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Inhibitory effects of long chain fatty acids on anaerobic sludge treatment: Biomass adaptation and microbial community assessment

Maria A. Szabo-Corbacho [a,b,c,f,*], Pragnya Sharma [a], Diana Míguez [b], Victoria de la Sovera [e], Damir Brdjanovic [a,d], Claudia Etchebehere [e], Hector A. García [a,f], Jules B. van Lier [a,c]

[a] Water Supply, Sanitation, and Environmental Engineering Department, IHE Delft Institute for Water Education, Westvest 7, 2611 AX Delft, the Netherlands
[b] Latitud – Fundación LATU, Laboratorio Tecnológico del Uruguay (LATU), Avda. Italia 6201, Montevideo, Uruguay
[c] Delft University of Technology, Faculty of Civil Engineering and Geosciences, Department of Water Management, Sanitary Engineering Section, Stevinweg 1, 2628 CN Delft, the Netherlands
[d] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, the Netherlands
[e] Microbial Ecology Laboratory, BioGem Department, Biological Research Institute Clemente Estable, Ministry of Education, Av. Italia, 3318 Montevideo, Uruguay
[f] Department of Sanitary Engineering, Central-South Regional Technological Institute, Technological University of Uruguay (UTEC), Maciel s/n esq. Luis Morquio, Durazno, Uruguay

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ABSTRACT

The study investigated the effects of long-chain fatty acids (LCFA) on anaerobic sludge treating lipid-rich wastewater. It involved batch experiments with three sludge samples: two acclimated to lipids and one non-acclimated. The experiments aimed to observe the degradation of LCFA, specifically oleate and palmitate, by dosing them at concentrations ranging from 50 to 600 mg/L. Measurements of the cumulative methane production and the LCFA concentration, quantified as fat, oil, and grease (FOG) were performed. To ensure the sludge was free from other biodegradable substrates, part of the samples was pre-incubated without feed. The tests were conducted with both pre-incubated and non-incubated inoculum sludge. The findings revealed that oleate was degraded more efficiently than palmitate across all sludge samples, with a greater conversion rate to methane. Sludge samples acclimated to lipids showed a superior capacity to degrade LCFA compared to non-acclimated ones. It was noted that at concentrations above 400 mg/L, the conversion of LCFAs to intermediate compounds was inhibited, although this did not affect the subsequent methane production. The study concludes with a recommendation for sludge adaptation strategies to boost the efficiency of anaerobic wastewater treatment systems dealing with lipid-rich waste. The presence of LCFA-degrading bacteria families like *Kosmotogaceae, Petrotogaceae*, and *Synergistaceae* in the acclimated sludge samples underscores the adaptation and potential for improved degradation performance.

* Corresponding author at: Department of Sanitary Engineering, Central-South Regional Technological Institute, Technological University of Uruguay (UTEC), Maciel s/n esq. Luis Morquio, Durazno, Uruguay.
E-mail addresses: M.szabocorbacho@tudelft.nl, alejandraszaboc@gmail.com (M.A. Szabo-Corbacho).

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1. Introduction

Anaerobic wastewater treatment exhibits distinct advantages compared to aerobic treatment, particularly, when treating wastewater with high concentrations of organic matter, as in food processing wastewater (van Lier, 2008). Wastewater produced by dairy, meat processing, and oil processing industries are characterized by high concentrations of both organic matter, including lipids (Perle et al., 1995; Sayed and de Zeeuw, 1988; Beccari et al., 1996). The largest group of lipids consists of triacyl glycerides, which are long chain fatty acids (LCFA) esterified to glycerol (Alves et al., 2009). Lipids are commonly measured using their biochemical properties, i.e., hydrophobicity, and are then identified as fats, oils, and grease (FOG). According to Hwu et al. (1998), the most commons LCFAs found in lipid rich wastewaters are palmitic acid (C16:0), a saturated LCFA; and oleic acid (C18:1), an unsaturated LCFA.

In the anaerobic treatment of wastewater with a high FOG content, the hydrolysis (by extracellular or membrane-bound lipases) of FOG occurs relatively fast resulting primarily in LCFAs and glycerol, when the FOG consists of primarily triacyl glycerides. However, the subsequent degradation of LCFA to acetate and hydrogen occurs at a low pace, potentially leading to the accumulation of LCFAs in the reactors (Pavlostathis and Giraldo-Gomez, 1991). The LCFA (saturated and unsaturated) conversion to acetate and hydrogen occurs via the β-oxidation reaction, in which an acetyl group is subsequently split off from the long aliphatic carbon chain of the LCFA. In the β - oxidation pathway, initially, the LCFA are transported into the bacterial cells (Mackie et al., 1991), converted into acyl-CoA thioesters by acyl-CoA synthetase, and then undergo β - oxidation. This process, detailed by DiRusso et al. (1999), cyclically shortens the acyl-CoA, producing acetyl-CoA and hydrogen (Sousa et. al, 2009).

However, there are differences in how the different LCFAs are degraded. Saturated LCFAs undergo immediate degradation via the conventional β - oxidation pathway (Supplementary information, S2). In contrast, the breakdown of unsaturated LCFAs may necessitate an initial step of hydrogenation or may follow a different degradation route, as indicated by research from Weng and Jeris (1976) and Roy et al. (1986). The degradation of unsaturated LCFAs involves two steps: hydrogenation to the saturated LCFA with the same chain length, like oleate (18:1) conversion to stearate (C18:0), and then followed by β -oxidation. The hydrogenation step, converting oleate to stearate, is often the limiting factor in this process, while the subsequent β - oxidation of stearate to palmitate (C16:0) typically proceeds more rapidly (Pereira et al., 2005). There is uncertainty whether these steps are carried out by a single microorganism or multiple species (Sousa et al., 2009). Observations from anaerobic bioreactors treating oleate-based effluents (Pereira et al., 2002) show palmitate accumulation outside cells, suggesting a bottleneck after one β - oxidation cycle. This could indicate that bacteria responsible for degrading oleate also handle saturated fatty acids like palmitate, but the reverse is not always true (Sousa et al., 2010).

Alves et al. (2009), Becker et al. (1999), and Broughton et al. (1998) reported that a high concentration of LCFAs in anaerobic reactors may lead to inhibition of the microorganisms involved in the β-oxidation pathway; thus, limiting the complete conversion of LCFA to acetate and possibly propionate. Notably, evenly numbered LCFAs only generate acetate, while non-evenly numbered LCFAs also produce propionate (Pereira et al., 2001). Potential inhibition reduces the treatment performance, as well as the potential amount of biogas that can be obtained by the subsequent conversion of acetate to methane. LCFA accumulation, therefore, limits the maximum amount of methane that can be obtained when treating lipid rich wastewater (Alves et al., 2009). Pereira et al. (2005) mention that inhibitory effects already can be visible at concentrations as low as 50 mg/L. However, this inhibition is not permanent; biomass can adapt, overcoming what is termed as "reversible or temporary inhibition." (Pereira et al., 2001). Bactericidal toxicity, on the other hand, may cause cell lysis due to interactions between LCFAs and microbial membranes (Rinzema et al., 1993). Studies have demonstrated that LCFA-laden biomass can still degrade LCFAs to methane when the mass transfer limitations are removed, thus maintaining the integrity and activity of the microbial cells (Pereira et al., 2005).

Anaerobic treatment of lipid-rich wastewater also poses challenges due to the adsorption of lipids onto biomass, which can cause issues like biomass flotation, biomass washout, and mass transport limitations (Hawkes et al., 1995; Hwu et al., 1998; Rinzema et al., 1989; Singh, 2019). Adsorption and accumulation of long-chain fatty acids (LCFA) on the biomass may result in prolonged lag phases in sludge batch experiments (Pereira et al., 2005).

The successful anaerobic degradation of lipid-rich wastewater, as studied by Silva et al. (2014), depends on a balance between LCFA-degrading bacteria and methane-producing archaea. Schink (1997) and Stams et al. (2006) described how certain bacteria break down LCFAs into acetate and hydrogen/formate, which are then converted into methane by archaea. This process is critically dependent on hydrogen-transfer rate between microbes, highlighting the essential nature of these microbial partnerships. The Gibbs' free energy change of acetogenic reactions becomes sufficiently negative at low partial pressure of hydrogen, maintained by a synergistic relationship between acetogenic bacteria and hydrogenotrophic methanogens. The partial pressure of hydrogen needs to be below 10 Pa for maintaining high conversion rates (Schink, 1997; Lalman and Bagley, 2002).

Research indicates enhanced degradation of LCFAs like oleic and palmitic acids, in the anaerobic treatment of lipid-rich wastewater using pre-acclimated biomass (Silva et al., 2014). Commonly, such wastewaters are characterized by concentrations ranges of oleic and palmitic acids from 100 to 900 mg/L (Cavaleiro et al., 2008). While most studies indeed use anaerobic sludge pre-acclimated to these common wastewater LCFAs, the impact of varying oleate and palmitate concentrations on LCFA degradation is less explored (Hanaki and Nagase, 1981). Notably, high concentrations (above 700 mg/L) of oleic and palmitic acids might inhibit their degradation in a different manner; an effect potentially influenced by their respective unsaturated and saturated characteristics (Pereira et al., 2005). Our present study aimed to evaluate the degradation of oleate and palmitate at varying concentrations using acclimated and non-acclimated sludge from different dairy wastewater treatment reactors. The research included methanogenic activity assays and microbial population analysis through 16 S rRNA gene sequencing to understand the roles of different microbes in the LCFA degradation pathway.

2. Materials and methods

2.1. Analytical methods

Chemical oxygen demand (COD) was determined using Spectroquant Kits (Merck Sharp & Dohme Corp., NJ, USA). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined using gravimetric analysis (APHA, 1989). The lipid content of the sludge was determined following the norm ISO 1443. A COD/ FOG theoretical ratio of 2.88 gCOD/gFOG for palmitic acid and 2.89 gCOD/gFOG was used for converting oleic acid into COD.

2.2. Sludge sources

This study utilized three distinct sludge samples. S1 came from a full-scale digester at a dairy plant where lipids are pre-removed (DAF unit), serving as a non-lipid-exposed baseline. Sludge sample S2, derived from a lab-scale AnMBR, was initially inoculated with S1, and later adapted by treating lipid-rich ice-cream wastewater for 635 days. Sludge sample S3, which was sourced from a full-scale anaerobic flotation reactor (AFR) (Biopaq®AFR, Hellendoorn, The Netherlands) treating FOG-rich dairy wastewater for over eight years, provided a second example of lipid-acclimated sludge. The S3 sample would have a potentially different microbial community due to its origin, coming from a brewery wastewater treating anaerobic high-rate reactor.

2.3. Assessment specific methanogenic activity

A modified specific methanogenic activity (SMA) test was conducted to assess the effects of selected LCFAs on the anaerobic sludge activity. Oleate and palmitate were selected as the representative LCFAs, considering both their abundance in industrial dairy wastewater and their potential toxic effects on the sludge (Karadag et al., 2015; Pereira et al., 2002). Different concentrations of synthetic reagent grade commercially available oleic acid and palmitic acid were added as substrates to the test vials.

Biodegradable substances possibly present in the original sludge samples could interfere during the SMA assessment carried out in batch assays. To determine such possible interferences, SMA assays were conducted using both non-incubated (NI) and pre-incubated (PI) sludge samples as inoculum. The PI sludge was incubated at 37 °C until no biogas production was observed, which lasted 15 days. The batch assays using the PI and NI sludge samples were carried simultaneously, at identical operational conditions.

For both oleate and palmitate, five batch experiments were carried out using PI sludge, applying initial concentrations of LCFA of 50, 100, 250, 450, and 600 mg /L. Using NI sludge, two batch experiments were carried out at initial concentrations of 250 and 600 mg/L of either oleate or palmitate.

Prior to the batch experiments, the sludge samples were characterized by determining the following parameters: TSS, VSS, COD, and FOG content. At the end of each batch test, the FOG content in the sludge in every batch test was again determined. In addition, the SMAs of the PI sludge samples were assessed (without adding any LCFA) by dosing acetate at an initial acetate concentration of 1.2 g COD/L. These experiments were carried out to assess the maximum methanogenic capacity of the sludge samples. For properly evaluating the LCFA degrading capacity by measuring the methane production rate, methanogenesis should not be the rate limiting step.

The batch experiments were conducted in 120 mL sealed serum bottles with a working volume of 65 mL. Buffer was provided by adding sodium bicarbonate at a concentration of 3.5 g/L in the bottles and the initial biomass concentration was 2.0 g VSS/L. The headspace of the bottles was flushed with nitrogen gas (99.99% N_2, Linde Uruguay LTDA, Montevideo, Uruguay) to create anaerobic conditions. The bottles were incubated at 37 °C (Memmert S25, Memmert GmbH + Co. KG) and shaken at 150 rpm in an orbital shaker (MaxQ Orbital shaker, Thermo Fisher Scientific). The batch experiments extended over a duration of approximately 36 days, while the SMA tests on the PI samples were conducted over a 5-hour period, utilizing 15-minute measurement intervals. The amount of produced biogas was determined using a pressure transducer at regular intervals (Colleran et al., 1991). A digital manometer (Flus, ET-922) was used to monitor the pressure increase. The methane content in the biogas produced was measured by liquid displacement, passing the biogas through a 2 M NaOH solution (Casallas-Ojeda et al., 2021). The methane content was corrected considering the non-standard temperature and pressure (STP) conditions in the test vials. The VSS specific methane production rate was calculated by linear regression, using the slope of the recorded methane production curve as suggested by Colleran et al. (1991) and was expressed in mg CH_4-COD/g VSS.d. The batch experiments were conducted in triplicate; and blanks were carried out in all experiments.

2.4. Microbial community analysis

Biomass samples were taken both from the sludge inoculum (S1, S2, and S3), as well as from each batch test carried out at 50 mg/L, 250 mg/L, and 600 mg/L of oleate and palmitate. Sludge sample S1 at 250 mg/L oleate, and sludge sample S2 at 250 mg/L palmitate were not taken for microbial community analysis. The samples were stored at − 20°C until performing the DNA extraction. For conducting the DNA extraction, the biomass was first separated by centrifugation (5000 rpm for 10 min).

The DNA extraction was conducted using the ZR Soil Microbe DNA MiniPrepTM kit (Zymo Research, CA, USA). 16 S rRNA (16 S ribosomal RNA) gene amplicons were obtained by PCR from the extracted DNA using adapters, barcodes, and the V4 Universal primers set 520 F 5´-AYTGGGYDTAAAGNG-3´and 802 R 5′-TACNNGGGTATCTAATCC-3′), as in Claesson et al. (2009). A specific primer set, (340 F 5 -CCCTAHGGGGYGCASCA-3 ´and 787 R 5 -´GGACTACVSGGGTATCTAAT −3), targeting the archaeal 16 S rRNA gene region was also used to improve the recovery of methanogens (Pinto and Raskin, 2012; Yu et al., 2005). The PCR products were visualized on

a 1% agarose gel electrophoresis. The amplicons were purified using a commercial kit (ZR Zymoclean™ Gel DNA Recovery Kit, USA). 16 S rRNA gene amplicon libraries were sequenced on an Ion Torrent PGM (Life Technologies, Thermo Fisher Scientific Inc., MA USA).

Bioinformatic processing was done using the QIIME Pipeline Version 1.9.1. Low quality reads were filtered (criteria: coefficient greater than 25) and sequences were trimmed to remove primers, barcodes, and adapters. Effective reads were obtained, which were further processed to remove chimera and noise. Operational taxonomic units (OTU) were made using the Uclust algorithm (Edgar, 2010) with a 97% identity threshold. The Silva database, release 132, was used for classification with a confidence threshold of 80%.

Principal coordinate analysis (PcoA) was performed to determine differences and similarities of the microbial communities from the following samples: (i) sludge samples taken as inoculum (S1, S2, and S3); and (ii) the sludge samples taken at the end of the different experiments as previously described. The PCoA was performed using the Bray-Curtis similarity index with the PAST software (Hammer et al., 2001).

3. Results

3.1. Physicochemical characterization and acetoclastic methanogenic activity of the inoculum sludge samples

The physicochemical characteristics of the inoculum sludge samples (S1, S2, and S3) were determined before (NI) and after (PI) incubation. The results are presented in Table 1. The incubation of the sludge samples resulted in a decreased VSS and COD content.

Activated sludge biomass ($C_5H_7O_2N$) typically has a COD/VSS ratio of 1.42 (Hoover and Porges, 1952). This ratio increases to 1.53 with anaerobic biomass ($C_5H_9O_2N$) (Batstone et al., 2000). Lipid/FOG accumulation can raise this ratio up to 2.0 - 2.9 (Ahnert and Krebs, 2021). Sludges S2 and S3 showed increased lipid content, which declined after pre-incubation, with S3 retaining more lipids than S2. The S1 COD/VSS ratio also reduced post-incubation, more than expected.

The maximum SMA values of the three PI sludge samples were determined using acetate as the substrate. The results showed that S1 and S2 exhibited similar SMA values of 1.11 ± 0.12 and 1.17 ± 0.15 g CH_4-COD/gVSS.d, respectively, while S3 showed a higher value of 1.65 ± 0.14 g CH_4-COD/gVSS.d. All the PI sludge samples showed a high methanogenic activity towards acetate, indicating that the three sludge samples were appropriate for testing the methanogenic activity using oleate and palmitate as the substrate (Pereira et al., 2005).

3.2. Methanogenic activity tests

3.2.1. Pre-incubated sludge evaluations

Fig. 1 compares cumulative methane production (CMP) from sludges when fed oleate versus palmitate. Higher methane yields and production rates were seen with oleate for all sludges, consistent with prior studies. All samples experienced a tree to four days lag phase before biogas production began, with peak methane rates in the first ten days, then plateauing. With palmitate, increased initial concentrations up to 250 mg/L enhanced methane production, which then declined at higher concentrations.

Fig. 2a presents maximum methane production from pre-incubated sludge, and Fig. 2b shows the corresponding production rates. Observed methane yields were significantly lower than theoretical maximums, particularly at LCFA concentrations above 250 mg/L. Methane yields were lower with palmitate except at 250 mg/L, where near-theoretical yields were seen. Sludge S3 showed better adaptation to LCFAs but still experienced inhibition at concentrations above 450 mg/L.

The methane production rates (Fig. 2b) indicated that oleate conversion rates increased with concentrations up to 450 mg/L, while palmitate did not show this trend and had overall lower rates. Early oleate inhibitory effects were less pronounced, while palmitate results were more variable.

3.2.2. Non-incubated sludge evaluations

Batch experiments using non-pre-incubated sludge were performed to evaluate the interferences of residual substrates present in the raw sludge, when dosing the LCFAs. Only two concentrations of oleate and palmitate were evaluated, i.e., 250 and 600 mg/L.

The results from the batch experiments shown in Fig. 3 indicated that the assessed CMP for the three evaluated sludge samples were always higher than the CMP observed in the PI sludge samples (Fig. 2). Strikingly, high CMP values were found for the blank, non-fed incubations.

Table 1
Physicochemical characterization of the inoculum sludge samples. The average values are presented.

Sludge	TSS (g/L)	VSS (g/L)	COD (g/L)	COD/VSS
S1-NI	41.8 ± 0.2	16.8 ± 0.1	23.1 ± 0.2	1.39
S1-PI	36.4 ± 0.2	15.3 ± 0.4	18.5 ± 0.2	1.21
S2 -NI	8.5 ± 0.1	6.5 ± 0.2	11.2 ± 0.4	1.72
S2-PI	6.6 ± 0.3	4.6 ± 0.2	7.0 ± 0.1	1.52
S3-NI	4.5 ± 0.1	3.2 ± 0.3	6.8 ± 0.2	2.13
S3-PI	4.1 ± 0.1	2.7 ± 0.3	5.4 ± 0.2	2.00

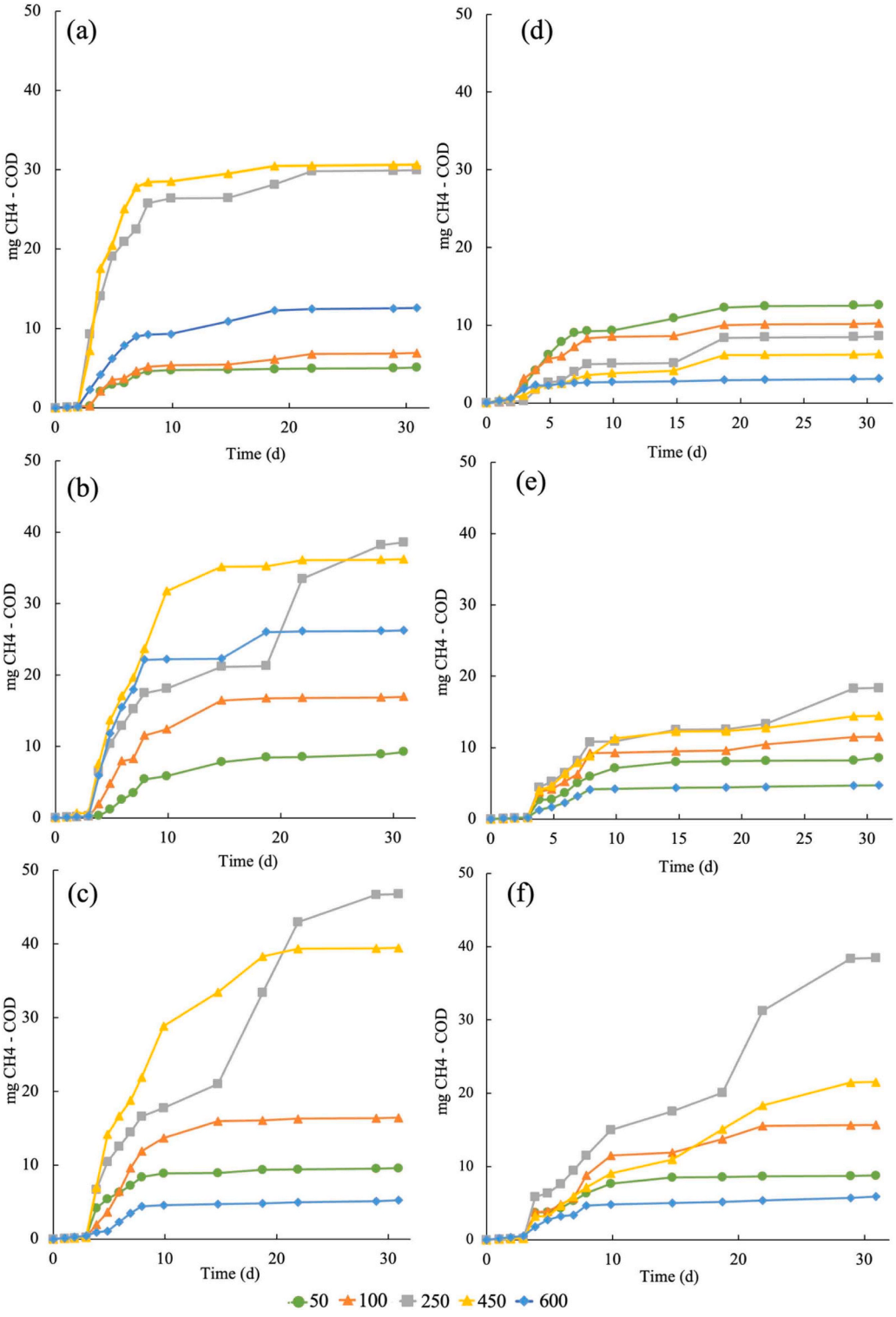

Fig. 1. Cumulative methane production exhibited by the PI sludge samples S1 (a, d), S2 (b, e) and S3 (c, f) at different oleate (a, b, c) and palmitate (d, e, f) concentrations. Average values are presented (maximum standard deviation of 10% were obtained – not shown in the figures); The PI blanks did not produce any methane.

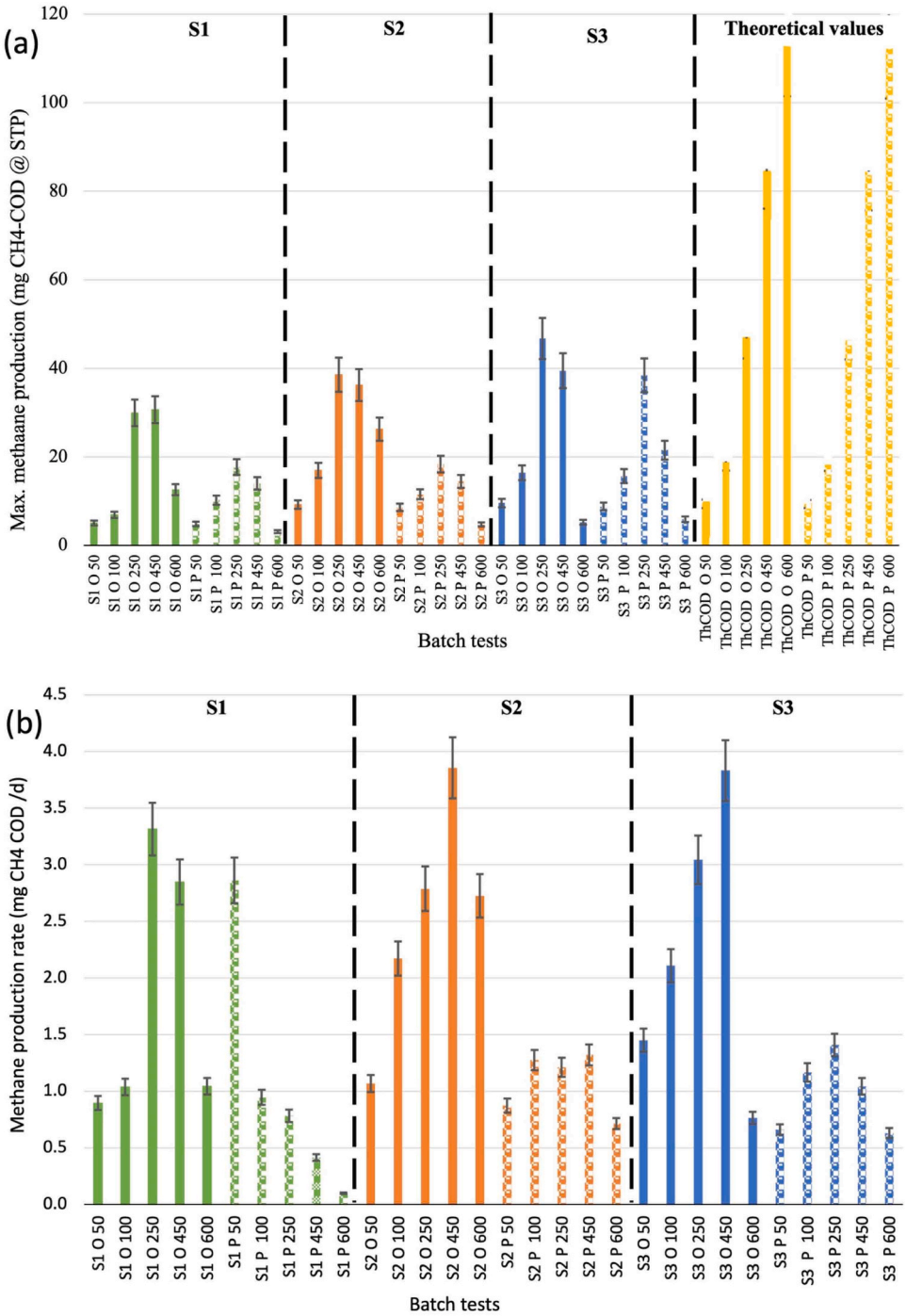

Fig. 2. (a) Maximum cumulative methane productions assessed with the PI sludge samples. S1: green; S2: orange; S3: blue. The solid color shows the experiments carried out with oleate, while the dashed color with palmitate. In yellow, the theoretical maximum cumulative methane productions based on the COD balance are shown; (b) Methane production rates for the PI sludge samples. S1: green; S2: orange; S3: blue. The solid color indicates oleate as the substrate, while the dashed color indicates palmitate. Average values are presented; the maximum standard deviation was 10% (not shown in the Figure).

3.3. FOG mass balances in PI sludge samples

Table 2 and Fig. 4 present the changes in FOG content in the sludge samples. After pre-incubating the sludge samples, residual FOG concentrations were still present (Table 1), likely consisting of non-biodegradable FOG under the prevailing pre-incubation conditions.

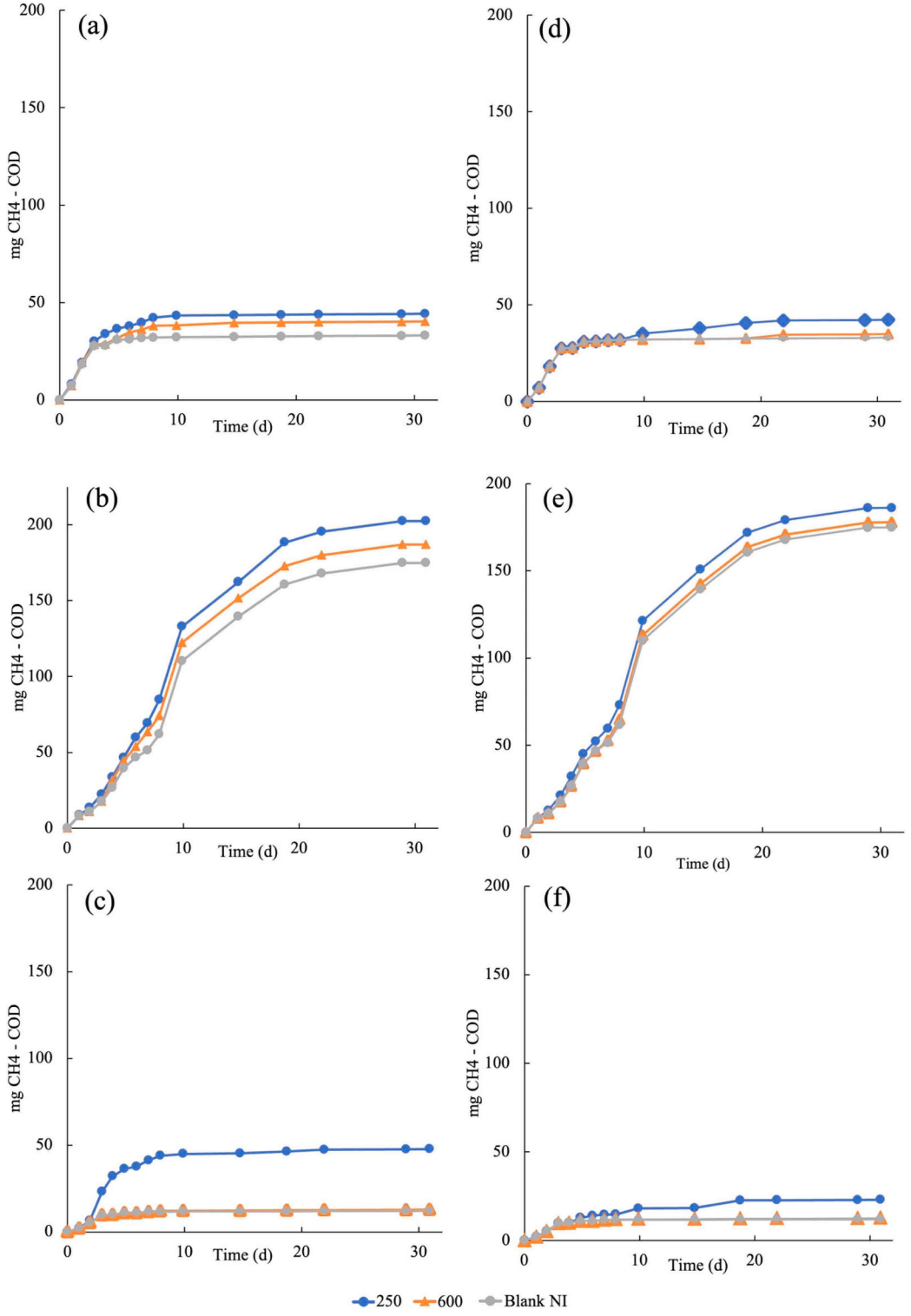

Fig. 3. Cumulative methane production exhibited by the NI sludge samples (S1, S2 and S3) at different oleate (a, b, c) and palmitate (d, e, f) concentrations. Average values are presented (maximum standard deviation of 10% were obtained – not shown in the figures). The NI blanks are shown in the figures.

Table 2
FOG mass balances, expressed as COD, for the PI sludge samples when dosing oleate (O) and palmitate (P). Average values are presented with a maximum standard deviation of 10%.

Batch test	Initial FOG-COD (mg/L)	Oleate/Palmitate addition COD (mg/L)	Initial Batch test FOG -COD (mg/L)	Final FOG -COD (mg/L)	Degradation COD (mg/L)	Oleate/Palmitate degradation (%)
S1 O50	144	144	288	201	87	60
S1 O250	144	720	864	420	444	62
S1 O600	144	1728	1872	1694	178	10
S2 O50	1134	144	1278	1080	144	100
S2 O250	1134	720	1854	1280	574	80
S2 O600	1134	1728	2862	2450	412	24
S3 O50	459	144	603	405	144	100
S3 O250	459	720	1179	400	720	100
S3 O600	459	1728	2187	2100	87	5
S1 P50	144	144	287	220	68	47
S1 P250	144	720	864	630	234	32
S1 P600	144	1728	1872	1820	52	3
S2 P50	1134	144	1278	1100	144	100
S2 P250	1134	720	1854	1580	273	38
S2 P600	1134	1728	2862	2790	72	4
S3 P50	459	144	603	460	143	100
S3 P250	459	720	1179	585	594	83
S3 P600	459	1728	2187	2100	87	5

At the start of the batch incubations, a known dose of LCFAs were added to each sludge sample, and the lipids content were determined again at the end of the batch experiments for assessing an FOG mass balance.

3.4. Microbial community analysis

3.4.1. Community analysis – bacteria

The microbial community composition was quite different at the phylum level for the three evaluated sludge samples. As shown in Fig. 5a, the *Thermotogae* phylum was dominant in the sludge samples S2 and S3 with 60% and 71% relative abundance, respectively. In the sludge sample S1, this phylum was practically absent at a relative abundance of only 3%. The sludge S1 exhibited other dominant phyla such as the *Proteobacteria*, *Acetothermia*, and the predominant phylum *Chloroflexi* with relative abundances of 15%, 30%, and 30%, respectively. Overall, the sludge S1 showed a broader variety of phyla compared to S2 and S3. Sludge samples S2 and S3 were acclimated to lipids, while S1 was not.

The relative abundance at the family level is shown in Fig. 5b. Also at this level, a more diverse microbial community was observed for the sludge S1 compared to the sludge S2 and S3. *Aquaspirillaceae* and *Burkholderlaceae* were the dominant families in the sludge S1, exhibiting a relative abundance of approximately 20% each. *Petrotogaceae* and *Synergistaceae* were the dominant families in the sludge S2 with a relative abundance of approximately 40% each. *Kosmotogaceae* was the predominant family in the sludge S3 at a relative abundance of approximately 80%.

3.4.2. Community analysis – archaea at genus level

The microbial populations were also determined for archaea. The universal primer used for bacterial analysis was not very effective detecting methanogens; hence, an archaeal primer was used instead (Fischer et al., 2016).

The *Methanosaeta* archaea were the dominant species in sludge samples S1 and S3 at a relative abundance of 80%, while the *Methanosarcina* archaea were the dominant species in the sludge S2 at a relative abundance of 60% (Fig. 5c). The *Methanosaeta* relative abundance increased in the sludge S1 at high concentrations of oleate and palmitate (600 mg/L) along with a decrease in the *Methanolinea* abundance.

Regarding the sludge S2, the *Methanosarcina* relative abundance decreased when dosing either oleate or palmitate at 600 mg/L, while the *Methanosaeta* and *Methanobacterium* relative abundance increased.

For the S3 sludge, the inoculum microbial community showed the highest relative abundance of *Methanosaeta* (about 80%) and *Methanobacterium* (20%). After the addition of the oleate and palmitate at 600 mg/L, a modest increase in relative abundance of *Methanobacterium* was observed. The genus *Methanobacterium* is commonly the predominant hydrogenotrophic methanogenic found in anaerobic digesters (Siegert et al., 2015).

The presence of *Methanobacterium* might be related to the ubiquitous presence of an electron sink in the syntrophic consortia, consisting of acetogens and methanogens, for the required β -oxidation reactions during LCFA degradation.

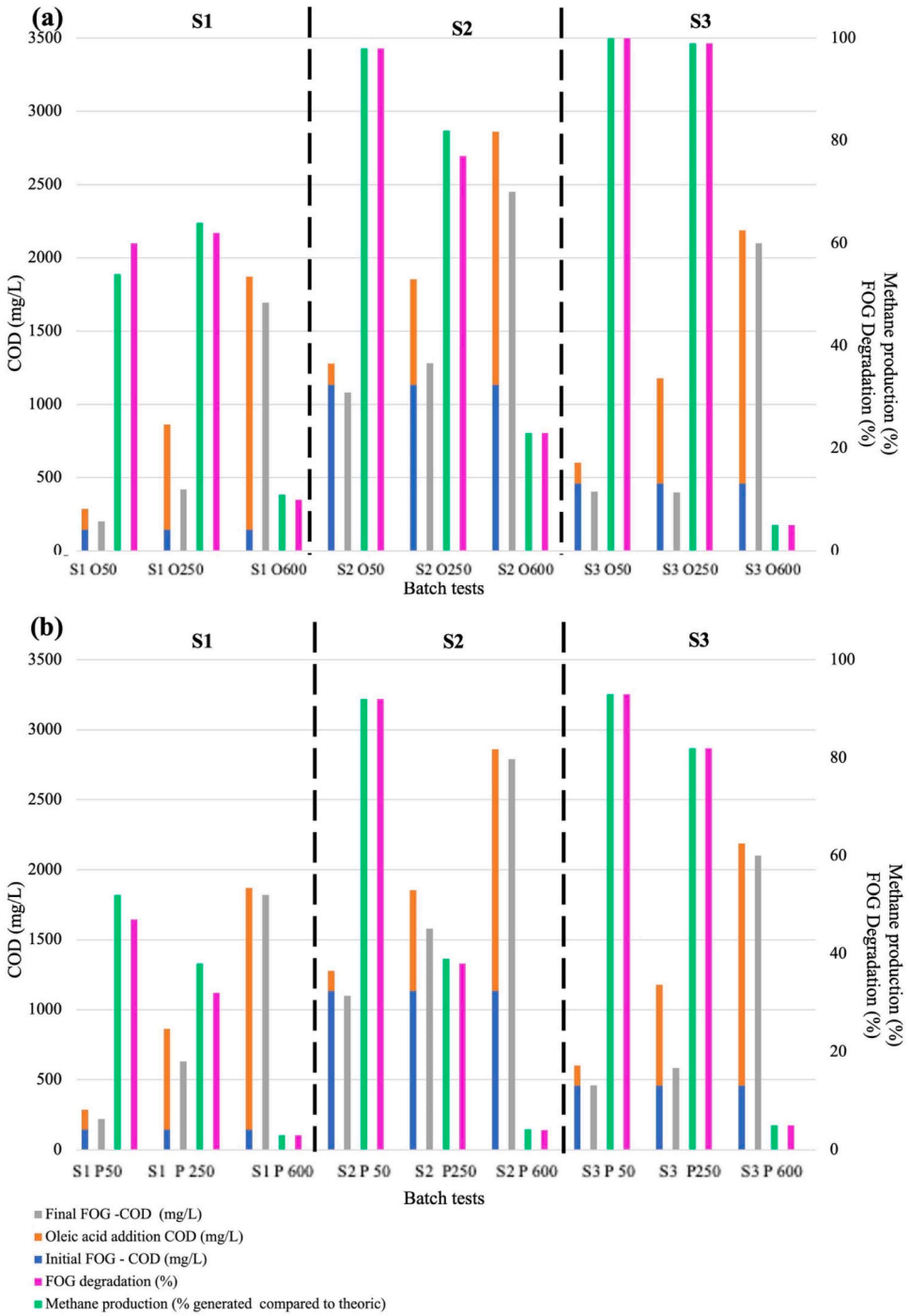

Fig. 4. FOG-COD mass balance for the PI sludge samples dosing (a) oleate; and (b) palmitate. From left to right bars: initial FOG-COD (blue) stacked with oleate/palmitate addition (orange); final FOG-COD (gray); methane production as % from theoretical amount (green); FOG degradation % (pink). X- axis: batch tests; sludge samples (S1, S2 and S3) at different oleate (O) and palmitate (P) at different concentrations (50, 250, 600 mgLCFA/L). Left y-axis: COD concentration in mg/L; right y-axis: Methane production in % and FOG degradation in %.

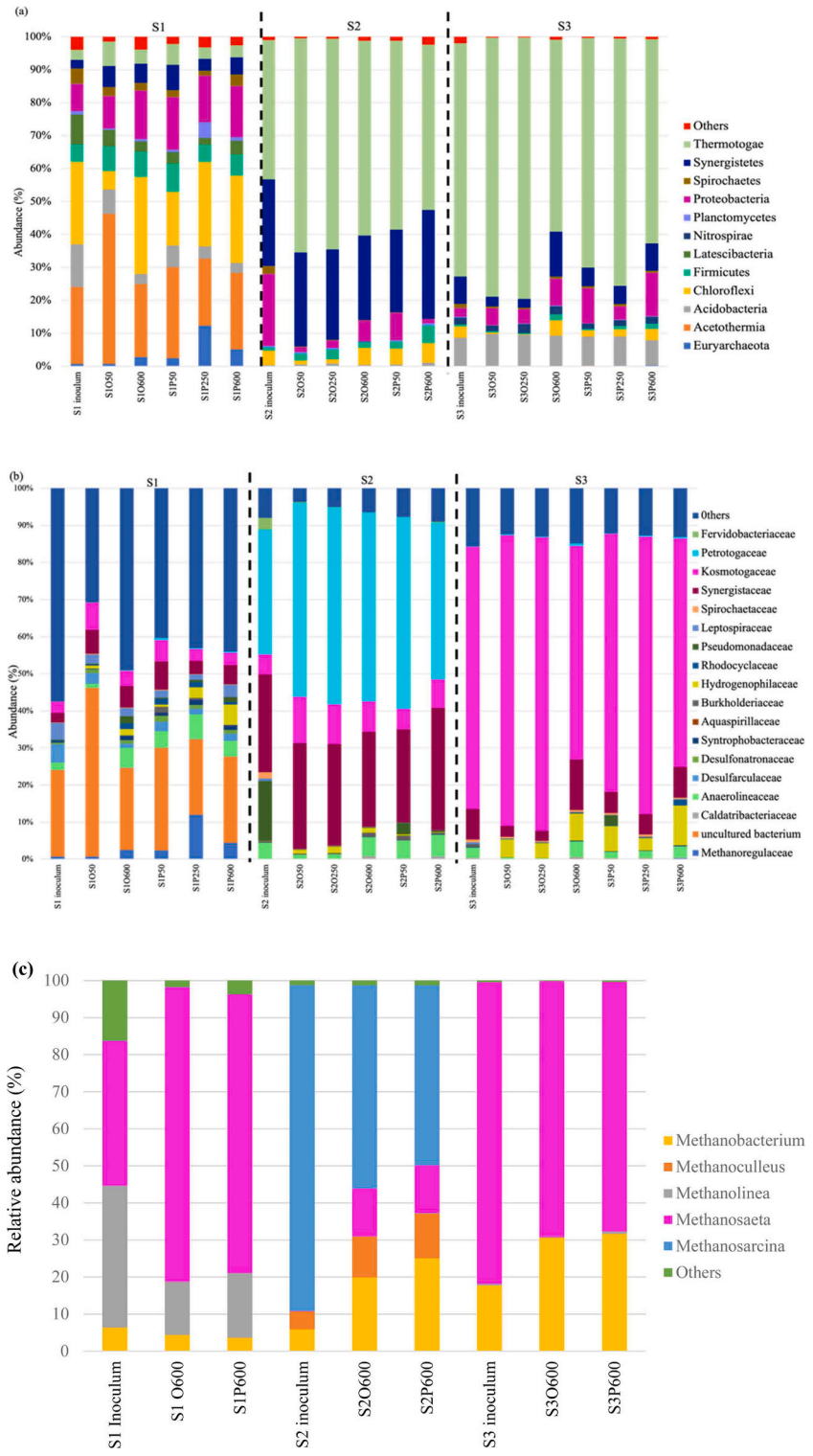

Fig. 5. Microbial community composition according to the 16S rRNA gene analysis using the Universal primers in the samples taken from the three inoculum and from the different experiments. The different colors represented the relative abundance at: (a) Phylum level; (b) Family level and (c) Microbial community composition according to the 16S rRNA gene analysis using archaeal primers in the samples taken from the three inoculum and from the different experiments. X-axis: inoculum for each sludge sample; batch tests; sludge samples (S1, S2 and S3) at different oleate (O) and palmitate (P) concentrations (50, 250, 600 mgLCFA/L). Y-axis: Abundance (%).

3.4.3. Principal coordinate analysis (PCoA) for bacteria

The PCoA used the Bray-Curtis index to evaluate differences and similarities in bacterial communities of sludge samples, based on 16 S rRNA gene data at the family level. Analysis across all samples revealed three distinct clusters (S1, S2, and S3) representing different sludges and their reactions to various LCFA concentrations. Separate analyses for each sludge (S2, S3, S1) showed groups corresponding to the original sludge, and those incubated with oleate and palmitate, highlighting shifts in microbial composition due to LCFA exposure.

4. Discussion

4.1. Physicochemical characterization and acetoclastic methanogenic activity of the inoculum sludge samples

Physicochemical characterization of the sludge samples showed differences in the characteristics of each inoculum, attributable to their different origins (Table 1). Sludge S1 derived from an anaerobic digester, not acclimated to lipids, was characterized by high TSS and COD values. Sludge samples S2 and S3, obtained from high-rate anaerobic treatment (HRAT) reactors, showed lower TSS and COD values compared to S1; COD/VSS ratios were according to expectations.

The incubation period was effective in reducing both the volatile solids and the COD content of the sludge in the three evaluated samples regardless their origin (Table 1). Some of the organic matter present in the sludge consisted of FOG (Table 2, Fig. 4). The pre-incubation period lasted until no methane production was observed. At the end of the pre-incubation period, the sludge samples still contained residual organic matter, which likely was non-biodegradable FOG-COD. The pre-incubation period was effective in conditioning the sludge samples for conducting the subsequent experiments. Regarding the assessed SMA values of the sludge samples, S3 showed the highest SMA compared to S1 and S2. Results indicated that all three sludge samples exhibited appropriate SMA values to be used as inoculums for further evaluating the LCFA degradation capacity (Alves et al., 2001; Cavaleiro et al., 2010; Silva et al., 2014).

4.2. Methanogenic activity using LCFA as substrate

4.2.1. Cumulative methane production tests using pre-incubated sludge

The batch incubations using oleate as the substrate produced more methane than the tests using palmitate. Alves et al. (2009) reported similar observations. Moreover, the same authors observed that unsaturated fatty acids (oleate) could be degraded by a wider range of bacteria compared to saturated fatty acids (palmitate). Recently, it has been shown that the biochemical pathways involved in the degradation of saturated fatty acids, such as palmitate, requires an additional step for degrading the fatty acids, compared to unsaturated fatty acids, such as oleate (Holohan et al., 2022; Pereira et al., 2001). Following current hypothesis, prior to the β - oxidation pathway, the saturated fatty acid needs to pass a preliminary dehydrogenation and hydration step at the alpha and beta carbons, eventually making the degradation process more complex. Such a dehydrogenation step is not observed for the unsaturated fatty acids, which explains the increased CMP when degrading oleic acid compared to palmitic acid.

At the lowest oleic acid and palmitic acid concentration range, i.e., from 50 to 250 mg/L, the three sludge samples performed much better, resulting in a higher CMP, than at LCFA concentrations of 450 and 600 mg/L. The S2 and S3 sludge samples, acclimated to lipids conversion, achieved full conversion of the low LCFA concentrations to methane, in agreement with the theoretically calculated values. Sludge sample S1, which was not acclimated to lipids, only partially converted LCFA, even at 50 mg/L. Several authors reported similar findings regarding the role of sludge acclimation in LCFA degradation (Kougias et al., 2016; Silva et al., 2014; Ziels et al., 2016). Although sludge samples S1 and S2 were originally from the same source, sample S2 was exposed to lipid rich substrate in an AnMBR during a period of 500 days.

Sludge sample S3 showed highest LCFA conversion to methane compared to S1 and S2 sludge, likely attributable to the inoculum origin. The S3 sludge was obtained from an industrial scale AFR, treating dairy ice cream wastewater with a COD concentration of 10 - 25 g/L consisting of 50% FOG (Frijters et al., 2014). Apparently, the S3 sludge was well adapted to oleate and to a lesser extent palmitate.

In the study by Cavaleiro et al. (2008), sludges acclimated to oleate and palmitate individually showed complete methane conversion at concentrations ranging from 100 to 900 mg/L, with no observed inhibition. In contrast, our study, which utilized sludges (S2 and S3) previously acclimated to a mix of lipids and long-chain fatty acids (LCFAs), demonstrated a decline in maximum methane production at concentrations exceeding 250 mg/L for both oleate and palmitate. Complete conversion to methane was only achieved at concentrations lower than 100 mg/L for each LCFA. These observations suggested that the prior acclimation to a complex mixture of wastewater components, despite pre-incubation, impacts subsequent degradation efficiency. The sludge in our study, which was exposed to a diverse range of lipids and LCFAs, developed a microbial community that, while more versatile, might lack the specialized efficiency for degrading high concentrations of specific fatty acids like oleate and palmitate (Ziels et. al, 2016).

In our study, a decline in oleate conversion rates was observed, leading to reduced methane production beyond a certain oleate concentration. This trend was also seen with palmitate. Unsaturated LCFAs, with their lower melting points and higher fluidity, transfer more easily to microorganisms (Wu et al., 2017), impacting both inhibition and degradation rates (Zonta et al., 2013). As a result, high concentrations of LCFA, especially oleate, inhibit both hydrogenotrophic and aceticlastic methanogenic activity and can limit the kinetics of syntrophic β-oxidizing bacteria (Hanaki et al., 1981; Hwu et al., 1996; Silva et al., 2016; Sousa et al., 2013).

The assessed methane production rates (Fig. 2b) were lower than reported by Cavaleiro et al. (2008), who also used oleate and palmitate as the substrate in concentrations ranging from 100 to 900 mg/L. The authors acclimated their sludge with oleate and palmitate for approximately 100 days before dosing oleate and palmitate. In our study, the inoculum sludges S2 and S3 that showed

best performance, were acclimated to lipid-rich wastewater with a variety of LCFAs present as well as skimmed milk.

4.3. FOG mass balances on PI sludge samples

Table 2 and Fig. 4 show the disappearance of LCFAs next to the methane production, relative to the theoretically expected value. Assuming that the initial FOG fraction of the PI sludge samples was non-biodegradable, then, only the added LCFA would be potentially biodegradable during the tests. With oleic acid as the substrate, the observed FOG removal agreed with the observed methane production, shown in Fig. 2a, b, and c. The S2 and S3 sludge samples exhibited higher FOG removal compared to the S1 sludge. In addition, FOG removals using S2 and S3 sludge samples were high, applying oleate concentrations of 50 and 250 mg/L, whereas very low removals were observed at 600 mg/L. Likewise, when dosing palmitate, observed FOG removal agreed with the observed methane production, being high at low concentrations and low at high concentrations.

The S1 sludge seemed less effective in degrading LCFAs compared to the S2 and S3 sludge samples. In addition, high concentrations of LCFAs (up to 600 mg/L), inhibited LCFA conversion. Results showed a clear correlation between LCFA removal expressed as FOG-COD, with the methane production expressed as relative value of the expected maximum theoretical methane production value (Fig. 4). For instance, for S1 sludge, FOG-COD removal efficiencies were 60%, 62% and 10% for oleate concentrations of 50, 250, and 600 mg/L, respectively. For the same concentrations, results in Fig. 2a showed relative methane productions of 54%, 63%, and 11%, respectively.

The FOG mass balances matched the CMP values obtained in the PI sludge incubations, indicating that the biodegradable LCFAs were indeed degraded and further converted into methane. The different pre-incubated sludge samples contained different initial concentrations of non-biodegradable FOG-COD after the pre-incubation step (Table 1). The S2 sludge exhibited the highest initial FOG-COD concentration after pre-incubating the sludge. However, S2 performed as good as the sludge sample S3 in degrading the LCFAs. S3 contained approximately half of the FOG-COD concentration after pre-incubation.

Sludge sample S1 contained the lowest concentration of the non-biodegradable FOG-COD after the pre-incubation period and exhibited the worse performance among the three evaluated sludge samples regarding the breakdown of the added LCFAs. Apparently, the remaining non-biodegradable FOG-COD content after the pre-incubation period did not exhibit any inhibitory effects on the breakdown of the added LCFAs and on their final conversion to methane.

4.4. Microbial community analysis

In our study, the three sludge samples exhibited distinct microbial compositions, influenced by their source. The sludges S1 and S3 originated from a full-scale system, whereas sample S2 was derived from a lab-scale AnMBR setup. Understanding the differences in these community compositions and their dynamics is crucial for enhancing wastewater treatment processes, as highlighted in Matsuda et al. (2010). The exposure to LCFAs in full-scale systems, as in lab-scale systems, causes notable shifts in microbial communities, marked by an increase in syntrophic LCFA-degrading bacteria like *Syntrophomonadaceae* (Sousa et al., 2009). These shifts affect the functional stability and adaptability of syntrophic and methanogenic populations, which are vital for anaerobic wastewater treatment (Ziels et al., 2017). Studies show methanogens' resilience to high LCFA levels, underlining the importance of monitoring microbial dynamics for optimal treatment performance (Salvador et al., 2013).

4.4.1. Community analysis – bacteria

The sludge samples S2 and S3, acclimated to degrade lipids, exhibited the presence of *Thermotogae, Synergistetes* and *Firmicutes* phyla at much higher relative abundances, compared to the non-acclimated sludge sample S1. The presence of those phyla was confirmed both in the sludge used as inoculum and in the samples taken after the incubation with different types and concentrations of LCFAs. The *Thermotogae* phylum, both thermophiles and mesophiles, are able to degrade a large variety of substrates producing hydrogen gas as a by-product (Gupta et al., 2014). Some *Thermotogae* species were found in the microbiota of animal gut, significantly increasing their abundance when the animals were exposed to lipid-rich/high-fat substrates (Ni et al., 2014). Similar observations were also reported for the *Synergistetes* phylum, which includes a group of 20 g-negative anaerobic bacteria (Roquetto et al., 2015). Kurade et al. (2019) reported a fivefold increase in the *Synergistetes* population in an anaerobic digester treating municipal wastewater sludge (primary sludge and aerobic secondary sludge, as well as anaerobically digested sludge) after the addition of FOGs. Similar findings were also reported by Callejas et al. (2019). Thus, the presence of these families could be related to the adaptation of the sludge to degrade LCFAs. On the other hand, the *Proteobacteria* phylum was clearly more abundant in the S1 sludge, which much less relative abundances in the S2 and S3 lipids-acclimated sludge samples. Previous studies associated the presence of the *Syntrophomonadaceae* and *Syntrophaceae* families, within the *Firmicutes* phylum, to the FOG digesting properties of the sludge (Palatsi et al., 2010; Sousa et al., 2007). At least fourteen acetogenic bacteria degrading LCFA in syntrophy with hydrogen scavengers have been reported to belong to those two families (Alves et al., 2009; Baserba et al., 2012; Callejas et al., 2019). Surprisingly, species of this phylum were barely found in the lipid-acclimated sludge samples S2 and S3 (not shown in Fig. 5a); the community size was more abundant in the non-acclimated sludge S1 (not shown in Fig. 5b). These types of bacteria are syntrophic, working in partnership with hydrogen scavengers.

The inoculum sludge S1 was taken from a digester equipped with an upstream preliminary treatment for removing FOG; so, only little FOG reached the anaerobic digester. Nonetheless, a small amount of fats could have passed to the digester promoting the development and presence of the *Syntrophomonadaceae* family. The S2 and S3 raw sludge samples did not exhibit the presence of this phylum. The composition of a specific microbial community in a particular acclimated sludge sample would be strongly influenced by

the microbial composition of the inoculum sludge. Still, the degradation of LCFAs would be possible due to the capacity of numerous species of acetogens to switch to the β - oxidation pathway when necessary (Kougias et al., 2016).

The *Petrotogaceae* and *Kosmotogaceae* families, both belonging to the *Thermotogae* phylum (Bhandari and Gupta, 2014), were the dominant families in the S2 and S3 sludge samples, respectively. The *Synergistaceae* family was also reported in these sludge samples. The inoculum sludge S2 also exhibited communities from the *Pseudomonaceae* family (*Proteobacteria* phylum). The relative abundance of this family seemed to decrease after dosing the LCFAs as also reported in previous studies (Baserba et al., 2012; Kurade et al., 2019).

The *Petrotogaceae*, *Kosmotogaceae*, and *Synergistaceae* families were not found, or found at a very low relative abundance, in the non-acclimated S1 sludge. In the S3 sludge, at the lowest LCFAs concentrations of 50 and 250 mg/L, the *Kosmotogaceae* seemed to dominate. However, when dosing 600 mg/L of either oleate or palmitate, the *Synergistaceae* family dominated. Moreover, as the dosage of the LCFAs increased, the relative abundance of the family *Hydrogenophilaceae* (*Chloroflexi)* also increased. These findings were also reported in other studies when exposing anaerobic sludge to lipid-rich wastewater (Ntougias et al., 2013). Likely, high LCFA concentrations exert different degrees of bactericidal effects leading to the observed differences in relative abundance.

In sludge S2, no major changes in the relative abundance were observed due to the addition of LCFAs; the most abundant families were *Petrotogaceae* and *Synergistaceae*. The occurrence and abundance of these two families were reported already in other studies when treating lipid rich wastewater (Bhandari and Gupta, 2014; Hatamoto et al., 2007a).

As reported in previous studies treating lipid-rich wastewater, this study also confirmed the presence of a relative high abundances of the *Kosmotogaceae*, *Petrotogaceae* (*Thermotogae* phylum), and *Synergistaceae* families in the lipid-acclimated sludge samples S2 and S3 (Baserba et al., 2012; Bhandari and Gupta, 2014; Hatamoto et al., 2007b; Palatsi et al., 2010). The *Anaerolineaceae* family within the *Chloroflexi* bacteria phylum, and the *Pseudomonadaceae* family within the phylum Proteobacteria also have been previously reported in sludge treating lipid-rich wastewater. However, these populations were not found in our studies at a high relative abundance (Nakasaki et al., 2020; Bialek et al., 2010; Shigematsu et al., 2006; Sousa et al., 2008).

The degradation of long-chain fatty acids (LCFA) involves a transition from acetogenic oxidation to β - oxidation, which is influenced by specific conditions. Saturated and unsaturated LCFA are degraded to acetate and hydrogen via β-oxidation (Sousa et al., 2009). However, the inhibitory effects of long-chain fatty acids on volatile fatty acid (VFA) degradation and β-oxidation can impact the process (Shin et al., 2003). Additionally, the kinetics of LCFA inhibition on acetoclastic methanogenesis, propionate degradation, and β-oxidation are crucial in determining the transition (Kim et al., 2004). Under methanogenic conditions, LCFA degradation requires a syntrophic association of LCFA-degrading anaerobes and hydrogenotrophic methanogens (Sousa et al., 2009). The oxidation of LCFA is thermodynamically unfavorable in such environments unless the consumption of reducing equivalents (hydrogen and formate) is coupled with this oxidation (Hatamoto et al., 2007a). LCFA feeding frequency also has been identified as an essential parameter for kinetics and microbial stability during anaerobic degradation of LCFA, where pulse feeding may trigger the activity of β - oxidizing bacteria and improve LCFA degradation (Ziels et al., 2016).

4.4.2. Community analysis – archaea at genus level

The dominant groups in the raw sludge S1 included mostly *Methanosaeta* (39%) and *Methanolinea* (29%); a small fraction of *Methanobacterium* was also reported. The addition of the LCFAs led to an increase in the relative abundance of the *Methanosaeta* population.

In sludge samples S2 and S3, the samples containing the largest concentrations of the added LCFAs, exhibited an increase in the relative abundance of the hydrogenotrophic *Methanobacterium*. Still the dominant species in S2 and S3 were *Methanosarcina* and *Methanosaeta*, respectively. Duarte et al. (2018) also reported the dominance of the hydrogenotrophic *Methanobacterium* over the acetoclastic *Methanosaeta* when anaerobically treating LCFA-rich wastewater under mesophilic conditions. That is, the more acclimated the sludge to LCFAs, the higher the relative abundance of *Methanobacterium.*

In the S2 sludge, the hydrogenotrophic *Methanobacterium* exhibited a higher relative abundance over the acetoclastic *Methanosaeta*. However, in the S3 sludge the *Methanosaeta* exhibited the highest relative abundance (81%) followed by the *Methanobacterium* (18%). In agreement, Raskin et al. (1994) reported that the acetoclastic *Methanosaeta* still exhibited an important relative abundance in the LCFA adapted sludge microbial community.

No major diversification in archaea genus level was observed for the sludge S3 after the LCFAs addition. However, the relative abundance of the *Methanobacteria* increased when dosing the LCFAs at concentrations of 600 mg/L. Likely, the *Methanobacteria* genus is less susceptible for high LCFA concentrations. *Methanobacteria* are the major hydrogenotrophic methanogenic genus commonly found in anaerobic digesters. In addition, their presence is crucial for the development of syntrophic relationships with acetogens to promote the β -oxidation process.

4.4.3. Principal coordinate analysis (PCoA) for bacteria

The PCoA analysis shown in Fig. S1a indicated the diverse nature of the different sludge types used in this research. It should be noted that the batch tests were conducted over a period of only 36 days following a single substrate dose at day 0. Likely, the short incubation time and the single substrate dose was insufficient to observe pronounced changes in the microbial community that can be ascribed to bacterial growth. However, high LCFA doses may exert different levels of bactericidal effects to bacteria and archaea. When observing the PCoA of the acclimated sludge samples S2 and S3 in Fig. S1c and d, respectively, three clusters were clearly observed corresponding to the inoculum and to the samples taken after dosing oleate and palmitate.

On the other hand, when observing the PCoA for the S1 sludge (Fig. S1b), some overlapping among the different communities were observed. Still, the sludge samples containing the LCFA addition in sludge S1 differed from the raw sludge. Another interesting observation was the grouping of the microbial populations when dosing the LCFA at the highest concentrations in the sludge samples

S3 and S1 (Fig. S1d y S1b – orange circles). The sludge samples that were exposed to 600 mg/L of oleate and palmitate exhibited similarities in the microbial community dynamics. Results indicated that the high LCFA dose negatively impacted specific species, having led to distinct changes in the microbial population.

4.5. Treatment implications

This study was part of a large research program on enhancing anaerobic wastewater treatment in the presence of high concentrations of FOG. Current results showed that the degradation of LCFAs is impeded when their concentration surpasses approximately 250 mg LCFA/L, equivalent to 720 mg COD/L, or 125 mg LCFA/g VSS. Retarded LCFA conversion may lead to LCFA accumulation in continuous-flow systems, causing issues like toxicity and operational challenges in high-rate anaerobic treatment (HRAT) systems, including biomass flotation and sludge degranulation. To mitigate these effects, process design modifications for anaerobic treatment are recommended. For HRAT systems, it's advisable to adjust the fat, oil, and grease (FOG) feed concentrations, keeping LCFA concentrations below 360 mg LCFA-COD/g VSS, in agreement with recommendations of Rinzema et al. (1989). Completely mixed reactor systems, although having lower substrate conversion rates than plug-flow reactors, can prevent high LCFA concentrations in the reactor's bulk liquid, while operating at reduced LCFA loading rates. AnMBRs are regarded a suitable treatment alternative for high-lipid wastewaters. They combine complete mixing and relatively high sludge concentrations, akin to HRAT, with ultrafiltration membranes for high-quality effluent.

Monitoring lipid degrading species *(Syntrophomonas and Syntrophus),* as well as integrating 16 S rRNA gene sequencing with multi-omics approaches, are considered useful tools for evaluating LCFA degradation effectiveness (Holohan et al., 2022). However, extended genomic databases are needed for comprehensive analysis.

5. Conclusions

In this study, oleate was found to degrade more efficiently than palmitate in all sludge samples, a critical observation for the treatment of LCFA. The disappearance of LCFAs, quantified as FOG, closely corresponded with methane production capacity across various sludge concentrations, highlighting LCFA conversion as the rate-limiting step. When LCFA concentrations exceeded 250 mg/L, inhibitory effects emerged, compromising their conversion to methane. This threshold is considered pivotal for managing LCFA levels in wastewater treatment. The research also revealed the importance of sludge adaptation strategies. Pre-acclimated sludge samples, S2 and S3, were more efficient in methane generation from LCFAs than the non-acclimated S1 sample, emphasizing the need for sludge adaptation in treating lipid-rich wastewater. Furthermore, the detection of bacterial families like *Kosmotogaceae, Petrotogaceae, and Synergistaceae* in these acclimated sludge samples indicated a biological adaptation to LCFA degradation, crucial for optimizing the anaerobic digestion process in high-lipid wastewater treatment.

CRediT authorship contribution statement

Victoria de la Sovera: Visualization, Writing – review & editing. **Diana Míguez:** Funding acquisition, Project administration, Resources, Writing – review & editing. **Claudia Etchebehere:** Writing – review & editing. **Damir Brdjanovic:** Writing – review & editing, Funding acquisition, Project administration, Resources. **Pragnya Sharma:** Data curation. **Maria A. Szabo-Corbacho:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Hector A. García:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Jules B. van Lier:** Conceptualization, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

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.eti.2024.103529.

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