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Understanding the effect of trace elements supplementation on volatile fatty acids production from proteins

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ABSTRACT

This investigation studies the impact of trace elements supplementation on protein anaerobic conversion into volatile fatty acids (VFAs). Two continuous reactors were operated with two model substrates, casein and gelatin, at acid (pH 5) and neutral (pH 7) conditions, with the addition of macro and micronutrients. Micronutrients increased the acidification degree of both proteins from 40% to 50% to more than 60% only at pH 7, which was consistent with a greater amino acid consumption at neutral conditions. Furthermore, trace elements modified the process selectivity, promoting valeric acids production and other variations dependant on protein composition. Isomerisation and chain elongation processes were identified as a consequence of the observed deviations between amino acid consumption and VFA production. Overall, this study demonstrated that the supplementation of micronutrients can be useful to enhance and steer the anaerobic fermentation of protein-rich streams.

1. Introduction

In the past years, many studies have been focusing on improving the anaerobic digestion performance by adding selected micronutrients [19,23,24], considering that many industrial sidestreams do not feature sufficient concentrations of these compounds to be suitable as feedstock for anaerobic bioprocesses [12,14,26]. The addition of certain trace metals appears to lead to an increase in methane yields [5]. Nickel (Ni), cobalt (Co), zinc (Zn), copper (Cu) and iron (Fe) have been reported to enhance methanogenesis, being essential for archaea cell growth and metabolism [13,17,27]. However, it should be also noted that above certain threshold concentrations the same compounds can become toxic (e.g. Co $\geq$ 70 mg/L), thus inhibiting the process [5].

The impact of trace element supplementation on the fermentation step has been studied as well, generally using model carbohydrates (e.g. glucose) and sugar-rich substrates. Kim et al. [16] demonstrated how Fe, Co and Ni addition favours the hydrolysis of synthetic sludge, thus increasing the volatile fatty acids (VFAs) production from the greater chemical oxygen demand (COD) solubilisation. Micronutrients can be also used to selectively steer the acidogenic process, as described in Dahiya et al. [8]: supplementing Co (6.3 mg/L) and Zn (10.4 mg/L) in the culture media resulted in higher titres of propionic acid during glucose fermentation. Yu and Fang [25] obtained comparable results fermenting dairy wastewaters with Zn concentrations lower than 10 mg/L, which not only promoted propionic acid production but also increased the overall conversion of the substrate to VFAs. Cu supplementation promoted propionic acid and ethanol production during sucrose fermentation [15] in detriment of n-butyric acid formation. Contrasting results were achieved by Zheng and Yu [28], who observed how increasing concentrations of Cu (0 – 400 mg/L) and Zn (0–500 mg/L) have a positive effect on n-butyric production from glucose, although slightly hindering propionic production in the process.

However, there is limited knowledge on the micronutrients requirement for the fermentation of proteins, albeit being a relevant fraction of many potential feedstocks (e.g. slaughterhouse waste [11]). Moreover, little information is available on the role of trace elements in the conversion of amino acids (AAs) to VFAs and how they can affect the underlying mechanisms. AAs are, in fact, converted to VFA through a complex array of reactions [20,21], but also directly uptaken for biomass growth, requiring a wide selection of enzymes and cofactors. For example, selenium (Se) is required for the production of glycine reductase [10], whose presence is fundamental for the conversion of this AA to acetic acid. Besides, several AAs feature pyruvate as a conversion reaction intermediate [21], meaning that many cofactors normally associated with increased VFA production from glucose, such as Cu and Zn [28], could be beneficial for protein fermentation.

Thus, the main objective of this investigation is to understand how amino acid conversion to VFA is affected by the presence of

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Table 1
Stoichiometry of AA conversion to VFAs for the casein and gelatin peptones used in the present study [21]. The stoichiometric coefficients are expressed in molar basis, assuming a value equal to 1.0 for each AA. Bold characters indicate relevant differences for the AA pathways between the two proteins.

Amino acid	Casein pathway	Gelatin pathway
Alanine	**0.9 Acetic + 0.1 Propionic**	**1.0 Acetic**
Arginine	1.0 Acetic + 1.0 Alanine	1.0 Acetic + 1.0 Alanine
Aspartic acid[a]	1.0 Propionic	1.0 Propionic
Cysteine	Acetic	Acetic
Glutamic acid[a]	1.0 Acetic + 0.5 n-Butyric	1.0 Acetic + 0.5 n-Butyric
Glycine	**1.0 Acetic**	**0.8 Acetic + 0.4 CO_2**
Histidine	1.0 Acetic + 0.5 n-Butyric	1.0 Acetic + 0.5 n-Butyric
Isoleucine	1.0 Iso-Valeric	1.0 Iso-Valeric
Leucine	1.0 Iso-Valeric	1.0 Iso-Valeric
Lysine	1.0 Acetic + 1.0 n-Butyric	1.0 Acetic + 1.0 n-Butyric
Methionine	**1.0 n-Butyric**	**1.0 Propionic**
Proline	0.5 Acetic + 0.5 Propionic + 0.5 n-Valeric	0.5 Acetic + 0.5 Propionic + 0.5 n-Valeric
Serine	Acetic	Acetic
Threonine	**Propionic**	**0.5 n-Butyric + 1.0 Glycine**
Valine	1.0 Iso-Butyric	1.0 Iso-Butyric

[a] Aspartic and glutamic acid respectively include asparagine and glutamine fermentation.

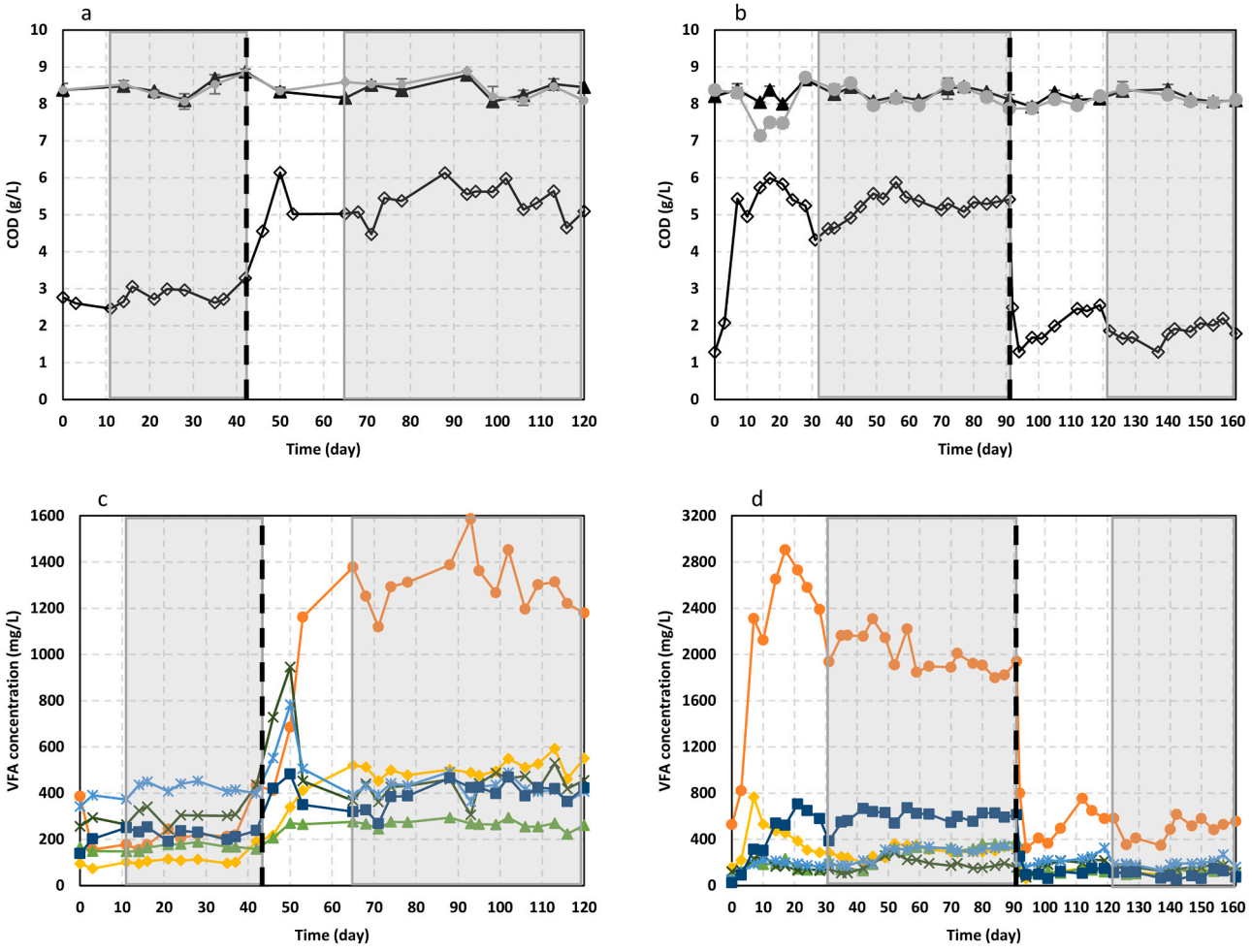

Fig. 1. COD balance (a, casein; b, gelatin: ▲ Influent total COD; ● Effluent total COD; ⋄ VFAs COD) and individual VFA concentrations (c, casein; d, gelatin: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; ∗ Iso-Valeric; ■ n-Valeric) in the reactors. The vertical segmented lines represent the pH shifts, from 5 to 7 in casein reactor (a, c) and from 7 to 5 in gelatin reactor (b, d), while the grayed areas indicate steady-state operation of the reactors.

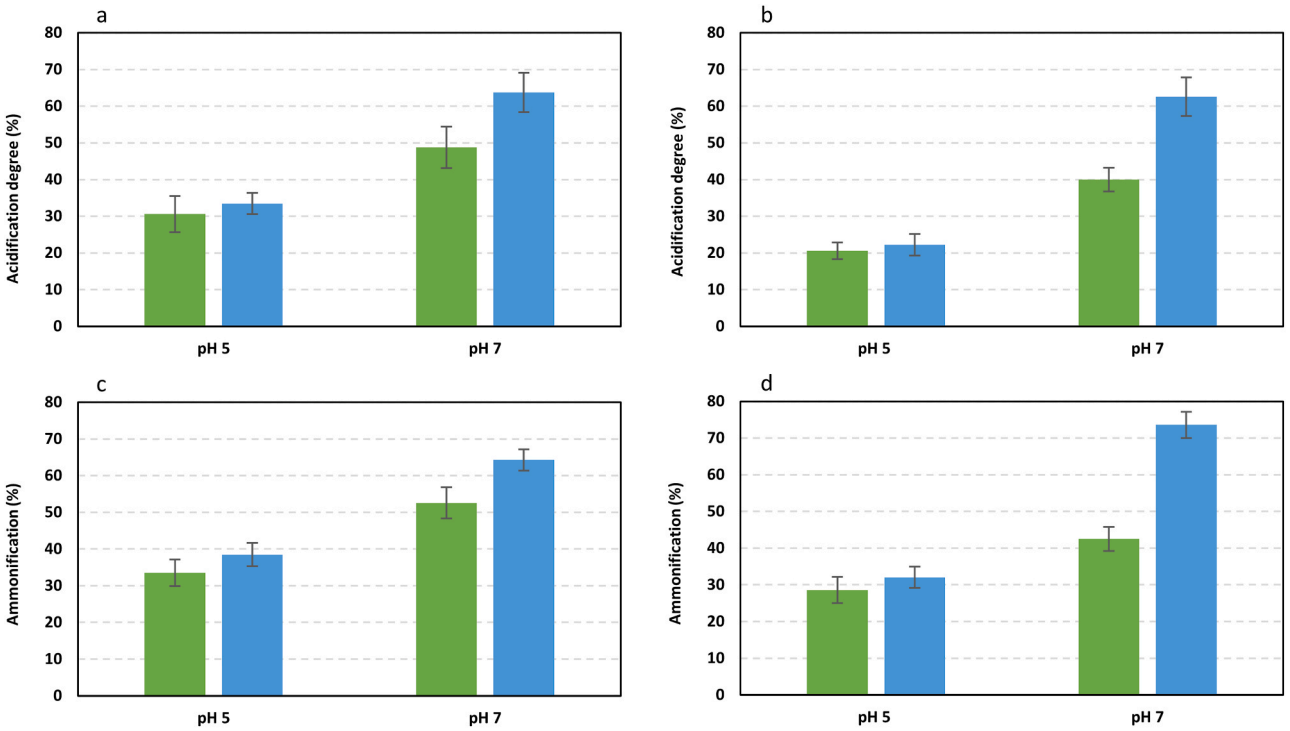

Fig. 2. Average acidification degree and ammonification achieved during casein (a, c) and gelatin (b, d) fermentation with ( ) and without ( ) micronutrients.

micronutrients during mixed-culture fermentation processes. The extent of this effect is also evaluated for different protein composition (i.e. amino acid profiles) and pH levels. Molar balances are established between the production of VFA and the consumption of AAs in order to better explain the variations in acidification degree, productivity and VFA selectivity. All this knowledge is useful in determining whether trace elements supplementation can be applied as an operational strategy to increase and potentially steer VFA production during the fermentation of protein-rich sidestreams.

2. Materials and methods

2.1. Reactors operation

Two continuous stirred tank reactors (CSTR) of 1 L made of glass were separately fed with casein and gelatin and operated for more than 400 days each without trace elements supplementation [2,3] before this study. The initial in-reactor biomass concentration was approximately 0.3–0.4 g VSS/L. Both reactors were operated with a hydraulic retention time (HRT) of 1.5 d, resulting in an organic loading rate (OLR) of 5.3 g COD/L·d. Magnetic stirring provided adequate mixing of the fermentation broth (200 rpm). The operational temperature was set at 25 °C via a temperature-controlled room, while pH was maintained at the chosen setpoint values (5 and 7) with HCl (3 M) addition regulated via a multiparametric analyser (CHEMITEC, Italy) connected to Hamilton probes. The liquid phase was constantly sparged with gaseous nitrogen (10 mL/min) to ensure anaerobic conditions inside the reactor and prevent hydrogen build-up.

The substrate used for the experiments was composed of either 7.500 g/L of hydrolysed casein (enzymatic digest, A2208,0500 PanReac) or 7.600 g/L of hydrolysed gelatin (enzymatic digest, 70951-1KG-F Sigma-Aldrich), supplemented with macronutrients (in g/L, NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530, Na_2SO_4 0.057; $MgCl_2.6H_2O$ 0.120) and micronutrients (in mg/L, $FeSO_4.7H_2O$ 3.100; $CaCl_2$ 0.600; H_3BO_4 0.1000; $Na_2MoO_4.2H_2O$ 0.100; $ZnSO_4.7H_2O$ 3.200; $CoCl_2{\cdot}H_2O$ 0.600; $CuCl_2.2H_2O$ 2.200; $MnCl_2.4H_2O$ 2.500; $NiCl_2.6H_2O$ 0.500; SeO_2 0.100). These liquid mixtures were refrigerated at 4 °C throughout the experiment to avoid degradation. The amino acid composition of casein and gelatin were already determined in a previous investigation [3].

The operation of the reactors was monitored through different parameters. Total COD of the feedstock and the mixed liquor, and soluble COD and solids content of the mixed liquor were determined once per week while pH was continuously measured inside the reactor via multiparametric analyser. VFA concentrations and total ammonia nitrogen (TAN) of the mixed liquor were determined two times per week, to respectively calculate the acidification degree and the ammonification percentage, a proxy for protein degradation. AA analysis of the reactors effluents was performed on selected frozen samples from steady state periods of operation.

2.2. Analytical methods

All the analytical methods were performed as in Bevilacqua et al. [2,3], according to the Standard Methods [1]. VFAs and secondary metabolites were respectively determined via gas chromatography and high-performance liquid chromatography (HPLC) [2].

After an acid hydrolysis pretreatment of 0.45 μm filtered mixed liquor samples, AA analysis is performed according to the AccQ-Tag method [6], using an HPLC with a fluorescence detector [2].

More detailed description of the analytical methods is provided in the electronic supplementary materials.

2.3. Calculations

The acidification degree, the ammonification and the molar balances between AA consumption and VFA production, used to describe the reactor operation and perform the data analysis, were calculated in the same way as in Bevilacqua et al. [2].

Biomass yield and activity were calculated to better understand how the presence of micronutrients affect the microbial communities responsible for the conversion of proteins to VFAs. These two parameters were respectively expressed as follows:

$$Biomass\ yield\ \ (g\ CODbm/g\ CODpr\ consumed) = \frac{C_{biomass}}{C_{pr\ consumed}} \quad (1)$$

Where $C_{biomass}$ is the concentration of biomass (in g COD_{bm}/L) measured in the effluents, and $C_{pr\ consumed}$ is the concentration of protein consumed (in g COD_{pr}/L), estimated from the ammonification parameter.

$$Biomass\ activity(g\ COD - VFA/g\ VSS{\bullet}d) = \frac{\Sigma\ \ C_{VFA}}{X_{biomass} \times HRT} \quad (2)$$

where C_{VFA} stands for the total concentration of the measured VFAs (in g COD-VFA/L) and $X_{biomass}$ refers to the total biomass concentration (in g VSS/L) in the effluent of the reactors. HRT refers to the hydraulic retention time, expressed in days, which is considered to be equal to the solid retention time as a continuous stirred tank reactor was used.

Given that the AAs stoichiometry proposed by Ramsay and Pullammanappallil [20] was found to be inaccurate depending on which VFA pathways are considered [3] and on the operational conditions applied to the fermentation process [2], a new prediction model for VFA production from proteins was developed [21]. The catabolism of glycine was updated to include, apart from its reduction to acetate, its conversion to CO_2 and ammonia with concomitant ATP generation by direct oxidation with NAD^+, via the glycine cleave system [22]. The overall stoichiometry varies according to the operational conditions and the AA profile of the protein considered (Table 1).

3. Results and discussion

3.1. Influence of micronutrients on reactors performance

Casein and gelatin reactors were continuously operated for 120 and 160 days in total (Fig. 1), with more than 40 days at each pH value,

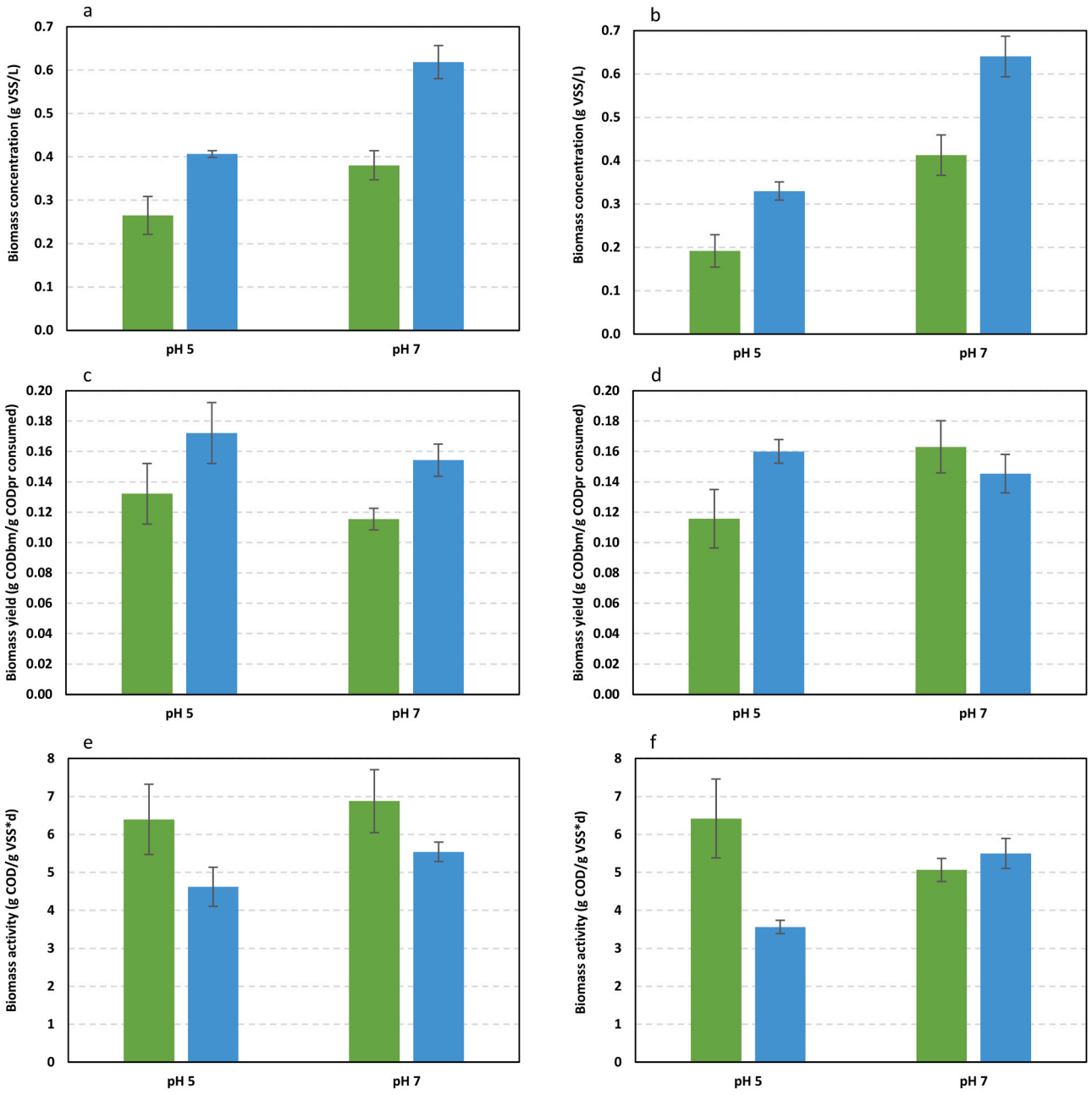

Fig. 3. Average biomass concentration, biomass yield and biomass activity achieved during casein (a, c, e) and gelatin (b, d, f) fermentation with (■) and without (■) micronutrients supplementation.

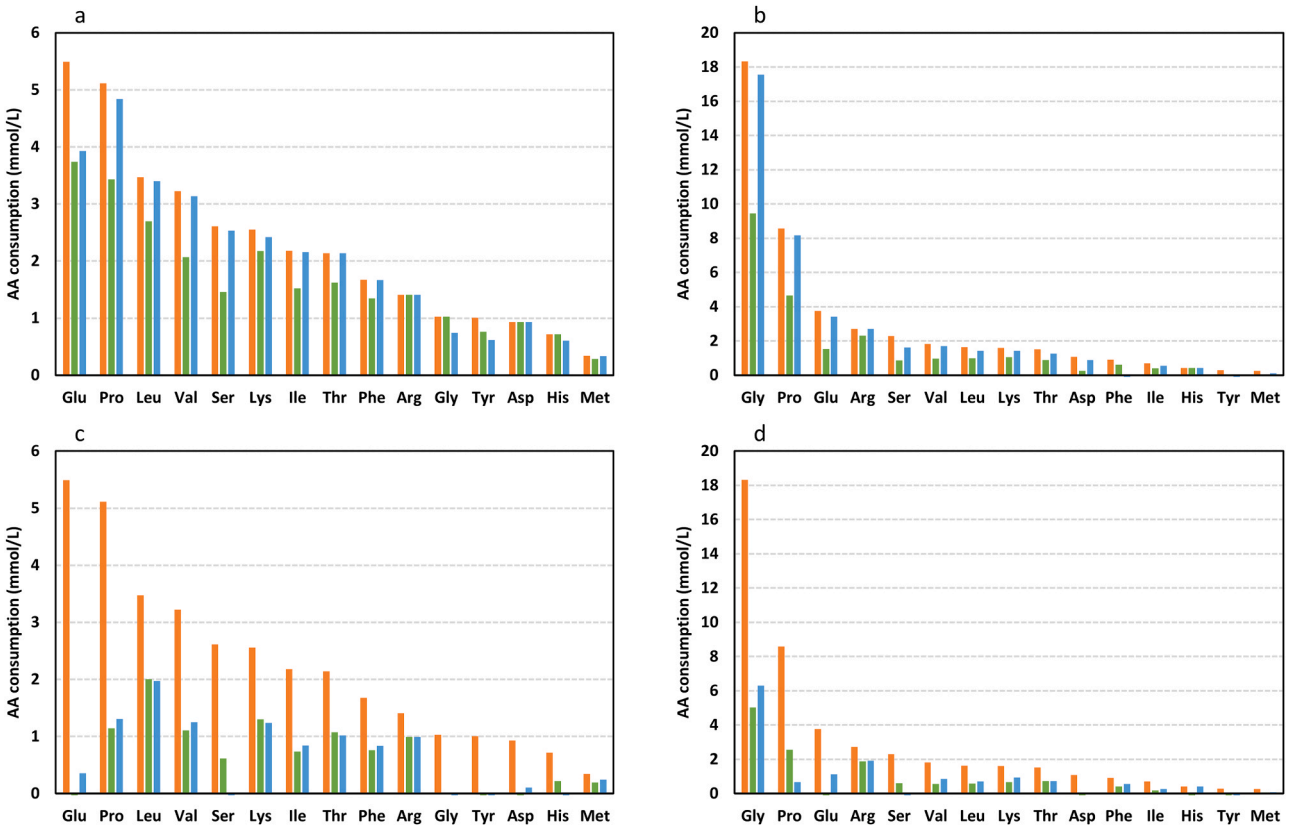

Fig. 4. Average amino acids consumption during casein (a: pH 7; c: pH 5) and gelatin (b: pH 7; d: pH 5) fermentation with ( ) and without ( ) micronutrients supplementation. Feedstock amino acid concentration. No data of alanine consumption is available, whereas the absence of bars indicates that no specific consumption was observed for a given amino acid.

ensuring steady state was achieved. Micronutrients presence accelerated reactor stabilisation after pH shift, reducing the acclimation time from 100 + days [2] to approximately 15 days (10 HRT times). To evaluate the impact of micronutrients presence on protein anaerobic fermentation, steady-state periods were selected for each experimental phase (Fig. 1): days 11–42 and 65–120 for casein at pH 5 and 7, respectively; and days 31–91 and 122–161 for gelatin at pH 7 and 5, respectively.

No differences between influent and effluent total COD concentrations were observed in any experimental phase (Fig. 1a and b), discarding the occurrence of methanisation. Higher VFA production was observed at neutral conditions for both proteins (≈5.5 g COD/L) than at the acid ones (≤3.0 g COD/L), as previously observed without micronutrients supplementation [2]. Acetic acid was the major product, with the exception of casein fermentation at pH 5, while the concentration of the other acids depended on both the protein composition and the operational pH. For example, propionic acid was the second major product of casein fermentation at pH 7 but was the minor product at pH 5 (Fig. 1c). A similar pattern was observed for n-valeric acid during gelatin fermentation (Fig. 1d). Medium chain VFAs were only detected during casein fermentation at rather low and variable concentrations (≤100 mg/L). In particular, iso-caproic acid formation was observed during pH 5 operation period, whereas the linear form (n-caproic acid) was measured at neutral conditions (data not shown). No secondary metabolites were ever detected, regardless of protein composition and pH conditions.

3.2. *Influence of micronutrients on substrate conversion and biomass growth*

The acidification degrees of both proteins increased due to the supplementation of trace elements (Fig. 2a and b). However, this positive effect was only significant at pH 7, especially for gelatin, whose acidification degree shifted from 40% to 62%. The latter indicates that trace elements effect is conditioned by both the pH conditions and the AA composition of the substrate. In fact, the variation in acidification degree is significant only at neutral conditions, to a greater extent for gelatin than for casein fermentation. Interestingly, the inhibition exerted by the acid conditions on the reactors operation outweighs the benefit posed by the micronutrients presence, discarding their supplementation as a viable strategy to increase the VFA production at low pH. Complete conversion of the proteins to VFAs was never achieved, which is consistent with previous studies [3,9]. The similar pattern observed for the ammonification percentage (Fig. 2c and d) confirms that all the consumed substrate was indeed transformed into VFAs.

Conversely, the effect of trace elements on the biomass growth is more generalised, as the protein composition and the operational pH do not seem to limit its extent. Micronutrients presence had a positive effect on the biomass growth (Fig. 3a and b), with increases in biomass concentrations above 40%. The biomass yields (Fig. 3c and d) increased compatibly, suggesting that the bacteria were more efficient at harvesting energy from the substrate for duplication purposes and/or at using the energy from catabolism in building new biomass. The only exception detected concerns gelatin fermentation at pH 7, being the values comparable with and without trace elements. In contrast, micronutrients supplementation lowered the biomass activities in comparison with the results obtained in their absence (Fig. 3e and f), due to the generalised increase in biomass concentration not being met with a compatible growth in VFA production. Only gelatin fermentation seemed to maintain a comparable biomass activity at neutral conditions, which would explain the similar increase observed in biomass concentration (+55.0%) as in protein conversion to VFAs (+56.5%). This exception could be due to gelatin being almost completely consumed,

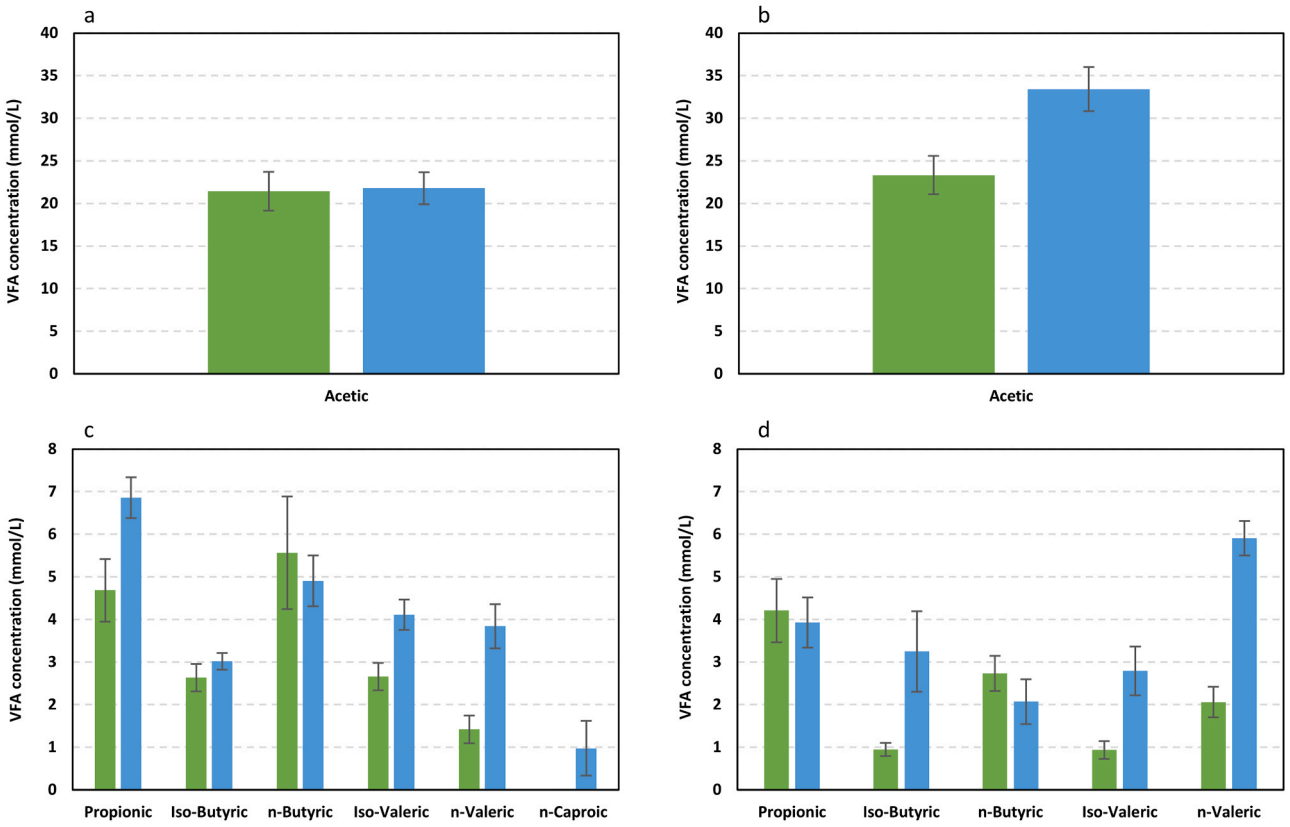

Fig. 5. Average individual VFA production during casein (a, c) and gelatin (b, d) fermentation with ( ) and without ( ) micronutrients supplementation.

leaving a residual fraction of AA whose energetic yield is too low to make their uptake and utilisation feasible.

In general, the supplementation of trace elements is more beneficial to the biomass growth rather than the production of VFA itself. Still, the two processes are interconnected, given that greater biomass concentrations can allow for greater substrate conversion and viceversa, making it difficult to discern whether the supplementation affects either the anabolism or the catabolism. More specifically, the supplementation of micronutrients might increase the energetic yield of AA conversion by enhancing the activity of the enzymes involved in catabolism and consequently reducing the associated energetic cost of enzyme production. Likewise, a net increase of harvested energy could be then used by the biomass for replication purposes, albeit it cannot be completely excluded that the presence of micronutrients could be improving biomass yields more directly, i.e. in anabolic reactions. The exception of gelatin neutral fermentation does not contradict this hypothesis, since the conversion of the residual AAs is not energetically viable for the microbial population, as previously mentioned.

3.3. Influence of micronutrients on amino acid consumption and VFA selectivity

The individual AA consumption (Fig. 4) confirmed the patterns observed in the previous section. At neutral conditions, the trace elements supplementation led to a generalised increase in AA utilisation, which was proportional to their abundance in the feedstock. As observed for the acidification degree, the effect was more relevant for gelatin (Fig. 4b) than for casein (Fig. 4a), as glycine (Gly) consumption experienced a 2-fold increase due to micronutrients presence. Conversely, neither significant variations nor patterns were observed in AA consumptions at low pH (Fig. 4c and d), confirming that the limitations exerted by the acid conditions outweighs the beneficial effects associated with the presence of trace elements.

The VFA selectivity of the fermentation process at neutral conditions was affected as well (Fig. 5). Trace element addition promoted the formation of both forms (i.e. branched and linear) of valeric acid (Figs. 5c and 5d), which was more marked in gelatin case (Fig. 5d). Other variations in VFA production were dependent on the protein composition. For example, acetic and iso-butyric acid formation increased only for gelatin fermentation (Figs. 5b and 5d), whereas propionic and n-caproic acid production was favoured only during casein conversion (Fig. 5c).

The positive effect of the trace elements supplementation on acetic acid production from gelatin (+10.1 mmol/L) was mainly justified by Gly increased conversion (+8.10 mmol/L), together with the contribution of glutamic acid (Glu) and proline (Pro). They also promoted the consumption of both Pro and threonine (Thr) during casein fermentation (+1.40 mmol/L), which explains the increased propionic acid formation (+1.7 mmol/L).

In contrast, the increased production of valeric acids and n-butyric acid is not justified by an increased consumption of the precursor AA (Fig. 6). The supplementation of trace elements led to a greater iso-butyric production than what is theoretically possible from valine (Val) consumption alone (Fig. 6a). However, the overall butyric acid balance appears to be closed, suggesting that isomerisation of n-butyric to iso-butyric acid might be occurring as a way of reducing the toxicity in the fermentative environment [4,18]. Similarly, n-valeric acid production during casein fermentation did not match proline consumption (Fig. 6b), while iso-valeric acid was underproduced. Interestingly, the isomerisation appears to be driving the interconversion in favour of the linear form rather than the branched one. In this case, however, the process seems to favour an equal concentration repartition between the two forms, rather than aiming to reduce the toxicity of the mixture. The fact that the trace elements supplementation promotes isomerisation is explained by a specific cofactor requirement of the associated enzymes. Both isobutyryl-CoA and isovaleryl-CoA, which catalyse the interconversion between iso and n-acids [7], have their activity completely

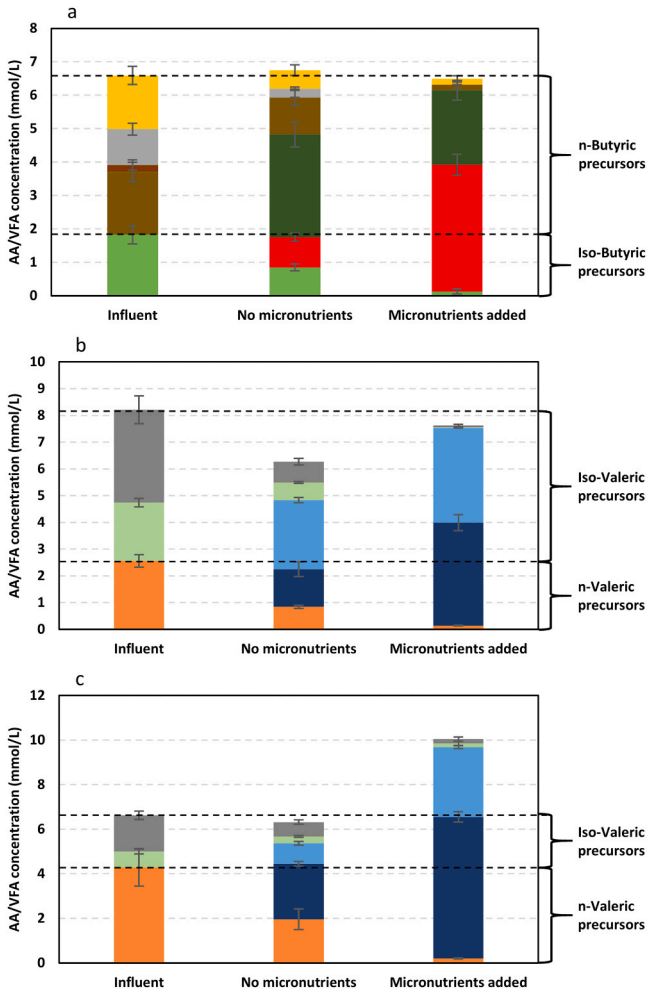

Fig. 6. Overall butyric (a, gelatin: Valine; iso-Butyric acid; n-Butyric acid; Glutamic acid; Histidine; Threonine; Lysine) and valeric (b, casein; c, gelatin: Proline; n-Valeric acid; Iso-Valeric acid; Isoleucine; Leucine) acid balance at pH 7. AA concentrations are expressed as VFA equivalents according to the stoichiometry described in section 2.4.

depending on Co ions presence, a compound which is indeed included in the mix of micronutrients supplemented during the present study.

The pattern is different during gelatin fermentation, since both forms of valeric acid were produced to a greater extent than what was expected based on the assumed stoichiometry (Fig. 6c). Although isomerisation cannot be completely discarded, it was hypothesised that the overproduction of these two VFAs might be due to elongation processes. This result is compatible with the n-caproic formation observed during casein fermentation, suggesting that the feasibility and the selectivity of the elongation reactions at neutral conditions depend on the trace elements supplementation and the protein composition.

4. Conclusions

This study successfully demonstrated the positive effect of micronutrients supplementation on protein fermentation, especially at neutral pH conditions. The extent of this effect is, however, dependent on the amino acid profile of the protein, as casein conversion increased from 49% to 64% whereas gelatin fermentation grew from 40% to 63%. Interestingly, trace elements presence modifies the process selectivity, as they can enhance chain elongation and isomerisation reactions. In fact, n-valeric acid production is tripled when trace elements are supplemented to the reactors, while iso-valeric acid benefits to a more variable extent (+40 to +200%). Other trends appear to be dependent on protein composition, resulting in a more flexible conversion system.

CRediT authorship contribution statement

Riccardo Bevilacqua: Methodology, Investigation, Writing - original draft, Writing - review & editing. **Alberte Regueira**: Methodology, Writing - original draft. **Miguel Mauricio-Iglesias:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Juan M. Lema:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Marta Carballa:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105934.

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