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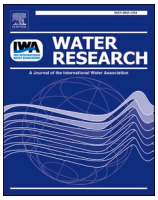

Consistent acidogenic co-fermentation of waste activated sludge and food waste under thermophilic conditions

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ABSTRACT

Acidogenic co-fermentation of waste activated sludge (WAS) and food waste (FW) under thermophilic conditions enhances process consistency, while overcoming the problem of acetic acid consumption due to growing methanogens. Two long-term continuous co-fermentation experiments were carried out with a WAS:FW mixture (70:30 % in VS) at organic loading rate of 8 gVS/(L·d). Experiment 1 assessed the impact of temperature (35 °C and 55 °C) and WAS origin (WAS_A and WAS_B) in two collection periods. Experiment 2 evaluated the consistency at 55 °C by testing three WAS origins (WAS_A, WAS_B and WAS_C) in 3 additional collection periods. Experimental results showed that at 55 °C, the solubilisation yield was enhanced compared to 35 °C, although this did not always lead to higher fermentation yield. The fermentation product profile was affected by the operating temperature, with 55 °C promoting the accumulation of acetic and butyric acids. Acetic acid consumption was only detected at 35 °C in fermenters treating WAS_A, whereas it was not observed in fermenters treating WAS_B. This consumption was prevented at 55 °C, as none of the 13 fermenters continuous operation showed acetic acid consumption. Acetic acid consumption was attributed to species midas_s_9557 (genus *Methanosarcina*), an aceticlastic methanogen, which did not grow under 55 °C. Temperature had a more significant effect on the microbial community structure than WAS origin. Functional redundancy was demonstrated by each fermenter having its own distinct microbial consortium while maintaining constant metabolic functions at 55 °C. Overall, the acidogenic co-fermentation of WAS and FW at 55 °C is regarded as a robust and consistent biotechnology.

1. Introduction

The biorefinery concept is widely acknowledged as the leading strategy to optimise wastewater and waste conversion into a wide range of value-added bioproducts (Leong et al., 2021; Puyol et al., 2017). To achieve this, wastewater treatment plants (WWTP) must be transformed into waste resource recovery facilities (WRRF) (Guven et al., 2023). Anaerobic fermentation, which transforms organic matter into easily assimilable compounds including volatile fatty acids (VFA), alcohols (XOH) and lactic acid, is a key process in WRRF (Fang et al., 2020; Yuan et al., 2019). These fermentation products serve as building blocks for other bioprocesses such as bioplastic production or used as carbon source in the activated sludge system (Lee et al., 2014; Ramos-Suarez et al., 2021). Co-fermentation, which refers to the simultaneous fermentation of two or more wastes, presents an opportunity for WWTPs to improve the fermentation yield of sludge mono-fermentation. To successfully transform WWTP into WRRF, it is necessary to study, understand, optimise the performance, and improve the robustness of the anaerobic fermentation and co-fermentation processes (Perez-Esteban et al., 2022).

One challenge associated with acidogenic fermentation is the sporadic and unpredictable acetic acid consumption, a phenomenon mostly reported in batch experiments (Pang et al., 2020; Peces et al., 2020; Perez-Esteban et al., 2024b, 2024a; Vidal-Antich et al., 2021), although it has also been reported in continuous experiments (Perez-Esteban et al., 2024b, 2024a). Although batch experiments are excellent screening tools, full-scale bioreactors predominantly operate under continuous conditions. In contrast to batch experiments, the performance of continuous fermenters is influenced by the long-term effect of: (i) microbial factors, including the development of a specialized

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microbial community, hydraulic selective pressure on the microbial community and microorganism immigration; (ii) operational parameters such as HRT, OLR and feeding regime; and (iii) process parameters such as pH, alkalinity, or the concentration of inhibitory compounds (Fang et al., 2020; Perez-Esteban et al., 2022; Ramos-Suarez et al., 2021; Zhou et al., 2018). Therefore, further research in continuous systems is necessary to gain a better understanding of the mechanisms behind the sporadic acetic acid consumption.

One hypothesis to explain the proliferation of net VFA consuming microorganisms (e.g., methanogenic archaea) is biomass migration from the substrate (e.g., WAS) (Perez-Esteban et al., 2022). Indeed, biomass migration has been shown to influence microbial community structure in other bioprocesses (Dottorini et al., 2021). Migrating microorganisms with the substrates might find favourable conditions to grow in the fermenters, consuming the fermentation products. Acetic acid consumption has been observed mostly under mesophilic conditions, despite having unfavourable conditions for aceticlastic methanogens growth such as acidic pH (5–6) and/or low HRT (~3 days) (Perez-Esteban et al., 2024b, 2024a). This fact compromises the robustness of the fermentation process and, in turn, could affect the reliability and reproducibility of the process.

Early studies have shown that working under thermophilic conditions could limit the growth of net acetic acid consuming microorganisms, particularly when using WAS as feedstock (Battista et al., 2022; Perez-Esteban et al., 2022). Working at thermophilic temperatures is an easy-to-implement strategy because it does not require a complete redesign or new infrastructure. However, it may lead to higher operational costs due to increased energy consumption. As a result, most fermentation research has been carried out under mesophilic conditions, leading to a knowledge gap about how thermophilic conditions affect to the fermentation process (Perez-Esteban et al., 2022). Previous research suggests that working under thermophilic conditions increases the solubilisation yield, as hydrolysis is improved. Nonetheless, the fermentation yield may be comparable to, or lower than, the achieved under mesophilic conditions (Moretto et al., 2019). Regarding the fermentation product profile, previous publications suggest that temperature does not highly impact the fermentation product profile (Battista et al., 2022; Fernández-Domínguez et al., 2020), while others have shown a distinct fermentation product profile depending on the operational temperature (Garcia-Aguirre et al., 2017; Perez-Esteban et al., 2024b). Therefore, there is insufficient knowledge on the effect of temperature on fermentation product profile (Ramos-Suarez et al., 2021).

Anaerobic co-fermentation emerges as a key biotechnology for biorefinery schemes, however, it must demonstrate robust performance to be considered a reliable biotechnology process. Consistency in co-fermentation means that under optimal operational conditions, despite the inherent heterogeneity of a waste substrate, the fermentation yield and final fermentation products remain relatively constant during the whole operation. Experiments focused on the continuous operation of the fermentation process allows for the investigation of the impact of operational conditions on the process performance and the long-term microbial response (Perez-Esteban et al., 2022).

The aim of this research is to evaluate the robustness and efficiency of acidogenic co-fermentation at thermophilic conditions in continuous mode to produce a consistent fermentation product profile and mitigate acetate consumption. Additionally, it evaluates the impact of microorganism migration on co-fermentation as a potential factor influencing the microbial community structure and process performance. To achieve these goals, two experiments were conducted. In Experiment 1, thermophilic and mesophilic co-fermentation reactors were run in parallel to establish a control baseline, using two WAS origins (WAS_A and WAS_B) and two collection periods (1 and 2). Experiment 2 focused on the consistency of thermophilic conditions, analysing the impact of three WAS origins (WAS_A, WAS_B, and WAS_C) and three collection periods (3, 4, and 5).

2. Materials and methods

2.1. Co-fermentation substrates

Thickened waste activated sludge (WAS) was collected from three different wastewater treatment plants (WWTP) equipped with mesophilic anaerobic digesters (labelled as WAS_A, WAS_B and WAS_C) from Barcelona metropolitan area (Catalonia, Spain). WAS_A originated from an aerobic membrane bioreactor, whereas WAS_B and WAS_C were obtained from a conventional activated sludge system with biological nitrogen removal. WAS were collected at different periods: January, March, April, November of 2023, and January of 2024. After collection, the samples were stored in a refrigerator at 4 °C until use. During the experiments, WAS was analysed once per week, including total and soluble chemical oxygen demand (tCOD and sCOD), volatile fatty acids (VFA) and alcohols (XOH), pH, alkalinity (Alk), total solids (TS) and volatile solids (VS), and total ammoniacal nitrogen (TAN) (see WAS characterisation in Table S1 in the Supplementary material). Samples for microbiological profiling were taken twice per week (see WAS microbiota in Fig. S1 in the Supplementary material). To ease the comparability between the different runs, WAS were diluted with deionised water to achieve a final concentration of 15 gVS/L.

Synthetic food waste (FW) based on Mediterranean diet was used as co-substrate to facilitate the reproducibility in co-fermentation experiments. It was prepared according to the composition reported by Vidal-Antich et al. (2021): vegetables (30 %), fruits (30 %), carbohydrates (20 %), meat (10 %), and fish and seafood (10 %) in weight basis, adding deionised water to achieve a 150 gTS/L concentration. The FW was blended with a cooking blender for 4 min to homogenise and minimise particle size, and then was stored at 4 °C until use. FW was freshly prepared every 2–3 days and analysed after preparation (pH, tCOD, sCOD, VFA and XOH, TS and VS, and TAN) (see FW characterisation in Table S1 in the Supplementary material).

2.2. Semi-continuous fermenters set-up

Jacketed reactors with a working volume of 4 L were equipped with a mechanical stirrer (RZR 2020, Heidolph) and Tedlar bag (Tedlar® Sample Bag, SKC) to collect the gases produced during the process. The operational temperature was controlled using a thermal bath (CORIO™ C-BT9 and Termotronic, JP Selecta), while the stirring was controlled by a timer which stopped 15 min after 45 min of working. The organic loading rate (OLR) was fixed at 8 gVS/(kg·d), consequently, a hydraulic retention time (HRT) of 2.6 days was applied. The reactor was fed with a WAS and FW mixture of 70:30 % in VS according to Vidal-Antich et al. (2021). Each fermenter was operated 28 days (ca. 10-HRT equivalents) to ensure that steady-state conditions were achieved. Steady-state conditions were considered for the last ~4 HRT equivalents (day 16 to 28). The process relied on WAS buffer capacity to maintain the pH between 4.5–6.0, therefore, the pH was not adjusted at the beginning nor during the co-fermentation process. The fermenters were started (day 0) adding 4 L of WAS, with a concentration of 15 gVS/L. To displace oxygen and create anaerobic conditions, the reactors' headspace was flushed with 99.99 % N_2 for 5 min at a rate of around 5 L/min. From day 1 onwards, the fermenters were fed daily with the WAS and FW mixture (influent). Effluent withdrawal and feeding were carried out by submerging a silicone tube connected to a syringe through the feeding port, which consisted of a glass tube immersed in the fermenter liquor to minimise the entry of air.

The following parameters were analysed three times per week for influent (WAS and FW mixture) and effluent: pH, tCOD, sCOD, VFA and XOH, Alk, TS, VS, and TAN (see influent characterisation in Table S2 in the Supplementary material).

Two experiments were run. In Experiment 1, four fermenters were operated in parallel, two operating at 35 °C and two operating at 55 °C. For each temperature, two WAS origins were studied (WAS_A and

Table 1
Experimental matrix indicating the experiment label, WAS origin, collection period, and operational temperature. Cells in the column temperature show the fermenters labels.

Experiment	WAS		Temperature	
	Origin	Collection period	35 °C	55 °C
Experiment 1	WAS_A	1	35_A1	55_A1
		2	35_A2	55_A2
Experiment 2		3		55_A3
		4		55_A4
		5		55_A5
Experiment 1	WAS_B	1	35_B1	55_B1
		2	35_B2	55_B2
Experiment 2		3		55_B3
		4		55_B4
		5		55_B5
Experiment 2	WAS_C	3		55_C3
		4		55_C4
		5		55_C5

WAS_B). In Experiment 1, two collection periods (1 and 2) were evaluated. In Experiment 2, three fermenters in parallel were operated under 55 °C. Each fermenter was fed with WAS from different origins (WAS_A, WAS_B and WAS_C). In addition, three different collection periods of WAS were evaluated (3, 4 and 5). Table 1 summarises and labels all the fermentation tested conditions.

2.3. Analytical procedures

The Standard Methods protocol 5520D (APHA, 2017) was used to analyse tCOD and sCOD, while TS and VS were determined according to the Standard Methods protocol 2540G (APHA, 2017). TAN was measured according to the 4500-NH3D Standard Method (APHA, 2017) using a Thermo Fisher Scientific ammonium ion-selective electrode (Orion 9512HPBNWP). Alkalinity was measured in accordance with 2320B Standard Method protocol (APHA, 2017) using pH-Burette 24 equipped with a pH-meter (CRISOL Basic 20) at pH 5.75 for partial alkalinity and at pH 4.3 for total alkalinity. The pH was measured with a pH electrode (CRISON Basic 20) connected to a pH-Burette 24. VFAs (i.e., acetic, propionic, i-butyric, n-butyric, i-valeric, n-valeric, i-caproic, n-caproic, heptanoic acid) and alcohols (XOH, i.e., ethanol, propanol, and butanol) were analysed following the Standard method protocol 5560D (APHA, 2017) using a gas chromatograph (GC) (Shimadzu GC-2010 Plus) equipped with an Agilent J&W DB-FFAP column (30m × 0.25 mm × 0.25 μm) and a flame ionisation detector. GC configuration and procedure is detailed in Perez-Esteban et al. (2024b). VFA isomers concentration (normal and iso) were summed in a single VFA species. Individual VFA and XOH concentration were expressed in COD equivalents using a theoretical value based on their elemental composition ($mgCOD/mg_{compound}$): 1.07 for acetic acid, 1.51 for propionic acid, 1.82 for butyric acid, 2.04 for valeric acid, 2.21 for caproic acid, 2.34 for heptanoic acid, and 2.09 for ethanol, 2.40 for propanol and 2.59 for butanol. Prior to VFA & XOH, sCOD and TAN analysis, the samples were centrifuged for 10 min at 16 600 × *g* (Sigma 1–14 microcentrifuge) and filtered through a 0.45 μm nylon syringe filter. To evaluate the fermentation process, the solubilisation yield (mgCOD/gVS) and fermentation yield (mgCOD/gVS) were calculated using Eq. (1) and Eq. (2), respectively.

$$Solubilisation\ yield = \frac{sCOD}{VS_{fed}} \quad (1)$$

$$Fermentation\ yield = \frac{COD_{VFA\ \&\ XOH}}{VS_{fed}} \quad (2)$$

Where sCOD stands for soluble COD (mgCOD/L), $COD_{VFA\&XOH}$ stands for the concentration of VFA and XOH on COD basis (mgCOD/L), and VS_{fed} stands for the influent (WAS and FW mixture) VS concentration (gVS/L).

2.4. Microbiology and data processing

To analyse the microbial communities, samples were collected during the steady-state periods (operational days between 16 to 28) (for Experiment 1; $n_{WAS} = 5$, $n_{fermenters} = 6$; for Experiment 2; $n_{WAS} = 4–5$, $n_{fermenters} = 6$). 2 mL of sample were stored in microcentrifuge tubes at -20 °C. DNA extraction was carried out using FastDNA kit for soil (MP Biomedicals, USA) and MiDAS field guide (https://www.midasfieldguide.org/guide/protocols). The extracted DNA was stored at −20 °C until 16S rRNA sequencing. The V3-V4 region was amplified using the primers (341F: 5′-CCTAYGGGRBGCASCAG-3′) and (806R: 5′-GGACTACNNGGGTATCTAAT-3′) conducted using the Illumina MiSeq platform by Novogene (Cambridge, UK). The raw 16S rRNA amplicon sequences were processed using DADA2 v.1.26.0 pipeline (Callahan et al., 2016) in R version (v.4.2.2.) (R Core Team, 2022). Forward and reverse reads were truncated at 220 bp to preserve sequences with a quality score >30. Reads with >5 expected errors were discarded. Dereplication, amplicon sequence variants (ASVs) inference and chimera removal were run with DADA2 default parameters. The taxonomic assignment of the resulting AVS was performed using the MiDAS database version 5.3 (Dueholm et al., 2023) implemented in the DADA2 function (assignTaxonomy()) with a minimum bootstrap confidence of 80 %. In Experiment 1, 8345 ASVs representing 44 phyla, 1601 genera, and 2416 species were identified. In Experiment 2, 8868 ASVs were detected across 44 phyla, 1667 genera, and 2609 species. Raw amplicon sequencing reads have been deposited in the European Nucleotide Archive (ENA) under the project code PRJEB77935 for Experiment 1 and PRJEB73832 for Experiment 2.

Microbial analysis was carried out in R version 4.2.2. (R Core Team, 2022) using the ampvis2 v2.7.32. package (Andersen et al., 2018). Microbial species relative abundance was evaluated through hetmaps. For unclassified ASVs at species level, the best taxonomix assigment is shown. The microbial community structure was evaluated by principal component analysis (PCA) using vegan package v.2.6–4 (Oksanen et al., 2022), previously the reads were Hellinger transformed. Permutational multivariate analysis of variance (PERMANOVA) was performed on the Euclidean distances obtained from the PCA to evaluate the temperature and WAS origin effect on microbial structure in the fermenters. This analysis was performed using the adonis2() function from the vegan package v.2.6–4 (Oksanen et al., 2022). Shannon and Simpson diversity were calculated using the function amp_alphadiv() of the package ampvis2 v2.7.32. package (Andersen et al., 2018).

A microbial mass balance was conducted between WAS and fermenters effluent to categorise the growth fate of WAS microorganisms in the fermenters, using the method described in Jiang et al. (2021) and Perez-Esteban et al. (2024b). Briefly, the microorganisms mass balance relies on the total biomass in the fermenters, along with the relative abundance of species in the fermenters compared to the total biomass load, and the relative abundance of species in the WAS. The detailed methodology can be found in Perez-Esteban et al. (2024b). The mass balance allows the microbial species to be categorised into growing ($k_{obs} > 0\ d^{-1}$) and non-growing ($k_{obs} < 0\ d^{-1}$). Species with relative abundance <0.01 % or a $k_{obs} = 0\ d^{-1}$ were classified as ambiguous

3. Results

3.1. Experiment 1: Effect of temperature and was origin

3.1.1. Acidogenic co-fermentation performance at mesophilic and thermophilic temperatures

Co-fermentation performance at 35 °C and 55 °C was compared by testing WAS collected from two WWTPs at two different collection periods. Fig. 1 shows the evolution of pH, sCOD, VFAs and XOH, TAN, solubilisation yield and VFA yield of the fermenters, and their steady-

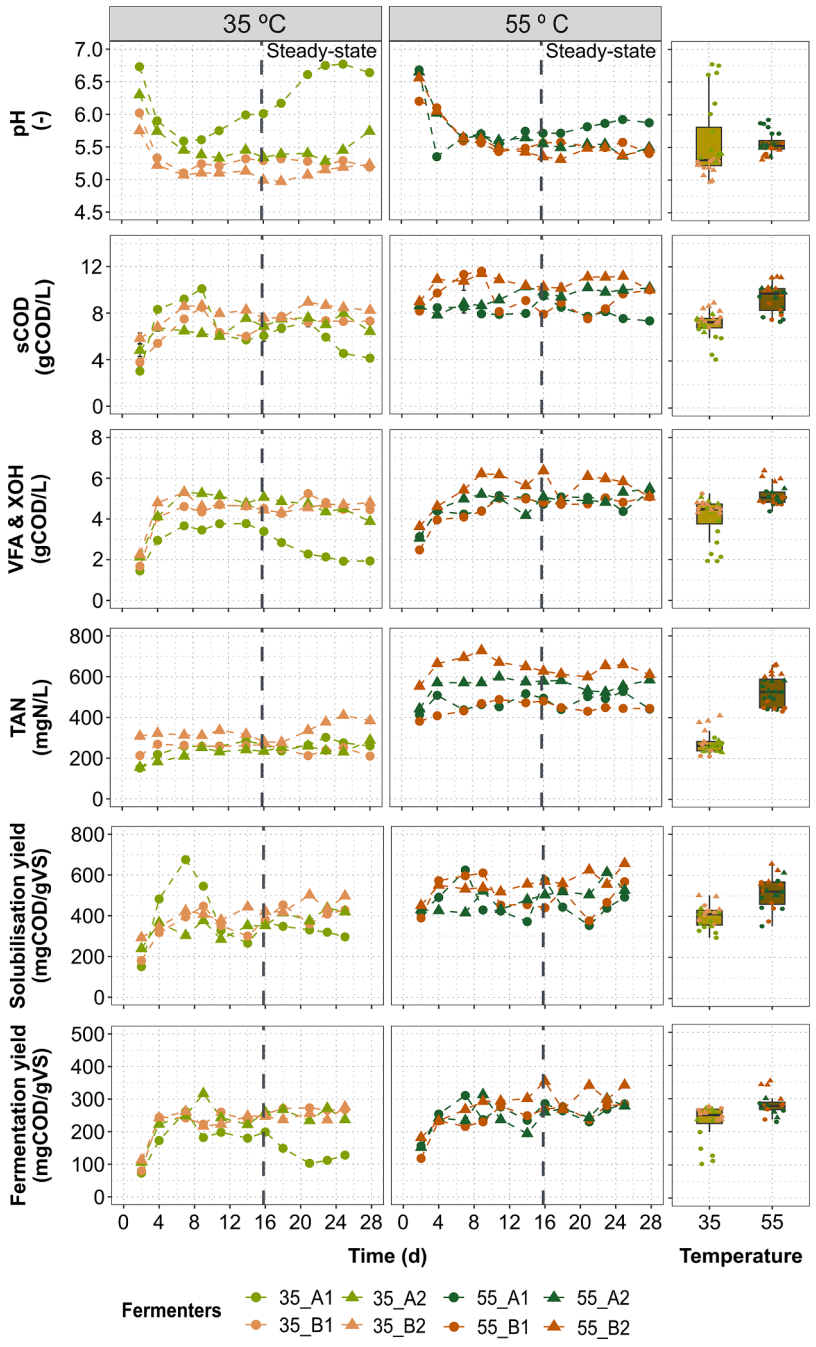

Fig. 1. Evolution of pH, sCOD, TAN, solubilisation yield, fermentation yield and total concentration of VFA & XOH for each fermenter in Experiment 1. Grey dashed line indicates the beginning of the steady-state period. Boxplot graph compares the steady-state values between temperatures (35 °C and 55 °C).

state boxplots from Experiment 1. The pH values obtained (5.3–5.8) were quite similar between the fermenters, except for mesophilic fermenters treating WAS_A (35_A1 and 35_A2), in which pH increased at the end of the experimental period due to acetic acid consumption (see effluent characterisation in Table S3 in the Supplementary material). The 35_A1 fermenter reached values close to 7.0, resulting in a wider pH range at 35 °C compared to 55 °C (Fig. 1). This increase in pH can be related to the sCOD depletion (mainly acetic acid consumption), which only occurred in 35A conditions (35_A1 and 35_A2). Regardless of the WAS origin and the collection period, the sCOD boxplot indicates that working under thermophilic temperature led to slightly higher sCOD values, resulting in higher solubilisation yields. The TAN concentration was also higher at 55 °C, reaching 627 mgN/L compared to 267 mgN/L at 35 °C (see effluent characterisation in Table S3 in the Supplementary material). At 55 °C, the fermentation yield ranged between 267–321 mgCOD/gVS, and VFA concentration ranged between 4.9–5.7 gCOD/L. These parameters were relatively constant during the steady-state for both WAS_A and WAS_B and the collection periods tested. At 35 °C, co-fermenters using WAS_B achieved a slightly lower fermentation yield (250–266 mgCOD/gVS) and VFA concentration (4.5–4.6 gCOD/L) compared to 55 °C. However, the mesophilic co-fermenters using WAS_A (35_A1 and 35_A2), which experienced a rise in pH and a depletion in sCOD, showed a downward trend in fermentation yield (139–254 mgCOD/gVS) and VFA concentration (2.6–4.6 gCOD/L) (Fig. 1). Overall, if acetic acid consumers do not proliferate in mesophilic conditions (Fig. 1), the results show no practical differences in the fermentation yield between the tested temperatures, WAS origin, and collection period.

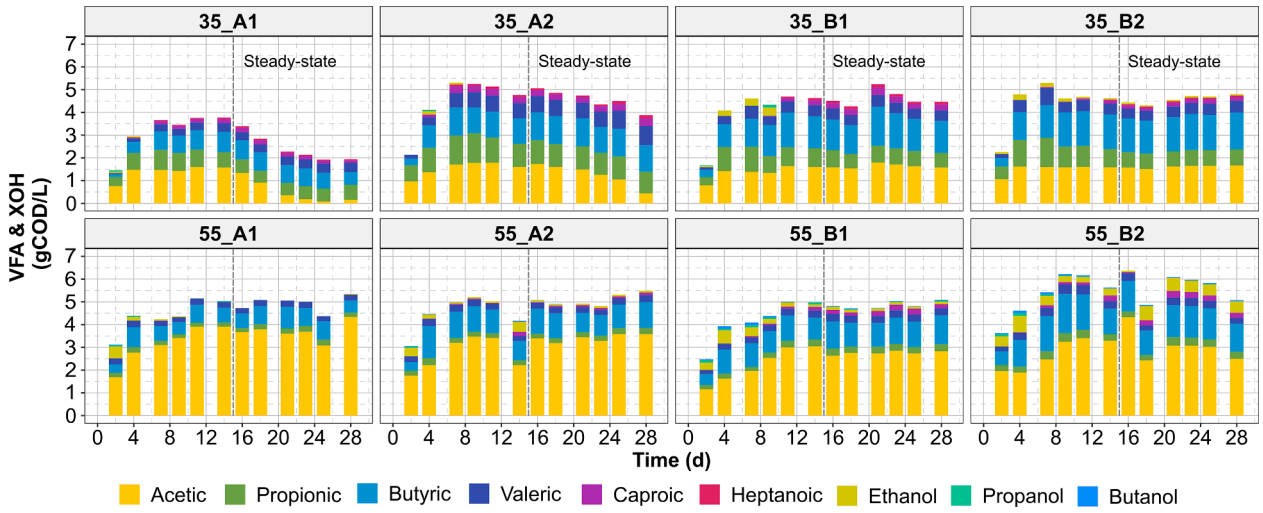

Fig. 2. Evolution of the fermentation product profile for each fermenter in Experiment 1. Grey dashed line indicates the beginning of the steady-state period.

Experiment 1 showed two different fermentation product profiles based on the operating temperature (Fig. 2). At 55 °C, the fermentation product profile was dominated by acetic acid and butyric acid. Fermenters 55_A1 and 55_A2 had acetic acid concentrations of ~4.0 gCOD/L, accounting for ~65 % of the fermentation products on COD basis, and butyric acid concentrations of ~2.0 gCOD/L, accounting for ~25 % of the fermentation products on COD basis (see fermentation product profile distribution Fig. S2 in the Supplementary material). Similarly, fermenters 55_B1 and 55_B2, obtained a concentration of acetic acid of ~3.5 gCOD/L, accounting for ~50 % of the fermentation products on COD basis, and butyric acid concentrations of ~2.0 gCOD/L, accounting for ~25 % of the fermentation products on COD basis (see fermentation product profile distribution Fig. S2 in the Supplementary material). At 35 °C, the fermentation product profile was dominated by acetic acid, propionic and butyric acid. In fermenters 35_B1 and 35_B2, acetic acid concentration was ~1.5 gCOD/L (~35 % in COD basis), propionic acid concentration was ~0.7 gCOD/L (~15 % in COD basis), and butyric acid was ~1.5 gCOD/L (~35 % in COD basis) (see fermentation product profile distribution in Fig. S2 in the Supplementary material). At 35 °C, differences in the fermentation product profile based on WAS origin were primarily driven by acetic acid consumption, while the concentrations of propionic, butyric and valeric acids remained similar across all mesophilic runs. Acetic acid consumption occurred only in fermenters fed with WAS_A (35_A1 and 35_A2) and was not observed in those fed with WAS_B (35_B1 and 35_B2). Therefore, given the similar concentrations of the other VFA, it cannot be concluded that WAS origin influences the product profile beyond the effect on acetic acid consumption.

3.1.2. *Microbial communities at mesophilic and thermophilic temperatures*

The microbial mass balance results show that, when using the same WAS origin, temperature had a strong impact on ASVs able to grow. Under thermophilic conditions, fewer ASVs migrating from WAS grew (13 % at 55 °C compared to 27 % at 35 °C). However, the ASVs that grew at 55 °C represented over 75 % of the cumulative relative abundance (Fig. 3A). Regarding the WAS origin, no differences in the proportion of

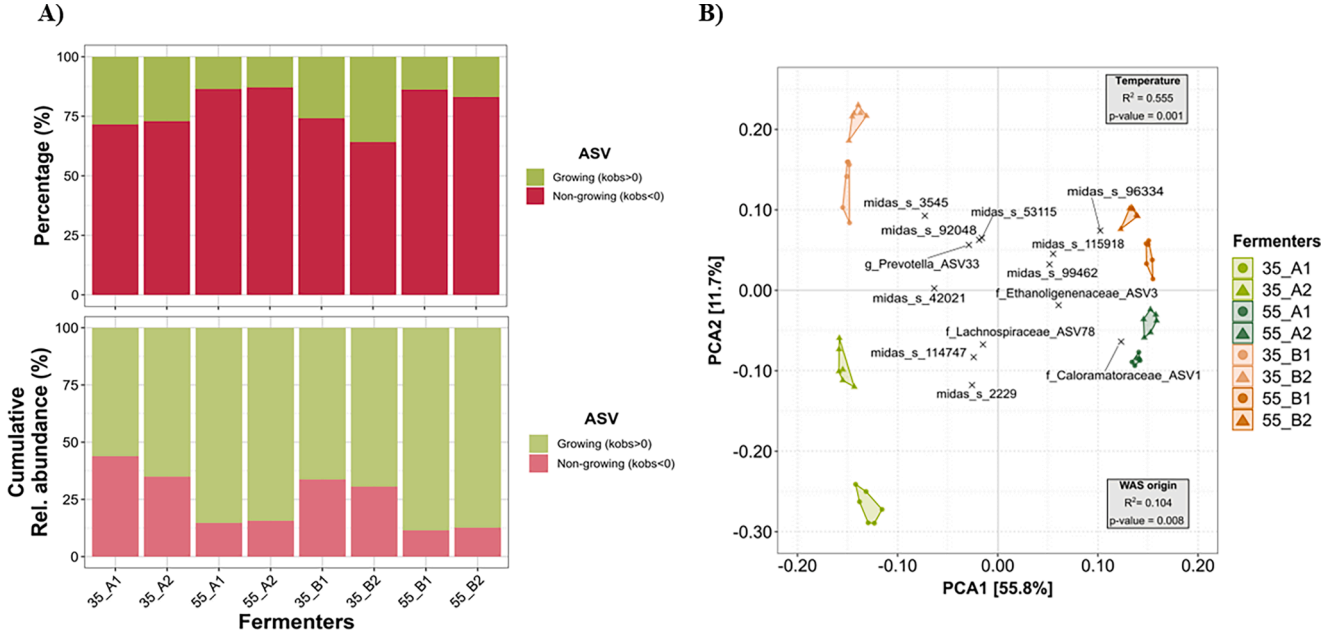

Fig. 3. A) Growing and non-growing microorganisms in Experiment 1. B) PCA of growing microorganisms for each fermenter. Colours represent WAS origin, and the different shapes represent the WAS collection period. Colour intensity represents the operational temperature. Grey box in the PCA shows the PERMANOVA results.

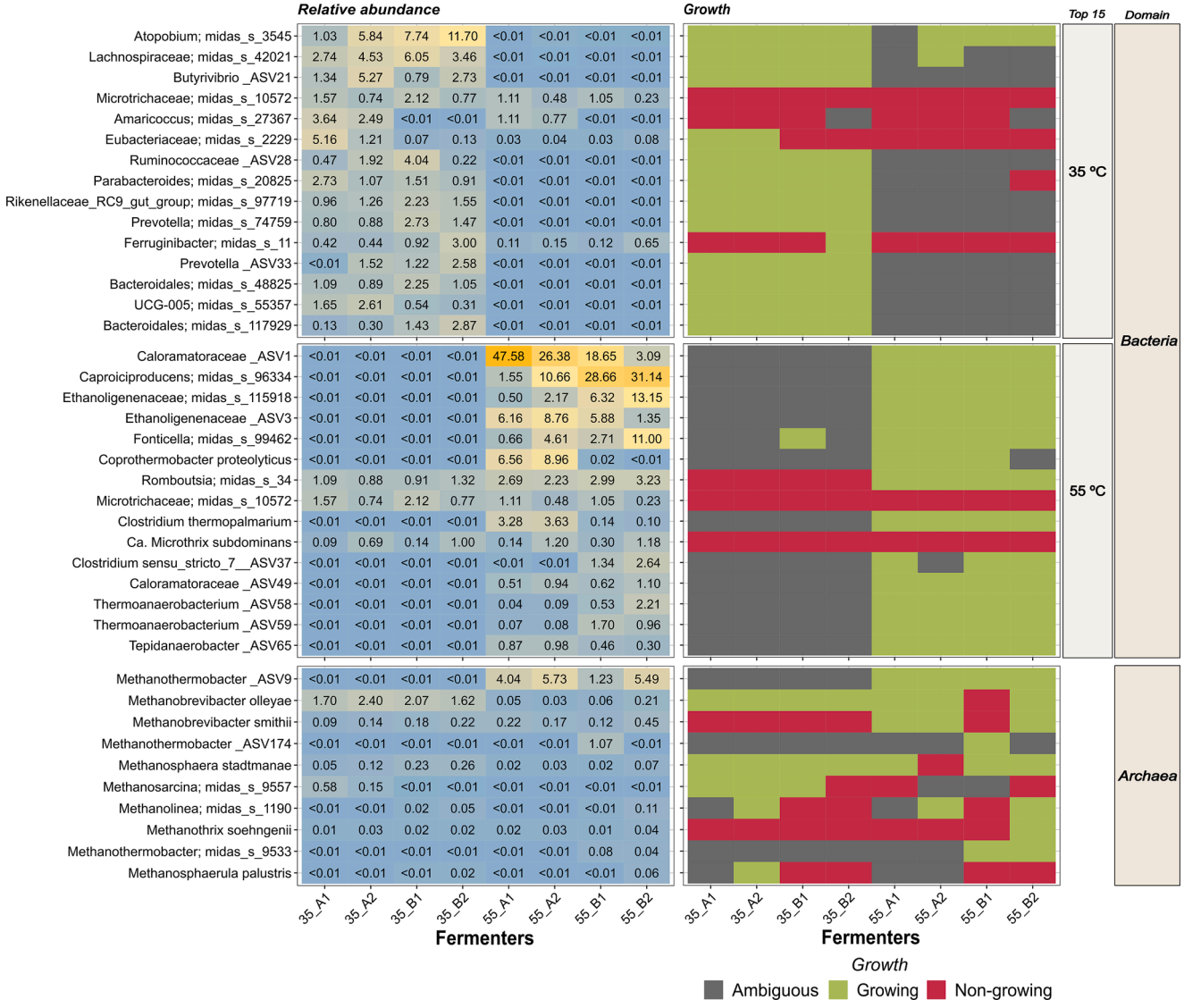

Fig. 4. Relative abundance heatmap (left) and growth fate of the species depending on the fermenter (right) in Experiment 1. The first two blocks represent the domain *Bacteria*, and the third block represents the domain *Archaea*. From top to bottom, the first block displays the 15 most abundant bacterial species at 35 °C, the second block shows the 15 most abundant species at 55 °C, and the third block shows the 10 most abundant *Archaea* species overall.

growing and non-growing microorganisms were observed between the WAS. Under the same operating temperature, the two WAS showed similar behaviour in terms of individual ASVs able to grow and the cumulative relative abundance they represented. Nonetheless, the co-fermentation microbial structure (beta-diversity, PCA) varied significantly with the operational temperature as visualised by the cluster's separation in PCA1 (Fig. 3B), which accounts for 55.8 % of the variance. Additionally, a different clustering pattern was observed based on the WAS origin, as shown by the separation along the PCA2 axis, which accounts for 11.7 % of the variance (Fig. 3B). PERMANOVA analysis confirmed that temperature had a greater impact on microbial consortium than WAS origin on fermenter's microbial structure. Specifically, temperature explained a $R^2 = 0.555$ (p-value = 0.001), while the WAS origin accounted for an $R^2 = 0.104$ (p-value = 0.008).

The differences observed in the microbial structure (identity and abundance) were mainly driven by the operating temperature. As for the microbial community of fermenters operated at 35 °C (35_A1, 35_A2, 35_B1 and 35_B2), microorganisms were more evenly distributed, whereas at 55 °C (55_A1, 55_A2, 55_B1 and 55_B2), the microbial community was dominated by 1 or 2 microorganisms (Fig. 4). The lower diversity under thermophilic conditions is evident from the lower diversity indices, with a Shannon diversity index of 3.16 ± 0.29 and a Simpson diversity index of 0.85 ± 0.07. In contrast, at 35 °C, the Shannon and Simpson indices were higher, at 5.21 ± 0.21 and 0.98 ± 0.01, respectively.

At 35 °C, the microbial community was mainly enriched in species midas_s_3545 (genus *Atopobium*), with a higher relative abundance in fermenters 35_B1 and 35_B2. This microorganism could also grow in thermophilic conditions; however, it did not thrive, with an abundance below 0.01 %. Species midas_s_42021 (genus *Lachnospiraceae*) was also enriched in all mesophilic fermenters with a relative abundance between 3–6 %. *Butyrivibrio*_ASV21 grew in all fermenters but it was more abundant in 35_A2. Species midas_s_2229 (family *Eubacteriaceae*) stands out because it only grew in the mesophilic fermenters fed with WAS_A (35_A1 and 35_A2). The family *Ruminococcaceae*_ASV28 also grew in mesophilic fermenters. At 55 °C, the microbial consortium of the fermenters 55_A1 and 55_A2 was dominated by the family *Caloramatoraceae*_ASV1, whereas the microbial community of 55_B1 and 55_B2 was dominated by midas_s_96334 (genus *Caproiciproducens*). The species midas_s_34 (genus *Romboutsia*) only grew under thermophilic conditions (55_A1, 55_A2, 55_B1 and 55_B2) with a relative abundance of 1–3 %.

In the *Archaea* domain, hydrogenotrophic archaea grew in all fermenters under both mesophilic and thermophilic conditions. *Methanothermobacter* (ASV9), with a relative abundance of 1–6 %, grew exclusively in the thermophilic fermenters (55_A1, 55_A2, 55_B1, and 55_B2). In contrast, *Methanobrevibacter olleyae* was present under both mesophilic and thermophilic conditions (except in 55_B1) but displayed a higher relative abundance in mesophilic conditions (1.5–2.5 %) compared to thermophilic conditions (<0.22 %). These two species are hydrogenotrophic methanogens that convert the H_2 produced during the fermentation process into methane. The aceticlastic methanogen, midas_s_9557 (genus *Methanosarcina)* grew in some mesophilic fermenters, but not at 55 °C. This microorganism grew in fermenters 35_A1, 35_A2 and 35_B1. Their relative abundance was greater than 0.01 % in fermenters 35_A1 and 35_A2 (0.58 % and 0.15 %, respectively). The proliferation of these microorganisms was related to the WAS origin. Although their relative abundance in the WAS was very low (<0.01 %), they were able to grow in the fermenters (see Top 10 most abundant Archaea Fig. S3 in the supplementary material).

3.2. Experiment 2: Co-fermentation consistency at 55 °C

3.2.1. Co-fermentation performance at thermophilic temperature

To evaluate the robustness of WAS and FW co-fermentation at 55 °C, nine fermentation runs were compared, comprising 3 WAS origins and 3 WAS collection periods for each WAS origin. All fermenters showed similar steady-state results (Fig. 5). The pH remained between 5.35–5.45 in fermenters A, B and C (see effluent characterisation in Table S4 in Supplementary material). There were no practical differences in solubilisation and fermentation yields among operational runs independently of WAS origin and collection period (Fig. 5). However, some minor differences were observed between WAS origin, and to a lesser extent, collection period. For example, fermenters A and B exhibited slightly higher sCOD accumulation (9.5 ± 0.1 gCOD/L and 9.9 ± 0.3 gCOD/L, respectively) than fermenters C (8.2 ± 0.3 gCOD/L) (see effluent characterisation Table S4 in Supplementary material). Regarding TAN, a slightly higher TAN concentration was observed in fermenters working with WAS_A (518–579 mgN/L) compared to those working with WAS_B and WAS_C (430–480 mg N/L). As for fermentation yields, fermenters A achieved 289 ± 28 mgCOD/gVS, fermenters B achieved 326 ± 10 mgCOD/gVS and fermenters C achieved 284 ± 8 mgCOD/gVS. The evolution of the fermentation product showed that no net VFA consumption occurred during the operation of these fermenters, and the total concentration was around 4.6–6.0 gCOD/L. Fermenters 55_A4 showed the lowest VFA concentration (4.6 ± 0.2 gCOD/L) and fermenter 55_A5 the highest (6.0 ± 0.2 gCOD/L) (see effluent characterisation in Table S4 in Supplementary material).

The fermentation product profile was also consistent across all fermenters, regardless of the WAS origin or WAS collection period (Fig. 6). In all scenarios the product profile was dominated by acetic acid and butyric acid. No noticeable effect of the WAS collection period was identified during fermenter's operation. However, minor differences on VFA concentration were observed when comparing fermenters operated with different WAS origins (WAS_A, WAS_B and WAS_C). The only noticeable difference was the lower concentration of acetic and butyric acids in the fourth collection period of fermenter A (55_A4), which led to the lowest fermentation yield.

Minor differences in VFA concentrations were observed between the fermenters. On the one hand, fermenters treating WAS_A produced more acetic acid (~4.0 gCOD/L) than those treating WAS_B and WAS_C (~2.7 gCOD/L and ~2.5 gCOD/L, respectively). On the other hand, the fermenter treating WAS_B accumulated more butyric acid (~2.5 gCOD/L) than those treating WAS_A and WAS_C (~2.1 gCOD/L and ~2.1 gCOD/L, respectively). A slight tendency to accumulate ethanol and caproic acid was observed in the fermenters treating WAS_B and WAS_C. Ethanol concentrations reached approximately 0.5 gCOD/L and 0.3 gCOD/L in fermenters treating WAS_B and WAS_C, respectively, representing <6 % and 4 % of the fermentation products on a COD basis (Fig. 6). This phenomenon was not observed in the fermenter treating WAS_A during the steady-state period (see fermentation product distribution in Fig. S4 in the Supplementary material).

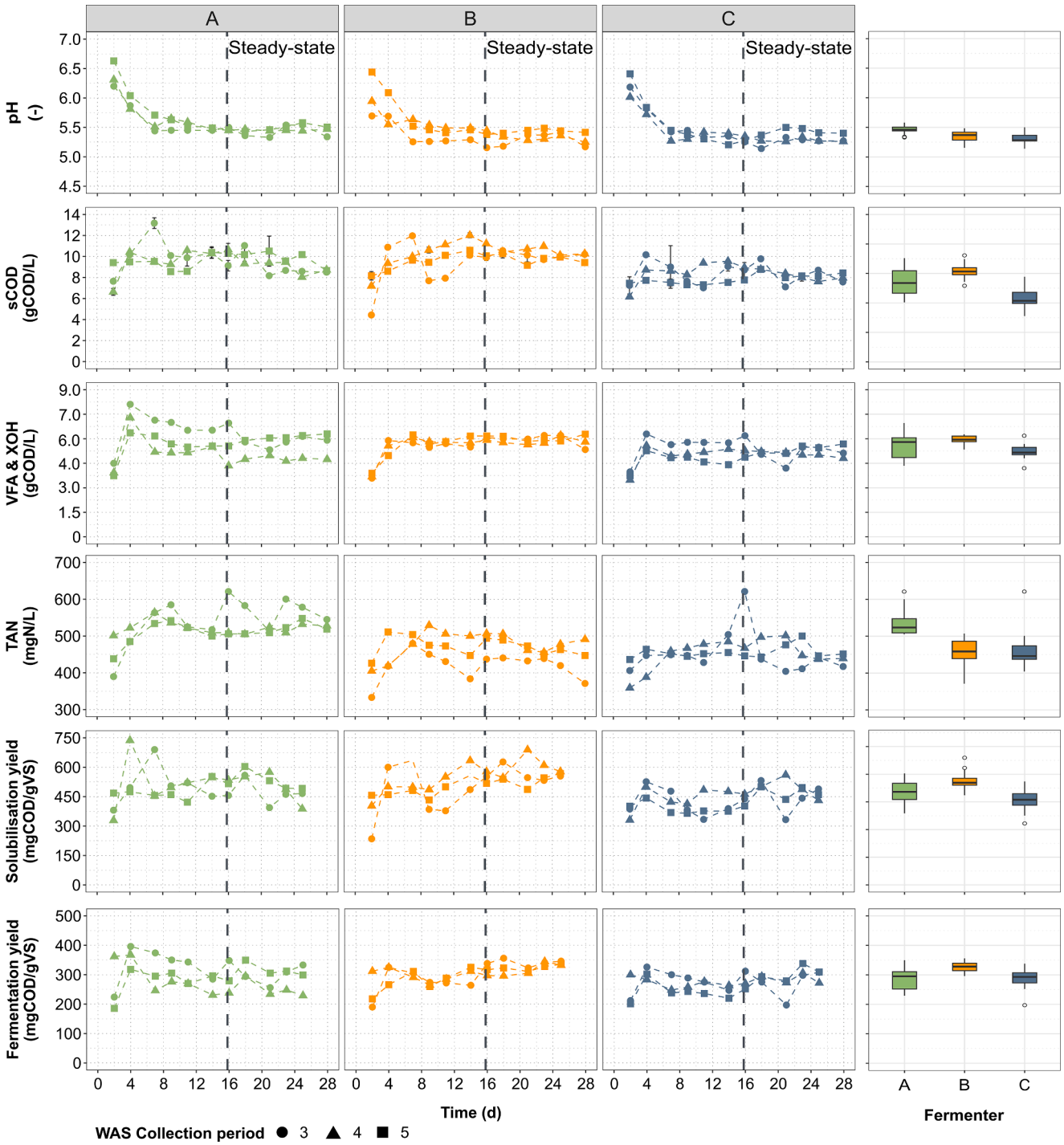

Fig. 5. pH, sCOD, TAN, solubilisation yield, fermentation yield and VFA & XOH concentration for each fermenter over time in Experiment 2. Grey dashed line indicates the beginning of the steady-state. Boxplot graph compares the steady-state values among WAS origin.

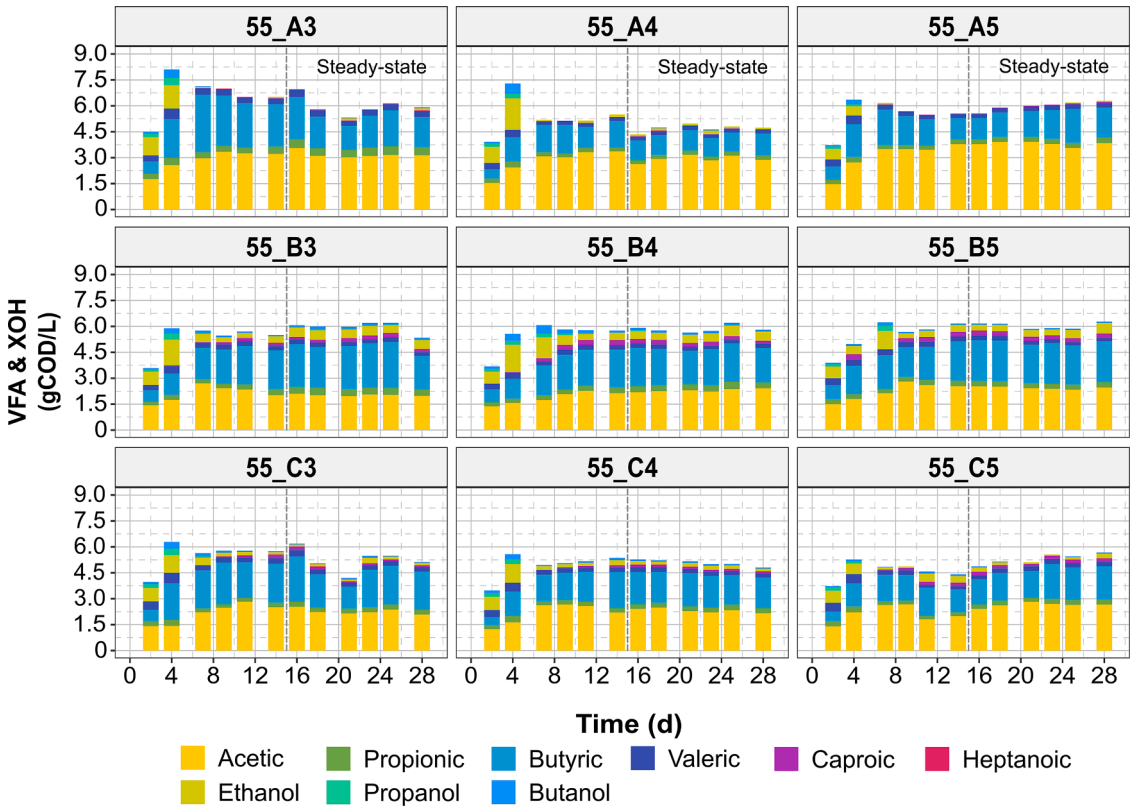

Fig. 6. Fermentation products profile for each fermenter over time in Experiment 2. Vertical dashed line indicates the start of the steady-state period.

3.2.2. *Microbial community at thermophilic temperature*

Around 10–20 % of the ASVs migrating from WAS were able to grow in the fermenters, according to the results of the microbial mass balance (Fig. 7A). However, the growing microorganisms accounted for almost 75 % of the cumulative relative abundance, indicating that WAS microorganisms can thrive in thermophilic fermenters.

The beta-diversity (PCA) of the growing microorganisms showed that each fermenter had its own microbial community (Fig. 7B). PERMANOVA analysis confirmed that WAS origin had a greater impact on microbial consortium. In Experiment 2, WAS origin explained an $R^2 = 0.527$ (p-value = 0.001). Furthermore, fermenters treating WAS_B and WAS_C exhibited similar microbial communities across all collection

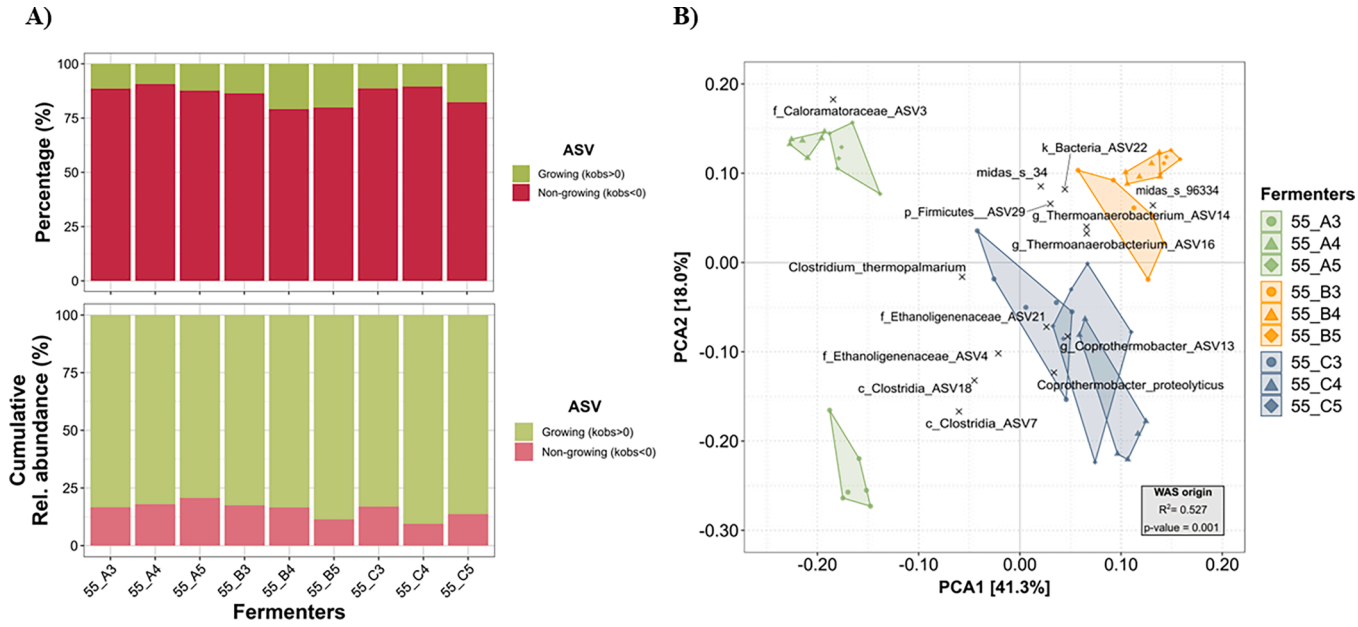

Fig. 7. A) Growing and non-growing microorganisms in Experiment 2. B) PCA of growing microorganisms for each fermenter in Experiment 2. Colours represent WAS origin, and the different shapes represent the WAS collection period. Colour intensity represents the operational temperature. Grey box in the PCA shows the PERMANOVA results.

periods. In case of the fermenter treating WAS_A, 55_A3 was distant from 55_A4 and 55_A5, indicating differences in their microbial consortium (Fig. 7B).

In fermenter 55_A3, the microbial consortium was dominated by *Clostridia* class, specifically by two unclassified ASVs, ASV7 (~23 %) and ASV8 (~13 %). In contrast, the family *Caloramatoraceae*_ASV3 flourished in fermenter 55_A4 and 55_A5, comprising ~50 % and ~35 % of relative abundance, respectively (Fig. 8). The species midas_s_96334 (genus *Caproiciproducens*) was the most abundant in fermenters treating WAS_B and WAS_C. *Caproiciproducens* midas_s_96334 also grew in fermenters treating WAS_A, but its relative abundance was lower (~ 6 %). In fermenters treating WAS_C, it was observed that the microbial community was also dominated by the genus *Caproiciproducens* (~24 %) and the family *Ethanoligenenaceae* (~19 %). Although midas_s_99762 (genus *Fonticella*) grew in all fermenters, its relative abundance was higher in fermenters B (~ 8 %) and fermenters C (6.7–12.5 %) compared to fermenters A (~ 4 %).

In reference to the *Archaea* kingdom, the genus *Methanothermobacter*_ASV9 grew in all fermenters with a relative abundance between 1–4 % (Fig. 8). An aceticlastic microorganisms, *Methanothrix soehngenii* was detected with a relative abundance <1 % but did not grow in any of the fermenters. *Methanothrix soehngenii* entered the

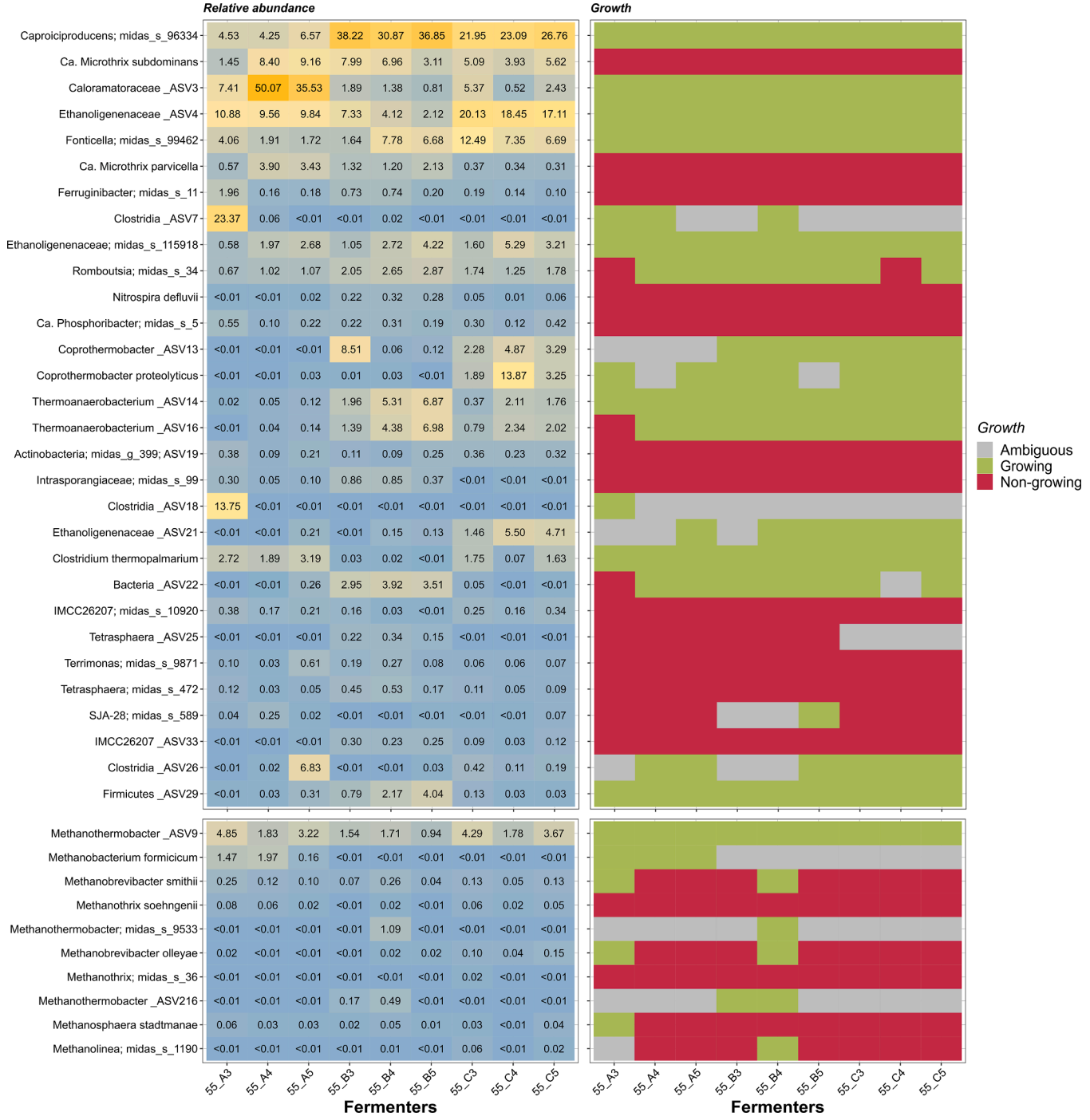

Fig. 8. Heatmap showing the most abundant microorganisms and their growth fates (growing, non-growing, and ambiguous) in all fermenters in Experiment 2. From top to bottom, top 30 most abundant in the *Bacteria* domain and followed by the top 10 species in the *Archaea* domain.

fermenters through biomass migration from the WAS, but it did not grow within the fermenters (see top 10 most abundance archaea in Fig. S5 in the Supplementary material).

In Experiment 2, genus *Methanosarcina* was not detected in the fermenters, in contrast to Experiment 1, where it was detected at a low relative abundance (< 0.6 %).

4. Discussion

4.1. Robustness of the fermentation process at 55 °C

Thermophilic co-fermentation performance was robust and consistent when compared to mesophilic conditions, considering most of the monitored parameters. At 35 °C, acetic acid consumption occurred in two of the four runs (35_A1 and 35_A2), resulting in an unwanted decrease in fermentation yield. The fact that this observation only took place in fermenters operated at 35 °C and WAS_A aligns with previous studies (Perez-Esteban et al., 2024b, 2024a). This behaviour was primarily attributed to acetic acid consumption by methanogenic archaea, as demonstrated in previous studies (Pang et al., 2020; Perez-Esteban et al., 2024b, 2024a). These results indicate that (i) the proliferation of methanogens from WAS migration have a noticeable impact on fermentation performance at 35 °C and (ii) the impact of WAS origin is mitigated in fermenters operated at 55 °C (55_A1 and 55_A2). Importantly, none of the fermenters operated at 55 °C (13 runs) showed episodes of acetic acid consumption. Regardless of the WAS origin, operating at 55 °C resulted in a higher solubilisation yield and higher TAN concentrations in the fermentation liquor in accordance with Garcia-Aguirre et al. (2017). Despite the higher hydrolysis rate at 55 °C, the fermentation yield did not improve significantly when compared to 35 °C. This could be caused by a higher solubilisation of the non-fermentable fraction.

The fermentation product profile obtained under thermophilic conditions was consistent with other studies that have co-fermented WAS and FW under thermophilic conditions (Garcia-Aguirre et al., 2017; Jiang et al., 2013; Perez-Esteban et al., 2024b). This fact indicates certain consistency in fermentation process at 55 °C. Small differences were observed according to the WAS origin. For example, 55_A accumulated more TAN in the fermentation liquor than 55_B and 55_C, which could be related to a higher protein content in WAS_A (Capson-Tojo et al., 2016; Xiao et al., 2017). Besides, fermenters 55_A accumulated more acetic acid than butyric acid, while 55_B and 55_C tended to accumulate alcohols. The differences in VFA concentration among fermenters A, B and C were around 1 gCOD/L, which is not considered a major difference for full-scale applications. At 55 °C, the WAS collection period from the same WWTP had no significant influence on co-fermentation performance. The only noticeable difference was the slightly lower concentration of acetic and butyric acid in 55_A4. The lower VFA concentration might be due to the lower biodegradability of the WAS compared to fermenters 55_A3 and 55_A4 (Marchetti et al., 2023).

4.2. Thermophilic temperature as a selective microbial community pressure to limit the growth of aceticlastic methanogens

At 35 °C, a higher microbial diversity was observed, while at 55 °C, the microbial communities were dominated by a lower number of microorganisms. This is caused by temperature exerting a higher selective pressure on the microbial consortium (Kim et al., 2010; Zeldes et al., 2015). In mesophilic conditions, the microbial communities were more evenly distributed, with no single species exceeding a maximum relative abundance of 11 %. The most abundant genera are putative fermenters producing short-chain fatty acids (*Atopobium, Lachnospiraceae, Butyvibrio, Eubacteriacea* and *Ruminococcaceae*) (Blasco et al., 2020; Brison et al., 2022; Gulhane et al., 2017; Sun et al., 2021). On the other hand, the microbial consortium at 55 °C was dominated by fewer genera, an unclassified *Clostridia* (ASV17 and ASV18) and *Caloramatoraceae* in fermenters treating WAS_A, *Caproiciproducens* and *Fonticella* in fermenters treating WAS_B, and *Caproiciproducens, Ethanoligenenaceae* and *Fonticella* in fermenters treating WAS_C. *Caloramatoraceae* are known producers of formic acid, acetic acid and ethanol (Zhu et al., 2023). The genus *Caproiciproducens* is associated with the production of carboxylic acids, such as butyric acid, acetic acid and caproic acid (Y. Zhang et al., 2023). *Caproiciproducens* is able to produce caproic acid. However, under the operational conditions of this study, either the metabolic pathway leading to caproic acid does not occur, or caproic acid is further degraded to acetic and butyric acids (Flaiz et al., 2020).

Clostridia is a highly polyphyletic class generally associated with fermentation reactions (Liu et al., 2012; Zahedi et al., 2016; Zhang et al., 2020), with some members involved in the degradation of long-chain fatty acids into acetate (Llamas et al., 2022). The genus *Fonticella* is known to produce acetic acid and could contribute to the accumulation of acetic acid in the fermentation liquor (Fraj et al., 2013; Zhu et al., 2023). Finally, the family *Ethanoligenenaceae* is known to produce ethanol and hydrogen, potentially contributing to ethanol accumulation in the fermentation liquor (Ndayisenga et al., 2022; Z. Zhang et al., 2023). Despite differences in microbial consortium, all fermenters demonstrated functional redundancy yielding in a similar fermentation product profile.

The acetic acid consumption episodes under 35 °C conditions were triggered by species midas_s_9557 (genus *Methanosarcina*). The methanogen *Methanosarcina* (midas_s_9557) had a relative abundance between 0.15–0.60 % in fermenters 35_A1 and 35_A2. The presence of this species was attributed to microbial migration from WAS (the main substrate) and subsequent proliferation in the fermenters. Despite its low relative abundance in WAS_A (<0.01 %), species midas_s_9557 was able to thrive in fermenters 35_A1 and 35_A2 (<0.01 %). *Methanosarcina* genera is a fast-growing mixotrophic methanogen with strong tolerance to high concentrations of TAN, salts and acetic acid concentration (De Vrieze et al., 2012). These characteristics implies that, under mesophilic conditions, limiting the growth of *Methanosarcina* that migrates with the influent is difficult (Conklin et al., 2006). Despite the higher operational costs, operating fermenters at 55 °C stands as a feasible solution to prevent the proliferation of aceticlastic methanogens in acidogenic fermenters treating WAS. Despite detecting *Methanosarcina* midas_s_9557 in conditions 55_A1 and 55_A2, it did not grow, and no net acetic acid consumption was recorded in any of the 13 fermentation runs operated at 55 °C. No thermophilic *Methanosarcina* species were detected in this study, neither in the WAS or the fermenters. Nonetheless, the identity and impact of net acetic acid consumers at thermophilic conditions if other substrates or fermentation operational conditions are used warrants further research.

5. Conclusions

Experiment 1 compared the effect of operational temperature (35 vs 55 °C) on the fermentation product profile of waste activated sludge (WAS) and food waste (FW). Under mesophilic conditions (35 °C), the product profile was dominated by acetic, butyric, and propionic acids, while at thermophilic conditions (55 °C), the product profile was dominated by acetic and butyric acids. A higher solubilisation yield was achieved at 55 °C (447–600 mgCOD/gVS) compared to 35 °C (332–454 mgCOD/gVS). In contrast, the fermentation yield was comparable, ranging from 249 to 340 mgCOD/gVS at 55 °C and 139–266 mgCOD/gVS at 35 °C. Regarding the microbial community, temperature had a more significant impact than WAS origin. The growth of midas_s_9557 (genus *Methanosarcina*) under mesophilic conditions was associated with acetic acid consumption periods. The growth species midas_s_9557 (genus *Methanosarcina*) was limited under thermophilic conditions, preventing the net consumption of acetic acid. Despite differences in the microbial communities under thermophilic conditions, the product profile was consistent across all fermenters, regardless of WAS origin and collection period.

CRediT authorship contribution statement

N. Perez-Esteban: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **R. Tully:** Writing – review & editing, Investigation, Data curation. **M. Peces:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **J. Dosta:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **S. Astals:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joan Dosta reports financial support was provided by Ministry of Science Technology and Innovations. Sergi Astals reports financial support was provided by Ministry of Science Technology and Innovations. Miriam Peces reports financial support was provided by Horizon Europe. Noemi Perez Esteban reports financial support was provided by Ministry of Science Technology and Innovations. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122970.

Data availability

Data will be made available on request.

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