



Insights on the production of volatile fatty acids from saline wastewater

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ABSTRACT

This work investigates how several marine salt levels typical of canning industry wastewaters (0, 10, 15, 20, and 35 g salt/L), mainly composed of proteins and sugars, impact the production of volatile fatty acids (VFAs), their selectivity and the acidogenic microbiome. Mixed-culture fermenters were operated in continuous (25 °C, pH = 7; HRT 1.5 d) and batch (25 °C, pH = 7, substrate-to-inoculum ratio 20 g COD/g VSS) regime. Low salinity levels (0–10 g/L) led to high VFA yields (74 %, COD basis), dropping to 46 % at higher salt concentrations. Acetic acid was always the main product (> 67 % molar basis), and butyric and valeric acid concentrations were negatively affected by high salinities (20–35 g/L). Salinity changes were also reflected in the microbial population: *Clostridia* was the dominant class in the whole salinity range although *Actinobacteria* increased its relative abundance as salinity increased. The experimental results were consistent with salt-in osmoregulation at low and medium salinities (0–15 g/L) while the compatible solutes strategy appears as more likely at high salinities (20–35 g salt/L). Given the needs for compatible solute strategy, these results enable the selection of co-substrates complementing wastewaters at the high salinity range to enhance the VFA production.

1. Introduction

In recent years, many investigations have been focused on harnessing the organic fraction of several types of wastes for resource recovery purposes [1], as it constitutes up to 80 % of the overall composition of some industrial solid wastes [2,3]. In the past years, the production of volatile fatty acids (VFAs) through anaerobic fermentation, which are intermediate products in anaerobic digestion (AD), has aroused great interest due to their multiple applications and greater profitability than biogas [4,5].

Many studies focus on mixed culture fermentations (MCFs) to produce VFAs, as they are more versatile and more resilient than pure culture fermentations [6,7]. They allow operating continuous systems with different substrates and under various operational conditions, thus being suitable for unknown compositions and complex feedstocks. Typical parameters in anaerobic fermentation processes, such as VFA selectivity and substrate conversion, are highly dependent on the operational conditions [6,7]. Some operational parameters have been extensively analysed, such as pH, which affects both substrate conversion and process selectivity [8–10]; or hydraulic retention time (HRT), which mainly influences substrate conversion [10–12].

Another parameter to take into account in anaerobic fermentation is the substrate composition. One of the inherent characteristics of some

food industry wastes, such as pickle or canning industry, is salinity. The canning industry consumes large water quantities, and its wastewater contains high salt concentrations and organic matter which can be valorised in order to obtain high value-added products such as VFAs, as reported in Fra-Vázquez et al. [13]. Moreover, a high saline effluent can facilitate the further purification of the VFA by liquid-liquid extraction with an organic solvent by the salting-out phenomenon, as it was probed as a low-cost and effective alternative for both purification and separation of VFA in a single-stage process, especially with the largest chain-size VFA [14]. The presence of salt was not only probed as a relevant parameter in extraction processes, as it has also been reported as a key factor in AD and anaerobic fermentation, but the literature did not explain the mechanisms which cause the increase or decrease of substrate conversion and therefore in VFA production. Table 1 summarises the literature found about the salinity effect under different substrates, processes and reactor modes.

These literature discrepancies about salinity impact, especially on anaerobic fermentation, might be explained by the different behaviour of the microorganisms to cope with salinity, which might be influenced by substrate composition and operational conditions. Two osmoregulation strategies were reported to be adopted by microorganisms to tackle high salt concentrations: the compatible-solutes strategy and the salt-in strategy [15–17]. For the compatible-solutes strategy, microbial

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metabolites, such as ectoine, glycine-betaine or glycerol, are intracellularly accumulated to compensate the osmotic pressure difference across the membrane. Alternatively, the salt-in strategy consists of the extracellular extrusion of sodium ions and intracellular accumulation of potassium ions via proton electrochemical gradient and at ATP expense [17]. Cells adopting the salt-in strategy must be tolerant to high osmotic pressure, as this strategy does not reduce it and the intracellular systems should be adapted to high salt concentrations [17]. Depending on the strategy chosen by the microorganisms, the effect on process performance varies. The salt-in strategy triggers a change in the concentration of sodium and potassium concentration on the medium. Alternatively, if the compatible-solutes strategy is adopted, a soluble COD loss would be observed in favour of the COD related to biomass fraction (particulate COD), as these compounds are produced and accumulated by the microorganisms [17]. Therefore, a study linking the behaviour of a saline fermentative system with the osmoregulation mechanisms of microorganisms is needed to understand their relationship and thus optimise the VFA production in a future stage using real saline wastewater.

Thus, this study aims to provide a sound hypothesis consistent with the contradictory results regarding the effect of salinity in anaerobic fermentation systems by linking macroscopic results (VFA composition), microscopic results (microbial profiles), the possible osmoregulation mechanisms and existing literature knowledge. Thus, a continuous reactor was operated to assess the impact of different salinity levels on the anaerobic (co-) fermentation of two model substrates (glucose and gelatine), in order to replicate a tuna wastewater fermentative system, as well as on the fermentative microbiome. In addition, batch experiments of individual substrates at each salinity level were performed to identify intermediate products and kinetic limitations during glucose and gelatine fermentation, respectively.

2. Materials and methods

2.1. Continuous reactor

A continuous 1.0 L (working volume) stirred tank reactor (CSTR) was inoculated with biomass from an acidogenic reactor (working volume: 5 L; HRT: 2 d; pH = 6.5) fermenting tuna cooking wastewater at mesophilic conditions, thus already adapted to a wide saline range (8–25 g salt/L). The CSTR was operated at constant pH (7.0, to boost maximum gelatine conversion [9]) and temperature (25 °C), with a hydraulic retention time of 1.5 d (which is equal to the solid retention time) and an organic loading rate of 6.7 g COD/(L·d), to ensure no kinetic limitations in gelatine conversion [18], which is converted at slower rates than glucose. The pH control and monitoring were performed through a multiparameter analyser (CHEMITEC, Italy) by the addition of 3 M HCl or 3 M NaOH. N₂ was sparged (≈ 10 mL/min) during the operation to ensure anaerobiosis and maintain the H₂ concentration limited within the reactor. The reactor setup is summarized in Fig. 1.

The reactor was continuously operated for 440 days and 5 periods can be distinguished according to the salinity levels: 0 g salt/L (days

0–53), 10 g salt/L (days 54–146), 15 g salt/L (days 147–181), 20 g salt/L (days 251–380) and 35 g salt/L (days 381–440). The operation was monitored via the measurement of the chemical oxygen demand (COD), total ammonia nitrogen (TAN) and VFA concentration twice a week, and the total and suspended solids concentrations were measured once a week. Biomass samples (in triplicates) were taken once a week at each salinity level, and microbial community analysis was performed when steady-state conditions were reached (steady state was considered when 6 consecutive samples differed <10 % of their average value in terms of substrate conversion and VFA concentration).

2.2. Feedstock description

The selected substrate was a mixture of two model compounds which simulate the typical carbon sources present in tuna-canning wastewaters: 7.60 g/L of hydrolysed peptone from gelatine (70951-1KG-F, Sigma-Aldrich) and 1.87 g/L of anhydrous glucose (A1422, PanReac). This corresponds to a sugar-to-protein ratio of 0.25 (g COD/g COD), which is also a typical composition of this type of wastewater. The reactor feedstock was supplemented with macronutrients and micronutrients as described in Table S1. Different salinity levels (no addition (0), 10, 15, 20 and 35 g salt/L) were applied throughout reactor operation using marine salt (AQ-AIUM SOLUTIONS, Spain) with the following ion concentration (molar %): Na⁺ 39.1 %, K⁺ 0.9 %, Mg⁺² 6.4 %, Ca⁺² 1.0 %, Cl⁻ 50.4 %, Br⁻ 0.1 % and SO₄⁻² 2.2 %.

2.3. Batch reactors

Several batch tests were performed by extracting biomass from the continuous reactor when steady state conditions at each salinity level were achieved. The experimental setup was similar to the continuous reactor: 1.0 L of working volume, magnetic stirring, 25 °C, pH = 7 and a constant N₂ sparging. The same macronutrient and micronutrient concentrations were added as in the continuous reactor feeding (Table S1). In particular, two batch experiments, one per carbon source, were conducted at different salinity levels (0, 10, 20 and 35 g salt/L).

For the batch experiments, the same concentrations of macronutrients and micronutrients were used, but substrate concentration was different: 9.60 g/L (10 g COD/L) of gelatine for protein batch test and 9.40 g/L (10 g COD/L) of glucose for sugar batch test.

The biomass from the continuous reactor was centrifuged and the resulting pellet was resuspended on the medium with the substrate (gelatine or glucose), micronutrients, macronutrients, and the salinity required. Experiments were carried out with a substrate-to-inoculum ratio of 20 g COD substrate/g VSS and a biomass concentration of 0.5 g VSS/L, as previously used by Bevilacqua et al. [18].

Initially, samples were taken at intervals of 2–3 h, decreasing this frequency progressively. The experiments were concluded when VFA spectra differed <10 % during at least 48 h. For the glucose tests, the optical density, glucose concentration, non-VFA products (lactic acid, formic acid, glycerol and ethanol) and VFA concentration were

Table 1

Literature summary about salt concentration effect under different conditions. The optimal conditions are referred to the conditions in which the VFA yield is optimised.

Substrate	Process	Reactor mode	Salt levels (g NaCl/L)	pH	T (°C)	Major products at optimal conditions	Ref
Food waste	Anaerobic digestion	Batch	0–15	7.0	37	Acetate & butyrate among 0–5 g/L	[36]
Food waste	Anaerobic fermentation	Batch	0, 30, 70	6.0	30	Acetate & propionate at 0 g/L	[34]
Food waste	Anaerobic fermentation	Batch	0–50	6.0	28	Butyrate & acetate at 40 g/L	[32]
Food waste	Anaerobic fermentation	Batch	0–70	6.0	30	Acetate & butyrate at 10 g/L	[27]
Kitchen wastewater	Anaerobic digestion	Batch	0–12	n.c	r.t	Acetate & butyrate among 0–0.5 g/L	[35]
Pretreated swine wastewater	Dark fermentation	Batch	10–35	9–10	36	Acetate & propionate among 30–35 g/L	[30]
Waste activated sludge	Anaerobic fermentation	Batch	0–29	6.8	25	Acetate & butyrate at 29 g/L	[31]
Cattle manure	Anaerobic digestion	CSTR	0.5–30.5	7.5	54	Acetate & propionate at 30.5 g/L	[24]
Molasses wastewaters	Anaerobic digestion	CSTR	0, 25*	n.c	34	Butyrate & acetate at 25 g/L	[37]

* Salinity estimated from conductivity; n.c: non controlled; r.t: room temperature.

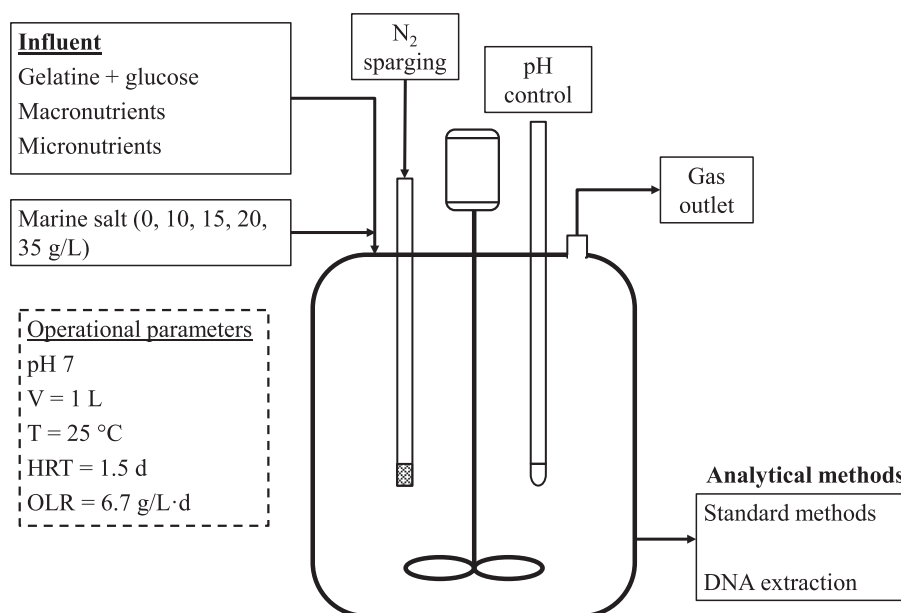


Fig. 1. Experimental setup of continuous reactor.

determined. For the gelatine tests, glucose concentration was discarded and TAN concentration was included.

2.4. Analytical methods

Standard Methods [19] were used for the determination of conventional physicochemical parameters. Raw samples were used to measure total (TS and VS) and suspended (TSS, VSS) solids (SM2540 B, D, E) and total COD (SM5220C modified), while the optical density (OD600) was calculated as in Bevilacqua et al. [18] to verify biomass concentration at the beginning of batch tests. Filtered samples were used to calculate TAN (SM4500-NH₃-F), soluble COD (SM5220C) and glucose concentration (SPINREACT kit 1001190).

A Shimadzu UV-1800 gas chromatograph with an Agilent Technologies' DB-Wax column (30 m × 0.250 mm × 0.25 μm) was used for the quantification of carboxylic acids from C2 (acetic acid) to C7 (heptanoic acid). The temperatures of the detector and injector were set to 300 °C and 200 °C, respectively, using N₂ as the carrier gas. The sample supernatant, which was obtained via centrifugation and filtration (0.45 μm), was acidified with 10 μM of H₃PO₄ (85 %) prior to analysis.

Lactic acid, formic acid, glycerol and ethanol, which are typical fermentative products referred to as non-VFA products, were quantified by the CLEFG1 technique based on high-performance liquid chromatography (HPLC) with an HP 1100 (IR HP1047A) detector. The AMINEX HPX-87H (300 × 7.8 mm) column was used with the detector set at 35 °C and the column at 30 °C, employing H₂SO₄ (5 mM) as isocratic eluent. Samples were prepared likewise for VFA determination.

2.5. Bacterial community study

The methodology employed for bacterial community composition analysis was the same as in Bevilacqua et al. [20]. To summarize, a Nucleospin Microbial DNA extraction kit (Macherey Nagel) was used for total genomic DNA extraction. After quantification, each sample was extracted by triplicate and pooled together, using both Nanodrop and Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA) for quality control. For bacteria analysis, the sequencing was performed by the Illumina NovaSeq PE250 platform at Novogene Co, Ltd. (UK), amplifying the V3-V4 hypervariable region [21].

Microbial Genomics module workflow (version 21.1) and workbench (version 21.0.3) were used to conduct the bioinformatic analyses. Once

the reads were filtered guaranteeing high quality, they were grouped into OTUs (Operational Taxonomic Units) at 97 % cutoff for sequence similarity and then classified using the Midas 4 database [22]. Only the bacterial OTUs that accounted for >1 % of all detected OTUs were taken into consideration for further study. Finally, unweighted UniFrac values between each pair of samples were used to calculate the beta diversity, which was represented in a principal coordinate analysis (PCoA) plot.

2.6. Calculations

Acidification degree was used to describe the substrate conversion to VFAs on COD basis (Table S2), based on the VFA concentration measured in the reactor effluent (in this case, aliphatic VFAs):

$$\text{Acidification degree (\%)} = \frac{\sum C_{VFA}}{C_{gel} + C_g} \times 100 \quad (1)$$

where C_{VFA} is the total concentration of the VFAs (in g COD-VFA/L) in the reactor effluent, C_{gel} stands for the gelatine concentration (in g COD/L) and C_g for the glucose concentration (in g COD/L) in the feeding of the reactor.

Ammonification degree was used to estimate the protein conversion, as NH₄⁺ release is related to the amino acid fermentation. It was expressed as follows [9]:

$$\text{Ammonification degree (\%)} = \frac{C_{TANeffluent} - C_{TANfeeding}}{C_{TANmaximum}} \times 100 \quad (2)$$

where $C_{TANeffluent}$ is the ammonium nitrogen concentration (mg N-NH₄⁺/L) in the reactor effluent, $C_{TANfeeding}$ stands for the ammonium nitrogen concentration (mg N-NH₄⁺/L) in the reactor feeding, as ammonium chloride was supplemented as macronutrient, and $C_{TANmaximum}$ is the maximum ammonium nitrogen concentration (mg N-NH₄⁺/L) achieved if complete degradation of gelatine happens. This parameter was estimated based on Total Kjeldahl Nitrogen measurements (SM4500C), which yielded 202 mg N-NH₄⁺/g protein for gelatine.

The reduction degree parameter was used to indicate the VFA chain size produced, since the greater the VFA length, the higher the COD and the higher the reducing power. It was expressed as below:

$$\text{Reduction degree} = \frac{\sum C_{\text{VFA}} \left(\frac{\text{g COD}}{\text{L}} \right)}{\sum C_{\text{VFA}} \left(\frac{\text{g}}{\text{L}} \right)} \quad (3)$$

where C_{VFA} indicates the total concentration of VFAs produced.

The selectivity was the parameter used to observe how salinity changes the product distribution within the VFA spectra. It was expressed as follows:

$$\text{Selectivity}(\%) = \frac{C_p \left(\frac{\text{g COD}}{\text{L}} \right)}{\sum C_s \left(\frac{\text{g COD}}{\text{L}} \right)} \times 100 \quad (4)$$

where C_p stands for a specific product concentration and C_s represents the concentration of all the measured soluble products derived from sugar and gelatine fermentation. The selectivity can be estimated either from this parameter or the product spectra, which indicates the variations of the products throughout the operation.

3. Results and discussion

3.1. Influence of salinity on the anaerobic cofermentation of glucose and gelatine

Throughout the continuous reactor operation, total COD concentrations in the influent and effluent were similar thus discarding methanisation and hydrogen production (Fig. 2). Biomass concentration remained constant at around 1.0 g VSS/L, which was also confirmed by the constant gap between total and soluble COD (Fig. 2).

Up to 15 g salt/L, the effluent soluble COD and the effluent VFA-COD were similar indicating the absence of unidentified products. During this period, the acidification degree, which stands for the VFA yield, was very high: $69 \pm 5\%$ at 0 g salt/L and $78 \pm 5\%$ at 10 g salt/L (Fig. 3), being the spectrum (Fig. 4) dominated by acetic acid (3.21–3.42 g COD/L), n-valeric acid (1.02–1.63 g COD/L), n-butyric acid (0.91–1.46 g COD/L) and propionic acid (0.45–0.79 g COD/L). Ammonification, which represents the gelatine conversion, was also high (around 70%), except for the period at 15 g salt/L, where it decreased to 50% and the acidification degree dropped to 65%. Glucose consumption was always

complete regardless of the salinity level since it was never detected in the effluent, which implies that ammonification stands for the overall substrate conversion. Moreover, the COD balance indicates that all soluble compounds in the reactor effluent were detected (Table S3), which corresponded to non-consumed gelatine and VFA.

A decrease in VFA production was observed during the first 20 days after a salinity change, suggesting that a microbial adaptation is needed. This adaptation entails either that the microorganisms needed some time to balance the osmotic pressure or that some populations could not cope with the new salinity level, and they were replaced by halotolerant populations.

On the contrary, at high salinity levels (20 and 35 g salt/L), the acidification degree decreased to 42–50% (Fig. 3), and protein conversion was also incomplete (49% and 46% respectively), since effluent soluble COD was higher than effluent VFA-COD (Fig. 2), and non-VFA products were never detected. Acetic acid remained as the main product, accounting for >70% (molar basis) of the total VFAs, but the production of propionic acid was favoured (0.79–0.91 g COD/L) in detriment of n-butyric (0.31–0.42 g COD/L) and n-valeric (0.37–0.41 g COD/L) acids. At these high salinities (20 and 35 g salt/L), the COD of converted substrate was higher than the amount of VFAs produced while no methanisation or production of hydrogen took place, which suggests non-detected products were produced.

All soluble compounds at low salinities are known and do not include compatible solutes, which are believed to be chemically distinct from VFAs. This behaviour suggests that the osmoregulation strategy adopted by the microorganisms at low salinity might be the salt-in strategy. On the contrary, at high salinities, it was hypothesised that a part of the substrate was being converted to unidentified compounds which could act as compatible solutes, such as ectoine or some amino acids (AAs) (e.g. glutamate, proline), which were directly used as compatible-solutes compounds to cope with the osmotic pressure [17]. The substrate consumption and VFA selectivity at low salinities were compatible with the literature [23–25] using different substrates (gelatine, cattle manure, and alkaline pre-treated sludge, respectively). Interestingly, the same pattern as Xiao et al. [25] was observed in this work: from 20 g salt/L on, acidification did not suffer noteworthy changes, suggesting that the minimum VFA concentration was reached in both studies.

The variations in the VFA spectrum led to a change in the average reduction degree, which revealed a shift towards the shortest VFAs

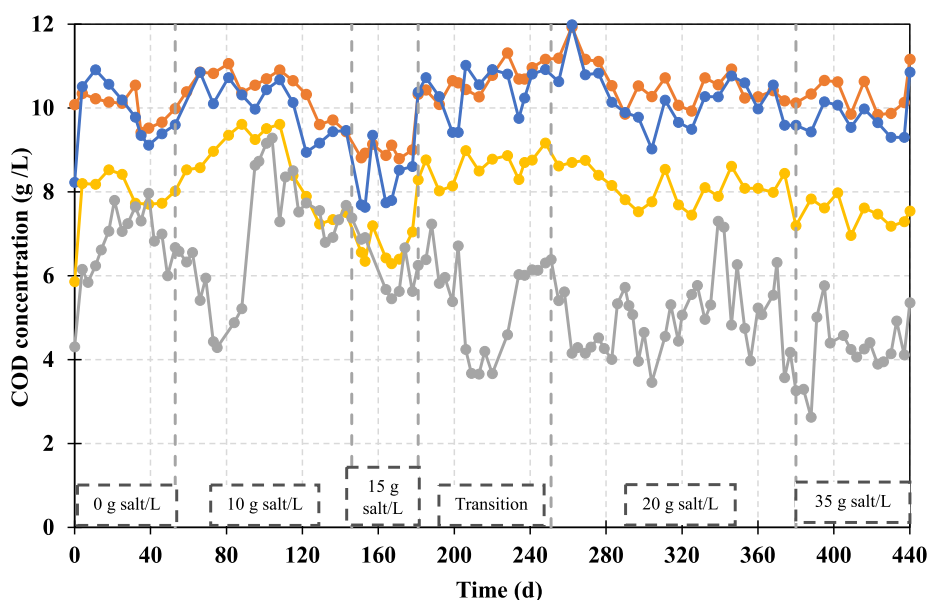


Fig. 2. COD balance (— total influent, — total effluent, — soluble effluent, — VFAs) in the continuous reactor at different salinity levels.

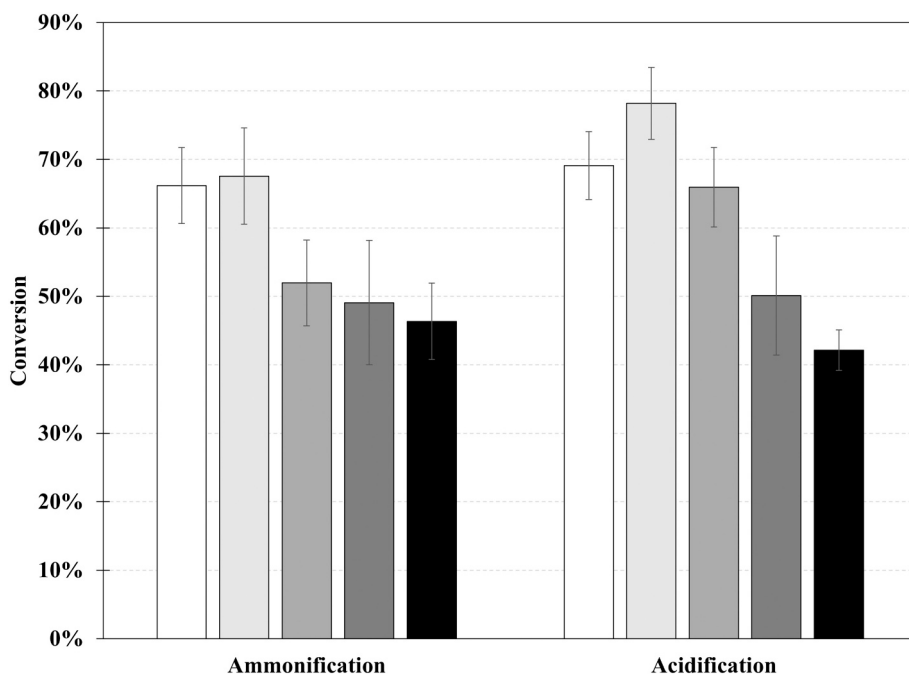


Fig. 3. Acidification and ammonification percentages achieved at different salinity levels in the continuous reactor (□ 0 g salt/L, ■ 10 g salt/L, ■ 15 g salt/L, ■ 20 g salt/L, ■ 35 g salt/L).

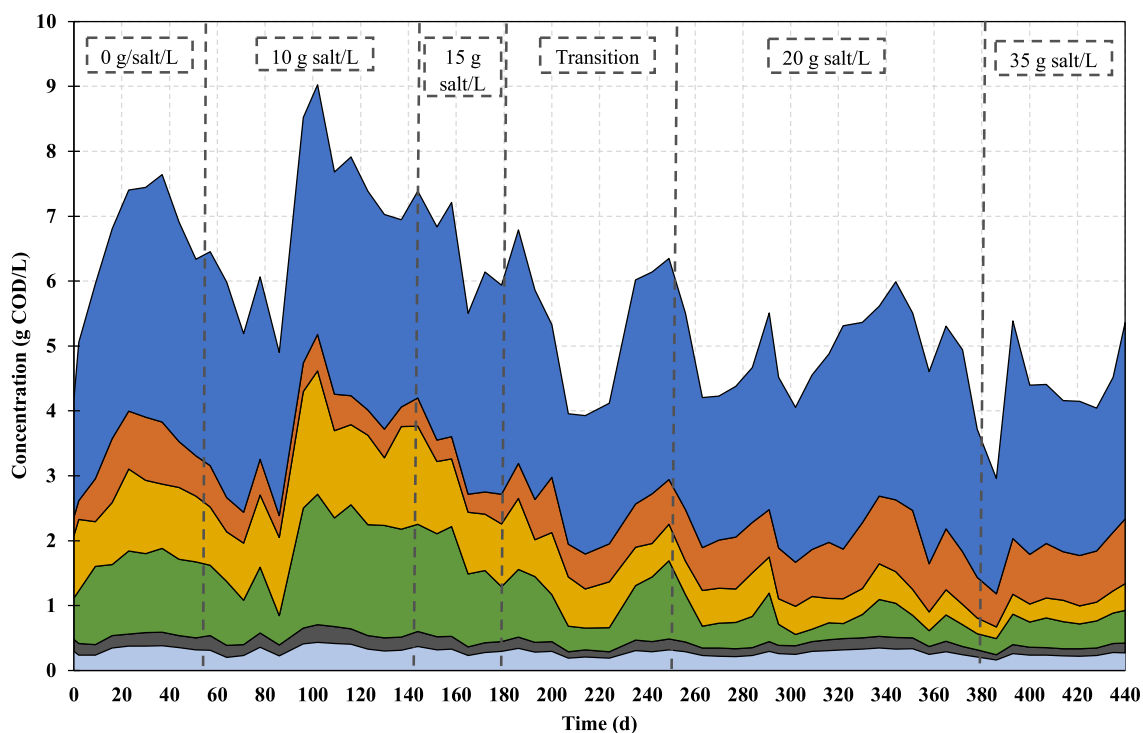


Fig. 4. VFAs concentration throughout the continuous reactor experiment at different salinity levels (■ acetic acid, ■ propionic acid, ■ n-butyric acid, ■ n-valeric acid, ■ isobutyric acid and ■ isovaleric acid).

(acetic and propionic acid) at high salinities, hence leading to a more oxidised spectrum. Although chemically diverse, many compatible solutes contain reduced carbon groups. Therefore, as salinity increases, a decrease of reduced VFAs, such as butyric and valeric acid, would be required to keep the electron balance if the compatible solute strategy is deployed. Isobutyric and isovaleric acid concentrations remained

constant throughout the operation. These iso-acids are originated from valine, leucine and isoleucine [26], suggesting that their consumption is not affected by the salinity.

Microbial community structure was affected by the presence of salt regardless of the salinity level. Principal Coordinates Analysis (PCoA) plot of unweighted UniFrac distances (Fig. S1) separated the samples

according to salt concentration, with those corresponding to 0 g salt/L being the most distinctive. All the rest of the samples were clustered according to the concentration of salt, with the lowest concentration (i.e. 10 g/L) being the one with the most similar structure.

Diversity progressively decreased (Table S4 and Fig. S2) and *Fusobacteria* was only detected during the period without salt (Fig. 5a). *Clostridia* is the predominant class and *Clostridiaceae* the dominant family at every salt level, except for the 35 g salt/L, in which *Peptostreptococcaceae* is the main family within *Clostridia* class and in the whole microbial community, increasing its abundance to 78 % (Fig. 5b). It is hypothesised that the *Peptostreptococcaceae* behaviour is related to a specific resilience to high salt concentrations probably due to a higher halotolerance, although it was not previously reported in literature. Following a similar trend as the acidification values and VFA spectra, clear differences were observed between moderate (10–15 g/L) and high (20–35 g/L) salinity levels. *Actinobacteria* class increased its relative

abundance at high salt concentrations as well as *Clostridia*, while *Bacilli* class reduced its proportion within the microbial community. From 0 to 20 g salt/L *Eggerthellaceae* is the dominant family within *Actinobacteria* class (Fig. 5c). However, the increase of the *Actinobacteria* class at 20 and 35 g salt/L is related to the growth of *Actinomycetaceae* family, being the second most abundant family at 35 g salt/L (Table S5). The growth of *Actinobacteria* class also meant the decrease of *Bacilli* class, specially marked in *Erysipelatoclostridiaceae* family, which was the most abundant *Bacilli* family at low and moderate salinities (0 g salt/L to 15 g salt/L) (Table S5). He et al. [27] obtained similar results operating with food waste, observing the predominance of *Firmicutes* phylum at different salinities and the growth of *Actinobacteria* phylum at high salinities (50 and 70 g salt/L), suggesting a specific resilience to these conditions given its rapid adaptation to many extreme environments [28].

The aforementioned variations in the fermentative microbiome are strongly related to the production of specific VFAs. More specifically,

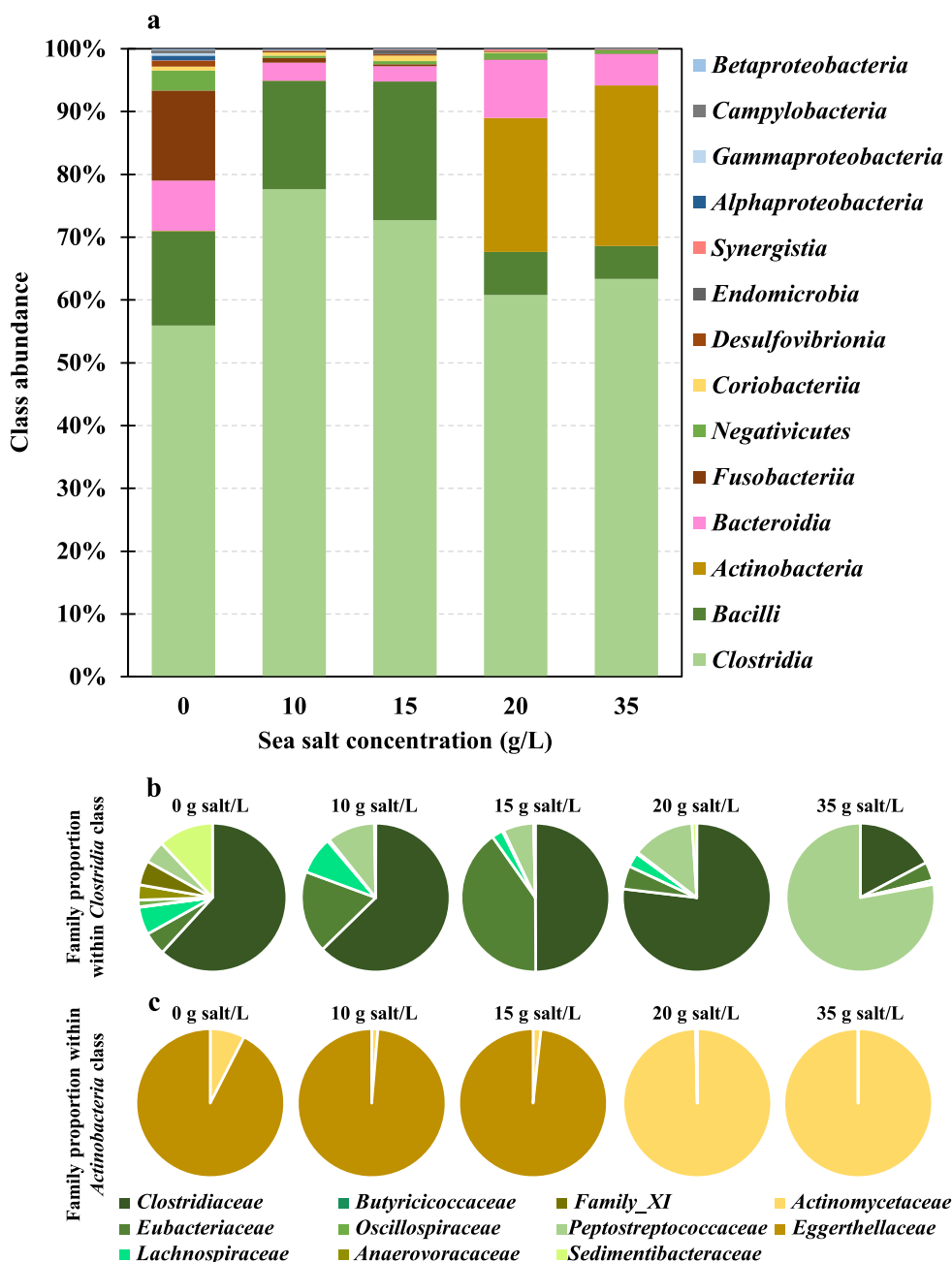


Fig. 5. Population abundance at class level (a), *Clostridia* family level (b) and *Actinobacteria* family level (c) in the continuous reactor at different salinities.

Clostridia is linked to acetic and butyric acid production and *Actinobacteria* with propionic acid production [27–29]. These findings suggest that the fermentative microbiome evolves according to each salinity level through a combination of the two osmoregulation strategies. As each strategy has a different impact on process performance, this community adaptation also results in a specific VFA profile.

The results obtained could explain the discordances in the aforementioned studies since the strategy embraced by the microorganisms leads to different results in the VFA spectrum and substrate conversion, which are dependent on the microbial community.

3.2. Influence of salinity on gelatine fermentation

Salinity affects both the average consumption rate (0.33, 0.21, 0.19 and 0.17 g gelatine/L·h) and ammonification (77 %, 63 %, 54 % and 56 %) of gelatine (Fig. 5a). The experiment with no salt was the only one in which lag phase was detected (6 h), and also reported the maximum ammonification (77 %) and the highest average consumption rate (0.33 g gelatine/L·h), consistent with previous studies using gelatine as substrate [23], in which an acidification of 73 % was reported. On the contrary, previous works on protein-rich substrate conversion to VFAs indicate no effect of salinity on substrate conversion or VFA selectivity [24,30,31], which does not correspond with the results observed in this study.

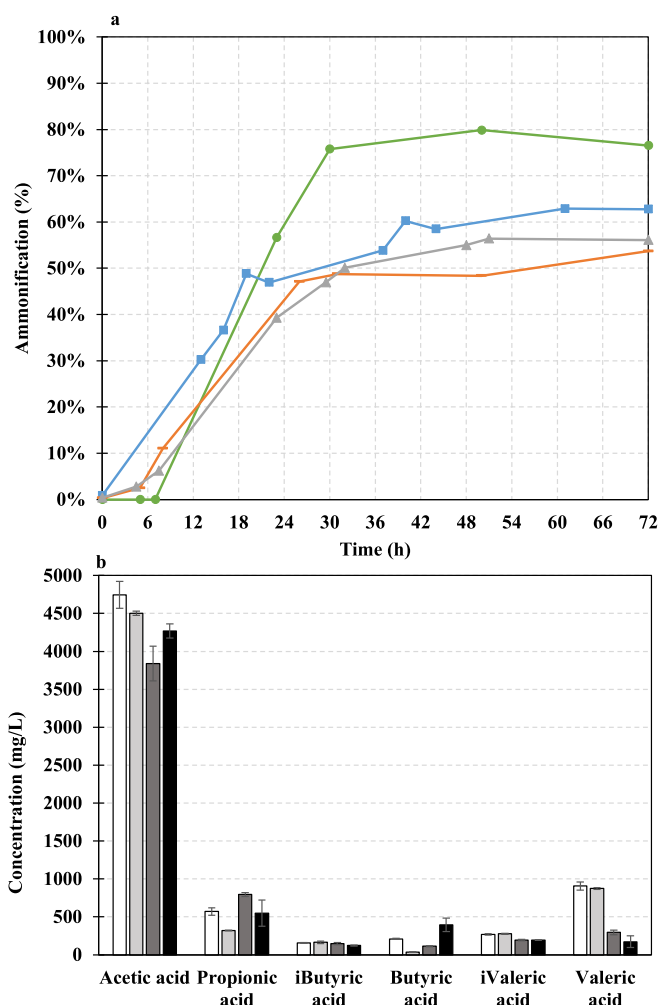


Fig. 6. Ammonification (a) in gelatine batch fermentation at each salinity level (—●— 0 g/L; —■— 10 g/L; —■— 20 g/L; —▲— 35 g/L) and VFA final concentration (b) at each salinity level (□ 0 g salt/L, ■ 10 g salt/L, ■ 20 g salt/L, ■ 35 g salt/L).

Acetic acid was the main product at the end of the gelatine test (Fig. 6b) at all salinity levels, with an average concentration of 4.3 g/L. Valeric acid was especially affected by the salinity increase, experiencing a concentration drop from 873 to 296 mg/L when salinity was increased from 10 to 20 g salt/L. This behaviour is consistent with the CSTR operation, in which valeric acid decreased similarly when salinity was raised from 10 to 20 g salt/L. Propionic and butyric acid present their lowest concentrations at 10 g salt/L, while isobutyric and isovaleric acids remain almost constant in all batch tests, resulting in a VFA spectrum similar at low salinities as in Bevilacqua et al. [23] operating with no salt.

Due to the unexpectedly high ammonification at 35 g salt/L (very similar to 10 and 20 g salt/L) and the differences in propionic and butyric acid concentration between the batch and the continuous reactor, the gelatine batch test at 35 g salt/L was replicated in order to verify these findings, obtaining the same results (data not shown).

The lack of a prolonged lag phase suggests that the AAs play a significant role in the microorganism adaptation (compatible-solutes strategy). Interestingly, similar ammonification results were obtained at all gelatine batch tests with salt, and the only change observed was between with (10, 20 and 35 g salt/L) and without salt (0 g salt/L), which indicates that the compatible-solutes strategy might be adopted from 10 to 35 g salt/L. So, it is hypothesised that the effect of salinity on gelatine fermentation does not depend on salt level, explaining the discordant results between this study and the previous literature using a protein-rich substrate, since the salt effect in anaerobic fermentation is different depending on the osmoregulation strategy embraced. At 10 g salt/L, the osmoregulation strategy is different from continuous operation, suggesting that the mechanism is not systematic, and it is hypothesised that the greatest presence of AAs leads to the adoption of the compatible-solutes strategy.

3.3. Influence of salinity on glucose fermentation

Glucose was completely consumed in all experiments (Fig. 6a), which is consistent with the results from the literature [8,32,33]. A lag phase was detected at all salinity levels, especially relevant at 35 g salt/L (54 h), suggesting that the AAs were playing a key role in the osmoregulation strategy at high salinities while hampering the microbial adaptation when absent. This behaviour is consistent with (some) AAs being used directly as compatible solutes or as precursors thereof. The average consumption rate after the lag phase also decreased with the salinity increase; 0.43, 0.35, 0.27 and 0.22 g glucose/L·h, respectively. Depending on the salinity level, different non-VFA products appeared as primary metabolites (Fig. 7 b, c, d, e). Formic acid was detected on every batch experiment, lactic acid was found on every test except for the 35 g salt/L one, ethanol was obtained on the 10 and 35 g salt/L and glycerol only appeared at 20 g salt/L. These non-VFA products were later consumed, except for ethanol at 10 and 35 g salt/L, which showed a final concentration of 330 and 400 mg/L, respectively. Ethanol has been reported to be produced at different salinities from 0 to 50 g salt/L [30,32] but it was not consumed during the experiment, and lactic acid prolonged its presence as the salt concentration increased [34]. However, the shift observed in the non-VFA products between the different salinities in this study has not been previously reported in the literature.

Overall, the main product was acetic acid (Fig. 8), followed by propionic acid as the second major product, having its major concentration at 20 g salt/L, and then by butyric acid as the third most abundant VFA. Valeric acid was only produced at 0 g salt/L at residual concentrations as it is not a direct product of glucose fermentation. Several studies operating on batch mode reported that VFA production from sugar-rich wastewater streams is affected by the salinity, which is consistent with the results obtained in this study, but they also stated that salinity does not affect the VFA spectrum [25,30,35]. In contrast, Su et al. [31] reported that the salinity increase favours butyric acid and affects negatively propionic acid production. Interestingly, Yin et al. [34] observed

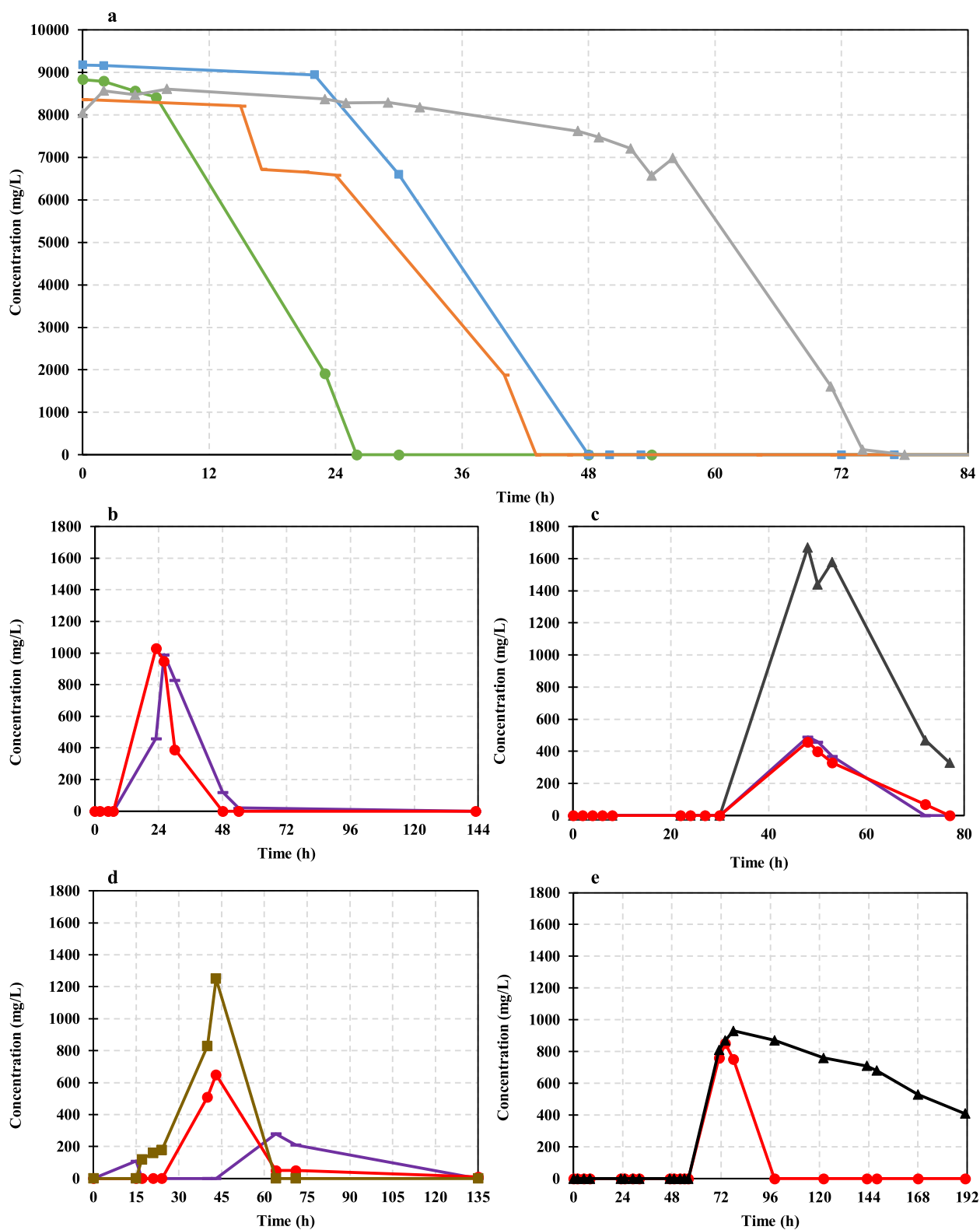


Fig. 7. Glucose consumption (a) in glucose fermentation at each salinity level (—●— 0 g/L; —■— 10 g/L; —■— 20 g/L; —▲— 35 g/L) and non-VFA products (—■— lactic acid; —●— formic acid; —▲— ethanol; —■— glycerol) at 0 g/L (b), 10 g/L (c), 20 g/L (d), 35 g/L (e) of salt.

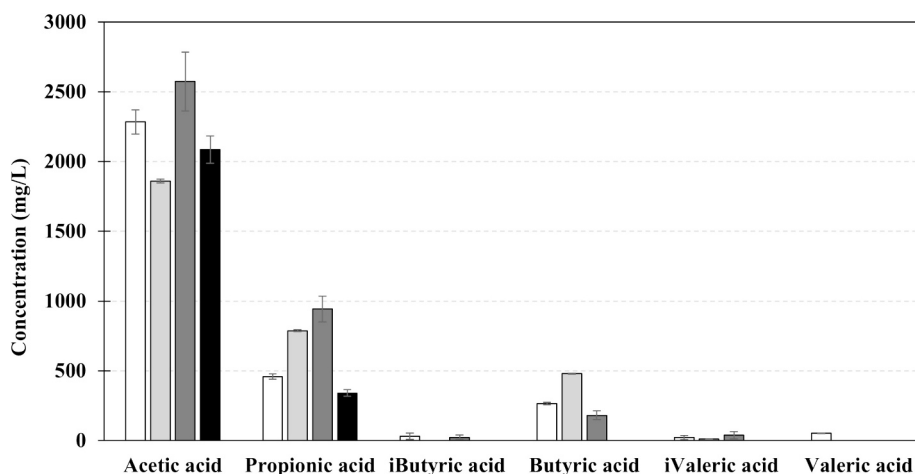


Fig. 8. VFA final concentration in glucose batch fermentation at each salinity level (□ 0 g salt/L, ■ 10 g salt/L, ■ 20 g salt/L, ■ 35 g salt/L).

that the lactate disappearance was related to the propionic acid production, which is consistent with the data collected in this study, except for the test with 35 g salt/L (Fig. S3), where lactic acid was not detected.

4. Conclusions

This work describes how salt concentration affects the VFA production and selectivity and the fermentative microbiome during the anaerobic (co-) fermentation of gelatine and glucose. The salt-in strategy seems to be adopted at low salinities, while at high salinities the compatible-solutes strategy is preferred. Yet, the osmoregulation strategy adopted is not systematic, since it depends on several parameters, such as the halotolerance of microorganisms, substrate composition or operation mode. This understanding helps enhance high saline-wastewater treatment processes since it allows assessing the potentiality of conversion of a given effluent. The different behaviours depending on the substrate fermented may open the door for the cofermentation of different high-salinity effluents (e.g. molasses wastewaters, pickle wastewater, etc.) which can fulfil the need for compatible solutes at high salinity and facilitate the microbial fermentation under these conditions.

CRedit authorship contribution statement

J. Iglesias-Riobó: Writing – review & editing, Writing – original draft, Methodology, Investigation. **S. Balboa:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **R. Bevilacqua:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **M. Mauricio-Iglesias:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **M. Carballa:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marta Carballa reports financial support was provided by European Regional Development Fund. Juan Iglesias-Riobó, Sabela Balboa, Riccardo Bevilacqua, Miguel Mauricio Iglesias, Marta Carballa report financial support was provided by Xunta de Galicia.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jwpe.2024.105689>.

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