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Multiple and flexible roles of facultative anaerobic bacteria in microaerophilic oleate degradation

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Summary

Anaerobic degradation of long-chain fatty acids (LCFA) involves syntrophic bacteria and methanogens, but facultative anaerobic bacteria (FAB) might have a relevant role as well. Here we investigated oleate degradation by a syntrophic synthetic co-culture of *Syntrophomonas zehnderi* (Sz) and *Methanobacterium formicicum* (Mf) and FAB (two oleate-degrading *Pseudomonas* spp. I1 + I2). Sz + Mf were first cultivated in a continuous bioreactor under strict anaerobic conditions. Thereafter, I1 + I2 were inoculated and microaerophilic conditions were provided. Methane and acetate were the main degradation products by Sz + Mf in anaerobiosis and by Sz + Mf + I1 + I2 in microaerophilic conditions. However, acetate production from oleate was higher in microaerophilic conditions (5% O_2) with the four microorganisms together (0.41 ± 0.07 mmol day^{-1}) than in anaerobiosis with Sz + Mf (0.23 ± 0.05 mmol day^{-1}). Oleate degradation in batch assays was faster by Sz + Mf + I1 + I2 (under microaerophilic conditions) than by Sz + Mf alone (under strict anaerobic conditions). I1 + I2 were able to grow with oleate and with intermediates of oleate degradation (hydrogen, acetate and formate). This work highlights the importance of FAB, particularly *Pseudomonas* sp., in anaerobic reactors treating oleate-based wastewater, because they accelerate oleate conversion to methane, by protecting strict anaerobes from oxygen toxicity and also by acting as alternative hydrogen/formate and acetate scavengers for LCFA-degrading anaerobes.

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Introduction

Anaerobic degradation of lipid/long-chain fatty acid (LCFA)-rich wastewater is not always effective, due to frequently reported operational problems (Alves *et al*., 2009). Additionally, a limited number of bacterial species are described as LCFA degraders under methanogenic conditions. These species belong to the genera *Syntrophomonas*, *Thermosyntropha* and *Syntrophus* (Sousa *et al*., 2009) and are dependent on the activity of hydrogenotrophic methanogens or other hydrogen-consumers to keep the H_2 partial pressure and formate concentration low, in order to make LCFA degradation thermodynamically feasible (Schink, 1997; McInerney *et al*., 2008). Up to now, only five *Syntrophomonas* species are described as LCFA degraders (Sousa *et al*., 2009), but in anaerobic bioreactors, they are usually detected in low percentages (Stams *et al*., 2012).

The presence of non-syntrophic bacteria in LCFA-degrading communities is usually associated to the conversion of metabolites formed during LCFA degradation [e.g. volatile fatty acids (VFAs)], which may enable these bacteria to outnumber the slowly growing syntrophic LCFA degraders (Sousa *et al*., 2009). Facultative anaerobic bacteria (FAB) are well known to play an important role in the fermentation/acidogenesis step of anaerobic digestion (Joubert and Britz, 1987; Botheju and Bakke, 2011; Nguyen and Khanal, 2018). The relevant role of microaerobic conditions and facultative microorganisms for a more efficient methane production and rate from LCFA has been suggested (Cavaleiro *et al*., 2016; Duarte *et al*., 2018). In bioreactors treating LCFA-based wastewaters, *Pseudomonas* species have been reported to be abundant (between 8% and 58%) (Pereira *et al*., 2002b; Baserba *et al*., 2012; Cavaleiro *et al*., 2016; Duarte *et al*., 2018). Their involvement in methanogenic LCFA degradation was also suggested previously (Cavaleiro *et al*., 2016), and likely they are important in oleate conversion to palmitate, which is the main LCFA that accumulates during oleate conversion in

methanogenic bioreactors (Pereira *et al*., 2002a). Also the presence of *Pseudomonas* spp. was strongly correlated with palmitate-to-total LCFA ratio in bioreactors fed with oleate (Duarte *et al*., 2018).

This work aims to investigate the microbial relationships between syntrophic LCFA-degrading bacteria, FAB and methanogens in oleate degradation, under anaerobic and microaerophilic conditions. A synthetic microbial consortium composed by *Syntrophomonas zehnderi* (a syntrophic LCFA-degrading bacterium), two *Pseudomonas* spp. (FAB isolated from an oleate degrading bioreactor) and *Methanobacterium formicicum* (a hydrogen/formate utilizing methanogen) was studied in continuous and batch bioreactors fed with oleate, under strict anaerobic and microaerophilic conditions.

Results

Oleate degradation by S. zehnderi*,* M. formicicum *and* Pseudomonas *spp. in a continuous bioreactor*

Oleate is an amphiphilic molecule that adsorbs easily to solid surfaces, including microbial aggregates, reactor walls, pumping tubes and feed vessel (which is possible to observe by naked eye). In the continuous reactor experiment, oleate is quantified in the bulk media, but a significant amount may remain in the reactor surfaces. Thus, mass balances based on oleate degradation were not possible (oleate consumption versus acetate and methane production). Consequently, in the experimental set up, operated under continuous mode, measurement of the products (acetate and methane) was the only rigorous way to assess the microbial activity occurring inside the bioreactor operated with the defined co-culture of *S*. *zehnderi* and *M*. *formicicum* (Period I–IV, Table 1) and with the synthetic consortium of *S*. *zehnderi*, *M*. *formicicum* and the two *Pseudomonas* isolates (Periods V–X, Table 1). Nevertheless, despite the possible under-quantification of oleate due to adsorption, the values measured throughout the experiment give information about the trend of oleate conversion and contribute to understand what is happening inside the bioreactor. Therefore, oleate concentrations measured in the continuous bioreactor during the 10 periods of operation are shown in Fig. 1, together with VFA concentrations, methane production, oxidation–reduction potential (ORP) and pH.

The bioreactor was operated for 10 periods that reflected the need for adjustment of the operational parameters (Table 1) in order to keep the community active. We highlight the results from Periods III–IV and IX–X that allow to compare the performance of the community under the same operational conditions (HRT and oleate concentration in the feed, Table 1), without and with the *Pseudomonas* spp. respectively. In the absence of *Pseudomonas* (Periods III and IV), more methane was produced; however, formate and oleate started to accumulate in the reactor (Fig. 1 and Table 2). By contrast, in the presence of *Pseudomonas* (in Periods IX–X), despite the lower methane production, more acetate was produced, poor oleate accumulation occurred and formate was not detected.

In Periods I and II, acetate concentration decreased over time and methane production stopped (Fig. 1A), which was accompanied by an increase in the pH (Fig. 1B). To counteract this, the bicarbonate concentration was decreased to 2 g L^{-1} in Period III, the headspace of the reactor was flushed with N_2/CO_2 and the HRT was decreased to approximately 10 days (Table 1). There was a recovery of the microbial activity, with acetate and methane accumulating in the reactor (Fig. 1). In this period, acetate and methane production was 0.14 ± 0.03 mmol day^{-1} and 0.03 mmol day^{-1} (Table 2) respectively.

In Period IV, the oleate concentration in the feeding was increased to 2.63 ± 0.87 mmol L^{-1} (Period IV, Table 1).

Table 1. Parameters set in the different operational periods of the continuous bioreactor: oleate concentration in the feed; HRT applied; mode: continuous or batch; percentage of oxygen in the gas phase of the feeding tank.

Microbial consortium	Period	Time (days)	Oleate in the feed (mmol $L^{-1)}$	HRT (days)	Mode	% O_2 in the feeding tank
Syntrophomonas zehnderi	I	0–13	0.25	18.6	Continuous	–
Methanobacterium	II	13–22	2.28 ± 0.09	18.6	Continuous	–
formicicum	III	22–70	1.32 ± 0.76	10.4 ± 0.8	Continuous	–
	IV	70–112	2.63 ± 0.87	11.5 ± 2.2	Continuous	–
Syntrophomonas zehnderi	V	112–133	3.21 ± 0.47	10.1 ± 1.2	Continuous	21
Methanobacterium	VI	133–160			Batch	–
formicicum	VII	160–165	4.67 ± 0.70	11.1 ± 0.3	Continuous	10
Pseudomonas isolates	VIII	165–176			Batch	–
	IX	176–216	1.36 ± 0.23	10.5 ± 0.9	Continuous	5
	X	216–245	2.13 ± 0.45	9.2 ± 0.7	Continuous	5

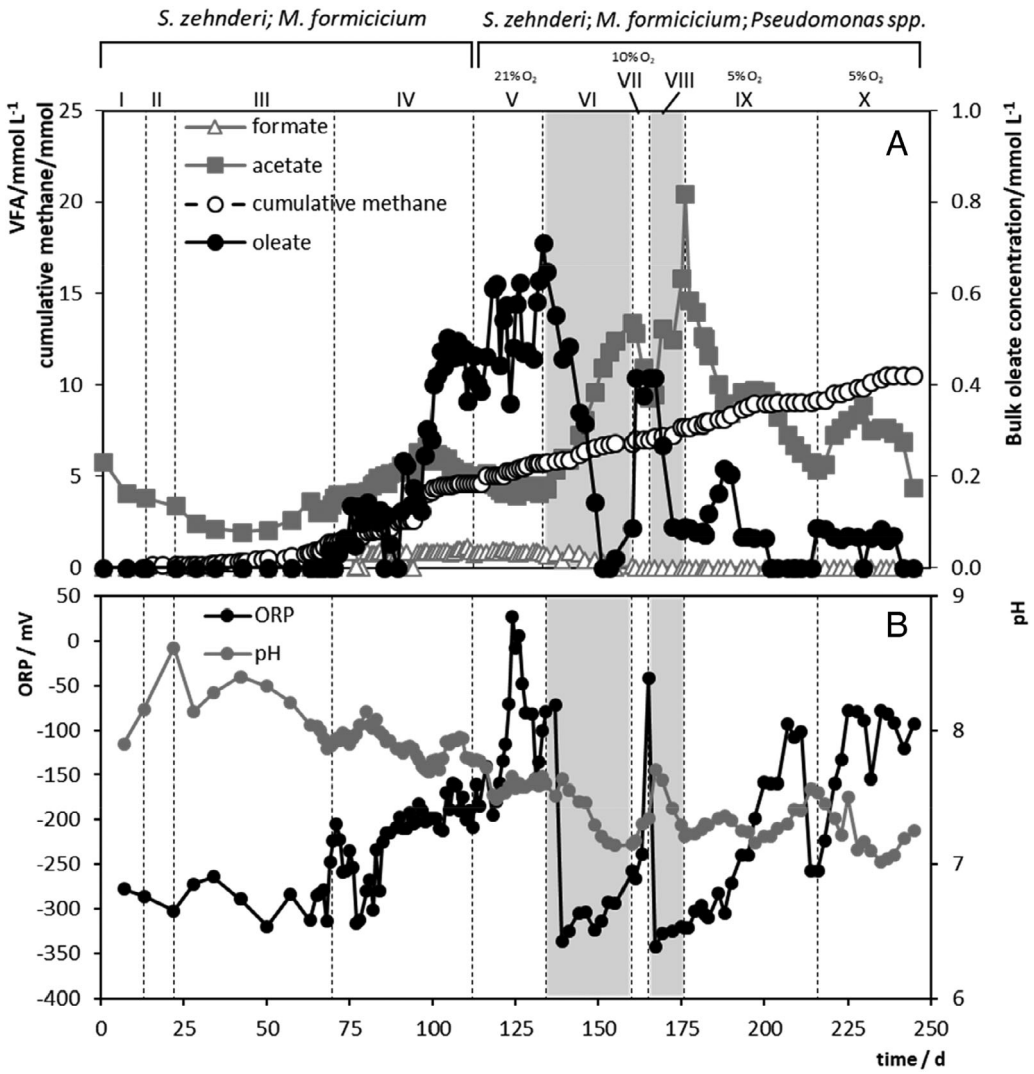

Fig. 1. Bulk oleate and VFA (acetate and formate) concentrations and cumulative methane production (A), ORP and pH (B) in the continuous bioreactor during the 10 periods of the operation. Batch periods are in grey.

Table 2. Acetate and methane productions in Periods III to X of the continuous bioreactor operation.

Period	Time (days)	Acetate (mmol day^{-1})	Methane (mmol day^{-1})
III	22–70	0.14 ± 0.03	0.03
IV	70–112	0.23 ± 0.05	0.08
V	112–133	0.22 ± 0.03	0.06
VI	133–160	0.16	0.04
VII	160–165	0.49 ± 0.07	0.00
VIII	165–176	0.40	0.03
IX	176–216	0.49 ± 0.14	0.03
X	216–245	0.41 ± 0.07	0.05

In this period (IV), the methane production increased to 0.08 mmol day^{-1} (Table 2) relatively to the previous period and was closer to what was theoretically expected (0.10 mmol day^{-1}) when compared with the acetate produced (0.23 ± 0.05 mmol day^{-1}), according to Equation 1. Formate (up to 1 mmol L^{-1}) and acetate (up to 6.6 mmol L^{-1}) concentrations increased in the reactor, and at Day 98, methane production almost ceased (Fig. 1A).

$$1\,oleate \rightarrow 9\,acetate + 3.75\,methane \quad (1)$$

When the *Pseudomonas* isolates (I1 + I2) were inoculated in the reactor (Day 112, beginning of Period V), the oleate concentration inside the reactor increased (up to a maximum of 0.65 mmol L^{-1}), and the methane production decreased (Fig. 1A), showing that the methanogens were inhibited. The reactor was set in batch mode (Period VI) to allow the recovery of the microbial community. After that, the reactor was again operated in continuous mode but with 10% (vol/vol) oxygen in the feeding tank (Period VII). Methane production ceased and oleate

started to accumulate (Fig. 1A). Therefore, the reactor was again set in batch mode (Period VIII) to allow the recovery of the community, which was achieved after 11 days. When the continuous operation was restarted (Period IX), the oxygen concentration in the feeding was reduced to 5% (vol/vol) to lower the impact of oxygen toxicity towards the strict anaerobes, and oleate concentration was decreased to 1.36 ± 0.23 mmol L^{-1} (Table 1), only then, stable operation was achieved under microaerophilic conditions. The methane production was similar to the former Period VIII (0.03 mmol day^{-1}). The acetate production was high, 0.49 ± 0.14 mmol day^{-1} (Table 2, Period IX), compared with the former periods, when the reactor was operated in continuous mode. In Period X all the operational parameters were maintained the same as period IX, with exception of oleate concentration that was increased to 2.13 ± 0.45 mmol L^{-1} because oleate had been degraded in the previous period. The methane production increased to 0.05 mmol day^{-1}, and the acetate production was 0.41 ± 0.07 mmol day^{-1} in this period (Table 2).

During the bioreactor operation, the pH varied between 7 and 8.6. Generally, the pH decreased with the increase in acetate concentration (Fig. 1). The changes of the operation mode were reflected in the ORP measured inside the reactor, with the ORP increasing during continuous operation (especially from Period V onwards) and decreasing during batch periods (reaching a minimum value of −343 mV in Period VIII) (Fig. 1B). This is justified by the fact that during continuous operation the feeding tank contained oxygen that consequently promoted the increase of the ORP inside the bioreactor. During Period V, when the percentage of O_2 in the feeding was the highest (21%, vol/vol), the ORP reached the highest value (+27 mV); in Period VII, when the oxygen in the feeding tank was kept at 10% (vol/vol), the ORP sharply increased from −266 to −42 mV.

During the experiment, samples were weekly collected for microscopic observations. Several microbial aggregates were observed (Fig. 2A). The curved rods are typical of *S. zehnderi* cells (Sousa *et al*., 2007) (Fig. 2B) and the long rods of *M. formicicum* cells, which typically appear as single cells or forming chains and filaments (Bryant and Boone, 1987) (Fig. 2A). The straight rods were the *Pseudomonas* isolates described before (Duarte *et al*., 2018) (Fig. 2C). Sometimes, under stress conditions, *Pseudomonas* I1 assumes a longer phenotype and forms inclusion bodies as may be observed in Fig. 2C.

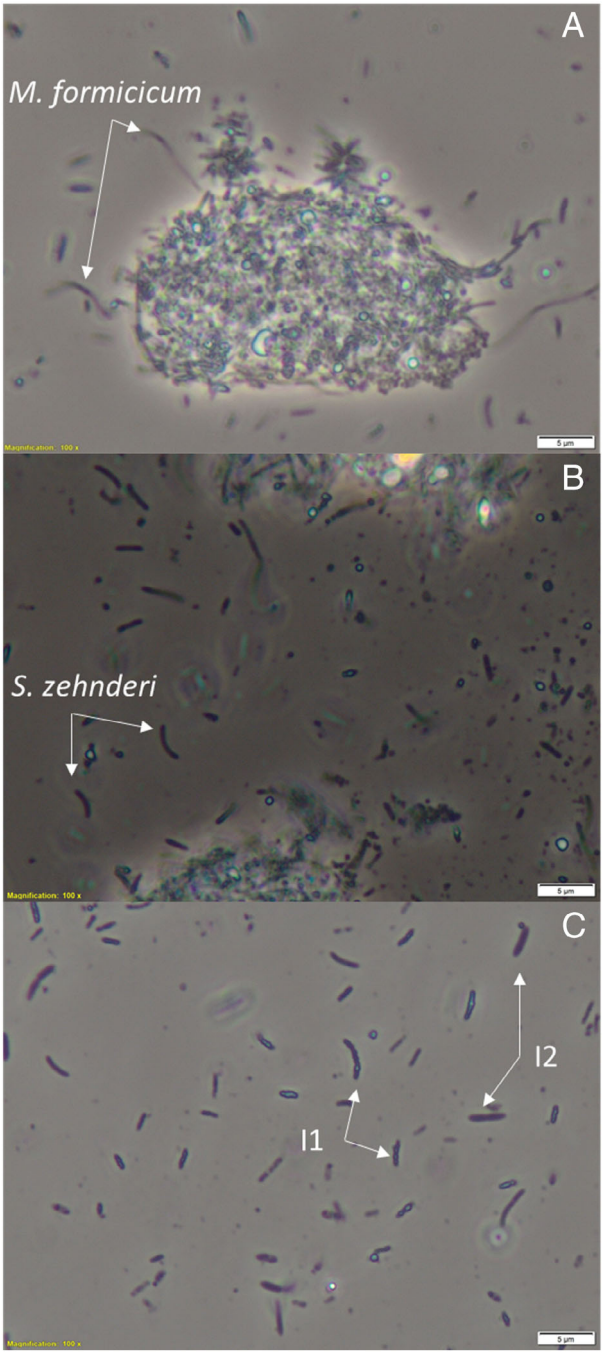

Fig. 2. Micrographs of the microbial consortium collected from the bioreactor at Day 225 in Period X, showing aggregates composed of oleate and different microbial morphotypes (A, B), where *M. formicicum* (A) and *S. zehnderi* (B) were identified. Micrograph of the *Pseudomonas* isolates I1 and I2 in the bioreactor at Day 195 in Period IX (C).

Degradation of oleate, acetate, hydrogen and formate by Pseudomonas *isolates I1 and I2*

To get insight into the role of the *Pseudomonas* isolates in the bioreactor, I1 and I2 were incubated in batch with oleate, as well as with the products formed from oleate by *S. zehnderi*. Assuming that oleate is degraded through beta-oxidation, acetate, hydrogen and formate may be formed (Sousa *et al*., 2007; Sousa *et al*., 2009). The two *Pseudomonas* isolates I1 and I2

growing separately in monoculture were able to degrade 16 mmol L^{-1} of acetate after approximately 24 h under aerobic conditions (Fig. S1). Oleate was also degraded aerobically by the isolates, and some acetate was detected as degradation product after 3 days of incubation (Fig. 3A). In contrast with the continuous bioreactor, oleate concentration measurements in batch tests (Fig. 3A) are reliable; by vigorous shaking, homogeneous samples can be taken. By the end of the incubations, 1 mmol L^{-1} of acetate was formed by the two strains. Not all oleate (0.9 mmol L^{-1}) was utilized by the *Pseudomonas* isolates, since 0.3 ± 0.1 mmol L^{-1} and 0.4 ± 0.0 mmol L^{-1} of oleate could still be detected at the end of the assays with I1 and I2 respectively (Fig. 3A). The degradation of oleate and formate together was also tested with the two isolates I1 + I2 under microaerophilic conditions. In the first 3 days, formate concentration decreased from 8.5 ± 0.5 mmol L^{-1} to 5.5 ± 0.6 mmol L^{-1}; from Day 3 onward, acetate started to be formed as a result of oleate degradation, reaching a final concentration of 0.7 ± 0.2 mmol L^{-1} (Fig. 3B).

Additional tests showed that the co-culture composed by isolates I1 and I2 was able to use both hydrogen and formate as substrate under microaerophilic conditions (Table 3). The amount of formate consumed (8.0 ± 1.2 mmol L^{-1}) was as expected according to the initial oxygen concentration (4.3 mmol L^{-1}) and assuming conversion to CO_2 (Equation 2). Regarding hydrogen consumption, a depletion of 6 mmol L^{-1} could be detected which represents only 60% of the theoretical expected (10 mmol L^{-1}) considering the amount of oxygen available (4.9 mmol L^{-1}) (Equation 3; Table 3).

$$2\,HCOOH + O_2 \rightarrow 2\,CO_2 + 2\,H_2O \quad (2)$$

$$2\,H_2 + O_2 \rightarrow 2\,H_2O \quad (3)$$

Oleate degradation by S. zehnderi*,* M. formicicum *and* Pseudomonas *in microaerophilic conditions in batch assays*

Batch assays were also performed with the constructed consortium composed by Sz + Mf + I1 + I2, to get better insights about the microbial interactions between the FAB (I1 and I2) and the syntrophic co-culture. *Syntrophomonas zehnderi* and *M. formicicum* co-cultures that were more active (H.A.) and less active (L.A.) were tested, since the parameters analysed, like the lag phase, may differ significantly when considering the initial activity of the syntrophic culture.

Methane production from the synthetic community cultivated under batch microaerophilic conditions (Mic-Sz + Mf + I1 + I2) presented a 1.4 times shorter lag phase than the anaerobic syntrophic co-culture An-Sz + Mf, in the assay performed with the H. A. co-culture, i.e. 10 and 14 days respectively (Fig. 4A). In the tests performed with L.A. co-culture, the Mic-Sz + Mf + I1 + I2 presented an estimated lag phase of 49 days, whereas the An-Sz + Mf was around 64 days (Fig. 4B).

After 22 days in the batch assay, both *Pseudomonas* isolates from the test Mic-Sz + Mf + I1 + $I2_{(H.A.)}$ were still viable as they grew well in less than 5 days on solid media.

Only residual methane production from oleate was obtained from the consortium composed by *M. formicicum* and *Pseudomonas* isolates (Mf + I1 + I2) under microaerophilic (0.16 ± 0.03 mmol L^{-1}) and under anaerobic conditions (0.13 ± 0.05 mmol L^{-1}) (Table S1). Under microaerophilic conditions, an oleate degradation of 0.19 ± 0.10 mmol L^{-1} was observed by Mf + I1 + I2, showing that oleate degradation by *Pseudomonas* may occur in the presence of the methanogens and without *S. zehnderi*. This result was similar to the control test performed in parallel, with only *Pseudomonas* I1 + I2, where

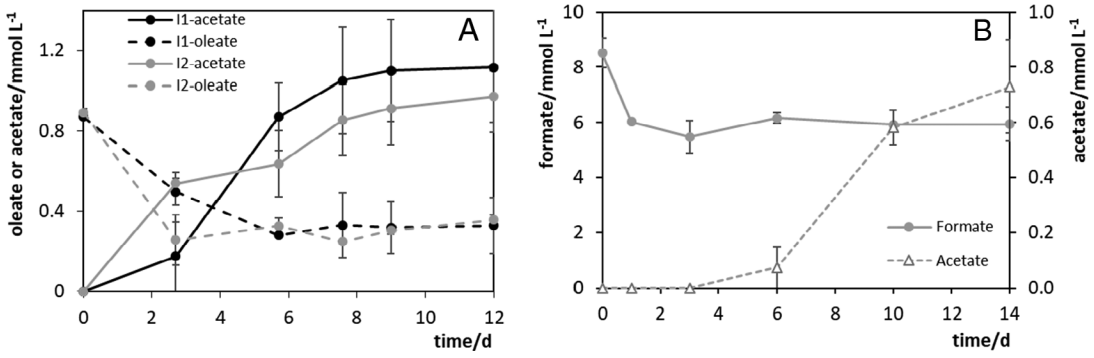

Fig. 3. Degradation of oleate and a mixture of oleate and formate by *Pseudomonas* isolates.
A. Oleate and acetate concentrations during the oleate degradation under aerobic conditions.
B. Formate and acetate concentrations during the degradation of a mixture of oleate and formate by *Pseudomonas* isolates, under microaerophilic conditions.

Table 3. Initial oxygen concentration and initial and final substrate (hydrogen or formate) concentrations in batch experiments performed with isolates I1 and I2.

Substrate	Time (days)	$[Oxygen]_{initial}$ (%, vol/vol)	$[Substrate]_{initial}$ (mmol L^{-1})	$[Substrate]_{final}$ (mmol L^{-1})
Formate	15	4	18.93 ± 0.90	10.92 ± 0.85
Hydrogen	6	5	60.37	54.37 ± 0.03

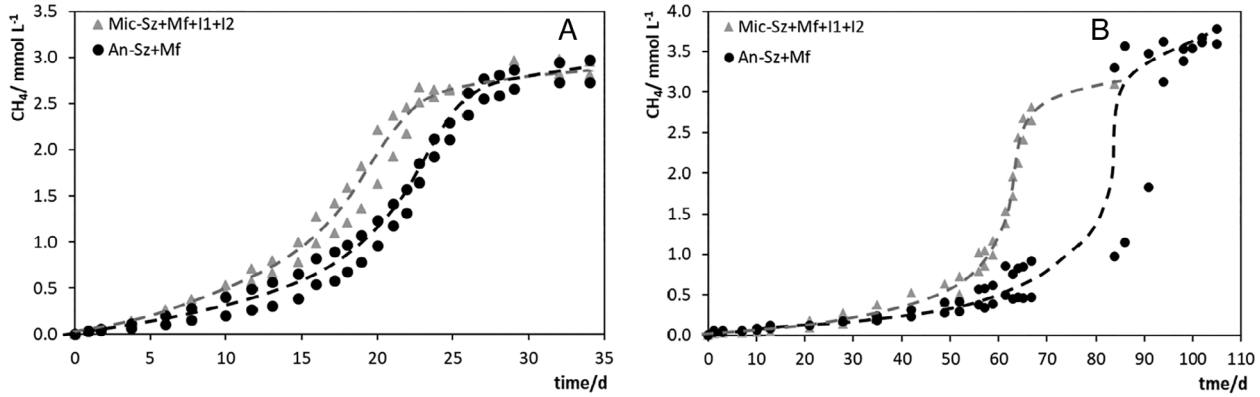

Fig. 4. Cumulative methane production in the batch assays. Mic-Sz + Mf + I1 + I2 and An-Sz + Mf inoculated with a syntrophic co-culture of *S. zehnderi* and *M. formicicum* with high (A) and low (B) metabolic activity.

oleate consumption was 0.21 ± 0.02 mmol L^{-1} (data not shown). Furthermore, methane produced (0.16 ± 0.03 mmol L^{-1}) was much lower than the expected 0.71 mmol L^{-1} from the oleate degradation (0.19 ± 0.10 mmol L^{-1}). These results suggest that the *Pseudomonas* and *M. formicicum* could not establish a syntrophic relationship. The co-culture of *S. zehnderi* and *M. formicicum* could not degrade oleate or produce methane (0.06 ± 0.03 mmol L^{-1}) under microaerophilic conditions (Table S1). The low methane productions in these tests probably resulted from the residual substrate (hydrogen) used to grow *M. formicicum* and not from oleate conversion to methane.

Discussion

In this work, the syntrophic LCFA degrading co-culture, *S. zehnderi* and *M. formicicum*, was maintained in a continuous anaerobic bioreactor converting oleate to acetate and methane (Periods III and IV) (Fig. 1A) at close to the stoichiometric values (Equation 1), i.e. taking into account that the oleate degradation resulted in 0.2 mmol day^{-1} of acetate, it would be expected approximately 0.08 mmol day^{-1} of methane. Under microaerophilic conditions (Periods V, IX and X) (Fig. 1A), oleate degradation was accomplished by a synthetic community composed by this culture together with two *Pseudomonas* isolates (Figs 1A, 2). In this case, acetate was the main product and methane was detected only in low amounts (Fig. 1A). At 5% O_2, higher oleate conversion to acetate was achieved (Table 2), especially when comparing anaerobic vs microaerophilic periods with similar OLR applied (i.e. Periods III vs IX, and IV vs X) (Tables 1 and 2). The results indicate that in the continuous bioreactor, operated under microaerophilic conditions, the presence of *Pseudomonas* is crucial to guarantee continuous oleate degradation because Sz + Mf, which are strictly anaerobic microorganisms, were unable to degrade oleate under microaerophilic conditions (Mic-Sz + Mf) (Table S1). FAB have been reported to protect strict anaerobes from oxygen toxicity (Gerritse and Gottschal, 1993), which is very important in real AD systems where the presence of vestigial amounts of oxygen is common. This protective role seems obvious in our batch assays where the presence of *Pseudomonas* resulted in 1.4 times shorter lag phases in microaerophilic conditions compared with the syntrophic co-culture alone (Fig. 4) under anaerobic conditions, showing that *Pseudomonas* isolates accelerated the conversion of oleate to methane by decreasing the lag phase.

Both *Pseudomonas* isolates (I1 and I2) consume oleate under aerobic (Fig. 3A) and microaerophilic conditions (Duarte *et al*., 2018), and therefore they may be directly involved in oleate degradation in the continuous bioreactor. Data from batch assays show that *Pseudomonas* produce approximately 2 mmol of acetate per mmol of oleate (Fig. 3A), and *S. zehnderi* + *M. formicicum* produce 9 mmol of acetate per mmol of oleate based on Equation 1. In the bioreactor, in Period IX, the acetate formed (0.49 ± 0.14 mmol day^{-1}, Table 2) is close to the expected

from syntrophic degradation of the oleate fed (Equation 1), i.e. 0.63 mmol day^{-1} of acetate considering the operational conditions applied (oleate concentration of 1.36 mmol L^{-1} and 10 days HRT—Table 1, Equation 1). Therefore, it is likely that the production of acetate from oleate in the continuous bioreactor, under microaerophilic conditions, occurred to a higher extent by the activity of *S. zehnderi*, showing the importance of *S. zehnderi* in the microbial consortia.

Oleate degradation by *S. zehnderi* is dependent on the activity of *M. formicicum* to keep the hydrogen partial pressure low. In our bioreactor, under microaerophilic conditions, methane production was low (Fig. 1A) compared with the amount of oleate degraded (Fig. 1A, Table 2), which allows to hypothesize that *Pseudomonas* may be an alternative syntrophic partner, explaining why oleate degradation proceeds even when methanogenic activity was negligible. Indeed, some *Pseudomonas* species were reported to grow on formate and hydrogen, for example *Pseudomonas putida* and *Pseudomonas pseudoflava* (Lee and Schlegel, 1981; Riis *et al*., 2003). In our study, *Pseudomonas* isolates (I1 and I2) were able to utilize formate and hydrogen in batch assays under microaerophilic conditions (Table 3). In the presence of formate and oleate (Fig. 3B), these isolates first degraded partially the formate (Day 1) and only then, at Day 3, the oleate started being degraded (i.e. acetate was formed). This ability of *Pseudomonas* isolates may explain the decrease in formate concentration in the continuous reactor in Period V (Fig. 1A), that occurred when the *Pseudomonas* isolates were inoculated.

We show that the presence of FAB, particularly *Pseudomonas* spp., is important for a stable and robust anaerobic treatment of oleate-based wastewaters. Besides protecting the strict anaerobic community from oxygen toxicity, the results obtained by us strongly suggest that *Pseudomonas* may act as alternative hydrogen/formate scavengers for syntrophic LCFA-degrading bacteria.

Experimental procedures

Oleate degradation by S. zehnderi, M. formicicum *and* Pseudomonas *spp. in a continuous bioreactor*

A continuous reactor of 0.5 L (working volume) with a headspace of 0.14 L was inoculated (10%, vol/vol) with a syntrophic co-culture of *S. zehnderi* (Sz) (DSM 17840) and *M. formicicum* (Mf) (DSM 1535) previously grown on 0.5 mmol L^{-1} of oleate. The glass reactor (Schott, Mainz, Germany) had a stainless steel 2 port connector to link the inlet and outlet marprene tubes (Watson-Marlow, Falmouth, UK) and a butyl rubber cap to sample the gas and the liquid. Ten small pieces (approximately 0.5 cm × 0.5 cm) of marprene tube were added to the reactor as a support for microbial cells attachment. The reactor was operated at mesophilic conditions (37°C). The biogas produced was kept in the headspace of the reactor, and the cumulative methane production was followed.

The bioreactor operation was divided into 10 different periods with different oleate concentrations, hydraulic retention time (HRT), and percentage of O_2 in the gas phase of the feeding tank (Table 1). During Periods I–IV, the bioreactor with the syntrophic co-culture (Sz + Mf) was operated under strict anaerobic conditions, until reaching a steady point where the culture was kept active inside the reactor, degrading oleate into acetate and methane, during the continuous operation. In those periods, the influent oleate concentration and HRT varied between 0.25 and 2.6 mmol L^{-1} and 19–10 days respectively (Table 1).

The feed consisted of anaerobic mineral salt medium (Stams *et al*., 1993) buffered with bicarbonate (4 g L^{-1} in Periods I–II and 2 g L^{-1} in Periods III–IV) and reduced with sodium sulfide (2 mmol L^{-1}). Sodium oleate (99%, Sigma, Darmstadt, Germany) was the substrate. The feeding was sterilized by autoclaving, and the feeding tank was kept sterile and anaerobic by using a syringe filter in the gas entrance from a bag containing nitrogen. The feeding tank was kept at 60°C to maintain the sodium oleate dissolved in the feed. The headspace of the bioreactor was initially pressurized with a mixture of N_2/CO_2 (80:20% vol/vol, 170 kPa).

In Period V, the two different *Pseudomonas* spp. (I1 + I2) (Duarte *et al*., 2018) were added to the bioreactor containing the syntrophic co-culture at a final optical density (OD) of 0.01. The main difference in the experimental set-up was that the headspace composition of the feeding tank changed from nitrogen to air (21% of oxygen) (Table 1). When methane production dropped, showing a decrease in methanogenic activity, the operation mode was changed from continuous to batch (Periods VI and VIII, Table 1). Also, oleate and oxygen concentrations were adjusted during the operation (Periods VII, IX and X, Table 1) to guarantee the microbial activity of the oleate-degrading consortium. From Periods V to X, bicarbonate concentration was set at 2 g L^{-1}. Although the conditions changed from anaerobic to aerobic or microaerophilic, sodium sulfide was still added (0.5 mmol L^{-1}) to the feed to provide a sulfur source for *M. formicicum* growth. Sulfide concentration was weekly measured in the feeding tank, and the concentration varied between 0.5 and 0.04 mmol L^{-1}, showing that not all the sulfide reacted with the oxygen available. Sporadically, when the ORP increased over −51 mV, and in the beginning of the batch periods, 0.5 mmol L^{-1} of sodium sulfide was added to the reactor. At Days 118 and 126 (Period V), approximately 5%

(vol/vol) of a well grown culture of Sz + Mf was added do the reactor.

The effluent flow rate was controlled using a peristaltic pump. Samples from the reactor were collected for methane, LCFA and VFA quantification, pH and ORP measurement, and for regular microscopic observations by phase-contrast microscopy (Olympus-CX41, Olympus Corporation, Tokyo, Japan). Prior to sampling, the reactor liquid was gently mixed.

Degradation of oleate, acetate, hydrogen and formate by Pseudomonas *isolates I1 and I2 in batch assays*

The two *Pseudomonas* isolates (I1 and I2) were characterized separately for their capacity to degrade oleate or acetate under aerobic conditions. For this purpose, each isolate was incubated in Erlenmeyer flasks containing basal medium (described in Duarte *et al*., 2018). Oleate was added at a concentration of 1 mmol L^{-1} and acetate at a concentration of 16 mmol L^{-1}. The consumption of hydrogen (60 mmol L^{-1}), formate (20 mmol L^{-1}) and a mixture of formate and oleate (10 mmol L^{-1} and 1 mmol L^{-1} respectively) by the co-culture composed by isolates I1 and I2 was also tested under microaerophilic conditions (5% O_2 in the hydrogen and formate + oleate tests and 4% in the formate test) as described in following section. LCFA, VFA and hydrogen were analysed during the experiments.

Oleate degradation by S. zehnderi*,* M. formicicum *and* Pseudomonas *under microaerophilic conditions in batch assays*

Oleate utilization as sole carbon and energy source by the microbial consortium of *M. formicicum* (M), *S. zehnderi* (S) and the *Pseudomonas* isolates (I1 + I2) was tested in batch assays under microaerophilic conditions (Mic-Sz + Mf + I1 + I2). This test was performed twice, using a syntrophic co-culture of *S. zehnderi* and *M. formicicum* with L.A. and H.A. respectively. The L.A. culture was slowly growing and took approximately 30 days to degrade 0.5 mmol L^{-1} of oleate, while the H.A. culture was growing faster and took 7 days to degrade the same amount of oleate.

The assays were performed in duplicate by using 120 ml closed serum bottles with 50 ml bicarbonate-buffered mineral salt medium supplemented with sodium sulfide (1 mmol L^{-1}). The cultures were cultivated as follows: (i) *M. formicicum* cultures were pre-grown with H_2/CO_2 (80:20% vol/vol, 170 kPa) until reaching the exponential phase; (ii) the headspace of the bottles was flushed four times under sterile conditions with N_2/CO_2 (80:20% vol/vol, 170 kPa) to remove the remaining hydrogen and the methane produced; (iii) *S. zehnderi* was inoculated (10% vol/vol) and oleate added to a final concentration of 0.5 mmol L^{-1}; (iv) after reaching the exponential phase, the headspace of the bottles was flushed four times under sterile conditions with N_2/CO_2 to remove the methane produced prior *Pseudomonas* inoculation; (v) at the moment of isolates inoculation, oleate was added to a final concentration of approximately 1 mmol L^{-1} and air was added under sterile conditions to obtain a concentration in the headspace of 2% (vol/vol) O_2. *Pseudomonas* isolates used to inoculate the bottles were pre-grown on SOB medium (Super Optimal Broth with added glucose), under aerobic conditions, at 37°C and at 100 rpm. *Pseudomonas* isolates (I1 + I2) were inoculated in order that the initial OD in the tests would be of approximately 0.01 (0.005 of isolate I1 plus 0.005 of isolate I2).

Control assays were performed as following: co-culture of *M. formicicum* (Mf) and *S. zehnderi* (Sz) incubated in oleate (1 mmol L^{-1}) under anaerobic (An-Sz + Mf) and microaerophilic conditions (Mic-Sz + Mf); *M. formicicum* (Mf) and *Pseudomonas* isolates I1 + I2 incubated in oleate (1 mmol L^{-1}) under anaerobic and microaerophilic conditions (An-Mf + I1 + I2 and Mic-Mf + I1 + I2); *M. formicicum* (Mf) incubated in H_2/CO_2 (80:20% vol/vol, 170 kPa) under anaerobic and microaerophilic conditions (An-Mf and Mic-Mf). The tests with the culture composed by *M. formicicum* and *Pseudomonas* (An-Mf + I1 + I2 and Mic- Mf + I1 + I2) followed the previously described Steps 1, 2 and 5. The controls with only *M. formicicum* (Mf) were pre-grown in H_2/CO_2 (80:20% vol/vol, 170 kPa) until reaching the exponential phase and then the headspace of the bottles was flushed four times under sterile conditions with H_2/CO_2 (80:20% vol/vol, 170 kPa) to remove the remaining hydrogen and the methane produced. In Mic-Mf test, air was added to the bottles to a final concentration of 2% (vol/vol) of O_2. Cumulative methane production was monitored during the assays.

Viability of the *Pseudomonas* isolates in the batch bottles was tested periodically by inoculation in 2% agar plates containing basal bicarbonate-buffered medium (without reducing agent) (Stams *et al*., 1993) and 1 mmol L^{-1} of sodium oleate (≥99%, Sigma-Aldrich, St. Louis, MO, USA) according to Duarte *et al*. (2018).

Analytical methods

Quantification of LCFA was carried out by gas chromatography (GC). Free LCFA from C12 to C18 were esterified with HCl:1-propanol at 100°C for 3.5 h. Extraction was carried out with dichloromethane, and the quantification was performed by GC-FID, as described by Neves *et al*. (2009). LCFA were separated using a Teknokroma TRB-WAX column (30 m × 0.25 mm × 0.25 μm) with helium as the carrier gas at 1 ml min^{-1}. Temperatures of the injector and detector were 220°C and 250°C

respectively. The initial oven temperature was 50°C, maintained for 2 min, followed by a 10°C min^{-1} ramp up to 225°C and finally isothermal conditions were maintained for 10 min. VFAs were analysed by high-performance liquid chromatography (Jasco, Tokyo, Japan), equipped with an Agilent Hi-Plex H column (300 mm × 7.7 mm), which was maintained at 60°C and with UV detection at 210 nm. Sulfuric acid was used as the mobile phase in a concentration of 5 mmol L^{-1} at 0.6 ml min^{-1}, and crotonic acid (1 g L^{-1}) was used as internal standard.

Methane content of the biogas was analysed by GC-2014 Shimadzu ATF (Kyoto, Japan), with a Porapak Q (80–100 mesh) (2 m × 3.75 mm) column and a flame ionization detector. Nitrogen was used as the carrier gas (30 ml min^{-1}). The detector, injector and oven temperatures were 35°C, 110°C and 220°C respectively. The gas sample had a volume of 0.5 ml. Hydrogen was quantified using a GC Bruker Scion 456 (Billerica, MA, USA) equipped with a thermal conductivity detector and a Molsieve packed column (13×, 80/100 mesh, 2 m of length, 2.1 mm of internal diameter). Argon was the carrier gas (30 ml min^{-1}), and the temperatures of the injector, column and detector were 100°C, 35°C and 130°C respectively.

ORP was measured using a multi-parameter analyser C533 (Consort, Turnhout, Belgium) equipped with an ORP electrode D 223 (VWR, Radnor, PA, USA). pH was measured with a Hanna (Woonsocket, RI, USA) pH meter.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Multiple and flexible roles of facultative bacteria in microaerophilic oleate degradation.