Engineering the outcome of cofermentation processes by altering the feedstock sugar-to-protein ratio

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ABSTRACT

This work investigates the impact of the sugar-to-protein (STP) ratio on the outcome of their anaerobic cofermentation in terms of substrate conversion and product selectivity. For this purpose, a continuous stirred tank reactor was operated at pH 7 and fed with casein and glucose at different STP ratios (0.25, 0.50, 0.75, 1.00 and 2.00 in COD basis). Casein conversion was unaffected by glucose presence as long as the ratio was lower or equal to 1. In this range of STP ratio, n-butyric and n-valeric acid production was promoted due to the occurrence and progressive intensification of chain elongation processes. Conversely, STP ratios greater than 1 are associated with lower amino acids consumption, inhibition of the elongation metabolism and lower volatile fatty acids production due to the formation of alternative end products (ethanol, lactate and formate) and unidentified compounds. Interestingly, these negative effects are reversible, as lowering the sugar-to-protein ratio allows to recover protein acidification degree, process productivity and the chain elongation. Overall, this work successfully demonstrates that sugar-protein cofermentation processes can be steered by adjusting their proportions in the feedstock.

KEYWORDS: amino acids; biorefinery; chain elongation; feedstock composition; glucose; volatile fatty acids

1 INTRODUCTION

Several studies[1–5] highlighted the potential of mixing different substrates to enhance the production of volatile fatty acids (VFAs) during mixed-culture fermentation (MCF) processes. The positive effect observed during the cofermentation of proteic streams with those rich in sugars is generally associated with a better balancing of micronutrients and carbon/nitrogen proportions, dilution of potentially toxic or inhibitory compounds, and/or an increase in hydrolysis rate due to the higher biomass yields achieved [6].

However, most literature examples dealing with anaerobic cofermentation are case studies involving mixtures of specific waste and wastewaters, as in this example studying waste activated sludge and potato peel waste[5]. Thus, the application of the resulting knowledge is limited to those specific substrates and the conclusions are not valid for the conversion of a generic mixture of proteins and carbohydrates into VFAs. Besides, all these previous results appear not to be conclusive concerning the influence of mixing different organic fractions. For example, Breure et al.[9] observed that the presence of a sugar (e.g. glucose) can partially inhibit the hydrolysis of proteins, and consequently their conversion into VFAs, when the two fractions loading were similar, whereas lower sugar loads did not show negative effects on protein fermentation. Conversely, Ma et al.[5] determined that increasing the carbohydrate fraction in the feedstock favours the consumption of proteins, with this synergistic effect being maintained even when carbohydrates were dominant over proteins. In disagreement with the two previous results, Tommaso et al.[10] observed that even minimal glucose presence induces a decrease in the degradation rate of the chosen model protein, bovine serum albumin.

Besides the conversion efficiency, sugars and proteins feature different VFA selectivity. The fermentation of sugars (e.g. glucose) mainly yields acetic, propionic and butyric acid[11] on proportions that can be steered through pH adjustments[12], similarly to lactose fermentation[13].

Conversely, proteins selectivity heavily depends on their composition,[14] given the potential combination occurring from the mix of the 20 main amino acids (AAs). Acetic acid tends to be the main product at neutral and alkaline conditions whereas low pH favours the conversion to longer chain VFAs[15]. Moreover, branched chain VFAs and n-valeric acid are mostly obtained through the fermentation of specific AAs rather than from sugars[16]. This substrate dependence suggests that the VFA distribution of a cofermentation process could be steered based on the feeding proportions between the two organic fractions. Yet, the literature concerning this effect is again contradictory. For example, supplementing gelatin fermentation with either glucose or lactose in equal proportions was associated to an increased production of n-butyric acid and ethanol[9]. Instead, Zhou et al.[17] observed an increase in acetic and propionic concentrations when progressively feeding greater proportions of carbohydrate-rich corn straw to the sludge-degrading reactor. In another case study, limiting the sugar fraction in the feeding mixture seemed to favour the formation of n-valeric acid[5].

The aforementioned information points out the need of a universal parameter to better understand the interaction between proteins and carbohydrates in a cofermentation process in order to engineer the process towards the desired outcome. Therefore, the present study proposes the sugar-to-protein ratio (STP), measured in chemical oxygen demand (COD) basis, as the parameter to assess and understand such interaction. The use of model protein and sugar compounds (i.e. casein and glucose, respectively) aims at facilitating results interpretation as well as their extrapolation to a generic protein-carbohydrate cofermentation process.

2 MATERIALS AND METHODS

2.1 Feedstock composition

Casein peptone (A2208,0500 PanReac) and D(+)-glucose anhydrous (131341.1211 PanReac) were the model compounds used in this study. Protein concentration was fixed at 7.50 g/L throughout

71 the experiment, while glucose concentration was progressively increased from 1.87 g/L to 14.96
72 g/L. The feedstock solution was supplemented with macro- and micro-nutrients, as described in
73 Bevilacqua et al.[18] , and it was maintained refrigerated throughout the experiment (4°C).

74 **2.2 Continuous reactors operation**

75 The continuous stirred tank reactor (CSTR) of 1 L used in the present study was the same as
76 described in Bevilacqua et al. [18] The pH was set at 7.0 for the whole duration of the experiment,
77 while the reactor was maintained at 25 °C through a temperature-controlled room. Being a CSTR,
78 the hydraulic retention time (HRT) and the solids retention time were both equal to 1.5 d. The
79 main difference between the two studies was glucose being included in the feedstock at increasing
80 concentrations in order to test several STP ratios (in COD basis): 0.25, 0.50, 0.75, 1.00, 2.00. Each
81 resulting STP ratio (Table 1) was maintained for at least 40 days, in order to evaluate its impact on
82 the cofermentation process after reaching a steady-state operation.

83 **Table 1.** Operational conditions of the different phases of the cofermentation reactor. STP: sugar-
84 to-protein ratio (COD basis); OLR: organic loading rate (g COD/L·d).

Phase	STP ratio	Casein OLR	Glucose OLR
I	0.25	5.33	1.33
II	0.50	5.33	2.67
III	0.75	5.33	4.00
IV	1.00	5.33	5.33
V	2.00	5.33	10.7

85

86 The reactor performance was monitored as described in Bevilacqua et al.[18] . In brief, the pH was
87 controlled at the 7.0 setpoint via a multiparametric analyser (CHEMITEC, Italy) and NaOH 3M
88 additions. VFA and Total Ammonia Nitrogen (TAN) concentrations were determined twice a week,
89 while COD (total and soluble) and solids concentrations were measured once a week. Amino acid

(AA) analysis was performed on samples specifically selected from steady state periods of operation.

2.3 Analytical methods

The analytical methods used are previously described in Bevilacqua et al. [15,18]. A summary is included in Supplementary Information.

2.4 Microbial community analyses

At each sugar-to-protein ratio, three biomass samples were taken, corresponding to three consecutive weeks of stable operation. Genomic DNA from 1 mL homogenized samples was extracted by triplicate using the Nucleospin Microbial DNA extraction kit (Machery-Nagel), according to the instructions of the manufacturer. The replica from eachsample were pooled together after quantification, ensuring quality control and normalization with Nanodrop and Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA). The V3-V4 hypervariable region for *Bacteria* was amplified using Bakt_341F (5' CCT ACG GGN GGC WGC AG 3') and Bakt_805R (5' GAC TAC HVG GGT ATC TAA TCC 3')[19]. DNA metabarcoding analyses of the region were carried out by AllGenetics & Biology SL (www.allgenetics.eu) in an Illumina MiSeq platform.

Bioinformatic analyses were performed using the Microbial Genomics module (version 21.1) workflow of the CLC Genomics workbench (version 21.0.3). Raw sequences were filtered to remove low-quality reads and then clustered into Operational Taxonomic Units (OTUs) at 97% cutoff for sequence similarity and classified against the non-redundant version SILVA SSU reference taxonomy (release 132; http://www.arb-silva.de)[20] . Only the most abundant bacterial OTUs (above 1 % of the total observed OTUs) were considered for further analysis.

Microbial abundance from phyla to genus level was analyzed, log-transformed and the statistical significance was determined for $p < 0.05$ by permutational multivariate analysis of variance

(PERMANOVA), including Bonferroni correction. Alpha diversity was estimated from the rarefaction analysis using the resulting phylogenetic tree of OTUs generated by the MUSCLE algorithm, with a maximum sampling depth to 26,618 reads. Beta diversity was measured by Bray-Curtis distances between each pair of samples applying principal coordinate analysis (PCoA) to the distance matrices. Significance was, likewise, assessed by PERMANOVA.

2.5 Calculations

Acidification degree was the parameter chosen to describe substrate conversion (in COD basis), while ammonification was also used as a proxy to monitor protein conversion to VFA, as amino acid fermentation is always related to NH_4^+ release. In addition, balances between AA consumption and VFA production were established to verify protein conversion stoichiometry. More details can be found in Supplementary Information.

3. RESULTS AND DISCUSSION

3.1 Cofermentation reactor operation

The cofermentation reactor was continuously operated for 344 days (Fig.1a). The first 56 days were jointly considered as a phase of start-up and acclimation to glucose presence (1.33 g COD/L·d), given that the inoculum was used to degrade only proteins during a previous experiment[18]. To inhibit methanogenesis, which began to occur at day 42, sodium 2-bromoethanesulphonate (BES, 137502, SigmaAldrich) was added to the reactor feedstock at a concentration of 0.5 g/L starting from day 45. At day 344, the reactor was stopped due to Covid-19 lockdown and restrictions on research activity and its content was stored at 4°C. The operation was then resumed after two months (Fig. 1b) by acclimating the stored biomass at the original conditions of pH, temperature and nitrogen sparging. The reactor was operated in batch mode for

136 the first 10 days by adding a diluted feedstock pulse to the vessel, in order to safely reactivate the
137 biomass activity. After having detected the occurrence of VFA production (Fig. 1d), continuous
138 feeding started at an hydraulic retention time (HRT) equal to 3 d (STP 1.00), to avoid potential
139 washout of the biomass. After one week it was lowered to 2 d, and finally set at the original value
140 of 1.5 d at day 24. On day 45, glucose concentration was increased to achieve the highest STP
141 value (2.00). The reactor operation was then finalised at day 88.

142 Biomass concentration rapidly grew from 0.6 to 1.0 g VSS/L when exposed at the lowest glucose
143 loading (STP 0.25), compatibly with the higher yields associated with sugar substrates[21]. Increasing
144 the STP ratio further favoured biomass growth, reaching 1.4 g VSS/L and 2.8 g VSS/L at STP ratios
145 of 1.00 and 2.00, respectively.

146 Methanisation was successfully inhibited from day 50 on, since no difference was detected
147 between the total COD concentrations in the reactor influent and effluent (Fig. 1a and b). The
148 difference between total and soluble COD in the effluents matched the biomass concentrations
149 achieved in the reactor. The overall concentration of VFA (COD basis) increased progressively with
150 the application of higher STP ratios, peaking at approximately 10 g COD/L (STP 1.00). VFA
151 production was 20% lower (8 g COD/L) after the reactor operation was resumed at the same
152 conditions (Fig. 1b), suggesting that the interruption and subsequent storage might have affected
153 the microbial population. Soluble COD concentration was systematically higher than the VFA-COD
154 concentration, suggesting the presence of non-converted substrate, alternative end products (e.g.
155 ethanol) and/or unidentified products. As glucose could not be detected in the reactor effluents,
156 only protein can account for the non-converted substrate.

157 As expected, global VFA production increased at higher STP ratios. However, the effect of STP ratio
158 on individual VFA production was acid-dependant (Fig. 1c and d). Acetic, n-butyric and n-valeric
159 acids were the main products for most of the reactor original operation (≥750 mg/L), progressively

160 increasing with the STP ratio. Interestingly, n-valeric acid production peaked at 1500 mg/L when
161 applying an STP value of 0.75, becoming the VFA with the highest concentration. Acetic acid
162 replaced it at STP 1.00, reaching 2000 mg/L. In comparison, n-butyric acid concentration grew
163 more steadily, stabilising at a final concentration of 1500 mg/L at STP 1.00. Conversely, iso-butyric
164 and iso-valeric acid production decreased from 330 to 250 mg/L and from 650 to 500 mg/L
165 respectively when applying an STP value greater than 0.25. n-Caproic acid was only detected for a
166 limited amount of time (STP 0.50) and only in small concentrations (≤ 150 mg/L). During the
167 resumed operation, the increase in STP ratio especially favoured acetic and propionic production
168 (≥2200 mg/L) in detriment of all the other VFAs, whose concentrations were equal or lower than
169 500 mg/L. Lactate, formate and ethanol production was not observed during the original
170 experiment and at variable concentrations during the resumed operation (data not shown).
171 To assess the impact of STP ratio on casein-glucose cofermentation, several steady-state periods
172 were identified: day 56 – 119, day 142 – 232, day 249 – 295 and day 312 – 344 for STP ratios of
173 0.25, 0.50, 0.75 and 1.00, respectively. For the resumed operation, the selected stable periods
174 were day 24 – 45 and day 52 – 88 for STP ratios of 1.00 and 2.00, respectively.
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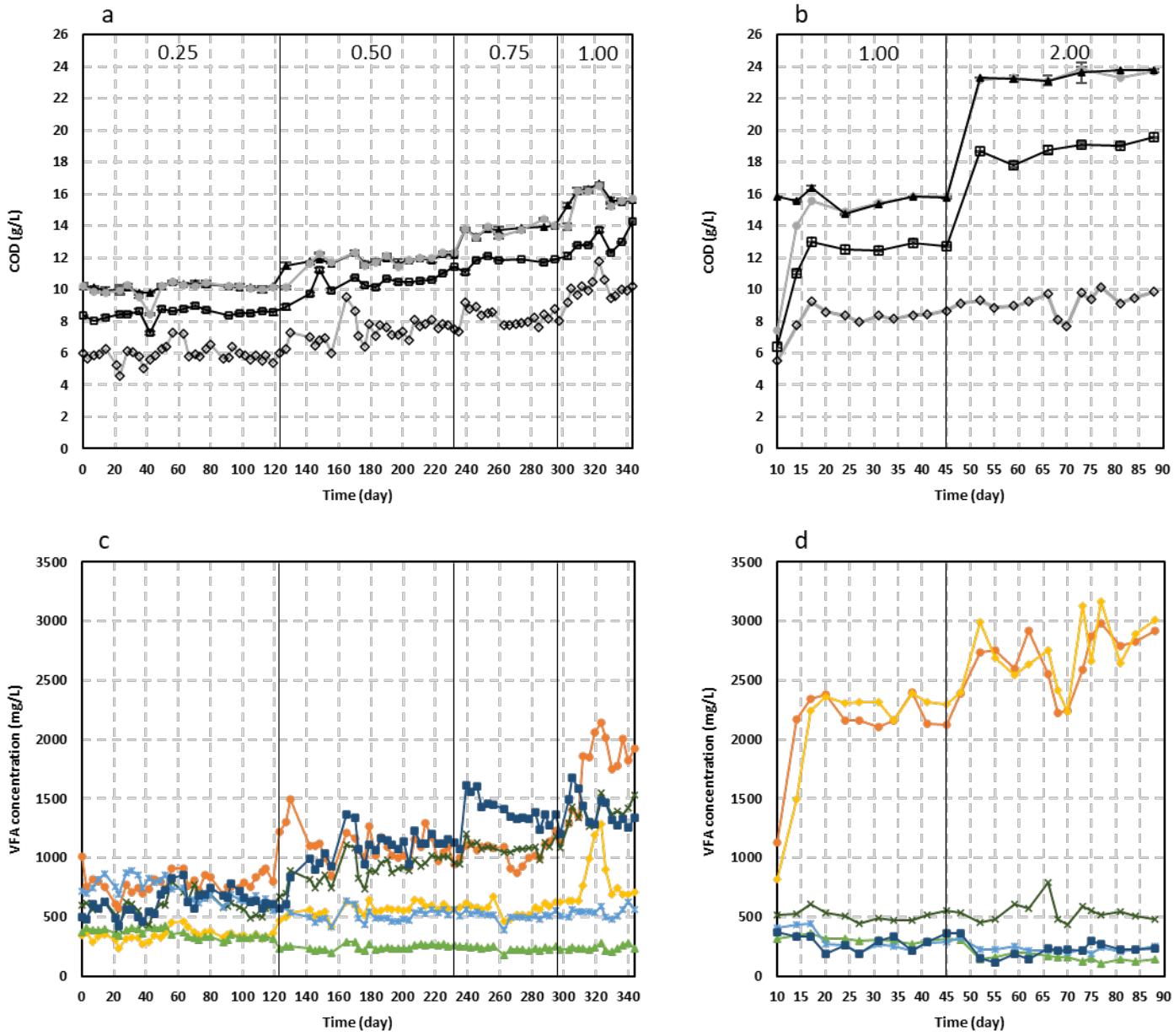


Figure 1. COD balance (a, original operation; b, resumed operation: ▲ Influent total COD; ● Effluent total COD; □ Effluent soluble COD; ◇ VFAs COD) and individual VFA concentrations in the cofermentation reactor (c, original operation; d, resumed operation: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; ✳ Iso-Valeric; ■ n-Valeric). The vertical black lines indicate the change in the STP ratio.

3.2 The influence of STP ratio on protein conversion and amino acid consumption

Glucose consumption was complete regardless of the STP ratio, while casein consumption was above 60% based on the ammonification parameter, except for the STP ratio of 2.00 (Fig. 2). Given

the little variation of this parameter from the value determined during casein monofermentation (STP 0.00[18]), it appears that glucose presence does not affect protein uptake and conversion as long as it is found in the same concentration as the protein (STP $\leq$ 1.00).

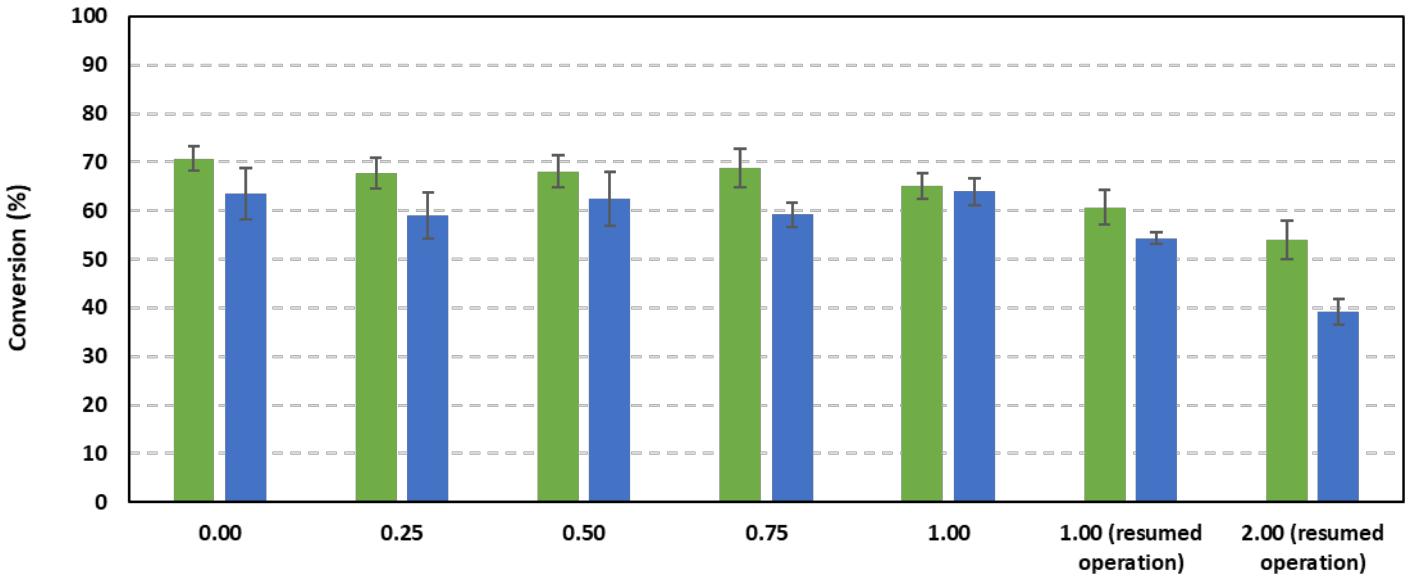


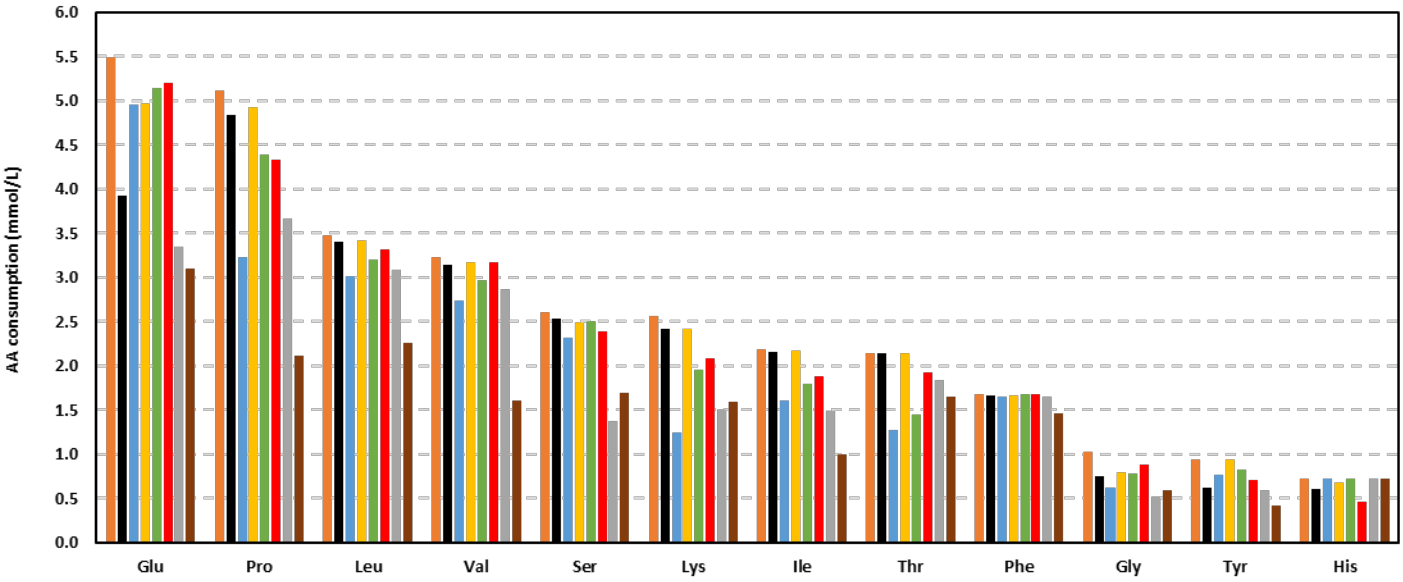
Figure 2. Comparison between ammonification (■) and acidification degree (■) at different sugar-to-protein ratios (0 – 2.00). The monofermentation values (sugar-to-protein ratio of 0.00) are obtained from a previous study[18].

The acidification degree follows the same pattern as the ammonification (Fig. 2). Moreover, the sum between the unconverted protein concentration (2.40 – 3.60 COD/L), estimated from the ammonification, and the VFA-COD concentration mostly close the soluble COD balance, except for STP 2.00 (Table 2). In this case, the concentrations of produced VFAs (9.20 g COD/L) and unconverted protein (3.68 g COD/L) does not account for all the measured soluble COD (18.8 g COD/L), suggesting that alternative end products could be acquiring more relevance in the products distribution. Indeed, ethanol together with lactate and formate became a much more relevant fraction of the soluble COD (2 – 5 g COD/L) at STP 2.00. The unidentified soluble fraction was mostly equal or lower than 1.00 g COD/L, becoming substantially greater at STP 2.00.

Table 2. Soluble COD fractioning at different STP ratios. All concentrations are expressed in gCOD/L. UP, AEP and UC respectively stand for Unconverted Protein, Alternative end products and Unidentified Compounds.

STP ratio	Soluble COD	VFA COD	UP	AEP	UC
0.25	8.62	6.04	2.40	0.00	0.17
0.50	10.6	7.44	2.40	0.00	0.71
0.75	11.9	8.16	2.40	0.00	1.33
1.00	13.2	10.2	2.80	0.00	0.17
1.00 (resumed)	12.7	8.33	3.20	0.50	0.63
2.00 (resumed)	18.8	9.20	3.68	2.00 -5.00	0.94 – 3.94

The effect of sugar presence on AA consumption is amino acid-dependant (Fig. 3). Some of them (leucine, valine, isoleucine, serine and glycine) follow the same behaviour as the ammonification, while others were already inhibited by the lowest STP ratio (proline, lysine) or not affected at all even at the highest STP ratio (phenylalanine, histidine). After resuming the reactor operation, the consumptions were equal or slightly lower than the values achieved during the original experiment, probably due to the interruption having affected the microbial community and its AA degradation capacity. Only the STP ratio of 2.00 seems to have substantially hindered some AA utilisation, which is consistent with the effect observed in the ammonification and acidification degree.



212 **Figure 3.** Amino acids consumption at different sugar-to-protein ratios: ■ Feedstock AA

213 concentration; ■ 0.00[18]; ■ 0.25; ■ 0.50; ■ 0.75; ■ 1.00; ■ 1.00 (resumed operation); ■ 2.00

214 (resumed operation). No data of alanine, arginine, methionine and tyrosine consumption is

215 available. Glu: glutamic acid; Pro: proline; Leu: leucine; Val: valine; Ser: serine; Lys: lysine; Ile:

216 isoleucine; Thr: threonine; Phe: phenylalanine; Gly: glycine; Tyr: tyrosine; His: histidine.

217 **3.3 The influence of STP on the VFA selectivity**

218 As seen in section 3.1, the influence of STP ratio on the VFA production was acid-dependant. In

219 fact, their molar fractions showed different behaviour in response to the increase in the STP ratio

220 (Fig. 4). The presence of glucose even at the lowest STP decreased acetic acid relevance in the

221 products distribution. However, values equal or greater than 1, directed the selectivity of the

222 process towards this acid again, reaching molar fractions comparable to the monofermentation

223 process (STP 0.00). Propionic acid fraction was mostly stable throughout the original experiment

224 (12-15%), becoming the second most relevant VFA after the reactor operation was resumed (40%).

225 The behaviour of these two acids contradicts the tendencies described by Zhou et al.[17], as acetic

226 and propionic acid should have benefitted from glucose presence even at the lowest STP ratio. Iso-

227 butyric and iso-valeric acid showed a similar behaviour, as their molar fractions progressively

decreased in response to the STP changes. This decrease in relevance is attributed to the increase in global VFA production determined by the greater glucose concentrations in the feeding, whereas these acids, being only produced from branched-chain AA conversion (Val, Ile and Leu), are limited by the protein fixed concentration. The only exception to the tendency was observed at STP 0.25, suggesting that isomerisation and/or chain elongation might be contributing to their production. In particular, iso-butyric acid could originate from the isomerisation of n-butyric acid, while iso-valeric acid could be either obtained from elongation pathways or from the isomerisation of n-valeric acid[18,22]. Finally, n-butyric and n-valeric acid behaved in the opposite way than acetic acid, as they both progressively benefitted from the increasing STP value during the original experiment whereas their molar fractions decreased considerably after the experiment interruption. n-Butyric acid production agrees with both the experimental results of Breure et al.[9] and the metabolic pathways associated with glucose fermentation[11,23]; in contrast, n-valeric acid behaviour contradicts the fact that its production is almost only associated with proteins fermentation[5]. It was consequently hypothesised that chain elongation (CE) could be progressively contributing to the formation of n-valeric acid, and possibly to n-butyric acid production as well. The decrease in n-butyric and n-valeric acid production would be then explained by the elongation process being suppressed by the interruption of the reactor operation, which could have negatively affected the microbial community responsible of this process. Yet, it is difficult to discern whether the CE suppression was solely caused by the interruption itself, as the excessive glucose loading associated with higher STP ratios might have played a role as well.

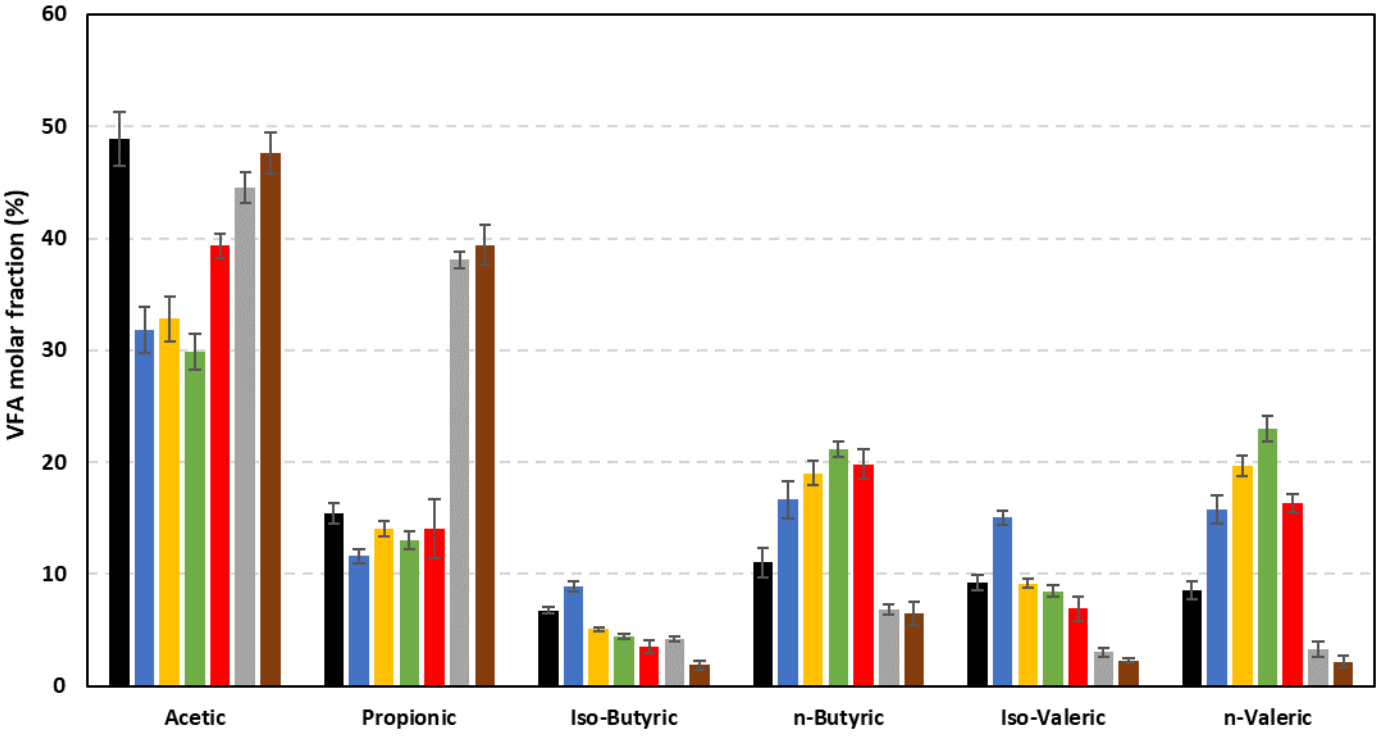


Figure 4. Comparison of VFA distributions at different STP ratios: ■ 0.00[18]; ■ 0.25; ■ 0.50; ■ 0.75; ■ 1.00; ■ 1.00 (resumed operation); ■ 2.00 (resumed operation).

It was hypothesised that the increase in STP ratio promotes CE processes as glucose is converted into adequate electron donor compounds, such as ethanol and/or lactate[11,24], while generating a surplus of reducing power[25]. These alternative end products are, in fact, suitable substrates for the elongation of acetic and propionic acid to n-butyric and n-valeric acid, respectively[26]. The formation of n-butyric and n-valeric acid by CE pathways is consistent with the fact that, after the reactor operation was resumed, n-butyric and n-valeric production decreased substantially whereas lactate and ethanol started to be detected in the reactor effluents. Moreover, the lack of acetic and propionic consumption could be at least partially responsible for the concentration of these short chain VFAs. The analysis of n-butyric acid production due to CE process is not straightforward as it can be yielded by glucose alone and by amino acids such as glutamic acid and lysine[16]. However, the combined production balance of iso and n-valeric acids (Fig. 5) unequivocally confirms the occurrence and relevance of CE, as the production of these VFA is

exclusively related to a limited number of AAs. In fact, the consumption of their precursor AAs does not justify their formation during the original operation with glucose. It also suggests that isomerisation between the two forms might be occurring in a similar way as seen for casein monofermentation[18]. At low STP (0.25) part of the n-valeric acid overproduction is converted to the branched form whereas at high STP (2.00) the opposite interconversion occurs, favouring the linear form. Overall, these results suggest that STP ratio can be adjusted to potentially steer the process towards the desired outcome in terms of VFA selectivity.

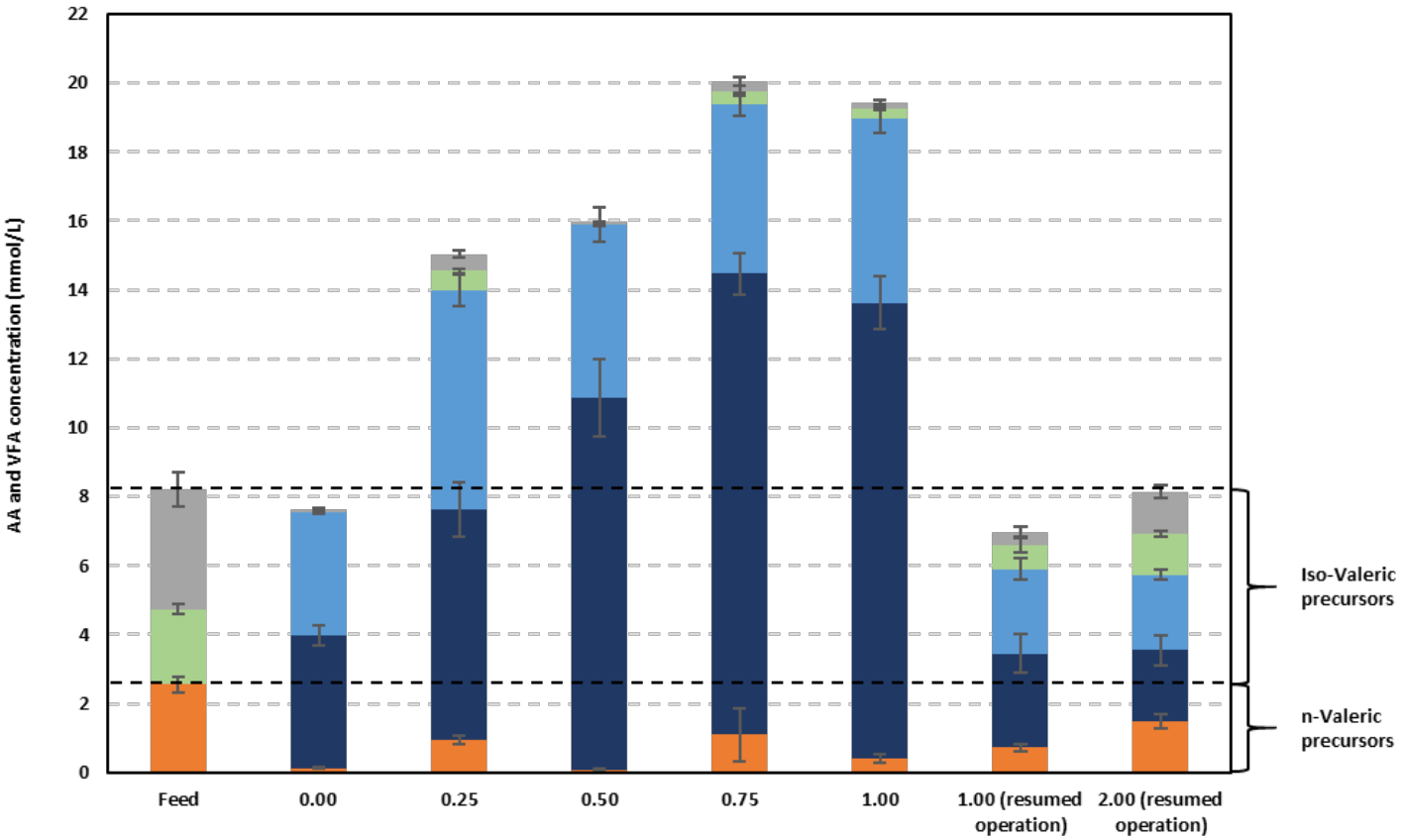


Figure 5. Iso and n-valeric acid balance in the cofermentation reactor: Proline; n-Valeric acid; Isoleucine; Leucine; Iso-Valeric acid. AA concentrations are expressed as VFA equivalents according to the stoichiometry described by Regueira et al.[16]

3.4 The influence of STP on the microbial community structure

A total of 296,998 reads were obtained after trimming and quality filtering, ranging from 46,105 to 32,006 with an average of 36,512 reads per sample, identifying 466 different OTUs (Table S1). In addition, the rarefaction curves obtained by the normalization of OTUs count for individual biological replicates reached plateau (Fig. S1), pointing out an adequate sample sequencing depth (26,618 reads).

Only OTUs with a minimum combined abundance of 1% were used for further analysis, resulting in 132 OTUs distributed in 16 classes among 10 phyla (Fig. 6). *Firmicutes* and *Actinobacteria* were the dominant phyla in all the samples (above 65%), particularly at STP ratio of 1.0 (above 97%). Both phyla, together with *Proteobacteria* and *Bacteroidetes*, are obligated or facultatively anaerobic bacteria well known by their ability to decompose polysaccharides and proteinaceous substrates to produce VFAs[27].

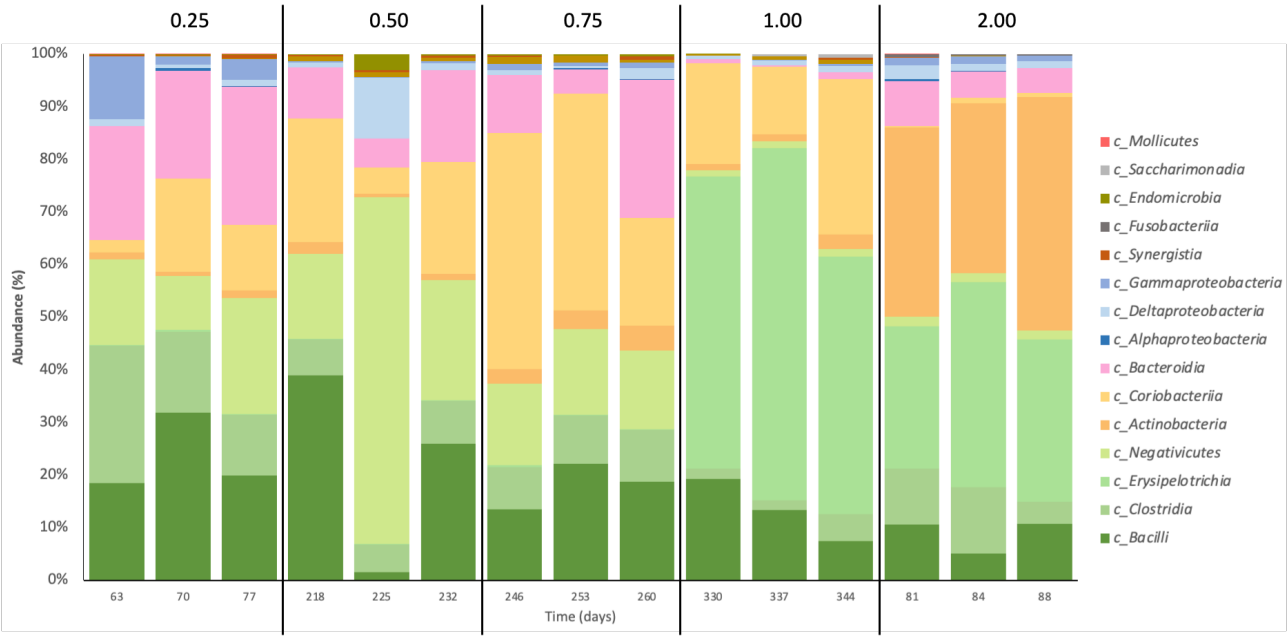


Figure 6. Bacterial classes with a total abundance higher than 1% at different STP ratios. The vertical black lines indicate the change in the STP ratio. Class abundances are colored according to the phyla they belong to.

However, a more clear influence of increasing glucose loads was observed at class level (Fig. 6). Overall, *Bacteroidia* abundance shows a decreasing trend, while the presence of *Actinobacteria* and *Erysipelotrichia* is favored. Interestingly, the microbial community composition was similar up to STP values of 0.75, but significant changes occurred when this ratio was increased to 1 and further to 2. This pattern was also confirmed by Beta diversity analysis (Fig. 7), where all samples belonging to STP ratios below 1.0 clustered together (moreover, PERMANOVA analyses showed no significant differences among STP ratios of 0.25, 0.50 and 0.75 (pseudo-f statistic 1.99, 6.90, 1.3, p-values > 0.1)) and separately from those belonging to STP ratios of 1 and 2, respectively.

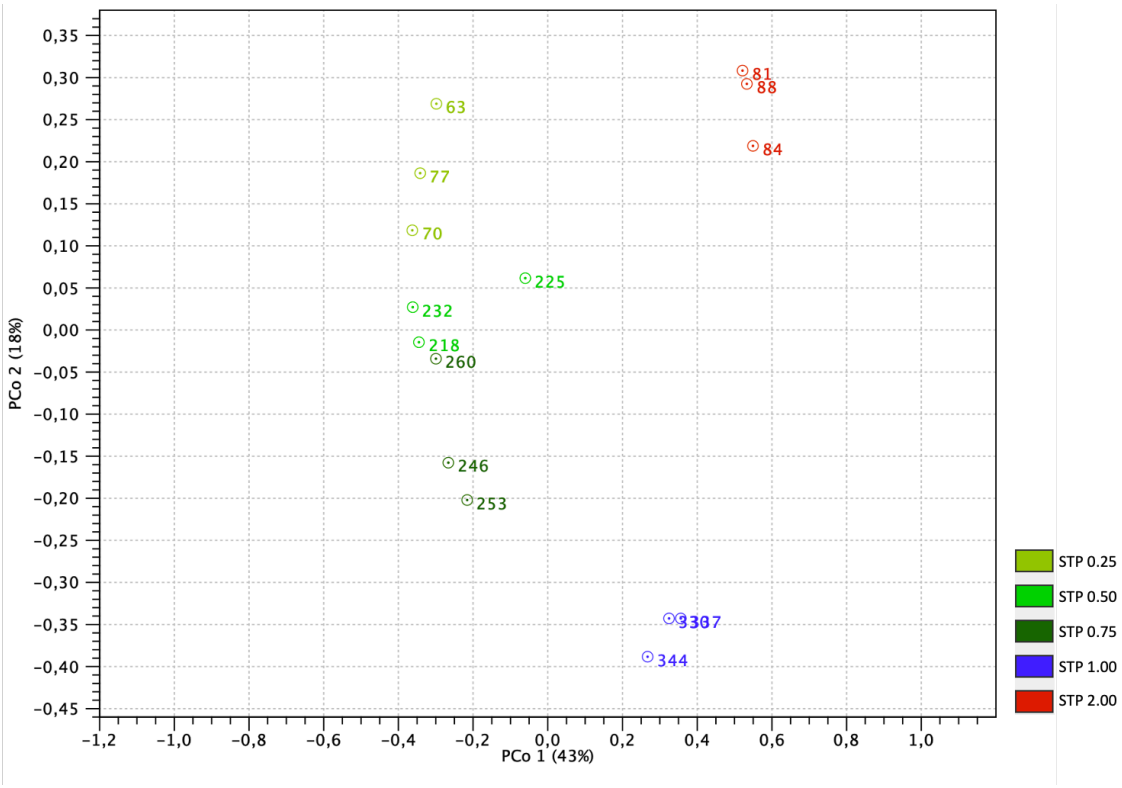


Figure 7. Principal component analysis (PCoA) showing the differences on the community composition related to increasing glucose loads. Points represent each sample and are coloured according to the STP value: 0.25 (light green), 0.50 (medium green), 0.75 (dark green), 1.0 (blue) and 2.0 (red).

Changing STP ratio from 0.75 to 1.0 lead to a very significant increase of *Erysipelotrichia* abundance (Fig. 6) in detriment of *Bacteroidia* and *Negativicutes* (Fig. S2). In addition, a decrease in Alpha diversity was observed (Fig. 8). Increasing further the STP ratio from 1.0 to 2.0 favored the presence of *Actinobacteria, Bacteroidia* and *Clostridia* in detriment of *Coriobacteriia* (Fig. S2), and also the overall diversity of the microbial community increases (Fig. 8). Combining these results with the different product selectivities observed during the reactor operation (Fig. 4), we could speculate the positive link between *Actinobacteria* and propionic acid production as well as the link between *Coriobacteriia* and the production of longer chain VFA (butyric and valeric acids).

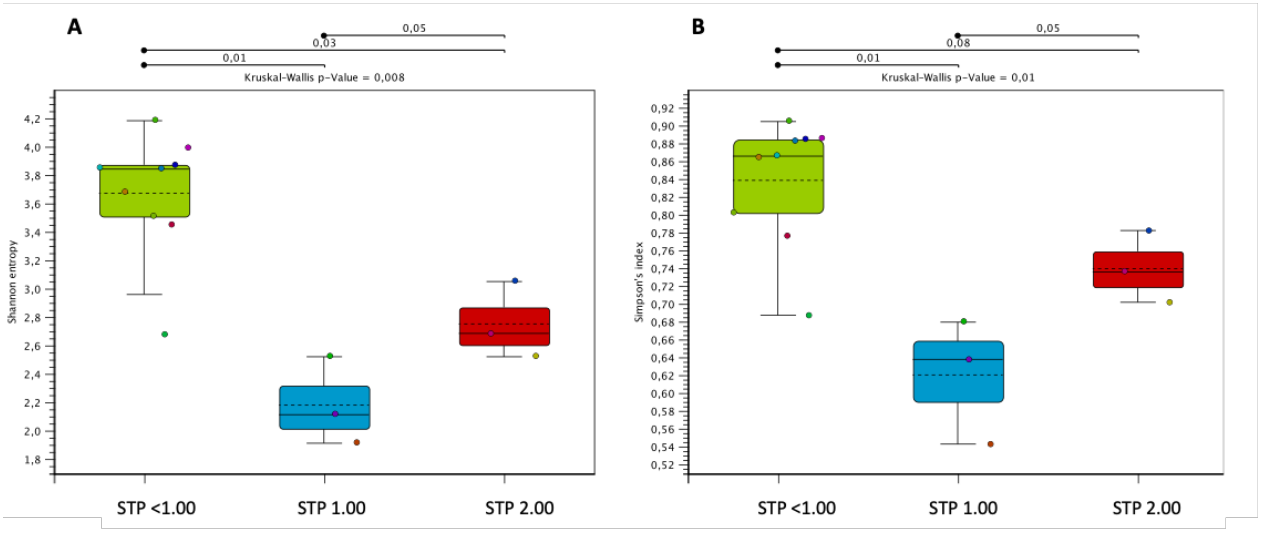
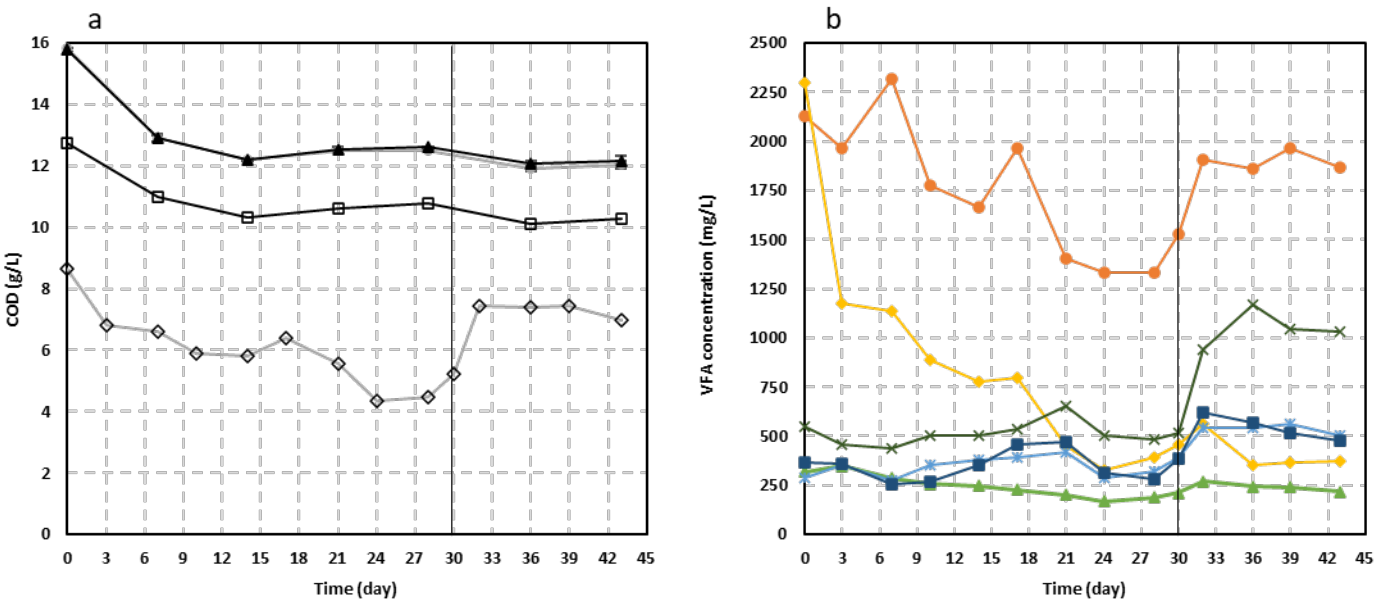


Figure 8. Summary of alpha diversity statistics shown as boxplot. A) Shannon entropy index; B) Simpson index. Statistical significance was measured by Kruskal-Wallis test.

3.5 CE can be recovered by lowering the STP ratio

To verify whether the CE process could be recovered by lowering the glucose loading, a parallel cofermentation reactor was inoculated with biomass taken from the main reactor on day 45 of the resumed operation (STP 1.00) and operated at an STP ratio of 0.50 (Fig. 9).



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321 **Figure 9.** Operation of the parallel reactor at an STP ratio of 0.50 to assess CE process recovery (a,

322 COD balance: ▲ Influent total COD; ● Effluent total COD; □ Effluent soluble COD; ◇ VFAs COD; b,

323 VFA concentrations: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; ✳ Iso-Valeric; ■ n-Valeric).

324 The vertical black lines separate the acclimation phase from the steady-state operation.

325 Both the total and the soluble COD of the reactor effluent decreased compatibly with the lower

326 STP applied to the reactor (Fig. 9a). Based on the VFA production (COD basis), it was possible to

327 identify two operational periods: from the start up to day 30 (acclimation stage) and from day 30

328 to 43 (steady-state operation). Interestingly, the values of all COD parameters were similar to

329 those previously obtained at STP 0.50 (Fig. 1a), providing the first proof concerning the

330 reversibility of excessive sugar supplementation.

331 In terms of VFA production (Fig. 9b), the acclimation period was associated with a decrease in

332 acetic and propionic acid concentrations, whereas the other VFAs remained mostly stable. In

333 contrast, except for propionic and iso-butyric acid, VFAs production increased between day 30 and

334 43. In particular, n-butyric, n-valeric acid and iso-valeric generation showed a two-fold increase

335 which, coupled with the absence of lactate and ethanol in the reactor effluents, further confirms

the reversibility of the effects caused by STP ratios greater than 1.00. Also, the balance of valeric acids (Fig. 10) indicates that CE process was recovered during the steady-state period, as proline consumption alone is not able to justify n-valeric acid production. This balance also highlights the occurrence of isomerisation from the iso to the n-form during the acclimation step.

Comparing these results with those described in the previous sections, it was hypothesised that the increased availability of glucose associated with higher STP ratios might be making further conversion of ethanol and lactate into VFAs less appealing to the microbial community due to kinetic limitations associated with high OLRs (16 g COD/L·d at STP ratio 2.00). Besides, the absence of substrate limitations might be making specialised metabolic pathways, such as CE, less appealing from a bioenergetics point of view. Still, the disruptive effect caused by the operation interruption cannot be completely discarded, as it might have accelerated the disappearance of the CE process at the highest STP ratios by altering the microbial community in first place. Besides, the VFA concentrations were not strictly the same as during the original experiment at STP 0.50 (Fig. 1c), suggesting that longer operation time might be required to fully recover the previously obtained steady state.

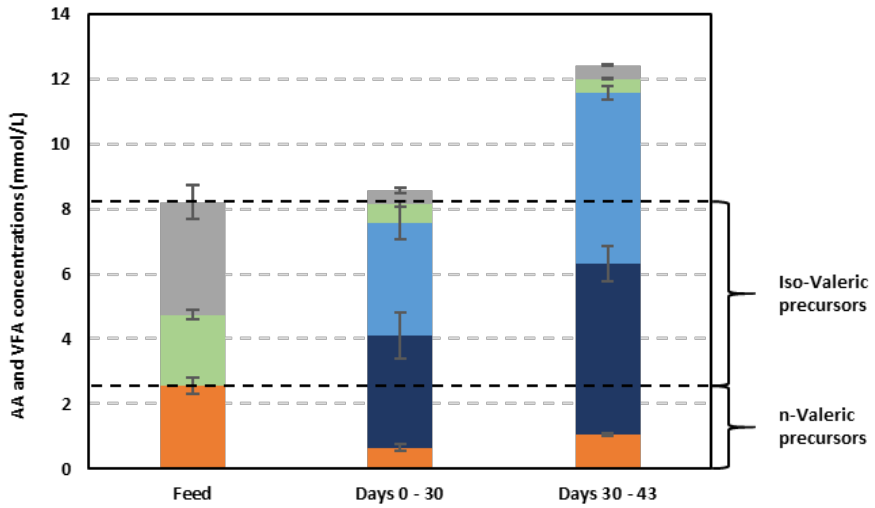

Figure 10. Iso and n-valeric acid balance in the parallel reactor at an STP ratio of 0.50: Proline; n-valeric acid; Isoleucine; Leucine; Iso-valeric acid. AA concentrations are expressed as VFA equivalents according to the stoichiometry described by Regueira et al.[16]

4. CONCLUSIONS

This study successfully investigated the interactions between amino acids and glucose during their cofermentation in order to understand the impact of the STP ratio on substrate consumption, acidification degree, product selectivity and microbial community structure. In particular, the main findings are:

- STP ratios equal or lower than 1.00 do not affect the extent of protein conversion, but excessive sugar loading hinders AA consumption and favours the production of alternative end products.
- The products distribution can be steered towards the production of n-butyric and n-valeric acid by increasing the sugar proportion up to the optimal STP ratio of 1.00, which promotes the occurrence of CE processes.

- The increasing load of glucose affected microbial community composition, especially at the highest STP ratio tested (2.0). Overall, the presence of *Actinobacteria* and *Erysipelotrichia* was favored in detriment of *Bacteroidia* abundance.
- The changes produced by excessive sugar loadings are reversible, as lowering the STP ratio allows to recover the longer chain VFA production to a certain extent.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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