Impact of monoterpenes in the stability of the anaerobic digestion of Mediterranean Wholesale Market Waste

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ABSTRACT

The stability and robustness of long-term anaerobic digestion process of fruit and vegetable waste (FVW) generated in Mediterranean wholesale markets were assessed, monitoring potential inhibitory volatile organic compounds (VOCs) such as volatile fatty acids (VFAs) and/or monoterpenes. Particle size (4 and 10 mm) and organic loading rate (OLR) were evaluated during the experimentation. Anaerobic digestion remained stable at OLR of 1.0 g VS L^{-1} d^{-1} (VS, total volatile solids) regardless of the feedstock particle size. At this OLR, methane yield was 254 ± 8 and 229 ± 11 mL CH_4 g VS^{-1} for 4 and 10 mm, respectively. As the OLR increased above 1.0 g VS L^{-1} d^{-1}, the system rapidly destabilized, and a high accumulation of VFAs was observed. In parallel of to the VFAs accumulation, other VOCs such as fenchone also reached concentrations that contributes to the destabilisation of the process. The destabilization process at an OLR of 3.0 g VS L^{-1} d^{-1} also resulted in a drastic reduction in the relative abundance and species diversity of methanogenic archaea in all digesters.

1. Introduction

The centralised generation of large quantities of fruit and vegetable wastes (FVWs) in wholesale markets can facilitate the implementation of more efficient management technologies for theses wastes [1]. Among all the technologies that have been investigated and evaluated as an alternative to FVW management, anaerobic digestion (AD) is considered one of the safest from an environmental perspective [2–4].

Previous research has reported some challenges to be addressed for the AD application of FVW [5–10]. For instance, requirement of pre-treatment to achieve an homogeneous feeding, the high seasonality of FVW, or the low organic loading rate (OLR) applied, among others. The rapid degradability of FVW can cause volatile fatty acids (VFAs) accumulation, which stresses and inhibits the activity of the methanogenic archaea and does not allow for high OLRs. Two-stage AD [11], co-digestion with other less biodegradable feedstocks [2,12], or a balanced fruit and vegetable mixture [4,13] have been evaluated as solutions. Another possible solution could be to decrease the particle size of FVW on the AD process as it could improve the hydrolysis stage,

and improve substrate homogeneity [14,15]. However, Trujillo-Reyes et al. [1] reported that particle size could positively or negatively affect methane production depending on the waste characteristics and/or the composition of a mixture of several wastes. Particle size reduction could accelerate the hydrolysis and acidogenesis stages, causing undesirable peaks of organic compounds in the reactor or releasing inhibitory compounds leading to a decrease in methane production [16–19].

Likewise, fruits, vegetables, and edible aromatic herbs are rich in volatile organic compounds (VOCs), such as terpenes. Many terpene compounds, mainly monoterpenes, present in fruits, vegetables, and aromatic herbs, such as limonene, fenchone, 4-terpineol, α-terpineol, and carvone, among others, have been associated to potent antibacterial, antimicrobial, and antioxidant activities [20–22]. The knowledge about the inhibitory potential of monoterpenes in the AD process is scarce [23], focusing almost only on limonene, found in citrus peel fruits and the seeds of some aromatic herbs such as fennel [24–27]. Moreover, although some experiments in batch have showed that the microbial populations in AD systems could be adapted against some terpenes [26,

28], there is a lack of knowledge on the potential effect on the long-term anaerobic digesters' stability.

The main objective of the research was to assess the stability and robustness of the long-term anaerobic digestion of FVW generated in the Mediterranean wholesale market. The process was evaluated at two particle sizes feedstock, i.e., 4 and 10 mm, varying the OLR throughout the experimental time. For assessing the stability and robustness of the process, methane production, stability parameters such as pH, and VOCs accumulation, including both VFA and monoterpenes, just as microbial population adaptation, were monitored.

2. Materials and methods

2.1. Feedstock composition and inoculum

A model FVW mixture generated in the autumn and winter seasons in a Mediterranean wholesale market was used as feedstock as described by Papirio et al. [29]. The FVW mixture consisted of a total of 23 products, including pomegranate (1.5%), coriander (2.0%), cucumber (2.0%), eggplant (2.0%), lemon (2.0%), pepper (2.0%), potato (2.0%), green beans (2.5%), celery (3.0%), courgette (3.0%), broccoli (4.0%), green cabbage leaves (4.0%), mandarin (4.0%), parsley (4.0%), pear (4.0%), apple (5.0%), cauliflower leaves (5.0%), grapefruit (5.0%), fennel leaves (6.0%), tomato (7.0%), carrot leaves (8.0%), onion leaves (8.0%) and orange (12.0%).

Fresh sludge from an industrial anaerobic reactor from the "COPERO" wastewater treatment plant in Seville (Spain) was used as inoculum source. The main anaerobic inoculum characteristics were pH = 7.4 ± 0.1; alkalinity = 8600 ± 78 mg $CaCO_3$ L^{-1}; total solids (TS) = 42 ± 1 g kg^{-1} and total volatile solids (VS) = 23 ± 1 g kg^{-1}.

2.2. Particle size reduction of feedstock

Two particle sizes of the FVW mixture were studied, i.e., 4 and 10 mm. FVW mixture was chopped to the desired particle size through a multi-functional slicer (*Seehoom*, model: B436–09) for 10 mm particle size and a mincer with a 0.5 L of capacity (*Moulinex*, model: Multi moulinette AT714G32) for 4 mm particle size, previously described by Trujillo-Reyes et al. [1]. FVW mixtures were prepared monthly following the recipe, except for the last mixtures that were prepared without fennel (feeding from day 78), and all were stored in plastic bags at − 20 °C until use. The main characteristics of the FVW mixture at different particle sizes are reported in Table 1, and the volatile organic compounds profile is found in Fig. 1 for both particle size, hereafter referred as feedstock.

Table 1
Physicochemical characterization of the FVW feedstocks according to particle size.

	FVW feedstocks	
Particle size (mm)	4	10
pH	4.5 ± 0.1	4.1 ± 0.1
Moisture (%)	89.1 ± 0.3	89.7 ± 0.4
TS (g kg^{-1})	109.4 ± 3.1	103.4 ± 4.3
VS (g kg^{-1})	94.9 ± 3.4	99.9 ± 2.9
C (%)	44.8 ± 0.3	46.5 ± 0.3
N (%)	2.3 ± 0.1	1.9 ± 0.1
C/N ratio	19	25
tCOD (g O_2 kg^{-1})	151.3 ± 1.7	175.6 ± 3.8
sCOD (g O_2 L^{-1})	66.7 ± 0.3	59.7 ± 0.7
Carbohydrates (mg L^{-1})	44.0 ± 0.6	28.9 ± 0.1

FVW: fruit and vegetable waste; TS: total solids; VS: volatile solids; tCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand.
(*) Results are given as means ± SD from triplicate samples.

2.3. Semi-continuous anaerobic digestion experimental procedure

The experiments were conducted in six semi-continuous anaerobic digesters, i.e., a triplicate of digesters for each studied particle size. All digesters had a working volume of 1.7 L (total volume of 2.0 L) and were inoculated with 10 g of VS L^{-1} of fresh sludge. The digesters were placed in a thermostatic chamber to maintain the operating temperature at 35 ± 2 ºC and kept under continuous agitation using a stirrer (KMO 2 basic model, IKA-WERKE, Germany). The methane production was measured daily by liquid displacement after removing CO_2 through hermetically sealed bubblers containing a 2 N NaOH solution and expressed under standard pressure and temperature conditions (25 ºC and 1 atm).

The experimental study was carried out using different OLRs (Table 2). The digesters were manually fed the feedstocks five times a week, from Monday to Friday. Additionally, on days 37 and 72, the digesters were supplemented with 1.7 mL of a macronutrient solution prepared as Raposo et al. [30] described. In addition, on day 42, the digesters were partially re-inoculated with 2 g VS L^{-1} of fresh inoculum.

2.4. Chemical analyses

By following the Standard Methods of the American Public Health Association (APHA) [31], pH, alkalinity, total solids (TS) and VS concentration, and total chemical oxygen demand (tCOD) were analysed. Elemental C and N were determined through a combustion carbon and nitrogen analyzer (*LECO CN828*) by Dumas's method according to the Standard Methods of APHA [31] drying the sample previously. The anthrone colorimetric method was used to determine water-soluble carbohydrates [32], applying previously a water extraction widely used to analyse soluble compounds in composted materials [33].

In the digesters, fed organic matter degradation was monitored by soluble organic matter accumulation, measured as soluble COD (sCOD), VFAs, and monoterpenes concentration. sCOD was analysed using the APHA method [31]. VFAs (C_2-C_5) were determined in duplicate using a gas chromatograph *Shimadzu* GC-2025 equipped with a Stabilwax-DA column (Crossbond Carbowax polyethylene glycol, *RESTEK*) of 0.25 mm i.d. x 30 m and a flame ionization detector (FID) at 250 ºC. The oven temperature gradually increases from 100 to 170 ºC at 5 ºC min^{-1}. Nitrogen (30 mL min^{-1}), hydrogen (40 mL min^{-1}), and air (400 mL min^{-1}) was used as carrier gas at a flow rate of 40 mL min^{-1} at 504 kPa. VOCs were analysed in duplicate using an *Agilent Technologies* HP-6890 gas chromatograph equipped with a DB-Wax capillary column (J&W, Scientific, Folsom, CA, USA) of 0.25 mm i.d. x 60 m and film thickness 0.25 μm. Liquid samples (0.5 mL) from feedstocks (after water extraction) and digesters at the end of each stage were collected and conditions to room temperature and then placed in a vial heater at 40 ºC. After 10 min equilibrium time, VOCs from headspace were adsorbed on an SPME fibre DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA, USA). The sampling time was 50 min at 40 ºC. Desorption of VOCs trapped in the SPME fibre was done directly into the GC injector. Compound identification was carried out on a Hewlet–Packard HRGC–MS 5975 series MSD equipped with the same column and oven program (more details on the operating conditions and compound identification are described in Abdallah et al. [34]). The concentration of most relevant identified VOCs, which corresponded to monoterpenes fenchone, limonene, α-terpineol, and 4-terpineol, were quantified by means of external calibration curves made using pure commercial standards. (+)-Fenchone, (R)-(+)-Limonene, (+)-α-terpineol and (+)− 4-terpineol were supplied by Sigma-Aldrich (St. Louis, MO, USA). The biogas composition (CH_4, CO_2, O_2, and N_2) was analysed from the digesters using a gas chromatograph *Shimadzu* GC-2014 described by Trujillo-Reyes et al. [1]. Each biogas sample was taken from the digesters head space using 1 mL plastic syringes fitted with a special gas valve (Mininert, *Supelco*). All measurements were made in triplicate to obtain mean and standard deviation values.
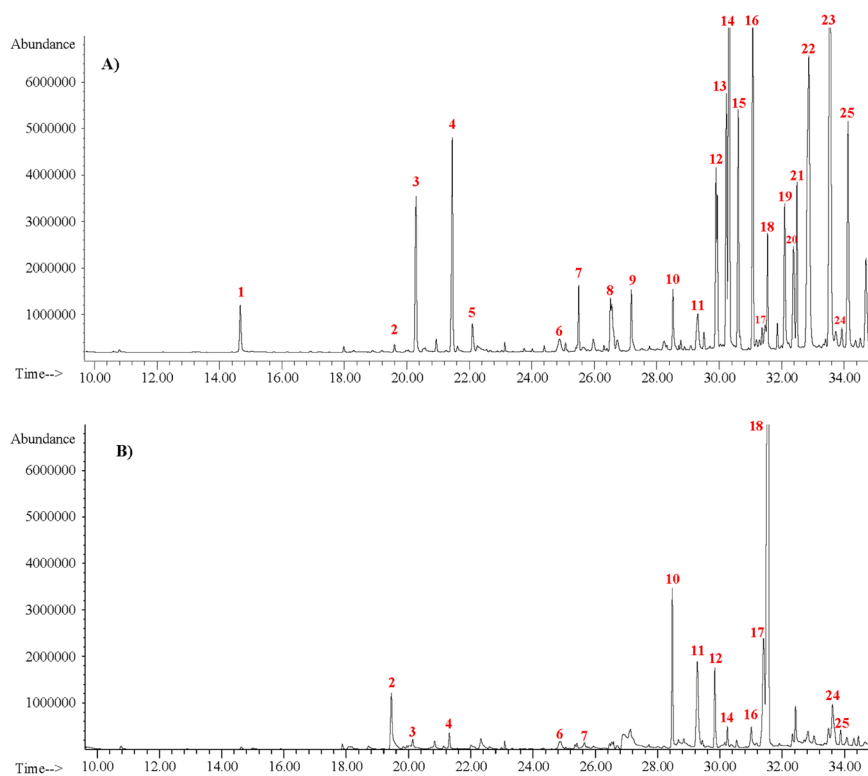
Fig. 1. Chromatograms of the volatile organic compounds identified by gas chromatography-mass spectrometry (GC-MS) in the feedstock characterization according to particle size **A)** 4 mm and **B)** 10 mm (1.Methyl disulfide; 2.Limonene; 3.Unidentified Hydrocarbon; 4.Unidentified Hydrocarbon; 5.Unidentified Hydrocarbon; 6.Hexanol; 7. Fenchone; 8.Styrene; 9.Unidentified Hydrocarbon; 10. Linalool; 11.Octanol; 12.4-Terpineol; 13.Carvone; 14. Menthadienol; 15.Dihydrocarvone; 16.Cis-Menthadienol; 17.p-Cresol; 18.α – Terpineol; 19.Unidentified Hydrocarbon; 20.Octane-2,4,6-triol; 21. Carvol; 22.Trans-Carveol; 23.Carvacrol; 24.Antioxidant Nº 33; 25. Cis-Carveol).

Table 2
Experimental design.

Experimental stage	Period (d)	OLR (g VS L^{-1} d^{-1})
Stage I	0 – 20	1.0
Stage II	21 – 29	3.0
Stage III	30 – 96	1.0
Stage IV	97 – 107	1.5

OLR: organic loading rate; VS: volatile solids.

2.5. Microbiology

2.5.1. DNA extraction and 16S rRNA gene sequencing

Samples for microbial analysis were collected from digesters at the beginning of the experiment and at the end of each stage. Samples were stored at − 80 °C until further processing. DNA extraction was performed using MagNA Pure LC DNA Isolation kit III (Bacteria, Fungi) (Roche Diagnostics GmbH, Germany) following the manufacturer's protocol. The V3-V4 region of the 16 S gene was amplified using the primers − 16S V3-V4 Forward (5´- TCGTCGGCAGCGTCAGATGTGTA-TAAGAGACAG CCTACGGGNGGCWGCAG - 3′) – 16 S V3-V4 Reverse (5´- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGG GTATCTAATCC - 3′) to target both bacteria and archaea [35]. 16 S rRNA amplicon sequencing was conducted using the Illumina MiSeq platform (Illumina, USA) using microbial genomic DNA (5 ng μL^{-1} in 10 mM Tris pH 8.5). 16 S rRNA amplicon sequences were processed with the DADA2 v.1.16.0 pipeline [36] implemented in RStudio (v.4.0.4) [37]. Forward reads were truncated at 280 bp to retain sequences with a quality score > 30. Reverse reads were truncated at 240 bp to retain sequences with a quality score > 20. Reads with > 1 expected error were discarded. Dereplication, amplicon sequence variants (ASV) inference, and chimera removal were run with DADA2 default parameters. Taxonomic assignment of the ASVs was performed using the *assignTaxonomy*() function in DADA2 with minimum bootstrap confidence of 80% using MiDAS 4 ecosystem specific database [38].

2.5.2. Downstream data processing and statistical analyses

Processing of 16 S rRNA gene data was carried out in R v4.0.4 (R Core Team, 2019, https://www.R-project.org/) using ampvis2 v.2.7.32 [39] and tidyverse v.1.3.6 [40] packages. The total reads per sample ranged from 61926 to 105737. The relative abundance of genera was visualised in heatmaps, where the putative function of know genera was assigned according to literature. Due to the different treatments, differences in microbial community beta-diversity were explored by principal component analysis (PCA) in ampvis2. Reads were Hellinger transformed previous PCA analysis. Heatmaps were used to visualise microbial composition during the different operational stages. Microbial species were assigned to known functional groups based on the main *in situ* metabolism described to occur in AD (https://www.mid-asfieldguide.org/guide/search) and literature sources. Wilcoxon test was performed to identify taxa differentially abundant between the two tested particle sizes.

3. Results

3.1. Stage I: start-up

During stage I (0–20 days), OLR = 1.0 g VS L^{-1} d^{-1}, a stable performance was maintained, obtaining methane productions with mean values of 254 ± 8 and 229 ± 11 mL CH$_4$ g VS^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Fig. 2A). The biogas composition remained almost constant and had only detected CH$_4$ and CO$_2$ with around a 50:50 ratio (Fig. 2B).

Concerning process stability, pH shortly varied between 6.5 and 7.5 for digesters, while the alkalinity decreased from 4000 to around 2000 mg CaCO$_3$ L^{-1} during this initial stage (Figs. 3A and 3B). The soluble organic matter accumulation measured as sCOD was maintained below 2000 mg O$_2$ L^{-1} for all digesters (Fig. 4A). In contrast, there was no accumulation of VFAs during these 21 days, i.e., the maximum VFAs total values detected during stage I only reached 1025 ± 28 and 986 ± 106 mg O$_2$ L^{-1} for digester fed with 4- and 10-mm particle sizes feedstocks, respectively, at day 8 (Figs. 4B and 4C). Three major VOCs of
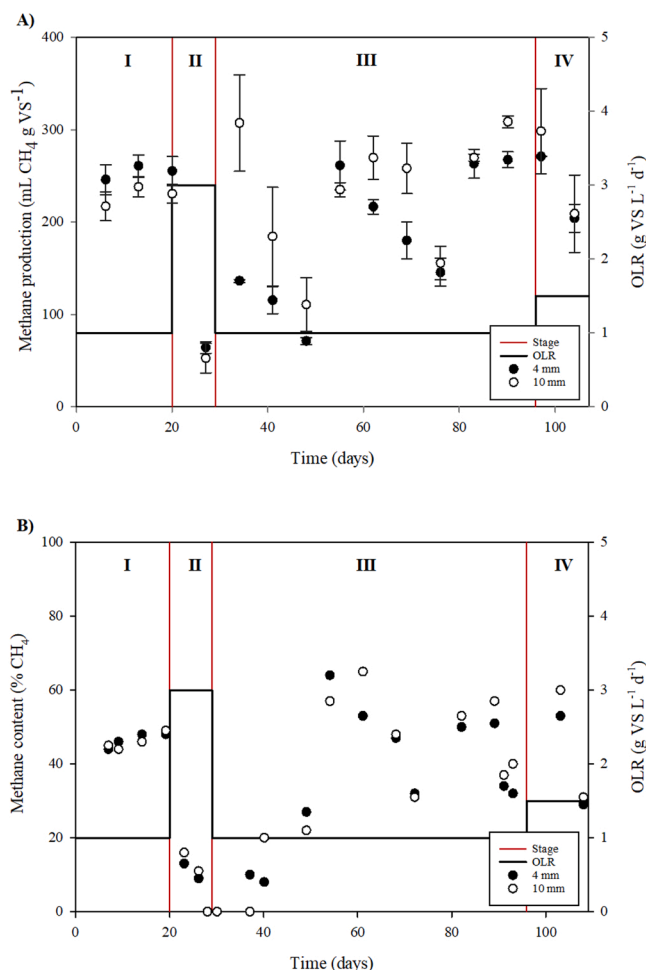
Fig. 2. A) Weekly variation of methane production (mL CH_4 g VS^{-1}) and **B)** daily variation methane content (% CH_4), according to particle size, under the different organic loading rates (OLR) during the experimental time.
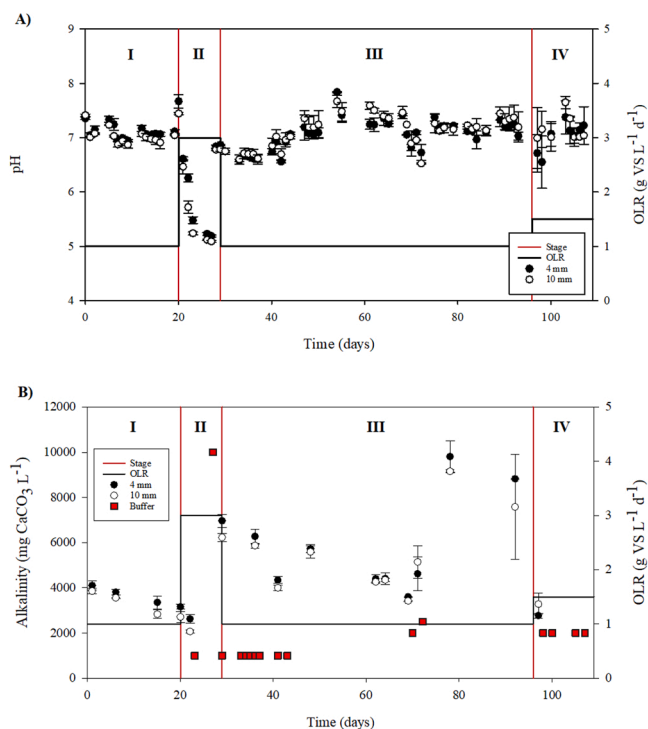

Fig. 3. Daily variation of **A)** pH and **B)** alkalinity (mg $CaCO_3$ L^{-1}), according to particle size, under the different organic loading rates (OLR) during the experimental time.

terpene group, i.e., fenchone, 4-terpineol, and α-terpineol, were identified for all digesters at the end of stage 1 (21-day). Fenchone and α-terpineol concentrations were twice as high in the digesters fed with the 10 mm particle size feedstock as with the 4 mm particle size feedstock (Table 3 and Fig. 1 S). Concretely, fenchone was the major compound, reaching a concentration of 4.44 mg L^{-1} and 8.03 mg L^{-1} for 4- and 10-mm particle size feedstocks, respectively. No limonene peak was detected in the digesters on day 21 (Table 3 and Fig. 1 S).

3.2. Stage II: Abrupt OLR change

In stage II (21–29 days), the OLR was increased from 1.0 to 3.0 g VS L^{-1} d^{-1}. The digesters showed a marked drop in methane production, i. e., around a 75% decrease than stage I (Fig. 2A). The methane content in the biogas composition also diminished, reaching almost 100% of carbon dioxide on day 28 (Fig. 2B).

The digesters showed a gradual decrease in pH and alkalinity, displaying pH values in a broader range than in the previous stage, i.e., 7.5–5.0. Thus, 10 g of $NaHCO_3$ L^{-1} buffer was added to each digester on day 27 to maintain the pH among the recommended range and avoid further acidification of the digesters (Figs. 3A and 3B). sCOD showed a significant increase achieving values around 10,000 mg O_2 L^{-1} for digesters fed with both particle sizes feedstocks (Fig. 4A). Most of the accumulated sCOD corresponded to the accumulation of VFAs, whose total values at the end of stage II were 10,120 ± 240 and 10,215 ± 255 mg O_2 L^{-1} for digesters fed with 4- and 10-mm particle sizes

feedstocks, respectively. Acetic and propionic acids comprised around 80% of the accumulated VFAs, reaching values of 3870 ± 180 and 5030 ± 100 mg O_2 L^{-1} for digesters fed with the 4 mm particle size feedstock and 3625 ± 90 and 4465 ± 190 mg O_2 L^{-1} for digesters fed with the 10 mm particle size feedstock, respectively, at the end of stage II (Figs. 4B and 4C). Fenchone remained the most abundant VOC in all digesters, with 80% of the total GC-MS quantified concentrations on day 29 (Table 3 and Fig. 1 S). At the end of this stage, fenchone, 4-terpineol, and α-terpineol concentrations increased significantly, being higher for digesters fed with 10 mm particle size feedstock than with 4 mm particle size feedstock. For digesters fed with the 4 mm particle size feedstock, the concentration of fenchone, 4-terpineol, and α-terpineol increased by 26%, 152%, and 41%, respectively, respect to the end of the previous stage 1. Whereas for digesters fed with the 10 mm particle size feedstock, the increased were 15% for fenchone and 200% for 4-terpineol, i. e., 9.20 mg L^{-1} and 0.96 mg L^{-1}, respectively, concerning the concentration of the end of stage I (Table 3 and Fig. 1 S). For 10 mm particle size substrate, α-terpineol showed a similar concentration than at the end of the previous stage 1, i.e., 1.33 mg L^{-1}. All limonene fed with the feedstocks was degraded according to the results obtained on day 29 (Table 3 and Fig. 1 S).

3.3. Stage III: recover of the system performance

In stage III (30–96 days), the OLR was reduced from 3.0 to 1.0 g VS L^{-1} d^{-1}. On day 37, Owing to the lack of positive response in the digesters operation to the decrease of OLR and buffer addition, a 1 mL L^{-1} of a micronutrient solution (composition described in Section 2.3) was added to each digester. After that, on day 40, approximately 850 mL of the digester working volume was extracted to remove part of the accumulated soluble compounds. The extracted volume was centrifuged at a low speed of 2000 rpm for 20 min to minimize the microorganisms' lysis. Subsequently, the liquid fraction was removed while the solid fraction was fed back into the digester by adding water to replace the removed liquid. After centrifugation, the concentration of sCOD
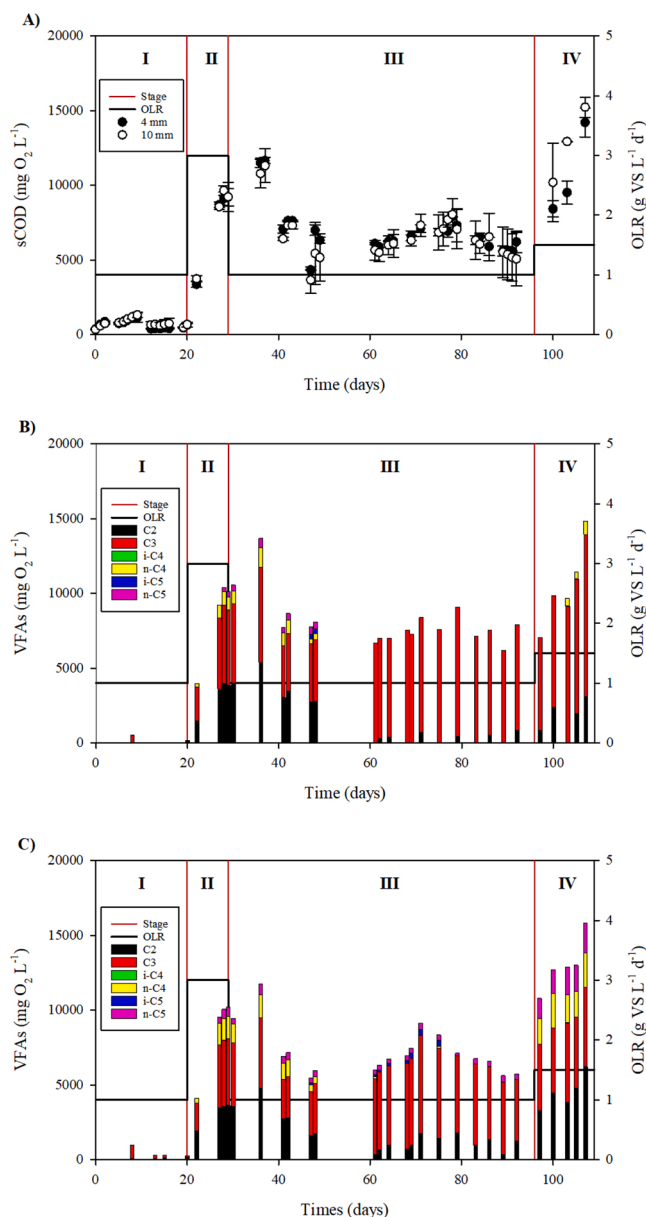
Fig. 4. Daily variation of **A)** sCOD (mg O$_2$/L), and the individual VFAs (mg O$_2$/L) according to particle size **B)** 4 mm and **C)** 10 mm, under the different organic loading rates (OLR) during the experimental time (C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; n-C4: butyric acid; i-C5: iso-valeric acid; n-C5: valeric acid).

Table 3
Concentration of quantified monoterpenes by GC-MS at the end of each stage of the experiment.

Compound	Retention time (min)	Concentrations for digesters fed with 4 mm particle size feedstock (mg L^{-1})			
		Day 21	Day 29	Day 96	Day 107
Limonene	19.450	n.d.	n.d.	n.d.	< 0.001
Fenchone	25.740	4.44 ± 0.18	5.61 ± 0.24	4.53 ± 0.19	2.91 ± 0.10
4-Terpineol	29.837	0.23 ± 0.01	0.59 ± 0.04	0.76 ± 0.05	0.85 ± 0.06
α-Terpineol	31.487	0.66 ± 0.04	0.93 ± 0.06	1.07 ± 0.07	0.89 ± 0.06
Compound	**Retention time (min)**	**Concentrations for digesters fed with 10 mm particle size feedstock (mg L^{-1})**			
		Day 21	**Day 29**	**Day 92**	**Day 107**
Limonene	19.450	n.d.	n.d.	n.d.	< 0.001
Fenchone	25.740	8.03 ± 0.33	9.20 ± 0.38	5.16 ± 0.22	3.86 ± 0.16
4-Terpineol	29.837	0.32 ± 0.02	0.96 ± 0.07	0.68 ± 0.05	0.76 ± 0.05
α-Terpineol	31.487	1.36 ± 0.08	1.33 ± 0.08	0.97 ± 0.06	1.63 ± 0.10

n.d.: not detected. Results are given as means ± SD from duplicate samples.

decrease in methane production was again observed between days 55 and 78, i.e., by 44% and 34% for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively. Still, these decreases were less pronounced than that observed during stage II. Finally, at the end of stage III (78–96 days), methane production improved to approximately the production values at the beginning of the experiment (stage I), i.e., 267 ± 9 and 309 ± 7 mL CH$_4$ g VS^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Fig. 2A). Following the same trend as methane production, the values of methane content in the biogas composition finally stabilized but did not reach the values obtained at stage I, i.e., only CH$_4$ and CO$_2$ were detected with around a 40:60 ratio (Fig. 2B).

As for pH and alkalinity values, after partial re-inoculation, the pH improved and remained constant at close to 7.5. As was observed for methane production, the alkalinity and pH values started to decrease on day 55 and adding 2.0 and 2.5 g of NaHCO$_3$ L^{-1} buffer was necessary on days 70 and 72 (Figs. 3A and 3B). At the end of stage III (between 78 and 96 days), pH values remained constant and stable, while alkalinity values increased significantly up to 9315 ± 700 and 8370 ± 1110 mg CaCO$_3$ L^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Figs. 3A and 3B). sCOD remained constant from day 42–60 at 6670 ± 670 and 6095 ± 780 mg O$_2$ L^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively, after the partial re-inoculation at day 42. However, from 60 to 78 days, an increase in sCOD was observed again, reaching values around 8000 mg O$_2$ L^{-1} for all digesters. At the end of stage III (78–96 days), a gradual decrease in sCOD values was observed, approximately 30% and 37% for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Fig. 4A). In line with that reported for sCOD, the VFAs accumulation followed the same trend. However, a variation in the complexity profile of accumulated acids for digesters fed with different particle sizes feedstocks became noticeable (Figs. 4B and 4C). For digesters fed with the 10 mm particle size feedstock, there was a sharp increase in the accumulation of VFAs from 6011 ± 946–9125 ± 2175 mg O$_2$ L^{-1} (61–71 days). Then, a gradual decrease of VFAs accumulation was achieved at the end of stage III (72–96 days), i.e., 9125 ± 2175–5615 ± 1890 mg O$_2$ L^{-1}. The predominant acid was always propionic acid being its concentration at the end of stage 4870 ± 845 mg O$_2$ L^{-1}. Although minor concentrations of acetic acid, just like butyric and valeric acids, were also quantified at the end of the stage (Fig. 4C). On the contrary, for digesters fed with the 4 mm particle size feedstocks, a slight increase in VFAs accumulation was observed between 61 and 79 days from 6697 ± 159–9075 ± 930 mg O$_2$ L^{-1}. However, between 80 and 96 days, no gradual and

decreased by about one-half, i.e., from 12,000–6500 mg O$_2$ L^{-1} (Fig. 4A). Similarly, the accumulation of total VFAs was also halved, showing a proportional decrease in the concentration of all individual acids, with acetic acid and propionic acid concentrations prevailing, i.e., 3045 ± 190 and 3465 ± 130 mg O$_2$ L^{-1} for digesters fed with 4 mm particle size feedstock and 2720 ± 645 and 2630 ± 130 mg O$_2$ L^{-1} for digesters fed with 10 mm particle size feedstock, respectively (Figs. 4B and 4C). Despite soluble organic matter reduction, methane production did not improve, with values of 115 ± 15 and 184 ± 53 mL CH$_4$ g VS^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Fig. 2A).

On day 42, the digesters were partially re-inoculated with 2 g VS L^{-1} of fresh inoculum. A marked improvement in methane production was observed between days 48 and 55, increasing from 71 ± 4–261 ± 27 mL CH$_4$ g VS^{-1} and 111 ± 28–235 ± 8 mL CH$_4$ g VS^{-1} for digesters fed with 4- and 10-mm particle sizes feedstocks, respectively (Fig. 2A). A

precise decrease of VFA concentration is observed as in the digesters fed with the 10 mm particle size feedstock. Even so, the VFA concentration decreased, reaching a value of 7905 ± 720 mg O_2 L^{-1} at the end of the stage. In distinction to the acid profile of the digesters fed with the 10 mm particle size feedstock, only propionic acid for digesters fed with the 4 mm particle size feedstock was quantified, except for some traces of acetic acid on some days, less than 900 mg O_2 L^{-1} (Fig. 4B). Fenchone was also the most abundant VOC at the end of stage III (day 92), reaching a concentration of 4.53 and 5.16 mg L^{-1} for digesters fed with the 4- and 10-mm particle size feedstocks, respectively. This concentration around 75% of the total GC-MS quantified concentrations all digesters (Table 3 and Fig. 1 S). For digesters fed with the 4 mm particle size feedstock, fenchone concentration decreased by 19%, while 4-terpinenol and α-terpineol concentrations increased by 30% and 15%, respectively respect to the end of stage II. For digesters fed with the10 mm particle size feedstock, fenchone, 4-terpinenol, and α-terpineol concentrations decreased by 44%, 29%, and 38%, respectively, concerning the concentration of the end of stage II (Table 3 and Fig. 1 S). No limonene was detected in the digesters on day 96 (Table 3 and Fig. 1 S).

3.4. Stage IV: OLR increased

In stage IV (97–107 days), following the improvement observed at the end of stage III (78–96 days), the OLR was increased from 1.0 to 1.5 g VS L^{-1} d^{-1}. During stage IV, methane production became unstable, although it did not drop as much as in stages II and III. Methane production decreased by 25% and 30% for digesters fed with 4- and 10-mm particle size feedstocks, respectively (Fig. 2A). The methane content in the biogas composition diminished up to around a 30:60 ratio of CH_4 and CO_2 (Fig. 2B).

As OLR increased, the system destabilized, and pH and alkalinity values dropped to 6.5 and approximately 3000 mg $CaCO_3$ L^{-1}, respectively, for all digesters at day 98 (Figs. 3A and 3B). 2 g of NaHCO₃ buffer L^{-1} were added to each digester for 98 and 107 days to maintain the pH among the recommended range and avoid further acidification (Figs. 3A and 3B). During this stage IV, sCOD values increased again, increasing more pronounced for the digester fed with the 10 mm particle size feedstock (Fig. 4A), going up to 15,230 ± 680 mg O_2 L^{-1}. VFAs followed the same trend as sCOD, although VFAs accumulation was less gradual and abrupt for digesters fed with the10 mm than 4 mm particle sizes feedstocks (Figs. 4B and 4C). Furthermore, the VFAs profile was more complex for the digesters fed with the 10 mm particle size feedstock, i.e., high concentrations of acetic (6225 ± 1165 mg O_2 L^{-1}) and propionic (5310 ± 1150 mg O_2 L^{-1}) acids were detected, followed by a considerable concentration of butyric (2305 ± 735 mg O_2 L^{-1}) and valeric (1985 ± 850 mg O_2 L^{-1}) acids. However, for digesters fed with the 4 mm particle size feedstock, the most abundant acid was propionic acid with 10815 ± 535 mg O_2 L^{-1} (Figs. 4B and 4C). Fenchone remained the most abundant VOC in the digesters, with 60% of the total for all digesters on day 107 (Table 3 and Fig. 1 S). For digesters fed with the 4 mm particle size feedstock, fenchone and α-terpineol concentrations decreased by 36% and 16%, respectively, whereas 4-terpinenol increased by 12% respect to the end of the previous stage III. for digesters fed with the 10 mm particle size feedstock, fenchone decreased by 25%, whereas 4-terpinenol and α-terpineol concentrations increased by 11% and 69%, respectively, concerning the concentration of the end of stage III (Table 3 and Fig. 1 S). All limonene fed with the feedstock was degraded according to the results obtained on day 107, observing trace values in the digesters fed with 4- and 10-mm particle sizes feedstocks (Table 3 and Fig. 1 S).

3.5. Bacterial and archaeal microbiome structure

The microbial composition analysis revealed little differences between the community structure at 4 mm and 10 mm (Fig. 2S) as well as in alpha-diversity (Fig. 3S). After an initial transition period (Stages I

and II) where Simpson and Shannon diversity indexes decreased, they soon after recovered to initial diversity values suggesting a similar evenness and dominance during the whole operational period. However, a decreasing trend in richness (reported as number of observed ASVs) was observed during the experimental time together with a change in identity and relative abundance of certain microbial species.

Species were classified according to their putative functional group in the AD presented in Figs. 5A, 5B, and 5C. The analysis of bacterial microbiome composition revealed that the most abundant phylum during the experimental time was Bacteroidota, where different species shifted abundance over time. Species midas_s_82 (genus DMER64) decreased in relative abundance in favour of species midas_s_738 (genus *Proteiniphilum*). However, Firmicutes, and more specifically species midas_s_35810 (genus *Lachonospira*) and species midas_s_33037 (family *Acidaminococcaceae*) dominated during stage II (day 29) and early stage III (day 41), corresponding to the bioreactors' instability period. Regarding functional groups of interest, a decrease in the relative abundance of taxa known as acetogens in Fig. 5B, e.g., *Syntrophomonas* and *Smithella* species, throughout the experimental time. Whereas in Fig. 5C, an enrichment of some genus/species of bacteria with aromatic and/or monoterpene degrading potential increased in relative abundance the later stages (III and IV, 92-106107 days), such as members of the *Proteobacteria* phylum, i.e., *Pseudomonas*, *Corynebacterium*, and *Thauera* genus [41–44].

Regarding the archaeal microbial populations, Fig. 5D shows a decline of acetoclastic methanogens of the Halobacterota phylum, e.g., *Methanothrix* and *Methanospirillum* genus, until their almost complete disappearance at the end of stage II (day 29). However, the development of a strict hydrogenotrophic methanogen classified as *Methanobrevibacter* genus was promoted for all digesters from stage III (day 41) to the end of the experiment (day 107).

4. Discussion

The process was stable at OLR of 1 g VS L^{-1} d^{-1} during stage I (0–21 days), keeping the pH (Fig. 3A) within optimal limits for methanogenic activity, i.e., 6.5 – 8.0 [45]. The stability of the digesters allowed an adequate methane production and biogas composition showing values in the range of 230–260 mL CH_4 g VS^{-1} and 50–60% CH_4, respectively (Figs. 2A and 2B). These values were similar to the reported for FVW by Edwiges et al. [6] and Masebinu et al. [13]. Despite the observed process stability, the alkalinity levels probably decreased due to feedstock acidity (Fig. 3A and Table 1), as previously described by Edwiges et al. [6]. The sCOD and VFAs accumulation was avoided in line with the detected methane production (Figs. 2A, 4A, 4B, and 4C), meaning that all metabolic pathways of the AD process satisfactorily operated, regardless of the fed particle sizes feedstocks. Moreover, performance under these conditions was not affected by the accumulation of VOCs at the detected concentrations. The absence of a limonene peak in the digesters (Table 3 and Fig. 1S) suggests that it was degraded into other metabolites such as 4-terpinenol and α-terpineol. Several authors have reported that under anaerobic conditions limonene can be degraded to α-terpineol by facultative anaerobic bacteria such as *Geobacillus stearothermophilus* and *Escherichia coli* [24,26,46,47], which also could explain the higher concentration of α-terpineol in digesters fed with the 10 mm particle size feedstock, which was richer in limonene than 4 mm particle size feedstock (Fig. 1). Furthermore, the observed difference in the VOCs profile of different particle sizes feedstocks fed could also explain the difference in VOCs concentrations accumulated in digesters fed with 4- and 10-mm particle sizes feedstocks (Table 3 and Fig. 1S).

The increase of OLR from 1.0 to 3.0 g VS L^{-1} d^{-1} entailed the total failure of the system for both particle sizes feedstocks. The drop in methane production (Fig. 2A) resulted from the rapid acidification of the digesters fed with both particle sizes feedstocks (Figs. 3A, 4B, and 4B). At this stage, the pH dropped to values outside the optimal range for methanogenic activity [45], derived from the accumulation of soluble

A)

	4mm 0	20	29	41	92	106	10mm 0	20	29	41	92	106
Bacteroidota; Proteiniphilum midas_s_738	0.00	0.01	0.69	3.24	14.93	21.94	0.00	0.00	0.66	2.15	9.25	6.59
Synergistota; Aminobacterium colombiense	0.01	0.00	0.00	0.61	7.32	3.89	0.01	0.00	0.00	0.27	15.26	8.43
Bacteroidota; DMER64 midas_s_82	19.41	28.65	8.93	4.19	7.06	1.26	18.15	28.22	10.00	8.63	7.11	6.53
Firmicutes; Lachnospiraceae NK4A136 group ASV12	0.00	0.00	0.00	0.00	5.63	6.65	0.00	0.00	0.00	0.01	2.72	2.81
Bacteroidota; Dysgonomonadaceae ASV16	0.00	0.00	0.00	0.01	0.09	0.14	0.00	0.00	0.00	0.00	6.31	6.53
Patescibacteria; midas_g_19454_ASV21	0.00	0.00	0.00	0.00	5.41	2.76	0.01	0.00	0.00	0.00	1.65	1.88
Bacteroidota; midas_g_8342; midas_s_8342	0.00	3.81	2.81	4.02	4.81	3.54	0.00	0.36	0.38	0.04	0.70	1.44
Patescibacteria; midas_g_546; midas_s_978	0.64	0.88	0.43	0.35	2.17	0.65	0.73	3.02	1.09	1.12	5.58	0.90
Bacteroidota; Proteiniphilum midas_s_6874	0.00	0.03	0.09	0.38	1.63	1.35	0.00	0.03	0.07	0.59	2.67	1.69
Firmicutes; Lachnospira midas_s_35810	0.00	0.12	22.41	14.95	0.61	4.74	0.00	0.48	9.57	10.01	1.08	0.57
Bacteroidota; Dysgonomonadaceae ASV38	0.00	0.00	0.00	0.00	0.03	0.06	0.00	0.00	0.00	0.00	2.76	3.30
Synergistota; midas_g_249; midas_s_249	1.31	1.79	0.35	0.24	1.13	1.19	1.85	1.28	0.66	0.34	1.59	2.23
Firmicutes; midas_g_52206; midas_s_52206	0.00	0.02	0.05	0.16	2.04	3.67	0.00	0.06	0.10	0.05	0.07	0.01
Firmicutes; Christensenellaceae R-7 group ASV44	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	2.32	3.16
Bacteria ASV41	0.00	0.00	0.00	0.00	1.85	3.60	0.00	0.00	0.00	0.00	0.02	0.04
Bacteroidota; Bacteroidales ASV40	0.00	0.00	0.01	0.16	4.30	0.87	0.00	0.00	0.03	0.06	0.06	0.09
Chloroflexi; midas_g_156; midas_s_156	1.43	1.84	0.96	2.12	1.32	0.75	1.07	1.79	1.69	2.04	2.01	1.08
Bacteroidota; DMER64 midas_s_35	1.11	2.53	0.56	0.57	1.17	0.38	1.22	2.20	0.63	0.62	1.74	1.59
Firmicutes; Lysinibacillus midas_s_15934	0.00	0.00	0.03	0.03	0.09	3.07	0.00	0.00	0.08	0.07	0.01	1.68
Firmicutes; Bacillales ASV59	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.02	0.00	0.01	0.00	4.34
Firmicutes; Lachnoclostridium midas_s_54273	0.00	0.12	1.06	2.39	0.40	0.26	0.00	1.41	0.36	1.10	2.42	1.44
Firmicutes; Ruminococcus ASV61	0.00	0.00	0.00	0.35	1.00	1.79	0.00	0.00	0.00	0.00	0.43	1.17
Firmicutes; Christensenellaceae R-7 group ASV55	0.00	0.00	0.01	0.15	0.64	1.79	0.00	0.00	0.00	0.17	0.50	1.45
Bacteroidota; Prevotella_7 midas_s_43135	0.00	0.88	14.60	1.06	1.23	1.44	0.00	2.36	12.33	4.49	0.95	0.72
Cloacimonadota; Ca_Cloacimonas midas_s_3011	0.08	0.12	0.23	0.96	1.88	1.06	0.05	0.11	0.28	0.26	0.54	0.66
Bacteroidota; Dysgonomonadaceae ASV57	0.00	0.03	0.01	0.79	2.63	0.58	0.00	0.00	0.01	0.00	0.70	0.14
Spirochaetota; Spirochaetaceae ASV58	0.00	0.11	0.09	0.11	1.53	1.04	0.00	0.11	0.12	0.09	0.68	0.71
Spirochaetota; Spirochaetaceae ASV66	0.00	0.00	0.00	0.00	0.45	1.49	0.00	0.00	0.00	0.02	0.97	0.78
Cloacimonadota; W5 midas_s_8265	0.07	0.05	0.04	0.10	0.47	0.36	0.01	0.04	0.05	0.07	0.85	1.35
Synergistota; midas_g_249; midas_s_2374	2.48	5.95	1.09	0.80	1.23	0.43	3.79	3.36	1.63	0.89	0.79	0.55

Operational time (day)

B)

	4mm 0	20	29	41	92	106	10mm 0	20	29	41	92	106
Firmicutes; Syntrophomonas midas_s_4716	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.30	0.05
Firmicutes; Syntrophomonas midas_s_15631	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.13	0.05
Firmicutes; Pelotomaculaceae ASV708	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.06	0.00
Firmicutes; Syntrophomonadaceae ASV715	0.00	0.00	0.00	0.00	0.03	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes; Syntrophomonas ASV1021	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.01
Firmicutes; Syntrophomonas midas_s_3034	0.41	0.19	0.15	0.12	0.03	0.01	0.31	0.17	0.13	0.12	0.01	0.00
Firmicutes; Syntrophomonas midas_s_5075	0.33	0.02	0.00	0.00	0.00	0.00	0.44	0.02	0.00	0.00	0.03	0.01
Firmicutes; Syntrophomonas ASV1581	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00
Firmicutes; Syntrophomonas ASV741	0.03	0.00	0.00	0.00	0.01	0.00	0.06	0.00	0.00	0.00	0.00	0.00
Desulfobacterota; Smithella midas_s_2155	2.44	0.94	0.09	0.05	0.01	0.00	2.40	0.59	0.15	0.09	0.00	0.00
Desulfobacterota; Syntrophobacter ASV399	0.12	0.06	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.00	0.00	0.00
Firmicutes; Syntrophomonas sapovorans	0.20	0.00	0.00	0.00	0.00	0.00	0.27	0.01	0.00	0.00	0.00	0.00
Desulfobacterota; Syntrophobacter midas_s_744	0.11	0.05	0.00	0.01	0.00	0.00	0.08	0.05	0.01	0.00	0.00	0.00

Operational time (day)

Fig. 5. Frequency heatmap of A) major bacterial communities, B) acetogenic bacterial communities, C) bacterial communities with potential to degrade aromatics/terpenes compounds, and D) archaeal communities at the taxonomic genus and species levels. The colour intensity shows the relative abundance.
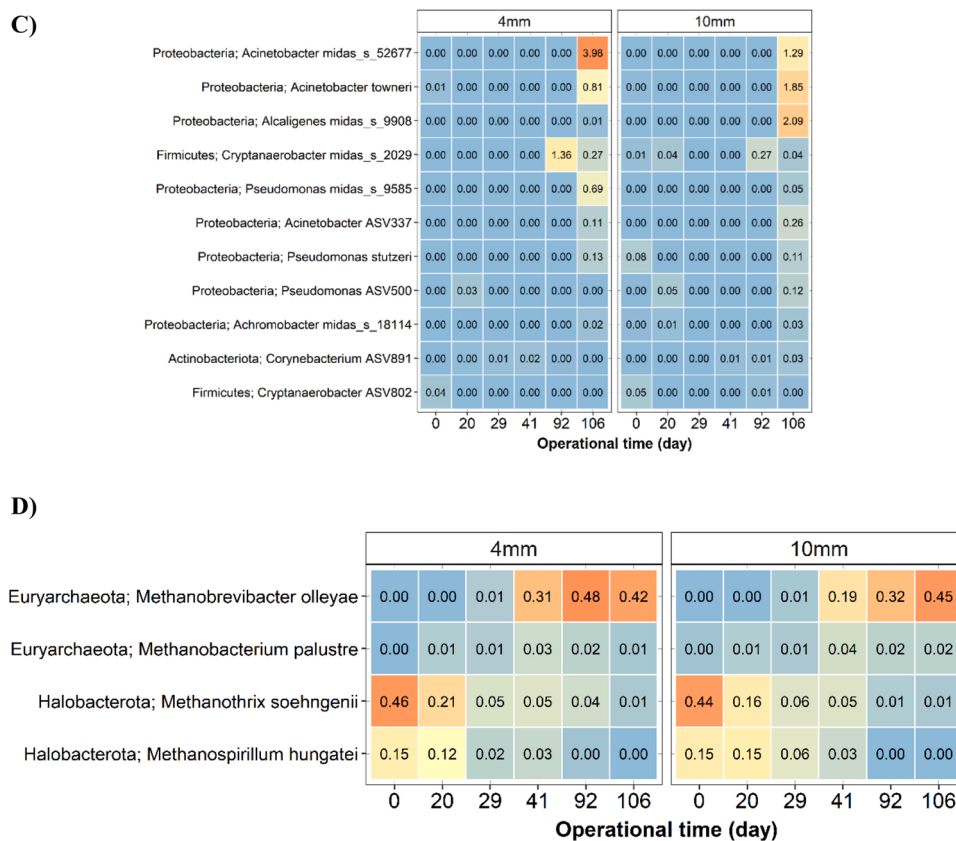
7

C)

Proteobacteria; Acinetobacter midas_s_52677		4mm						10mm				
Proteobacteria; Acinetobacter midas_s_52677	0.00	0.00	0.00	0.00	0.00	3.98	0.00	0.00	0.00	0.00	0.00	1.29
Proteobacteria; Acinetobacter towneri	0.01	0.00	0.00	0.00	0.00	0.81	0.00	0.00	0.00	0.00	0.00	1.85
Proteobacteria; Alcaligenes midas_s_9908	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	2.09
Firmicutes; Cryptanaerobacter midas_s_2029	0.00	0.00	0.00	0.00	1.36	0.27	0.01	0.04	0.00	0.00	0.27	0.04
Proteobacteria; Pseudomonas midas_s_9585	0.00	0.00	0.00	0.00	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.05
Proteobacteria; Acinetobacter ASV337	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.26
Proteobacteria; Pseudomonas stutzeri	0.00	0.00	0.00	0.00	0.00	0.13	0.08	0.00	0.00	0.00	0.00	0.11
Proteobacteria; Pseudomonas ASV500	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.12
Proteobacteria; Achromobacter midas_s_18114	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.03
Actinobacteriota; Corynebacterium ASV891	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.03
Firmicutes; Cryptanaerobacter ASV802	0.04	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.01	0.00
	0	20	29	41	92	106	0	20	29	41	92	106

Operational time (day)

D)

		4mm						10mm				
Euryarchaeota; Methanobrevibacter olleyae	0.00	0.00	0.01	0.31	0.48	0.42	0.00	0.00	0.01	0.19	0.32	0.45
Euryarchaeota; Methanobacterium palustre	0.00	0.01	0.01	0.03	0.02	0.01	0.00	0.01	0.01	0.04	0.02	0.02
Halobacterota; Methanothrix soehngenii	0.46	0.21	0.05	0.05	0.04	0.01	0.44	0.16	0.06	0.05	0.01	0.01
Halobacterota; Methanospirillum hungatei	0.15	0.12	0.02	0.03	0.00	0.00	0.15	0.15	0.06	0.03	0.00	0.00
	0	20	29	41	92	106	0	20	29	41	92	106

Operational time (day)

Fig. 5. (*continued*).

organic compounds (Figs. 4A, 4B, and 4C). The impact of acidification was clearly observed in the microbial diversity, decreasing the methanogens below detection limits at the end of stage II (Fig. 5D). That almost all the sCOD was as VFAs (Figs. 4A, 4B, and 4C) suggested that hydrolytic and acidogenic bacteria maintained their activity, increasing their relative abundance in the microbial community (Fig. 5A). These unbalances of AD metabolism allowed VFAs to continue to be generated (Figs. 4B and 4C), which, as they were not consumed, led to further lowering of the pH and accelerated the system destabilisation [48,49]. Other authors have reported that from an OLR equal to or higher than 3.0 g VS L^{-1} d^{-1}, the AD of FVW was disestablished [4–6,9,13]. Likewise, the increment in the concentration of monoterpenes from the end of stage I could have contributed to the observed system destabilisation due to their antimicrobial properties [21,22,50,51]. Despite impaired methanogenesis, the digesters were still able to degrade the added limonene. However, the degradation of the limonene would result in the accumulation of secondary metabolites, as already reported by other authors, such as α-terpineol, and sometimes 4-terpineol [47], whose inhibitory potential would have contributed to the system destabilisation. Ruiz et al. [52] reported that the microbial effect of α-terpineol is between 1000 and 5000 times higher than that of limonene, according to its minimum inhibitory concentrations for microorganisms such as *E. coli* and *Staphylococcus aureus*. Since the inhibitory concentration for limonene in continuous anaerobic reactors has been reported to be in a range of 24–75 mg L^{-1} [26], the increase in the concentration of α-terpineol at the end of stage II (Table 3) would have been one of the responsible of the destabilisation of the systems. The actual impact of α-terpineol and 4-terpineol in the performance of the digesters is not easy to discuss since the available literature is restricted to batch assays, where the reported inhibition limits use to be several orders of magnitude higher than for continuous systems. For example, limonene inhibition limits were reported to be 24–75 mg L^{-1} for continuous systems and 423 mg L^{-1} for batch systems [26].

At the beginning of stage III (30–42 days), different subsequent attempts were made to restore the digesters' stability by adding $NaHCO_3$ buffer to increase the pH (day 27), nutrients to enrich the medium (day 37), and centrifuging part of the volume of the digesters to reduce the VFAs concentration in the medium (day 40). An improvement in methane production for all digesters was observed compared to the end of stage II, which could be related to hydrogenotrophic methanogens enrichment, such as sp. *Methanobrevibacter olleyae* (Fig. 5D). Hydrogenotrophic methanogens were also correlated with VFAs accumulation in an overloaded reactor by Lerm et al. [53]. However, a decrease in the relative presence of acetoclastic methanogens, such as sp. *Methanothrix soehngenii* and sp. *Methanospirillum hungatei*, would explain that methane production did not reach the same level as at the end of stage I and that there was no decrease in VFAs (Figs. 2A, 4B, 4C, and 5C). The reduction in VFAs concentration observed was related to centrifugation, not the biological effect. The methanogenic core and microbial population dynamics have been previously investigated in the AD of similar feedstocks such as food waste (FW) and municipal solid waste (MSW). These investigations showed that the accumulation of VFAs resulted in blocked acetoclastic methanogenesis and a shift to the CO_2 reduction pathway reflected in the enrichment of hydrogenotrophic methanogens in the system [12,54–56].

After partial re-inoculation (day 42), an improvement in methanogenic activity was observed (48–55 days), as shows the improvement in the methane production and methane content in biogas (Figs. 2A and 2B). The improvement in the methanogenic activity also entailed an increment in pH and alkalinity (Figs. 3A and 3B). A complete and balanced AD process caused pH and alkalinity values to improve, as previously reported by Chen et al. [57]. Despite the observed improvement, VFAs were still accumulated, mainly propionic acid (Figs. 4B and 4B). This VFAs accumulation could have affected the acetogenic activity in the digesters, which would explain the absence of acetoclastic archaea at the end of stage III (day 92), despite the previous

re-inoculation on day 42 (Figs. 5B and 5D).

Following improvement after partial re-inoculation, a gradual decrease in methane production was observed for digesters fed with 4- and 10-mm particle sizes feedstocks (55–78 days), followed by recovery (78–96 days) (Fig. 2A). The methane production recovery occurred simultaneously with the fennel removal of the fed FVW mixtures (day 78), since the fennel was the primary fenchone source [25,51]. Fenchone was attributed to powerful antibacterial and antimicrobial properties against a wide range of microbes [22,50,58]. Kotan et al. [21] studied the response of 63 bacterial strains to the antibacterial potential of 21 oxygenated monoterpenes, with the result that fenchone is one of the few compounds that affect a large number of bacteria studied. However, to our best knowledge, nothing has been reported on the inhibitory potential of fenchone in the AD process. As reference, the concentration of fenchone in the digesters at the end of the stage II was in the same order of magnitude that the inhibition limit described for limonene in continuous anaerobic digestion systems, i.e., 24–75 mg L-1 [26], so the reduction of the fenchone at the end of stage III (Table 3) would be related with the observed enhancement of the methane production (Fig. 2A). In the same line, Mohamed [25] evaluated the anaerobic degradation of fennel plant seeds and found that the biogas produced by fennel was significantly reduced compared to the control, attributing these results to the chemical components of fennel.

Despite similar behaviour and characterisation of digesters fed with 4- and 10-mm particle sizes feedstocks, differences in the complexity of the VFAs profile and the VOCs accumulation started to be observed from day 60 to the end of stage III (day 96). The higher monoterpenes accumulation in digesters fed with the 4 mm than the 10 mm particle size feedstock could be related to a lower relative presence of bacterial genera/species with the potential to degrade aromatic/monoterpene compounds in an anaerobic environment at the end of the stage III (Fig. 5D). However, this hypothesis could not be confirmed since the relative abundance of species with reported potential such as genera *Pseudomonas*, *Corynebacterium*, *Thauera*, *Azoarcus*, and *Aromatoleum* [51,59,60] was similar between the two particle sizes feedstocks. Despite the similarity between the two bioreactors few species were statistically more abundant at 10 mm (Fig. 5S), such as *Aminobacterium colombiense*, two unclassified ASVs belonging to the *Dysgonomonadaceae* family or species midas_s_2192 (genus *Lentimicrobium*). However, ecophysiological studies should be carried to identify if those microorganisms are able to degrade aromatic compounds.

The final methane production 309 ± 7 and 267 ± 9 mL CH$_4$ g VS^{-1} values were slightly higher for the digesters fed with the 10 mm particle size feedstock than for the 4 mm particle size feedstock (Fig. 2A), respectively, which could be due to differences in the relative presence of monoterpene degrading microorganisms between the digesters mentioned above. Rashama et al. [61] have reported studies assessing terpenes' degradability in the ruminant system and their effect on methanogenesis. These authors concluded that although biological degradation of terpenes is possible, these compounds can inhibit methanogenesis depending on the compound type, microbial diversity, and conditions.

Finally, the increase in OLR from 1.0 to 1.5 g VS L^{-1} d^{-1} (day 96) during stage IV again caused an accumulation of soluble organic matter (Fig. 4A), mainly VFAs (Figs. 4B and 4C), destabilizing the process by decreasing pH (Fig. 3A), methane production (Fig. 2A), and methane content (Fig. 2B). The pH was artificially maintained by adding buffer (Figs. 3A and 3B) during this stage IV, whereas the accumulation of destabilisation was observed in VFAs (Figs. 4B and 4C). The difference in the accumulated VFA profile for digesters fed with both particle sizes feedstocks would be the trigger for the observed differences in the hydrogenotrophic methanogens population of the digesters at the end of the experiment. Tonanzi et al. [55] reported that a critical problem in the AD of FW is the progressive drop in the hydrogenotrophic microorganisms' activity resulting in a degradative accumulation of propionic acid due to thermodynamic limitations. The limitation of the OLR at

values relatively low was previously described for the semicontinuous anaerobic digestion of olive mill solid waste, another easily biodegradable and acidic substrate, reporting that the operation was stable only at an OLR of 1.0 g VS L^{-1} d^{-1}, whereas the increment of the OLR resulted in a rapid acidification of the system [62]. Similarly, Fiore et al., [63] reported an OLR of 1.5 g VS L^{-1} d^{-1} for the stable operation of a pilot-scale anaerobic reactor fed with food waste. Some authors attribute to the high protein, sugars and lipids contents in the food waste the limitation of the performance to low OLR, since this kind of substrate can easily release inhibitory levels of ammonia, long chain fatty acids or phenols [64,65]. Despite that, an energy balance demonstrated that the anaerobic digestion of olive mill solid waste, for an OLR of 1.0 g VS L^{-1} d^{-1}, with methane yield coefficients of 200–350 mL CH$_4$ g VS^{-1} generates a net energy production above 2500 kJ per kg of substrate [66].

At the end of the experiment (day 107), the increase in some of the quantified VOCs could be related to the system's overfeeding with feedstocks rich in these compounds, as seen in Fig. 1 of the VOCs profile for each particle sizes feedstock studied. There is evidence that with proper digester management, the microbial community/digester functioning may overcome critical periods of destabilization. However, it will require necessary monitoring of many factors/parameters and maintaining exceptional attention to monoterpenes that might be playing a higher toxicity role than expected.

5. Conclusions

The stability and robustness of long-term AD process of FVW was strongly affected by the increases of the OLR. Methane yield was 254 ± 8 and 229 ± 11 mL CH$_4$ g VS^{-1} with an OLR of 1.0 g VS L^{-1} d^{-1} for 4 and 10 mm, respectively. This OLR was stable, with low influence due to particle size. The system was destabilized as the OLR increased from 1.0 to 3.0 and 1.5 g VS L^{-1} d^{-1}, and a high VFA accumulation was observed. The variation of the particle size of the FVW influenced the VFA profile, although it did not result in differences in the stability of the operation. The accumulation of monoterpenes, mainly fenchone, reached concentrations higher enough to be found as potential contributors to the acidification of the process at increasing the OLR. The episodes of destabilisation were reflected in a drastic reduction in the relative abundance and species diversity of methanogenic archaea at all digesters, especially at an OLR of 3.0 g VS L^{-1} d^{-1}. This research evidenced that the AD is a complex process that is affected by a wide range of compounds and, among them, monoterpenes must be monitored due to their potential contribution to the process destabilization.

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CRediT authorship contribution statement

Ángeles Trujillo-Reyes: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Antonio Serrano:** Investigation, Supervision, Writing – review & editing. **Ana G. Pérez:** Methodology, Data curation, Formal analysis, Investigation, Writing – review & editing. **Miriam Peces:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Fernando G. Fermoso:** Supervision, Writing – review & editing, Funding acquisition.

Conflicts of interest

The authors have no relevant financial or non-financial interest to disclose.

Data Availability

Data will be made available on request.

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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.jece.2023.109653.

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