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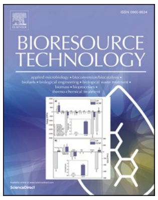

Thermal hydrolysis pre-treatment has no positive influence on volatile fatty acids production from sewage sludge

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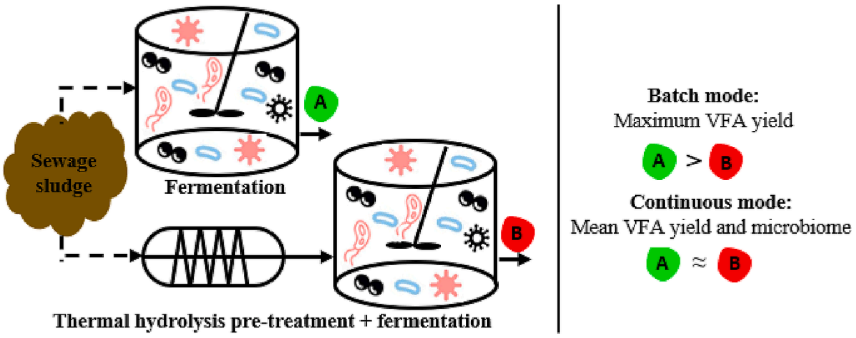
HIGHLIGHTS:

- Raw and thermally pre-treated sludge were compared in terms of VFA production.
- In batch mode, pH 8 resulted the most favourable one for VFA production.
- VFA yield in batch tests resulted lower when sludge was thermally pre-treated.
- In continuous operation, raw and pre-treated sludge achieved similar VFA yield.
- Microbial key-players were alike independently of applying thermal pre-treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The study compares the potential to produce volatile fatty acids (VFA) from sewage sludge, both raw and thermally pre-treated in two modes of operation. In batch mode, raw sludge at pH 8 obtained the highest maximum VFA yield (0.41 g COD-VFA/g CODfed) whereas pre-treated sludge achieved a lower value (0.27 g COD-VFA/g CODfed). The operation of 5-L continuous reactors showed that thermal hydrolysis pre-treatment (THP) did not have any significant influence on VFA yields, averaging 15.1 % g COD-VFA/g COD with raw sludge and 16.6 % g COD-VFA/g COD with pre-treated one. Microbial community analysis showed that phylum *Firmicutes* was predominant in both reactors and that the enzymatic profiles involved in VFA production were very similar regardless of the substrate fed.

1. Introduction

Over the last decades, the growing population connected to sewerage, the building of new wastewater treatment plants (WWTPs) and the enhancement of current ones to comply with severe water treatment legislation led to a dramatic increase in sewage sludge production (Zheng et al., 2021), and this tendency is expected to continue in the future.

Sewage sludge disposal usually accounts for around 60 % of the total operating costs of WWTPs (Maragkaki et al., 2018). As a consequence, anaerobic digestion (AD) has been broadly implemented; at first to decrease the amount of sewage sludge produced but then also for biogas

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production, a renewable energy that is normally used in the installation to warm up the digester and produce electricity, which reduces energy costs (Pramanik et al., 2019).

The first -and in many cases limiting- stage of sewage sludge AD is hydrolysis that consists of the breakdown of particulate organic matter into smaller compounds. To boost this stage, a vast diversity of pre-treatment technologies such as ultrasonic, microwave, acid/alkali, ozone, and thermal ones have emerged with the aim not only to accelerate the digestion process but also to increasing biogas production (Zhen et al., 2017). Amongst them, thermal hydrolysis pre-treatment (THP) has evolved into one of the most popular because it also achieves: (i) odour reduction and pathogen sterilisation; (ii) improvement of sludge dewaterability and (iii) diminution of sludge viscosity (Bougrier et al., 2006). However, some authors have also reported some disadvantages such as the dependence on centrifugal separators and polymers for sludge thickening to make the process profitable and the potential generation of recalcitrant and inhibitory compounds in the centrate (Barber, 2016; Eskicioglu et al., 2006).

In recent years, intentions to go beyond biogas have sped up, focusing the attention on producing bioproducts such as volatile fatty acids (VFA). This interest is both economical (VFA present higher added-value than biogas and wide variety of applications, for instance as preservatives in the pharmaceutical and food industry and in the production of esters, bioplastics, and bioenergy (Kleerebezem et al., 2015)) and environmental, since commercial production of VFA is mainly based on fossil resources (Chen et al., 2017).

The impact of pre-treatment technologies on sewage sludge transformation into VFA has been less studied than into biogas, but in general literature shows that THP is advantageous for the production of VFA from sludge as well. Morgan-Sagastume et al., (2011) found that the production capacity of VFA from hydrolysed sludge increased by 2–5 times in comparison with raw sludge. However, the comparison was made at different hydraulic retention times (HRT) and this is one of the parameters that was reported to affect most the fermentation, together with others like pH, temperature and substrate (Yuan et al., 2019). Zhang et al., (2019) also studied the effect of THP on VFA production, getting the best results with pre-treated sludge at 35 °C, 44.6 % higher than those reported with raw sludge.

There are some works in the literature which have already studied the influence of THP on VFA production from sewage sludge. For instance, Morgan-Sagastume et al., (2011) reported advantages of THP regarding VFA production. However, they established different organic loading rate (OLR) between reactors (16 vs 42 g COD/L·d), being this affected both by the different HRT applied and by the concentration of organic sludge, what makes difficult to determine which parameter influenced the most VFA generation. In their work, Zhang et al., (2019) also studied the effect of THP on VFA production, but they did so in semi-continuous mode reactors and the batch/continuous mode of operation may be a key factor in fermentation. Wen et al., (2022) studied the microbial fermentative populations in batch mode while Iglesias-Iglesias et al., (2019) conducted the same analysis of microbial communities involved in raw and pre-treated sludge fermentation, but without including the research of VFA-related enzymes. Thus, the novelty of this work lies in the fact that it encompasses two modes of operation and the microbiological study in continuous reactors, going beyond through the comparison of predicted profiles of VFA-related enzymes.

The objective of this work is to evaluate: i) the effect of pH and THP on maximum VFA production yield and selectivity in batch mode and ii) how THP affects VFA production in continuous operation and the implication of this pre-treatment on bacterial dynamics.

2. Materials and methods

2.1. Sludge samples and inoculum

Sludge samples (raw and thermally pre-treated) were picked up in a WWTP located in north-western Spain. The facility has a full-scale high-pressure THP in which a stream composed by a mix of primary sludge (70 % COD basis) and biological sludge (30 % COD basis) is first thickened (15 % dryness), heated and subsequently thermal hydrolysed by submitting it to 6 bar and 165 °C for 30 min. After that, the stream is conducted to a flash tank where the temperature and pressure are suddenly diminished. The inoculum used was collected from the anaerobic digester of the same WWTP operating in mesophilic range, being therefore fed with thermally pre-treated sludge.

2.2. Acidification test in batch mode

A batch acidification experiment was conducted in triplicate utilising bottles of 0.5 L of working volume. The food/microorganism ratio was 2 g COD substrate/g VS of inoculum. The addition of $NaHCO_3$ provided a mineral medium and by establishing an alkalinity of 1 g $CaCO_3$/g COD substrate, an immediate fall in pH was avoided. 2-Bromoethanesulphonic acid sodium salt (BES) was added in order to inhibit methanogenesis (2 g/L), using distilled water to fill up the volume to 0.5 L. pH in the bottles was initially adjusted to 8 or 10 adding NaOH 50 % (v/v) and blanks at both pHs were used to study the production of VFA from the inoculum. The decision to test these pH values was because Yuan et al., (2006) conducted batch fermentation tests at pH ranging from 4 to 11, reporting that VFA production at alkaline pH was much higher than at acidic and neutral conditions due to the increase in protein degradation efficiency and the lack of VFA consumption by methanogens.

Before being sealed, the bottles' headspace was purged with N_2 for two minutes to ensure an anaerobic environment. To carry out the experiments, a modified AMPTS II equipment from Bioprocess Control allowing liquid sampling, was employed. The bottles were placed inside a thermostatic bath at a temperature of 37 ± 1 °C (mesophilic operation).

2.3. Continuous stirred-tank reactors

Two continuous stirred-tank reactors (CSTR) with 5 L of working volume were operated maintaining a temperature of 37 ± 1 °C using a thermostatic bath and an agitation speed of 170 rpm (IKA RW Digital 60). The reactors were firstly inoculated with 5 L of inoculum, for later withdrawing and feeding twice a day the corresponding volume in order to operate at an HRT of 10 days using the Masterflex L/S series pump. Aiming at operating at the same OLR, feedings of both reactors, raw sludge one (reactor RS) and pre-treated sludge one (reactor PS), were diluted to around 50–60 g COD/L, keeping the pH controlled at 8.5 during the complete operation period (80 days) with an OLR ranging between 4.8 and 6.5 g COD/L·d due to fluctuations in the characterization of WWTP's streams. Samples were analysed in both the influent and the effluent twice a week.

In order to compare both the VFA yield obtained in both reactors as well as the acidification yield, it was used the t-Student test for independent samples of equal variance with $\alpha = 0.05$. Student's *t*-test is commonly utilised when two independent groups are compared, being a parametric test and assuming normality of the data and equality of variances across comparison groups. In order to check normality, it was used the IBM SPSS Statistics 28 software through the Shapiro-Wilk statistic (number of samples less than 50), being also confirmed homoscedasticity through Levene's test.

2.4. Analytical methods

The determination of total and volatile solids (TS and VS) was done as indicated in Standard Methods (APHA, 2012). Total and soluble chemical oxygen demand (tCOD and sCOD), Total Ammonia Nitrogen (TAN), total nitrogen (TN) phosphate (P-PO_4^{3-}) and Total Phosphorus (TP), were analysed with a Hach LCK cuvette test system. Determination of tCOD, TN, and TP was carried out with the raw sample, but to analyse sCOD, TAN, P-PO_4^{3-} and VFA it was necessary to pre-treat the samples centrifuging at 6,000 rpm for 10 min and then filtrating through Whatman GFC filters of 0.45 µm pore size.

VFA concentration was determined through gas chromatography (AGV-DB1 method). As equipment, it was used an Agilent 6850 with a flame ionization detector (FID) equipped with a column DB-Wax from Agilent Technologies (30 m × 0.250 mm × 0.25 µm) and with an injector at 200 °C, being N_2 the carrier gas.

2.5. Calculations

The VFA yield in batch mode (expressed in g VFA-COD/g CODfed) was calculated with Eq. (1):

$$VFA\ yield\ (t) = \frac{\Sigma VFA\ (t) - \Sigma VFAblank\ (t)}{tCOD,\ i} \tag{1}$$

where:

ΣVFA(t) represents the amount of the different VFA (as g COD) that is obtained at a fixed reaction time (t) in each bottle, ΣVFAblank (t) represents the amount of the different VFA (as g COD) that is obtained at a fixed reaction time (t) in the blank and tCOD is the amount of substrate added (g COD) for each assay.

When referring to continuous operation, VFA yield was expressed as a percentage in g VFA-COD/g COD influent, being calculated with Eq. (2):

$$VFA\ yield\ (t) = \frac{\Sigma VFAo\ (t) - \Sigma VFAi\ (t)}{tCODi(t)} \cdot 100 \tag{2}$$

where:

ΣVFA(t) represents the concentration of VFA (as g COD/L) measured in the inlet (i) and outlet (o) at a particular moment and tCODi (t) correspond to the total COD concentration of the inlet.

The acidification yield, expressed as mg VFA-COD/g VS was calculated as Eq. (3):

$$Acidification\ yield\ (t) = \frac{\Sigma VFAo(t) - \Sigma VFAi(t)}{VSi(t)} \tag{3}$$

where:

ΣVFA(t) represents the concentration of VFA (as mg COD/L) measured in the inlet and outlet at a particular moment and VS is the concentration of volatile solids in the inlet (g/L).

2.6. Analysis of the microbial community

Once reached steady operation, six samples were taken both from the inlets and outlets of the two continuous reactors (from day 21 of operation to 79).

The extraction of total genomic DNA was conducted in duplicate using Nucleospin Microbial DNA extraction kit (Macherey Nagel) and pooled together after quantification, and quality control using Nanodrop and Qubit fluorometer (Thermo Fisher Scientific Waltham, MA, USA). For Bacteria analysis, The V3-V4 hypervariable region was amplified using Bakt_805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′) and Bakt_341F (5′ CCT ACG GGN GGC WGC AG 3′) (Herlemann et al., 2011). DNA metabarcoding analyses of the region were executed in an Illumina NovaSeq PE250 platform, generating paired ends reads (2 × 250) by AllGenetics & Biology SL (https://www.allgenetics.eu).

Bioinformatic analyses were conducted through the Microbial Genomics module workflow (version 21.1) of the CLC Genomics workbench (version 21.0.3). After filtration of unprocessed sequences to discard low-quality reads, sequences were clustered into OTUs (Operational Taxonomic Units) at 97 % cutoff for sequence similarity and grouped upon the non-redundant version Midas 4 database (https://www.midasfieldguide.org/guide). For further analysis, only the bacterial OTUs with more presence (above 1 % of the total observed OTUs) were considered.

Beta diversity was determined by Bray-Curtis distances between each pair of samples and plotted in a principal coordinate analysis (PCoA). Significance was assessed by PERMANOVA.

Alpha diversity was predicted from the rarefaction analysis utilising phylogenetic tree of OTUs obtained from the MUSCLE algorithm, with a maximum sampling depth of 36,846 reads.

The functional pathways related to VFA metabolism were predicted using PICRUSt software (Lawrence et al., 2017), which provides functional annotations based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases using the closest relative of the OTUs whose genome has been sequenced.

3. Results and discussion

3.1. Batch operation

The aim of the acidification batch tests has been to find out the most favourable pH for obtaining the maximum VFA yield from sewage sludge, both raw and pre-treated. In addition, it was intended to evaluate the effect of pH on the selectivity of the different assays.

3.1.1. Physicochemical characterization of inoculum and substrate

The physicochemical characterization of the inoculum and substrate used for the acidification test is summarized in Table 1. As it can be perceived, the pH of pre-treated sludge is lightly higher than that of raw one probably due to the breakdown of proteins, which provokes an increase in ammonia and alkalinity (Barber, 2016). Total COD of raw sludge is considerably higher than those conventionally reported for raw mixed sludge (Zhu et al., 2021) because, in WWTP with THP, sludge must be thickened to make the process energetically efficient (Taboada-Santos et al., 2019). Pre-treated sludge shows lower TS, VS, and COD concentrations because of the dilution caused by steam condensation. As expected, a higher fraction of COD was solubilized in pre-treated sludge (15.3 %) in comparison with raw sludge (4.8 %). The ratio VS/TS (0.82 and 0.81 g/g for raw and pre-treated sludge, respectively) and tCOD/VS (around 2 g/g in both cases) are also in line with those reported by other authors for sewage sludge (Zhang et al., 2019), meaning that THP did not remove organic compounds and total solids in the substrate.

Inoculum characterization is similar to that of other anaerobic digesters treating thermally pre-treated sludge (Gong et al., 2021), but an accumulation of sCOD indicates that refractory or recalcitrant compounds could be being formed may be due to the occurrence of Maillard reaction (Yan et al., 2022), which happens between reducing sugars an amino acids under certain thermal situations.

3.1.2. Effect of pH

pH is one of the crucial parameters influencing VFA generation

Table 1
Characterization of both substrates and inoculum used in the batch acidification experiment.

	Raw sludge	Pre-treated sludge	Inoculum
pH	6.12 ± 0.00	6.31 ± 0.01	8.02 ± 0.00
tCOD (g/L)	200.8 ± 2.0	162.5 ± 1.1	58.6 ± 1.0
sCOD (g/L)	9.6 ± 0.2	24.9 ± 0.4	5.4 ± 0.2
TS (g/L)	114.2 ± 1.1	91.0 ± 1.5	46.7 ± 0.3
VS (g/L)	94.0 ± 1.3	73.4 ± 1.4	26.5 ± 0.1
TP (g/L)	2.7 ± 0.0	1.9 ± 0.1	2.0 ± 0.2
P-PO_4^{3-} (g/L)	0.146 ± 0.001	0.133 ± 0.002	0.094 ± 0.002

(Varghese et al., 2022). It could affect the efficiency of substrate conversion, the utilization of the carbon source, and the microorganisms' growth rate, apart from having a strong impact on the VFA profile generated through mixed culture fermentation (Temudo et al., 2007).

Fang et al. (2020) compiled the results of several works and found that the pH range to obtain higher VFA yields from sewage sludge was between 8 and 11. Accordingly, an acidification batch test was conducted at pH 8 and 10 to determine the effect of pH on VFA production from raw and pre-treated sludge. Working in this alkaline condition, hydrolysis rate and biodegradation are enhanced due to the disintegration and solubilization of proteins and carbohydrates of bacterial cells; moreover, this condition largely inhibits the methanogens' activity, which convert VFA to methane letting their accumulation.

Fig. 1 clearly shows that pH 8 resulted in more favourable in terms of VFA production than pH 10. At pH 8, the maximum VFA yields obtained were 0.41 and 0.27 g COD-VFA/g CODfed for raw and pre-treated sludge, respectively, whilst for pH 10 were 0.26 for raw sludge and 0.15 g COD-VFA/g CODfed for pre-treated. Presti et al. (2021) stated that the optimum condition to produce VFA from sewage sludge was an initial pH adjustment to 10, but they registered variations of pH during the batch test of around 2.5 units, while in this work the addition of alkalinity avoided a pH decrease higher than 0.5 units, thus the optimal average fermentation pH being very similar in both cases. The results obtained are also in line with those reported by Feng et al., (2009), who registered the highest VFA production yields at pH 8 treating secondary sludge. The most favourable effect of pH 8 in comparison with pH 10 could be attributed to the fact that higher pH promotes the formation of hard-biodegradable organic matter (lignin-like and humic-like substances) with refractory nature which hinders the fermentative process (Ma et al., 2019).

pH resulted crucial in VFA production yields as stated Atasoy et al., (2019), although in their case better VFA productions were obtained at initial pH of 10 using a synthetic medium, while in this work a real sample of sewage sludge was used. Fig. 1 shows that after reaching the maximum VFA production on day 11, in experiments at pH 8 the yield started to decrease and a small volume of methane was registered, indicating that the inhibitory effect of the BES was just temporary and Archaea were partially active (Ward et al., 2008). Contrary, at pH 10 this decrease was not observed since this pH range is not favourable for Archaea populations.

THP presented a negative influence on the production of VFA from sewage sludge, a behaviour that has not been identified previously in literature working with mixed sludge. A possible explanation could be the use of an inoculum not adapted to THP as it is a key factor in the fermentation's pathways (De Gioannis et al., 2013). However, in this case, the inoculum was obtained from a digester operating with pre-treated sludge and therefore well adapted to the substrate.

THP could result not only in a higher organic matter solubilisation but also in the production of hardly biodegradable structures which can be even inhibitory for biotransformation. These compounds such as melanoidin and melanin produced through the Maillard reaction are found in the bottles of the batch assays at their higher value from the beginning of the experiment and could provoke a decrease in the expected yield as they preferentially affect fermentative microorganisms (Chandra et al., 2008).

Another hypothesis to explain the better VFA yield obtained with raw sludge could be the generation of humic acids during THP, compounds that can reach concentrations in the range of 8–15 g/L in pre-thickened sludge digestion (Ghasimi et al., 2016) as the used in this work, causing important inhibitions on cellulose hydrolytic enzymes (Yap et al., 2018).

Moreover, Vidal-Antich et al., (2021) found that hydrolysis pretreatments (in their case at lab-scale and a temperature of 55 °C) did not improve fermentation yields in batch mode when using a mixture of sludge and biowaste as a substrate. The production of toxic and non-fermentable compounds during THP could be the reason for the results obtained, so strict temperature control in THP is vital to balance the enhancement in sludge solubilization and melanoidins generation (Yan et al., 2022).

Fig. 2 shows the distribution of the VFA mixture produced at both pHs tested, 8 and 10. Fig. 2 A and B show that there was no noticeable difference between the VFA spectrum obtained with raw sludge and the pre-treated one considering the error bars. With raw sludge, the presence of acetic acid on the VFA mix is 72 % (COD basis), whereas with pre-treated sludge it accounts for 78 %. Contrarily, the proportion of propionic is slightly higher in raw than in pre-treated sludge (20 % versus 15 % on a COD basis). The rest of the VFA account for a much lower fraction. These results are similar to those presented by Iglesias-Iglesias et al., (2019), who reported that working at the same food to microorganism ratio, the main acid was also acetic acid (accounting for

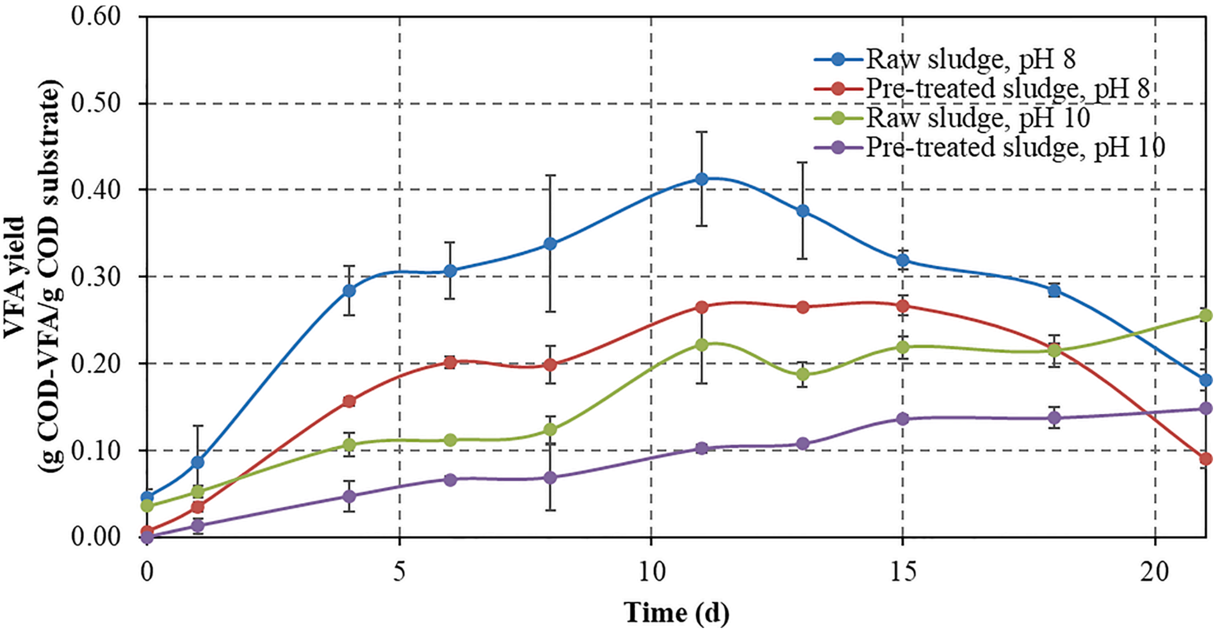

Fig. 1. Acidification yield of raw and pre-treated sludge at both pH tested.

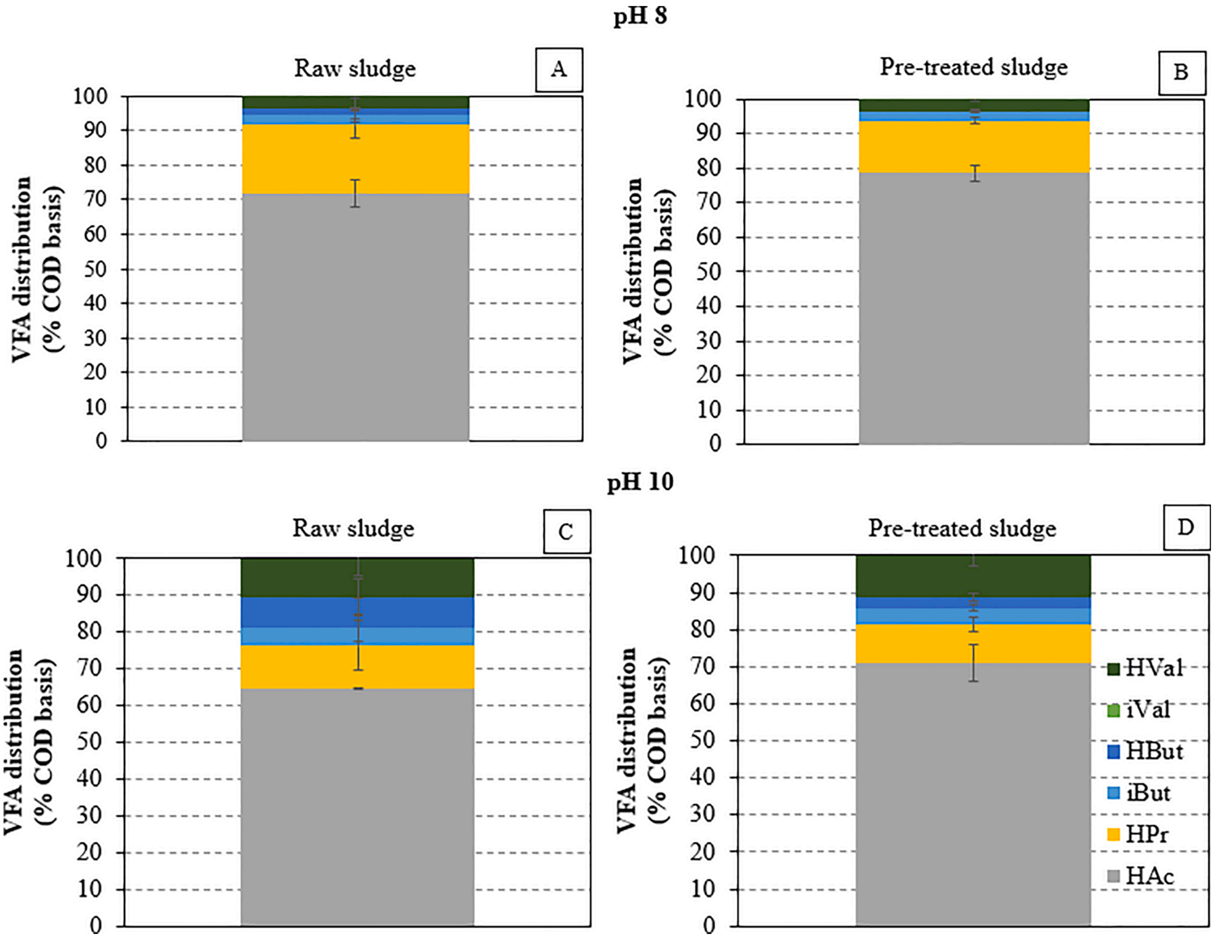

Fig. 2. VFA distribution of the produced mix on the batch tests.

50 % of the COD of the VFA mix) followed by propionic acid. The lower proportion of acetic acid might be attributed to the fact that they worked at uncontrolled pH and acidic pH favours the production of other VFA such as butyric, while acetic is the main acid at neutral and alkaline pH (Cokgor et al., 2009).

Fig. 2 C and D also show that at pH 10 there are no major differences between the VFA mix produced with raw and pre-treated sludge, being acetic acid again the dominant VFA with around 65 % of the distribution (COD basis). These results correspond with those reported by Morgan-Sagastume et al., (2011), who stated that the VFA spectrum is highly subjected to the type of waste fermented and pH rather than on the application of pre-treatments. Contrary to results obtained at pH 8, valeric acid is the second VFA in abundance (accounting for up to 11 % of the VFA mix on COD basis with and without pre-treatment). Valeric acid production could be associated with protein fermentation through the Stickland reaction whose stoichiometry is very much affected by pH (Bevilacqua et al., 2021).

In summary, the acidification test showed that pH 8 was more favourable in terms of VFA yield and that the raw sludge obtained better results. At both pHs, acetic acid was predominant in the mix, always exceeding 60 % since alkaline conditions favour its production (Fang et al., 2020). Moreover, the addition of the BES has not been effective working at pH 8 in the long term as a partial methanisation occurred after day eleven of the experiment (Fig. 1).

3.2. *Continuous operation*

Two continuously stirred reactors fed with raw and pre-treated sludge (Reactor RS and PS, respectively) were operated setting pH at 8.5 to ensure a high VFA production and a less favourable condition for methanogenesis. HRT was set at 10 days because it was the time of maximum VFA production yield in batch operation, apart from being the most favourable HRT in terms of VFA production according to Yuan et al., (2009). The process stability was evaluated as well as the VFA yields and selectivity. Moreover, aiming at elucidating the influence of THP on bacterial community fermentation, six samples were taken and analysed.

3.2.1. *VFA production reactors' performance*

Table 2 shows the characterization of the inlet and outlet as well as the metrics of VFA production performance. Inlets were taken from the same WWTP as those used in batch mode, although they have been diluted to around 60 g COD/L, operating in an OLR interval of 4.8–6.5 g COD/L·d, values similar to those applied by Wu et al., (2018). As can be expected, the sCOD of the PS reactor's inlet is much higher than that of the RS reactor (corresponding with 19.9 % of tCOD versus 8.8 %) as a result of THP. TAN and P-PO_4^{3-} concentration increased after THP due to the breakdown of cell material.

Fig. 3A and B depict the COD distribution of the inlet and outlet of both continuous reactors. The graph shows a period of operation in which the reactors are already working at a steady state after a stabilization period of more than 30 days.

For reactor RS (Fig. 3A), 8.1 g COD-VFA/L were obtained, which implies a VFA yield of 16.6 % g COD-VFA/g COD substrate. These results are slightly higher than those reported by Zhang et al., (2019) who also run semi-continuous reactors with an inoculum adapted to the THP process (mean VFA yield of 10 % g COD-VFA/g COD substrate). Besides, Iglesias-Iglesias et al., (2019) also ran a continuous reactor with raw

Table 2
Mean characterization of raw and pre-treated sludge fed in the continuous operation reactors and reactors performance.

	Reactor RS	Reactor PS
Inlet characterization		
pH	6.42 ± 0.09	6.65 ± 0.10
tCOD (g/L)	48.7 ± 4.1	53.2 ± 4.3
sCOD (g/L)	4.3 ± 0.7	10.6 ± 1.0
TS (g/L)	40.7 ± 1.4	42.2 ± 1.6
VS (g/L)	29.3 ± 1.1	30.6 ± 1.3
TAN (g N/L)	0.096 ± 0.042	0.183 ± 0.031
P-PO$_4^{3-}$ (g/L)	0.019 ± 0.007	0.034 ± 0.009
Outlet characterization		
pH	8.44 ± 0.12	8.42 ± 0.10
tCOD (g/L)	40.8 ± 4.0	47.6 ± 4.6
sCOD (g/L)	12.4 ± 0.5	13.3 ± 1.1
TS (g/L)	38.0 ± 1.9	43.3 ± 1.1
VS (g/L)	20.7 ± 1.4	24.3 ± 1.6
TAN (g N/L)	0.582 ± 0.121	0.622 ± 0.123
P-PO$_4^{3-}$ (g/L)	0.066 ± 0.005	0.088 ± 0.010
Reactor performance		
VFA concentration (g COD/L)	8.1 ± 0.8	8.0 ± 0.9
VFA yield (% g COD-VFA/g COD substrate)	16.6 ± 0.9	15.1 ± 1.3
Acidification yield (mg COD-VFA/g VS)	294.3 ± 4.9	263.9 ± 5.3
COD-VFA/sCODout (%)	66.3 ± 2.1	60.3 ± 1.6

sludge, reaching a similar average conversion. Finally, Yuan et al., (2009) obtained a mean VFA yield of 14.1 % g COD-VFA/g COD substrate operating a semi-continuous reactor with secondary sludge at HRT of 10 days, which is in line with this work, but the slightly lower yield could be related to the composition of waste activated sludge, containing mostly bacterial mass and having the cell lysis as the rate-limiting step.

A mean acidification yield of 294.3 mg COD-VFA/g VS was obtained in reactor RS, which is higher than that reported by Morgan-Sagastume et al., (2011) and Zhang et al., (2019) working with raw sludge in the mesophilic range (150 and 100 mg COD-VFA/g VS, respectively). In line with the acidification yield reported in this work, Da Ros et al., (2020) obtained a value of 322 mg COD/g VS operating a sequencing batch reactor fed with primary sludge. The COD-VFA/sCODout ratio was 66 %, very similar to that reported by Zhang et al., (2019), who reported a value of 71 %, showing that most of the soluble organic matter is turned into VFA.

Regarding reactor PS (Fig. 3B), an average yield of 15.1 % g COD-VFA/g COD substrate was obtained, comparable to the one reported by Zhang et al., (2019) (14 % g COD-VFA/g COD substrate), who also worked with pre-treated sludge and an inoculum adapted to THP. Morgan-Sagastume et al., (2011) also reported a VFA yield of 15 % g COD-VFA/g COD when they fed their reactors with thermal hydrolysed sludge. The mean acidification yield in reactor PS was 263.9 mg COD-VFA/g VS, which is almost the same as that reported by Liu et al., (2019) working at a pilot scale with alkaline pre-treated sewage sludge (261.3 mg COD/g VS). For this PS reactor, the COD-VFA/sCODout ratio was 60 %, 52 % as reported by Zhang et al., (2019) with pre-treated sludge.

It was detected that for both continuous reactors, between 10 and 15 % of the total COD fed was lost (likely as hydrogen), which indicates that operating pH and HRT have been effective in inhibiting methanogenic archaea (Yuan et al., 2019). Moreover, the COD-VFA/sCODout ratio was higher in the RS reactor, which indicates that soluble carbonaceous compounds released by THP have a lower biodegradability than that of raw sludge, a fact that could be related to the generation of bio-refractory compounds during Maillard reaction as it was discussed previously.

Statistical analysis performed through Student's *t* test concluded that the difference between mean VFA yield obtained in reactor RS and PS was not significant. It was also found that there was no significant difference between the acidification yield obtained in the two reactors. Therefore, working in continuous mode, THP has any influence on VFA production from sewage sludge, unlike the results obtained in batch mode in which a negative effect was observed.

During the operation of the continuous reactors, the composition of the VFA mixture obtained was analysed (Fig. 4) and there was no major difference between the mix obtained with raw and with pre-treated sludge; Tayou et al., (2022) also observed that THP applied before a mesophilic fermentation did not have a relevant effect on the VFA mix produced. In both cases, acetic acid was the dominant product (presence in the mix based on COD higher than 60 %), followed by propionic and other VFA. Comparable results were reported by other authors such as Iglesias-Iglesias et al., (2019) using raw sludge in a continuous reactor. However, Zhang et al., (2019) comparing the fermentation of raw and pre-treated sludge in semi-continuous mode at uncontrolled pH, saw that the proportion of VFA in their mix was practically the same with both substrates although the weight of acetic acid based on COD did not exceed 30 %. In their work, Ma et al., (2019) operating semi-continuous fermenters fed with secondary sludge at pH 9 (close to the operating pH of 8.5), observed that the proportion of acetic acid based on COD was around 35 % of the mix distribution, with about the same propionic, followed by isovaleric. Yang et al., (2014), operating a CSTR with hydrolysed waste-activated sludge, observed that the acetic-acid type fermentation was predominant for almost the entire time of operation, being acetic acid the predominant acid with 51.8 % of presence in the mix on COD basis.

Thus, obtained results show a proportion of acetic acid exceeding that usually seen in the literature, highlighting the influence of both the substrate used and the specific operating conditions chosen in the fermentative process (Yuan et al., 2019).

A priori, better VFA yields were expected when using pre-treated sludge as substrate, since the hydrolytic step was already boosted in THP. However, sometimes refractory organic compounds are produced and they cause a lower biodegradability of organic matter, preventing the fermentation process (Bougrier et al., 2007).

It seems that the negative effect of THP observed in batch mode practically disappears in continuous operation. Indeed, batch mode assays execution could imply a higher concentration of inhibitory compounds generated from the beginning of the assay due to the high food/microorganism ratio applied. However, the particularities of operating in continuous mode favour a lower concentration of these compounds since these reactors were fed with diluted sludge, both raw and pre-treated. This is consistent with the fact that works involving refractory compound generation have been carried out in batch mode (Eskicioglu et al., 2006; Penaud et al., 2000).

A similar behaviour when comparing VFA yields in operation modes was observed by Iglesias-Iglesias et al., (2019) and Owusu-Agyeman et al., (2020), who reported that a change from a batch system to a continuous one implied a remarkable drop in VFA production (from a maximum of 45 % g COD-VFA/g CODfed in batch to around 20 % in semi-continuous mode).

3.2.2. *Bacterial community characterisation*

Once continuous reactors were running stably, six sludge samples of inlets and outlets were taken to study the impact of THP on microbial communities.

A total of 1,894,431 reads were registered after trimming and quality filtering, ranging from 41,275 (PS-O day 21) and 110,395 from RS-I day 42 (see supplementary material). In order to obtain the rarefaction curves, a normalization of OTUs count for individual biological replicates was done, showing that all of them reached the plateau and that 36,846 reads are the adequate sample sequencing depth to analyse the diversity.

The beta diversity measured by Bray-Curtis index separated the samples according to the treatment (raw or pre-treated sludge) and

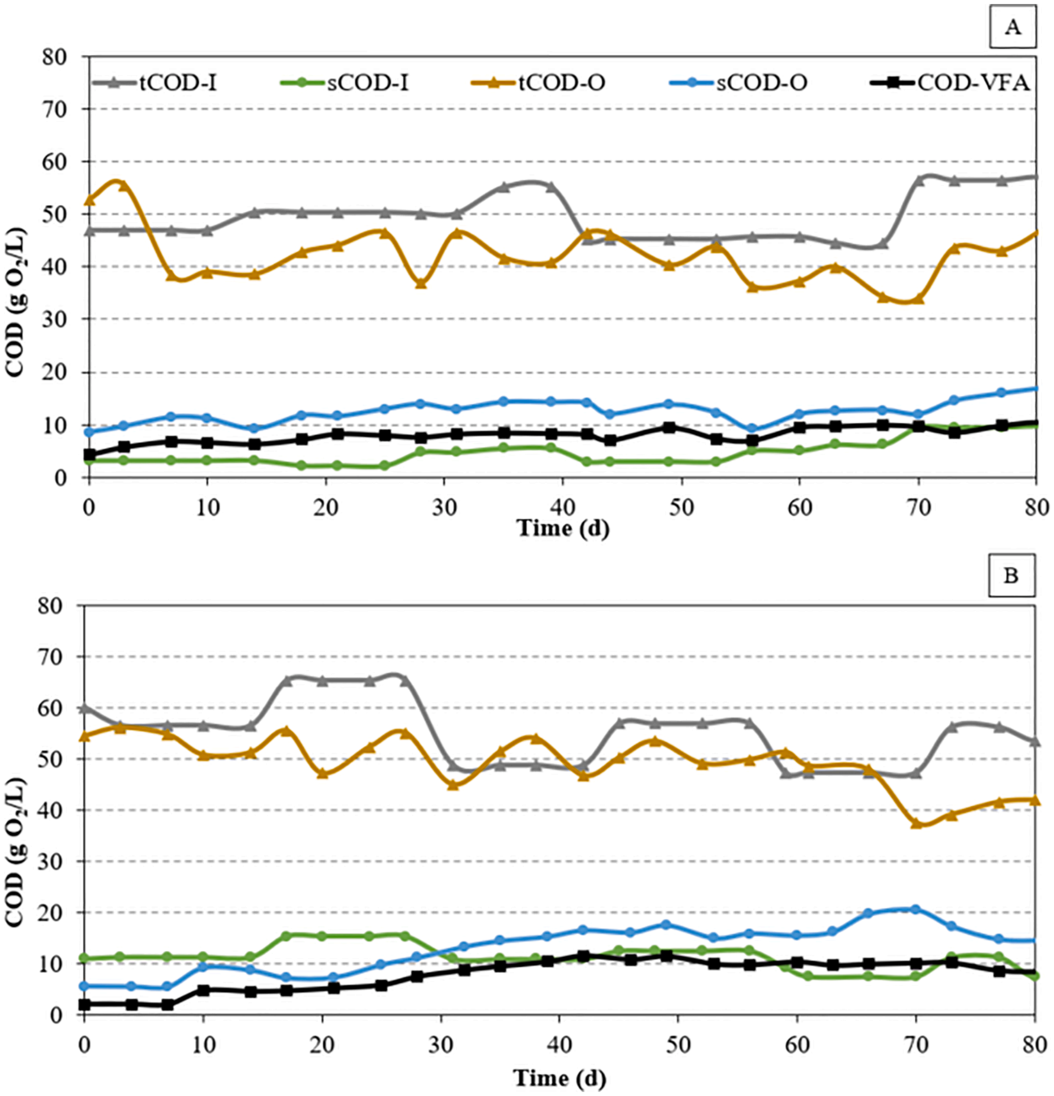

Fig. 3. COD distribution of the inlet (I) and outlet (O) of continuous reactors. A) Reactor RS. B) Reactor PS.

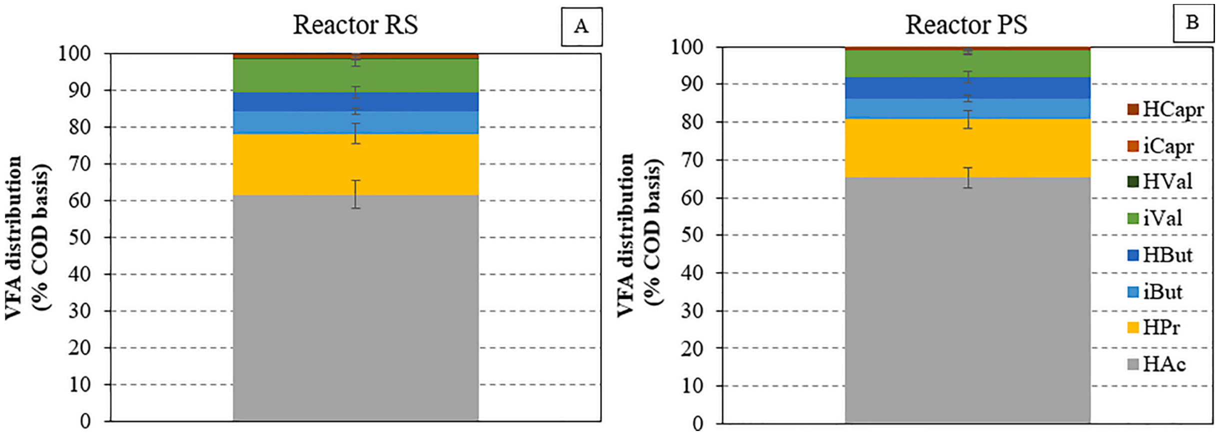

Fig. 4. VFA distribution of the produced mix in the continuous reactors. A) Reactor fed with raw sludge (RS). B) Reactor fed with pre-treated sludge (PS).

location (inlet or outlet of reactors), as shown in supplementary material, and thus 4 groups were defined. Moreover, PERMANOVA analysis showed significant differences among those groups using both a phylogenetic method, unweighted UniFrac distances (*Pseudo-F* = 26.737; p = 0.00001) and non-phylogenetic methods, Bray Curtis method (*Pseudo-F* = 10.753; p = 0.00001), demonstrating that the origin of the sample has a strong influence on the microbiome.

The alpha diversity measures (see supplementary material) suggest a specialization of the microbial communities present in both reactors converging towards similar hydrolytic and acidogenic populations which give rise to the same VFA yields in continuous operation, independently of the pre-treatment applied to the inlet. Bacterial richness measured as the total number of OTUs was significantly higher in the reactor RS (both in the inlet and outlet) because it was fed with sludge not thermally hydrolysed. When the richness is calculated considering Shannon entropy and Simpson indexes, there was no significant difference between the outlets of both reactors; only if Chao 1 index is considered, which is focused on rare OTUs, there was a significant difference between the outlets from both reactors.

Fig. 5A shows the relative abundance of the main phyla detected in the six samples of reactors' outlets. The most abundant phylum in reactor RS was *Firmicutes*, whose mean relative abundance was 56.7 ± 11.6 %, followed by *Proteobacteria* (33.5 ± 9.7%) and *Actinobacteriota* (4.9 ± 1.7 %). In reactor PS, the main phylum was also *Firmicutes* (62.3 ± 10.9 %), followed by *Actinobacteriota* (23.2 ± 8.5 %) and *Proteobacteria* (8.5 ± 4.6 %). *Firmicutes* can secrete lipases, proteases and cellulases as well as other extracellular enzymes involved in lipid, protein and polysaccharide hydrolysis and acidogenesis stages (Chen and Chang, 2017). The difference between the bacterial populations in the two reactors could be related to the generation of certain compounds such as humic acid, which could explain the higher abundance of *Actinobacteria* in reactor PS, a group highly resilient to humic acid (Yap et al, 2018). The higher presence of *Proteobacteria* in reactor RS could be because this phylum produces hydrolytic extracellular enzymes such as polysaccharase, protease and lipase (Jang et al., 2014), which implies that fermentation of raw sludge may require a higher presence of hydrolytic populations to disintegrate particulate matters, being unnecessary for pre-treated one.

Fig. 5B outstands that the predicted profile of VFA-related enzymes showed very similar trends in both reactors, being the majority of routes related to acetic metabolism (blue colour), followed by enzymes of propionic routes (yellow colour), the two most abundant VFA produced as shows Fig. 4. The pattern seen during the different days of sampling is very similar, and the following enzymes belonging to Enzyme Commission number (EC) 6 stand out: propionyl-CoA carboxylase (EC 6.4.1.3), acetate-CoA ligase (EC 6.2.1.1) and phenylacetate-CoA ligase

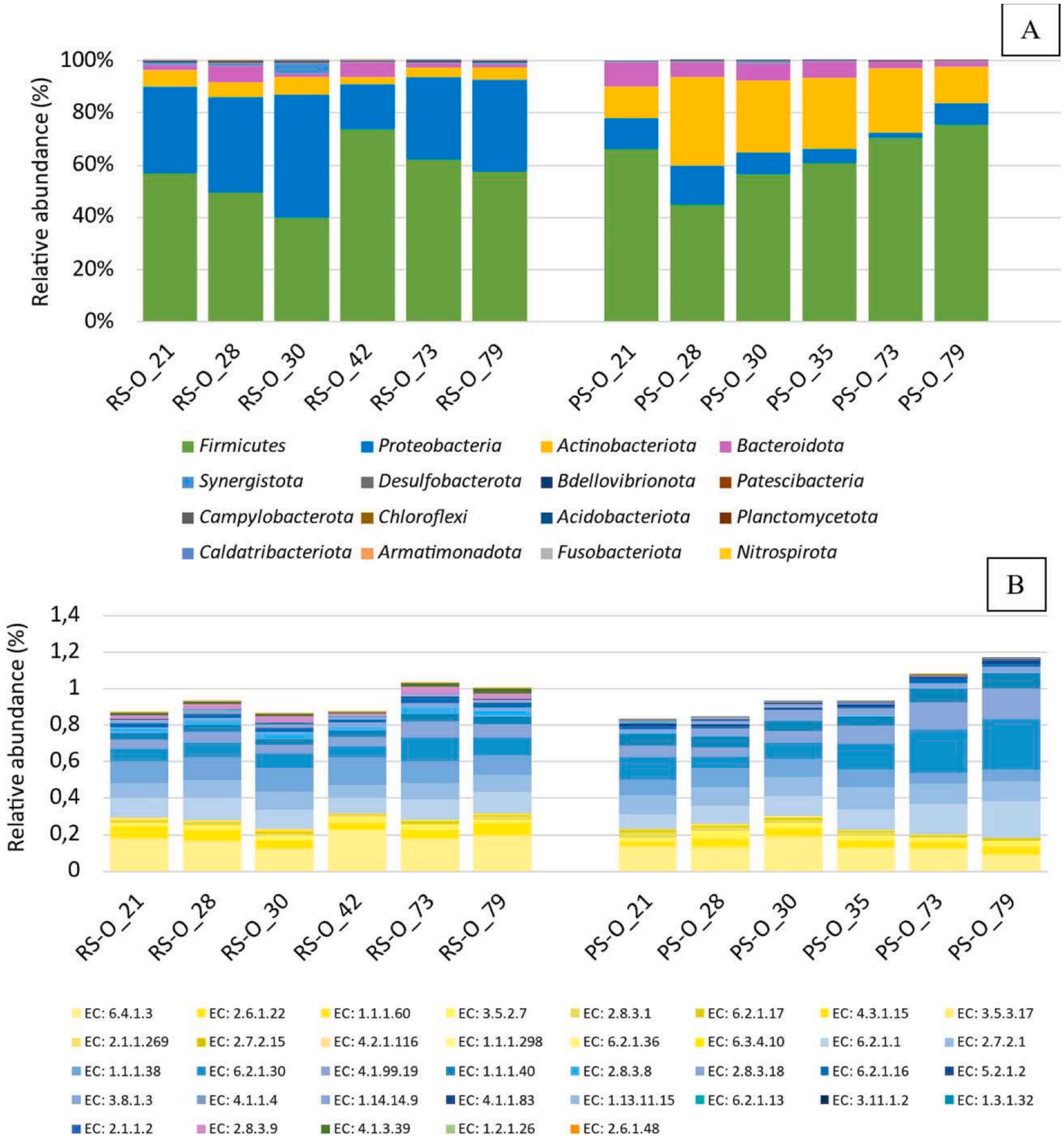

Fig. 5. A) Bacterial community composition at phylum level considering only OTUs with total abundance higher than 1 % and B) Detailed enzymes with their EC number related to VFA metabolism. Numbers represent day of operation.

(EC 6.2.1.30), whose common function is joining together two molecules by synthesizing bonds with ATP's breakdown (Feng et al., 2022). This similarity reaffirms the equality both in the production yields and in the selectivity of the acidogenic process, being the same as those key players involved in the VFA metabolic pathways.

Although THP has any effect on VFA production in continuous mode, it has other benefits like the sterilisation and sanitation of the sewage sludge by eliminating most of the bacteria with presence in feedstock, affecting also to physicochemical properties of the sludge such as solubilisation and viscosity's reduction (Bougrier et al., 2006).

4. Conclusions

Raw sludge was compared with thermally pre-treated sludge to observe the effect of THP on VFA production in batch and continuous operation. In batch mode, THP resulted in lower VFA yields at both tested pH, being pH 8 the most favourable in terms of VFA yield. In continuous reactors, the negative effect of THP disappeared, obtaining similar VFA yields and distribution mix independently of the pretreatment of substrate. Continuous mode microbiology analysis confirmed that both reactors converged into similar bacterial populations with *Firmicutes* phylum as the most abundant one, being the VFA-related enzymes also alike.

E-supplementary data for this work can be found in e-version of this paper online.

CRediT authorship contribution statement

Ander Castro-Fernandez: Investigation, Conceptualization, Visualization, Writing – original draft, Software. **Anton Taboada-Santos:** Project administration, Supervision, Writing – review & editing. **Sabela Balboa:** Investigation, Formal analysis. **Juan M. Lema:** Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

The data that has been used is confidential.

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