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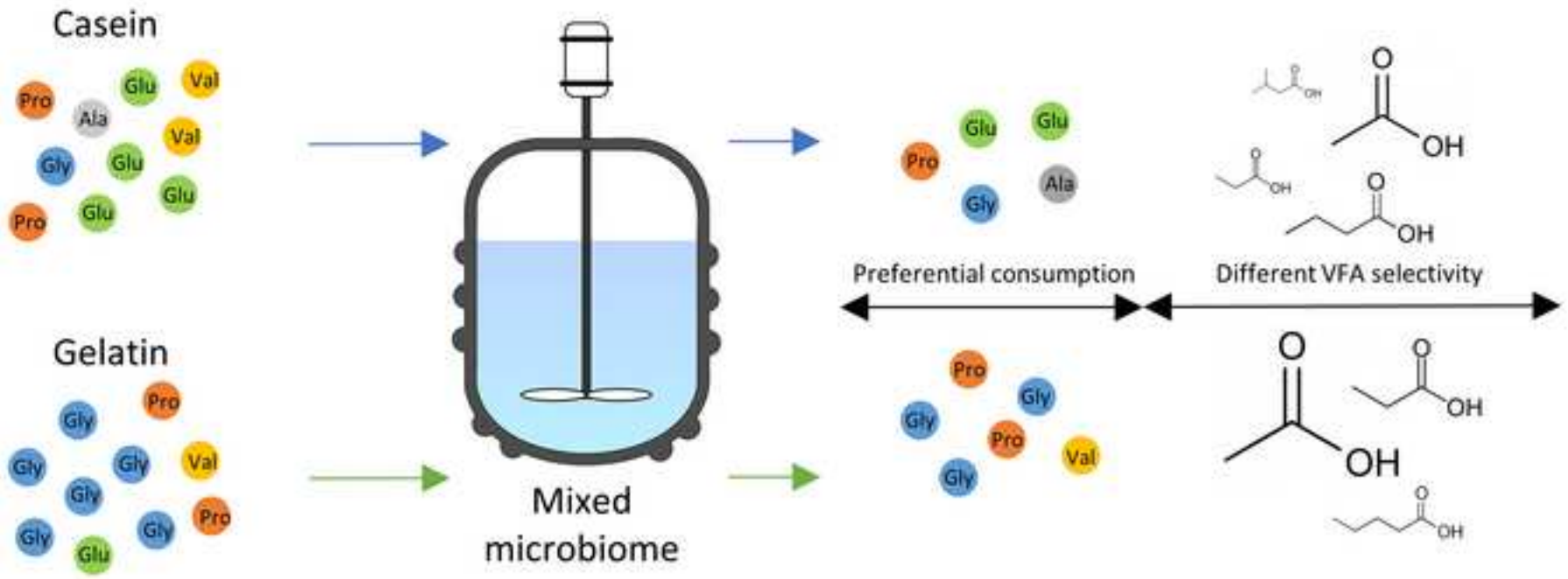
Highlights

- Protein fermentation generally results in incomplete acidification
- Balanced amino acid redox roles do not guarantee higher acidification degrees
- The preferential consumption of amino acids was demonstrated
- Protein composition affects acidification, selectivity and preferential consumption
- The accepted stoichiometry is not sufficient to describe protein conversion to VFA

Graphical Abstract



Protein composition determines the preferential consumption of amino acids during anaerobic mixed-culture fermentation

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ABSTRACT

The valorisation of protein-rich residual streams by anaerobic mixed-culture fermentation (MCF) has been barely studied in contrast to carbohydrate-rich wastes. The aim of this work was, therefore, to investigate how protein composition, i.e. the amino acid (AA) profile, affects the individual consumption of amino acids and, consequently, the outcome of the process. Mixed-culture fermentations were performed with two model proteins (casein and gelatin) using continuous and batch reactors at neutral pH values and 25°C. The acidification was incomplete for both proteins, with casein achieving a higher value than gelatin. Albeit dominated by acetic acid, product spectra were different as well, with n-butyric acid as the second major product for casein and propionic acid for gelatin. The preferential consumption of amino acids was demonstrated, which interestingly depends on protein composition. The previously accepted stoichiometry accurately describes iso and n-butyric acid production, but it fails for propionic, iso and n-valeric acid generation. Overall, this study offers a better understanding of protein fermentation

mechanisms, which will help to improve degradation models and to design fermentation processes, based on optimal substrate selection.

KEYWORDS: acidification; biorefinery; productivity; protein composition; selectivity; volatile fatty acid production

1 INTRODUCTION

Anaerobic fermentation of solid wastes and industrial sidestreams leading to the production of carboxylic acids, solvents and hydrogen is an appealing alternative to biogas generation due to their application as precursors of biofuels, bioplastics, cosmetic products, dietary supplements, etc. (Sauer et al., 2008; Kandylis et al., 2016; Bathia & Yang, 2017). Moreover, market prices indicate higher profitability of volatile fatty acids (VFAs) compared to methane or biogas (Moscoviz et al., 2018).

Mixed culture anaerobic fermentation (MCF), a resilient and versatile process for multiple substrates, results in a variety of products with different concentrations, fundamentally depending on the microbiome, substrate composition and operational conditions (Domingos et al., 2017). Since microbiology can be engineered only to a limited extent, both operational parameters and especially substrate composition are the important factors to consider when designing an MCF process (Bathia & Yang, 2017). Many studies have been focusing on sugars (Gujer & Zehnder, 1983; Pavlostathis & Giraldo-Gomez, 1991; Skiadas et al., 2000, Temudo et al., 2007), mainly glucose, due to the well-known metabolic pathways (González-Cabaleiro, 2015) and their wide availability and high biodegradability. In contrast, albeit being a relevant fraction of many industrial sidestreams and wastes, proteins and their fermentation mechanisms have not been studied so thoroughly. Their main complexity lies in the fact that they can be considered as a mix of some 20 different substrates, i.e. the amino acids (AAs), as opposed to glucose, which is only one substance.

Stickland coupled redox reactions are currently the accepted metabolic pathway for AAs fermentation (Ramsay & Pullammanappallil, 2001), accounting for up to 90% of the overall degradation process (Nagase & Matsuo, 1982). Based on these reactions, Ramsay and Pullammanappallil (2001) proposed a fixed stoichiometry to describe MCF of AAs. This means that

each AA is converted to specific VFAs, regardless of the protein composition or the operational conditions. Therefore, this degradation model does not take into account possible imbalances between AA redox roles, related to the protein composition, excluding alternative metabolic pathways that fermentative bacteria might opt for if a surplus of either electron donor or acceptors accumulates in the reactor. In addition, the flexibility of some AAs, which can potentially act both as electron donor or acceptor (e.g. leucine and arginine), is not compatible with the proposed fixed stoichiometry. Moreover, the experimental evidence of incomplete protein consumption, both in continuous and batch experiments (Breure & van Andel, 1984; Duong et al., 2019), might indicate the preferential consumption of some AAs due to bioenergetics motivation (Regueira et al., 2019), refuting the assumption of the proposed fixed stoichiometry, by which all AAs are completely and equally degraded.

From the abovementioned hypotheses and model limitations, the main aim of this work is to assess how AA composition of proteic substrates affects their consumption and interaction, and consequently, the VFA selectivity and productivity of the process. The gathered knowledge contributes to the understanding of protein degradation mechanisms during anaerobic mixed-culture fermentation.

2 MATERIALS AND METHODS

2.1 Inoculum and proteic substrate characteristics

The inoculum was obtained from an acidogenic reactor inoculated with anaerobic biomass from a mesophilic sewage sludge digester and digestate from an anaerobic digester fed with brewery wastewaters. This reactor was fed with a mix of three different substrates, glucose, casein and sodium oleate, which represented respectively 60, 30 and 10% of the total influent chemical oxygen demand (COD). It was operated at room temperature (approximately 25°C), with

controlled pH (5.7 ± 0.1) and at an organic loading rate (OLR) and hydraulic retention time (HRT) of 8 g COD/L·d and 2 days, respectively. Only macronutrients were supplemented, with the following concentrations (g/L): NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530, Na_2SO_4 0.057; $MgCl_2 \cdot 6H_2O$ 0.120. The operational conditions were chosen to promote the inhibition and washout of methanogenic biomass, consequently developing an acidogenic-only microbial community to be used in the experiments described in this study.

The proteic substrates were composed of a synthetic hydrolysed protein (peptone from casein (A2208,0500 PanReac) or peptone from gelatin (70951-1KG-F Sigma-Aldrich)) as sole carbon source, supplemented with macro nutrients. The composition of the feedstock solution was as follows (g/L): hydrolysed casein or gelatin 7.500-7.600; NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530, Na_2SO_4 0.057; $MgCl_2 \cdot 6H_2O$ 0.120. The feedstocks were kept at 4ºC throughout the experiment. The AA composition of the chosen proteins, casein and gelatin, was analysed in order to better relate the results of the fermentation with protein composition (Table 1).

<Table 1 should be placed approximately here>

2.2 Continuous reactors

Two continuous stirred tank reactors (CSTR) of 2 L (1 L working volume) were used. The reactors were inoculated with an initial in-reactor biomass concentration of around 1.0 g VSS/L and they were operated with an HRT of 1.0 d and an OLR of 8.0 g COD/L·d. On day 44, the HRT of the gelatin reactor was increased to 1.5 d (OLR of 5.3 g COD/L·d) in order to surpass possible kinetic limitations. Stirring was provided via magnetic agitators (200 rpm). The reactors were placed in a temperature-controlled room at 25°C and pH was continuously monitored with Hamilton probes through a multiparametric analyser (CHEMITEC, Italy). Constant nitrogen sparging (approximately

10 mL/min) of the liquid phase was conducted to ensure anaerobic conditions and minimize hydrogen saturation.

COD (total and soluble) and VFA concentrations were determined three times per week, while Total Ammonia Nitrogen (TAN) and solids concentrations were measured once a week. AA content was measured on selected samples from steady state periods of operation.

2.3 Batch experiments

2.3.1 Maximum substrate biodegradability

Biochemical methane potential (BMP) tests were performed with both proteins as described by Holliger et al. (2016) in order to determine their maximum anaerobic biodegradability. Bottles of 0.5 L total volume (0.375 L of working volume) with rubber stoppers were used. The bottles were inoculated with anaerobic biomass coming from a mesophilic lab-scale reactor fed with sewage sludge. Inoculum (8.0 g VS/L) and substrate (4.0 g/L) concentrations were selected in order to achieve a non-inhibitory inoculum to substrate ratio (ISR) of 2. A blank assay (only inoculum) was also included to monitor residual biogas production from the inoculum. The tests were conducted by triplicates at 37.5°C in an orbital shaker for 16 days. Biogas production and composition were monitored daily.

2.3.2 Acidification tests

Several batch fermentation tests were also conducted in order to: i) understand incomplete protein consumption, and ii) estimate kinetic parameters for protein-degrading biomass. Bottles of 0.5 L total volume (0.375 L of working volume) with rubber stoppers were used and the operational conditions were similar to the continuous reactors (25°C, N_2 sparging), although pH was controlled with HCl 2M addition to maintain a constant value of 7. The inoculum came from the continuous reactors. Mixed liquour samples were centrifuged to separate the VFA-rich

supernatants and the obtained biomass pellets were used after being washed with fresh (inorganic) medium. Macronutrients were added with the same concentrations as in the reactors feedstock. 10 mL samples were taken at increasing time intervals (initially 2-3 hours). Half of the sample volume was centrifuged and filtered for TAN and VFA determination, with the surplus being frozen for AAs analysis. The remaining 5 mL were centrifuged, and the biomass solid pellets were then resuspended in 5 mL of a 0.7% w/w solution of NaCl and distilled water for optical density determination. At the end of each experiment, VSS concentration of the fermentation broth was also measured.

2.4 Analytical methods

Conventional physicochemical parameters were determined according to Standard Methods (APHA, 2017). Mixed liquor samples from the reactor were used for total (TS and VS), suspended (TSS and VSS) solids (SM2540B, D and E) and total COD (modified SM5220C) content analysis. Filtered (0.45 μm) mixed liquor samples were used to determine the soluble chemical oxygen demand (SM5220C) and TAN (SM4500-NH_3.F). All spectrophotometric measurements were performed with a Shimazdu UV-1800.

VFAs from C2 to C7 were measured through gas chromatography (AGV-DB1 method), though nC6 and C7 compounds were never detected and iC6 only occasionally identified. The equipment used was an Agilent 6850 with a flame ionization detector (FID). The column used was a DB-Wax, from Agilent Technologies (30 m x 0.250 mm x 0.25 μm). The injector had a temperature of 200°C while the detector was set at 300°C. The carrier gas was nitrogen. The samples were filtered (0.45 μm) and then acidified with 10 μM of concentrated H_3PO_4 (85%) prior to analysis.

158 Formic acid, lactic acid and ethanol were measured through high performance liquid
159 chromatography (GLEFG1 method) with a HP 1100 equipped with an IR HP1047A detector. The
160 column used was an AMINEX HPX-87H (300 x 7.8 mm) using H_2SO_4 (5 mM) as an isocratic eluent.
161 The set temperature for the column was 30°C while for the detector was 35°C. The samples were
162 prepared in the same way as for VFA determination.

163 Gas composition was determined by gas chromatography with a HP 5890 Series II. Gas syringes of
164 1 mL were used to extract the gaseous samples through silicon rubber septa attached to the
165 outflow gas tubes of the reactors and through the bottle rubber stoppers.

166 For total AA determination, samples underwent acid hydrolysis for 24 hours at 110°C using HCl 6
167 N. Then, AccQ-Tag method was used to convert them to stable fluorescent derivatives (Cohen et
168 al., 1993) which were finally analysed through HPLC with a Waters 2695 equipped with a
169 fluorescence detector (Waters 2475).

170 Turbidity (i.e. optical density) was determined using a spectrophotometer set at 600 nm and
171 calibrated with actual VSS measurements.

172 **2.5 Calculations**

173 Acidification degree was the parameter chosen to describe substrate conversion (in COD basis),
174 based on the concentration of measured VFA (in this case aliphatic VFA) and expressed as:

$$Acidification\ degree\ (\%) = \frac{\Sigma\ C_{VFA}}{C_{pr}} \times 100 \qquad (1)$$

175 where C_{VFA} stands for the total concentration of the measured VFAs (in g COD-VFA/L) in the
176 reactor effluent and C_{pr} for the total protein concentration (in g COD/L) in the reactors feedstock.

177 Anaerobic biodegradability (BMP) was calculated in COD basis as a ratio between the methane
178 produced and the total substrate used in the test, as described in the following equation:

$$Anaerobic\ biodegradability\ (\%) = \frac{M_{CH4}}{M_{pr}} \times 100 \quad (2)$$

where M_{CH4} stands for the total production of methane (in g COD-CH_4) and M_{pr} for the initial protein mass (in g COD).

Combining Stickland stoichiometry (Ramsay & Pullammanappallil, 2001) and the measured anaerobic biodegradability, it was possible to differentiate the methane produced from aliphatic VFA, aromatic ones and hydrogen.

2.6 Kinetic parameters estimation

Available kinetic parameters on AA fermentation in literature were determined mainly in methanogenic assays (Ramsay, 1997; Angelidaki et al., 1999; Flotats et al., 2006). Since the environmental conditions are different, it is likely that those parameters are not suitable to describe the dynamics of AA fermentation. A kinetic model was built to estimate the kinetic parameters of protein-degrading biomass (maximum specific growth rate (μ_{max}), yield (Y), decay constant ($k_{dec,X}$) and the different VFA stoichiometric factors (F_{VFA})) in fermentative environments. It was assumed that AA conversion to VFA was performed by a single population of microorganisms (AA degraders) and that the feeding consisted of a mixture of AAs. AA conversion was modelled following a Monod equation, with a fixed half saturation constant (1.5 g COD-AA/L). To reflect the observed non-complete consumption of AA, the model includes the possibility of converting the substrate to an inert fraction. Aromatic VFA and H_2 yields were determined based on the AA composition of the fermented protein and following the stoichiometry proposed by Ramsay and Pullammanappallil (2001), as they were not measured experimentally.

The calibration procedure was done following the non-linear least squares method (Eq. 3) in MATLAB 9.0 (R2016a) (Mathworks Inc., Natick, MA, USA) using the *lsqnonlin* command (trust-

200 region reflective algorithm). A Bootstrap methodology was followed to ensure a robust parameter
201 estimation, as described in Gonzalez-Gil et al. (2018).
202

$$\hat{\theta} = \arg\min\left(\sum_{k}\left(\sum_{j}\left(\sum_{i}\left(\frac{y_{j,i}(\theta) - y_{j,i,exp}}{\sigma_{j,i}}\right)^2\right)\right)\right) \quad (3)$$

203
204

205 where $\hat{\theta}$ is the set of parameters to estimate, *y* is the simulated concentration, y_{exp} is the
206 experimentally measured concentration and σ is the experimental standard deviation. The
207 subscript *i* refers to the different compounds, the subscript *j* refers to the different measurements
208 over time and the subscript *k* refers to the different batch experiments.
209

210

211 # 3. RESULTS AND DISCUSSION

212 ## 3.1 Reactors operation

213 Casein and gelatin reactors were continuously operated for 140 and 170 days, respectively (Fig. 1a
214 and b). No pH control was needed since it naturally adjusted on neutral values (7.2-7.4) due to the
215 joint effect of ammonia release (buffering VFA production acidification) and nitrogen sparging
216 (partially stripping the CO_2 from the system). Biomass concentration rapidly decreased from
217 above 1.0 g VSS/L to 0.35 – 0.40 g VSS/L in both CSTRs during the first seven days, remaining
218 constant afterwards. No methanisation occurred during the experiment since no differences were
219 observed between the total COD concentrations in the influent and effluent in any of the two
220 reactors (Fig. 1a and 1b). Analysis of the gas composition of the headspace volume of the reactors

confirmed the absence of methane, with nitrogen (close to 100%) and carbon dioxide (up to 2%) as the only detected gases. Hydrogen was not detected either throughout the whole experiment.

Acidification degree increased over time from 31.3% (days 40-85) to 48.8% (days 100-140) in casein reactor and from less than 10% (days 15-70) to 40% (days 145-170) in gelatin reactor. In the latter case, different strategies were adopted in order to improve substrate conversion. The addition of selenium dioxide (1 µM on day 91), to satisfy the requisite for glycine reductase production (Dürre & Andreesen, 1981), an enzyme especially relevant to gelatin degradation because of its high content in glycine (Table 1), and an increase in HRT from 1.0 to 1.5 days (day 44) were not successful (Fig. 1b). In contrast, cross inoculation with methanogenic biomass to increase the diversity of the microbial population inside the reactor (days 70 and 133) resulted in VFA peak production followed by a gradual decrease and stabilization with a 2-fold improvement of the acidification degree. This suggests that gelatin conversion was hindered either by a low microbial diversity and/or by the lack of some micronutrients.

Even though acidification degree appeared to be stable, product composition (VFA spectra) varied more during the experimental period (Fig. 1c and d). Acetic acid was the major product in both reactors. However, the second major product differed between them, being n-butyric acid in casein reactor and propionic acid in gelatin reactor. Other possible fermentation products, such as lactic acid, formic acid and ethanol, were not detected during the operational period.

In order to assess the influence of protein composition on fermentation performance, the following periods, day 100-140 and day 147-170, were selected as stable periods for casein and gelatin, respectively. Steady-state periods were identified as those where the variability of the VFA relative molar fractions was lower than 15% (measured as the coefficient of variation).

244 *<Figure 1 should be placed approximately here>*

245

246 **3.2 Influence of protein composition on acidification degree and selectivity**

247 Protein composition affects acidification degree, with higher values being achieved for casein
248 (around 50%) than for gelatin (around 40%). This, combined with the lower HRT applied in the
249 casein reactor, derived in higher productivities (4.1 ± 0.5 g COD-VFA/L·d; 11.6 ± 3.0 g COD-VFA/g
250 VSS·d) when compared to the values obtained for gelatin (2.1 ± 0.2 g COD-VFA/L·d; 5.1 ± 1.1 g
251 COD-VFA/g VSS·d). In general, lower values were reported in literature for casein fermentation
252 (30% of the influent carbon), though the HRT was lower as well (0.4 d, Ramsay, 1997). In contrast,
253 literature data indicate higher acidification of gelatin (around 50%) regardless of the HRT applied
254 (Breure & van Andel, 1984; Breure et al., 1986). This difference in conversion efficiencies might be
255 attributed to a number of factors (e.g. protein composition, inocula type), among which
256 micronutrients presence seems to be the most influential one.

257 To explain the limited conversion achieved during continuous experiments, biochemical methane
258 potential tests of the chosen proteins were performed to evaluate their maximum anaerobic
259 biodegradability. The results showed that the conversion of both proteins to methane is very
260 similar, with values close to 90%. According to the fixed stoichiometry (Ramsay &
261 Pullammanappallil, 2001), 73-83% of the methane produced is related to aliphatic VFA, while non-
262 measured products (aromatic VFAs and hydrogen) only account for approximately 5-15%. There
263 are different and possibly concurrent explanations to explain these higher values compared to the
264 acidification degrees achieved in the reactors: the higher temperature applied to the batch tests
265 (37.5 against 25°C), the presence of micronutrients in the inoculum, higher microbial diversity
266 covering all possible metabolic niches, absence of product (VFA) inhibition due to their conversion

267 to methane and longer reaction time. Temperature role was discarded as similar acidification
268 degrees were obtained regardless of its value (Yu & Fang, 2003).

269 To verify whether product inhibition and/or limited reaction time were responsible for the
270 incomplete CSTR conversions, three casein batch experiments were carried out varying the
271 substrate-to-inoculum ratios (SIR) from 5 to 20 (g COD protein/g VSS inoculated), while the gelatin
272 batch test was only performed at a value of 10. The acidification degree of casein was close to 50%
273 in all the three cases (Fig. 2a) after 96 h, as also observed in the continuous CSTR operation,
274 highlighting the SIR and HRT values as being uninfluential on the outcome. Given that the actual
275 VFA concentration at the end of the tests was 1, 2 and 4 gCOD-VFA/L, respectively, potential
276 product inhibition was ruled out as well as the cause of the limitation in substrate conversion. On
277 the contrary, the acidification degree of gelatin after 192 h was double than the one achieved in
278 the continuous reactor (Fig. 2b) meaning that reaction time might play a more important role in
279 this case.

280

281 Protein composition determined process selectivity as well (Fig. 3). In all cases, acetic acid was the
282 main product, followed by either propionic acid (in gelatin) or n-butyric acid (in casein). Iso-butyric
283 and iso- and n-valeric acids were minor products accounting for less than 10% of the total VFA
284 molar percentage. The main difference between casein and gelatin was that more reduced
285 products were obtained from casein in detriment of acetic acid. No significant differences were
286 observed between continuous and batch experiments (Fig. 3b and 3c). These results are
287 comparable to those previously described in literature (Breure & van Andel, 1984; Ramsay, 1997).

288 This VFA selectivity can be explained taking into account the AA composition of the two proteins
289 (Table 1). The large proportion of glycine in gelatin, a precursor of acetic acid, is likely responsible

290 for the predominance of this acid in all the gelatin tests. Similarly, valine is the sole responsible of
291 iso-butyric acid production and it is more abundant in casein than in gelatin.

292 *<Figure 3 should be placed approximately here>*

293

294 As protein composition affects both the acidification degree and the VFA selectivity, knowing the
295 average AA profile of different suitable substrates will be interesting/crucial when designing
296 and/or operating a VFA recovery installation from proteinaceous sidestreams and wastewaters
297 because it will enable the definition of the most suitable feedstock composition to achieve the
298 desired goal (greater yields and/or required product distribution).

299 **3.3 Influence of protein composition on amino acid consumption**

300 The previous section showed that the protein acidification was incomplete and varied depending
301 on the substrate composition. The consumption of individual AAs is evaluated in this section, both
302 for continuous (Fig. 4a) and batch (Fig. 4b) experiments, as the root cause for the different
303 observed acidifications.

304 *<Figure 4 should be placed approximately here>*

305

306 First of all, it can be observed that the amino acid degradation differs from the acidification
307 degrees of casein (50%) and gelatin (40%). During casein fermentation, the majority of the AAs
308 were largely consumed (≥70%), with some even reaching the complete utilisation (e.g. Arg and
309 Asp). The least consumed AA was Ser, whose conversion only reached 55%. In comparison, gelatin
310 fermentation led to generally lower and more variable consumptions: Arg and His were
311 extensively converted (≥80%), while the other AAs were consumed between 40 and 60%. The only

312 exceptions were Asp and Tyr, whose limited fermentation only reached the 20%, together with
313 Met being not even metabolised by the mixed culture. Comparing the two reactor configurations,
314 the batch system did not extensively alter the consumption patterns observed in the CSTR
315 operation, only with overall higher values, especially for gelatin fermentation due to the greater
316 acidification degree achieved during the discontinuous test. The failed conversion of Tyr in both
317 gelatin and casein batch test fermentation was considered as a strategy to avoid further
318 accumulation of aromatic VFAs (more toxic than the aliphatic ones), while the lower consumptions
319 of Gly and His during batch fermentation of casein were probably related to experimental noise,
320 given their limited abundance in casein composition (Table 1).

321 From these results, it can be concluded that AAs are not consumed evenly, and that the
322 preferential consumption depends on the protein composition. Recognising Stickland reactions as
323 the main route for AA conversion into VFA (Nagase and Matsuo, 1982; Ramsay and
324 Pullammanappallil, 2001) leads to the hypothesis that AA redox roles (i.e. electron donors and/or
325 acceptors) should be equilibrated. It was consequently hypothesised that the preferential
326 consumption of AA might respond to a strategy to compensate the overall redox balance. Indeed,
327 the redox balances calculated from measured AA consumptions (Table 2), expressed as mmoles of
328 hydrogen equivalents per C-mmole of degraded protein, are close to zero for both proteins, thus
329 supporting the aforementioned hypothesis. Still, a surplus of electron donor AA, as in the case of
330 casein (Table 3), might prove beneficial to the overall conversion to VFA due to both higher
331 acidification degree and AA consumptions than in the case of gelatin.

332 *<Table 2 should be placed approximately here>*

333

334 *<Table 3 should be placed approximately here>*

335 **3.4 Balancing AA consumption with VFA production**

336 Balancing the AA consumption with the VFA produced is a manner to understand better the
337 transformation routes and how these may change as a response to the substrate composition. To
338 do this, the stoichiometry proposed by Ramsay and Pullammanappalil (2001) is taken as a starting
339 point and other possible routes are discussed. Acetic acid was left out of this analysis as it is
340 yielded by many AAs, hindering the identification of the metabolic pathways.

341 Iso-butyric production, being linked only to valine degradation, was well described by this
342 stoichiometry during both casein and gelatin fermentation (Fig. 5a and b). The same applied to n-
343 butyric acid production which appears to be correctly related to the degradation of four specific
344 AAs, namely glutamate, threonine, histidine and lysine (Fig. 5c and d).

345 *<Figure 5 should be placed approximately here>*

346

347 However, discrepancies arose with propionic, iso- and n-valeric balances (Figure 6). Iso-valeric was
348 produced to a lower than expected extent (1:1 molar ratio) in comparison with the degradation of
349 the related AAs, isoleucine and leucine (Fig. 6a and b), especially in casein case. In the case of
350 propionic and n-valeric acids, both acids should be produced at equal molar ratios from arginine
351 and proline (1 mmol AA = 0.5 mmol Pr + 0.5 mmol nVal), although propionic acid is also generated
352 from methionine (1:1 molar ratio). Even though the balance appears to be closed (Figure 6c and
353 d), the production of n-valeric acid was much lower than the one of propionic acid.

354 *<Figure 6 should be placed approximately here>*

355

356 To explain the abovementioned discrepancies, it was hypothesised that either the stoichiometric
357 coefficients are incorrect or other unknown metabolic pathways should be considered. For
358 example, leucine can also be converted to iso-caproic acid (Regueira et al., 2019), though it was
359 rarely detected and only at low concentrations in this study. Also, arginine can be converted to
360 alanine and acetyl-CoA (Fonknechten et al., 2010) and, ultimately, to acetic and propionic acid
361 rather than going through 5-aminovalerate route (Barker et al., 1987); and, both aspartic acid, via
362 fumarate (Unden et al., 2016), and threonine (Sawers, 1998) could potentially generate propionic
363 acid. As a general recommendation, energetic criteria appear to be the most effective ones to
364 identify the routes linking AA and VFA (Regueira et al., 2019).

365

366 **3.5 Kinetic parameters of protein degrading microorganisms**

367 The data gathered during the fermentation batch tests were used to obtain kinetic parameters for
368 casein and gelatin fermentation (Table 4). Gelatin root-mean-squared error (RMSE) was 5.3% and
369 it lied between 5.5 and 7.6% for the three casein experiments, showing the good validity of the
370 estimated parameters. Moreover, in the case of casein, the parameters have shown to be suitable
371 for the different SIR applied in the batch experiments.

372 *<Table 4 should be placed approximately here>*

373

374 The values of the estimated parameters show significant differences depending on the fermented
375 protein. Casein fermenters have maximum growth rates almost two times higher than gelatin
376 fermenters, while the biomass yield in both cases is comparable. These values are higher than the
377 few available data in literature (Ramsay, 1997) and are similar to values reported for sugar
378 fermenters (Batstone et al., 2002). The ATP production per gram of COD of some AAs was

379 determined and, in fact, is comparable to the ratio found in glucose fermentation (data not
380 shown). Decay values are 20% and 5% with respect to the μ_{max} value for casein and gelatin,
381 respectively, which are usual values for anaerobic biomass. The stoichiometry coefficients show
382 that the selectivity on the different VFA is influenced by the fermented protein, in agreement with
383 the results of section 3.2. Overall, acetic acid dominates the product spectra in both cases but to a
384 greater extent in gelatin fermentation. Consequently, in casein fermentation the yields of the
385 secondary VFA have greater values than in the case of gelatin. Finally, gelatin was converted
386 almost completely to VFA and biomass and only 13.9% was converted to inert substrate while
387 almost half of the casein was converted to inerts, underlying the differences in acidification degree
388 depending on the substrate composition. This difference cannot be attributed to different batch
389 test duration since in both experiments VFA concentrations were stable. The effect of the test
390 length is reflected in the different μmax values, instead. In consequence, to properly design
391 processes centred on VFA production, models need trustworthy kinetic parameters estimated
392 specifically in fermentative environments and considering substrate composition.

393 ## 4. CONCLUSIONS

394 To the best of our knowledge, this study investigated for the first time the impact of protein
395 composition on individual amino acid consumption in mixed-culture anaerobic fermentations,
396 linking it to VFA production. In particular, the main findings are:

397 - Protein fermentation results in an incomplete acidification, which depends on protein
398 composition.
399 - A balanced AA composition, in terms of redox roles, does not guarantee a higher
400 acidification.

- Acetic acid is the major product, regardless of the protein composition, but casein fermentation results in a higher fraction of reduced products than gelatin fermentation.
- Preferential consumption of AAs was demonstrated, which interestingly depends on protein composition.
- The known stoichiometry accurately describes iso and n-butyric acid production, but fails for propionic, iso- and n-valeric acids. Therefore, further studies are required to upgrade it.

CONFLICTS OF INTEREST

There are no conflicts to declare

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Table 1. Measured AA composition (molar fraction in %) of casein and gelatin, redox roles (RR) and VFA produced according to the stoichiometry proposed by Ramsay and Pullammanappallil (2001). D/A are AAs that can act as both donor and/or acceptor. Uncoupled AAs are not involved in Stickland reactions.

Amino acid	Casein	Gelatin	RR[1]	RR[2]	Stickland-related VFA(s)
Alanine (Ala)	7.64	13.5	Donor	Donor	Acetic
Arginine (Arg)	3.83	5.11	Donor	D/A	Acetic/propionic/n-valeric
Aspartic acid (Asp)*	2.54	2.03	Donor	Acceptor	Acetic
Cysteine (Cys)	0.00	0.00	Donor	Donor	Acetic
Glutamic acid (Glu)*	15.0	7.07	Donor	Donor	Acetic/n-butyric
Glycine (Gly)	2.80	34.5	Acceptor	Acceptor	Acetic
Histidine (His)	1.96	0.80	Uncoupled	Donor	Acetic/n-butyric
Isoleucine (Ile)	5.94	1.33	Donor	Donor	Iso-valeric
Leucine (Leu)	9.46	3.07	D/A	D/A	Iso-valeric
Lysine (Lys)	6.96	3.02	Donor	Donor	Acetic/n-butyric
Methionine (Met)	0.93	0.49	Donor	Donor	Propionic
Phenylalanine (Phe)	4.56	1.73	D/A	D/A	Aromatic VFA
Proline (Pro)	13.9	16.1	Acceptor	Acceptor	Acetic/propionic/n-valeric
Serine (Ser)	7.12	4.32	Donor	Donor	Acetic
Threonine (Thr)	5.83	2.87	D/A	Donor	Acetic/n-butyric
Tryptophan (Trp)	0.00	0.00	D/A	D/A	Aromatic VFA
Tyrosine (Tyr)	2.74	0.55	D/A	D/A	Acetic/aromatic VFA
Valine (Val)	8.79	3.44	Donor	Donor	Iso-butyric

[1] Redox roles according to Ramsay (1997). [2] Redox roles according to De Vladar (2012). *Glu and Asp also include the fraction related to Glutamine and Asparagine, respectively.

Table 2. Experimental reducing power (H_2 equivalents) balance from amino acid degradation in casein and gelatin reactors, based on the fixed stoichiometry proposed by Ramsay and Pullammanappallil (2001) and assuming fixed AAs redox roles.

Amino acid	H_2 mole/consumed AA mole	Casein H_2 mmoles	Gelatin H_2 mmoles
Alanine (Ala)	2	4.9794	5.5816
Arginine (Arg)	-1	-1.4067	-2.3109
Aspartic acid (Asp)	2	1.8616	0.5340
Cysteine (Cys)	0.5	0.0000	0.0000
Glutamic acid (Glu)	0	0.0000	0.0000
Glycine (Gly)	-1	-1.0269	-9.4557
Histidine (His)	0	0.0000	0.0000
Isoleucine (Ile)	2	3.0450	0.7971
Leucine (Leu)	2	5.3937	1.9703
Lysine (Lys)	0	0.0000	0.0000
Methionine (Met)	1	0.2834	0.0000
Phenylalanine (Phe)	2	2.6928	1.2204
Proline (Pro)	-1	-3.4354	-4.6597
Serine (Ser)	1	1.4626	0.8570
Threonine (Thr)	-1	-1.6244	-0.8858
Tryptophan (Trp)	2	0.0000	0.0000
Tyrosine (Tyr)	1	0.7615	0.0505
Valine (Val)	2	4.1340	1.9586
	Sum	17.1206	-4.3427
	H_2 mmoles/protein C-mmole	0.1207	-0.0405

Table 3. Protein composition in terms of electron donor and acceptor AAs, according to the stoichiometry proposed by Ramsay and Pullammanappallil (2001).

Role	Casein	Gelatin
e^- acceptor (%)	26.4	58.6
e^- donor (%)	71.7	40.6
Uncoupled (%)	1.96	0.80

Table 4

Table 4: Estimated kinetic parameters (average [estimated confidence interval with α = 0.05]) for protein-degrading biomass (BM)

Parameter	Casein	Gelatin
μ_{max} (h^{-1})	0.034 [0.030, 0.039]	0.019 [0.017, 0.021]
Yield (g_{COD} BM/g_{COD} AA)	0.192 [0.170, 0.225]	0.165 [0.146, 0.188]
k_{decay} (h^{-1})	$6 \cdot 10^{-3}$ [$5 \cdot 10^{-3}$, $9 \cdot 10^{-3}$]	$9 \cdot 10^{-4}$ [0, $2 \cdot 10^{-3}$]
F_{Ac} (g_{COD} Ac/g_{COD} AA)	0.338 [0.327, 0.350]	0.571 [0.556, 0.587]
F_{Pro} (g_{COD} Pro/g_{COD} AA)	0.141 [0.134, 0.148]	0.177 [0.167, 0.187]
F_{But} (g_{COD} But/g_{COD} AA)	0.223 [0.214, 0.233]	0.131 [0.121, 0.140]
F_{Val} (g_{COD} Val/g_{COD} AA)	0.136 [0.128, 0.145]	0.076 [0.068, 0.084]
Inert AA (%)	45.2 [42.7, 47.4]	13.9 [8.4, 19.0]

Figure 1

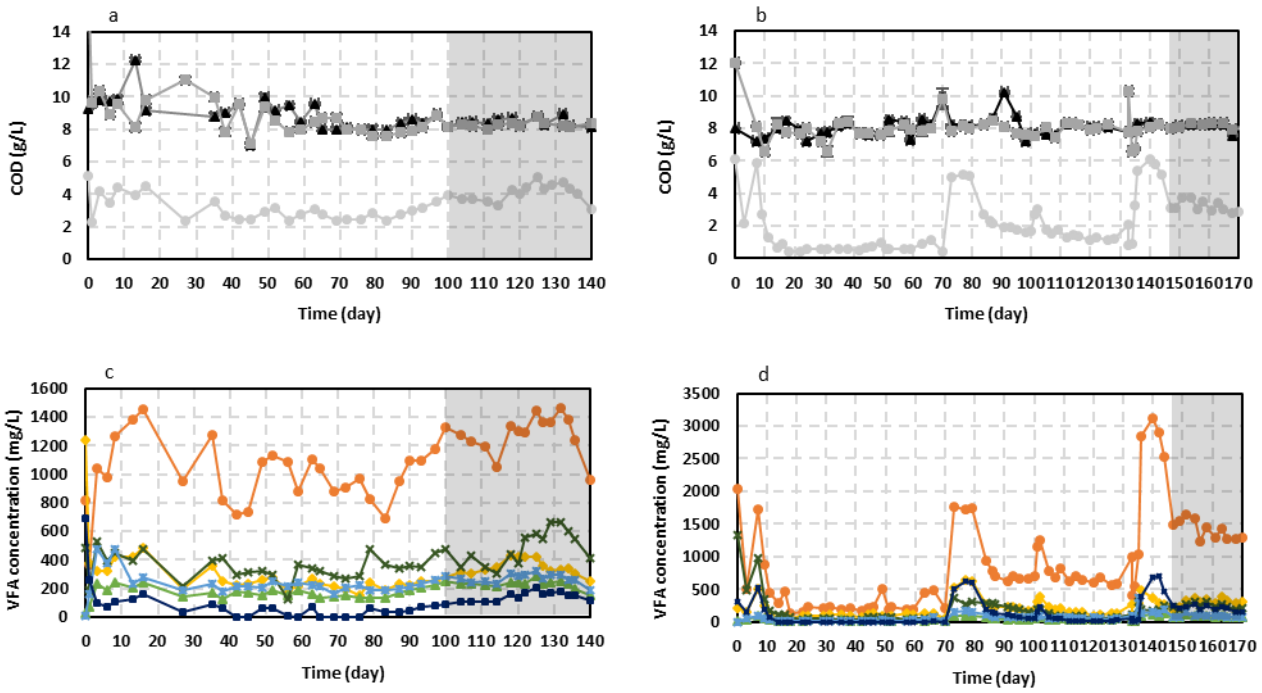

Figure 1. COD balance (a, casein; b, gelatin: ▲ Influent total COD; ■ Effluent total COD; ● VFAs COD) and individual VFA concentrations in the reactors (c, casein; d, gelatin: ● Acetic; ◆ Propionic; ▲ Iso-Butyric; x n-Butyric; * Iso-Valeric; ■ n-Valeric), with the shadowed areas corresponding to the identified steady-state periods

Figure 2

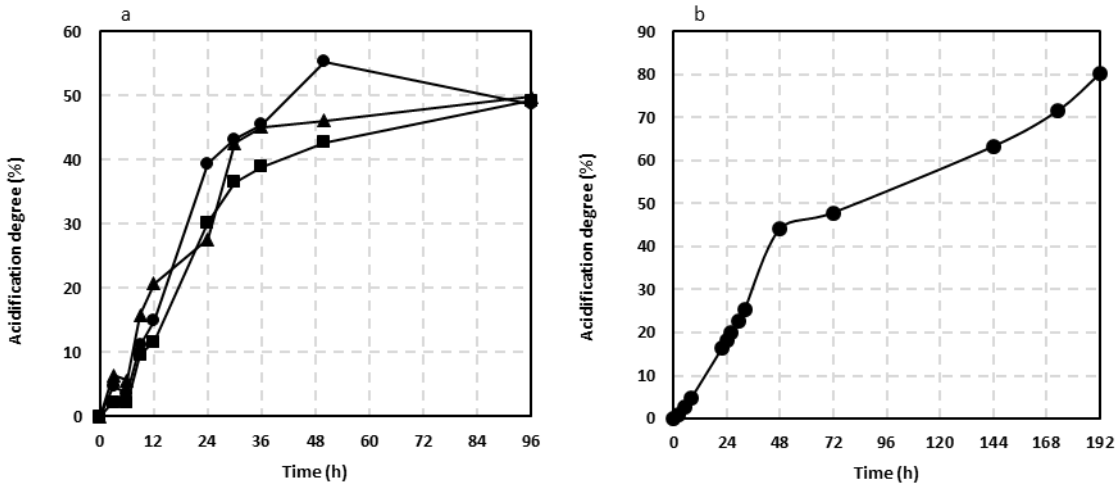

Figure 2. Acidification degree evolution over time for casein conversion (a, at three substrate-to-inoculum ratios, ■ SIR20; ● SIR10; ▲ SIR5) and gelatin (b, only ● SIR10)

Figure 3

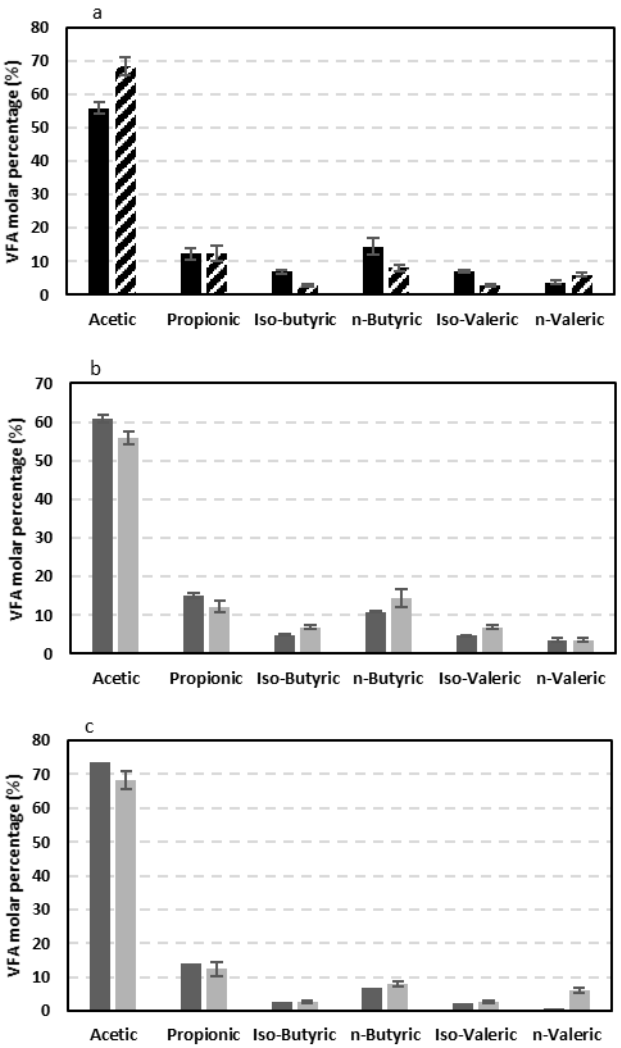

Figure 3. Comparison of VFA spectra between casein (■) and gelatin (▨) during CSTR operation (a) and between batch (■) and continuous (■) operation (b, casein; c, gelatin)

Figure 4

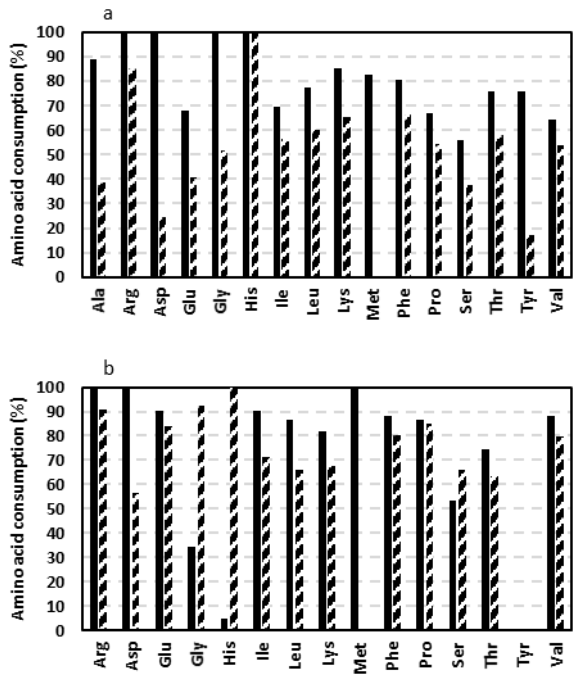

Figure 4. Comparison of amino acid consumption during casein (■) and gelatin (▨) fermentation in continuous reactor (a) and batch tests (b). No data of alanine consumption is available for the batch tests.

Figure 5

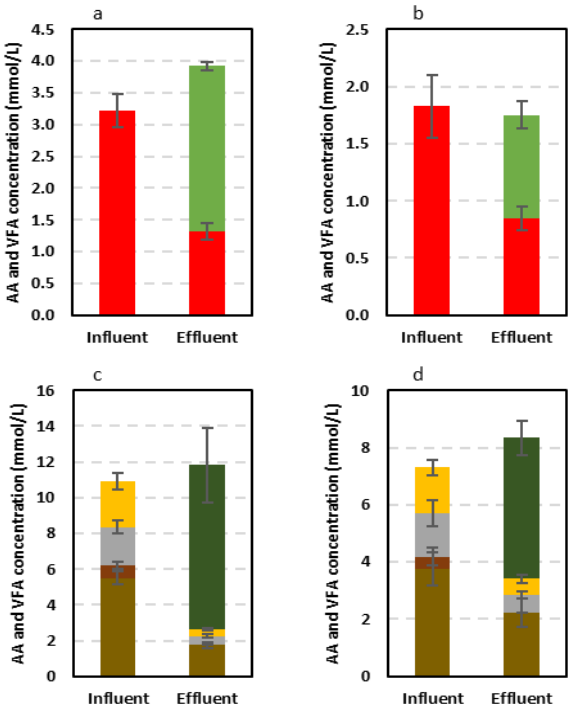

Figure 5. Iso-butyric (a, casein; b, gelatin: ■ Valine; ■ Iso-Butyric acid) and n-butyric (c, casein; d, gelatin: ■ Glutamic acid; ■ Histidine; ■ Threonine; ■ Lysine; ■ n-Butyric acid) acid balance in the continuous reactors.

Figure 6

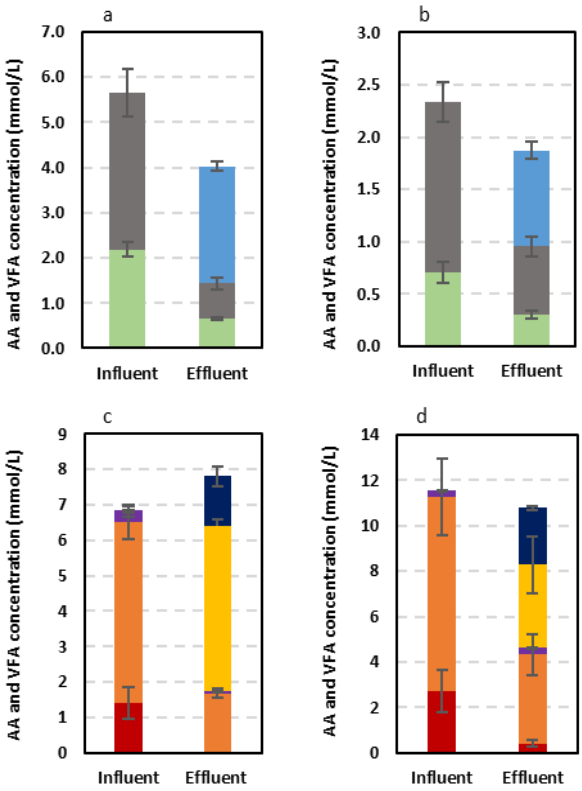

Figure 6: Iso-valeric (a, casein; b, gelatin: ■ Isoleucine; ■ Leucine; ■ Iso-Valeric acid), propionic and n-valeric (c, casein; d, gelatin: ■ Arginine; ■ Proline; ■ Methionine; ■ Propionic acid; ■ n-Valeric acid) acid balance in the continuous reactors.