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Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste

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ABSTRACT

Volatile fatty acids (VFAs) revalorisation from waste products are key in achieving industrial sustainability and circular economic goals. Hence, the objective of this work was to correlate the adaptability of the microbial community in olive mill solid waste (OMSW) anaerobic fermentation processes, to the production of VFAs under different pH conditions, i.e. under acidic (pH 4 &5), neutral (pH 6 & 7) and alkaline conditions (pH 9 & 10). At neutral conditions, anaerobic digestion exhibited minimal accumulation of VFAs, as they were primarily biotransformed to methane, where no significant changes in the microbial community were observed. At acidic conditions, a diverse profile of VFAs were present in the reactors, although the VFA production was limited to around 20 % of fed OMSW. Despite the low accumulation, the VFA profile at pH 5 was more complex than those at alkaline conditions, accounting propionic acid as the main VFA compound produced at pH 5 (60 % of the total VFAs). Acidic conditions entailed a shift in the microbial composition compared to the initial inoculum, although the reactors maintained similar diversity indices. At alkaline conditions, around 50 % of the fed OMSW was accumulated as VFAs, mainly as acetic acid. Overall, a lower diversity and higher dominance corresponded to a less diverse VFAs profile, such as the preponderance of acetic acid correlated with a microbial diversity decrease and the increased dominance of *Tissirella*.

1. Introduction

Olive oil consumption has spread worldwide; however, production is centred around Mediterranean countries. Most of the olive oil industry generates one major waste flow, i.e. the olive mill solid waste (OMSW) (Ducom et al., 2023). OMSW is mainly composed of the vegetation water, the fibrous part of the mesocarp, parts of the stone and the olives skin (Najla et al., 2022). The composition of the OMSW is water (60–70 %), lignin (13–15 %), cellulose and hemicellulose (18–20 %), olive oil retained in the pulp (2.5–3 %), and mineral solids (2.5 %). Its organic components include sugars (3 %), volatile fatty acids (VFAs) (C-2 to C-7) (1 %), polyalcohol (0.2 %),

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Table 1
Physicochemical characterization of olive mill solid waste (OMSW) and the anaerobic inoculum.

	OMSW	Anaerobic inoculum
pH	4.6 ± 0.1	7.3 ± 0.1
Alkalinity (mg $CaCO_3$ L^{-1})	n.d	5401 ± 154
VS/TS (%)	94.0	52.0
Moisture (%)	74.7 ± 0.3	96.0 ± 0.4
tCOD (g O_2 L^{-1})	325.6 ± 31	27.0 ± 0.6
sCOD (g O_2 L^{-1})	13.9 ± 0.1	0.5 ± 0.2
TOC (mg L^{-1})	3795 ± 10	7150 ± 10
C/N	35 ± 1.0	7 ± 1.0
Total phenols (mg gallic acid eq. L^{-1})	6135 ± 131	n.d.
3,4-DHPG (mg L^{-1})	296 ± 6	n.d.
HT (mg L^{-1})	2750 ± 65	n.d.
Ty (mg L^{-1})	799 ± 11	n.d.

VS: volatile solids; TS: total solids; tCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand; TOC: total organic carbon; 3,4-DHPG: 3,4-Dihidroxiphenilglycol; HT: Hidroxytyrosol; Ty: Tyrosol; n.d.: not determinated.

proteins (1.5 %), polyphenols (0.2–2 %), and different pigments (0.5 %) (Kourmentza et al., 2017). The olive sector generates four tons of OMSW for each ton of olive oil produced, which is creating a management challenge due to the high volume generated, but also an opportunity as it can be used as raw material for different bioprocesses. An alternative approach worth considering is obtaining VFAs from OMSW. The latter are short-chain fatty acids (from 2 to 7 carbons) and are key building blocks in the chemical industry, hence generating high value-added chemicals or biofuel generation (Fang et al., 2017). In particular, acetic acid and its derivatives are used in various industrial processes, such as food preservation (Budiman et al., 2016). In the cosmetic industry, ethyl acetate is used as an odour enhancer in different products (Bhatia and Yang, 2017), and in the textile industry, where polyvinyl acetate is used in the manufacture of synthetic fibres (Pal and Nayak, 2017). Other VFAs such as propionic, butyric or isovaleric acid are used in the food industry as preservatives and flavour enhancers (Bhatia and Yang, 2017). Other current industrial applications of VFAs include medication such as antispasmodics or cancer drugs (Dahiya et al., 2023) or the production of printer inks (Bhatia and Yang, 2017). Thus, acquiring VFAs through microbial processes from OMSW is a renewable and sustainable approach to creating chemical building blocks or oil-based by-products, otherwise obtained from fossil sources.

Although the VFAs content of OMSW is low, these compounds are intermediate metabolites of the anaerobic digestion process (AD). VFAs can be obtained from this type of fermentation via the control of operating parameters, influencing the flow of metabolic pathways, specifically increasing hydrolysis and acidogenesis, while inhibiting methanogenesis (Fang et al., 2017). Several studies have shown that controlling operational parameters such as temperature, pH, hydraulic retention time (TRH) or organic loading rate (OLR) could facilitate the accumulation of VFAs in anaerobic bioreactors (Cabrera et al., 2019; Chen et al., 2017; Garcia-Aguirre et al., 2017; Jankowska et al., 2015; Niero et al., 2022; Yuan et al., 2006). pH control during fermentation could be a highly effective strategy for VFAs accumulation, based on the microbiome involved in the AD process. Among them, microorganisms involved in methanogenesis usually have an optimum pH range of around 6.8–7.8, presenting a very limited activity outside this range. Whereas microorganisms in the hydrolytic and acidogenic stages might effectively function at more extreme pH values, thus, improving the accumulation of VFAs (Dahiya et al., 2023; Xu et al., 2021) To enhance VFAs accumulation working on such extreme pH values, the microbial community should increase the relative quantity of bacteria with hydrolytic and acidogenic activity (Zapata Martínez et al., 2019, 2019) while decreasing the relative abundance of acetoclastic and hydrogenotrophic archaea (Jabłoński et al., 2015).

Therefore, the objective of this work was to relate the adaptability of the microbial community in anaerobic fermentation processes from OMSW for VFAs production under different pH conditions. Specifically, this work has correlated the operation of two acid pH (pH 4 and 5), two neutral pH (pH 6 and 7) and two alkaline pH (pH 9 and 10) with their microbial community.

2. Materials and methods

2.1. OMSW substrate and anaerobic inoculum

OMSW was supplied by an olive oil producing company called “Oleícola El Tejar Ntra. Sra. de Araceli”, located in Marchena (Seville), Spain. The OMSW was stored at −20 °C after collection in containers to avoid uncontrolled substrate fermentation.

The anaerobic inoculum used in the experiments was obtained from an industrial anaerobic reactor at the “COPERO” wastewater treatment plant in Seville, Spain. The main characteristics of the OMSW and the anaerobic inoculum are summarized in Table 1.

2.2. Batch-fed acid Fermentation Set-Up and operation

OMSW batch-fed per duplicate reactors were evaluated for each pH condition. Six pH conditions were studied, i.e., 4, 5, 6, 7, 9 and 10. The experimental design of this assay was carried out following the procedure reported by Cabrera et al. (2019). The capacity of the

reactors was 2.0 L, with a working volume of 1.6 L, and were inoculated with 10 g VS L^{-1} of fresh inoculum. The operational pH for each reactor was controlled with HP series proportional flow pumps (4–20 mA) (*MYTHO technologies, Hunenberg, Switzerland)* connected to a *46D - Series* controller, sending signals to the pump when the pH fell below or above the pH thresholds, causing the addition of acid (HCl 3 N) or base (NaOH 2 N) for pH regulation. This signal is provided by an XS PRO-VP 120 pH electrode (*Labprocess Distribuciones S.L*, Barcelona, Spain) at each reactor. The operation temperature (35–37 ºC) was maintained by means of jacketed reactors and a water bath Digiterm-TFT *(J.P.Selecta,* Barcelona, Spain). The methane generated was measured by a liquid displacement after CO_2 removal with bubblers containing a 2 N NaOH solution. Batch feeding was 2 g VS OMSW L^{-1} in each reactor. For all pH conditions evaluated, soluble organic matter was monitored to ensure if it had stabilized prior to a next feeding. Batch feeding was performed six times throughout the 45-day experiment. The feedings took place at regular intervals of seven days, specifically on days 0, 7, 14, 21, 28, and 35.

2.3. Chemical analysis

The characterization of the substrate and anaerobic inoculum were determined after the following analytical tests. Solid concentrations [Total (TS), Mineral (MS), Volatile (VS)], total chemical oxygen demand (tCOD), soluble chemical oxygen demand sCOD have been determined according to APHA standard methods (Baird et al., 2017). Elemental C and N were determined through a combustion with LECO CN828 by Duma′s method according to APHA. Individual VFA compounds were determined using a Shimadzu gas chromatograph (GC-2025) equipped with a 30 m × 0.25 mm (i.d.) Crossbond Carbowax polyethylene glycol column and a flame ionization detector (FID) at 250 ºC. The total soluble phenols were determined by the Folin-Ciocalteu method (Trujillo-Reyes et al., 2019). The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high performance liquid chromatography equipment coupled with a diode array detector and an Agilent 1100 automatic injector (20 μL samples were injected). The chromatographic column used was Teknokroma Tracer Extrasil OSD2 of 5 μL particle size and dimensions of 25 m x 0.45 mm internal dimeter. The individual soluble phenolic compounds quantified were 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol (HT), tyrosol (Ty), 4-metilcatechol (4-MC) and 4-ethylphenol (4-EP).

2.4. Molecular analyses of microbial communities

2.4.1. RNA extraction, amplicon PCR and sequencing

Three digestate samples were extracted directly from each of the reactors on day 0 (initial conditions – T0), around the mid operation time, between day 22 and day 30 (mid HRT – T1) and day 45 (the last operating day – T2). The samples were centrifuged 13 min at 1000 rpm to separate the solid phase of the digestate. Subsequently 0.5 g wet solid phase was transferred to the lysing matrix and immediately frozen with liquid nitrogen and stored at −80 °C. RNA was extracted from the solid phase of the digestate, using the FastRNA™ Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24™ Classic bead beating system/MP Biomedicals). RNA was pretreated with DNaseI, and retrotranscribed to cDNA using SuperScript™ II RT (Invitrogen) with random primers according to the manufacturer's instructions. Amplicon sequencing of the V3/V4 region of the 16 S rDNA was performed by the Genomic Unit at the Instituto de Parasitologia y Biomedicina-Lopez Neyra (CSIC, Granada, Spain) from the cDNA. Briefly, DNA concentration was measured using Qubit® 3.0 Fluorometer (Invitrogen,Carlsbad, CA, USA) and normalized to the same concentration. A 2-step PCR was performed on the DNA samples. Primers 16S_ProV3V4_forward (5′-CCTACGGGNBGCASCAG-3′) and 16S_ProV3V4_reverse (5′-GACTACNVGGGTATCTAATCC-3′), each with the adaptor (5′-CTGTCTCTTATACACATCT-3′), were used to amplify the V3/V4 region with the following cycling conditions: 95 ºC, 3 min; (95ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s) x 25 cycles; 72ºC, 5 min; and 4ºC, hold. A second PCR step was performed to index the samples using the Nextera XT Index Kit v2, for a further 8 cycles. Bidirectional sequencing was carried out on a MiSeq™ System (Illumina, 2 ×275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeq™ System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Primers were removed using cutadapt (Martin, 2011), pair-end reads were joined using vsearch (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400 bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignation at the species level was applied, and OTUs were classified according to their last known taxon.

2.4.2. Bacterial and archaeal quantitative analysis (qPCR)

Bacterial and archaeal 16 S rRNA gene quantitative PCR (qPCR) using primers 341F and 534R (Muyzer et al., 1993) and primers ARCH915 and UNI-b-rev (Yu et al., 2008) were used as proxies for the total *Bacteria* and *Archaea* abundances, respectively. Amplification reactions and PCR conditions were followed according to Correa-Galeote et al. (2021). As standards for the quantification of the genes, dilution series of cloned linear fragments developed previously in our lab were employed. Both qPCR reactions were run on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA) by triplicate.

2.4.3. Statistical analysis

Non-metric multi-dimensional scaling (NMDS) was performed using the software PRIMER-E, v.6.0, (Plymouth, UK), and analysis of similarities (ANOSIM) was based on Bray-Curtis distance.

The R packages Pheatmap (Kolde, 2015) and RColorBrewer were used to create the heatmaps. Euclidean clustering was performed to show similarity patterns on taxonomic units. Clustered taxa at the species level were labelled "s_" before their classification. Unclassified clustered taxa at the species level were labelled according to their last classification, i.e. g_genus, f_family, o_order, c_class,

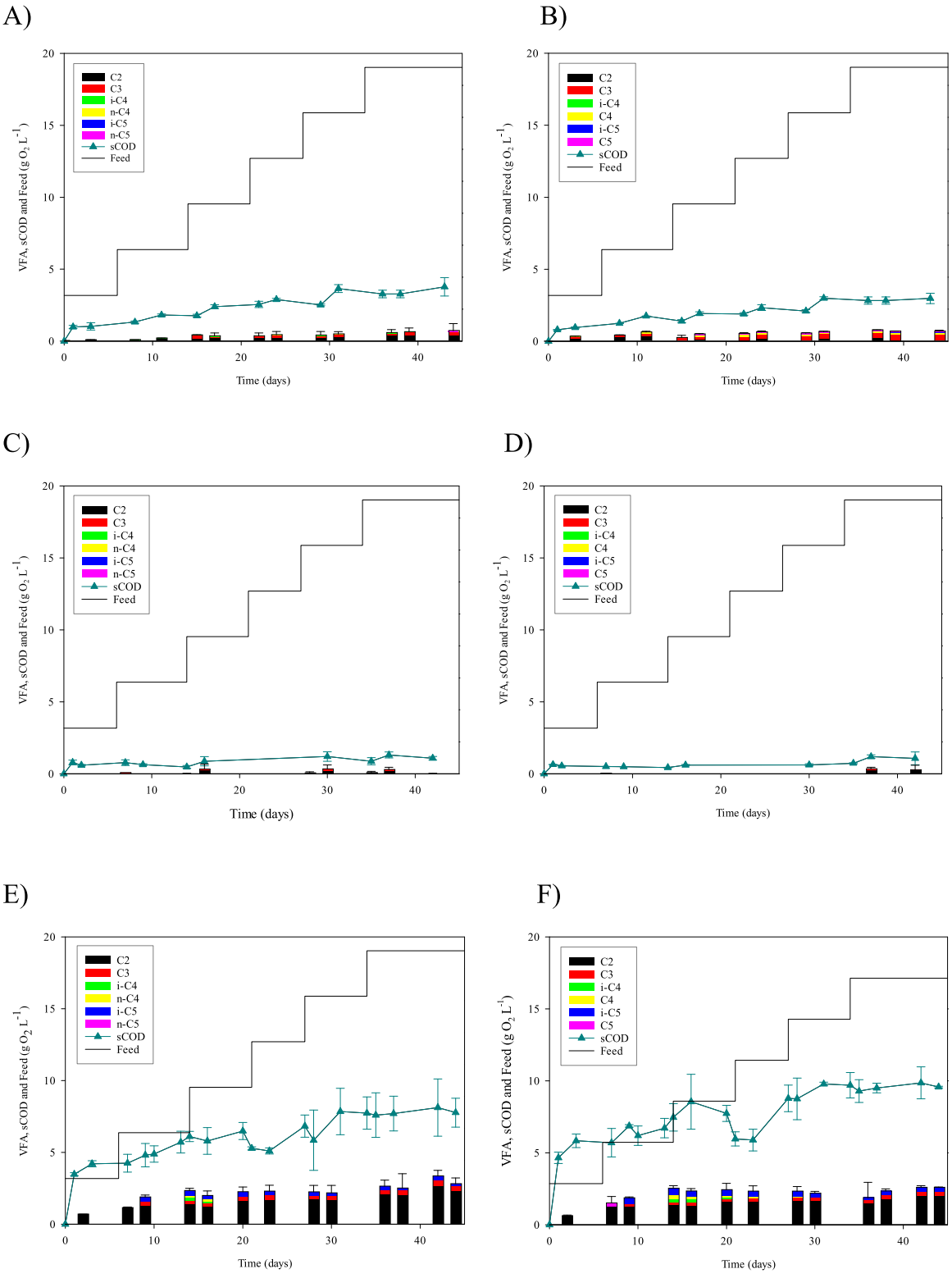

Fig. 1. Volatile fatty acids and total organic carbon generation (g O_2 L^{-1}), according to the feed (expressed as g O_2 L^{-1}) olive mill solid waste during the experimental time. A) pH 4, B) pH 5, C) pH 6, D) pH 7, E) pH 9, F) pH 10.

Table 2
Total and individual phenolic compounds for all conditions in mg per litre of reactor.

	Time (days)	3,4-DHFG (mg L^{-1})	HT (mg L^{-1})	Ty (mg L^{-1})	4-MC (mg L^{-1})	4-EP (mg L^{-1})	Total Phenols (mg gallic acid eq. L^{-1})
OMSW	0	4.0	37.6 ± 0.9	10.8 ± 0.1	n.d	n.d	82.9
pH 4	3	4.2 ± 0.4	21.2 ± 0.5	traces	n.d	n.d	93.5 ± 4.8
	11	5.7 ± 1.8	31.4 ± 4.7	traces	n.d	n.d	157.9 ± 58.0
	17	5.9 ± 0.8	34.8 ± 4.7	traces	n.d	n.d	172.5 ± 57.0
	24	7.7 ± 0.6	41.2 ± 5.8	traces	n.d	n.d	188.9 ± 30.0
	31	7.8 ± 0.3	47.8 ± 5.5	traces	n.d	n.d	166.4 ± 2.0
	40	11.5 ± 3.3	47.3 ± 2.2	n.d	n.d	n.d	196.9 ± 14.0
pH 5	3	4.4	21.2 ± 0.5	n.d	traces	n.d	60.2 ± 5.7
	11	traces	21.1 ± 4.7	9.4 ± 1.4	traces	9.4 ± 1.4	93.0 ± 0.4
	17	1.7 ± 2.4	21.4 ± 4.7	9.8 ± 0.4	traces	9.8 ± 0.4	107.4 ± 10.3
	24	4.2 ± 0.0	24.3 ± 5.8	9.5 ± 0.9	traces	9.5 ± 0.9	169.0 ± 77.4
	31	3.9 ± 1.0	25.3 ± 5.5	9.2 ± 0.6	traces	9.2 ± 0.6	156.5 ± 26.2
	40	4.8 ± 0.2	26.8 ± 2.2	8.5 ± 0.4	traces	8.5 ± 0.4	179.2 ± 12.0
pH 6	9	3.7 ± 0.1	n.d	n.d	n.d	n.d	43.4 ± 2.0
	14	3.8 ± 2.4	n.d	n.d	n.d	traces	52.5 ± 3.2
	21	4.4 ± 1.0	n.d	n.d	n.d	traces	60.9 ± 3.0
	28	4.4 ± 0.7	n.d	n.d	n.d	traces	74.5 ± 3.9
	37	4.7 ± 0.3	n.d	n.d	n.d	traces	81.3 ± 7.0
pH 7	9	n.d	n.d	n.d	n.d	n.d	40.8 ± 2.2
	14	n.d	n.d	n.d	n.d	n.d	60.4 ± 70.8
	21	n.d	n.d	n.d	n.d	n.d	67.4 ± 0.2
	28	n.d	n.d	n.d	n.d	n.d	76.9 ± 1.0
	37	n.d	n.d	n.d	n.d	n.d	83.7 ± 2.4
pH 9	3	n.d	traces	traces	n.d	n.d	120.7 ± 24.0
	9	n.d	traces	traces	13.6 ± 6.6	traces	179.1 ± 43.0
	16	1.8 ± 2.5	traces	traces	13.9 ± 7.5	traces	184.9 ± 75.8
	23	3.7 ± 0.1	traces	traces	14.5 ± 7.7	traces	215.8 ± 62.4
	30	3.9 ± 0.1	traces	traces	14.4 ± 8.1	traces	245.9 ± 68.8
	37	3.9 ± 0.1	n.d	n.d	14.3 ± 7.6	traces	256.5 ± 64.5
pH 10	3	n.d	n.d	n.d	n.d	n.d	179.1 ± 6.0
	9	n.d	traces	traces	23.9 ± 3.1	traces	226.3 ± 8.2
	16	n.d	traces	traces	23.7 ± 0.2	traces	267.7 ± 19.7
	23	n.d	traces	traces	25.1 ± 1.8	traces	297.1 ± 8.5
	30	3.6 ± 0.2	traces	traces	23.6 ± 3.9	traces	315.5 ± 10.3
	37	3.7 ± 0.9	traces	traces	22.5 ± 0.3	traces	324.7 ± 3.3

n.d: not detected

p_phylum and k_kingdom.

Shannon, Simpson and Chao1 were used to describe the microbial communities (Hill, 1973; Chao, 1984), with the R packages vegan (Oksanen et al., 2017) and fossil (Vavrek, 2011) using data at the ASV level. The Shannon-Wiener (H) index is a measure of the diversity, where across natural systems, values are between 0.5 and 5. Simpson index evaluates the dominance of individual species within mixed populations via evaluating the probability of two random individuals belonging to the same type, where a value of 1-D = 0 represents complete dominance within the sample. The Chao1 index, an abundance-based coverage estimator, adjusts observed richness by adding a correction factor to obtain an estimated number of "absolute" species.

3. Results and discussion

3.1. Influence of pH on organic matter solubilization during anaerobic fermentation derived from OMSW

3.1.1. sCOD and VFAs accumulation at different pH conditions

Following each OMSW feeding, there was an immediately increase in the sCOD value in all reactors. Notably, sCOD increases slightly more at acidic (4 and 5) and alkaline (9 and 10) pH levels compared to neutral pH (6 and 7) (Fig. 1 A-F). At pH 9 and 10, sCOD exhibited the highest accumulation at the end of the operational time, with values of 7.8 ± 1 g O_2 L^{-1} and 9.6 ± 0.5 g O_2 L^{-1}, respectively (Fig. 1E and 1F) which is around 50 % of the total fed organic matter. Dahiya et al. (2015) stated that proteins and carbohydrates are favourably decomposed by the release of carboxylic groups at alkaline conditions, thus facilitating the solubilization of organic matter. Additionally, alkaline conditions have also been reported to hinder activity of acetoclastic and hydrogenoclastic methanogens, avoiding VFAs consumption (Cui et al., 2019; Dahiya et al., 2015). pH 4 and 5 also induced sCOD accumulation (Fig. 1A and 1B), although to a lesser extent compared to the alkaline conditions (pH 9 and 10), approximately 35 % of the total fed organic matter. These values were in the same range than the reported for other lignocellulosic substrates. For instance, in the case of the biological VFAs production from strawberry extrudate, only 55 % at pH 5 % and 50 % at pH 9 of the fed COD was hydrolysed to sCOD (Cubero-Cardoso et al., 2022). In the case of OMSW, Cabrera et al. (2019) described that at pH 5 and 9 the hydrolysis for OMSW only reached values of 50 % and 56 %, respectively, in line with the present study. Despite of the previous limited hydrolysis values, at pH 7

a 90 % degradation of organic matter was reported by the latest study (Cabrera et al., 2019). According to data published by Serrano et al. (2021), the biodegradability range of OMSW is between 57.3 % and 93.5 % at pH 7 operating at mesophilic temperature. Similarly, in the present study, at neutral pH (6 and 7) VS reduction was approximately 80–90 % (Data not shown). Likewise, less than 10 % of the total fed organic matter accumulated as sCOD (Fig. 1C and 1D), indicating that most of the fed OMSW was transformed into biogas (methane yields coefficient at pH 6 and 7 ranged between 380 and 420 mL CH_4·g VS^{-1} feed). This support that, at neutral pH conditions the methanogenic stage in the anaerobic digestion process was, as expected, favourable (Latif et al., 2017; Sun et al., 2020).

The accumulation of VFAs showed a similar increasing trend as that observed in the accumulation of sCOD (Fig. 1). The highest VFA accumulation was achieved at the end of the experimental time at operational pH 9 and 10, with values of 2.8 ± 0.4 g COD-VFA L^{-1}, and 2.6 ± 0.1 g COD-VFA L^{-1}, respectively (Fig. 1E and 1F). These values of VFAs correspond with 36 % and 27 % of sCOD, respectively. At pH 4 and 5, at the end of the experimental time, VFA accumulation reached concentrations around 0.7 g COD-VFA L^{-1} (Fig. 1A and 1B), corresponding to 19 % and 23 % of sCOD, respectively. Therefore, for the two controlled acidic conditions (pH 4 and 5), approximately 75 % less VFAs were obtained than at the alkaline conditions (pH 9 and 10). Operation at pH 6 and 7 did not result in a relevant accumulation of VFAs, with values below 0.3 g COD-VFA L^{-1} throughout the experimental time (Fig. 1B and 1C). Jiang et al. (2013) operated batch reactors using organic fraction of municipal solid wastes as substrate at different pHs, i.e. 5, 6, 7 and uncontrolled pH, obtained 17.08, 39.46 and 37.09 g VFA L^{-1}, respectively. The VFA production obtained by Jiang et al. (2013) was higher than in the present study, possibly due to the type of substrate, since OMSW is highly lignocellulosic. Despite this, at pH 5 the VFA/sCOD ratio reported by these authors was 20 %, similar to the obtained in the present research, around 23 %.

As shown in Fig. 1A, 1B, 1E, 1F, the individual VFA profile was more diverse at acidic conditions (pH 4 and 5) than at alkaline conditions (pH 9 and 10). The major VFA at pH 5 was propionic acid representing 63 % of total VFAs, whereas at pH 9 and 10 acetic acid was mainly accumulated, representing 82 % and 77 % of total VFAs at the end of the experimental time, respectively. Owusu-Agyeman et al. (2021) and Chen et al. (2013) reported a high abundance of propionic acid obtained at acid pH with high presence of carbohydrates in the substrate used. Khiewwijit et al. (2015) reported that, during VFA production from sewage sludge at pH 9 and 10, the VFA composition was dominated by acetic acid (58–63 % of the total VFAs). VFA production also was minorly composed by propionic acid (25–30 %) and butyric acid (10–15 %). These VFA profiles were in line to those obtained in similar studies (Cheah et al., 2019; Jie et al., 2014).

3.1.2. *Evaluation of phenolic compounds in anaerobic fermentation at different pH condition*

Individual and total soluble phenolic compounds were determined in the batch reactors due to the high concentration contained in the fed OMSW (Table 2). These compounds present high microbial toxicity that could result in the inhibition of bioprocesses (Chen et al., 2008; Poirier and Chapleur, 2018). At neutral pH (6 and 7) there is neither accumulation nor removal of phenolic compounds; in fact, approximately the same amount of total soluble phenols fed from OMSW were obtained on day 37 (Table 2). At both acidic and alkaline conditions phenols were accumulated (Table 2). The highest accumulation occurred at alkaline conditions, where at pH 10 reached concentration values up to three times the phenol concentration in the OMSW (Table 2). The increase of the phenolic compounds has been related to the disruption of lignocellulosic fibres (Hendriks and Zeeman, 2009), like in the hydrolysis of OMSW (Serrano et al., 2017b). The phenolic compounds are released during the breaking down of the olive pulp cell walls, whose main components are cellulose and pectic polysaccharides, and where the phenolic compounds can be found as minor components (Rubio-Senent et al., 2013). Although the concentration of phenols increased throughout the experimentation in the digesters at acidic and alkaline conditions, the values obtained were lower than the limits of inhibition for the fermentation process at the different pH tested. According to Calabrò et al. (2018), the concentrations of phenols that can be considered inhibitory in the AD process were values greater than 2000 mg L^{-1}.

The total concentration of each of the individual phenols coming from OMSW and added during the whole experimental time to each reactor were accounted by 38.34 ± 2.0 mg 3,4-DHPG L^{-1}, 360 ± 17.0 L^{-1} mg HT L^{-1} and 103.5 ± 3.0 mg Ty L^{-1}. Considering the measured concentration of these individual compounds in the reactors at the end of the experimental time, these phenols have been highly degraded across all pH conditions tested (Table 2). Remarkably, removal efficiencies exceeding 70 % were achieved for all of these individual phenols with some of them reaching removal efficiencies up to 100 % (Table 2). It is worth to notice that during anaerobic fermentation at pH 5, 9, and 10, two discernible phenolic compounds were identified which were absent in the original fed OMSW. At pH 5, the detected compound was 4-EP, while at pH 9 and 10, 4-MC was observed. In the similar study with OMSW by Cabrera et al. (2019), the authors reported the presence of 4-EP in their anaerobic fermenters at pH 5 as an outcome of their experiment. 4-EP and 4-MC have been described as intermediate metabolites in the anaerobic degradation of more complex phenols (Serrano et al., 2017a). For example, 4-EP can be generated by decarboxylation of some phenolic acids from OMSW, such as *p*-coumaric, ferulic or caffeic acids (Milheiro et al., 2019; Rubio-Senent et al., 2013).

3.2. *Impact of the operational pH in the microbial populations*

Three samples were extracted directly from each of the reactors on day 0 (initial conditions – T0), around the mid operation time, between day 22 and day 30 (mid HRT – T1) and day 45 (the last operating day – T2). The samples were taken for further microbial profile analysis. RNA extraction, followed by retrotranscription and 16 S rDNA sequencing was performed in order to observe correlations between operational conditions, and VFA production with the activity of microbial population.

All samples at the different pH conditions at T0 had high similarity values in terms of diversity as observed in Fig. 2 and Table 3. At neutral pH (6 and 7), diversity of the microbial community remained stable throughout the experimental time, showing similar values of diversity indices (Table 3). Only a slight change in diversity indices was observed in the neutral pH at T1and T2 which could be

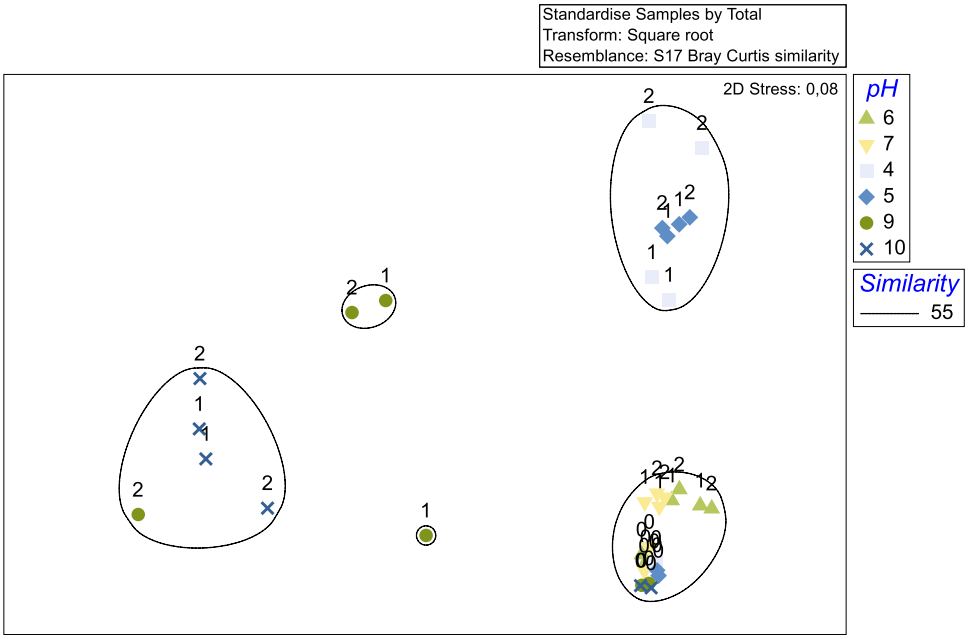

Fig. 2. Multi-dimensional scaling (MDS) of bacterial communities at species level based on Bray-Curtis dissimilarity index. Each data point label is the time at which the sample was taken (0 – initial, 1 – mid, 2 – final experimental time points). Replicates are observed for each condition.

Table 3
Diversity, dominance and Species richness indices.

	Samples	Shannon Index	Simpson 1-D	Chao1
pH 4	T0	5.56 ± 0.09	0.989 ± 0.001	1068.5 ± 6.36
	T1	4.28 ± 0.1	0.967 ± 0.005	533.5 ± 28.99
	T2	3.68 ± 0.39	0.945 ± 0.01	281 ± 46.67
pH 5	T0	5.47 ± 0.09	0.987 ± 0.002	1054 ± 46.67
	T1	3.6 ± 0.05	0.901 ± 0.002	340 ± 29.7
	T2	4.11 ± 0.13	0.962 ± 0.011	349.5 ± 3.54
pH 6	T0	5.48 ± 0.07	0.986 ± 0.002	1040.5 ± 31.82
	T1	4.96 ± 0.09	0.981 ± 0.002	717 ± 26.87
	T2	4.88 ± 0.23	0.981 ± 0.007	657.5 ± 26.16
pH 7	T0	5.42 ± 0.02	0.987 ± 0.001	905 ± 32.53
	T1	5.12 ± 0.07	0.984 ± 0.003	674 ± 11.31
	T2	5.08 ± 0.04	0.986 ± 0.001	642.5 ± 9.19
pH 9	T0	5.43 ± 0.03	0.989 ± 0.001	887 ± 41.01
	T1	4.33 ± 0.6	0.959 ± 0.029	385 ± 72.12
	T2	3.42 ± 0.22	0.881 ± 0.022	248.5 ± 222.74
pH 10	T0	5.41 ± 0.1	0.988 ± 0.001	850.5 ± 21.92
	T1	2.9 ± 0.16	0.832 ± 0.058	92.5 ± 34.65
	T2	2.79 ± 0.45	0.794 ± 0.094	116.5 ± 37.48

T0: day 0 (sample initial conditions); T1: sample mid TRH; T2: sample last operational day.

explained by the adaptation of the inoculum from treating sewage sludge from a wastewater treatment plant to OMSW. The latter was also observed in Fig. 2, where all neutral pH samples were clustered with the T0 data points. Therefore, the maintenance of a similar microbial structure can explain that methanogenic activity was maintained in the reactors operating at pH 6 and 7, thus, leading to VFA consumption (Fig. 1 and supplementary material, Table S1). The species' relative abundances at T0, did not present large variations in the different pH conditions studied (Fig. 3). Species' relative abundances at neutral pH conditions for all time points, were similar to those at T0. The species with the highest relative abundances in all samples at T0, and all samples analysed for neutral pH (6 and 7) were as follows: *f_Peptostreptococccaceae, o_Bacillales_2, g_Acetobacterium, c_Gammaproteobacteria, p_Bacteroidetes* and *o_Bacteroidales.* Species *c_Deltaproteobacteria, p_Proteobacteria, c_Gammaproteobacteria, k_Bacteria,* whose relative abundances at neutral pH (6 and 7) were high throughout the experiment, may be involved in syntrophic methanogenesis processes (Pannekens et al., 2019).

Opposite trends were observed from the neutral pH reactors with acidic (4 and 5) and alkaline (9 and 10) pH reactors, such as a drop in Shannon Index at T1 and T2 (Table 3). The diversity reduction of the Shannon index values was accompanied by a decrease in the Simpson (1-D) and Chao1 indices. Hence, the latter indicates a decrease in species number (ChaoI) and an increase in dominant

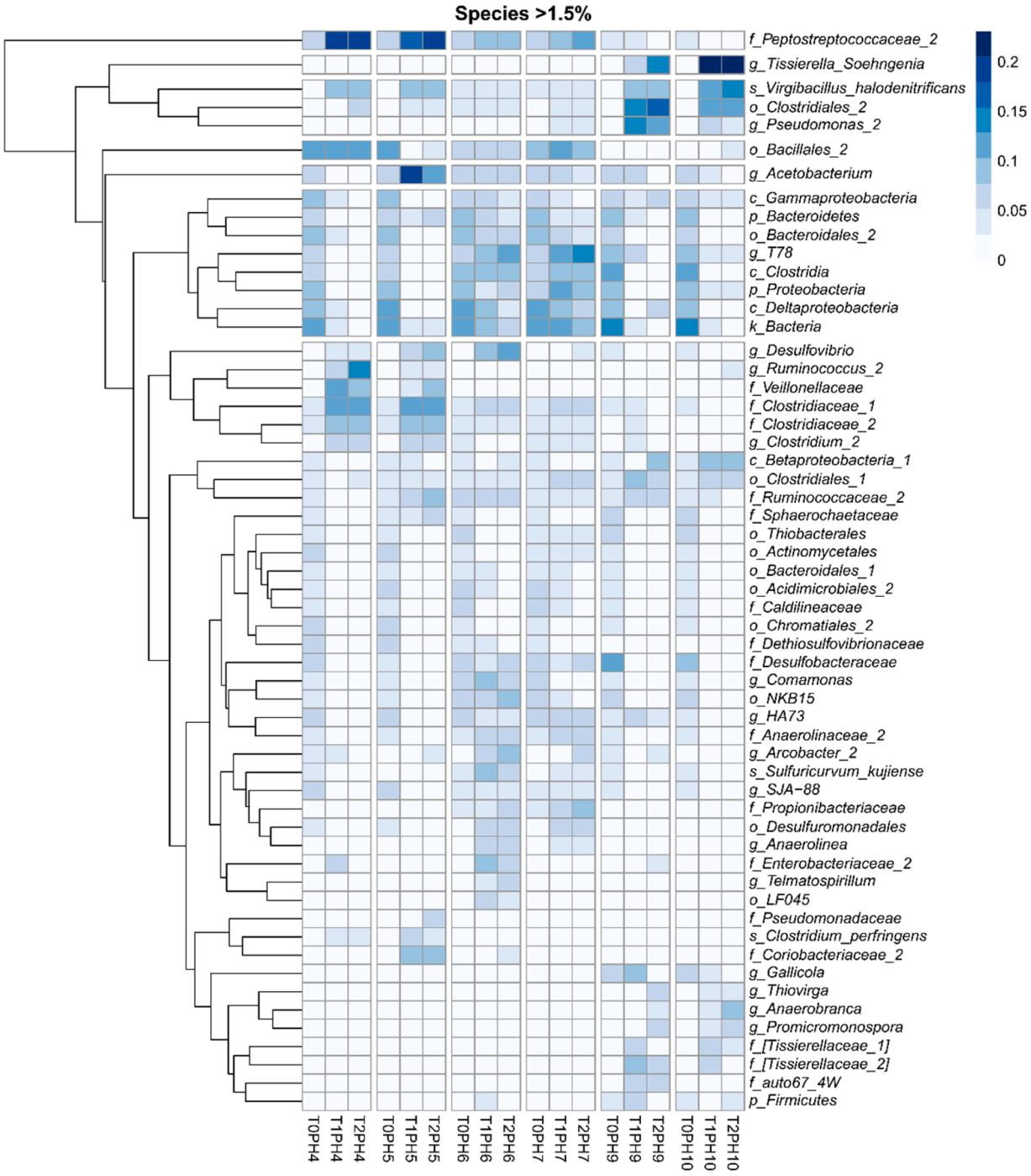

Fig. 3. Cluster analysis of microbial species in the digestate samples at different pH conditions. The colour scale indicates the $\log_{10}$ of the square root of the mean relative abundance. The included species were limited to OTUs with a relative abundance of $>$ 1.5 %, in at least 1 sample. Taxa labels are based on the last known taxon classification. Rows are clustered using Euclidean distance.

species (Simpson 1-D) as a consequence of the adaptation occurring within the microbial communities in the digesters at the more extreme operational pH (Harirchi et al., 2022). This described specialization would explain the methanogenic activity cessation, usually related to neutral pH conditions (Latif et al., 2017; Sun et al., 2020), and the increment in VFA accumulation (Fig. 1 and supplementary material, Table S1). The highest decrease in the Shannon and Simpson (1-D) index was observed in the reactor operating at alkaline conditions (Table 3). Both acidic and alkaline conditions resulted in specialization of the microbial structure for each condition, as can be observed in Fig. 2, where each extreme pH is clustered together, especially at acidic conditions (>55 %

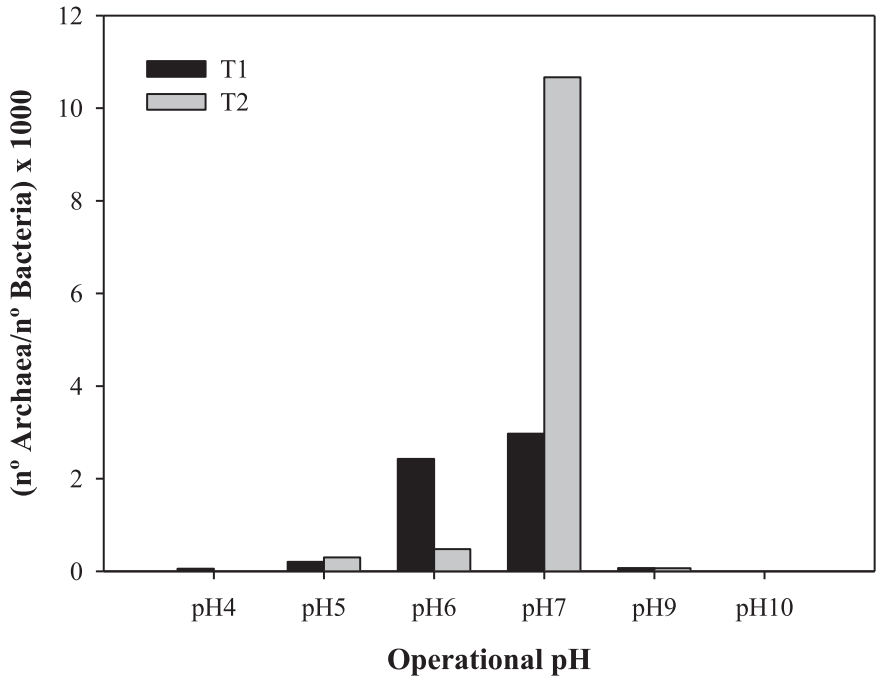

Fig. 4. Ratio between the number of archaea and bacteria (x1000) at the intermediate and final time at each operational pH, quantified by qPCR (n = 6), where T1: mid TRH sample; T2: last operational sample.

similarity) at the end of the experimental period (T2). In acidic conditions there was a marked increase in the relative abundance of *f_Peptostreptococcaceae_2,* and to a lesser extent, an increase in *s_Virgibacillus_halodenitrificans* and *f_Clostridiaceae* (Fig. 3). The *Peptostreptococcus* genus contains coccobacillus and cocci that are producers of acetic, butyric, iso-butyric, iso-valeric and iso-caproic acids (Downes and Wade, 2006; Murdoch, 1998). This may explain the greater diversity in the VFA profile at pH 4 and 5 (Fig. 1 and supplementary material, Table S1).

Although microbial populations at alkaline conditions were similar, clustering of these samples was much lower (35 %) than at acidic conditions (Fig. 2). At alkaline conditions, lower diversity and higher dominance values were observed, i.e. lower Shannon and Simpson indices values (Table 3). Hence, a less complex VFA profile was correlated with a lower number of microorganisms performing most of the microbial activity at these alkaline conditions. The species with the highest relative abundance were *g_Tissierella_soegngenia, s_Virgibacillus_halodenitrificans, o_Clostridiales_2* and *g_Pseudomonas_2* (Fig. 3). The enrichment of the microbial population in *Tissirella* would explain the high production of acetic acid, since this is one of the major metabolic end products of this genus, along with butyric and iso-valeric acids (Alauzet et al., 2014; Lawson, 2019). Some species of genus *Tissirella,* such as *Tissierella creatinini*, present an optimal growth pH in the alkaline range (Farrow et al., 1995; Lawson, 2019), that would explain the enrichment in *Tissierella* observed at pH 9 and 10 (Fig. 3).

The total bacterial and archaeal abundance of the inoculum were 1.02×10^{10} and 5.99×10^{7} copies per g of biomass, respectively. The variation of operational pH entailed a high change in the ration between archaea and bacteria in the reactors (Fig. 4 and supplementary material, Table S2). The operation at pH 7 favoured a higher prevalence of the archaeal population respect to the number of total bacteria, reaching a archaea/bacteria ratio (x 1000) higher than 10. At pH 6 at T1, the archaea/bacteria ratio at intermediate time was similar than the obtained at pH 7, although a longer operation resulted in a decrease of the ratio (approximately 0.48 at T2). The operation at both acidic and alkaline conditions strongly affected the archaeal population, resulting in archaea/bacteria ratios (x 1000) values below 0.3 (Fig. 4). These results were expected since the methanogenic activity has been widely associated to neutral pH (Latif et al., 2017; Sun et al., 2020). Therefore, the VFA accumulation resulted in a negative effect on the total archaeal abundances without strong impact on those of *Bacteria* (Figs. 1 and 4), suggesting that the control of the operational pH can be a suitable strategy to inhibit the methanogenic activity and, thus, to promote the VFA accumulation.

4. Conclusions

The anaerobic fermentation of OMSW has been investigated across a broad pH range. It has been observed that in acidic conditions a diverse profile of VFAs were present in the reactors. Alkaline condition presented the highest VFA production but with a much a less diverse VFA profile, where almost all the generated VFAs corresponded to acetic acid. Phenols were extensively removed at all the tested conditions. The differences in the microbial composition among reactors at acidic and alkaline conditions could explain the higher diversity generated in the VFA profile at acidic conditions with respect to alkaline conditions. Thus, a lower diversity and higher dominance corresponded to a less diverse VFA profile.

CRediT authorship contribution statement

E. Jiménez-Páez: Methodology, Formal analysis, Writing - Original Draft. **A. Serrano:** Conceptualization, Writing - Original Draft, Funding acquisition. **J. Purswani:** Formal analysis, Resources, Writing - Review & Editing. **D. Correa-Galeote:** Methodology, Resources. **Juan Cubero-Cardoso:** Formal analysis, Writing - Review & Editing. **F.G. Fermoso:** Conceptualization, Funding acquisition, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonio Serrano reports financial support was provided by Spanish Ministry of Economy, Industry and Competitiveness. Antonio Serrano reports financial support was provided by Consejería de Transformación Económica, Industria, Conocimiento y Universidades. Juan Cubero-Cardoso reports financial support was provided by Spanish University Ministry.

Data availability

The sequence data from this study have been submitted to NCBI BioProject (http://www.ncbi.nlm.nih.gov/bioproject) under BioProject number PRJNA1019715.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2023.103409.

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