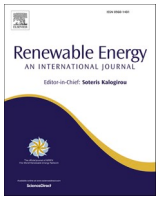
Renewable Energy 225 (2024) 120270

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene



Bioaugmentation strategies based on bacterial and methanogenic cultures to relieve stress in anaerobic digestion of protein-rich substrates

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ARTICLE INFO

Keywords:
Anaerobic co-digestion
Hydrogenotrophic methanogens
Bioaugmentation
Ammonia inhibition
Casein
Maize silage

ABSTRACT

Anaerobic co-digestion of protein-rich substrates is a prominent strategy for converting valuable feedstocks into methane, but it releases ammonia, which can inhibit the overall process. This study developed a cutting-edge combined culturomic and metagenomic approach to investigate the microbial composition of an ammonia-tolerant biogas plant. Newly-isolated microorganisms were used for bioaugmentation of stressed batch reactors fed with casein, maize silage and their combination. A co-culture enriched with proteolytic bacteria was isolated, selected and compared with the proteolytic collection strain *Pseudomonas lundensis* DSM6252. The co-culture and *P. lundensis* were combined with the ammonia-resistant archaeon *Methanoculleus bourgensis* MS2 to boost process stability. A microbial population pre-adapted to casein was also tested for evaluating the digestion of protein-rich feedstock. The promising results suggest combining proteolytic bacteria and *M. bourgensis* could exploit microbial co-cultures to improve anaerobic digestion stability and ensure stable productivity even under the harshest of ammonia conditions.

1. Introduction

Renewable energy sources capable of replacing fossil fuels are considered fundamental strategies to face the energy and environmental crisis [1]. Biogas produced by digesting organic material is a fuel gas rich in methane, rated as one of the unquestioned protagonists of the green economy [2]. It can generate heat and energy [3] and, when enriched in methane content (>95%), can be used as a vehicle fuel or injected into the gas grid [4]. The environmentally friendly anaerobic digestion (AD) process, by generating biogas and fertilising digestate, represents an interesting solution for the management of waste materials and residues from agriculture and industry (e.g. manure and crop biomass), municipal solid waste, sewage sludge, and urban wastewater, considering the potential of those products to replace fossil fuels and mineral fertilisers starting from valueless wastes [5,6].

AD is a biologically mediated process in which the whole microbial community cooperates in the degradation of organic substrates, resulting in biogas and digestate production. The functionality of a biogas system is strictly related to the microbial community [7]. The knowledge of microbial hierarchy, particularly in AD systems, has been recently obtained with high throughput sequencing techniques but some limitations towards the full microbiome picture are still present [8]. Microbial species present at low abundance are difficult to be detected, a threshold value for the taxonomic assignment at the species level is difficult to be set [9], and some copies of the 16S rRNA gene can be acquired by horizontal gene transfer [10]. For these reasons, culturomics is considered a valid and complementary strategy to metagenomics for microbial studies. However, culturable microbes represent only a small fraction of the microbiota, with an estimation of approximately 95–99.9% of the organisms of interest not readily culturable [11].

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https://doi.org/10.1016/j.renene.2024.120270
Received 26 April 2023; Received in revised form 5 February 2024; Accepted 5 March 2024
Available online 5 March 2024

Anyhow, sequencing approaches (like 16S rRNA gene sequencing applied in this study) became essential to support microbiomes' and isolates' characterisation.

Therefore, a focus on AD microbial features is necessary, as it allows to identify microbes with potential resilience in unique environments as those distinguished by inhibiting conditions. In numerous organic wastes, such as those generated by whey, cheese, fish, and some vegetables industries, proteins are abundantly present [12,13]. During AD these macromolecules determine the release of ammonia, of which high levels in the system are estimated to be the primary cause of process inhibition [14,15]. Although NH_3 concentrations below 0.2 g/L are necessary for the microbial cells to grow, levels higher than 2 g/L can hinder biogas production [16]. In particular, ammonia concentrations ranging from 3.5 to 7 g/L were reported to drastically reduce methanogens activity [17].

The inhibiting effects of ammonia are related to its hydrophobic form which penetrates the membranes causing intracellular damage [17]. For this reason, FAN (Free Ammonia Nitrogen: NH_3) is more toxic than TAN (Total Ammonia Nitrogen: $NH_3 + NH_4^+$). Since the two forms are in dynamic equilibrium, their concentrations depend on pH and temperature [18]: the increase of both these parameters leads to higher levels of free ammonia. Indeed, thermophilic cultures have been shown to be more sensitive to ammonia stress [19]. Within the microbial population, acetoclastic archaea are the most sensitive regarding this stress, as their metabolic activity is reduced and consequently, fermentation intermediates (volatile fatty acids (VFA)) accumulate in the system leading to process inhibition [20,21]. When acetoclastic methanogenesis can no longer be performed, a shift in the acetate utilisation occurs: the oxidation of acetate to H_2 and CO_2 via the reverse Wood-Ljungdahl pathway by syntrophic acetate-oxidising bacteria (SAOB, more robust to ammonia stress) rises, sustaining hydrogenotrophic methanogenesis [22,23].

To relieve the inhibition of high ammonia in AD, different solutions have been tested, including the ammonia extraction, control of process parameters and adjustment of operational ones (as C/N ratio, pH, temperature) [24], addition of exogenous materials (e.g. carbon-based additives, iron-based additives, adsorbent materials), co-digestion, microbial acclimatation and bioaugmentation [15,25–27]. Some of these approaches, such as pH control and temperature regulation, proved to be inefficient or economically expensive. On the contrary, the adaptation of the methanogenic population to high levels of ammonia, and the correlated change in the methane production pathways, was found to be effective [27]. Bioaugmentation represent an interesting solution, enriching the AD system with a microbial consortium or a pure culture pre-adapted and/or tolerant to ammonia-related stress [28].

In pursuit of optimising the AD for protein-rich substrates, this research focused on the isolation of novel bacterial strains or co-cultures for their potential application when the ammonia produced during protein hydrolysis reaches microbes-inhibiting levels. An interesting co-culture mainly represented by the proteolytic bacterium *Tissierella praeacuta* was characterised and used to facilitate the digestion of casein in AD settings. Several bioaugmentation strategies were then pursued exploiting the novel and promising co-culture as well as strains of a proteolytic bacterium or a hydrogenotrophic archaea obtained from cultures collection, in addition to a culture acclimated to casein. Variations in methane yields and environmental parameters, such as pH and ammonium levels, were then monitored to assess the bioaugmentation efficiency in a protein-fed AD process. This study lays the groundwork for the translation of knowledge obtained from studies on reactors' microbial compositions into practical applications, specifically the bioaugmentation of selected and/or adapted microorganisms in other inhibited reactors.

2. Materials and methods

2.1. Sampling of an industrial-scale biogas plant

The mesophilic (42 °C) biogas plant considered in this study is located in Chiari (Brescia, Italy), and equipped with three digesters: hydrolysis (H), fermenter (F) and post-fermenter (P). At the sampling time the plant was fed with a mixture of chicken manure, maize silage, and pig slurry. The substrate mix is transferred from the pre-hydrolysis tank to the first fermenter, where the main digestion takes place, and subsequently to the post-fermenter where the residual materials are degraded. The biogas plant has been working for years in stable conditions with an average ammonium level of 5.5 g NH_4^+-N/L, a pH of 8.0 and a Flüchtige Organische Säuren/Totales Anorganisches Carbonat (FOS/TAC) ratio of 0.23. Samples from each digester were kept at 42 °C to perform species isolation, and one aliquot for each of them was sequenced for molecular characterisation of the microbiota.

2.2. DNA extraction and 16S rRNA sequencing

Genomic DNA of the microbial communities collected from H, F and P digesters, together with pure microbial strains, was extracted with the DNeasy PowerSoil kit (QIAGEN, Germany). In order to recover the pellet from microbial isolates, samples of 10 mL were previously centrifuged at 4000 rpm, 4 °C for 15 min. The concentration and quality of purified genomic DNA were estimated using NanoDrop 2000 (ThermoFisher Scientific, MA, USA) and Qubit 2.0 fluorometer (Invitrogen, MA, USA), using the Qubit dsDNA High Sensitivity assay kit (Invitrogen).

Amplification of hypervariable region V4 of the 16S rRNA gene of digesters samples was performed using the degenerated primers pair 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) as suggested by Parada et al. [29].

For isolates identification, the combination of universal primer 10F (5′-AGTTTGATCCTGGCTCAG-3′) [30] and degenerated primer 806R were used to amplify the rRNA 16S gene sequences. The PCR conditions used for all the reactions were: 32 cycles of a program with a denaturation phase of 45 s at 98 °C, an annealing of 40 s at 58 °C, and an elongation of 50 s at 72 °C. A final extension phase at 72 °C for 5 min was performed at the end of the amplification.

Processing and sequencing of the H, F and P amplicons were performed with the Illumina MiSeq paired-end platform at the NGS facility of the Biology Department of the University of Padova (Padova, Italy). Raw reads (250 + 250 bp) have been submitted to the sequence read archive database (SRA) of NCBI (https://www.ncbi.nlm.nih.gov/sra) under the BioProject PRJNA866107 with accession numbers SRX16984443, SRX16984444, and SRX16984445. The amplicons of the 10–806 region of the 16S rRNA gene of isolates were sequenced with the Sanger method at BMR genomics S.r.l. (Padua, Italy), after being purified using AMPure XP beads (Beckman Coulter, CA, USA) to remove the primers following the instructions of the supplier.

2.3. Bioinformatic analyses

Raw reads in fastq format from H, F and P were analysed with CLC Genomics workbench software V.20.0.4 with microbial genomics module plug-in (CLC Bio, QIAGEN), using SILVA 138 as the amplicon reference database. The taxonomic assignment of each Operational Taxonomic Unit (OTU) and Sanger sequence was manually verified with similarity search through an alignment with NCBI Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using 16S ribosomal RNA sequences (Bacteria and Archaea) as database. Multi experiment viewer software (MeV 4.9.0) [31] was used for visual representation of the high abundant OTUs as a heatmap, and the Pearson's correlation was adopted for the hierarchical clustering, highlighting OTUs with similar behaviour.

The Sanger sequences were visualised with the Chromas 2.6.6

software (http://www.technelysium.com.au/chromas.html).

2.4. Isolation and cultivation of microorganisms

Enrichment and isolation of the microorganisms were carried out under an anaerobic glove box with N_2 atmosphere (MBRAUN MB200B, Germany). To isolate the highest number of bacterial strains, basal anaerobic (BA) medium [32] was specifically supplemented with three different carbon sources: nutrient broth (NB) powder (No. 3 Sigma-Aldrich, MO, USA, 13 g/L) to support the growth of general anaerobic bacteria, acetate (2.5 g/L) with the aim of isolating archaea and/or SAOB, and skim milk (SM) powder (Sigma-Aldrich, 10 g/L) to facilitate the selection of proteolytic microbes. Yeast extract (YE) (LP0021, 26 Oxoid Limited, Cheshire, England) and a double volume of vitamin solution, with respect to that suggested in the BA medium preparation protocol, were added to better sustain the microbial growth.

The three samples from H, F and P reactors were filtered to remove larger particles. The suspensions were then serially diluted, plated on agar medium with acetate, NB or SM as carbon source and incubated at 42 °C under anaerobic conditions for 21 days, after which CFU (colony forming units) were determined. Anaerobic conditions were obtained in anaerobic jars (Oxoid) flushed with CO_2 and H_2 gas in a ratio of 1:4. All steps were conducted in triplicates.

From each sample, colonies were purified through consecutive streakings. Colonies of interest were then inoculated in liquid medium with the same carbon source used for their isolation, and the volumes were subsequently increased to the final working volume of 50 mL in 120 mL bottles. Bottles were closed with butyl-rubber stoppers and sealed with aluminium crimps to avoid any possible gas exchange and oxygen presence. Cultures have been incubated in the dark at 42 °C, and biogas and VFA productions have been measured to metabolically characterise the microorganisms. The isolates were kept active by continuous subcultures (approximately every 30 days), while long-term storage at −80 °C was performed by adding 20% glycerol (v/v) suspension.

The protein-acclimated culture was obtained as previously described [33].

2.5. Bioaugmentation experiment set up

The bioaugmentation experiment was applied on an AD system selectively fed with maize silage, casein, and their co-digestion. The inoculum used to start up the batches derived from a mesophilic lab-scale reactor in Thessaloniki (Greece), fed with bovine manure as the main substrate. The characteristics of the inoculum utilised for the experiment are described in Table 1.

Four sets of cultures were used for the bioaugmentation. A hydrogenotrophic archaea, *Methanoculleus bourgensis* MS2 (DSM3045), and a proteolytic bacterial strain, *Pseudomonas lundensis* DSM6252, were purchased from DSMZ GmbH (Leibniz Institute, Germany). *M. bourgensis* MS2 was grown at 37 °C in 120 mL (40 mL working volume) anaerobic batch reactors in the medium DSMZ 332, with the addition of H_2 and CO_2 gas mixture (80/20, v/v) as substrate. Growth was weekly monitored by measuring methane production and optical density (OD_{600nm}). *P. lundensis* DSM6252 was aerobically grown in NB at 25 °C. A co-culture enriched with *Tissierella* species, obtained in the current work, was also selected for its proteolytic capabilities. At last, the third generation of the casein-acclimated culture [33] was also tested.

Batch reactors with an organic loading (OL) of 4 g VS/L were set up in triplicate using 120 mL bottles, operating at inoculum to substrate (I:S) ratio of 9.5:1. Substrates were mixed with 24 g of mesophilic inoculum and an amount of distilled water required to reach the final working volume of 30 mL. Bioaugmentation cultures have been added to a concentration of 10% (v/v) of the final working volume. According to our previous experience, additional control with sterilised bioaugmentation cultures was not needed due to the low added volume of them. Additionally, since these cultures already efficiently degraded most of the carbon matter present into the media at the moment of bioaugmentation, the residual one was negligible in comparison to the organic loading of the tests. To establish anaerobic conditions, both liquid and headspace were flushed with N_2 for 3 min each. The bottles were immediately closed with rubber stoppers, sealed with aluminium crimps, and incubated at 42 °C. Once per day the bottles were manually shaken to keep the solution well homogenised and every two days biogas production was monitored using the water displacement method. Biogas composition in terms of H_2, CO_2 and CH_4 was measured by a gas chromatograph, and values expressed under the standard conditions of temperature and pressure, i.e., at a temperature of 0 °C and a pressure of 1 atm. Benchmark experiments, containing only inoculum and distilled water, were also performed. Methane values were expressed in mL CH_4/g VS.

Table 1
Characteristics of the inoculum and substrates (NA: not available; DM: dry matter).

Parameters	Unit	Inoculum	Maize silage	Casein
TS	(% of DM)	6.52 ± 0.06	37.60 ± 0.31	91.59 ± 0.21
VS	(% of DM)	4.72 ± 0.01	36.71 ± 0.24	90.12 ± 0.19
pH		8.05		
VFA	(mg/L)	138.79 ± 9.95		
NH_4^+-N	(mg/L)	4692.30 ± 100.98	NA	NA

2.6. Analytical methods and statistical analysis

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN) and pH were determined according to APHA Standard Methods for the Examination of Water and Wastewater [34].

CH_4, H_2 and CO_2 content in biogas were determined three times per week using a gas chromatograph (490 Micro GC, Agilent Technologies, CA, USA) equipped with a thermal conductivity detector (TCD) and two different capillary columns, one using argon as carrier gas and the other using helium, operating at 145 °C, 30 psi and 100 °C, 28 psi, respectively. Data were analysed by SOPRANE 2 software (S.R.A. Instruments, France). For VFA analysis, samples were previously filtered using 0.22 μm cellulose acetate membrane and analysed through Shimadzu Nexera HPLC system, equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The chromatographic separations were performed using a Phenomenex Rezex ROA-Organic Acid H^+ (8%) column (300 mm × 7.8 mm) set at 65 °C. The analysis was performed at a flow rate of 0.6 mL/min using isocratic elution, with 5 mM H_2SO_4 as a mobile phase. VFA Mix 10 mM (Sigma-Aldrich) was used as a reference standard. Analytes were identified by comparing their retention times and the concentrations were calculated by exploiting calibration curves of the corresponding external standard. All the determinations were performed in triplicate.

Statistical evaluation of the data set was also assessed using RStudio v3.6.3 (https://www.rstudio.com/), and the fmsb package v0.7.3 (https://cran.r-project.org/web/packages/fmsb/index.html). A *t*-test assuming a normal distribution of data has been performed to evaluate statistical significance of methane yield of single conditions compared to the others. The *p*-value has been corrected considering non-independent tests, through FDR (false discovery rate) [35].

3. Results and discussion

3.1. Microbiome analysis of the digesters

A preliminary outline of the microbial composition of hydrolysis (H), fermentation (F) and post-fermentation (P) mesophilic digesters of a full-scale biogas plant located in Chiari (Brescia, Italy) was obtained by 16S rRNA gene sequence analysis. Total raw data includes 142566,

121276 and 102521 filtered reads for H, F and P, respectively. Up to 76% of the microbial community consists of Bacteria, with Firmicutes as the most abundant phylum (62–65%) followed by Bacteroidetes (18–27%) and Euryarchaeota (6–17%); furthermore, also Synergistetes and Deinococcus-thermus are identified at low levels (2 and 1%, respectively) (**Supplementary Data**). Euryarchaeota is the unique phylum of the Archaea domain with a marked difference in abundance across the three microbial systems, with the highest value (17%) detected in the post-fermenter. This amount highly exceeds the one in the fermenter, confirming the continuous and intensive activity of methanogenic archaea also in the post-fermentation reactor.

The eighteen most abundant families are reported in Fig. 1a. The 25% of the OTUs belong to the family Oscillospiraceae, followed by Clostridiaceae and Methanomicrobiaceae, particularly abundant in the post-fermenter, and Erysipelotrichaceae, Dysgonomonadaceae and Marinilabiliaceae. Since the taxonomy of the family is not strictly related to the metabolic function of the microorganism, a classification at the genus level is required, resulting more valid from a functional perspective. Although 16S analysis exhibits limitations in taxonomic and functional assignment, this technique was sufficient to provide valuable insights into the microbial community under investigation. Despite differences in microbial communities of three reactors, a microbial core composed by *Ercella*, *Methanoculleus*, *Clostridium* and *Proteiniphilum* is shared by all digesters, representing half of the community in the post-fermenter (**Supplementary Data**). In fact, the hydrolytic and fermentative reactors are characterised by similar microbial compositions. In particular, *Ercella* and *Proteiniphilum* respectively are a carbohydrate-fermenting and a proteolytic bacterial genus [36,37], which are fundamental for the discussed reactors. Since many OTUs did not have a high level of similarity with reference sequences in the public database, only few OTUs can be tentatively classified to the species level. Considering the highly divergent functions of the different reactors, considerable variations in OTUs distribution were expected and can be observed between H and P digesters (Fig. 1b). Moreover, P was also found to be rich in *M. bourgensis* OTU13 and Clostridiaceae and Bacteroidales species. *M. bourgensis* is affiliated to Methanomicrobiaceae

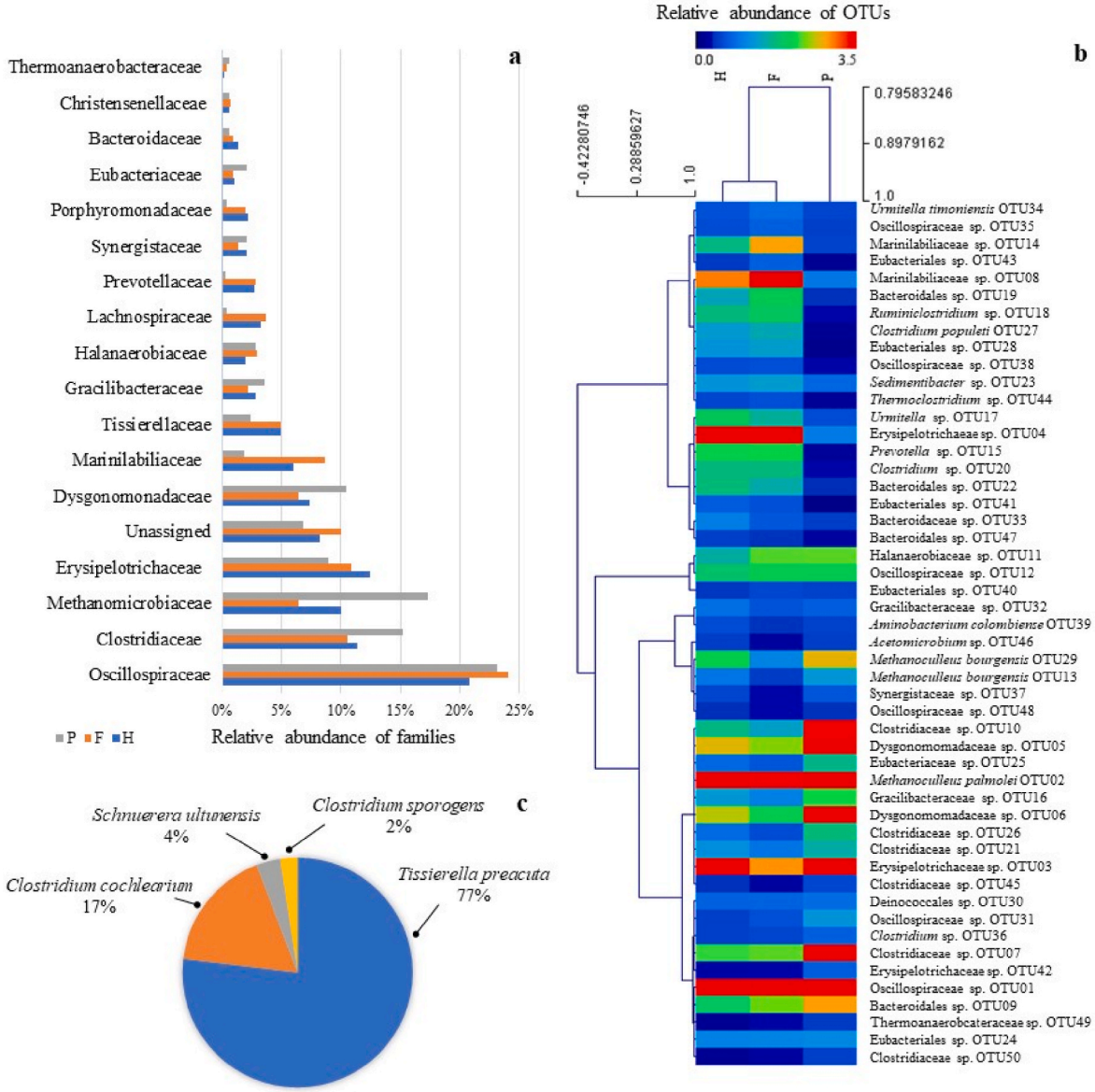

Fig. 1. (**a**) Phylogenetic assignment based on 16S rRNA sequence analysis related to families (%) in H, F and P digesters, (**b**) heatmap of OTUs relative abundance in each reactor and (**c**) relative abundance of species of the enriched co-culture used for bioaugmentation. Hierarchical clustering in (**b**) was made with Pearson's correlation coefficient.

family and it is frequently reported to play a central role in biomethanation [38]. It is worth noting that the dominance of the *Methanoculleus* identified in these reactors indicates that the predominant methanogenic pathway was hydrogenotrophic. This is in agreement with studies of microbial population of biogas plants treating protein-rich substrates [13,39] as the ammonia released by their digestion drives the methanogenic process from the most common acetoclastic pathway to the hydrogenotrophic one [27].

3.2. Isolation and characterisation of enriched microbial consortia and single species

During the isolation procedure, the highest number of CFUs (nearly 3×10^6 CFU/mL) was detected on NB and SM media, whereas lower CFUs (5×10^5 CFU/mL) were obtained from acetic acid. A total of 40 representative isolates from the three media, in single culture or co-culture, were tested for their ability to produce VFA, H_2, CO_2, and methane from different carbon sources. Isolates having the phenotypic properties of interest were selected and identified by 16S rRNA sequencing (Table 2). All of them were assigned to Firmicutes phylum, and, more specifically, to classes of Clostridia (four isolates), Bacilli (two isolates) and Tissierellia (three isolates). *Schnuerera ultunensis* is indeed a SAOB, as it establishes a syntrophy with hydrogen-consuming archaea [40]. It is also commonly abundant in AD systems releasing ammonia from substrate degradation, supporting methane production via hydrogenotrophic pathway, usually dominated by *Methanoculleus* spp [27]. High throughput 16S rRNA amplicon sequencing of this co-culture disclosed that it is mostly composed by *T. praeacuta* (Fig. 1c). The total number of 16S rRNA amplicon reads for the sample of interest was 264910, with an average length of 289 bp. In the selected culture, the OTU assigned to *T. praeacuta* (100% identity) showed a 66.9% relative abundance, whereas *C. cochlearium* (100% identity) and *S. ultunensis* (94.9% identity) were also assigned to OTUs with 14.6% and 2.8% relative abundance, respectively. All the selected isolates were able to produce various levels of CO_2 and H_2 from different substrates. H_2 production varies from 1.8 to 15% whereas CO_2 from 5.7 to 25.7% (Table 2). The highest levels of H_2 were produced by the isolates L6 and L21, as well as by the co-culture I4. When acetate was used as a carbon source, *S. ultunensis* L21 produced 25.7% of CO_2 and 12.2% of H_2. This result agrees with other studies describing *S. ultunensis* as able to convert acetate into CO_2 and H_2 through the reverse Wood-Ljungdahl pathway [41,42]. Although *Bacillus thermoamylovorans* I6 mostly produced CO_2 and limited amounts of H_2, it can be used to boost H_2 production from biomass if co-cultures with *Clostridium* sp. strains [43]. Substantial quantities of CO_2 and H_2 were also released by *Caproiciproducens galactitolivorans* L6, supporting what was previously described by Kim et al. [44], according to which this species can convert skim milk into simple molecules, such as gases and VFA.

Despite the strong variability observed, all the selected strains produced a significant amount of VFA (Table 2). Regarding the VFA profile, acetate was the most abundant, while butyric, formic and isovaleric acids were identified in some cultures. *C. galactitolivorans* L6 produced the largest VFA profiles as formic, acetic, butyric and isovaleric acids were detected in the spent broth containing skim milk as the main carbon source.

C. cochlearium L3, a potential proteolytic organism involved in cheese spoilage [45] produced butyric and acetic acid from NB. This is in accordance with other papers describing this species as producer of both VFA from various protein-rich carbon sources [46], which are abundantly present in the NB formulation. As expected, isovaleric and acetic acids were produced by *S. ultunensis* L21 in agreement with other studies [42].

A co-culture named I4 showed promising technological traits, being able to produce the highest levels of H_2 and CO_2 (15 and 25.5%, respectively) from skim milk (Table 2). Furthermore, the production of interesting amounts of formic, acetic, and butyric acids was observed by this co-culture along with proteolytic activities, which were confirmed on plate (data not shown), suggesting the potential of these microbes to be adopted for AD of protein-rich organic waste streams.

3.3. Bioaugmentation strategy to optimise AD of protein-rich substrates

A previous experiment of co-digestion of maize silage and casein involving the same inoculum used in this work and the addition of the proteolytic bacteria *P. lundensis* DSM6252 resulted in a methane yield decrease (data not shown). No biogas enhancement was observed during the experiment while increase in ammonia was prominent. Considering the toxicity of ammonia produced during the AD process, a combination of *M. bourgensis* MS2, *P. lundensis* DSM6252 and co-culture I4 were tested to enhance protein hydrolysis. The selection was based on a previous study indicating hydrogenotrophic methanogens, specifically *Methanoculleus* spp. [27], as the most resistant archaea to ammonia. Besides, *P. lundensis* DSM6252 and the co-culture I4 could enhance protein hydrolysis. In this way ammonia-tolerant methanogen, or a community acclimated to a double casein OL, was adopted to increase the methanation rate.

Casein and maize silage were here selected as representative substrates for proteinaceous and agricultural AD feedstocks, respectively.

Table 2
16S rRNA gene sequencing of selected bacterial strains anaerobically isolated from acetate, SM and NB and their technological properties in terms of VFA and gas production from different carbon sources.[a] Partial pressure. [b]Residual acetic acid after bacterial growth on acetate.

Strain	Main carbon source	Closest related NCBI GenBank entry			VFA (g/L)				Final products (%)[a]	
		Class	Species	16S rRNA identity (%)	Formic acid	Acetic acid	Butyric acid	Isovaleric acid	H_2	CO_2
I2	Acetate	Bacilli	*Kyrpidia tusciae*	97.9		1.2[b] ± 0.1			1.8	5.7
L1	Acetate	Tissierellia	*Clostridium tepidum*	99.2		1.9[b] ± 0.1			3.5	9.8
L21	Acetate	Tissierellia	*Schnuerera ultunensis*	95.4		1.5[b] ± 0.1		0.4 ± 0.1	12.2	25.7
L3	NB	Clostridia	*Clostridium cochlearium*	99.1		1.3 ± 0.1	0.3 ± 0.1		4.8	11.1
L14	NB	Tissierellia	*Tissierella praeacuta*	99.6		1.4 ± 0.1			5.0	13.9
L15	NB	Clostridia	*Clostridium tepidum*	99.0		1.5 ± 0.1			6.7	15.9
L6	Skim milk	Clostridia	*Caproiciproducens galactitolivorans*	97.2	0.8 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.1 ± 0.02	9.9	19.3
I6	Skim milk	Bacilli	*Bacillus thermoamylovorans*	95.8		0.8 ± 0.1	0.1 ± 0.02		6.5	20.9
I4 (Co-culture)	Skim milk		*Tissierella praeacuta* (as main species)	95.8	0.7 ± 0.1	2.4 ± 0.2	0.6 ± 0.1		15.0	25.5

The several bioaugmentation approaches adopted are briefly described below. The choice of *P. lundensis* DSM6252 leans on previous findings about its relevance in the degradation of protein substrates, mainly derived from cheese whey and wastes [47]. Furthermore, the functional analysis of a MAG (Metagenome-Assembled Genome) taxonomically assigned to *P. lundensis* DSM6252 reported the presence of a complete β-oxidation pathway and 21 proteases [47,48]. Likewise, since the prevalent species in the co-culture I4 (Fig. 1c), *T. praeacuta*, was predicted to be proteolytic, this culture was used to compare its effectiveness with that of the collection strain of *P. lundensis* DSM6252. Moreover, the presence of *C. cochlearium* in the same culture (Fig. 1c) must be taken into account, given its implication in degradation of dairy products. As a parallel strategy, the bioaugmentation with the culture adapted to casein was meant to potentially improve the AD, since a selected casein-degrading consortium is expected to have better performances when casein is the sole carbon source. The choice of *M. bourgensis* MS2 lies in its ability to thrive under high ammonia concentrations without hampering the methane yield, demonstrating high resilience in conditions that can be stressful for several methanogens [27,49].

3.3.1. Production of methane from batch reactors under different bioaugmentation approaches

The four bioaugmentation strategies above described, namely M (*M. bourgensis*), PM (*P. lundensis* and *M. bourgensis*), EM (enriched co-culture I4 and *M. bourgensis*), and A (casein acclimatised culture), were compared to the benchmark not bioaugmented (NB) in batch reactors for the production of biogas from maize silage, casein and their combination. Their methane productivity was monitored in a time-course experiment (Fig. 2), together with pH, VFA profiles, and TKN levels (Fig. 3).

The initial kinetics of all the experimental conditions per each substrate appear to be similar. The differences in methane yield become more evident while reaching the stationary phase (Fig. 2). In all three tested cases, bioaugmentation strategies were revealed to effectively increase biogas production.

The highest methane production from maize silage was achieved in the case of the EM bioaugmentation, which peaked at 707 ± 55 mL CH_4/g VS, corresponding to 1.48-fold the NB control condition (477 ± 44 mL CH_4/g VS). PM followed the lead, inducing a production 37% higher than NB (654 ± 9 mL CH_4/g VS). Overall, both conditions resulted in methane yields statistically higher ($p < 0.05$) than those detected for NB, M and A. In fact, both involve the bioaugmentation with a combination of *M. bourgensis* and a proteolytic bacterium/consortium: the effect of the high release of ammonia from the proteolytic activity can be mitigated by the resilience of *M. bourgensis*, which in turn drives the archaeal community towards a more efficient methanogenesis [28].

Reactors fed with maize silage evidenced a similar trend for M and A (Fig. 2a). Considering the high specialisation of the culture A for a different, protein-rich feedstock (casein), the main contribution to the system was due to methanogenic activity, which appears to be comparable to that measured in the case of M bioaugmentation. The greater bioaugmentation effects observed with EM and PM cultures are likely due to their microbial architecture, since both contain hydrolytic and proteolytic bacteria which can efficiently degrade macronutrient added to the reactors. Focusing on EM, results may indicate a higher degradation rate of maize silage, resulting in larger nutrient availability, and consequently in a greater methane yield. Even though methanogenesis is the rate-limiting step, it is possible that the high presence of *M. bourgensis* in the bioaugmentation culture pushed toward the higher consumption of molecules generated in the other AD steps by bacterial strains. VFA and pH profiles seem to support this finding since the highest values of total VFA were detected in the bioaugmented batches, and pH levels decreased accordingly (Fig. 3d). Nitrogen content did not change significantly in the different inoculation strategies (Fig. 3a).

Methane production from casein was faster than in maize silage with, on average, almost 66% of the biogas that was already produced after 7 days of incubation (Fig. 2b). As expected, the NB condition resulted in methane yield higher than that obtained from maize silage ($p < 0.05$). Moreover, the most efficient bioaugmentation was found to be with the A culture (745 ± 103 mL CH_4/g VS, +26% on average). Significantly lower methane yield and productivity values were displayed by the EM approach (661 ± 62 mL CH_4/g VS, +11%). The proficient proteolytic activities of both A and EM were evident considering the increase in total nitrogen content (Fig. 3b) and the highest total VFA profiles (Fig. 3e). Noteworthy, both bioaugmentations were more productive than the batch experiments inoculated with PM and M. This suggests a lower hydrolytic activity of *P. lundensis* (PM) at such high OL of casein, in comparison to the one of the enriched co-culture I4 (EM).

Checking biochemical parameters, higher TKN values were expected, since casein contains much more nitrogen than maize silage. Total VFA are similar in all the conditions, except for the reactors bioaugmented with A (Fig. 3e), which shows lower values. This further indicates that the VFA produced during the acidogenesis phase were

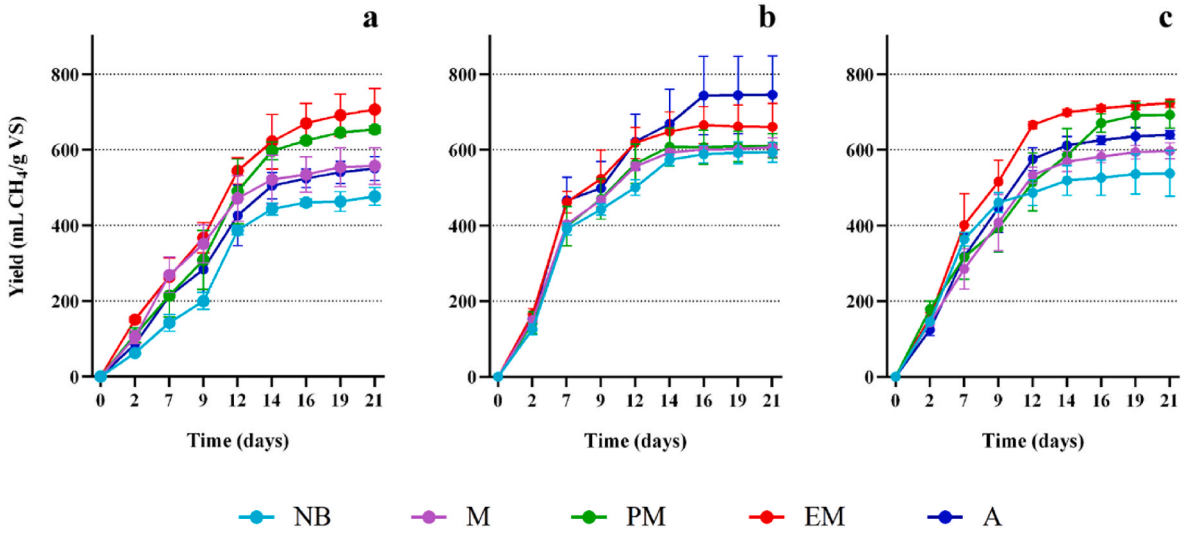

Fig. 2. Methane production yields (mL CH_4/g VS) of batch reactors fed with maize silage (**a**), casein (**b**), and their combination (**c**). The different conditions are reported as: not bioaugmented (NB), *M. bourgensis* (M), *P. lundensis* and *M. bourgensis* (PM), enriched culture and *M. bourgensis* (EM), casein acclimatised culture (A). Error bars denote standard deviation from the mean of triplicate measurements ($n = 3$).

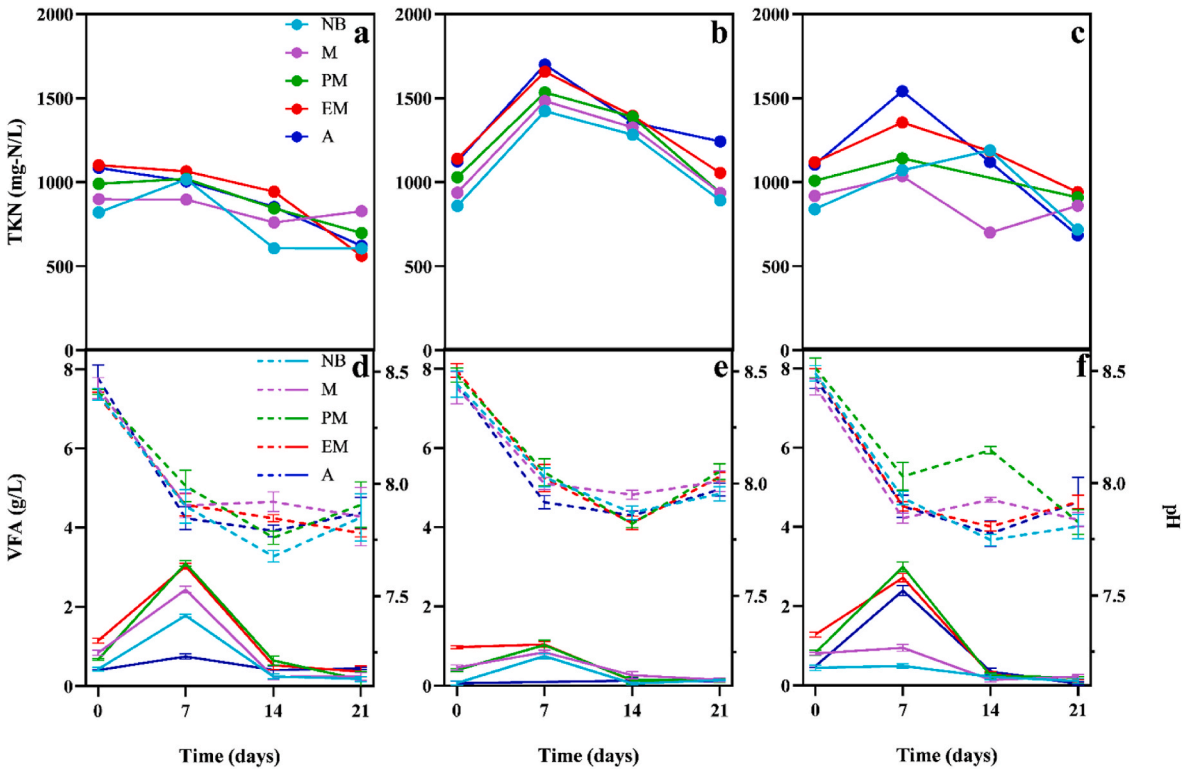

Fig. 3. TKN (mg-N/L), total VFA (full lines) and pH (dashed lines) values during AD of maize silage (**a**, **d**), casein (**b**, **e**) and their combination (**c**, **f**).

immediately converted into methane, especially when A was used for bioaugmentation, confirming its higher casein conversion efficiency.

Considering now the co-digestion of casein and maize silage, EM and PM cultures once again triggered biogas productions higher than that of NB ($p < 0.05$), with increments in methane yields corresponding to 34 and 19%, respectively. As such, the presence of *P. lundensis* or the newly isolated enriched culture was efficient in supporting the feedstock hydrolysis (Fig. 2c) and then the production of methane in a co-digestion context.

As expected, the bioaugmentation with A showed an approximately intermediate methane yield (640 ± 11 mL CH_4/g VS) between those observed with maize silage (550 ± 32 mL CH_4/g VS) and with casein (745 ± 103 mL CH_4/g VS) alone. The same observation is also found in the TKN trend (Fig. 3c).

On the contrary, the bioaugmentation with M did not result in increased methane yields compared to the benchmark. Overall, it is noteworthy that the use of a mixed culture with an archaeon and a hydrolytic bacteria avoided the inhibition of the system. In fact, their methane yields never fell behind the NB ones. Similarly, TKN has intermediate values between the single feedstocks digestion (Fig. 3a and b). Moreover, the steepest decrease in TKN measured in the A condition after 7 days may indicate a certain incorporation of the ammonia in the cell biomass, thus suggesting a higher growth rate of the community.

Considering that the co-digestion of maize silage and casein can be representative of many agricultural anaerobic digesters treating different feedstocks, the results obtained in this condition are the most interesting ones and indicated that the EM and PM bioaugmentations were the most promising. In fact, they were effective in alleviating the stressing conditions triggered by casein supplementation in the benchmark AD (NB). Besides, the combination of microorganisms is a quite easy procedure that allows to finely select and mix single strains according to the goal to be reached, with a stronger control on the operating conditions. On the contrary, the acquisition of an adapted culture is a long and harsh process, as it has to be specific for the single application, and not always the expected results can be achieved. Concluding, the bioaugmentation with an adapted community can result in proficient effect on a specific AD condition, whereas with the combination of microbes more flexibility is possible.

3.3.2. Effect on production of VFA and process parameters on batch reactors under different bioaugmentation approaches

Intermediate products such as VFA and the process parameter pH were measured once per week. The pH of the reactors fluctuated from 8.5 to 7.65 with a decreasing trend (Fig. 3d–f), hence remaining in the optimal range for methane production [50]. All the experimental conditions have similar trends and evidence a reduction in pH as a consequence of substrate degradation and VFA production. Specifically, it is noteworthy that a pH reduction to approximately 7.7 corresponds to an increase in methane production till it reaches the steady state (days 14–16) (Fig. 2). In batch settings, VFA generally accumulate in the first phase of the process and sharply decrease in the following steps when methanogenesis occurs very rapidly. This is in agreement with the total VFA profile detected over time also in this study, which is dominated by acetic acid, followed by propionic and valeric acids (**Supplementary Data**).

The initial increase in TKN values (in the casein and substrate combination conditions) is reasonably due to the degradation of complex substrate molecules that were retained in the filter during the preparation of samples for TKN analysis. On the contrary, their decrease in every condition is likely due to the microbial growth and the incorporation of nitrogen into their biomass [51].

4. Conclusions

In this study, newly isolated strains and a co-culture from an anaerobic digester were investigated for their potential in biogas production. A combination of genomic and biochemical analysis was used to characterise the selected inoculum and co-cultures. The bioaugmentation approach was successful in boosting methane production from difficult substrates that more than TKN itself represent a burden for

AD systems. The casein consortium showed the best effect in bioaugmenting the casein digestion, with a +26% methane yield, and a high contribution in the co-digestion (+19%). The results obtained in the co-digestion are the most encouraging, with a boost of +34% methane yield given by the bioaugmentation with the proteolytic co-culture with *M. bourgensis*. Such bacterial and archaeal combination is currently under study on continuous reactors to further assess the feasibility of this bioaugmentation strategy at larger scale towards an efficient and stable AD of protein-rich feedstocks.

Supplementary data related to this work can be found in the online version of the paper.

CRediT authorship contribution statement

Sara Agostini: Data curation, Investigation, Visualization, Writing – original draft. **Luca Bucci:** Data curation, Investigation, Visualization, Writing – original draft. **Davide Doni:** Resources. **Paola Costantini:** Resources. **Ameya Gupte:** Investigation. **Bettina Müller:** Resources. **Fabrizio Sibilla:** Resources. **Marina Basaglia:** Resources. **Sergio Casella:** Resources. **Panagiotis G. Kougias:** Conceptualization, Resources. **Stefano Campanaro:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Lorenzo Favaro:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Laura Treu:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – review & editing.

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.

Acknowledgments

We acknowledge Valentino Pizzocchero (MSc) for technical assistance in GC and HPLC analysis. Fundings: This project was supported by Fondazione Cariverona under the project “Più-BIOGAS APP”, the funding BIRD210708/21, the project “Sviluppo Catalisi dell’Innovazione nelle Biotecnologie” (MIUR ex D.M.738 dd 08/08/19) of the Consorzio Interuniversitario per le Biotecnologie” (CIB) and by the project LIFE20 CCM/GR/001642 – LIFE CO2toCH4 of the European Union LIFE + program. The microGC (Agilent Technologies, CA, USA) was funded by Progetto di Eccellenza “Centro per l’Agricoltura, la Sostenibilità e gli Alimenti” (CASA), CUP C26C18000190001, MIUR, Italy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2024.120270.

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