spirito et al., 2018).

Among the metabolites generated during fermentation, only lactate and ethanol can serve as EDs in the CE whereas all the other VFAs act excusively as EAs. Considering that lactate and ethanol production via fermentation is typically limited (Roghair et al., 2018a; Contreras Davila et al., 2021) - when present at all –a pronounced imbalance between EDs and EAs is commonly observed in the metabolites pool. This imbalance costitutes a major bottleneck in the elongation process (Grootscholten et al., 2014). Consequently, in most applications EDs are externally supplied, leading to increased operational cost and a higher environmental impact (Chen et al., 2020; Kucek et al., 2016).

To address this limitation, numerous studies have focused on identifying optimal conditions to enhance ED production (Contreras Davila et al., 2020, 2020b). In this context FW, unlike most substrates, has demonstrated a strong capacity to produce substantial amount of lactate during the fermentation step, thereby emerging as a promising substrate for CE (Luo et al., 2025; Perez-Rangel et al., 2026).

FW is an organic waste whose global production in 2022 amounted to 1052 millions of tonns (UN, 2024); as such, FW represents a key resource in the framework of the circular economy (Pei et al., 2026). Composting and anaerobic digestion are widely recognized as environmental sustainable options for FW management. In contrast, FW disposal in landfill can lead to significant methane emissions (Zhang et al., 2025b), a potent greenhouse gas whose emissions are mostly released untreated in the atmosphere.

Emerging technologies such as CE could therefore represent a valid option for FW valorization; in this context, gaining insights into the metabolic mechanisms governing lactate production during FW fermentation is of primary importance.

To this aim, metabolic models are a valid tool for estimating the stoichiometric fractionation of the metabolites (González-Cabaleiro et al., 2015; Regueira et al., 2020). These models are based on the maximizations of the ATP yield from glycolysis; consequently, they tend to underestimate the lactate production pathway, as it is associated to a lower ATP production compared to that of other metabolites such as acetate, butyrate and ethanol. Nevertheless, it was observed that the ATP yield is not the sole driver of fermentation stoichiometry (Rombouts et al., 2020). In fact, under high organic loads, microorganisms with faster substrate uptake rates are favoured over those with higher ATP production efficiency, in a tradeoff between growth rate and energy efficiency. This phenomenon is disregarded in the metabolic models and this may explain the discrepancies observed in lactate production predictions. However, it is worth noting that lactate production is largely favoured when proteins are present, since Lactic Acid Bacteria (LAB) are auxotrophic for amino acids, meaning that they require amino acids and peptides for their growth because they cannot produce these compounds from the carbon substrate (Regueira et al., 2021a, 2021b).

To overcome these limitations, these models were integrated with the resource allocation theory proposed by Regueira et al. (2021 a). According to this framework, microbial growth is constrained by the competition for a limited pool of amino acids, which are utilized as enzymes for the microorganisms metabolism. Therefore, under conditions where the total protein concentration is limiting, such as at high growth rates, pathways with lower enzyme requirements are favoured.

Regueira et al. (2021 a, 2021 b) demonstrated that LAB, owing to their auxotrophic anabolism, can directly utilize the amino acids present in the anaerobic media, thereby reducing the need for enzymes synthesis. Indeed, the enzymatic requirement for the pyruvate to lactate pathway is as low as 0.007 mg Prot (mmol pyruvate h^{-1})$^{-1}$, which is two to three orders of magnitude lower than the all the other glycolytic pathways (Regueira et al. 2021 b), resulting in more efficient proteome allocation and higher growth rates.

Consequently, when amino acids are available LAB can outcompete other microorganisms although being less efficient in terms of energy utilization. Considering that FW is a carbohydrate-rich substrate, with proteins accounting for approximately 20 % of its total composition (Braguglia et al., 2017), the substantial lactate production observed during FW fermentation can reasonably be attributed to this characteristic.

To investigate this hypothesis, it is essential to quantify to which extent lactate production is associated to the proteins content. To this goal, a synthetic substrate was formulated to reproduced the typical sugars/proteins ratio of the FW, corresponding to 4:1 on a COD basis (Gallipoli et al., 2024). Using a synthetic substrate was fundamental, since real FW is a lignocellulosic feedstock composed by an extremely variable pool of sugars and protein, in addition to other organic recalcitrant constituents (Braguglia et al., 2017); consequently, the use of a simplified substrate allows several biological processes (e.g., hydrolysis), which may influence the overall process outcome, to be excluded. Although this approach inevitably provides a simplified representation of the complex composition of FW and the overall fermentation process, it enables a more targeted investigation of the phenomenon of interest, namely the relationship between proteins content and lactate production. In this view, glucose and albumin were selected as the most suitable compounds to represent the sugars and proteins pool, respectively. This substrate was continuously fed to a Chemostat run at a Hydraulic Retention Time (HRT) of 20 h and an Organic Loading Rate (OLR) of 6 gr COD L^{-1} day $^{-1}$; these operational conditions were selected in analogy to similar experiments reported by Temudo et al. (2008).

It's worth noting that, despite the use of a simplified substrate, the mechanisms identified herein have broader implications for the management of FW and agro-industrial residues. In particular, understanding how the carbohydrate-to-protein ratio affects lactate formation and its subsequent conversion can inform the characterization, blending, and allocation of waste streams to fermentation or chain-elongation processes. This is particularly relevant because MCFAs production via chain elongation often relies on external electron donors, whose cost can hinder process implementation at industrial scale. In this context, the fermentative production of electron donors, such as lactate, from biowastes represents a promising strategy to overcome this technological bottleneck and facilitate the large-scale deployment of chain-elongation technologies. Ultimately, this knowledge can support the development of more predictable waste biorefineries that divert biodegradable carbon from landfilling or low-value energy recovery towards the production of recoverable carboxylates.

To facilitate the identification of the metabolic pathways shift induced by the presence of proteins, a substrate composed of sole glucose was also used as a control in two parallel experiments. Furthermore, to specifically investigate the fate of lactate, a third substrate comprised of glucose (70 %), albumin (18 %) and lactate (12 %) was also fed to two additional reactors operated under the same operational conditions.

Mathematical modeling is a fundamental tool for investigating biochemical processes. With regard to anaerobic processes, the most widely used model in the literature is the Anaerobic Digestion Model nr 1 (ADM1) developed by the International Water Association (Batstone et al., 2002). ADM1 is a kinetic model, primarily designed to predict methane production; although it also accounts for fermentation, it fails to adequately capture the most peculiar aspects of the fermentative

pathway.

To gain more insights into the distinctive features of the fermentation process, kinetic models with alternative approaches have been proposed in the literature; among the others, the most reliable ones were based on variable stoichiometry(Antonopoulou et al., 2012; Nabaterega et al., 2023).

These models aimed to identify the stoichiometric fractionation of the substrate - typically glucose - in relation to the amount of metabolites produced throughout the process. Compared with metabolic models, kinetic models are more widely applied and more easily calibrated against traditional experiments; however, unlike metabolic models, they do not account for energy fluxes and metabolic constraints, such as the conservation of intracellular electron carriers.

Consequently, when they are applied to a system with multiple degrees of freedom - as is the case of the fermentation process, where calibration is focused on a broad pool of metabolites - they can lead to unrealistic estimation of the stoichiometric parameters. To address this issue, the most important metabolic constraint accounted for in the metabolic models, namely, electron carriers (i.e NADH) conservation, was added to a kinetic model based on the variable stoichiometry approach; the model was aimed at the identification of the glycolytic stoichiometric paramers. Although being based on the ADM1 approach, the fermentative process was extend to two additional compounds, namely ethanol and lactate. Furthermore, the acrylate pathway, which is the only metabolic pathway involving lactate and propionate for this simplified synthetic substrate - other to glycolysis - was included in the model. This implementation allowed to estimate which of biochemical pathways (glycolysis or acrylate pathway) took place with the highest probability, thereby assessing the impact of the proteins on the process.

To the best of our knowledge, this is the first study to incorporate this metabolic constraint in a variable stoichiometry model, thereby making the model outcomes far more realistic. While future biological analyses may provide further support for the model predictions, the present findings demonstrate that this approach can already yield significant and informative results for understanding the fermentation process.

2. Materials and Methods

2.1. Experimental setup

The experiments were performed in a thermostated 1-L chemostat reactor operated in continuous mode and coupled with an ADI 1010 Bio Controller integrated with an ADI 1025 Bio Console (Applikon Biotechnology, The Netherlands) for continuous monitoring and adjustment of pH, temperature, medium level and agitation speed. The pH levels (5 or 5.5) were automatically maintained by adding 3 M NaOH or 4.5 M HCl, as required. Six fermentation experiments were performed by feeding the reactor with three synthetic substrates (Table 1) at a flow rate of 0.05 L h^{-1} corresponding to a constant HRT of 20 h.

Substrate concentration amounted to 5 gr COD L^{-1}, resulting in an OLR of 0.25 g COD L^{-1} h^{-1} (6 g COD L^{-1} day^{-1}). All experiments were carried out at 37 °C under controlled pH conditions of 5 and 5.5 and 160 rpm agitation speed, until steady-state conditions were achieved.

Three synthetic substrates were formulated with the following component compositions: (i) sole glucose (Exp_1 and Exp_2); (ii) glucose (80 %) and albumin (20 %) (Exp_3 and Exp_4); (iii) glucose (70 %), albumin (18 %) and lactate (12 %) (Exp_5 and Exp_6). The substrates used for Exp_3 and Exp_4 are intended to represent the FW extract described in Gallipoli et al. (2024). Bovine serum albumin was used as the albumin source in lyophilized powder form (A3294, Sigma-Aldrich). The synthetic substrates were supplemented with a mineral medium containing $(NH_4)_2SO_4$ (0.5 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.1 g L^{-1}), $CaCl_2$ (0.05 g L^{-1}), K_2HPO_4 (0.11 g L^{-1}), KH_2PO_4 (0.51 g L^{-1}), $FeCl_3$ (0.002 g L^{-1}), and NaEDTA (0.003 g L^{-1}). Lab-scale methanogenic digested sludge was used as the inoculum. The anaerobic biomass was acclimated for 10 days before starting the experiments by daily feeding (and withdrawal) 0.15 L of synthetic feed (glucose and albumin mix).

In analogy with Temudo et al. (2008), other experiments were carried out with an HRT of 8 h. However, these experiments failed to achieve a steady-state conditions owing to biomass washout (data not shown).

Operational and feeding conditions are summarized in Table 1.

Table 1
Feeding and operational conditions of the experiments.

Experiment	Substrate	HRT [h]	OLR [g COD L^{-1} day^{-1}]	pH
1	Glucose	20	6	5.5
2	Glucose	20	6	5
3	Glucose (80 %) Albumin (20 %)	20	6	5.5
4	Glucose (80 %) Albumin (20 %)	20	6	5
5	Glucose (70 %) Albumin (18 %) Lactate (12 %)	20	6	5.5
6	Glucose (70 %) Albumin (18 %) Lactate (12 %)	20	6	5

2.2. Chemical analysis

Total and Volatile Solids (TS, VS) content of the fermentation broth was used to verify steady-state attainment and was measured according to APHA Standards Methods. Total and soluble organic content (CODt and CODs), total nitrogen content and colloidal carbohydrate and protein levels (measured on 1.2 μm filtered samples) were determined following the analytical procedures described in Tonanzi et al. (2020). VFAs and ethanol concentrations were quantified by injecting 0.5 mL filtered (0.2 μm porosity) and acidified liquid samples into a GC-FID (Agilent, 8860) as previously reported by Montecchio et al. (2024). Lactic acid was analyzed on 0.45 μm filtered soluble phase using an HPLC Ultimate 3000 system (Dionex, Thermo Fisher Scientific, USA) equipped with a UV detector (VWD-3400RS). Separation was achieved on an Acclaim OA column (250 × 4.0 mm, 5 μm). The mobile phase was 100 mM Na_2SO_4, adjusted to pH 2.65 with methanesulfonic acid (flow rate of 0.6 mL min^{-1}, isocratic mode). The column temperature was maintained at 30 °C, the injection volume was 5 μL, and detection was carried out at 210 nm. VFAs, ethanol and lactic acid quantification were performed using external standards. Calibration curves were constructed over the concentration range 50-400 mg/L for VFAs and ethanol and 50-1000 mg/L for lactic acid. Samples exceeding the calibration range were diluted and re-analyzed; concentrations below the lowest calibration standard were considered as below the calibration range and therefore negligible for the purpose of this study. All reagents were of analytical grade and purchased from VWR International (Radnor, USA).

2.3. Model description

The model is a kinetic one and is based on mass conservation and perfect mixing, following the framework of traditional models for wastewater treatment and anaerobic digestion, such as the Activated Sludge Model No. 1 (ASM1) by Henze et al. (2000) and the ADM1. Lactate and ethanol were incorporated as additional variables into the ADM1 fermentation metabolites pool, following the approach proposed by García-Gen et al. (2013). The selection of the substrate and the experimental configuration was aimed at simplifying the process representation. Given albumin characteristics (see sub. 2.1), it was treated as a soluble compound and represented by the soluble state-variable Saa, in accordance with the ADM1 framework. Consequently, hydrolysis was not included in the model. Similarly, the methanogenic step was not considered, owing to the absence of methane throughout the experiments.

It's worth noting that when methane is absent from the liquid phase - and consequently from the biogas - hydrogen partial pressure can rise to values as high as 1 atm. Under these conditions, the oxydation of VFAs to acetate, commonly referred to as acetogenesis, becomes endergonic and therefore cannot take place in the anaerobic media (Paton and Rodriguez, 2019). The remaining processes accounted for were fermentation (of sugars and amino acids), the acrylate pathway, and CE; biomass decay was considered negligible given the short HRT. The potential occurrence of the acrylate pathway was supported by both previous findings reported in the literature (Rymuszka et al., 2026) and the experimental results obtained in this study. The Wood-Werkman cycle represents an alternative pathway for propionate uptake; however, this pathway requires Vitamin B_{12} as an essential co-factor for methylmalonyl-CoA mutase (Dank et al., 2022). Since Vitamin B_{12} was not supplied to the reactor (and was absent in the substrate), the involvement of the The Wood-Werkman cycle can be ruled out. However, in the absence of microbial community characterization, the activity of the acrylate pathway cannot be unequivocally verified.

The Petersen Matrix of the organic variables is reported in Table 2; process kinetics were formulated with Monod equations similarly to Batstone et al. (2002). Inorganic carbon and nitrogen were represented with separate variables in analogy with the ADM1.

The metabolites produced through the fermentation steps were hydrogen, acetate, propionate, butyrate, valerate – all of which were accounted for in the ADM1 – plus lactate and hydrogen. The stoichiometric coefficients for these metabolites were treated as variables and were estimated through the calibration procedure described in Subsection 2.4.

The acrylate pathway was based on eq. (1) (Rymuszka et al., 2026), whereas the two possible CE reactions, based on lactate elongation to butyrate, are displayed in eq. (2) and eq. (3) (Wu et al., 2018), respectively:

$$C_3H_6O_3 \rightarrow 2/3\ C_3H_6O_2 + 1/3\ C_2H_4O_2 + 1/3\ CO_2 + 1/3\ H_2O \quad (1)$$

$$2\ C_3H_5O_3 + H^+ + e^- \rightarrow C_4H_7O_2 + 2\ CO_2 + 2\ H_2 \quad (2)$$

$$C_3H_5O_3 + C_2H_4O_2 \rightarrow C_4H_7O_2 + CO_2 + H_2O \quad (3)$$

For simplicity, a single variable was used to represent the biomass of fermentative microorganisms. The biogas was assumed to consist of hydrogen, carbon dioxide and water vapour.

Overall, the model comprised 17 variables (including the gaseous phase), four biochemical processes and the liquid/gas mass transfer. The system of 17differential equations was resolved using the ODE15s solver function in Matlab®.

2.4. Parameters calibration

Sensitivity analysis was not needed since for each process all the most relevant parameters were calibrated.

Calibration was focused on both the kinetic and stoichiometric parameters governing the fermentation step. The procedure was carried out in two stages. First, the maximum uptake rate for sugars and amino acids were calculated by fitting model predictions to experimental data. Second, the distribution of glycolytic fluxes was determined by optimizing the stoichiometric parameters associated to the production of the target variables, namely acetate, lactate, ethanol, propionate, butyrate and valerate. This approach constitutes the basis of the variable stoichiometry framework (Antonopoulou et al., 2012). In Exp_5 and Exp_6, the parameters related to the Chain Elongation process were also calibrated. The only parameter that could not be reliably estimate through calibration was the maximum lactate uptake rate for the acrylate pathway, due to the low lactate concentrations detected throughout the experiments.

Calibration was performed by minimizing the Root Mean Square Error (RMSE) of the residuals, as defined in eq. (4):

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(X_{obs}{}^{i} - X_{sim}{}^{i}\right)^2}{n}} \quad (4)$$

where X^i_{obs} and X^i_{sim} are the observed and simulated average concentration of the target variables, respectively, whereas n is the number of data points (n = 5).

The goodness of fit was further evaluated using additional cost functions reported in the literature (Montecchio et al., 2019), including the Nash Sutcliffe Efficiency Coefficient, the Normalized RMSE and the Correlation Coefficient. The choice of the function did not significantly influence the outcomes of the calibration.

To account for the metabolic characteristics of the fermentation step, metabolic constraints were incorporated into the calibration of the glycolytic stoichiometric coefficients. This aspect, although disregarded in most applications, was essential to prevent unrealistic predictions. In particular, the conservation of the electron carriers (i.e., NADH), was imposed throughout the glycolysis step.

Accordingly, the calibration step was based on a constrained numerical optimization procedure, where the objective function was the RMSE of the residuals and the constraints were defined in eqs. (5) and (6).

$$\sum_{i=i}^{n} v_i = 1 \quad (5)$$

Table 2
Petersen matrix of the model.

Processes ↓	Organic Variables												
	Ssu	Saa	Sac	Seth	Slac	Spro	Sbu	Sva	Sh2	Xglu	Xaa	Xacry	Xce
Uptake of Sugars	−1		(1-Ysu) ·fac_su	(1-Ysu) ·feth_su	(1- Ysu) ·flac_su	(1- Ysu) ·fpro_su	(1- Ysu) ·fbu_su		(1- Ysu) ·fh2_su	Ysu			
Uptake of Aminoacids		−1	(1- Yaa) ·fac_aa	(1- Yaa) ·feth_aa	(1- Yaa) ·flac_aa	(1- Yaa) ·fpro_aa	(1- Yaa) ·fbu_aa	(1- Yaa) ·fva_aa	(1- Yaa) ·fh2_aa		Yaa		
Acrylate pathway			(1- Yacry) ·0.222		−1	(1- Yacry) ·0.778						Yacry	
Lactate elongation					-N1		N1· (1-Y_ce)· 0.833		N1· (1-Y_ce) · 0.167				N1·Yce
Lactate and acetate elongation			-(1-N1) ·(1-Y_ce)· 0.667		-(1-N1)		(1-N1)· (1-Y_ce)· 1.667						(1-N1) · Yce

Parameters nomenclature in accordance with Batstone et al. (2002), García-Gen et al. (2013) and Xu et al. (2026).
N1 is the Lactate fraction utilized for self elongation to butyrate in the CE process.

$$\sum_{i=i}^{n} v_i NADH_i = 0 \quad (6)$$

where

v_i is the molar stoichiometric fraction of the ith metabolic branch (Table 3);

n is the number of the metabolites produced along glycolysis (n = 5);

$NADH_i$ is the NADH production associated to the production of the ith metabolite (Table 3).

The optimization algorithm was implemented using the pattern-search function available in MATLAB®; for validation purposes the results were compared with those obtained using the "Optimization" tool of gPROMS (Process Systems Enterprise, London, UK). Both algorithms provided nearly identical results; the results presented in this study are those obtained using MATLAB®.

3. Results and discussion

3.1. Glucose, albumin and lactate uptake

The concentration profiles of the compounds constituting the three different substrates, namely glucose, albumin, and lactate, during the experiments are presented in Fig. 1; as shown, steady-state conditions were reached after approximately 5–6 HRTs.

In all the experiments glucose uptake was very fast and removal efficiency was always higher than 95 % (Fig. 1); the calibrated value of the maximum sugars uptake rate exceeded 200 d^{-1} for exp_1 to exp_4 (Table 4), and was slightly lower than 200 d^{-1} when lactate was supplied with the substrate. These rates are extremely high considering that the default ADM1 value is 30 d^{-1} (Batstone et al., 2002).

On the other hand, when albumin was supplied in the substrate (exp_3 and exp_4), its uptake was markedly less efficient than glucose; only in exp_3 half of the albumin content was consumed. Under lower pH conditions, as in exp_4, albumin consumption was nearly negligible (<10 %). In the experiments fed with the mixture, albumin removal remained very low, never exceeding 30 %. Overall, albumin uptake rate ranged between 16 and 27 d^{-1}, substantially lower than the default value of 50 d^{-1} reported for amino acids (Batstone et al., 2002). These results suggest that, under the adopted operational conditions, microorganisms responsible for albumin uptake are less competitive than those utilizing sugars.

The utilization of lactate directly supplied in the mixture was extremely effective, as no measurable lactate was detected in the fermentation medium in exp_5 and exp_6. Regardless of the metabolic pathway responsible for the uptake of this metabolite, which is further discussed in subsection 3.3, this finding confirms that lactate consumption under anaerobic conditions is rapid. Even when lactate production is significant, it is common to shift from the net production to the net consumption of lactate (González-Hernández et al., 2022), and consequently its presence in the anaerobic environment can be neglected and the associated biochemical pathways may remain undetected (Sathapy et al., 2016).

Table 3
Stoichiometry of the metabolic constraint.

Metabolic branch	Stoichiometry [mol metabolite mol^{-1} glucose]	NADH generation [mol NADH mol^{-1} glucose]	COD-to-NADH ratio [gr_{COD} metabolite mol^{-1} NADH]
butyrate	1	−1	160
acetate	2	2	64
lactate	2	0	0
propionate	2	−2	112
ethanol	2	−2	96

3.2. Metabolites production

The experimental data used for evaluation were calculated as the average concentrations measured over the final three HRT periods, as displayed in Fig. 2.

Butyrate, acetate, and propionate were the most abundant metabolites detected, while ethanol, lactate, and valerate were present at markedly lower concentrations.

The most peculiar aspect of this study, which was aimed at estimating lactate production during the simultaneous fermentation of sugars and proteins, is that lactate remained at very low concentrations throughout all the experiments - and sometimes it was undetectable. This finding appears to rebut the hypothesis that proteins play a major role in driving the microbial community dynamics towards the selection of LAB.

However, throughout experiments 1, 2, 5 and 6 the metabolite detected at the highest concentrations was butyrate, followed by acetate. On the other hand, in exp_3 and exp_4 - where albumin was supplied - propionate accumulation was observed; this metabolite was detected at far lower concentrations when the substrate was comprised of pure glucose, as in exp_1 and exp_2. Propionate was detected at concentrations as high as 1.500 – 2.000 mgCOD L^{-1} along both exp_3 and exp_4; these levels exceeded the combined concentrations of acetate and butyrate. Notably, in exp_4 the metabolic shift toward propionate production could not be attributed to albumin uptake, as this compound was scarcely consumed during the fermentation process. Given the metabolic framework of the glycolytic process, it is highly unusual for propionate production to exceed that of acetate and butyrate. Indeed, the propionate pathway yields fewer ATP moles than both acetate and butyrate and, moreover, it does not regenerate NAD^+ - which are consumed during acetate formation - to the same extent as butyrate (Table 3).

It's reasonable to assume that propionate generation was driven by a metabolic pathway other to glycolysis. The most relevant, if not the only, microbial pathway capable of accounting for the propionate levels observed in these experiments is the acrylate pathway, where 1 mol of lactate is converted in 2/3 of mole of propionate and 1/3 mol of acetate, as reported in eq. (1). If confirmed, the occurrence of this pathway would support a metabolic shift of glycolysis toward lactate production and further corroborated the hypothesis that proteins were utilized for the growth of LAB, given their auxotrophic anabolism.

With regard to exp_5 ed exp_6, acetate and propionate were detected at low concentrations whereas a substantial increase of butyrate was observed. In this experimental setup, externally lactate supplied - along with the fraction potentially produced via glycolysis – was converted in different compounds. Considering the significant butyrate production, which far exceeds that of any other metabolite, the most plausible explanation is that lactate was converted into acetyl-CoA and consequently utilized as an ED in the reverse β oxydation cycle, which is the framework of the CE pathway. Lactate utilization as an ED in the CE processes has been described in the literature, among other authors, by Spirito et al. (2014). Two possible EAs may have been utilized for lactate oxydation: protons, in lactate self elongation to butyrate - with concurrent hydrogen production – and acetate (Montecchio et al., 2024).

3.3. Modeling results

As displayed in Figs. 3 and 4, there was a good agreement between experimental and simulated results, suggesting that the calibration strategy was adequate and that the most significant biochemical pathways occurring in the reactor were properly represented in the model.

In this study a constant value was adopted for the kinetic parameter km_lac, as calibration was not feasible due to the low lactate concentrations observed. Therefore, a value in line with the literature data under mesophilic conditions was used, corresponding to 40 d^{-1} (Xu et al., 2026). The other kinetic and stoichiometric parameters (e.g.,

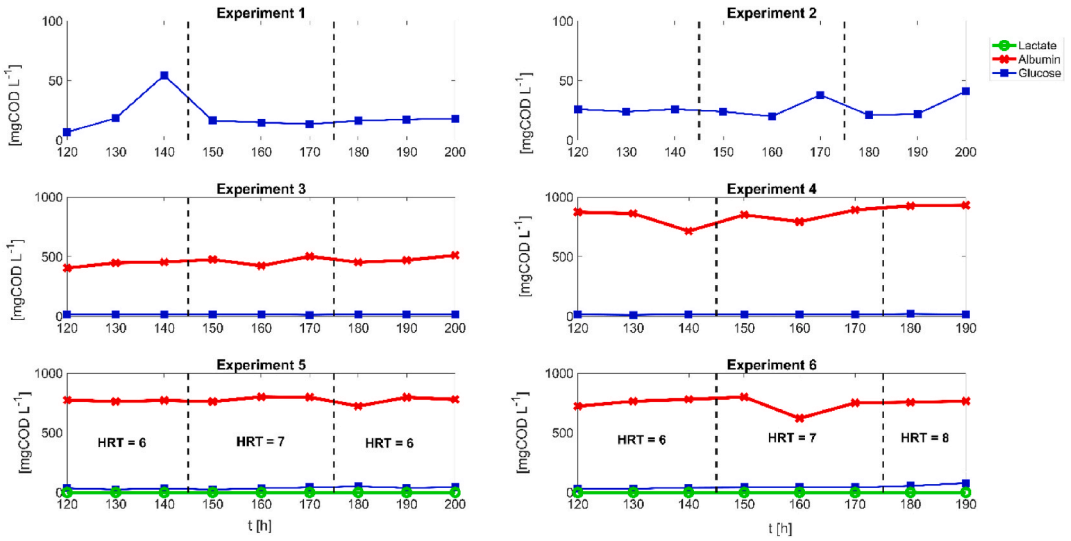

Fig. 1. Glucose and albumin concentrations under steady-state conditions.

Table 4
Kinetic and stoichiometric parameters resulting from calibration.

Parameter	Experiment					
	1	2	3	4	5	6
km_su [d^{-1}]	331	238	348	335	178	198
km_aa [d^{-1}]			24	16	19	27
km_lac_CE[a] [d^{-1}]					64	42
f_ac_su	0.23	0.21	0.20	0.10	0.19	0.18
f_eth_su	0.02	0.03	0.06	0.05	0.05	0.09
f_pro_su	0.05	0	0.23	0.04	0.05	0.06
f_lac_su	0	0.07	0.36	0.64	0.22	0.35
f_bu_su	0.49	0.48	0.07	0.11	0.33	0.19
f_h2_su	0.21	0.21	0.08	0.06	0.16	0.13
f_ac_aa			0			
f_eth_aa			0			
f_pro _aa			0.4			
f_lac _aa			0.19			
f_bu _aa			0			
f_va _aa			0.31			
f_h2_aa			0.1			
N1					0.93	1

[a] Maximum lactate uptake rate of the CE process.

affinity constants, biomass yield, and other related parameters), apart from those subjected to calibration (Table 4), were adopted from (Batstone et al., 2002).

The calibrated stoichiometric parameters indicated that, at equivalent pH, the lactate-yielding pathway (f_lac_su) was more prominent in the presence of albumin, regardless of the extent of its consumption. In particular, in exp_4—where albumin uptake was minimal—the lactate pathway accounted for more than 60 % of the glycolytic electron flux (Fig. 5). This observation suggests that, in protein-rich conditions, LAB can outcompete other microbial communities due to their ability to utilize proteinaceous substrates for their growth task (Kieliszek et al., 2021).

Kinetic parameters and fractionations coefficients derived from calibration are reported in Table 4 and Fig. 5.

This result was achieved through the adoption, within the calibration framework, of the mathematical constraint representing electron carriers conservation. This finding further indicated that the propionate production observed across exp_4 was not associated with direct glycolytic activity. Rather, the glycolytic flux was primarily directed toward the production of lactate, which was subsequently converted to propionate via acrylate pathway. To be more precise, it should also be noted that, from a theoretical perspective, it would have been possible to

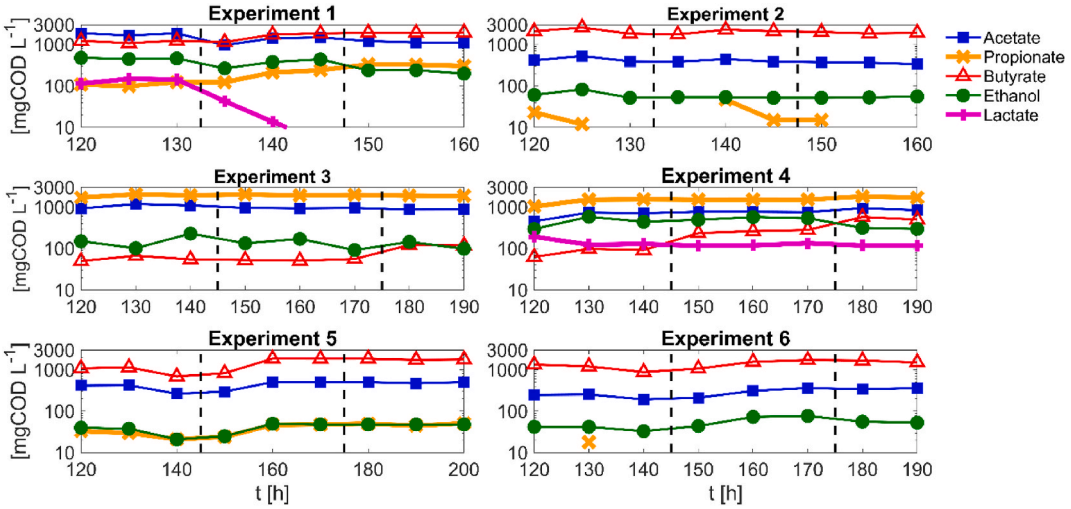

Fig. 2. Acetate, propionate, butyrate, ethanol and lactate concentrations detected under steady-state conditions.

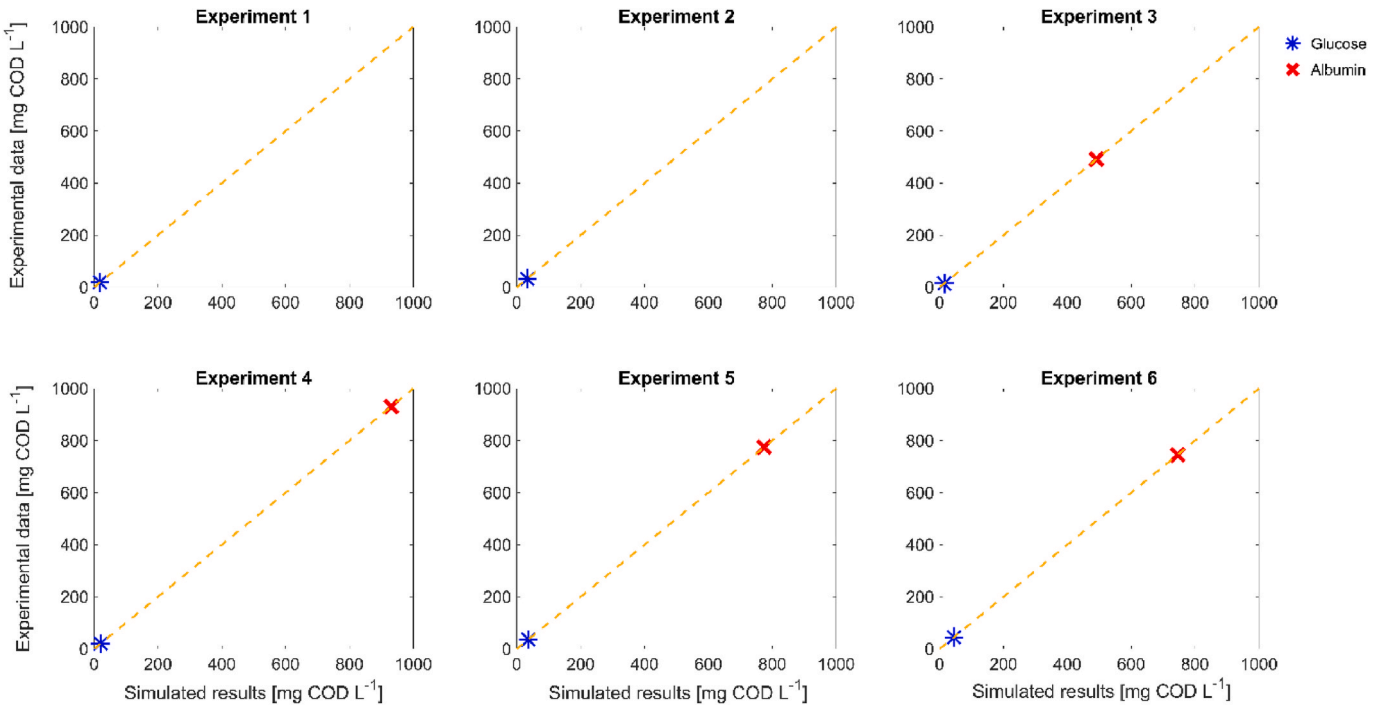

Fig. 3. Parity plot for glucose and albumin obtained using the calibration procedure.

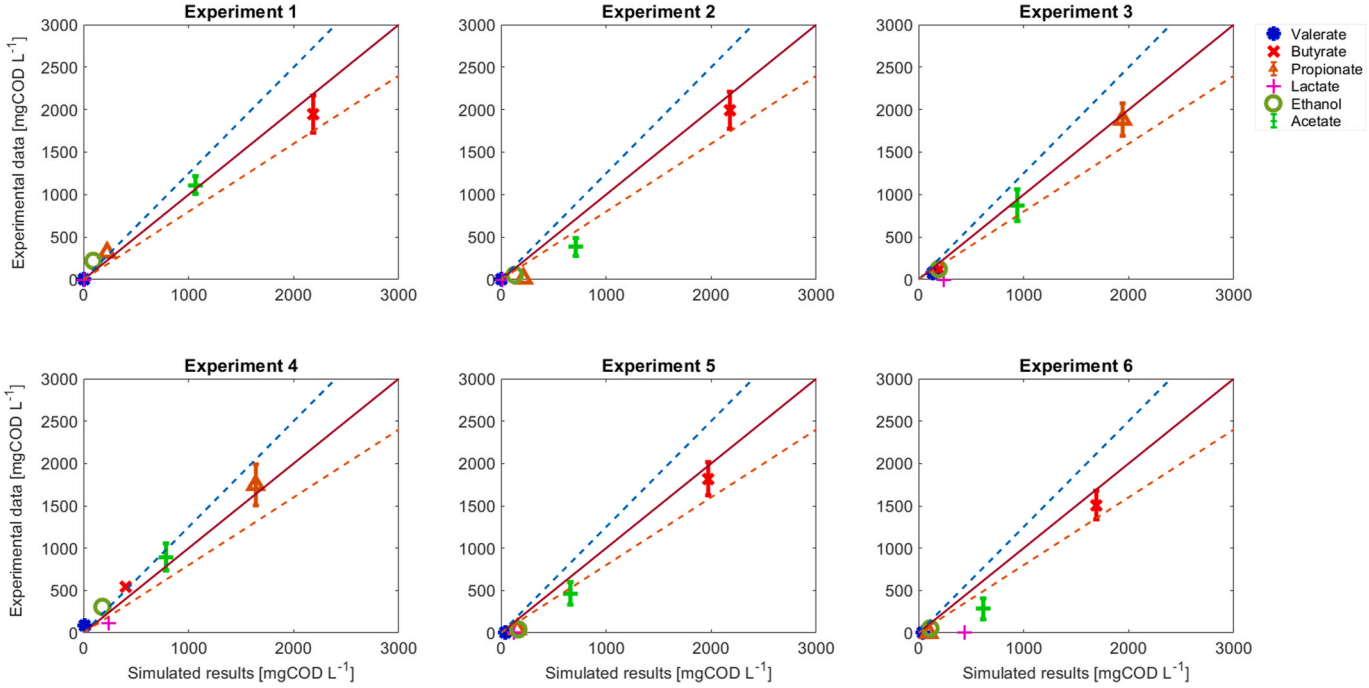

Fig. 4. Parity plot for fermentation metabolites obtained using the calibration procedure.

force the model to reproduce the high propionate production through direct glycolysis rather than via the acrylate pathway. However, to achieve this outcome a very low value of km_lac should have been adopted, in order to force the model to consume lactate under experimental conditions characterized by low HRT values. Moreover, this approach would have resulted in a higher acetic acid production than that observed experimentally, in order to compensate for the NAD^+ generation associated with propionate production.

In fact, from a metabolic point of view, the low acetate yield observed in exp_4, where calibrated f_ac_su was 0.1, resulted in a limited NADH generation, which was reflected in a similar low production of NADH consuming metabolites, such as butyrate and ethanol; consistently, calibrated f_bu_su and f_eth_su values were 0.11 and 0.05, respectively. For this reason, assuming a significant propionate production via glycolysis would be unrealistic, as this pathway would have led to an additional NADH consumption, thus violating electron carriers conservation. In constrast, lactate was the only metabolite whose production was neutral in terms of NADH utilization (Table 3), making this pathway the only other possible in these conditions. This explains the high calibrated value of f_lac_su, thereby supporting the hypothesis that

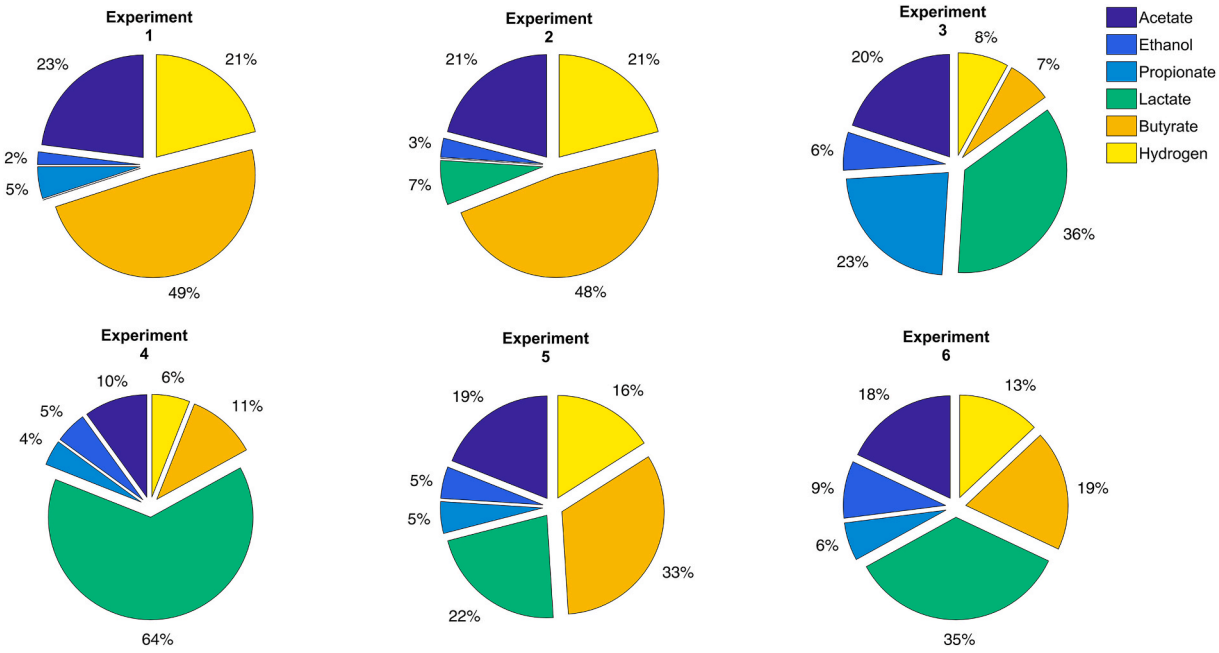

Fig. 5. Metabolites fractionation during glycolysis.

propionate production occurred via acrylate pathway. Although this value appears to be very high, it is still in line with other calibrations reported in the literature (Couto et al., 2020).

Similar considerations apply to exp_3; however, acetate production was twofold higher than in exp_4 (f_ac_su = 0.2). In this case, a fraction of the electron carriers reduced during acetate production may also be reoxidized via the glycolytic propionate pathway (Table 3). Nevertheless, substantial propionate fraction was associated with the acrylate pathway, as lactate accounted for approximately one third of glucose uptake (Table 4). It's worth noting that in this experiment albumin was consumed up to 50 % of the supplied amount, thereby increasing the degrees of freedom of the calibration, as stoichiometric parameters associated with albumin fermentation also contributed to the final results.

In experiments 5 and 6, where the substrate was supplemented with lactate, the metabolic pathway responsible for lactate uptake shifted from the acrylate pathway to reverse β-oxidation, as discussed in Subsection 3.2. The underlying cause of this metabolic transition remains unknown. The mathematical model was adapted to this new condition; in particular, the acrylate pathway was suppressed and replaced by lactate elongation to butyrate. The CE process is very complex and comprises multiple reactions (Scarborough et al., 2018), many of which are likely still unidentified. However, in this study no medium chain fatty acid was detected, as for example caproate - which is the most typical metabolite of CE – suggesting that the elongation process did not proceed beyond butyrate.

In this context, only the two reactions reported in eqs. (2) and (3) were incorporated into the model. In addition to the glycolytic stoichiometric parameters, the calibration procedure was designed to estimate the maximum lactate uptake rate via CE (km_lac_CE) and to quantify the fraction of lactate oxidized through self elongation (N1) versus that utilizing acetate as an EA (1 – N1).

The maximum lactate uptake rates via CE were 64 d^{-1} and 42 d^{-1} in exp_5 and exp_6, respectively, exceeding all the default kinetic parameters for VFAs oxidation adopted in the ADM1. However, the model reproduced observed results less accurately than in the other experiments, as it predicted a residual concentration of lactate, ranging between 120 and 220 mgCOD L^{-1}, that was not detected experimentally (Figs. 3 and 4), suggesting that the calibrated values were underestimated.

Lactate self elongation was simulated as the predominant reaction within the CE process, accounting for nearly all of the flux through the reverse β-oxidation pathway (Table 4). According to the simulated stoichiometric fractionation, glycolysis was predominantly butyric, with lower acetate production; the formation of the remaining metabolites, including lactate, was predicted to occurr at nearly negligible levels. These results further confirm that, under these environmental conditions, metabolic pathways can significantly change also in response to limited variations of the substrate composition.

4. Conclusions

Proteins supplementation induced a significant shift in the pool of metabolites produced via glycolysis, although their uptake was considerably slower — if not negligible — compared to that of glucose.

However, lactate was not detected at significant concentrations in any of the experiments conducted, thereby challenging the hypothesis that the addition of proteins to a predominantly sugar-based substrate could favor the growth of LAB, given their auxotrophic metabolism.

Nevertheless, substantial propionate production was observed in the experiments where albumin was added to the substrate; this finding is noteworthy, as propionate – via the acrylate pathway – can be generated from the lactate produced during glycolysis.

Modeling indicated that the observed propionate production was incompatible with the electron carriers conservation, a fundamental metabolic constraint, unless the lactate uptake rate was reduced to presumably unrealistic values. Therefore, given the absence of other alternative pathway for propionate production in this intentionally simplified substrate, it is reasonable to hypothesize that the high concentrations of this metabolite resulted from the production of lactate occurred during the glycolytic process. Model calibration estimated that when albumin was present in the substrate, lactate production accounted for ~ 35 – 65 % of the stoichiometric glucose flux.

On the contrary, in the experiments where lactate was directly supplied with the substrate, the process shifted towards the CE pathway, resulting in butyrate accumulation. Future research should focus on the operational and microbial factors governing the partitioning of lactate between the acrylate and chain-elongation pathways. However, the

proposed NADH-constrained model proved to be a valuable tool for interpreting complex fermentative systems, particularly when detailed microbiological information is unavailable. In the future, such a tool could be readily extended to investigate fermentative systems comprising mixtures of biowaste and biosubstrates, with the aim of optimizing lactate production for its subsequent use as an electron donor in the chain elongation process.

CRediT authorship contribution statement

D. Montecchio: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **S. Angelini:** Writing – review & editing. **C. Braguglia:** Writing – review & editing, Funding acquisition. **D. De Cata:** Writing – review & editing, Validation, Software. **A. Gianico:** Writing – review & editing. **S. Grandinetti:** Software, Data curation. **V. Piemonte:** Writing – review & editing. **M. Sbicego:** Writing – review & editing, Investigation, Data curation. **A. Gallipoli:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation.

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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Data availability

Data will be made available on request.

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