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Effect of ammonia on anaerobic digestion of municipal solid waste: inhibitory performance, bioaugmentation and microbiome functional reconstruction

Miao Yan\textsuperscript{a}, Laura Treu\textsuperscript{b,*}, Stefano Campanaro\textsuperscript{b,c,*}, Hailin Tian\textsuperscript{a,d}, Xinyu Zhu\textsuperscript{a}, Benyamin Khoshnevisan\textsuperscript{a,e}, Panagiotis Tsapekos\textsuperscript{a}, Irini Angelidaki\textsuperscript{a,§}, Ioannis A. Fotidis\textsuperscript{a,f,§}

\textsuperscript{a} Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet Bygning 115, DK-2800 Kgs. Lyngby, DK

\textsuperscript{b} Department of Biology, University of Padova, Via U. Bassi 58/b, 35121 Padova, Italy

\textsuperscript{c} CRIBI Biotechnology Center, University of Padua, 35131, Padua, Italy

\textsuperscript{d} NUS Environmental Research Institute, National University of Singapore, 1 Create Way, 138602, Singapore

\textsuperscript{e} Key Laboratory of Non-point Source Pollution Control, Ministry of Agriculture, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

\textsuperscript{f} School of Civil Engineering Southeast University Nanjing, China 210096

*Corresponding authors: Laura Treu laura.treu@unipd.it, Stefano Campanaro stefano.campanaro@unipd.it

§equally contributing
Abstract

The bioaugmentation is crucial to improve the energy-efficient process for anaerobic digestion of organic wastes at high ammonia levels. Genomic insights into the intricate microbial networks at a high ammonia level remain underexplored. The present study showed that the addition of *Methanoculleus* sp. DTU887 remarkably enhanced the methane production yield of organic fraction of municipal solid waste by 21% and decreased the volatile fatty acids by 10% when compared to the period before bioaugmentation. Genome-centric metagenomics reports the functional contribution of microbial members during organic waste degradation under the extremely high level of 13.5 g NH$_4^+$-N/L. Specifically, metabolic reconstruction revealed that these organisms have the potential to perform fermentative and acetogenic catabolism, a process facilitated by energy conservation-related with H$_2$/CO$_2$ metabolism. *Peptococcaceae* spp. (DTU903, DTU900, and DTU895). and *Tissierellales* sp. DTU879 could degrade the organic waste hydrolysis product, i.e., glucose to acetate and H$_2$. *Tissierellales* sp. DTU879 and *Syntrophaceticus* sp. DTU783 could degrade the derived acetate. The H$_2$ scavenging *Methanoculleus* sp. DTU887 performs complementary metabolic reactions with *Peptococcaceae* spp., *Tissierellales* sp. and *Syntrophaceticus* sp., indicating syntrophic glucose and acetate degradation. This research offers the first insight that the key organisms form a syntrophy-supported food web in response to the bioaugmentation with ammonia tolerant methanogens performed in an AD system subjected to severe ammonia inhibition.
Graphical abstract

Keywords

Bioaugmentation; metagenomics; ammonia tolerance; energy-converting mechanisms; interspecies interaction; metabolic reconstruction
1. Introduction

The increasing amount of organic waste and its consequent disposal has been a major worldwide environmental and economic problem [1]. Inappropriate treatment, such as landfill, leads to greenhouse gas emissions, odors, and sanitary concerns [2]. To reach the United Nations’ Sustainable Development Goals (SDGs) by 2030, organic waste should be integrated into a circular economy concept including nutrients recovery and bioenergy production [3, 4]. Therefore the focus has been given to anaerobic digestion (AD) as a key technology, which treats residual biomasses properly and produces renewable energy (i.e., biogas). The biogas can substitute fossil fuels in heat production, electricity generation and transportation and, at the same time, preserve nutrients which can be retrieved for soil applications [5]. The efficient conversion of organic waste into biogas requires a well-balanced microbiome performing syntrophic interactions among four main steps of the AD process (i.e. hydrolysis, acidogenesis, acetogenesis, methanogenesis) This syntrophic association may contribute to (i) maintain the microbiome balance [6], (ii) prevent volatile fatty acid (VFA) and hydrogen accumulation, and (iii) convert intermediate compounds (acetate, H₂, CO₂) to methane [7]. Moreover, the energy potential of the current worldwide organic waste is estimated between 10,100 and 14,000 TWh equivalent to 26-37% of the current natural gas consumed globally (World Biogas Association 2019); therefore even small improvements in biogas production may be extremely relevant [8].

However, degradation of N-rich organic wastes, especially effluents from many industries, aquaculture production units, food processing plants, etc., can represent a major challenge in maintaining a balanced process [9]. Specifically, when the concentration of ammonia exceeds a certain limit (approximately 1.5 g NH₄⁺-N /L
during AD), it becomes toxic for the microbial consortium [10]. To be noticed, the methanogenic archaea are directly responsible for biogas production and more susceptible to ammonia compared to bacteria, excluding syntrophic acetate oxidizers (SAOB) [11-13]. The reduced methanogenic activity induces inefficient hydrogen/formate removal, and as a consequence, SAOB does not harvest enough energy to support self-growth at high H₂ concentrations. Besides, accumulation of additional intermediate compounds (e.g. VFA, H₂) happens because the reduction in the activity of bacteria doesn’t occur to the same degree as that of methanogens [14, 15]. This accumulation further inhibits methanogenesis lowering methane production rates, and can eventually lead to AD failure.

The supplementation of ammonia tolerant methanogens is one of the key strategies applied for the recovery from ammonia inhibition. This can be achieved by bioaugmentation, i.e. introducing specific acclimatized pure strain or enriched cultures into an under-performing anaerobic digester [16, 17]. Additionally, another advantage of using the bioaugmentation is that the microbial cultures incorporated in the digestate can be directly used as a soil amendment or fertilizer [18]. In fact, other ammonia alleviation strategies, such as magnetite or zeolite addition, require extra separation steps before discharge [19, 20]. The syntrophic mechanisms occurring due to bioaugmentation imply that the addition of specific microbes enhances the removal of self-limiting products (e.g. H₂ or acetate), which are used by archaea as electron donors/acceptors during methanogenesis [21]. The removal of these intermediates stimulates the growth of bacteria, improving the substrate consumption rates and funneling the substrate fermentation toward methane production [7]. Yang et al. (2019) have proved that the bioaugmentation performed with the simultaneous addition of
Methanobrevibacter smithii and Syntrophaceticu schinkii could efficiently improve methane production by 71% at 4 g NH$_4^+$-N/L. To pinpoint the key role of specific consortia in the AD process [17], Westerholm et al. (2012) proved that the addition of SAOB cultures alone couldn’t improve the performance at an ammonia load of 11 g NH$_4^+$-N/L [22]. However, bioaugmentation with hydrogenotrophic methanogens alone could dramatically stimulate the growth of SAOB, rapidly alleviating the acetate-ammonia co-inhibitory effect in thermophilic and mesophilic reactors [23, 24]. Despite this, the metabolic pathways and microbial interactions induced by bioaugmentation and being responsible for improving methane production remain unknown. Recently, the metagenomics approach has been applied to unravel genomic information for diverse unknown microorganisms in AD microbiomes and offer insights into their potential functions creating a global reference database [12, 25, 26]. However genomic insight into interconnected networks of effective microbes growing at high ammonia level is still lacking.

The present study aimed to decipher holistic AD syntrophic interactions in response to the bioaugmentation with ammonia tolerant methanogen performed in an AD system subjected to severe ammonia stress. To achieve that, the bioaugmentation strategy was employed in an inhibited continuously stirred anaerobic reactor (CSTR) fed with organic fraction municipal solid waste (OFMSW) at 13.5 g NH$_4^+$-N/L. Specifically, bioaugmentation was performed with a pure culture of one ammonia-tolerant Methanoculleus bourgensis, belonging to a well-known resistant methanogenic genus [25]. An in-depth analysis of the microbial responses was performed by metagenomics and the metabolic pathways encoded by the most abundant taxa were reconstructed in detail.
2. Material and methods

2.1. Inoculum and feedstock

The inoculum used to start two CSTRs obtained from a mesophilic (37°C) anaerobic digester used for OFMSW degradation at total ammonia nitrogen (TAN) of 9.5 g NH$_4^+$-N/L, which had been operated stably for 10 months [27]. The OFMSW, collected from Gemidan Ecogi A/S, was used as substrate after treated with the bio pulping process [28]. The substrate was preserved at -20°C and thawed at 4°C before use, then diluted with distilled water to reach a fixed total solid (TS) content of 80 g VS/kg. The characteristics of the inoculum and of the substrate are presented in Table 1.

Table 1. Characteristics of inoculum and substrate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Inoculum (SD)</th>
<th>Biopulp (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS)</td>
<td>g/kg</td>
<td>49.04 (0.92)</td>
<td>79.39 (0.51)</td>
</tr>
<tr>
<td>Volatile solids (VS)</td>
<td>g/kg</td>
<td>39.75 (0.71)</td>
<td>67.66 (0.21)</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>7.8(0.01)</td>
<td>3.9 (0.1)</td>
</tr>
<tr>
<td>Total ammonia nitrogen (TAN)</td>
<td>g NH$_4^+$-N/L</td>
<td>9.50 (0.01)</td>
<td>1.10 (0.01)</td>
</tr>
<tr>
<td>Free ammonia nitrogen (FAN)</td>
<td>g NH$_3$-N/L</td>
<td>0.896 (0.01)</td>
<td>0.10 (0.00)</td>
</tr>
<tr>
<td>Total Kjeldahl Nitrogen (TKN)</td>
<td>g N/L</td>
<td>5.38 (0.25)</td>
<td>NM</td>
</tr>
<tr>
<td>Total Carbohydrate</td>
<td>g/L</td>
<td>NM</td>
<td>42.1 (1.58)</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/L</td>
<td>NM</td>
<td>12.11 (0.45)</td>
</tr>
<tr>
<td>Total lipid</td>
<td>g/L</td>
<td>NM</td>
<td>10.49 (0.39)</td>
</tr>
</tbody>
</table>

* NM: Not Measured.
2.2. Bioaugmentation culture

*M. bourgensis* MS2 culture (DSM 3045) was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, and routinely maintained for more than five years in the Bioenergy laboratory at Denmark Technical University. The sequential cultivation was done obtaining sub generation from previous seed generation at the exponential growth phase. Prior to bioaugmentation, the culture was acclimatized to 14 g NH$_4^+$-N/L (increment with 2 g NH$_4^+$-N/L each step) at 37 °C. The characteristics of the cultivation medium (CM) and growth curve for *M. bourgensis* are shown in Table S1.

2.3. Experimental setup

The experiment was performed in two identical mesophilic CSTR reactors with a total and working volume of 4.5 and 3 L, respectively (Fig. 1). $R_{bio}$ and $R_{con}$ represent high ammonia loaded reactors with bioaugmentation (addition of *M. bourgensis*) and abiotic augmentation (without the addition of *M. bourgensis*), respectively. With a hydraulic retention time (HRT) of 20 days, the two reactors were fed with OFMSW at an organic loading rate of 3.4 g VS/L/day for 135 days. The whole experiment was divided into five periods (P1-P5) as presented in Table 2. During P1 to P3, the TAN levels of the influent and the reactor content were increased concurrently from 9.5 to 13.5 g NH$_4^+$-N/L (increment with 2 g NH$_4^+$-N/L each step) with the addition of urea (CO(NH$_2$)$_2$) and ammonium chloride (NH$_4$Cl). The ammonia load of 13.5 g NH$_4^+$-N/L was kept for all the remaining periods (P4-P5). The bioaugmentation with *M. bourgensis* resuspended in CM for $R_{bio}$ was performed for four consecutive days at P4. Specifically, 240 mL of culture (volatile suspended solids (VSS): 67 mg/L) were centrifuged at 4000 rpm (37°C) for 10 minutes; afterward, the 180 ml of supernatant
was removed under nitrogen gas. The pellet was resuspended by vortexing in 60 ml of residual CM and was injected in the reactors with a sterile syringe. Meanwhile, the same volume of CM containing 14 g NH₄⁺-N/L was introduced to R_{con} as abiotic augmentation. The mixing in CSTR reactors was stopped for 4 hours after each injection to allow the culture to settle in the reactor. Besides, the substrate feeding was stopped during P₄, avoiding any washout of the injected microbial culture.

Fig. 1. Schematic diagram of the continuously stirring tank reactors.
Table 2. The CSTR reactors experimental design

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>TAN (g NH₄⁺-N/L)</th>
<th>Extra added ammonia CO(NH₂)₂ (g NH₄⁺-N/L)</th>
<th>NH₄Cl (g NH₄⁺-N/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0-5</td>
<td>9.5</td>
<td>4.5</td>
<td>3.9</td>
</tr>
<tr>
<td>P2</td>
<td>6-22</td>
<td>11.5</td>
<td>5.5</td>
<td>4.9</td>
</tr>
<tr>
<td>P3</td>
<td>23-50</td>
<td>13.5</td>
<td>6.5</td>
<td>5.9</td>
</tr>
<tr>
<td>P4 (Bioaugmentation)</td>
<td>51-54</td>
<td>13.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P5</td>
<td>55-120</td>
<td>13.5</td>
<td>6.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* "-": No extra ammonia addition

2.4. Chemical analyses

Methane concentration and VFA were measured twice per week using two different gas chromatographs as previously described by Yan et al. (2019) [27]. pH was measured with PHM99 LAB pH meter (RadiometerTM). TS, Volatile solid, VSS, and TAN were measured according to the standard methods [29].

2.5. Microbial analysis

To explain how the bioaugmentation inocula affected the microbiome both immediately and steady-state after bioaugmentation, the sludge samples for genomic DNA extraction were collected from Rbio on days 31 (P3), 56 (P4), and 91 (P5) and were used for metagenomic analysis. A first step was added to the regular DNA extraction where Phenol:Chloroform: Isoamyl Alcohol (25: 24: 1) was used to further improve the quality of the DNA, afterward the samples were extracted using DNeasy PowerSoil® (QIAGEN GmbH, Hilden, Germany) with minor modification [30]. Finally, DNA concentration and purity were checked using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).
2.6. Metagenomic sequencing and binning analysis

A sequencing platform including both Oxford Nanopore MinION single-molecule and Illumina sequencers was performed. Nextera DNA Flex Library Prep Kit (Illumina Inc., San Diego CA) and SQK-RBK004 rapid sequencing kit (Oxford Nanopore Technologies, Oxford, UK) were applied for library preparation. Libraries sequencing was performed with FLO-MIN106 R9 flow cell on a MinION device (Oxford Nanopore Technologies, Oxford, UK) and Illumina NextSeq 500 platform (Illumina Inc., San Diego CA) with paired-end at the CRIBI biotechnology center sequencing facility (University of Padova, Italy). The total raw data includes 26997584, 25494946, and 21089120 filtered reads for P3, P4, and P5 respectively. Trimmomatic (v0.39) was used to filter Illumina reads with low quality or ambiguous bases [31]. Spades (v3.13.0) [32], Unicycler (v0.4.8-beta) [33] and OPERA-MS [34] were independently used for high-quality reads assembly. Illumina reads alone and Illumina reads bound with Nanopore data via MEGAHIT (V1.2.4beta) software [35], were utilized during the assembly process. Afterwards, all the scaffolds longer than 1 kb were kept and Quality Assessment Tool (QUAST _1, V4.1) was used for the binning [36]. The alignment of the reads per each sample back to the assembly was performed using Bowtie 2 (v2.2.4) [37] to determine scaffolds’ coverage. Metagenomic binning was achieved using MaxBin2 (v2.2.6) and Metabat2 (v2.12.1) software [38]. The best MAGs among the different assemblies and binning results were chosen using metaWRAP [39] and finally 26, 21 and 20 MAGs were obtained from metaspades, OPERA-MS and unicycler, respectively. The basic metrics, contamination, and completeness of the final MAGs were evaluated using CheckM (v1.0.3). GTDB-Tk [40] and CAT [41] provided taxonomical assignment and functional analysis. The identification of protein-encoding
genes was achieved using Prodigal (v2.6.2) [42, 43] in normal mode and connected with KEGG IDs with Diamond (v0.9.22.123) [44]. Protein sequences predicted using Prodigal (v2.6.3) were annotated by similarity search on COG and Pfam databases using RPS-BLAST (v2.2.26) using self-written perl scripts as previously described [45]. Functional completeness based on KEGG IDs was assessed using the KEGG mapper-reconstruct pathway. Hierarchical clustering analyses were done with the Multiexperiment viewer (v4.9.0). The characteristic of the microbiome collected from each period was visualized using Anvi’o [46]. The details regarding metagenomic results are described in Table S2. Raw sequencing data have been uploaded to the NCBI Sequence Read Archive database under the Bioproject PRJNA613576, with specific SRA codes per sample: SRR11356280, SRR11356279, and SRR11356278.

2.7. Data analysis

All tests were measured in duplicate, and the significance of each result was determined by analyses of variance (ANOVAs) and Student's t-test. A p value <0.05 means statistically significant.

3. Results and discussion

3.1. Reactors’ performance

From P1 to the end of P3, both reactors experienced inhibition with an average methane production loss close to 25% compared to the results obtained from previous research (Fig. 2). The experiment was performed in the same reactor and operating conditions treating the same OFMSW under steady-state without ammonia addition with an average methane yield of 368 CH₄/g VS [27]. During bioaugmentation (P4), methane production yield in R_bio and R_con decreased slightly because of the
discontinuity in substrate feeding. After bioaugmentation, the average methane production (from day 54 to 120) in R_{bio} reactor showed an increase by 21% compared to P3 (Fig. 2). Moreover, during the final steady-state of P5 (days 70–120), the R_{bio} had an average 11% (p < 0.05) higher methane yield than R_{con}. To our knowledge, this is the first time that bioaugmentation of ammonia tolerant methanogen significantly improved methane productivity of CSTR fed with OFMSW under the extremely high level of 13.5 g NH_{4}^{+}-N/L.

The gap in VFA concentrations between the two reactors, during the P5 at 13.5 g NH_{4}^{+}-N/L, was consistent with the different methane production (Fig. 2, Fig.3). More specifically, the VFA levels in R_{bio} decreased continuously for over 50 days from 15000 to 5398 mg/L. Each point (Fig.2 and 3) presents the average of consecutive two days, thus the data points of 15000 and 5398 are representative of the VFA reduction profile at P5. In particular, the acetate consumption indicated a faster degradation compared to propionate. On the contrary, VFA accumulation (above 7500 mg/L) in R_{con} progressed as expected in reactors exposed to ammonia (Fig. 3). Hence, the findings in this study confirmed that bioaugmentation of the ammonia tolerant inocula did successfully recover the methane yield and determined a decrease in VFA levels compared to R_{con}. 
Fig. 2. CH$_4$ production in $R_{con}$ and $R_{bio}$, and TAN levels throughout the experimental period. The maximum methane yield means average methane yield in a reactor treating the same OFMSW under the steady-state condition without ammonia addition. The arrow (blue) represents the sampling point for microbial analysis. Each point presents an average of two consecutive days.
3.2 The microbiome composition dynamics

The role of the microbiome in reactor performance was determined using genome-centric metagenomics, which allowed the identification of 67 MAGs as the most
abundant members (≥99.5% of cumulative relative abundance) of the microbiome (Table S2 and S3). 42 out of 67 MAGs were of high-quality (completeness ≥ 90% and contamination ≤ 5%), while the remaining 25 MAGs were of medium quality (50% ≤ completeness ≥ 90% and 5% ≤ contamination ≤ 10%) referring to Genomic Standards Consortium about a Metagenome-Assembled Genome (MIMAG) [44] (Fig. 4; Table S2). The 67 MAGs were taxonomically assigned to five phyla, namely *Firmicutes*, *Actinobacteria*, *Cloacimonadota*, *Bacteroidetes* and *Euryarchaeota* (Fig. 4). The bioaugmentation process induced a marked change in the microbial composition throughout the P4 to P5 (Fig. 4; Table S3). During P3, within the *Firmicutes* phylum, *Peptococcaceae* spp. (DTU 903, DTU900, and DTU895), *Tissierellales* sp. DTU879 and *Syntrophaceticus* sp. DTU783 (55%, 3.7% and 4.4% of cumulative relative abundance, respectively) were the most relevant members identified (Fig. 4; Table S3). In the archaeal community, *Methanoculleus* spp. DTU887, DTU888 and *Methanomassiliicoccales* sp. DTU778 were present at low relative abundance (0.42%, 0.23% and 0.27%) and were assumed to pull OFMSW degradation through methanation of intermediate compounds derived from fermentation (i.e. H₂ and methanol). The low abundance of methanogens was the main cause of the under-performing AD process (e.g. low methane production) during the inhibited P3. Afterwards, the bioaugmented culture triggered the whole microbiome shift during the transition from P3 to P4 (Fig. 4, Fig.5). Specifically, an increment of *Tissierellales* sp. DTU879 (3.3 fold when compared to P3) was observed after bioaugmentation, suggesting that positive interactions were established among these microbes (Fig. 5; Table S3). During P5, the relative abundance of *Methanoculleus* sp. DTU887 was 6.1 fold higher than P3, indicating a successful establishment of the exogenous archaea (*M. bourgensis*) in the
endemic community of R\textsubscript{bio} (Fig. 5; Table S3). Correspondingly, \textit{Peptococcaceae} spp. (DTU 903, DTU900, and DTU895), \textit{Syntrophaceticus} sp. DTU 783 and \textit{Tissierellales} sp. DTU879 increased in abundance by 4.7, 2.4 and 1.5 fold, respectively, when compared to P3 (Fig. 5; Table S3).

Fig. 4. Microbial samples collected from R\textsubscript{bio} at different time points: on days 31 (P3), 56 (P4), and 91 (P5). The characteristics (taxonomic assignment, coverage and MIMAG values) of 67 MAGs composing the microbiome are presented. The outer layer represents the taxonomy at the phylum level. The four middle layers represent the relative abundance (%) of each MAG in the different phases. The four inner layers
represent completeness (%), contamination (%), number of scaffolds, and genome size (Mbp) colored in pink, purple, grey and black, respectively. The middle tree represents the euclidean clustering of MAGs based on their relative abundances.

Fig. 5. The specific variation trends of dominant MAGs was obtained by calculating the fold change (log2) between the different periods.

3.3. Species interactions among key microorganisms

The functional role of individual MAGs was determined based on the information of their metabolic potential combined with relative abundances in the different conditions. In this way, a network of metabolic interactions occurring in the microbiome at high ammonia levels was manually reconstructed (Fig. 6; Table S4).

After substrate hydrolysis, amino acids, fatty acids and sugars are generally degraded...
into acetate, \( \text{H}_2 \), and \( \text{CO}_2 \), which are the main feed sources for SAOB and methanogens. The roles of microbes involved in the key metabolic pathways related to acetate and \( \text{H}_2 \) formation/oxidation were established.

### 3.3.1 Acetogenic-related related community

The cumulative relative abundance of dominant bacteria (Peptococcaceae sp. DTU903, Peptococcaceae sp. DTU895, Peptococcaceae sp. DTU900, and Tissierellales sp. DTU879) accounted for more than 50% through the entire period (Fig. 4; Table S3), suggesting their major role in degrading OFMSW. The high content of carbohydrates (65%) [28] in OFMSW and the presence of two distinct glucose metabolic pathways in these dominant bacteria reveals that glucose is a necessary intermediate product during organic waste catabolism (Fig. 6; Table S4 and Table S5). More specifically, in the first glucose degradation pathway (identified as complete only in Tissierellales sp. DTU879) (Fig. 6; Table S4), pyruvate produced through the Embden-Meyerhof-Parnas (EMP) process was directly converted to \( \text{H}_2/\text{CO}_2 \) via the glycine cleavage system and tetrahydrofolate pathways, as previously reported [26]. The incomplete pathway in Peptococcaceae spp. (DTU895, DTU900 and DTU903) intriguingly suggests a possible interspecies exchange of nutrients and intermediates products occurring during glucose degradation. The second glucose degradation pathway starts from pyruvate production through the EMP process, and follows with acetate and \( \text{H}_2 \) as the end products (Fig. 6; Table S4). The genes of rhodobacter nitrogen fixation complex (\( rnf \)) harbored in Peptococcaceae spp. and Tissierellales sp. DTU879 had the potential to produce reduced ferredoxin. The utilization of these high-energy electron carriers is essential to achieve the endergonic electron transfer in AD systems, specifically facilitating the reactions of \( \text{NAD}^+ \) reduction to NADH, \( \text{H}^+ \).
reduction to H₂ and pyruvate reduction to acetyl-CoA (Fig. 6; Table S4). It is consistent with previous finding of the key role of reduced ferredoxin for driving redox reactions [47]. The presence of rnf potentially supports the degradation of organic matter to acetate, H₂ and ATP, and possibly confers an advantage to the dominant bacteria (Peptococcaceae spp. related members, and Tissierellales sp. DTU879). However, both glucose conversion pathways are dependent from the low concentrations of H₂ and acetate, as previously reported by Zhu et al. (2019) [26]. More specifically, H₂ and NAD⁺ should be always regenerated from NADH during the above processes; in fact, NAD⁺ is an essential electron acceptor for the anaerobic degradation of sugars, amino acids, and fatty acids. Nevertheless, NAD⁺ formation is impossible at a high H₂ partial pressure and thus the produced CO₂/H₂ needs to be scavenged by hydrogenotrophic methanogen for methanogenesis which thereby drives glucose degradation at real reactor conditions [48]. In the present study, MAGs reconstruction provides evidence of cooperation between Tissierellales sp. DTU879/Peptococcaceae spp. and Methanoculleus sp. DTU887 (Fig. 4, Fig. 6), which is consistent with previous findings [26, 27]. Thereby, syntrophic glucose degrading-communities exploited possible thermodynamic reactions to conserve the maximum amount of energy.

Additionally, Syntrophomonadaceae and Syntrophothermaceae groups might participate in the syntrophic butyrate and propionate oxidation. Functional annotation of MAGs assigned to Syntrophaceticus and Syntrophomonadaceae revealed a partially complete butyrate/propionate degradation pathways, probably due to the incompleteness of some genomes (Table S5). As evidenced by a previous study, Syntrophomonas related members were obligate syntrophic bacteria able to utilize various fatty acids, especially butyrate [49]. However, in the present study, their low abundance (less than
2% suggests a minor role in the global community, and the scarce butyrate detected in the reactor suggests a negligible contribution to OFMSW degradation.

### 3.3.2 Acetate degraders

The extremely high concentrations of acetate throughout P1 to P3 (Fig. 3) indicates that acetate degraders were less active than fermenting bacteria. Acetogenic microbes are assumed to play an important role in the AD system, scavenging the acetate produced during fermentation, which pulls the degradation of glucose/VFA and alleviates acetate-ammonia synergistic inhibition [50, 51]. For example, *Syntrophaceticus* sp. DTU783 encodes two complete gene sets involved in the reversed Wood-Ljungdahl pathway and the alternative acetate degradation through the glycine cleavage system (Fig. 6; Table S4). The hydrogenase involved in energy conservation in these two pathways was different. Specifically, during acetate degradation through glycine cleavage system, 1 Fdred was employed for electron transport, instead of NADH, thus 1 more ATP was generated per each molecule of acetate compared to that of the reversed Wood-Ljungdahl pathway.

During the P5, the increased relative abundance of *Syntrophaceticus* sp. DTU783 (2.4 fold when compared with P3), occurring in response to bioaugmentation, greatly lowered the acetate concentration (Fig. 3, Fig. 5; Table S3). This reduction subsequently promoted VFAs and/or glucose degradation due to reactions equilibrium, which is consistent with previous findings [52]. As evidenced by the negative correlation between the abundance of *Syntrophaceticus* sp. DTU783 and acetate concentration (Fig. S2), we proposed DTU783 as the key acetate metabolizer in the system, possibly acting in syntrophy with the bioaugmented *Methanoculleus* sp. DTU887 (Fig. 6). This result highlights again that the exogenous addition of *Methanoculleus* sp. DTU887 represents
a prime remedy to guarantee the optimized performance in ammonia-inhibited mesophilic processes. Interestingly, also the other dominant bacteria (Tissierellales sp. DTU879, Peptococcaceae spp.) harbored the genes for acetate degradation via glycine cleavage pathway (Fig. 6; Table S5). As Nobu et al. (2015) proposed, in comparison to the Wood-Ljungdahl pathway, there is no endergonic 5-methyl-THF oxidation in this alternative acetate degradation, whereby intermediates produced could be easily used for biosynthesis [47]. Therefore, acetate oxidation through the acetate-glycine pathway might be another biological solution to overcome the thermodynamic limitations, especially when hydrogenotrophic methanogens are present at low relative abundance [53, 54]. The multiple strategies to degrade carbon sources and regulate energy conversions could be the winning strategy put in place by microbes to optimize their energy metabolism when a suitable partner is absent, or in response to varying environmental conditions.

3.3.3 Methanogenesis

*Methanoculleus* spp. are known to be involved in the utilization of hydrogen produced by syntrophic acetate-producing species or by fatty acid-oxidizing microorganisms [55, 56]. The complete pathway for H\(_2\)-mediated reduction of CO\(_2\) to CH\(_4\) was identified in *Methanoculleus* sp. DTU887 (Fig. 6). The first step of this pathway is the reduction of CO\(_2\) with H\(_2\) to formyl-MFR, which is subsequently reduced to CH\(_4\), in a process catalyzed by Fwd, Ftr, Mch, Mtd, Mer, Mtr and Mvh-Hdr respectively (Fig. 6; Table S4). The presence of methanol dehydrogenase (mdh) and its electron acceptor - a specific c-type cytochrome (Fig. 6; Table S4), is associated with the high affinity for H\(_2\), which confirms the high growth yield on H\(_2\) of *Methanoculleus* sp. found in the previous study [57].
Besides, the presence of genes encoding multiple energy regulating complexes performing H\(^+\) and Na\(^+\) translocation (mtr, hydrogenase subunits (eha), the energy-converting hydrogenase (ech), Na\(^+\)/H\(^+\) antiporter (mnh) and V/A type ATP synthase) were proposed to contribute to *Methanoculleus* sp. DTU887 robustness (Fig. 6; Table S4). Specifically, Mtr, Eha hydrogenase subunits, and Na\(^+\)/H\(^+\) antiporter are assumed to be sodium ion translocating, while Ech hydrogenase subunits, as well as the ion pump and V/A type ATP synthase, should be proton translocating [58]. Overall, the presence of multiple ion-translocating electron transfer complexes, and membrane-bound Fe-S proteins with connected heterodisulfide reductase in the genome of *Methanoculleus* sp. DTU887, can modulate the energy status of the cell in response to varying thermodynamic conditions. This confirms the previous observations of the flexible energy conversion approach, which is taking advantage of the positive energy margin [59, 60]. Besides, *Methanoculleus* sp. DTU887 harbors genes potentially involved in osmolytes uptake system (*kdp, putp, and proW/V*), as well as the *mnhBCDE* complexes responsible for pH homeostasis and balancing external H\(^+\)/Na\(^+\) ratios. These genetic characteristics of *Methanoculleus* sp. DTU887 (the bioaugmented culture) enable its high tolerance to extreme ammonia levels and drive the syntrophic substrate degradation in situations of ammonia stress.

Additionally, the increased abundance of *Methanoculleus* sp. DTU887 (from P4 to P5) decreased the inter-microbial distances occurring between H\(_2\)-producing bacteria and methanogens. As a consequence, the H\(_2\) diffusion distance diminished, thus the interspecies transfer rate increased and methanogenic activity was enhanced. Hydrogen consumption by methanogens can lower the partial pressure, and enhance the rate of acetate degradation promptly [61, 62]. Moreover, the addition of *Methanoculleus* sp.
DTU887 perturbed the microbiome structure, triggering modification of the indigenous microbial community with the increase of hydrogenotrophic methanogens (Fig. 4), which finally resulted in a huge increase in methane yield (21%) (Fig. 2).

Hence, *Methanoculleus* sp. was critical to recovering the AD system from ammonia inhibition: its increase in abundance accelerated the consumption of VFA and other intermediates by strengthening the electron transfer between syntrophic microbes. Therefore, the resume of a complete chain of energetic electron transfer among the OFMSW-degrading microbiome renders the successful bioaugmentation.
Fig. 6. Metabolic reconstruction of the OFMSW degrading syntrophic community. Metagenomic data revealed pathways involved in substrate degradation, in the utilization of intermediates compounds (H₂/CO₂, acetate) and in methanogenesis. All the relevant genes included can be found in Table S4.

4. Conclusions

This study is the first comprehensive investigation supported by metagenomics analysis revealing the interconnected metabolic pathways induced by the addition of Methanoculleus sp. to AD of OFMSW. Implementation of cutting-edge technology based on microbial functional reconstruction and insights into the varying conditions of AD allowed a more clear investigation of the OFMSW-degrading community. This study proved the importance of a critical amount of methanogens for a fully-functional AD process. Additionally, it also provided evidence for the occurrence of important interactions among different microbiome members by revealing the presence of the genes (e.g. gcvp, metF and ackA) related to glucose and acetate degradation pathways. The presence of multiple pathways might provide enough flexibility to some species, such as Peptococcaceae spp., Tissierellales sp. DTU879, and Syntrophaceticus sp. DTU783, to recover the AD efficiency under extremely high ammonia levels. This study provides strong evidence regarding the importance of Methanoculleus sp. to enhance the ability of the anaerobic reactor to withstand the shock determined by VFA over-load and ammonia inhibition.

List of abbreviations

MAG Metagenome assembled genome
PTA-ACKA phosphotransacetylase-acetate kinase pathway
ACS  forming acyl-CoA synthetase
acs  acyl-CoA synthetase gene
ack  acetate kinase gene
mcrA  methyl coenzyme M reductase gene
mta, mtb  coenzyme M methyltransferase genes
coo  carbon monoxide dehydrogenase gene
gcv  glycine decarboxylase genes
grd  glycine reductase gene
Vho  methanophenazine-reducing hydrogenase
Frh  coenzyme F₄₂₀-reducing hydrogenase
Ech  energy converting hydrogenase
Fpo  F₄₂₀H₂ dehydrogenase
hdr  membrane-bound heterodisulfide reductase gene
Ehb  energy-conserving hydrogenase B
WL  Wood-Ljungdahl
Rnf  Rhodobacter nitrogen fixation complex
EMP pathway  Embdene Meyerhofe Parnas pathway

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