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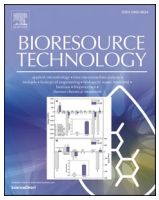

Facultative anaerobic bacteria enable syntrophic fatty acids degradation under micro-aerobic conditions

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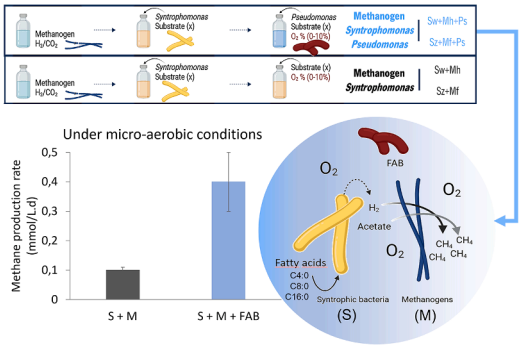
HIGHLIGHTS

- Low oxygen concentrations (0.5%) inhibit the activity of syntrophic co-cultures.
- With *Pseudomonas*, syntrophic cultures converted fatty acids to CH_4, at O_2 up to 2%.
- *Pseudomonas* spp. play a crucial role in mitigating oxygen toxicity.
- Facultative anaerobes (FAB) enable resilient and functional syntrophic consortia.
- FAB's activity is more relevant for less easy-to-degrade substrates.

GRAPHICAL ABSTRACT



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ABSTRACT

Trace amounts of oxygen stimulate facultative anaerobic bacteria (FAB) within anaerobic bioreactors, which was shown to correlate with enhanced methane production from long-chain fatty acids. The relationship between FAB and fatty acid-degrading syntrophic communities under micro-aerobic conditions is still unclear. In this work, two syntrophic co-cultures, *Syntrophomonas wolfei* + *Methanospirillum hungatei* and *Syntrophomonas zehnderi* + *Methanobacterium formicicum*, were assembled and incubated with short, medium and long-chain fatty acids, with 0–10 % O_2, in the presence and absence of FAB, here represented by *Pseudomonas* spp. Without *Pseudomonas*, the syntrophic activity was inhibited by 79 % at 0.5 % O_2, but with *Pseudomonas*, the syntrophic co-cultures successfully converted the fatty acids to methane with up to 2 % O_2. These findings underscore the pivotal role of FAB in the protection of syntrophic fatty acid-degrading communities under micro-aerobic

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conditions and emphasizes its significance in real-scale anaerobic digesters where strictly anaerobic conditions may not consistently be maintained.

1. Introduction

Energy is an indispensable life requirement. In the search for alternatives to fossil fuels, anaerobic digestion (AD) is seen today as a sustainable technology for methane-production, leveraging the valorization of organic waste and wastewater (European Commission, 2022; European Biogas Association, 2024). In recent years, the controlled addition of vestigial amounts of oxygen (micro-aeration) has been applied as a strategy to improve AD processes. This approach has demonstrated multiple advantages, including H_2S removal, increased hydrolysis, prevention of volatile fatty acids (VFA) accumulation and enhanced methane production (Johansen and Bakke, 2006; Jeníček et al., 2017; Nguyen et al., 2019).

Although oxygen is considered harmful to strict anaerobic microorganisms, studies have shown that some methanogens can deploy different mechanisms to withstand oxygen exposure. These include the synthesis of enzymes capable of scavenging reactive oxygen species (ROS), strategies to minimize ROS formation, and inherent self-repair mechanisms (Kaster et al., 2011; Khademian and Imlay, 2021). Therefore, the tolerance levels to oxygen will vary among different microorganisms (Lu and Imlay, 2021).

Oxygen stimulates the growth of facultative anaerobic bacteria (FAB). FAB are associated with higher hydrolysis efficiency and maintenance of the reduced redox environment required for effective methanogenesis (Zitomer and Shrout, 1998; Duarte et al., 2018; Nguyen and Khanal, 2018). Moreover, in anaerobic granular sludge, FAB are normally present in the external layers of the granules, allowing the protection of the methanogens, which are generally located in the inner layers of these aggregates (Nguyen and Khanal, 2018). A different role for FAB in the AD of lipids was reported by Duarte et al. (2018), which demonstrated that the conversion of oleate (C18 unsaturated long chain fatty acid, LCFA) to methane was facilitated in the presence of small amounts of oxygen. Micro-aerobic reactors presented higher accumulation of palmitate (C16 saturated LCFA), which is less toxic and easier to degrade than oleate, resulting in faster conversion to methane. In addition, it has been shown that bacteria of the *Pseudomonas* genus were statistically correlated with the palmitate that accumulated in the system (Duarte et al., 2018), and that these bacteria were also able to scavenges hydrogen/formate, possibly acting as syntrophic partners of the oleate-degrading organisms, thereby accelerating syntrophic oleate conversion to methane (Duarte et al. 2020).

Although the potential of FAB to enhance methane production from LCFA has been shown in engineering applications, current understanding of the microbial interactions between FAB and LCFA-degrading syntrophic communities, comprising fatty acid-degrading bacteria and methanogens, is limited. The high complexity of these engineered systems makes it difficult to clearly understand and prove this role. Therefore, in this work, a model system was assembled, that was as simple as possible (i.e., batch and with very specific co-cultures), but would still allow to deepen this knowledge. Two synthetic microbial consortia were assembled, simulating natural syntrophic communities involved in the degradation of fatty-acids. The objective was to investigate the impact of FAB, here represented by *Pseudomonas* spp., on the syntrophic degradation of different fatty acids under micro-aerobic conditions. This research endeavors to expand current knowledge about the complex microbial interactions involved in these processes.

2. Materials and methods

2.1. Microbial strains and routine cultivation

Methanospirillum hungatei DSM 864^T (Mh) and *Methanobacterium formicicum* DSM 1535^T (Mf) were grown in mineral salt medium (basal medium, BM) supplemented with 3 g/L bicarbonate, 0.3 g/L acetate, 1 mmoL/L $Na_2S{\cdot}9H_2O$ (reducing agent), salts, and vitamins (prepared as described by Stams et al., 1993), in serum bottles sealed with butyl rubber stoppers and aluminum crimp caps. The headspace of the bottles was flushed and pressurized with a mixture of H_2/CO_2 (80:20 % v/v, 1.7 $\times$ 10^5 Pa). Incubations were performed at 37 °C, 100 rpm and in the dark.

Syntrophomonas wolfei subsp. *wolfei* DSM 102351^T (Sw) and *Syntrophomonas zehnderi* DSM 17840^T (Sz) were pre-grown in co-culture with methanogens (Mh or Mf, respectively), following the procedure described by Duarte et al. (2020). These syntrophic partnerships are well stablished, and the methanogenic partner chosen was the same used for the isolations of the syntrophic bacteria in order to perform the experiments with active and stable syntrophic co-cultures (McInerney et al., 1979; Sousa et al., 2007).

In routine cultivation and in the experiments, unless otherwise started, sodium salts of butyrate (C4:0, 20 mmol/L) and caprylate (C8:0, 2.5 mmol/L) were added as substrate to the Sw + Mh cultures; caprylate (C8:0, 2 mmol/L) and palmitate (C16:0, 1 mmol/L) were added as substrate to the Sz + Mf cultures. Incubations were made at 37 °C, with no agitation, in the dark.

Pseudomonas spp. isolates I1 and I2 were previously isolated with oleate as substrate (Duarte et al., 2018). These isolates are affiliated within the *Pseudomonas oleovorans* and *Stutzerimonas stutzeri* (formerly *Pseudomonas stutzeri* (Gomila et al., 2022)) groups. *Pseudomonas* spp. I1 and I2 were grown separately in S.O.C. medium (NZYtech, Portugal), for approximately 16 h, at 37 °C and 100 rpm. Then, cells were centrifuged and eluted in BM before inoculation in the assays.

2.2. Syntrophic fatty acids degradation under micro-aerobic conditions

Degradation of the three selected fatty acids (C4:0, C8:0, C16:0, as mentioned in the previous section) was studied in batch assays, under anaerobic (0 % O_2) and micro-aerobic conditions (0.5–10 % O_2). The maximum O_2 concentration of 10 % was chosen considering that microaerophilic metabolism has been reported for members of the *Pseudomonas* genus (Vaz et al., 2016; Planet, 2018; Tribelli et al., 2018), and that microaerophilic microorganisms typically show optimal growth in environments with O_2 concentrations up to 10 % (Prescott et al., 1999). The assays were performed in triplicate, in 120 mL serum bottles containing 55 mL BM supplemented with bicarbonate, salts and vitamins (Stams et al., 1993), following the procedure described by Duarte et al. (2020). Serum bottles were sealed with butyl rubber stoppers and aluminum crimp caps.

The syntrophic co-cultures and a mixture of the two *Pseudomonas* spp. (final OD_{600nm} = 0.01) were used to inoculate the assays, which were designated as Sw + Mh + Ps or Sz + Mf + Ps. In parallel, assays inoculated only with the syntrophic consortia (Sw + Mh or Sz + Mf) were prepared. Additionally, controls were also set-up, either co-inoculated with the methanogens and *Pseudomonas* spp. (Mh + Ps or Mf + Ps), or solely inoculated with the two *Pseudomonas* isolates (Ps). Controls with the pure cultures of methanogens (Mh or Mf) were prepared as well, but using H_2/CO_2 (80:20 % v/v).

The experimental layout is presented in Fig. 1. Briefly, the experiment was divided into three phases (Fig. 1), and at each phase transition,

the headspace of the bottles was flushed under sterile conditions by using a manifold apparatus (Paralab, Porto, Portugal) connected to a vacuum pump and to gas cylinders, which performs alternate cycles of vacuum and gas injection. The final gas composition after flushing was N_2/CO_2 (80:20 % v/v) with 1.7×10^5 Pa final pressure. In the assays in which air/O_2 was added, the pressure to be applied with the manifold apparatus was lower, and was previously calculated for each condition so that, after air/O_2 injection, the final pressure in all the bottles was 1.7×10^5 Pa.

Phase I: growth of the methanogens with H_2/CO_2 (80:20 % v/v, 1.7×10^5 Pa), until the end of the exponential phase of methane production (i.e., methane production reached the theoretical expected – 15 mmol/L, and methane production curve reached the stationary phase, lasting approximately 5 days);

Phase II: inoculation with the syntrophic co-cultures (Mh + Sw or Mf + Sz − 10 % v/v) and addition of the fatty acids. When all conditions reached the end of the exponential phase of methane production, as theoretically predicted (i.e., 10 mmol/L, 3.75 mmol/L and 3.5 mmol/L of methane for C4:0, C8:0 and C16:0, respectively – see Table 1), and methane production curve reached the stationary phase (~10–13 days), phase III was started;

Phase III: inoculation with *Pseudomonas* spp., followed by new substrate addition and supplementation with oxygen (added only once, as previously tested by Duarte et al. (2020), in the beginning of phase III). Micro-aerobic conditions were created with different oxygen concentrations in the bottles' headspace: 0 %, 0.5 %, 1 %, 2 % and 10 % (corresponding to the addition of 0, 11, 21, 44 and 218 mL O_2 per L of working volume, respectively) (see **supplementary materials**). Final pressure in the bottles was 1.7×10^5 Pa. Air (containing 21 % of O_2) was used for 0.5 % − 2 % O_2, and pure oxygen was used for 10 % O_2 (in order to avoid having in this case, bottles in vacuum between phases II and III). Air or O_2 were manually added, at atmospheric pressure (1.013×10^5 Pa) with a gas-tight syringe (SGE™ Luer lock valve, ThermoFisher Scientific, Waltham, MA, USA) through a PES sterile 0.22 μm syringe filter. O_2 addition was done in the headspace to ensure O_2 dissolution due to gas–liquid equilibrium and at the same time avoid damaging the strict anaerobes (Nguyen and Khanal, 2018; Costanzo et al., 2024).

One should bear in mind that, although the relative percentage of O_2 in air remains constant at approximately 21 %, the absolute O_2 concentration may vary with the barometric pressure and temperature (Bugbee and Blonquist, 2006). However, considering that air was added only once, over a short period of time, to all the bottles, the atmospheric pressure and temperature did not vary significantly and thus, no important differences are expected between the different assays. The amount of O_2 from air that was added to the bottles was calculated by applying the ideal gas law, PV = nRT (P is pressure, V is volume, n is the total number of moles, T is temperature, and R is the ideal gas constant), assuming $P = 1.013 \times 10^5$ Pa, T = 21°C and n_{O2} = 21 %.

A higher initial concentration of *Pseudomonas* spp. was also tested in assays with C16:0, to evaluate its effect in the velocity of oxygen

Table 1
Stoichiometry of the reactions involved in syntrophic fatty acids' oxidation coupled to hydrogenotrophic methanogenesis.

N°	Reactant	Equation	$\Delta G^{0'}$ (kJ reaction^{-1})[(a)]
	Fatty acids oxidation to acetate and H_2		
(1)	Butyrate (C4:0)	$Butyrate^- + 2\ H_2O \rightarrow 2\ acetate^- + 2\ H_2 + H^+$	+48
(2)	Caprylate (C8:0)	$Caprylate^- + 6\ H_2O \rightarrow 4\ acetate^- + 6\ H_2 + 3H^+$	+129
(3)	Palmitate (C16:0)	$Palmitate^- + 14\ H_2O \rightarrow 8\ acetate^- + 14\ H_2 + 7H^+$	+353
	Hydrogenotrophic methanogenesis		
(4)	Hydrogen	$H_2 + 0.25HCO_3^- + 0.25H^+ \rightarrow 0.25\ CH_4 + 0.75\ H_2O$	−34
	Syntrophic fatty acids oxidation to acetate and methane		
(1 + 4)	Butyrate (C4:0)	$Butyrate^- + 0.5H_2O + 0.5HCO_3^- \rightarrow 2\ acetate^- + 0.5\ CH_4 + 0.5H^+$	−19
(2 + 4)	Caprylate (C8:0)	$Caprylate^- + 1.5H_2O + 1.5HCO_3^- \rightarrow 4\ acetate^- + 1.5\ CH_4 + 1.5H^+$	−74
(3 + 4)	Palmitate (C16:0)	$Palmitate^- + 3.5H_2O + 3.5HCO_3^- \rightarrow 8\ acetate^- + 3.5\ CH_4 + 3.5H^+$	−121

[(a)] Gibbs free energy changes calculated at standard conditions (solute concentration of 1 mol/L, gas partial pressure of 10^5 Pa, T = 25 °C) and pH = 7.

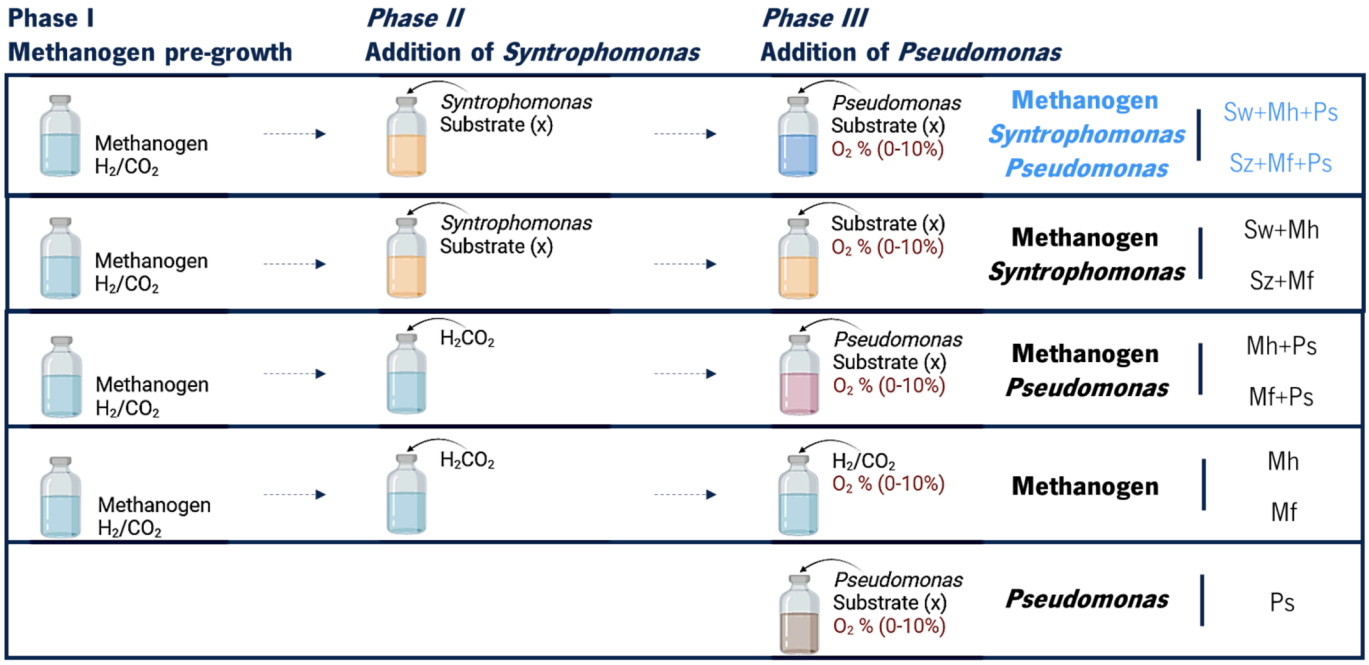

Fig. 1. Experimental layout of the assays.

consumption and potential facilitation of the syntrophic relationships under micro-aerobic conditions. For that, *M. formicicum* was pre-grown and the headspace flushed (as previously described). Syntrophic co-culture (Sz + Mf) (10 % v/v) and *Pseudomonas* spp. cells (OD_{600nm} = 0.1) were added to the methanogen culture. Then 1 mmol/L of C16:0 was supplemented, and oxygen concentrations of 0 %, 0.5 %, 1 % and 2 % were applied.

All inoculations, transfers and manipulations were performed under aseptic conditions. The incubations were made at 37 °C, statically and in the dark. Cumulative methane production was monitored throughout the three phases. Fatty acids were monitored during phases II and III. Additionally, pH and ORP were monitored during phase III. Cultures were checked weekly by phase contrast microscopy. *Pseudomonas* spp. viability in the constructed tri-cultures was verified at the end of the assays by streaking on oleate (0.5 mmol/L) agar plates. Oxygen consumption was monitored during phase III in the cultures supplemented with palmitate.

2.3. Second incubation of the grown tri-cultures

The tri-cultures that were able to grow with C8:0 and C16:0 at phase III were transferred (10 % v/v) to a pre-grown culture of the methanogen (after headspace flushing with N_2/CO_2) and supplemented with the same substrate in which they grew before. O_2 concentrations ranging from 0-2 % were applied (see **supplementary material**). Assays were performed in triplicate. The bottles were incubated at 37 °C, statically and in the dark. Cumulative methane production and fatty acids were monitored over time.

2.4. Aerobic degradation of fatty acids by Pseudomonas isolates I1 and I2

The *Pseudomonas* isolates were tested separately for their capacity to degrade the different fatty acids under aerobic conditions (21 % O_2). Each isolate was incubated in 250 mL Erlenmeyer flasks containing 100 mL BM, supplemented with 3 g/L bicarbonate, salts, and vitamins (Stams et al., 1993). *Pseudomonas* isolates were pre-grown as previously described (in S.O.C. medium) and were added to the medium with an initial OD_{600nm} of 0.005. Inoculations and transfers were performed under sterile conditions. Assays were performed in triplicate. Incubations took place at 37 °C and 100 rpm. Substrate consumption, VFA formation and culture growth (OD_{600nm}) were followed over time.

2.5. Analytical methods

Gas samples (0.5 mL) were withdrawn from the bottles with a gas-tight syringe. Methane quantification was performed in a gas chromatograph GC 2014 (Shimadzu, Kyoto, Japan) with a flame ionization detector and a Porapak Q (80–100 mesh) (2 m × 3.75 mm) column. The injector and detector temperatures were 35 °C and 220 °C, respectively. The nitrogen flow was set at 30 mL/min. Oxygen and hydrogen quantification was performed with a GC chromatograph Bruker Scion 456 with a MolSieve column (MS-13x 80/100 mesh) and a thermal conductivity detector (Bruker, Billerica, MA, USA) with argon (60 mL/min) as the carrier gas. The injector, column and detector temperatures were 100 °C, 35 °C and 130 °C respectively.

VFA (C2 to C5) were determined by HPLC (Jasco, Tokyo, Japan) with an Aminex HPX-87 column and a UV-2075 detector at λ = 210 nm (Jasco, Tokyo, Japan). The column temperature was kept at 60 °C. The mobile phase was 5 mmol/L sulfuric acid (HPLC grade) at 0.6 mL/min. The samples were centrifuged (10 min, 10.000 *g*) and filtered with a 0.22 µm microfilter before HPLC analysis. Crotonic acid was used as internal standard. Quantification of medium- and long-chain fatty acids was carried out by gas chromatography (GC). Free fatty acids from C6:0 to C18:0 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). Medium chain and long chain fatty acids (MCFA and 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. The injector and detector temperatures were 220 °C and 250 °C, respectively. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °C/min ramp up to 225 °C and finally isothermal conditions were maintained for 10 min.

pH and ORP were monitored using multiparameter analyzers (Consort, Turnhout, Belgium). Additional procedures were adopted to minimize O_2 diffusion to the medium in each sampling withdrawal, *e.g.*, all the used material was previously flushed with sterile N_2 gas. Optical density (OD_{600nm}) was measured by a DR 2800 spectrophotometer (Hach-Lange GmbH, Dusseldorf, Germany). Phase contrast microscopy was performed using an Olympus CX41 RF microscope, and micrographs were obtained with an Olympus Altra 20 microscope camera and the software AnalySIS getIT (Olympus soft imaging solutions GmbH, Münster, Germany).

2.6. Data analysis

The experimental methane production data were fitted by the modified Gompertz equation (**Equation (1)** (Zwietering et al., 1990). For that, the results from the three replicates were combined into a single dataset and treated independently, to provide a more robust estimation of the parameters and reduce the potential impact of variability in individual replicates.

$$M(t) = P \times \exp\left\{ -\exp\left[\frac{MPR \times e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

M (t) represents the cumulative methane production (mmol/L), P is the maximum cumulative methane production (mmol/L), MPR corresponds to the methane production rate (mmol/L d^{-1}), e = 2.7182818, λ is the duration of the lag phase (d). The standard error for each variable and the coefficient of determination (R^2) were calculated.

The statistical significance of the differences ($p < 0.05$) observed in the MPR results was evaluated using an easy-to-use statistical software package available online (MedCalc Software Ltd, 2024). For each consortium and substrate, MPR obtained under different O_2 concentrations were compared with the control (0 % O_2). MPR in the presence and absence of *Pseudomonas* spp. were compared as well, for the same consortium/substrate/O_2 concentration.

The MPR of the assays with increasing O_2 concentrations (MPR_{O2}) were compared to the average value from the anaerobic assays ($MPR_{0\%O2}$), and the inhibitory effect (%) of O_2 was calculated **Equation (2)**.

$$Inhibition(\%) = \frac{MPR_{0\%O2} - MPR_{O2}}{MPR_{0\%O2}} \times 100 \quad (2)$$

3. Results

Initially, C4:0 degradation by *S. wolfei* was studied, in the presence of 0 %, 2 % and 10 % oxygen. Based on the results obtained (Fig. 2), 10 % O_2 was considered too high since methanogenesis inhibition occurred even in the presence of *Pseudomonas* spp., and therefore the experiments with C8:0 were performed with 0 %, 1 % and 2 % O_2, for both *S. wolfei* + *M. hungatei* and *S. zehnderi* + *M. formicicum* consortia. In the assay with C16:0 (*S. zehnderi* + *M. formicicum*), a new condition with 0.5 % O_2 was included. All this information is presented in **Supplementary Materials**. Given that phases I and II served as preparatory stages for phase III, and that the results obtained under the various conditions in these preliminary phases were highly similar (good replicates), only the results from phase III will be presented.

Cumulative methane production obtained in the experiments with the consortia Sw + Mh and Sz + Mf is shown in Fig. 2 and Fig. 3, respectively. Regardless of the consortia involved and of the substrate tested, the tri-cultures, which include *Pseudomonas* spp., exhibited

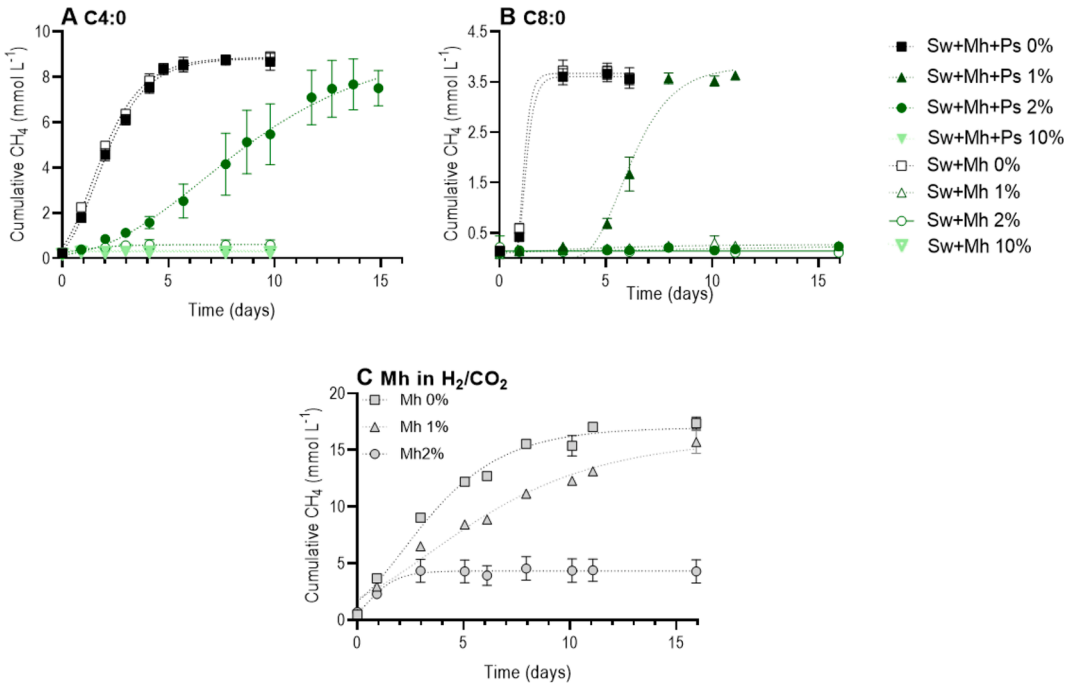

Fig. 2. Effect of micro-aerobic conditions in the cumulative methane production from C4:0 (**A**) and C8:0 (**B**) by the tri-culture Sw + Mh + Ps (closed symbols) versus the co-culture Sw + Mh (open symbols). Squares 0 % O_2, up-triangles 1 % O_2, circles 2 % O_2, down-triangles 10 % O_2. Control assays performed with pure cultures of *M. hungatei* (Mh) in H_2/CO_2 and different O_2 concentrations are also shown (**C**). Each data point is the average of triplicates ± standard deviation. The lines show the fitting of the modified Gompertz equation to the data.

visible growth and consistently produced methane under micro-aerobic conditions (except for 10 % O2) (Figs. 2 and 3). With 2 % O_2, methane production in the tri-culture Sw + Mh + Ps started only after 15 days, and was highly variable among replicates (data not shown). Conversely, without *Pseudomonas* spp., the Sw + Mh co-culture failed to produce methane under the tested micro-aerobic conditions (Fig. 2); for the Sz + Mf co-culture, minimal methanogenic activity was observed at 1 % O2 in C8:0 (Fig. 3-**A**), thriving only under 0.5 % O2 in C16:0 (Fig. 3-**B**). With the increase in oxygen concentrations, MPR tended to decrease (Table 2), and the inhibition of the MPR was consistently higher for the syntrophic co-cultures than for the tri-cultures (Fig. 2, Fig. 3, Table 2). At 0 % O_2, the tri-culture and the co-culture exhibited identical behavior, across all substrates and consortia (Fig. 2 and Fig. 3, Table 2). In addition to methane, acetate was produced from the degradation of all fatty acids (see **supplementary material**), with no other products being detected.

Pure cultures of methanogens grown with H_2/CO_2 exhibited sensitivity to oxygen (Fig. 2-**C,** Fig. 3-**C,** Table 2). However, Mh cultivated at 1 % O_2 and Mf at 0.5 % O_2 were able to utilize the H_2/CO_2 present in the headspace, showing their ability to overcome oxidative challenges. Still, this was done at a lower MPR than in the assays without oxygen (0 % O_2) (Table 2). When exposed to 1 % O_2, Mh was able to produce more methane than Mf (Fig. 2-**C** and Fig. 3-**C**), suggesting its greater resistance to O_2 toxicity.

When higher initial number of *Pseudomonas* cells (10x more, i.e., OD = 0.1) was tested in the tricultures with palmitate, no important differences were observed in methane or acetate production (Magalhães, 2023), showing that the abundance of *Pseudomonas* spp. cells was not a limiting factor under the conditions studied.

Each culture was frequently checked by microscopic observations. The activity of the *Pseudomonas* spp. in the constructed tri-cultures was confirmed at the end of the assays since they could form colonies when incubated aerobically in agar plates.

No direct relationship was established between methanogens and *Pseudomonas* isolates (Mh + Ps or Mf + Ps), since no methane or other compounds were detected in these conditions.

The two *Pseudomonas* isolates could consume all the tested substrates under aerobic conditions, but it was not possible to validate if they also did it in micro-aerobic conditions, since substrate consumption was not evident (see **supplementary material**). This was probably due to the relatively low oxygen concentrations added – *e.g.*, only 0.12 and 0.24 mmol/L of caprylate could have been degraded using 1 % and 2 % O_2, respectively; for palmitate, only 0.06 and 0.12 mmol/L could have been degraded with 1 % and 2 % O_2, respectively (**Equations 3–5**).

$$C_4H_8O_2 + 5\ O_2 \rightarrow 4\ CO_2 + 4\ H_2O \quad (3)$$

$$C_8H_{16}O_2 + 11\ O_2 \rightarrow 8\ CO_2 + 8\ H_2O \quad (4)$$

$$C_{16}H_{32}O_2 + 23\ O_2 \rightarrow 16\ CO_2 + 16\ H_2O \quad (5)$$

Moreover, it is also not possible to confirm whether the *Pseudomonas* spp. utilized the added oxygen to consume acetate or H_2, which might have been produced through the syntrophic degradation of the fatty acids in the assays with the tri-cultures, as previously proposed by Duarte et al. (2020). To assess the potential occurrence of aerobic metabolism in the assays, oxygen concentrations were measured over time in the bottles supplemented with C16:0 (Table 3). After 3 days of incubation, oxygen was completely consumed by the tri-culture Sz + Mf + Ps and by the Mf + Ps control, at all tested concentrations, while oxygen concentrations close to the initial values were detected in the assays with Sz + Mf.

Throughout the experiment, pH values remained stable, ranging between 6.9 and 7.1 across all tested conditions. Increasing oxygen concentrations had a direct effect on ORP (Magalhães, 2023), leading to slightly higher values with the addition of more oxygen to the bottles at t_0, as expected. For all the conditions and O_2 concentrations tested, except for the control assays inoculated solely with *Pseudomonas* spp. (Ps assays), ORP values at t_0 ranged between −343 mV and −225 mV, with an average value of −265 ± 24 mV. Ps assays generally displayed higher ORP values (averaging −137 ± 38 mV) (Magalhães, 2023). Over the course of the experiments, ORP values tended to increase, this being more pronounced in the assays involving syntrophic co-cultures (ΔORP

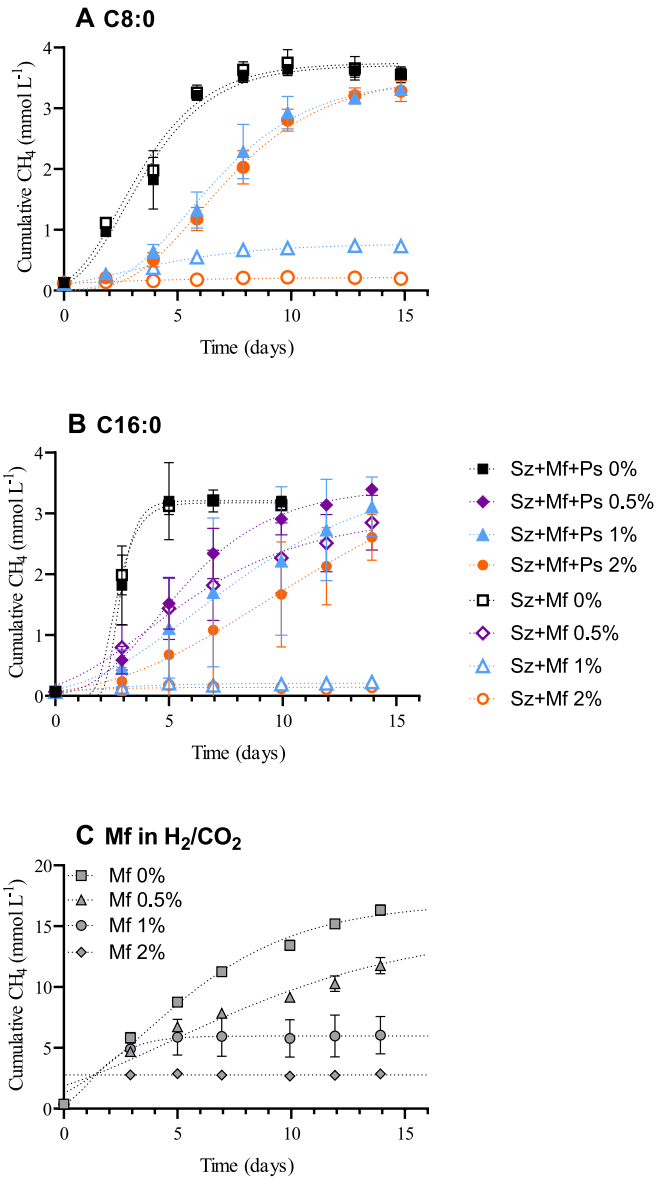

Fig. 3. Effect of micro-aerobic conditions in the cumulative methane production from C8:0 (**A**) and C16:0 (**B**) by the tri-culture Sz + Mf + Ps (closed symbols) versus the co-culture Sz + Mf (open symbols). Squares 0 % O_2, diamonds 0.5 % O_2, up-triangles 1 % O_2, circles 2 % O_2. Control assays performed with pure cultures of *M. formicicum* (Mf) in H_2/CO_2 and different O_2 concentrations are also shown (**C**). Each data point is the average of triplicates ± standard deviation. The lines show the fitting of the modified Gompertz equation to the data.

= 12–191 mV) than those with tri-cultures (ΔORP = −26 to 39 mV) (Magalhães, 2023).

A second incubation of the tri-cultures thriving in Phase III was performed (see **supplementary material**). Almost all the cultures effectively utilized the added fatty acids, resulting in close-to-stoichiometric acetate and methane production (see **supplementary material**), with the exception of culture Sw + Mh + Ps in C8:0 that, having been previously incubated with 0 % and 1 % O_2, did not produced methane upon transfer to 2 % O_2 (see **supplementary material**). During this second incubation, increasing oxygen concentrations led again to increased inhibition of MPR (see **supplementary material**).

4. Discussion

Syntrophic fatty acid-degrading bacteria are generally detected in low percentages in AD communities (Sousa et al., 2009), but are responsible for an extremely important step of AD. These bacteria work in syntrophy with methanogens, to break down intermediary products, such as fatty acids, into acetate, hydrogen (H_2) or formate, and carbon dioxide (CO_2) (Agne et al., 2021). Potential interactions between these syntrophic consortia and FAB under the influence of small amounts of oxygen, are still unknown.

Methanogens and other so called obligate anaerobes were first thought to be deprived of strategies to deal with oxidative stress (Khademian and Imlay, 2021). This idea has been surpassed, and the results here obtained in the controls Mh or Mf, as well as in the co-culture assays (Sw + Mh or Sf + Mf), corroborate that. Several enzymes are known to scavenge oxidative radicals and control oxidative stress in these organisms (Lu and Imlay, 2021). Among those enzymes, it has been proposed that the presence of one or both superoxide dismutase (SOD) and catalase (KAT) activities determines whether an anaerobe is aerotolerant (Pedone et al., 2004). The genomes of the two methanogens and two *Syntrophomonas* species studied in this work were analyzed using the National Center for Biotechnology Information (NCBI) genomic platform (https://www.ncbi.nlm.nih.gov/). This analysis allowed to confirm that the methanogens possess genes encoding both SOD and KAT enzymes, while the studied *Syntrophomonas* species contain genes only for SOD enzyme production. Besides SOD and KAT, other enzymes known to scavenge oxidative radicals and control oxidative stress can be synthesized by these microorganisms (Magalhães, 2023). For example, both Mf and Sz have encoded in their genomes the ability to synthesize superoxide reductase (SOR), peroxidase (PX), rubrerythrin (RBR), type A flavoprotein (Fpr A, $F_{420}H_2$ oxidase), thioredoxin (Trx), thioredoxin reductase (TrxR) and peroxiredoxin (Prx). Additionally, chemical compounds such as sulfide, cysteine or glutathione are also able to scavenge oxidative radicals.

Nevertheless, despite this potential, in this work micro-aerobic conditions influenced the dynamics of methane production in the tested cultures (Fig. 2, Fig. 3 and Table 2). Even low concentrations of oxygen (0.5 % O_2) proved to be critical for the activity of syntrophic co-cultures (Sw + Mh or Sz + Mf), leading to MPR inhibition always higher than 79 % under oxygen exposure. In the presence of *Pseudomonas* spp. (Sw + Mh + Ps or Sz + Mf + Ps), inhibition still occurred but it was always lower (Table 2). For example, MPR inhibition of 29–30 % was calculated for Sz + Mf + Ps with C8:0 and 1–2 % O2, while these values reached 91 % and 100 % for this co-culture in the absence of the *Pseudomonas* (Sz + Mf). In general, cultures containing *Pseudomonas* spp. exhibited higher MPR (Table 2), faster oxygen removal (Table 3), and more stable ORP, demonstrating that *Pseudomonas* spp. play a crucial role in mitigating oxygen toxicity. Faster oxygen removal decreases the risk of permanent cell damage by the accumulation of reactive oxygen species (ROS). In addition, *Pseudomonas* can also scavenge oxidative radicals and regulate oxidative stress, maintaining the reduced conditions required for the survival of strict anaerobes, assuring internal cell redox stability (Banerjee, 2012). On the other hand, cultures without the presence of *Pseudomonas* spp. have higher ORP values, suggesting unbalancing cellular redox state and further inhibition. Therefore, *Pseudomonas* spp. act as a protective shield (Botheju and Bakke, 2011), providing protection and safeguarding the individual cells or the syntrophic consortium from the potential damages resulting from the presence of O_2 or ROS. This study also shows that high numbers of *Pseudomonas* cells are not necessary to achieve this shield effect over syntrophic activity. Moreover, in a second incubation this effect was maintained (see **supplementary material**), highlighting its relevance for the consortia's performance under repeated contact with micro-aerobic conditions.

The data collected in this work reinforce that the substrate used and the microbial consortia composition are crucial factors that influence

Table 2
Methane production parameters, determined by fitting the modified Gompertz equation to the data, for all cultures and conditions. Inhibition (%) of MPR in the assays with oxygen relative to the anaerobic assays (0 % O_2) is also shown.

	Culture	Substrate	O_2 (%)	P (mmol/L)	MPR (mmol/L d^{-1})	λ (d)	R^2	Inhibition (%)
Experiments with the Sw + Mh consortium			**0**	8.8 ± 0.2	2.5 ± 0.1	0.2 ± 0.0	0.99	–
	Sw + Mh + Ps	**C4:0**	**2**	8.8 ± 0.1	0.9 ± 0.2 [(a)]	3.2 ± 0.1	0.99	74
			10	–	–	–	–	100
	Sw + Mh		**0**	8.9 ± 0.1	2.5 ± 0.3	0.3 ± 0.2	1.00	–
		C4:0	**2**	–	–	–	–	100
			10	–	–	–	–	100
	Sw + Mh + Ps		**0**	3.7 ± 0.1	3.0 ± 0.2	0.9 ± 0.0	0.99	–
		C8:0	**1**	3.6 ± 0.0	0.9 ± 0.0 [(a)]	4.7 ± 0.2	0.96	70
			2	–	–	–	–	–
	Sw + Mh		**0**	3.7 ± 0.2	3.0 ± 0.3	0.8 ± 0.0	0.99	–
		C8:0	**1**	–	–	–	–	100
			2	–	–	–	–	100
	Mh	**H_2/CO_2**	**0**	16.7 ± 0.3	2.7 ± 0.0	0.0 ± 0.0	0.98	–
			1	15.5 ± 0.4	1.3 ± 0.0	0.0 ± 0.0	0.97	53
			2	5.0 ± 0.8	*	*	*	*
			10	–	–	–	–	–
Experiments with the Sz + Mf consortium	**Sz + Mf + Ps**		**0**	3.9 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.97	–
		C8:0	**1**	3.5 ± 0.1	0.4 ± 0.1 [(b)]	2.5 ± 0.3	0.97	29
			2	3.5 ± 0.2	0.4 ± 0.0 [(a)]	3.0 ± 0.3	0.98	30
	Sz + Mf		**0**	4.0 ± 0.1	0.6 ± 0.0	0.3 ± 0.0	0.98	–
		C8:0	**1**	0.7 ± 0.0	0.1 ± 0.0 [(a),(b)]	0.0 ± 0.0	0.98	91
			2	–	–	–	–	100
	Sz + Mf + Ps	**C16:0**	**0**	3.2 ± 0.1	1.3 ± 0.4	1.3 ± 0.2	0.92	–
			0.5	3.5 ± 0.0	0.4 ± 0.1 [(a)]	1.5 ± 0.2	0.97	67
			1	3.4 ± 0.0	0.5 ± 0.0 [(a)]	1.6 ± 0.1	0.99	64
			2	3.2 ± 0.2	0.3 ± 0.0 [(a)]	5.9 ± 0.0	0.99	69
	Sz + Mf	**C16:0**	**0**	3.2 ± 0.1	1.3 ± 0.4	1.3 ± 0.3	0.99	–
			0.5	3.1 ± 0.2	0.3 ± 0.1 [(a)]	1.3 ± 0.3	0.99	79
			1	–	–	–	–	100
			2	–	–	–	–	100
	Mf	**H_2/CO_2**	**0**	16.9 ± 0.2	1.7 ± 0.0	1.0 ± 0.0	0.99	–
			0.5	14.4 ± 0.8	1.1 ± 0.1	1.1 ± 0.1	0.95	38
			1	5.9 ± 1.3	*	*	*	*
			2	2.8 ± 0.3	*	*	*	*

* Cumulative methane production stopped after 2–5 days. [(a)] Statistically significant differences with $p < 0.05$, compared to the corresponding control assays (0% O_2). [(b)] Statistically significant difference with $p < 0.05$, between the tri-culture and co-culture assays.

Table 3
Oxygen concentration measured in the headspace of the bottles, in the assays supplemented with C16:0, at the beginning of the assay and after 3 days of incubation. The data are the average of triplicates ± standard deviation.

	O_2 (%)	O_2 concentration measured (mmol/L) [(a)]	
		t = 0 days	t = 3 days
Sz + Mf + Ps	0	0.00 ± 0.00	0.00 ± 0.00
	0.5	0.35 ± 0.05	0.00 ± 0.03
	1.0	0.73 ± 0.06	0.00 ± 0.01
	2.0	1.39 ± 0.08	0.00 ± 0.02
Sz + Mf	0	0.00 ± 0.00	0.00 ± 0.00
	0.5	0.33 ± 0.02	0.18 ± 0.02
	1.0	0.52 ± 0.03	0.53 ± 0.02
	2.0	1.26 ± 0.11	1.25 ± 0.03
Mf + Ps	0	0.00 ± 0.00	0.00 ± 0.00
	0.5	0.31 ± 0.08	0.00 ± 0.04
	1.0	0.69 ± 0.05	0.00 ± 0.04
	2.0	1.36 ± 0.05	0.00 ± 0.08
Ps	0	0.00 ± 0.00	0.00 ± 0.00
	0.5	0.12 ± 0.03	0.03 ± 0.04
	1.0	0.49 ± 0.04	0.07 ± 0.03
	2.0	0.99 ± 0.07	0.26 ± 0.06

[(a)] Expressed relative to the volume of headspace.

this approach (Song et al., 2021). In fact, cultures Sz + Mf + Ps and Sz + Mf showed lower resistance to oxygen toxicity with C16:0 than with C8:0 (Table 2), pointing that the substrate may be acting as an extra inhibitory factor, in this case, most probably due to the different chain length. LCFA have been reported as toxic/inhibitory to anaerobic microbial communities, affecting mostly Gram-positive bacteria and methanogens (Holohan et al., 2022). This toxic effect increases with the carbon chain length, which is associated to the increase in hydrophobicity of the molecule, affecting membrane integrity, fluidity and permeability (Holohan et al., 2022). As such, possible membrane damage caused by palmitate may potentiate the toxic effect of oxygen or reactive oxygen species.

Additionally, each syntrophic consortia studied in this work exhibit different sensibility to oxygen – *e.g.*, with C8:0, at 1 % O_2, cultures Sz + Mf + Ps (29 % inhibition) or Sz + Mf (91 % inhibition) show lower inhibition percentages than Sw + Mh + Ps (70 % inhibition) and Sw + Mh (100 % inhibition) (Table 2). Moreover, longer lag phases preceded the onset of methane production by the tri-culture Sw + Mh + Ps (i.e., 4.7 days and > 15 days with 1 % and 2 % O_2, respectively, Table 2) than by Sz + Mf + Ps (lag phases of 2.5 and 3.0 days with 1 % and 2 % O_2). This is interesting because the higher resilience of Mh pure cultures (53 % inhibition at 1 % O_2), relatively to Mf (poor methane production), could suggest the opposite. Therefore, it seems that *S. zehnderi*, or the syntrophic collaboration between *S. zehnderi* and *M. formicium* (Sz + Mf), is more efficient in overcoming oxygen toxicity (showing 91 % inhibition under 1 % O_2) than *S. wolfei* (Sw + Mh, 100 % inhibition).

Duarte et al. (2018, 2020) showed that *Pseudomonas* spp. facilitated the conversion of oleate (C18:1) to methane under micro-aerobic conditions (2) % O_2 v/v, added one time in the headspace), attaining higher MPR than under strict anaerobic conditions. Here, MPR did not improve under micro-aerobic conditions, relatively to the anaerobic assays. This is probably related to the fact that the substrates tested are easier to degrade and less toxic than oleate. Moreover, the cultures studied are known to be well able of degrading the studied substrates. This suggests that micro-aeration, and the role of FAB, are probably more crucial and

noticeable in the degradation of substrates which are more difficult to degrade. Even so, *Pseudomonas* spp. proved essential for the stability of syntrophic activity under micro-aerobic conditions.

The comprehensive understanding of how micro-aeration influences the various potential functions of *Pseudomonas* spp. (or FAB) in AD systems, and their relation with the substrate being degraded is essential to further enhance and optimize methane yields in full-scale anaerobic digesters. Additionally, the role of micro-aeration in promoting the co-occurrence of aerobic and anaerobic microbial respiration at both individual and community levels, and the biochemical mechanisms that regulate the switch between aerobic/anaerobic respiration, must be uncover. Furthermore, the long-term effects of micro-aeration on the adaptation of strict anaerobes and its role in shaping synergistic interactions between FAB, anaerobic bacteria and methanogens is essential.

5. Conclusions

Pseudomonas spp. are crucial in syntrophic fatty acids degradation to methane under micro-aerobic conditions, by protecting microbial consortia from potential oxygen damage. This protective shield effect was maintained during repeated micro-aerobic exposure. Micro-aerobic conditions did not improve the MPR, when compared to strict anaerobic conditions, probably due to the easy degradation of the substrates tested. Therefore, micro-aeration, and the role of FAB, seem to be more important in the degradation of difficult substrates. Nonetheless, considering that most large-scale AD plants operate under non-strict anaerobic conditions, *Pseudomonas* spp. have an essential role in maintaining stable, resilient and functional syntrophic communities.

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CRediT authorship contribution statement

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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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131829.

Data availability

No data was used for the research described in the article.

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