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Short communication

Influence of cysteine, serine, sulfate, and sulfide on anaerobic conversion of unsaturated long-chain fatty acid, oleate, to methane

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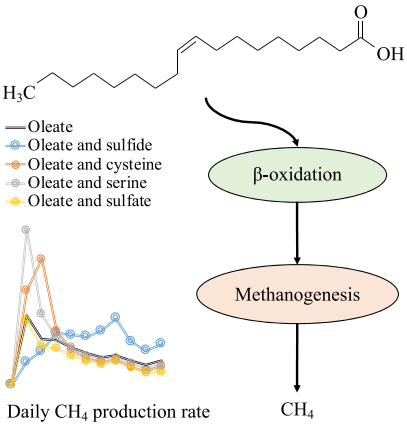
HIGHLIGHTS

- Cysteine, serine, and sulfide accelerate conversion rate of oleate to methane.
- Microbial community alters differently upon cysteine, serine, or sulfide amendments.
- Sulfide has the potential to abiotically react with unsaturated bonds of LCFA.
- Sulfide toxicity can be mitigated in the presence of unsaturated LCFA.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to elucidate the role of sulfide and its precursors in anaerobic digestion (i.e., cysteine, representing sulfur-containing amino acids, and sulfate) on microbial oleate conversion to methane. Serine, with a similar structure to cysteine but with a hydroxyl group instead of a thiol, was included as a control to assess potential effects on methane formation that were not related to sulfur functionalities. The results showed that copresence of sulfide and oleate in anaerobic batch assays accelerated the methane formation compared to assays with only oleate and mitigated negative effect on methane formation caused by increased sulfide level. Nuclear magnetic resonance spectroscopy of sulfide-exposed oleate suggested that sulfide reaction with oleate double bonds likely contributed to negation of the negative effect on the methanogenic activity. Methane formation from oleate was also accelerated in the presence of cysteine or serine, while sulfate decreased the cumulative methane formation from oleate. Neither cysteine nor serine was converted to methane, and their accelerating effects was associated to different mechanisms due to establishment of microbial communities with different structures, as evidenced by high-throughput sequencing of 16S rRNA gene. These outcomes contribute with new knowledge to develop strategies for optimum use of sulfur- and lipid-rich wastes in anaerobic digestion processes.

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1. Introduction

Waste lipids are attractive substrates for use in anaerobic digestion (AD) processes due to their high methane potential and energy density (Alves et al., 2009). Microbial degradation of lipids leads to formation of long-chain fatty acids (LCFA) such as palmitate, stearate, and oleate, which often accumulate in digesters due to their limited mass transfer and slow growth of the LCFA-degrading microorganisms (Elsamadony et al., 2021). At high concentrations, LCFA tend to adsorb on microbial cells that restricts the exchange of nutrients and microbial products with the surroundings, perturbing the microbial activity and methane formation (Usman et al., 2020). LCFA also encompass surface active properties, thus foaming is another complication observed at high loads of lipids (Kougias et al., 2013). In this context, kinetics of LCFA degradation sets a limit on the capacity of anaerobic digesters for handling lipid loads without encountering process disturbances.

LCFA are anaerobically degraded to acetate and hydrogen by acetogenic bacteria via β-oxidation pathway (Sousa et al., 2009b). Production of hydrogen by this pathway is endergonic and proceeds only if the hydrogen partial pressure is maintained at low levels. This in turn necessitates syntrophic partnership of the LCFA-degrading bacteria with hydrogen-utilizing microorganisms, such as methanogens, for obtaining an efficient LCFA conversion to methane (Duarte et al., 2018). Among different environmental variables, sulfide formation has been shown to substantially influence the degree and kinetics of LCFA degradation (Shakeri Yekta et al., 2019). In anaerobic environments, sulfide is formed via microbial sulfate reduction and/or mineralization of S-containing organic compounds such as the S-containing amino acids and peptides. Sulfate reduction in AD processes is often associated with a declined methane production from organic wastes, including waste lipids, due to utilization of the organic acids (and/or hydrogen) by sulfate-reducing bacteria and a declined availability of substrates for methanogenesis (Raskin et al., 1996; Wu et al., 2021). Nevertheless, a large group of sulfate-reducers are capable of degrading LCFA (Alves et al., 2020), offering a potential to remove LCFA upon AD of lipid-containing wastes.

Formation of sulfide is also associated with microbial toxicity in AD systems, as unionized sulfide (H_2S) may diffuse across the cell membrane and upset the cell growth and activity (O'Flaherty et al., 1999). Furthermore, reaction of sulfide by micronutrient trace metals such as Co and Ni may induce nutrient deficiency and methanogenic inhibition (Shakeri Yekta et al., 2017a). However, recent studies demonstrated that the occurrence of sulfide ions, often positively, influences the kinetics of unsaturated LCFA conversion (e.g., oleate, $C_{18:1}$) to methane and/or acetate in anaerobic digesters (Shakeri Yekta et al., 2019; Shakeri Yekta et al., 2017b). Such effect has not been observed for saturated LCFA (e.g., stearate, $C_{18:0}$) (Shakeri Yekta et al., 2019). It has been further observed that organic acid-degrading syntrophic associations within the microbial network are promoted at an elevated sulfide level together with the conversion rate of unsaturated LCFA to methane, likely due to an improved hydrogen-utilizing capacity of the methanogenic community (Shakeri Yekta et al., 2021). Despite this evidence, mechanisms behind the interactions of sulfide with LCFA conversion to methane are not fully understood.

This study aimed to elucidate the role of sulfide in LCFA conversion to methane and the undelaying mechanisms. More specifically, effects of sulfide and potential precursors of sulfide (i.e., cysteine, representing S-containing amino acids, and sulfate) on conversion of unsaturated LCFA (represented by oleate) to methane were investigated. Furthermore, abiotic effects of sulfide on chemical structure of LCFA that might affect the LCFA conversion to methane were assessed by nuclear magnetic resonance (NMR) spectroscopy of sulfide-exposed oleate. The outcomes of this study extend our current understanding of the underlying mechanisms behind the effects of sulfide on anaerobic oleate conversion to methane as well as interactions of other S species and microbial LCFA degradation in AD processes.

2. Materials and methods

2.1. Oleate conversion to methane

Conversion of oleate to methane was assessed using an automatic methane potential test system (AMPTS, Bioprocess control, Sweden), inoculated with 250 ml sludge (per batch bottle) from a full-scale municipal sludge digester at Henriksdal wastewater treatment plant (Stockholm, Sweden). The experimental setup included triplicate batch assays supplemented with 5 mM oleate (final concentration) with and without 20 mM cysteine, sodium sulfate, or sodium sulfide in separate bottles. Concentrations were chosen in a way to represent elevated S and LCFA levels in municipal sludge digesters, while avoiding methanogenic inhibition based on our previous studies (Shakeri Yekta et al., 2019). Triplicate control assays were also set up by adding the same amounts of cysteine, sodium sulfate and sodium sulfide without oleate supplementation. In addition, oleate-amended assays with and without supply of serine (20 mM) were included. Serine has the same chemical structure as cysteine but with a hydroxyl (-OH) functional group instead of thiol (-SH). Assessment of methane formation from oleate in the presence of serine allowed to determine potential influences that were not related to S functionalities in the amino acid structures. Cumulative methane production from oleate was calculated based on differences in cumulative methane production in oleate-amended and control assays. Theoretical methane potential of the total amount of the added oleate, cysteine and serine were calculated based on the conventional Buswell equation (Buswell and Mueller, 1952).

2.2. Microbial community analysis

Microbial community composition was evaluated by next generation amplicons sequencing of 16S rRNA gene in inoculum and in samples collected after termination of the batch assays (i.e., 55 days). Procedure for retrieval of the sequencing data was described in Shakeri Yekta et al. (2021). In short, DNA extraction was performed by using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) and concentration of DNA was determined by a Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). A two-step polymerase chain reaction (PCR) was carried out for amplification of the 16S rRNA genes by primer pair 515′F(GTGBCAGCMGCC GCG GTAA)/805R(GAC TAC HVGGG TAT CTA ATC C) for retrieving bacteria sequences and 516F(TGY CAG CCG CCG CGG TAA HACCVGC)/915R(GTG CTC CCC CGC CAA TTC CT) for archaea (Hugerth et al., 2014; Takai and Horikoshi, 2000). Sequence data from the DNA extracts was obtained by Illumina MiSeq technology at the SNP&SEQ Technology Platform of the SciLifeLab in Uppsala, Sweden. DADA2 software and the rRNA database SILVA, release 132, were used to assign taxonomic profiles based on amplicon sequence variants (ASVs) (Callahan et al., 2016; Edgar, 2018; Quast et al., 2013). Forward and reverse sequences were cut to lengths 119 and 210 bp, respectively, for bacteria with the quality threshold of maxEE = c(1,2) and truncQ = 2, and to 291 and 232 bp for archaea with maxEE = c(2,3) and truncQ = 2, according to in silico calculation by FIGARO (Sasada et al., 2020). R software together with vegan (Oksanen et al., 2016) package were used for statistical analysis. The sequencing data can be accessed via the National Center for Biotechnology Information database (identification number: PRJNA764191).

2.3. NMR spectroscopy

Among inorganic S species, sulfide is particularly susceptible for reaction with organic matter due to its high reactivity (Yu et al., 2015). Therefore, we investigated potential abiotic effects of sulfide on chemical structure of oleate using NMR spectroscopy. A solution of 5 mM oleate and 20 mM sulfide (i.e., sodium sulfide in de-aerated ultrapure water) were prepared and stirred for 6 or 24 h in a sealed glass vial at room temperature using a magnetic stirrer. Thereafter, sulfide-exposed oleate was transferred to a new vial and dissolved in 0.5 ml of $CDCl_3$ solvent using a vortex mixer. Solution-state NMR spectra of oleate dissolved in $CDCl_3$

were acquired on a Bruker 600 MHz Avance III HD spectrometer equipped with a 5 mm BBO cryoprobe with *Z*-gradients at 25 °C. The ^{1}H NMR spectra were obtained with 8 scans and an excitation pulse at 30 degree (sweep-width of 20 ppm and relaxation delay of 1.5 s). Two dimensional $^{1}H^{13}C$ heteronuclear single-quantum coherence (HSQC) spectra were obtained using sweep-widths of 12 and 165 ppm in the ^{1}H and ^{13}C dimensions, respectively, a relaxation delay of 1 s with 4 scans and 512 data-points in the indirect dimension (using non-uniform sampling with 25% actual sampling-points). 90-degree shifted squared sine window functions were applied in both dimensions before Fourier transformation. Spectral data were processed with Topspin 3.2 (Bruker Biospin, Germany) or ACD/Spectrus Processor 2015.2.5 (ACD/Labs, Canada). Chemical shift assignments are based on previous NMR data of oleate and phosphatidylcholine recorded in-house.

3. Results and discussion

It has been previously reported that the kinetics of oleate β-oxidation to acetate and oleate conversion to methane are enhanced as a result of sulfide amendment to municipal sludge digesters (Shakeri Yekta et al., 2019; Shakeri Yekta et al., 2017b). Similarly, methane formation from oleate in the anaerobic batch assays in this study was faster in the presence of sulfide,

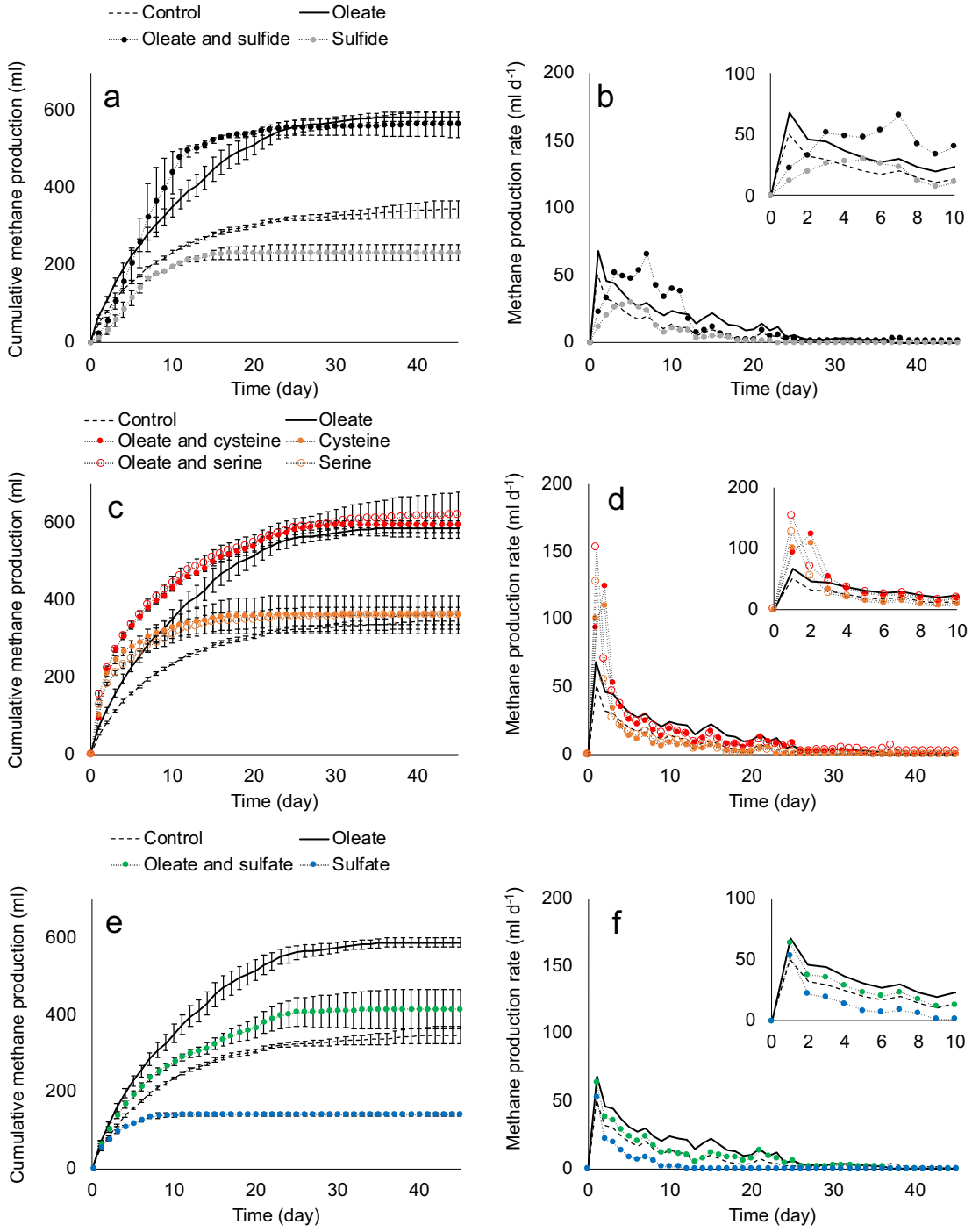

Fig. 1. Average cumulative methane production (ml) and production rate (ml d^{-1}) in triplicate batch assays supplemented with oleate in the presence or absence of sulfide (a and b), cysteine or serine (c and d), and sulfate (e and f). Data are also presented for assays that contained only inoculum (control), and supplied with sulfide, cysteine, serine, or sulfate (a-f).

where the time to reach 90% of the final cumulative methane production from oleate decreased from 21 to 14 days upon sulfide addition (Fig. 1a). The enhanced kinetics of methane formation in sulfide-amended assays was maintained over approximately 12 days after start of the incubation (Fig. 1b). Methane formation in the oleate-amended assays were also faster in the presence of cysteine or serine compared to the corresponding assays without the amino acids (Fig. 1c), where the maximum methane formation rate was achieved 2–3 days after the incubation start followed by a decline (Fig. 1d). There were no significant differences (*t*-test, $p > 0.05$) in cumulative methane production of the oleate-amended assays in the absence (585 ± 10 ml methane) or presence of sulfide, cysteine, or serine (570 ± 35, 595 ± 10, and 620 ± 60 ml methane, respectively) after 55 days of incubation. In contrast, sulfate reduced the cumulative methane formation in oleate-amended assays (from 585 ± 10 to 415 ± 50; Fig. 1e), with an overall slower kinetics compared to the assays without sulfate (Fig. 1f). Evidently, methane formation pattern in sulfide-amended assays was different from the assays that were supplied with cysteine, serine, or sulfate, implying that different mechanisms may lie behind their effect on oleate conversion to methane. Potential mechanisms underlying the impacts of cysteine, serine, sulfide, and sulfate on methane formation from oleate are explored in the following sections.

3.1. Effects of cysteine and serine on oleate conversion to methane

Cumulative methane production in batch assays with cysteine and serine was similar to control assays that contained only inoculum (~350 ml methane; Fig. 1c), indicating that these amino acids were not converted to methane during 55 days of incubation despite their theoretical methane potential of ~150 ml (calculated based on Buswell equation). In the same way, cumulative methane production from oleate was similar in assays with and without addition of the amino acids (~245 ml methane based on differences in cumulative methane formation in oleate-amended and control assays without oleate amendment; Fig. 1c). It can therefore be inferred that neither cysteine nor serine were sources of methane in batch assays, and they did not affect the degree of oleate conversion to methane, which was approximately 45% of the theoretical methane potential of the oleate added (i.e., ~540 ml methane based on Buswell equation).

In contrary to these observations, amino acids can be anaerobically degraded to organic acids and serve as precursors of methane via two main microbial routes. A range of bacteria implement Stickland pathway coupled to NAD^+/NADH redox reaction, that requires access to specific pairs of amino acids as electron donors and acceptors (Nisman, 1954). In this pathway, one amino acid is oxidized to an acid that is one carbon shorter and the other is reduced to its corresponding fatty acid. Single amino acids can also be transformed via reductive and oxidative deamination, presumably when there is a deficiency of amino acid redox pairs for the Stickland pathway (Mead, 1971). The fact that cysteine and serine were not converted to methane in the batch assays suggests absence of appropriate amino acid pairs for the Stickland pathway and/or a lack of microbial capacity for single cysteine or serine degradation to organic acids, which otherwise could serve as methanogenic substrates. In this regard, it may also be inferred that cysteine addition to the batch assays did not result in a formation of sulfide that is expected upon cysteine degradation. Consequently, the observed positive effect by cysteine on kinetics of oleate conversion to methane could not be attributed to formation of sulfide.

Nonetheless, both cysteine and serine enhanced the rate of methane formation in batch assays. Rate of methanogenesis is closely entangled with interspecies electron transfer via diffusion of metabolites (e.g., hydrogen or formate) that is often a rate-limiting step for organic acids degradation and methane formation (Dolfing, 1992). It has been previously suggested that cysteine has the potential to mediate the electron transfer between syntrophic partners and accelerates the rate of methane formation, e.g., in acetate- and propionate-degrading methanogenic cultures (Kaden et al., 2002; Zhuang et al., 2017). This was attributed to the ability of cysteine to take part in cysteine-cystine redox cycle that shuttles electrons between organic acid degraders and methanogens. In this process, oxidation of cysteine to cystine transfers electrons to methanogens for reduction of carbon dioxide, while reduction of cystine to cysteine occurs through electron transfer from organic acid oxidizing bacteria. Accordingly, a cysteine-accelerated electron transfer between the syntrophic partners could potentially favor the rate of LCFA β-oxidation and hydrogenotrophic methanogenesis, which are generally dependent on the hydrogen/formate transfer between LCFA-degrading bacteria and hydrogen-utilizing methanogens.

There were no apparent differences in the degree and the rate of methane formation between assays supplied with cysteine or serine, indicating that the structural differences of these compounds, i.e., presence of thiol or hydroxyl functional groups, did not impact the oleate conversion to methane. Despite similar methane formation patterns, microbial community compositions were different in batch assays supplied with cysteine or serine. This is reflected in the weighted UniFrac PCoA of the bacterial ASVs obtained from the samples retrieved after termination of the batch experiment (Fig. 2). Bacterial community in cysteine-amended assays with and without oleate were similar to each other and had a close phylogenetic proximity to the bacterial community in the control assays that contained only inoculum. However, the bacterial community of the serine-amended assays with and without oleate showed a high degree of dissimilarity to those supplied with cysteine and the control assays (Fig. 2). Furthermore, the phylogenetic dissimilarities between the bacterial community in cysteine- and serine-amended assays with those supplemented with sulfate or sulfide suggests that the bacterial community evolved differently in the presence of these compounds (Fig. 2).

The initial bacterial community structure in the inoculum was dominated by the order SJA-15 (60 ± 11% of bacteria), belonging to cellulose-degrading class Anaerolineae (Xia et al., 2016), as well as genus DMER64 (17 ± 7.0% of bacteria), belonging to the fermentative family Rikenellaceae (Graf, 2014). After 55 days of incubation, the overall community structure in all cysteine-amended assays resembled those in the inoculum, while differed from serine-amended assays partly due to a lower abundance of the order Anaerolineae SJA-15 in the assays supplemented with serine (Fig. 2). The observed differences were however less pronounced among the archaeal communities of cysteine- and serine-amended assays (Fig. 3). The archaeal community in the inoculum was dominated by acetoclastic genus *Methanosaeta* (69 ± 3.0% of archaea), followed by genera *Candidatus Methanofastidiosum*, *Methanolinea*, and *Candidatus Methanomethylicus* (9.0 ± 0.5, 7.8 ± 1.0 and 6.4 ± 0.2% of archaea, respectively). At the end of incubation, relative abundances of *Candidatus Methanomethylicus* together with less-abundant hydrogenotrophic genera, *Methanobacterium*, *Methanospirillum*, *Methanoculleus* and the order *Methanomicrobiales* were marginally lower in the serine-amended assays compared to those that received cysteine (Fig. 3).

Amino acids in anaerobic medium may affect microbial growth both as inhibitory or stimulating compounds (Javed and Baghaei-Yazdi, 2016). Serine in particular is a precursor of multiple metabolites (e.g., cysteine, glycine, and tryptophan) and activities of a suite of enzymes, such as serine dehydrase (catalyzing serine dehydration and deamination to pyruvate) or serine hydroxymethyltransferase (SHMT; catalyzing interconversion of serine and glycine), may affect the fate of serine in biological systems (Sawers, 1998; Song et al., 2020). Accordingly, an enhanced rate of methane formation in cysteine and serine-amended assays might be related to their function in essential metabolic pathways. In addition, some methanogens such as *Methanobacterium thermoautotrophicum* and *Methanosphaera stadtmanae* (order Methanobacteriales) can convert serine to methane, likely due to possession of tetrahydromethanopterin-dependent SHMT activity, while other methanogens such as *Methanosarcina barkeri* (order Methanosarcinales) and *Methanospirillum hungatei* (order Methanomicrobiales) lack such ability (Lin and Sparling, 1998). Several species belonging to the order Methanomicrobiales (e.g., *M. barkeri*, *Methanosaeta concilii*, and *Methanolobus tindarius*) also lack the tetrahydromethanopterin-dependent SHMT activity for conversion of serine to methane. In our study, the dominant archaeal genera in the serine-amended assays were *Methanosaeta* (56–66% of archaea), *Methanolinea* (10–14% of archaea), *Candidatus*

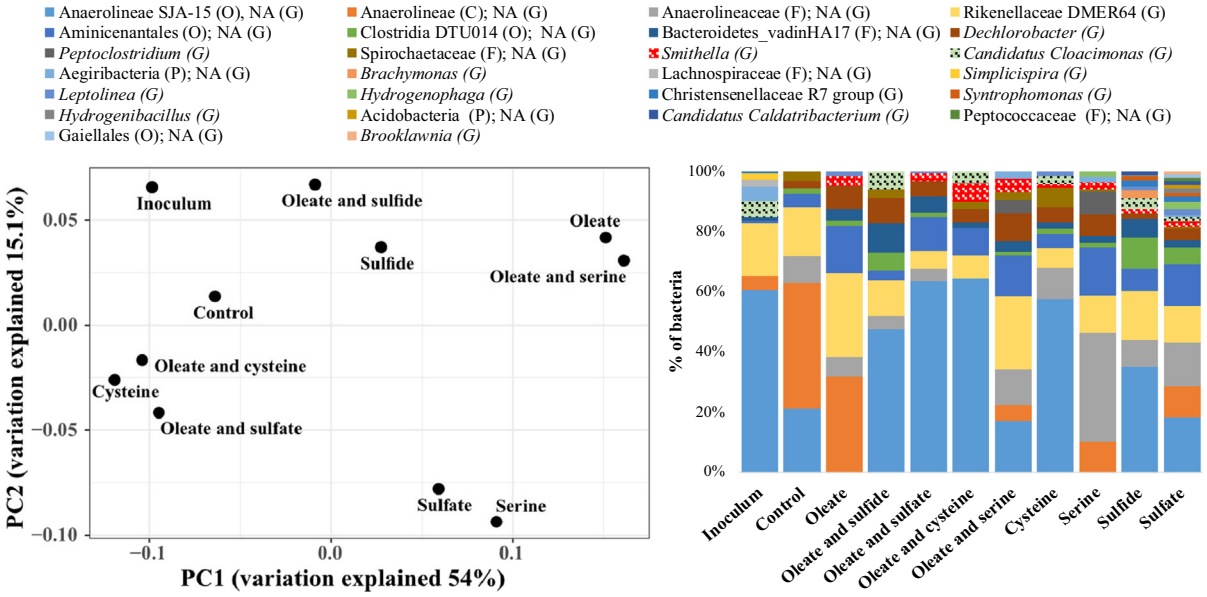

Fig. 2. Phylogenetic distance of bacterial communities determined by weighted UniFrac principal coordinate (PC) analysis of ASV read counts. Relative abundances of bacterial 16S rRNA genes at genus level are presented, based on the average ASV reads from triplicate samples of inoculum and those collected after 55 days of incubation. Where genus name could not be assigned to the sequences (marked by NA), the closest classified taxonomic level is depicted; kingdom (K), phylum (P), class (C), order (O), family (F), genus (G). Bacteria with relative abundances ≥1.0% in at least one sample are depicted.

Methanofastidiosum (14–16% of archaea), *Candidatus Methanomethylicus* (4–6% of archaea), and *Methanobacterium* (1–4% of archaea). Although the ability of these methanogens to convert serine to methane is not well known, the fact that serine was not converted to methane in our study, suggests the potential absence of SHMT activity for the use of serine as a methanogenic substrate.

3.2. Effect of sulfate and sulfide on oleate conversion to methane

Addition of sulfate decreased the amount of cumulative methane production in the batch assays that contained only inoculum (from 360 ± 20 to 140 ± 10 ml methane). The overall efficiency of oleate conversion to methane declined in the presence of sulfate from 45 to 10% of the theoretical methane potential (Fig. 1e). These results point at a reduced methane yield caused by competition of sulfate-reducing bacteria with methanogens for substrates (Sousa et al., 2009a). Potential sulfate-reducing bacteria in the dataset with relative abundances ≥1.0% of bacteria belonged to genera *Smithella* (phylum Desulfobacterota; Waite et al., 2020). The relative abundances of syntrophic organic acid-oxidizing genus *Smithella* in sulfate-amended assays were increased over the incubation period (from 0 up to 3% of bacteria; Fig. 2). However, an increase in the relative abundance of *Smithella* was not unique for the sulfate-added community and the abundances of this genus were also relatively high in cysteine- and serine-amended assays with and without oleate (1–6% of bacteria; Fig. 2). *Smithella* is a known propionate oxidizer in AD processes (De Bok et al., 2001), and the key role of *Smithella* in supporting the interspecies electron transfer between organic acid degraders and hydrogenotrophic methanogens during LCFA degradation has been previously highlighted (Shakeri Yekta et al., 2021). The capability of *Smithella* for LCFA degradation is unclear, yet potential contribution of *Smithella* to LCFA degradation

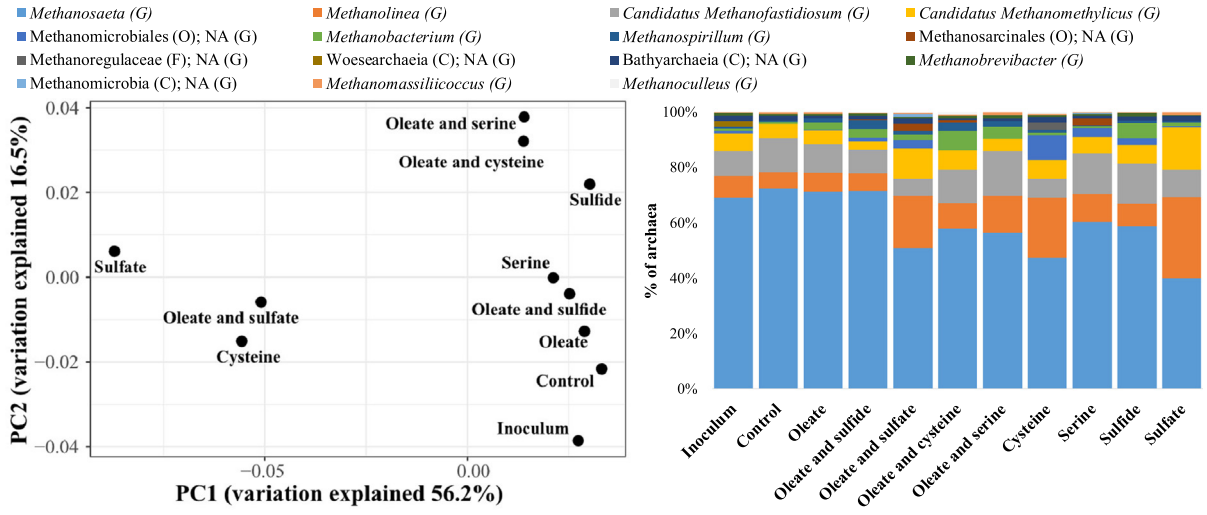

Fig. 3. Phylogenetic distance of archaeal communities determined by weighted UniFrac principal coordinate (PC) analysis of ASV read counts. Relative abundances of archaeal 16S rRNA genes at genus level are presented, based on the average ASV reads from triplicate samples of inoculum and those collected after 55 days of incubation. Where genus name could not be assigned to the sequences (marked by NA), the closest classified taxonomic level is depicted; kingdom (K), phylum (P), class (C), order (O), family (F), genus (G). Archaea with relative abundances ≥1.0% in at least one sample are depicted.

is plausible due to its association with the Syntrophaceae family and its phylogenetic proximity to genus *Syntrophus* that is capable of LCFA degradation (Gray et al., 2011). Nevertheless, a suite of bacteria emerged with low relative abundances particularly in the assays amended only with sulfate, in which the acetoclastic *Methanosaeta* showed the lowest relative abundance (Figs. 2 and 3). Therefore, a diverse group of bacteria might have benefited from the availability of sulfate as an electron acceptor for utilization of organic acids (e.g., acetate) at the expense of acetoclastic methanogenesis.

Sulfide supplementation alone also decreased the cumulative methane production in assays that contained inoculum (from 360 ± 20 to 235 ± 20 ml methane; Fig. 1a), but to a lower extent compared to sulfate addition. This observation indicates that sulfide supplementation imposed a partial inhibition on methane formation, likely related to its toxic effect on microbial activity. Interestingly, combined oleate and sulfide addition resulted in the same amount of methane formation as in the oleate-amended assays without sulfide (Fig. 1a). Apparently, the partial inhibition of methane formation caused by sulfide addition was mitigated in the presence of oleate. The ^{1}H and HSQC NMR spectroscopy were used to investigate whether this mitigating effect could be related to a direct chemical reaction between oleate and sulfide, uncoupled from microbial activity.

Analysis of the NMR data from sulfide-exposed oleate samples demonstrated a decline in the ratio between the olefinic peak (δ_H: 5.4 ppm) and the terminal CH_3 peak (δ_H: 0.9 ppm) from 2:3 to 1.3:3, suggesting an addition over the olefinic double-bonds (Fig. 4). Furthermore, peaks from two new methylene groups (CH_2) appeared at δ_H/δ_C 1.92;1.80/34.4 ppm and δ_H/δ_C 1.57;1.40/26.6 ppm (Fig. 4c and d). The fact that the protons in both of the methylene groups are diastereotopic further corroborates addition over the double-bonds, resulting in at least one stereogenic center. The exact structure of the addition products cannot be discerned, but the

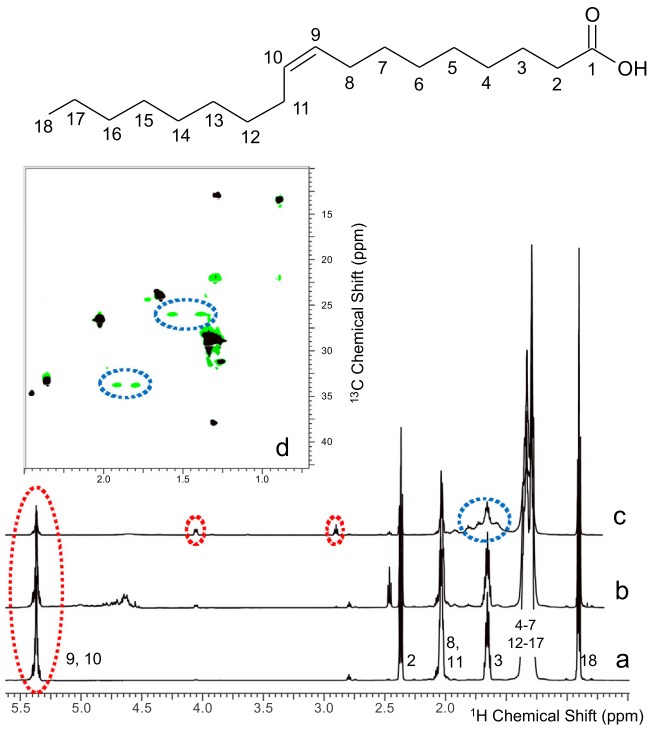

Fig. 4. Liquid-state ^{1}H NMR spectra acquired from oleate dissolved in $CDCl_3$, before (a) and after exposure to sulfide for 6 and 24 h (b and c). ^{1}H chemical shifts assigned to hydrogen in different positions of the carbon chain of oleate are depicted by numbers. d) ^{1}H,^{13}C HSQC NMR spectra of oleate before (black) and after 24 h exposure to sulfide (green). The dotted rings mark chemical shift regions that represent alterations in structure of oleate upon exposure to sulfide.

^{13}C chemical shift of the formed CH group is more consistent with thiol formation than alcohol formation, suggesting an addition of sulfide. Peaks at δ_H: 2.9 and 4.05 ppm have increased in intensities after sulfide-exposure (also observed with very low intensities in the original oleate sample) and their origin is unclear. Altogether, the NMR spectroscopy provided strong evidence on conversion of CC to CC in conjunction with possible formation of thiol groups attached to the oleate structure after sulfide exposure, which can be analogous to the known mechanism of free radical addition of hydrogen sulfide to C double bonds (Schwab et al., 1968). Assuming the occurrence of this reaction in AD systems, it can be postulated that the unsaturated bonds of oleate has the potential to deplete sulfide ions. This reaction might be the reason behind mitigation of the negative effect of sulfide on methane formation in batch assays amended with both sulfide and oleate.

Moreover, the observed increase in the rate of methane formation from oleate in sulfide-amended assays might also be related to the observed abiotic effect of sulfide on oleate chemical structure. The rate and the degree of oleate conversion in anaerobic digesters are often correlated to the relative abundance of genus *Syntrophomonas*, which has been suggested as one of the main contributors to LCFA degradation in AD processes with a wide range of substrate profiles and operational conditions (Ziels et al., 2018). However, we did not detect the occurrence of this genus in any of the oleate-amended assays (Fig. 2), which implies that the enhanced kinetics of oleate conversion upon addition of sulfide (as well as cysteine or serine) was likely not related to an improved activity of *Syntrophomonas*. Further investigation is therefore needed to be able to differentiate the effects of sulfide-induced alterations in oleate chemical structure on the rate of methane formation from those caused by changes in the microbial community. Nevertheless, it can be concluded that the inhibitory impact of sulfide on methane formation can be mitigated in the presence of oleate, and sulfide addition has the potential to enhance the kinetics of unsaturated LCFA conversion to methane.

4. Concluding remarks

The outcomes of this study provide novel evidence on the interactions of sulfide and its precursors with the conversion of LCFA to methane in AD systems. Sulfide, cysteine, S- containing amino acid, and serine, as its non-S amino acid counterpart, enhanced the rate of methane formation from oleate, apparently through different mechanisms. An accelerated LCFA conversion to methane may provide prerequisites for efficient use of waste lipids during AD by tackling the slow kinetics of LCFA conversion to methane. As amino acids and sulfide formations are expected in AD processes due to protein hydrolysis and mineralization of proteinous S, co-digestion of protein-rich organic wastes (e.g., food and slaughterhouse wastes) together with waste lipids may be considered as strategies for optimization of the rate of LCFA β-oxidation and methane production. Nevertheless, composition of the pool of LCFA in term of carbon chain saturation needs be taken in to account as sulfide effect is apparently linked to its reaction with unsaturated carbon bonds of LCFA. Furthermore, mixture of amino acids is found upon AD of organic wastes, necessitating further research to discern how the catalytic function of amino acids, e.g., cysteine and serine, can be maintained under operational environment of AD processes. Our results further suggest that the use of waste lipids as co-substrates and corresponding formation of unsaturated LCFA in AD processes with high sulfide levels may be considered for mitigating the negative effects of sulfide and, at the same time, improve the methane production. However, since sulfate presence reduced the amount of methane formation from oleate, methane yield of waste lipids may be compromised if used as co-substrates in AD systems treating sulfate-rich streams (e.g., stillage from bio-ethanol production, industrial wastewater from pulp and paper production and petroleum refining).

CRediT authorship contribution statement

Sepehr Shakeri Yekta: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Visualization, Funding

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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.

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