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Conversion of C_n-Unsaturated into C_{n-2}-Saturated LCFA Can Occur Uncoupled from Methanogenesis in Anaerobic Bioreactors

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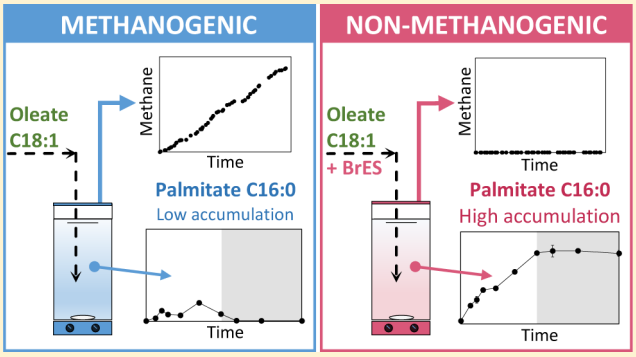
Supporting Information

ABSTRACT: Fat, oils, and grease present in complex wastewater can be readily converted to methane, but the energy potential of these compounds is not always recyclable, due to incomplete degradation of long chain fatty acids (LCFA) released during lipids hydrolysis. Oleate (C18:1) is generally the dominant LCFA in lipid-containing wastewater, and its conversion in anaerobic bioreactors results in palmitate (C16:0) accumulation. The reason why oleate is continuously converted to palmitate without further degradation via β-oxidation is still unknown. In this work, the influence of methanogenic activity in the initial conversion steps of unsaturated LCFA was studied in 10 bioreactors continuously operated with saturated or unsaturated C16- and C18-LCFA, in the presence or absence of the methanogenic inhibitor bromoethanesulfonate (BrES). Saturated C_{n-2}-LCFA accumulated both in the presence and absence of BrES during the degradation of unsaturated C_n-LCFA, and represented more than 50% of total LCFA. In the presence of BrES further conversion of saturated intermediates did not proceed, not even when prolonged batch incubation was applied. As the initial steps of unsaturated LCFA degradation proceed uncoupled from methanogenesis, accumulation of saturated LCFA can be expected. Analysis of the active microbial communities suggests a role for facultative anaerobic bacteria in the initial steps of unsaturated LCFA biodegradation. Understanding this role is now imperative to optimize methane production from LCFA.



INTRODUCTION

Biogas production from waste lipids is achieved when bacteria degrading long chain fatty acids (LCFA) and methanogenic archaea couple their activity in mixed microbial communities.[1] However, accumulation of high LCFA concentrations is frequently reported during the continuous operation of anaerobic bioreactors at high organic loads, which results in methanogenesis inhibition and process failure.[2−4] Besides metabolic inhibition, LCFA tend to adsorb to the surface of microbial cells, creating a physical barrier that can induce mass transfer limitations.[5] These effects are especially stringent when treating unsaturated LCFA, from which oleate (C18:1) and linoleate (C18:2) are the most abundant in waste materials and wastewaters.[5]

Pereira and co-workers showed that palmitate, a C16 saturated LCFA, accumulates during oleate degradation in continuous bioreactors, and further conversion of the saturated LCFA was promoted by interrupting oleate feeding.[6] Saturated LCFA are degraded through several cycles of β-oxidation; in each β-oxidation cycle a C_{n-2} fatty acid, acetate, and hydrogen are formed.[7] Details on the biochemistry of LCFA biodegradation can be found in the review from Sousa and co-workers.[1] β-oxidation is only thermodynamically feasible when the hydrogen partial pressure is kept low, which is accomplished through syntrophic cooperation with hydrogenotrophic archaea (Table 1). Syntrophic metabolism implies that the chemical energy released during the partial reactions is shared between the syntrophic partners, in conditions that are close to thermodynamic equilibrium.[8,9]

For unsaturated LCFA, Weng and Jeris suggested a degradation mechanism that starts with chain saturation followed by β-oxidation,[7] although the possibility of direct β-oxidation was never ruled out.[12,13] Stearate (C18:0) was only occasionally detected mostly in low concentrations,[4,14] while the accumulation of about equal amounts of stearate and palmitate was reported by Broughton et al. during the anaerobic digestion of sheep tallow,[15] and by Cavaleiro et al. during the stepwise feeding start-up of a reactor treating an oleate-based wastewater.[16] Independent of this, accumulation of C_{n-2} fatty acids is not expected because it suggests an interruption of the β-oxidation after just one cycle. Therefore, the initial steps in the conversion of unsaturated fatty acids are still unclear.

Pereira et al. showed that the conversion between oleate (C18:1) and palmitate (C16:0) did not occur when sludge was

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Table 1. Energetic of Syntrophic Growth on LCFA

reactant	equation	$\Delta G^{0\prime}$ (kJ reaction^{-1})[a]	$\Delta G'$ (kJ reaction^{-1})[b]
Fatty Acids Oxidation Reactions:			
linoleate (C18:2)	linoleate$^-$ + 16H_2O → 9acetate$^-$ + 14H_2 + 8H^+	+247	−240
oleate (C18:1)	oleate$^-$ + 16H_2O → 9acetate$^-$ + 15H_2 + 8H^+	+326	−190
palmitoleate (C16:1)	palmitoleate$^-$ + 14H_2O → 8acetate$^-$ + 13H_2 + 7H^+	+275	−174
stearate (C18:0)	stearate$^-$ + 16H_2O → 9Acetate$^-$ + 16H_2 + 8H^+	+404	−139
palmitate (C16:0)	palmitate$^-$ + 14H_2O → 8acetate$^-$ + 14H_2 + 7H^+	+353	−124
myristate (C14:0)	myristate$^-$ + 12H_2O → 7acetate$^-$ + 12H_2 + 6H^+	+303	−108
Methanogenic Reactions:			
acetate	acetate$^-$ + H_2O → HCO_3^- + CH_4	−31	
hydrogen	4H_2 + HCO_3^- + H^+ → CH_4 + 3H_2O	−136	
Oleate Oxidation to Palmitate:			
oleate (C18:1)	oleate$^-$ + 2H_2O → palmitate$^-$ + acetate$^-$ + H_2 + H^+	−28	−73

[a]Gibbs free energies calculated at standard conditions (solute concentrations of 1 mol L^{-1}, gas partial pressure of 10^5 Pa, T = 25 °C, pH 7). Standard Gibbs energies of formation of LCFA (in aqueous solution, pH 7 and T = 25 °C) were estimated from the structure of the compounds, using a group contribution method described by Mavrovouniotis;[10] standard Gibbs energies of formation of other compounds involved in the reactions were obtained from Thauer et al.[11] [b]Gibbs free energies (at 25 °C, pH 7) for LCFA concentrations of 1 mmol L^{-1}, considering acetate stoichiometric accumulation (9 or 8 mmol L^{-1} for linoleate/oleate/stearate and palmitoleate/palmitate degradation, respectively) and H_2 depletion to a partial pressure of 1 Pa.

Table 2. Operational Parameters Applied in the Reactors Operated in the Presence or Absence of the Methanogenic Inhibitor BrES

					time (days)	
reactor	substrate	Cn:d[a]	BrES (mmol L^{-1})	OLR (mol LCFA m^{-3} day^{-1})	continuous	batch
MR LIN	linoleate	C18:2	0	2.5	0−11	11−42
IR LIN	linoleate	C18:2	20	2.5	0−11	11−42
MR OL	oleate	C18:1	0	2.5	0−24	24−80
IR OL	oleate	C18:1	20	2.5	0−24	24−80
MR OL+GLY	oleate + glycerol	C18:1	0	2.5	0−24	24−80
IR OL+GLY	oleate + glycerol	C18:1	20	2.5	0−24	24−80
MR PALOL	palmitoleate	C16:1	0	2.8	0−26	26−70
IR PALOL	palmitoleate	C16:1	20	2.8	0−26	26−70
MR STE+PAL	stearate + palmitate	C18:0 + C16:0	0	1.8 + 0.7	0−27	27−48
IR STE+PAL	stearate + palmitate	C18:0 + C16:0	20	1.8 + 0.7	0−27	27−48

[a]Cn:d, n is the number of carbon atoms and d is the number of double bonds in the LCFA.

inactivated by heat treatment, proving that these reactions were mediated biologically.[17] Further palmitate degradation did not proceed unless oleate was removed from the medium.[6] Based on these observations and on the fact that palmitate accumulation was recurrently observed in bioreactors fed with oleate, we suggest that oleate to palmitate conversion is fast and nonlimiting in oleate catabolism, whereas further palmitate degradation proceeds slowly and is hindered by continuous oleate feeding. The reason why oleate is not completely degraded to acetate through several β-oxidation cycles, and instead is partially oxidized to palmitate, was investigated in this work. Our hypothesis is that the first steps of unsaturated LCFA degradation in bioreactors can happen without the participation of methanogenic interactions. This hypothesis was tested in bioreactors inoculated with methanogenic granules and operated in parallel with bioreactors inoculated with granules submitted to previous treatment with a methanogenesis inhibitor. Saturated or unsaturated C16 and C18 LCFA were used as substrates. LCFA, volatile fatty acids (VFA) and methane were monitored.

MATERIALS AND METHODS

Experimental Setup. Ten upflow bioreactors with approximately 1 L working volume were operated in continuous mode, at mesophilic conditions (37 °C), and fed with different LCFA as energy and carbon source (Table 2). Agitation was provided through magnetic stirring (2× per day, 5 min). Five bioreactors were inoculated with methanogenic granules (MR, methanogenic reactors) and operated in parallel with five bioreactors inoculated with granules submitted to previous treatment for methanogenesis inhibition (IR, inhibited reactors). Biogas production and methane composition were monitored during the experiment. LCFA and volatile fatty acids (VFA) in the bulk of the bioreactors were also analyzed.

Inoculum Source, Acclimation and Inhibition of the Methanogenic Activity. Anaerobic granular sludge, collected from a pilot-scale upflow anaerobic sludge blanket (UASB) reactor treating winery effluent (Santiago de Compostela, Spain), was used as inoculum for the bioreactors at a final volatile solids (VS) concentration of 10 g L^{-1}. Sludge acclimation was performed in the bioreactors during approximately one month, through continuous feeding with a mixture of skim milk (1.5% v/v) and LCFA (approximately 5 mmol L^{-1}). After sludge acclimation, specific methanogenic activity in the presence of acetate and H_2/CO_2 was measured as described by Alves et al.,[18] and was 73 ± 7.6 and 320 ± 13.4 mL g^{-1} day^{-1}, respectively. Subsequently, sludge treatment for inhibition of the methanogenic activity was applied in five bioreactors. This treatment consisted of a 48 h batch contact with a 30 mmol L^{-1}

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solution of 2-bromoethanesulfonate (BrES). The absence of methanogenic activity was further confirmed in batch incubations with H_2/CO_2.

Substrates and Operating Mode. Sodium salts of linoleate (*cis*,*cis*-9,12 C18:2, ≥ 99%, Sigma-Aldrich), oleate (*cis*-9 C18:1, purum, min. 82% assay of fatty acids, Riedel-de Häen), palmitoleate (*cis*-9 C16:1, 99%, Acros) and a mixture of stearate (C18:0) and palmitate (C16:0) (71:24% total LCFA w/w, respectively; min. 88% assay of fatty acids, Sigma-Aldrich) (Table 2) were used as substrate in the experiments. To simulate a feeding with lipids posthydrolysis composition, a mixture of glycerol and oleate (1:3 molar composition simulating a typical fat triolein) was also tested. Macronutrientes, micronutrients and sodium bicarbonate were supplemented to all the reactors as previously described by Alves et al.[18] In order to guarantee an adequate COD/N/P ratio, NH_4Cl was also supplemented (0.4 g L^{-1}).

The reactors were operated at an organic loading rate (OLR) of approximately 2 kg COD m^{-3} day^{-1}, correspondent to approximately 2.5 mol m^{-3} day^{-1} (Table 2), and hydraulic retention time (HRT) of 2 days. IR were also continuously supplied with BrES at 20 mmol L^{-1}. After a period of continuous operation, the feeding was stopped and the reactors were kept in batch.

Analytical Methods. Biogas production was measured with a Ritter MilliGascounter (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany) and methane content was analyzed in a Micro-GC CP-4900 (Varian Inc.). A 10 m PPU column heated at 80 °C, helium (at 150 kPa) as carrier gas, and a thermal conductivity detector (55 °C) were used. The temperature of the injection port was 110 °C. Mixed liquor samples were periodically withdrawn from the reactors for VFA and LCFA quantification. VFA were analyzed by high performance liquid chromatography (HPLC, Jasco, Japan) using a Chrompack organic analysis column (30 × 6.5 mm) with a sulfuric acid mobile phase of 5 mmol L^{-1} at a flow rate of 0.7 mL min^{-1}. The column temperature was set at 60 °C and the detection was made spectrophotometrically at 210 nm. LCFA were extracted from the mixed liquor samples and quantified as previously described by Neves et al.[19] Esterification of free fatty acids was performed with propanol, in acid medium (3.5 h at 100 °C). Propyl esters were further extracted with dichloromethane and analyzed in a gas chromatograph (Varian 3800) equipped with a flame ionization detector (FID) and an eq CP-Sil 52 CB 30 m x 0.32 mm × 0.25 μm capillary column (Teknokroma, TR-WAX). Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. Initial oven temperature was set at 50 °C for 2 min and final temperature of 225 °C was attained with a ramp rate of 10 °C min^{-1}. Injector and detector temperatures were 220 and 250 °C, respectively. Extraction and quantification was performed in duplicate for all samples. LCFA identification was further confirmed by gas chromatography coupled to mass spectrometry (GC-MS). Following esterification and extraction, as just described, fatty acid propyl esters (FAPE) were analyzed using a GC-FID Varian 4000 coupled with a detector 240-MS/4000 Mass Spec. A capillary column VF-WAX (Varian, 30 m x 0.15 mm) was used for the separation of the FAPE. Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. For GC analysis, initial oven temperature was set at 60 °C for 1 min; final temperature of 260 °C was attained with a ramp rate of 8 °C min^{-1}, and kept at 260 °C for 15 min. Injector temperature was 285 °C. The MS detection conditions were as follows: electronic impact mode (70 eV) with an acquisition range (m/z) from 38 to 650, and an acquisition frequency of 610 ms. Identification of the FAPE in the analyzed samples was performed using the mass spectra library NIST (National Institute of Standards and Technology) and retention times of standard.

Microbial Composition of Bioreactor Sludges Fed with Monounsaturated LCFA. Sludge samples were collected at the beginning and end of the continuous operation, from all the reactors fed with monounsaturated LCFA (oleate and palmitoleate). These samples were immediately preserved for further DNA or RNA extraction, with the aim of studying microbial diversity and composition of the total or active bacterial communities, respectively.

Bacterial Diversity Profiling of Bioreactor Sludges. DNA Extraction and Amplification. Aliquots of well homogenized sludge were collected and immediately frozen at −20 °C. Total genomic DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals). For denaturing gradient gel electrophoresis (DGGE), bacterial 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using the primers set U968GC-f/L1401-r.[20] Size and yield of PCR products were estimated using a 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) via 1% (w/v) agarose gel electrophoresis and safe green staining.

DGGE Analysis. DGGE analysis of the PCR amplification products was performed as previously described by Sousa et al.,[21] using the Dcode system (Bio-Rad, Hercules, CAA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30−60%. A 100% denaturing solution was defined as 7 mmol L^{-1} urea and 40% formamide. Electrophoresis was performed for 16 h at 85 V in a 0.5x TAE buffer at 60 °C. DGGE gels were stained with $AgNO_3$ as described by Sanguinetti et al.[22] Gels were scanned at 400 dpi and DGGE profiles were compared using the Bionumerics software (version5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity indices of the densitometric curves of the compared profiles were calculated using Dice's coefficient.

Diversity and Composition of Active Microbial Communities. RNA Extraction, Reverse Transcription to cDNA, PCR Amplification and DGGE Analysis. Samples were immediately suspended in 5 mL RNAlater (Sigma), corresponding approximately to 10 mg of sample total solids per mL RNAlater, and stored at −20 °C. Total RNA was extracted using the FastRNAPro Soil-Direct Kit (MP Biomedicals). The resulting crude RNA was digested with RNase-free DNase I (Invitrogen, Carlsbad, CA) and the presence of residual DNA was tested by PCR using the 16S rRNA gene as a target for amplification with the bacterial and archaeal specific primers sets U968GC-f/L1401-r[20] and A109(T)-f/515GC-r.[23,24] DNA-free RNA was used as template for complementary DNA (cDNA) synthesis using SuperScript reverse transcriptase III (Invitrogen, Carlsbad, CA). DGGE analysis was performed as described in previous section.

16S rRNA Gene Pyrosequencing. cDNA samples were sent for 16S rRNA gene 454-pyrosequencing at LifeSequencing S.L. (Valencia, Spain) on a Roche 454 FLX GS System, according to standard protocols (Roche 454 Life Sciences, Branford, CT). For bacterial and archaeal 16S rRNA gene pyrosequencing, amplification was performed using random primers. The amount of DNA amplified from each sample was quantified using the Quant system - Invitrogen It PicoGreen, and this information was used to prepare an equimolar pool that was further sequenced. Sequencing data underwent quality control, which

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included the removal of sequences with a length lower than 100 bp. Taxonomic assignment of each trimmed sequence was performed using a BLASTN (Standard Nucleotide Basic Local Alignment Search Tool-like alignment tool) search in a database containing a collection of bacterial 16S rRNA sequences, mostly from the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/). This database has been cured to minimize alignment errors and reduce the rate of false positives. All identifications were made using a maximum e-value of 1×10^{-5} and a minimum identity cutoff of 70%. The relative abundance of different taxa within a sample was estimated by the relative number of reads corresponding to each taxon.

Batch Assays with Pure Cultures. The ability to degrade oleate, and the possible conversion of this compound to palmitate, was studied in batch assays performed with (i) a pure culture of *Rheinheimera pacifica* (DSM 17616^T), incubated under aerobic or strict anaerobic conditions, and (ii) a coculture of *Syntrophomonas zehnderi* (DSM 17840^T) and *Methanobacterium formicicum* (DSM 1535^T) in anaerobic conditions. Cultures were grown in bicarbonate-buffered mineral salt medium.[21] Aerobic assays were performed in Erlenmeyer flasks, while all anaerobic incubations were made in closed bottles flushed with a gas mixture of 80:20% N_2/CO_2 (1.7×10^5 Pa final pressure), reduced with 0.8 mmol L^{-1} Na_2S and supplemented with salts and vitamins.[21] Sodium oleate (≥99%, Fluka) was added to the medium from a sterile stock solution. In the assays with *R. pacifica*, a final oleate concentration of 2 mmol L^{-1} was provided. Incubations were performed at 30 °C with shaking at 150 rpm, and samples were collected overtime for LCFA analysis. In the experiments with *S. zehnderi* + *M. formicicum*, triplicate bottles were amended with oleate (1 mmol L^{-1}) and another set of three bottles was supplemented with the same concentration of oleate plus BrES (20 mmol L^{-1}). Methane concentration in the headspace of the bottles, VFA and LCFA were monitored during the experiment. Incubations were performed at 37 °C, statically and in the dark.

Thermodynamic Analysis. The standard Gibbs free energy changes ($\Delta G^{0\prime}$) associated with the partial reactions possibly involved in LCFA biodegradation have been calculated from free energy of formation data and the relationship $\Delta G^{0\prime} = \sum \Delta G_f^0$ (products) $- \sum \Delta G_f^0$ (substrates), where ΔG_f^0 refers to the standard free energy of formation for substrates and products. Standard free energies of formation of LCFA (in aqueous solution, pH 7 and 25 °C) were estimated from the structure of the compounds, using a group contribution method described by Mavrovouniotis;[10] standard energies of formation of other compounds involved in the reactions of LCFA degradation were obtained from Thauer et al.[11]

Gibbs free energy changes at nonstandard conditions were calculated according to the following equation:[11]

$$\Delta G' = \Delta G^{0'} + 2.3 \times RT \times \log\left(\frac{[C]^c[D]^d}{[A]^a[B]^b}\right) \quad (1)$$

where $\Delta G'$, Gibbs free energy change (25 °C, pH 7) calculated at nonstandard conditions; $\Delta G^{0\prime}$, Gibbs free energy change calculated at standard conditions (solute concentrations of 1 mol L^{-1}, gas partial pressure of 10^5 Pa, 25 °C) and pH 7; $[A]$, $[B]$, substrates concentrations (mol L^{-1}); $[C]$, $[D]$, products concentrations (mol L^{-1}); a, b, c, d, stoichiometric coefficients of substrates and products; R, ideal gas constant (8.314 J mol^{-1} K^{-1}); T, temperature (K).

RESULTS

Saturated and unsaturated LCFA were converted to acetate and methane in all the methanogenic reactors (MR), with transient LCFA accumulation (Figure 1a, c, e, g, i, and Supporting Information (SI) Figure S1). Biogas with an average methane content of 70% (v/v) was produced in these reactors. Sludges submitted to selective inhibition of methanogenic activity (IR reactors) converted the unsaturated substrates to saturated LCFA-intermediates (Figure 1b, d, f, h), which accumulated consistently and achieved 2−4 times higher concentrations than in the methanogenic reactors. No methane was produced in these reactors, and VFA were not detected. IR STE+PAL did not show any catalytic activity toward the saturated LCFA that accumulated in the bulk in similar concentrations to the ones in the feed (Figure 1j). Maximum concentration measured for LCFA accumulating in the bulk and their relative distribution (expressed as % of total LCFA) are presented in Table 3.

Palmitate (C16:0) was the main intermediate of oleate (C18:1) degradation, identified by gas chromatography and further confirmed by GC-MS. Palmitate accounted for approximately 79 ± 6% of total LCFA accumulated in reactors MR OL, IR OL, MR OL+GLY, and IR OL+GLY (Figure 1c−f; Table 3). Higher palmitate concentrations were quantified in the IR reactors, reaching a maximum concentration of 11 ± 1 mmol L^{-1} (Table 3). Stearate (C18:0) represented less than 13% of total LCFA.

Degradation of monounsaturated C16 followed a similar pathway; it was mainly converted to myristate (C14:0), that represented 31 ± 9% and 52 ± 1% of total LCFA quantified in reactors MR PALOL and IR PALOL, respectively. However, in these reactors palmitate was also an important intermediate, making up 34 ± 6% of total LCFA (Figure 1g, h; Table 3).

When linoleate (C18:2) was used as substrate, palmitate (C16:0) and oleate (C18:1) were the main LCFA quantified, representing 56 ± 7% and 20 ± 3% of the total LCFA, respectively, as shown in Figure 1a, b and Table 3. Palmitoleate (C16:1) was never detected during the degradation of unsaturated C18 LCFA (i.e., C18:2 and C18:1).

No intermediary LCFA were detected in the MR and IR fed with the mixture of saturated LCFA, where only stearate (C18:0) and palmitate (C16:0) were quantified in relative amounts similar to the feeding mixture supplied, that is, 76 ± 3% and 23 ± 2% of total LCFA, respectively (Figure 1i, j; Table 3).

VFA were not detected in the IR reactors, and acetate was the only VFA measured in the methanogenic reactors. Acetate accumulated steadily during the continuous operation, reaching maximum concentrations between 1.4 and 7 mmol L^{-1} in the different MR reactors, and was further degraded in the batch period (SI Figure S1).

Differences in the bacterial communities in MR and IR bioreactors fed with monounsaturated LCFA were evaluated. First, DNA was extracted from the samples collected at the beginning (t0) and end (tf) of the continuous operation in reactors MR OL and IR OL, and DGGE of PCR amplified 16S rDNA fragments was performed (SI Figure S2). The four DNA-derived DGGE profiles were highly similar (>84%, SI Figure S2), and thus further analysis were made based on bacterial activity, rather than on presence. For that, RNA was extracted from MR OL, IR OL, MR PALOL, or IR PALOL samples collected at tf, and RNA(cDNA)-targeted DGGE analysis was performed to evaluate the differences between the active LCFA-degrading communities developed in the presence or absence of BrES. Significant differences were evident in

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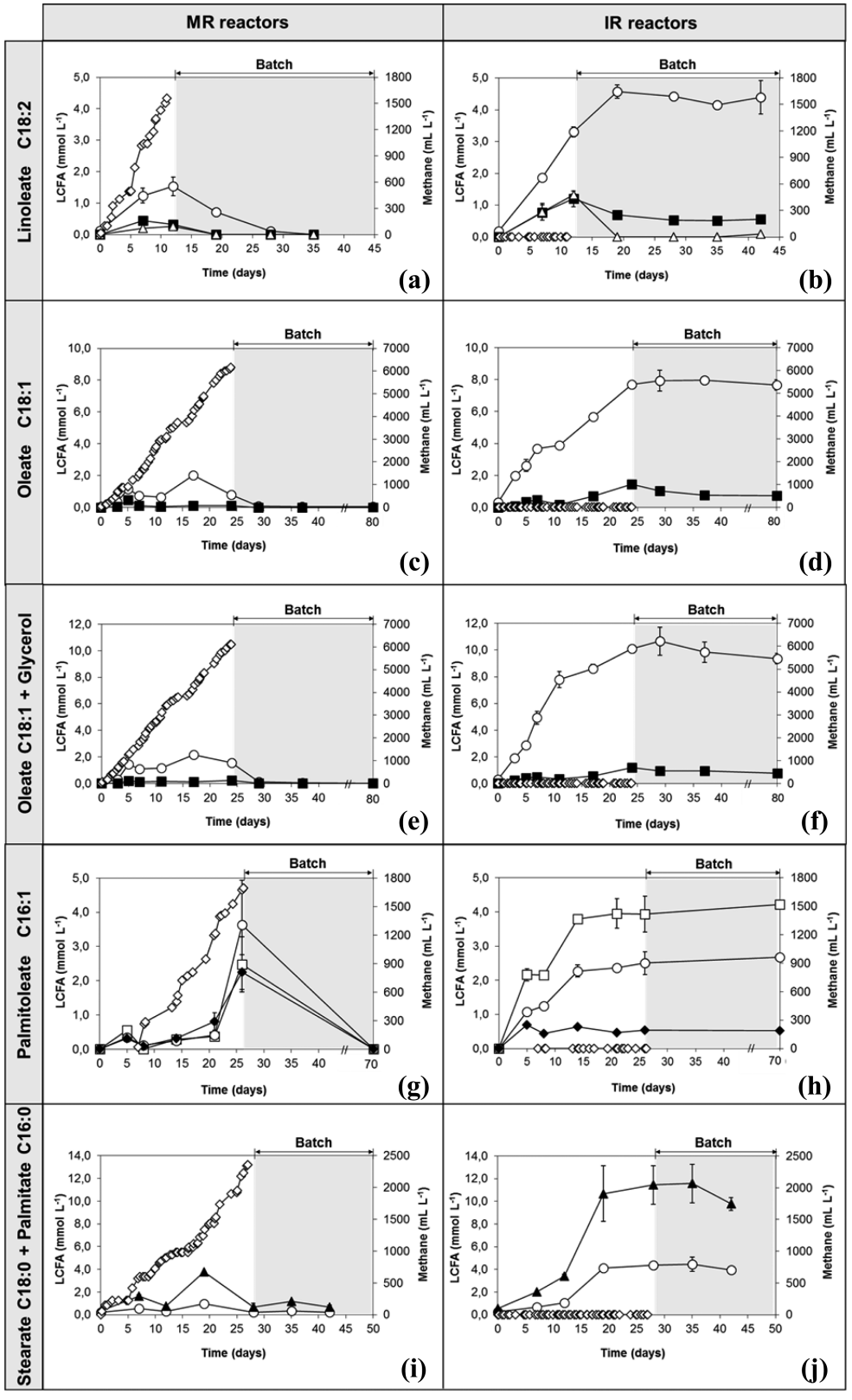

Figure 1. Main LCFA accumulated during the experiment and cumulative methane production in reactors (a) MR LIN, (b) IR LIN, (c) MR OL, (d) IR OL, (e) MR OL+GLY, (f) IR OL+GLY, (g) MR PALOL, (h) IR PALOL, (i) MR STE+PAL and (j) IR STE+PAL. C18:2 (△), C18:1 (■), C18:0 (▲), C16:1 (♦), C16:0 (○), C14:0 (□), and methane (◊).

the communities' fingerprints from MR and IR reactors (SI Figure S3). Samples from IR OL and IR PALOL clustered together and presented 88% similarity. Sludges from these four reactors operated with monounsaturated LCFA were sequenced by RNA(cDNA)-targeted 454-pyrosequencing and the results obtained are shown in Table 4 and SI Figure S4.

At the genus level, the majority of the 16S rRNA gene sequences retrieved from the microbial communities developed

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Table 3. Maximum LCFA Concentrations (mmol L^{-1}) and relative distribution (% of total LCFA, in parentheses) during the Whole Operation Time

reactor	C18:2	C18:1	C18:0	C16:1	C16:0	C14:0
MR LIN	0.3 ± 0.0 (10−12%)	0.4 ± 0.0 (15−23%)	nd[a]	nd	1.5 ± 0.3 (58−66%)	< 0.2
IR LIN	1.3 ± 0.1 (22−23%)	1.2 ± 0.3 (21−23%)	< 0.2	nd	3.3 ± 0.2 (50−51%)	< 0.3
MR OL	nd	0.5 ± 0.1 (4−28%)	0.1 ± 0.0 (0−7%)	nd	2.0 ± 0.3 (62−84%)	< 0.2
IR OL	nd	1.4 ± 0.1 (4−14%)	1.5 ± 0.1 (5−13%)	nd	8.0 ± 0.2 (68−85%)	< 0.7
MR OL+GLY	nd	0.2 ± 0.0 (5−13%)	0.1 ± 0.0 (0−6%)	nd	2.2 ± 0.2 (76−87%)	< 0.2
IR OL+GLY	nd	1.2 ± 0.1 (4−12%)	0.9 ± 0.1 (5−8%)	nd	11 ± 1.1 (76−88%)	< 0.5
MR PALOL	nd	nd	nd	2.3 ± 0.5 (27−52%)	3.6 ± 1.3 (27−46%)	2.5 ± 0.8 (21−43%)
IR PALOL	nd	nd	nd	0.7 ± 0.0 (7−17%)	2.7 ± 0.1 (30−38%)	4.2 ± 0.1 (52−54%)
MR STE+PAL	nd	nd	3.8 ± 0.0 (64−80%)	nd	1.0 ± 0.0 (19−36%)	nd
IR STE+PAL	nd	nd	11.6 ± 1.7 (71−78%)	nd	4.4 ± 0.6 (22−30%)	< 0.3

[a]nd, not detected.

Table 4. Phylogenetic Affiliation of the Microbial Reads Identified at the Genus Level (at >0.1% abundance)

MR OL		IR OL		MR PALOL		IR PALOL	
taxon (genus)	% of reads	taxon (genus)	% of reads	taxon (genus)	% of reads	taxon (genus)	% of reads
Methanosaeta	37	*Pseudomonas*	33	*Methanosaeta*	21	*Rheinheimera*	33
unknown/uncultured	4.4	*Clostridium*	23	*Pseudomonas*	14	*Pseudomonas*	25
Methanobacterium	4.2	*Acinetobacter*	11	*Clostridium*	4.3	*Delftia*	7.8
Methanospirillum	3.7	*Sporanaerobacter*	4.2	*Methanobacterium*	3.8	*Clostridium*	6.6
Levilinea	3.1	*Aminiphilus*	3.2	unknown/uncultured	3.5	*Sporanaerobacter*	2.8
Clostridium	3.0	*Delftia*	2.4	*Bellilinea*	2.3	*Methanobacterium*	2.4
Syntrophomonas	2.9	*Propionibacterium*	2.1	*Delftia*	2.0	*Aminiphilus*	1.5
Spirochaeta	2.4	*Acidovorax*	2.0	*Levilinea*	2.0	*Janthinobacterium*	1.4
Longilinea	2.0	*Sporobacter*	2.0	*Curtobacterium*	1.9	*Curtobacterium*	1.3
Bellilinea	1.9	*Streptomyces*	1.7	*Spirochaeta*	1.7	*Aminobacterium*	1.2
Thermanaerovibrio	1.9	*Methanobacterium*	1.4	*Rheinheimera*	1.7	*Desulfovibrio*	0.89
Thermanaerothrix	1.5	*Desulfovibrio*	1.4	*Thermanaerothrix*	1.6	*Acidovorax*	0.73
Methanolinea	1.3	*Aminobacterium*	1.1	*Methanolinea*	1.5	*Candidatus* Odyssella	0.23
Anaerolinea	1.2	*Eubacterium*	1.0	*Candidatus* Odyssella	1.4	*Spirochaeta*	0.16
Syntrophobacter	1.1	*Curtobacterium*	1.0	*Petrotoga*	1.3	*Anaerolinea*	0.15
Aminobacterium	0.89	*Janthinobacterium*	1.0	*Anaerolinea*	1.2	*Sporobacter*	0.14
Pseudomonas	0.81			*Longilinea*	1.0	*Acinetobacter*	0.13
Aminiphilus	0.67			*Syntrophobacter*	0.83		
Desulfovibrio	0.39			*Desulfovibrio*	0.78		
Sporobacter	0.24			*Syntrophomonas*	0.76		
Petrotoga	0.17			*Janthinobacterium*	0.55		
				Aminobacterium	0.48		
				Methanospirillum	0.41		
				Aminiphilus	0.38		
				Eubacterium	0.36		
				Sporobacter	0.36		
				Thermanaerovibrio	0.20		

in the absence of BrES were assigned to *Methanosaeta* (37% and 22% in the presence of oleate and palmitoleate, respectively). Hydrogenotrophic methanogens were also present in these communities, even though less abundant than acetoclasts according to pyrosequencing results, and the most represented genera were *Methanobacterium*, *Methanospirillum* and *Methanolinea* (Table 4). In the absence of methanogenic activity, microbial communities were dominated by microorganisms of the genera *Pseudomonas*, *Clostridium* and *Rheinheimera*. Bacterial members of the genus *Sporanaerobacter* were present in the bioreactors where methanogenesis was inhibited, but not in the methanogenic bioreactors.

Aerobic oleate biodegradation by *Pseudomonas* spp. has been previously reported, and these bacteria are capable of anoxic metabolism in the presence of nitrate.[25,26] To assess the ability of *Rheinheimera* spp. to degrade unsaturated fatty acids, aerobic and strict anaerobic batch incubations were performed with a pure culture of *Rheinheimera pacifica* in the presence of 2 mmol L^{-1} oleate. Oleate was not degraded in the anaerobic batch vials, but was completely mineralized in the presence of oxygen (Figure S5).

The ability of *Syntrophomonas zehnderi*, in coculture with *Methanobacterium formicicum*, to perform the first steps of oleate degradation to palmitate was also studied in batch bottles amended with 1 mmol L^{-1} oleate and 20 mmol L^{-1} BrES. *S. zehnderi* is an important oleate degrader that was isolated from an anaerobic bioreactor treating oleate.[27] Oleate degradation, followed by acetate accumulation and methane production, only occurred in the bottles without BrES. Stearate or palmitate were not detected in the bottles, neither with nor without methanogenic activity (SI Figure S6).

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DISCUSSION

Sludge flotation and washout and LCFA inhibition of the microbial communities have been identified as the major problems in anaerobic bioreactors treating lipids or LCFA-rich wastewater.[2,28] Since these problems arise from LCFA accumulation onto the biomass, a deeper knowledge on the mechanisms of LCFA biodegradation can contribute to improve the anaerobic treatment and enhance methane production from these substrates. In this work, we hypothesized that the initial steps of unsaturated LCFA biodegradation may occur decoupled from methanogenesis.

For all the tested compounds, accumulation of saturated LCFA-intermediates was observed both in the absence and in the presence of the inhibitor of methanogenesis. However, further β-oxidation to acetate only occurred in sludges with methanogenic activity (SI Figure S1). In the absence of active methanogens, degradation of saturated LCFA did not proceed, not even when a prolonged batch phase was applied (Figure 1, Table 3). Moreover, no LCFA conversion was observed in reactor IR STE+PAL fed with the mixture of saturated LCFA (Figure 1j). These results indicate that in anaerobic bioreactors the first steps of unsaturated LCFA biodegradation do not depend on the activity of the methanogenic archaea. A previous work of Pereira et al. also reported palmitate accumulation and low methane production in an anaerobic bioreactor fed with palmitate at a concentration of 4 g COD L^{-1} and 1 day HRT.[17]

Palmitate (C16:0) was the major intermediate quantified in the IR reactors during unsaturated C18 LCFA degradation (IR LIN, IR OL and IR OL+GLY), accounting for more than 50% of total LCFA and reaching values as high as 88% when glycerol was added (Table 3). Hydrogenation was also experimentally verified, with the quantification of oleate C18:1 (22 ± 2%) and stearate C18:0 (<13%) in the reactors fed with linoleate (C18:2) and oleate, respectively. Degradation of monounsaturated C16 followed the same pathway leading to the accumulation of C16:0 and C14:0 as the main intermediates.

From a thermodynamic point of view (SI Figure S7), hydrogenation of unsaturated LCFA is favorable at standard temperature and pressure conditions ($\Delta G^{0\prime} = -79$ kJ mol^{-1}), but β-oxidation cycles are not ($\Delta G^{0\prime} = +51$ kJ mol^{-1}), therefore requiring syntrophic cooperation of the LCFA-degrading bacteria with hydrogenotrophic microorganisms to maintain low hydrogen partial pressure (PH_2). Considering nonstandard conditions (1 mmol L^{-1} for reagent LCFA, products stoichiometric accumulation, at 25 °C and pH 7) these reactions only become favorable for PH_2 lower than 100 Pa (10^{-3} atm). Nevertheless, linoleate conversion to palmitate is still thermodynamically feasible at nonstandard conditions ($\Delta G^{0\prime} = -107$ kJ mol^{-1}) and is not limited by the hydrogen partial pressure ($Linoleate^- + 2H_2O \rightarrow Palmitate^- + Acetate^- + H^+$). Oleate to palmitate conversion is thermodynamically feasible even at standard temperature and pressure conditions ($\Delta G^{0\prime} = -28$ kJ mol^{-1}, Table 1), whereas further palmitate degradation to acetate is not. For the nonstandard conditions previously defined, $\Delta G'$ of oleate to palmitate conversion is negative for PH_2 as high as 10^{10} kPa, in contrast with the PH_2 < 100 Pa calculated for the complete palmitate degradation to acetate, thus pointing to the possibility of palmitate accumulation in the medium. This suggests that linoleate/oleate to palmitate conversion may be predominant in unsaturated C18-fed bioreactors, and that palmitate degradation to acetate will only occur when the hydrogen partial pressure is low.

In real wastewater treatment systems complex mixtures of substrates are degraded. This situation was simulated through oleate plus glycerol feeding to reactors MR OL+GLY and IR OL+GLY. The presence of the cosubstrate contributed to an extensive oleate conversion to palmitate, leading to the accumulation of higher palmitate concentrations in the IR (11 ± 1 mmol L^{-1}, Table 3). Glycerol may have stimulated other microbial groups present in the microbial community, or may have functioned as an additional hydrogen source. This is consistent with the pathway proposed in this work, where oleate conversion to palmitate is practically not constrained by PH_2 values below 10^{10} kPa.

Unsaturated LCFA biodegradation pathway in anaerobic bioreactors can now be redefined, considering two major phases:

(i) Phase 1: fast, nonlimiting and nondependent of methanogenesis, with the production of a saturated C_{n-2} LCFA;
(ii) Phase 2: slower and syntrophic, corresponds to the classical pathway of saturated LCFA biodegradation. The saturated C_{n-2} LCFA produced in the previous phase are converted to acetate.

Under continuous operation, as long as high LCFA accumulation is avoided, both phases can occur simultaneously. For that, low hydrogen partial pressure must be guaranteed to avoid the buildup of saturated LCFA intermediates.

Phase 1 and phase 2 can be performed by one or more microorganisms. Some syntrophic LCFA-degrading bacteria can use unsaturated LCFA as sole carbon and energy source, and some fermentative bacteria are able to hydrogenate the double bonds of the fatty acids aliphatic chain, although lacking the enzymes required for β-oxidation.[29] Moreover, microbial communities specialized on unsaturated LCFA also degrade saturated LCFA, but the opposite does not occur.[1]

In this work, microbial diversity and composition was assessed in the bioreactors supplemented with monounsaturated LCFA in the presence or absence of BrES. Despite the presence of similar bacterial communities, as shown by the analysis of DNA-derived PCR amplicons (SI Figure S2), rRNA derived PCR products showed that different bacterial populations were contributing to the RNA pool when methanogens were active or inhibited (SI Figure S3). Thus, different microorganisms were active and likely involved in the metabolic reactions occurring in these reactors.

Regardless of previous reports on high sensibility of *Methanosaeta* spp. to unsaturated LCFA, high abundance of *Methanosaeta*-like microorganisms was found in the two MR operated with monounsaturated LCFA (Table 4). Similar results were previously reported by Salvador et al.[30] during the operation of a continuous bioreactor with high oleate loads. In the reactors where methanogenesis was inhibited, aerobic or facultative anaerobic microorganisms, namely *Pseudomonas* and *Rheinheimera* spp., became predominantly active, and no metabolically active syntrophic bacteria such as *Syntrophomonas* sp. was detected (Table 4). These observations indicate that bacteria of the genus *Syntrophomonas* were not involved in the conversion of oleate to palmitate in the studied bioreactors. The fact that palmitate was not detected in batch incubations of *S. zehnderi* + *M. formicicum* in the presence of BrES (SI Figure S6), reinforces this conclusion.

The detection of active *Pseudomonas* and *Rheinheimera* species in the IR was unexpected, since these genera are known as aerobic or facultative anaerobic bacteria. *Rheinheimera pacifica* was able to degrade oleate aerobically, but not in strict anaerobic

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conditions (SI Figure S5). This result may, however, be due to the different environments that are created in batch or continuous bioreactors, and/or be related with distinct functions that this microorganism might exhibit in pure culture or integrated in a mixed microbial community. Further research is thus needed to elucidate the role of the detected *Pseudomonas* and *Rheinheimera* spp. in the conversion of unsaturated fatty acids.

This work shows for the first time that in anaerobic bioreactors C_n-unsaturated LCFA are converted to C_{n-2}-saturated LCFA by microorganisms other than syntrophic bacteria (facultative anaerobic bacteria), and do not depend on the presence of active hydrogenotrophic methanogens. This opens new perspectives to improve biogas formation from lipid-containing wastewater streams.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03204.

Acetate concentration in the MR reactors; DNA-derived and RNA(cDNA)-targeted DGGE analysis of bacterial 16S rRNA gene fragments; microbial sequence reads at the phylum level from pyrosequencing analysis in the reactors fed with monounsaturated LCFA; results from the pure cultures assays; possible intermediates during unsaturated LCFA catabolism and Gibbs free energy changes of each metabolic step (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BLASTN	standard nucleotide basic local alignment search tool
BrES	2-Bromoethanesulfonate
COD	chemical oxygen demand
DGGE	denaturing gradient gel electrophoresis
FAPE	fatty acid propyl esters
FID	flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
HPLC	high-performance liquid chromatography
HRT	hydraulic retention time
LCFA	long chain fatty acids
NIST	National Institute of Standards and Technology
OLR	organic loading rate
PCR	polymerase chain reaction
RDP	ribosomal database project
rRNA	ribosomal ribonucleic acid
r.p.m	revolutions per minute
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acids
VS	volatile solids

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