Preprints.org

Article

Not peer-reviewed version

Comparison of Different Carbon Sources on Biomethane Pro-Duction With Clostridium cellulovorans and Methanogens

Haruki Sawada , Hisao Tomita , Fumiyoshi Okazaki , Yutaka Tamaru *

Posted Date: 12 May 2023

doi: 10.20944/preprints202305.0930.v1

Keywords: methanogenesis; pig manure; carbon sources; C. cellulovorans; methanogens

Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

Article

Comparison of Different Carbon Sources on Biomethane Production with *Clostridium cellulovorans* and Methanogens

Haruki Sawada [1], Hisao Tomiya [1], Fumiyoshi Okazaki [1,2,3] and Yutaka Tamaru [1,2,3,*]

1 Department of Life Sciences, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie 514-8507, Japan
2 Department of Bioinfomatics, Mie University Advanced Science Research Center, Mie University, 1577 Kurimamachiya, Tsu, Mie 514-8507, Japan
3 Smart Cell Innovation Research Center, Mie University, 1577 Kurimamachiya, Tsu, Mie 514-8507, Japan
* Correspondence: ytamaru@bio.mie-u.ac.jp; Tel.: +81-59-231-9560

Abstract: Methane (CH_4) has attracted attention as not only one of the hydrogen carriers in terms of energy density, but also synthetic natural gas. In nature, there exist bacterial ecosystems that can decompose organic compounds to produce CH_4 and CO_2. In this study, *Clostridium cellulovorans* as a decomposer was cultivated with pig manure (PM) as an unused biomass. As a result, acetate and butyrate were increased in the *C. cellulovorans* medium containing 0.5% PM by high-performance liquid chromatography (HPLC), while formate and lactate were decreased in it. Accordingly, in comparison with carbon sources such as glucose, cellobiose, and acetate, cocultivation of *C. cellulovorans* and *Methanosarcina mazei* or microbial flora of methane production (MFMP) was performed in the *C. cellulovorans* medium. These results showed that 0.5% acetate as the sole carbon source produced CH_4 only by cocultivating *C. cellulovorans* and MFMP. Moreover, MFMP was only cultivated with 1% acetate or 1% methanol as a carbon source after precultivated with 0.5% glucose medium for 12 h. As a result, methane productivity of MFMP with 1% methanol medium was approximately eight times higher than that with 1% acetate medium. After cultivation with 1% acetate or 1% methane, next-generation sequencing (NGS) analysis of MFMP was carried out. Interestingly, *Methanofollis* (0.211%) belonging to methanogens through CO_2 reduction pathway was dominant in the 1% acetate medium for 72 h cultivation, while *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in 1% methanol medium for 72 h cultivation. Since *Methanosarcina* spp. are belonging to acetoclasts (acetoclastic pathway), methanol could promote to grow *Methanosarcina* spp. rather than acetate. Therefore, it seemed *Methanosarcina* spp. may play a key methanogenesis in MFMP. Thus, these results will provide important information for low cost biomethane production.

Keywords: methanogenesis; pig manure; carbon sources; *C. cellulovorans*; methanogens

1. Introduction

Anaerobic digestion (AD) consists of a series of biochemical processes such as hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis performed by various interacting microorganisms, including bacteria such as acidogens and acetogens, and archaea (methanogens). It is also clear that the cumulative CH_4 production from the three different substrates varied significantly and was not in agreement with the expected according to the theoretical value calculated (Table 1) (formate 82.35 N mLCH_4/gVS, acetate 273.17 N mLCH_4/gVS, H_2/CO_2 414.81 N mLCH_4/gVS) [1]. Since methanogenesis is the final step in anaerobic carbon transformation and is of critical concern in thawing permafrost peatland systems where CH_4 release is increasing rapidly, predicting the magnitude of carbon loss as CO_2 or CH_4 is hampered by our limited knowledge of microbial metabolism of organic matter in these environments [2]. Genome-centric metagenomic analysis of

© 2023 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

microbial communities provides the necessary information to examine how specific lineages transform organic matter during permafrost thaw [3]. The biomethanation process in nature relies on the microbial interactions between three main metabolic groups of anaerobes such as fermentative, acetogenic, and methanogenic microorganisms [4–6]. Whereas the first two groups decompose complex organic matters to acetate, H_2 and CO_2, which are the key precursors for methanogenesis, methanogens further convert these metabolites to CH_4 by two major routes such as acetoclastic pathway and CO_2 reduction pathway [7]. On the other hand, although the growth behavior of a donor bacterium, *Sulfurospirillum multivorans* in the modified *Methanococcus voltae* (acceptor) medium with pyruvate alone as substrate was similar to that in the medium originally used for cultivation of *S. multivorans*, the morphology of *S. multivorans* cells was unaltered in the *M. voltae* medium and independent from the type of cultivation—fermentatively or respiratory [8]. In this case, the new medium with lactate as the sole growth substrate instead of formate and acetate could not promote growth for pure *S. multivorans* cultures. Furthermore, 15mM lactate was consumed in approximately 2 weeks while methane was produced in the corresponding coculture, indicating lactate fermentation by *S. multivorans* and H_2 transfer to *M. voltae* as syntrophic partner. Therefore, the coculture system seems to include system unique advantages, composition, products, and interaction mechanisms.

Table 1. Methanogenic reactions from typical substrates.

Reactions	$\Delta G^{0'}$ (kJ/mol CH_4)	Microorganisms
I. Hydrogen $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135	Most methanogens
II. Formate $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-130	Many hydrogenotrophic methanogens
III. Acetate $CH_3COOH \rightarrow CH_4 + CO_2$	-33	*Methanosarcina* and *Methanosaeta*

Elaboration of the underlying mechanism in microbial communities such as the exchange of intermediate metabolites, cell-to-cell electrical connections, communications, etc. would guide the design of artificial microbial consortia and further improve the robustness and stability of the cocultivation systems [9–12]. Therefore, these artificial microbial consortia interact mutually through the interaction of synergism, commensalism, competition, mutualism, and so on [12]. Diverse microbial communities within the same or different species have been set up to realize more complicated tasks [8,13,14]. In particular, the greatest advantage of coculture systems consists of the combination of the metabolic capacity of two or more microorganisms that allows for the utilization of more complex substrates, and the production of specific products [14]. In addition, treatment of waste-water, biodegradation of textile azo dye and dispose of contaminated soil have also applied to produce biofuels, bulk chemicals, and natural products recently by the cocultivation systems [15–26].

Cellulose is most abundant on the Earth and not easily degraded and utilized. In addition to cellulosic sources, various other carbohydrates, carbon monoxide and syngas can also be processed using these systems [27]. The cellulolytic system of *Clostridium cellulovorans* mainly consists of a cellulosome which synergistically collaborates with non-complexed enzymes [28,29]. IBE (isopropanol-butanol-ethanol) fermentation by the cocultivations of *C. cellulovorans* and *C. beijerinckii* was performed using mandarin orange wastes [30], methane produced from sugar beet pulp [31], and mandarin orange peel under cocultivation with *C. cellulovorans* and methanogens [32]. Furthermore, two coculture models combining *C. cellulovorans* with *Methanosarcina barkeri* Fusaro or *M. mazei* Gö1 were established for the direct conversion of cellulose to CH_4 [33]. Coculturing *C. cellulovorans* with *M. barkeri* or *M. mazei* not only enabled direct conversion of cellulose to CH_4, but also stabilized pH for *C. cellulovorans*, resulting in a metabolic shift and enhanced cellulose degradation. The other approach was by implementing nanotechnology in combination with *C. cellulovorans* through consolidated bioprocessing (CBP) method to produce hydrogen from raw corn cob [34].

In this study, we observed the cocultivation of *C. cellulovorans* and *M. mazei* or microbial flora of methane production (MFMP) for the different carbon sources between sugars such as glucose and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

cellobiose that are the products from cellulose degraded by *C. cellulovorans* and acetate metabolized from glucose through TCA cycle. Furthermore, pig manure (PM) was used for the *C. cellulovorans* cultivation and was analyzed with organic acids. In addition, we investigated the cultivation manner of MFMP in comparison with acetate and methanol as the sole carbon source. Finally, 16S rRNA analysis in MFMP was performed by next generation sequencing (NGS) after cultivations with acetate or methanol as a carbon source.

2. Materials and Methods

2.1. Microorganism

C. cellulovorans 743B (ATCC35296) was anaerobically grown as described previously [28], with pig manure (PM) (Mie University, Tsu, Japan) as a carbon source. *M. mazei* (DSM# 3647) was purchased from the German Collections of Microorganisms and Cell Cultures (DSMZ, Germany) and was cultivated with the JCM230 medium [35].

2.2. Culture conditions

0.5% (w/v) PM, 0.5% (w/v) glucose, 0.5% acetic acid (FUJIFILM Wako Chemicals, Japan), and 0.5% (w/v) cellobiose (Sigma, MO, USA) were used as the sole carbon source in 10 ml or 50 ml of *C. cellulovorans* media and was anaerobically cultivated. The microbial flora of methane production (MFMP) was obtained from methane fermentation digested liquid on January, 2017 at Gifu in Japan [32]. *C. cellulovorans* was precultured with 0.5% cellobiose for 12 h at 37 °C and *M. mazei* and MFMP were done with 0.5% glucose for 12 h at 37 °C, respectively. Co-cultivation was performed as approximately 1000 RLU of *C.c* cells and approximately 20000 RLU of MFMP cells (*C. cellulovorans*:MFMP = 1:20) and approximately 1000 RLU of *C. cellulovorans* cells and approximately 3000 RLU of *M. mazei* cells (*C. cellulovorans*:*M. mazei* = 1:3), respectively. In comparison of cocultivations with 1% acetate and 1% methanol media, *C. cellulovorans* was precultured with 0.5% cellobiose for 12 h at 37 °C and *M. mazei* and MFMP were done with 0.5% glucose for 12 h at 37 °C, respectively, the precultured cells were inoculated into each medium with 1% acetate or 1% methanol. After the cells were collected by centrifugation, the supernatants and the cells were used for each experiment.

2.3. 16S rRNA sequencing

Samples for bacterial cells cultivated in the culture medium were crashed by Shake Master Neo (bms, Tokyo, Japan) and DNA was extracted by Fast DNA spin kit (MP Bio, CA, USA). iSeq 100 (Illumina, CA, USA) was used for sequencing under the condition of 2 × 150 bp. The 16S Metagenomics App performs taxonomic classification of 16S rRNA targeted amplicon reads using a version of the GreenGenes taxonomic database curated by Illumina. The primer sequences used in the protocol are: PCR1_Forward (50 bp): 5′–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3′ and PCR1_Reverse (55 bp): 5′–GTCTCGTGG GCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3′, respectively. The 16S rRNA sequences of MFMP previously reported [31] has been deposited in the DDBJ database (accession no. DRR160954).

2.4. Gas and organic acid concentrations

The total gas amount and the concentration of organic acids were measured as previously described [31]. The produced gas after the cultivation was recovered by downward displacement of water by a syringe (Terumo, Tokyo, Japan) and measured by gas chromatography (Shimadzu, Kyoto, Japan). The concentration of organic acids was measured by high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with UV detector. The data represent at least three independent experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

3. Results

3.1. Cultivation of C. cellulovorans with pig manure

In order to promote the utilization of pig manure (PM) as an unused biomass, the cultivation of *C. cellulovorans* was carried out. PM was pretreated with 0.45 μm filter to remove the inhibitor for bacterial cell growth and 0.5% (w/v) pretreated PM was used as the sole carbon source in the *C. cellulovorans* medium. *C. cellulovorans* was inoculated into the PM medium and then organic acids were measured by HPLC. The result suggested *C. cellulovorans* was able to grow in the 0.5% PM medium and acetate and butyrate were increased, while formate and lactate were decreased after once increased at 1 day cultivation (Figure 1). Total concentrations of acetate and butyrate at 14 days was approximately 2300 mg/L and 820 mg/L, respectively, resulting that PM would be an excellent biomass for methanogenesis.

3.2. Co-cultivation of C. cellulovorans with methanogens or M. mazei

CH_4 production by coculturing *C. cellulovorans*–methanogens (MFMP) was examined with 0.5% (w/v) glucose, 0.5% (w/v) cellobiose, and 0.5% (v/v) acetate, respectively, while cocultivation of *C. cellulovrorans*–*M. mazei* was done with 0.5% cellobiose as the sole substrate. As shown in Figure 2A, the cell growth in each coculture was observed and different patterns. On the other hand, the cocultivation of *C. cellulovorans*-MFMP showed CH_4 production only with 0.5% acetate, whereas the cocultivation of *C. cellulovorans-M. mazei* with the 0.5% cellobiose medium led to no methanogenesis during the cultivation period, resulting that *M. mazei* could never use cellobiose for its growth (Figure 2B). These results suggested methanogenesis promotes not sugars such as glucose or cellobiose but acetate as the carbon source.

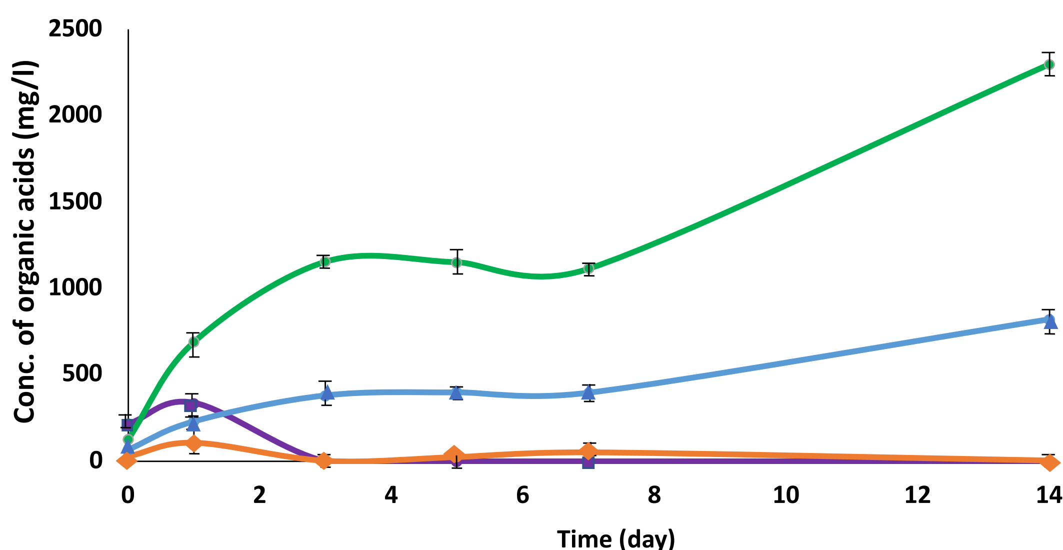

Figure 1. Measurement of organic acids from 0.5% pig manure (PM) cultivated by *C. cellulovorans*. Symbols: rhombus, formate; square, lactate, circle, acetate; triangle, butyrate. The data represent at least three independent experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

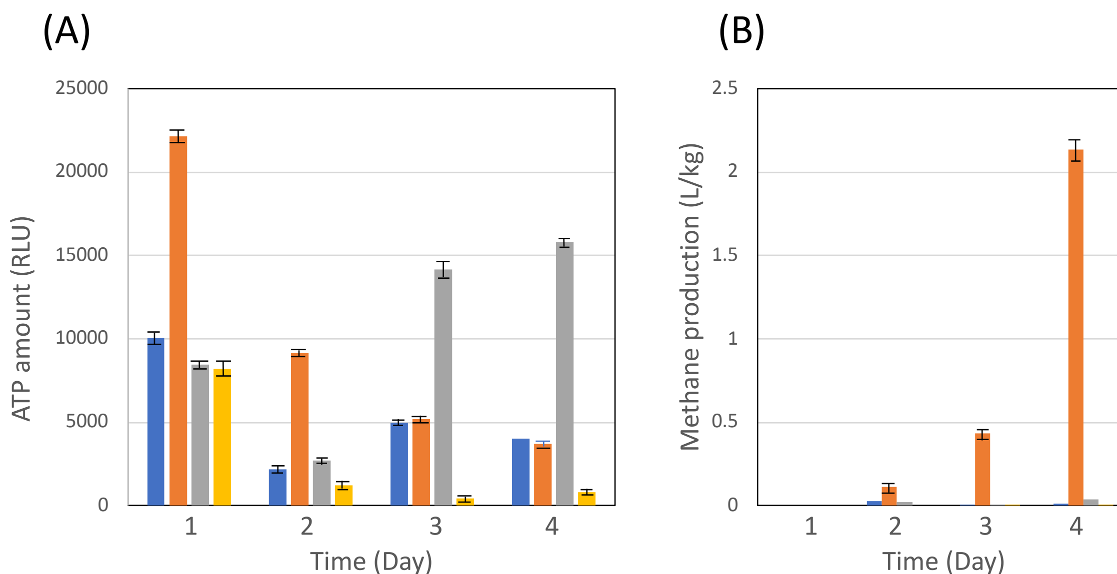

Figure 2. Measurement of ATP amount (RLU) and methane production (B) with cocultivation of *C. cellulovorans* and MFMP or *M. mazei*. Bars: blue, 0.5% cellobiose cultivated with *C. cellulovorans* and MFMP; orenge, 0.5% acetate cultivated with *C. cellulovorans* and MFMP; gray, 0.5% glucose cultivated with *C. cellulovorans* and MFMP; yellow, 0.5% cellobiose cultivated with *C. cellulovorans* and *M. mazei*. The data represent at least three independent experiments.

3.3. Effect of carbon sources with methanogens

In order to produce CH_4 efficiently, MFMP was examined with the culture media of 1.0% (v/v) acetate and 1.0% (v/v) methanol, respectively (Figure 3). The cell growth in the medium of 1.0% acetic acid was a peak at 1 day, while that in the medium of 1.0% methanol was a peak at 16 days (Figure 3A). On the other hand, CH_4 production on the methanol medium was increased from 8 days, and then the maximum production of methane was a peak at 16 days (Figure 3B). In case of the acetic acid medium, CH_4 production was lower than that of the methane medium, resulting in the difference of metabolic pathway of methanogenesis in MFMP. These results indicated methanogenesis easily occurs for not acetate but methanol and the production of methane by 1.0% methanol was 8 times higher than that by 1.0% acetate.

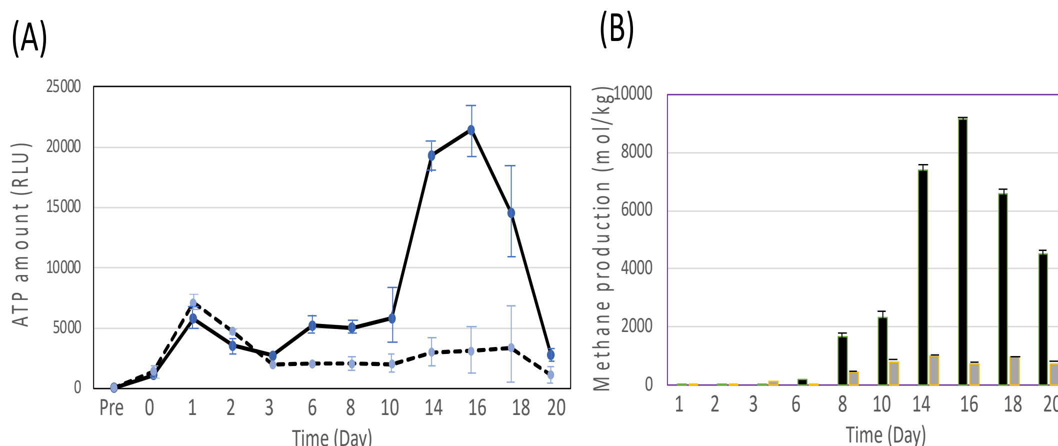

Figure 3. Measurement of ATP amount (RLU) (**A**) and methane production (**B**) in MFMP cultivation. (**A**) black line, 1% methanol; wavey line, 1% acetic acid. (**B**) black bar, 1% methanol; gray bar, 1% acetic acid. The data represent at least three independent experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

3.4. Identification of methanogens cultivatied with the different carbon sources

MFMP was precultivated with 0.5% glucose medium for 12 h at 37 °C and then 1,000 RLU of MFMP cells was inoculated into the *C. cellulovorans* medium containing 1% acetate or 1% methanol at 37 °C for 72 h. After DNA extraction from the growth cells of each medium, 16S rRNA analyses were carried out by next generation sequencer. As shown in Figure 4, *Methanofollis* was a majority of archaea and was 0.211% in 1% acetate medium for 72 h cultivation. On the other hand, *Methanofollis* in 1% methanol medium was found, i.e., 0.007% for 24 h cultivation and 0.490% for 72 h cultivation, respectively. On the other hand, *Methanosarcina barkeri* was a typical methanogen and was 0.011% for 24 h cultivation and 0.015% for 72 h cultivation, respectively, in 1% acetate medium. Interestingly, for 72 h cultivation 0.004% of *M. mazei* was found in 1% methanol medium, while 0.571% of *M. barkeri* was detected in the same medium. These results indicated the growth of methanogens was dependent on the carbon sources and their growth trend of individual methanogens seemed remarkably different under the sole carbon sources.

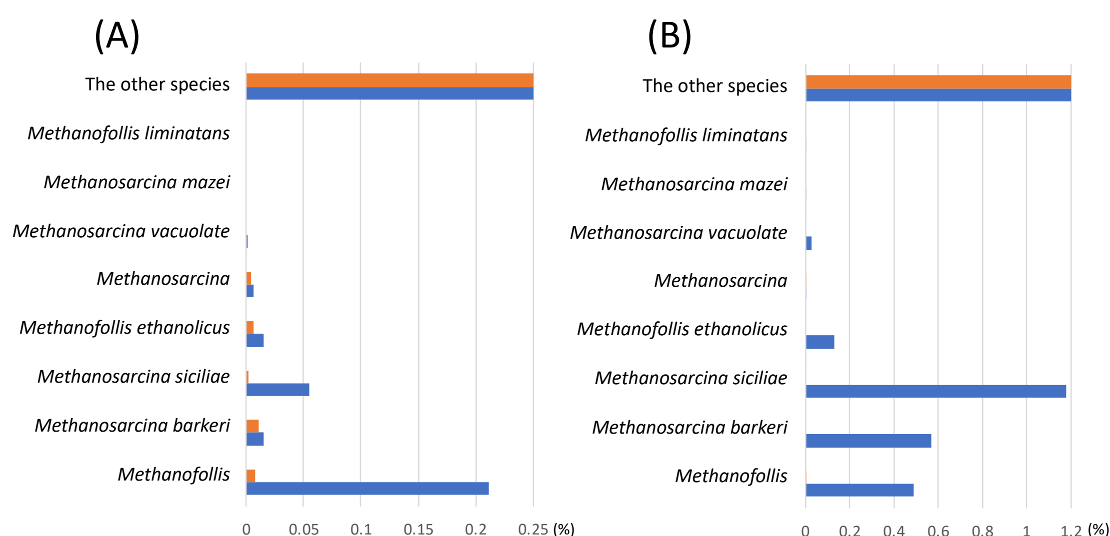

Figure 4. Relative abundance ratio of archaea in MFMP after cultivated with the different carbon sources. After 16S rRNA analysis, the idetified archea were compaied. (**A**) 1% acetate cultivation; (**B**) 1% mathanol cultivation. Bars indicate orenge (after 24 h cultivation) and blue (after 72 h cultivation), respectively. The others show the rest of total percentage.

4. Discussion

In Japan, around 25.31 million tons of food waste was generated in 2018 from consumer households, food manufacturing and retail [36]. In addition to maximize social and economic benefits, such appropriate food waste management should be implemented to minimize the environmental impacts. Although recycling food waste as is preferred to compost and animal feed in Japan, composting of food waste still presents high-quality demanded by farmers, relatively low price, and a shortage of cropland for application [37–39]. Therefore, since the most successful application at the commercial scale has so far been anaerobic digestion (AD), which has been widely adopted for waste treatment, a plentiful source of organic compounds such as pig manure (PM) can be used as feedstock in AD. Namely, since the fermented liquid feed (FLF) for pigs contains several nutrients required for bacterial growth, recycling food waste was considered a possible alternative for many years. Also, PM having a high buffering capacity possibly protects AD against failures due to the accumulation of volatile fatty acids (VFAs) [40–42]. It was reported that the effect of varying PM with food waste mixing ratio was evaluated on methane yield, suggesting that the feedstock composition of 60:40 (volatile solid basis) significantly enhanced methane yield [43]. On the other hand, the other group reported that using vegetable processing wastes as co-substrate with a feedstock ratio of 50:50 (dry weight basis) could improve methane yield up to 3-fold [44]. Thus, since

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

several potential co-substrates have been examined to assess the effect of varying feedstock composition on improving the AD process performance and increasing methane yield, the VFAs of the *C. cellulovorans* medium containing PM were measured in this study. As a result, acetic acid (approx. 2300 mg/mL) and butyric acid (approx. 820 mg/mL) were accumulated for 14 days, respectively (Figure 1). Since the high ammonia concentration might inhibit bacterial activity in AD [45–49], PM was pretreated with 0.45 μm filtration before inoculation of *C. cellulovorans* in this study to enhance methane production. By adjusting the carbon-to-nitrogen (C/N) ratio, co-digestion of PM with organic wastes including high carbon dilute seemed to improve the inhibitory effect on ammonia and to enhance the macro- and micro-nutrient balance in the feedstocks [50,51]. On the other hand, cow manure (CM) is rich in nutrients and can provide strong buffer capacity, and thus, CM seems more robust than other manures in AD [52]. Therefore, the alleviation of ammonia inhibition when CM is used in AD seems not that urgent and should not be the priority of co-digestion. Additionally, CM is categorized as lignocellulosic waste due to its high amount of lignocellulose (50% in dry matter), which is relatively low in other types of manure [53]. Hence, to make full use of CM to produce more methane via co-digestion, attention should be paid to how to improve the degradation of recalcitrant lignocellulose in CM. In addition, the current study determined biogas production in single-stage and two-stage AD using sheep manure (SP) as substrate and yak rumen fluid as the inoculum. Yak rumen fluid is rich in hydrolytic bacteria [54] and, consequently, its inclusion should improve the degradation of lignocellosic biomass, leading to high biogas production.

All archaeal metagenome-assembled genomes (MAGs) could reveal the reconstruction of pathways related to methanogenesis and relevant energy conservation systems [55]. Furthermore, the holistic microbial community activity could be evaluated by the average RPKM of genes in each KEGG module [56]. Thus, by maintaining the methanogenic activity of the microbial community, such a syntrophic behavior is required to synthesize numerous metabolites. An overall shift of the microbial activity was observed in the majority of the KEGG modules after H_2 addition. Moreover, H_2 also enhanced the activity of the glyoxylate cycle and the biosynthesis of lipids and specific amino acids. In addition to H_2, formate such as a similarly formed product during fermentative metabolism, is an important electron carrier in the syntrophic fatty acid-degrading methanogenic consortia [57]. In fact, formate was low concentration and immediately consumed in the PM medium (Figure 1). Therefore, other anaerobes might utilize both formate and H_2 as an electron donor for methanogenesis or sulfate respiration.

Clostridium coculture systems are typically used to produce biofuels such as H_2 and CH_4, solvents, and organic acids [58]. Because cellulosic materials are commonly found in nature [18], the specific metabolic capacities of cellulolytic strains and producers in coculture systems have attracted significant attention and offered many long-term prospects for development. Furthermore, since the combination of genome-centric metagenomics and metatranscriptomics successfully revealed individual functional roles of microbial members in methanogenic microcosms, these results assigned a multi-trophic role to *Methanosarcina* ssp., suggesting its ability to perform simultaneous methanogenesis from acetate, CO_2 and methanol/methylamine [55]. MFMP used in this study originally consisted of *C. butyricum* (0.005%) identified as the same genus of *C. celulovorans* and *M. mazei* (1.34%) found among methanogens [32]. Furthermore, other methanogens such as *Methanosaetaceae*, *Methanosaeta*, and *Methanospirillaceae* were also identified in MFMP. The genus *Methanosaeta*, which utilizes only acetate, was a large portion of ratio next to *Methanosarcina*. On the other hand, 1% acetate or 1% methanol was used as the sole carbon source for MFMP cultivation in this study. As a result, while *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in the 1% methanol medium for 72 h cultivation, *Methanofollis* (0.211%) was dominant in the 1% acetate medium for 72 h cultivation (Table 2). It is thought that all methanogens are physiologically specialized and able to scavenge the electrons from H_2, formate, acetate, and methanol, having CH_4 as the final product [49]. The *Clostridium* coculture system can also produce CH_4 in addition to producing H_2 and solvents, in particular the coculture of cellulolytic Clostridia and methanogens including *M. barkeri* Fusaro, *M. mazei*, and *Methanothermobacter*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

thermautotrophicus, the methanogens utilized H_2 and CO_2, acetate, and even formate that was generated by the cellulolytic Clostridia from cellulose to produce CH_4 [33,59]. In this study, CH_4 production by cellobiose was not found in the cocultivation of *C. cellulovorans*-*M. mazei* (*C.c*:*M.m* = 1:3), while only acetate led to methanogenesis in the cocultivation of *C. cellulovorans*-MFMP (Figure 2). In addition, since *M. barkeri* was more dominant than *M. mazei* in MFMP cultivation according to the 16S rRNA analysis (Table 2), it seemed that *Methanosarcina* spp. may play a key role on the methanogenesis of MFMP. So far, it has been reported that CH_4 production was investigated with sugar beet pulp [16] and mandarin orange peel [17] in the cocultivation of *C. cellulovorans*-MFMP (*C.c*:MFMP = 1:20). Therefore, carbon sources such as acetic acid and methanol were compared by the production of CH_4 in this study. As expected, CH_4 production from methanol was approximately eight times higher than that from acetic acid, with related to the cell growth of MFMP (Figure 3). Thus, methanogens seemed to be altered in their flora dependent on the sole carbon source.

5. Conclusions

In this study, *C. cellulovorans* was cultivated with PM and cocultivation of *C. cellulovoroans-M. mazei* or *C. cellulovorans*-MFMP was performed with different carbon sources. Since the cultivation of *C. cellulovorans* with PM had much acetic acid, it was thought to be one of excellent biomass for methane production. On the other hand, methanol was a best carbon source for CH_4 production with MFMP. Regarding next generation sequence analysis of MFMP, *Methanosarcina siciliae* (1.178%), *M. barkeri* (0.571%), and *Methanofollis* (0.490%) were major species in 1% methanol medium for 72 h cultivation, while *Methanofollis* (0.211%) was dominant in the 1% acetic acid medium for 72 h cultivation. Therefore, it seemed *Methanosarcina* spp. may play a key role on the methanogenesis of MFMP.

Author Contributions: Performed the experiments: H.S. and H.T.; experimental design: Y.T.; writing—original draft preparation, H.S. and F.O.; writing—review and editing, Y.T.; supervision, Y.T.; funding acquisition, Y.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was a part supported by Chubu Electric Power Co., Inc., Mitsui Chemicals, Inc., and Yanmar Holdings Co., Ltd.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Mie University.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Profs. Dr. Hiroki Matsui and Dr. Tomomi Ban at Mie University for providing PM. The authors also thank Ms. Mayu Hayashi and Ms. Midori Kosaka at Tamaru's laboratory for technical support of experimental studies.

Conflicts of Interest: The authors have no conflicts of interest relevant to the content of this article.

References

1. Pan, X.; Angelidaki, I.; Alvarado-Morales, M.; Liu, H.; Liu, Y.; Huang, X.; Zh, G. Methane production from formate, acetate and H2/CO2; focusing on kinetics and microbial characterization. *Bioresour. Technol.* **2016,** *218*, 796–806.
2. Christensen, T.R.; Johansson, T.; Åkerman, H.J.; Mastepanov, M.; Malmer, N.; Friborg, T.; Crill, P.; Svenssonet, B.H. Thawing sub-arctic permafrost: effect sonvegetation and methane emissions. *Geophys. Res. Lett.* **2004,** *31*, L04501.
3. Woodcroft, B.J.; Singleton, C.M.; Boyd, J.A.; Evans, P.N.; Emerson, J.B.; Zayed, A.F.Z.; Hoelzle, R.D.; Lamberton, T.O.; Mccalley, C.K.; Hodgkins, S.B.; Wilson, R.M.; Purvine, S.O.; Nicora, C.D.; Li, L.; Frolking, S.; Chanton, J.P.; Patrick, M.; Crill, P.M.; Saleska, S.R.; Rich, V.I.; Tyson, G.W. Genome-centric view of carbon processing in thawing permafrost. *Nature* **2018,** *560*, 49–58.
4. Hattori, S. Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ.* **2008,** *23*, 118–127.
5. Thauer, R.K.; Kaster, A.K.; Seedorf, H.; Buckel, W.; Hedderich, R. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Microbiol.* **2008,** *6*, 579–591.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

6. Garcia, J.L.; Patel, B.K.C; Ollivier B. Taxonomic, phylogenetic, and ecological diversity of methanogenic Archaea. *Anaerobe* **2000,** *6*, 205–226.
7. Deppenmeier, U.; Müller, V.; Gottschalk, G. Pathways of energy conservation in methanogenic archaea. *Arch. Microbiol.* **1996,** *165,* 149–163.
8. Kruse, S.; Goris, T.; Westermann, M.; Adrian, L.; Diekert, G. Hydrogen production by *Sulfurospirillum* species enables syntrophic interactions of Epsilonproteobacteria. *Nat. Commun.* **2018**, *9*, 4872.
9. Fischbach, M.A.; Segre, J.A. Signaling in host-associated microbial com- munities. *Cell* **2016,** *164,* 1288–1300.
10. Kenny, D.J.; Balskus, E.P. Engineering chemical interactions in microbial communities. *Chem Soc Rev.* **2018,** *47*, 1705–1729.
11. Brenner, K.; You, L.; Arnold, F.H. Engineering microbial consortia: a new frontier in synthetic biology. *Trends Biotechnol*. **2008**, *26*, 483–489.
12. Ding, M.Z.; Song, H.; Wang, E.X.; Liu, Y.; Yuan, Y.J. Design and construction of synthetic microbial consortia in China. *Synth Syst Biotechnol.* **2016,** *1*, 230–235.
13. De Bok, F.A.; Plugge, C.M.; Stams, A.J. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res.* **2004,** *38,* 1368–1375.
14. Du, Y.; Zou, W.; Zhang, K.; Ye, G.; Yang, J. Advances and applications of *Clostridium* co-culture systems in biotechnology. *Front. Microbiol.* **2020,** *11*, 560223.
15. He, F; Hu, W.; Li, Y. Biodegradation mechanisms and kinetics of azo dye 4BS by a microbial consortium. *Chemosphere.* **2004,** *57,* 293–301.
16. Khouni, I.; Marrot, B.; Amar, R.B. Treatment of reconstituted textile waste-water containing a reactive dye in an aerobic sequencing batch reactor using a novel bacterial consortium. *Sep Purif Technol.* **2012,** *87,* 110–119.
17. Safonova, E.; Kvitko, K.V.; Iankevitch, M.I.; Surgko, L.F.; Afti, I.A.; Reisser, W. Biotreatment of industrial wastewater by selected algal-bacterial consortia. *Eng Life Sci.* **2004,** *4*, 347–353.
18. Xu, X.H.; Liu, X.M.; Zhang, L.; Mu, Y.; Zhu, X.Y.; Fang, J.Y.; Li, S.P.; Jiang, J.D. Bioaugmentation of chlorothalonil-contaminated soil with hydrolytically or reductively dehalogenating strain and its effect on soil microbial community. *J Hazard Mater.* **2018,** *351,* 240–249.
19. Sabra, W.; Dietz, D.; Tjahjasari, D.; Zeng, A.P. Biosystems analysis and engineering of microbial consortia for industrial biotechnology. *Eng Life Sci.* **2010,** *10,* 407–421.
20. Bertrand, S.; Bohni, N.; Schnee, S.; Schumpp, O.; Gindro, K.; Wolfender, J.L. Metabolite induction via microorganism co-culture: a potential way to enhance chemical diversity for drug discovery. *Biotechnol Adv.* **2014,** *32,* 1180–1204.
21. Eiteman, M.A.; Lee, S.A.; Altman, R.; Altman, E. A substrate-selective co-fermentation strategy with Escherichia coli produces lactate by simultaneously consuming xylose and glucose. *Biotechnol Bioeng.* **2009,** *102,* 822–827.
22. Wang, E.X.; Ding, M.Z.; Ma, Q.; Dong, X.T.; Yuan, Y.J. Reorganization of a synthetic microbial consortium for one-step vitamin C fermentation. *Microb Cell Fact.* **2016,** *15,* 21.
23. Schroeckh, V.; Scherlach, K.; Nützmann, H.W.; Shelest, E.; Schmidtheck, W.; Schuemann, J.; Martin, K.; Hertweck, C.; Brakhage, A.A. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc Natl Acad Sci USA.* **2009,** *106,* 14558–14563.
24. Xin, F.X.; He, J.Z. Characterization of a thermostable xylanase from a newly isolated *Kluyvera* species and its application for biobutanol production. *Bioresour Technol.* **2013,** *135,* 309–315.
25. Tsai, S.L.; Goyal, G.; Chen, W. Surface display of a functional minicelluloome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production. *Appl Environ Microbiol.* 2010, 76, 7514–7520.
26. Lovley, D.R. Happy together: microbial communities that hook up to swap electrons. *ISME J.* **2016,** *11,* 327–336.
27. Charubin, K.; Bennett, R.K.; Fast, A.G.; Papoutsakis, E.T. Engineering *Clostridium* organisms as microbial cell-factories: challenges & opportunities. *Metab. Eng.* **2018,** *50*, 173–191.
28. Tamaru, Y.; Miyake, H.; Kuroda, K.; Nakanishi, A.; Kawade, Y.; Yamamoto, K.; Uemura, M.; Fujita, Y.; Doi, R.H.; Ueda, M. Genome sequence of the cellulosome-producing mesophilic organism *Clostridium cellulovorans* 743B. *J. Bacteriol.* **2010,** *192,* 901– 902.
29. Tamaru,Y.; Miyake, H.; Kuroda, K.; Nakanishi, A.; Matsushima, C.; Doi, R.H.; Ueda, M. Comparison of the mesophilic cellulosome- producing *Clostridium cellulovorans* genome with other cellulosome-related clostridial genomes. *Micro. Biotechnol.* **2011,** *4*, 64–73.
30. Tomita, H.; Okazaki, F.; Tamaru, Y. Direct IBE fermentation from mandarin orange wastes by combination of *Clostridium cellulovorans* and *Clostridium beijerinckii*. *AMB Express* **2019,** *9*,1.
31. Tomita, H.; Okazaki, F.; Tamaru, Y. Biomethane production from sugar beet pulp under cocultivation with *Clostridium cellulovorans* and methanogens. *AMB Express* **2019,** *9*, 28.
32. Tomita, H.; Tamaru, Y. The second-generation biomethane from mandarin orange peel under cocultivation with methanogens and the armed *Clostridium cellulovorans*. *Fermentation* **2019,** *5*, 95.
33. Lu, H.; Ng, S.-K.; Jia, Y.; Cai, M.; Lee, P.K.H. Physiological and molecular characterizations of the interactions in two cellulose-to-methane cocultures. *Biotechnol. Biofuels* **2017,** *10,* 37.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

34. Singhvi, M.; Kim, B.S. Green hydrogen production through consolidated bioprocessing of lignocellulosic biomass using nanobiotechnology approach. *Biores. Technol.* **2022,** *365*, 128108.
35. Goevert, D.; Conrad, R. Effect of substrate concentration on carbon isotope fractionation during acetoclastic methanogenesis by *Methanosarcina barkeri* and *M. acetivorans* and in rice field soil. *Appl. Environ. Microbiol.* **2009,** *75,* 2605–2612.
36. Ministry of the Environment. MOE Discloses the Estimated Amount of Japan's Food Loss and Waste Generated in FY 2018. 2021. Available online: https://www.env.go.jp/en/headline/2515.html (accessed on 10 November 2022).
37. Watanabe, E.; Seike, N.; Motoki, Y.; Inao, K.; Otani, T. Potential application of immunoassays for simple, rapid and quantitative detections of phytoavailable neonicotinoid insecticides in cropland soils. *Ecotoxicol. Environ. Saf.* ***2016,*** *132*, 288–294.
38. Cheung, H.N.B.; Huang, G.H.; Yu, H. Microbial-growth inhibition during composting of food waste: Effect of organic acids. *Bioresour. Technol.* **2010,** *101,* 5925–5934.
39. Nakasaki, K.; Hirai, H. Temperature control strategy to enhance the activity of yeast inoculated into compost raw material for accelerated composting. *Waste Manag.* **2017,** *65,* 29–36.
40. Wang, H.; Lim, T.T.; Duong, C.; Zhang, W.; Xu, C.; Yan, L.; Mei, Z.; Wang, W. Long-Term Mesophilic Anaerobic Co-Digestion of Swine Manure with Corn Stover and Microbial Community Analysis. *Microorganisms* **2020,** *8*, 188.
41. Córdoba, V.; Fernández, M.; Santalla, E. The effect of different inoculums on anaerobic digestion of swine wastewater. J. Environ. Chem. Eng. **2016,** *4*, 115–122.
42. Cuetos, M.J.; Fernández, C.; Gómez, X.; Moran, A. Anaerobic co-digestion of swine manure with energy crop residues. Biotechnol. *Bioprocess Eng*. **2011,** *16*, 1044–1052.
43. Dennehy, C.; Lawlor, P.G.; McCabe, M.S.; Cormican, P.; Sheahan, J.; Jiang, Y.; Zhan, X.; Gardiner, G.E. Anaerobic co-digestion of pig manure and food waste: Effects on digestate dewaterability, and microbial community dynamics. *Waste Manag*. **2018,** *71,* 532–541.
44. Molinuevo-Salces, B.; González-Fernández, C.; Gómez, X.; García-González, M.C.; Morán, A. Vegetable processing wastes addition to improve swine manure anaerobic digestion: Evaluation in terms of methane yield and SEM characterization. *Appl. Energy* **2012,** *91,* 36–42.
45. Mata-Alvarez, J.; Dosta, J.; Macé, S.; Astals, S. Codigestion of solid wastes: A review of its uses and perspectives including modeling. *Crit. Rev. Biotechnol***. 2011,** *31,* 99–111.
46. Yenigün, O.; Demirel, B. Ammonia inhibition in anaerobic digestion: A review. *Process Biochem.* **2013,** *48,* 901–911.
47. Astals, S.; Nolla-Ardèvol, V.; Mata-Alvarez, J. Anaerobic co-digestion of pig manure and crude glycerol at mesophilic conditions: Biogas and digestate. *Bioresour. Technol.* **2012,** *110,* 63–70.
48. Wang, X.; Yang, G.; Feng, Y.; Ren, G.; Han, X. Optimizing feeding composition and carbon–nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw. *Bioresour. Technol.* **2012,** *120,* 78–83.
49. Moestedt, J.; Müller, B.; Westerholm, M.; Schnürer, A. Ammonia threshold for inhibition of anaerobic digestion of thin stillage and the importance of organic loading rate. *Microb. Biotechnol.* **2016,** *9,* 180–194.
50. Hartmann, H.; Ahring, B.K. Strategies for the anaerobic digestion of the organic fraction of municipal solid waste: An overview. *Water Sci. Technol.* **2006,** *53,* 7–22.
51. Zamanzadeh, M.; Hagen, L.H.; Svensson, K.; Linjordet, R.; Horn, S.J. Biogas production from food waste via co-digestion and digestion- effects on performance and microbial ecology. *Sci. Rep.* **2017,** *7,* 17664.
52. Font-Palma, C. Methods for the treatment of cattle manure. A review. *C-J. Carbon Res.* ***2019,*** 5, 27.
53. Kafle, G.K.; Chen, L. Comparison on batch anaerobic digestion of five different live-stock manures and prediction of biochemical methane potential (BMP) using different statistical models. *Waste Manag.* ***2016,*** 48, 492–502.
54. Rabee, A.E.; Sayed Alahl, A.A.; Lamara, M.; Ishaq, S.L. Fibrolytic rumen bacteria of camel and sheep and their applications in the bioconversion of barley straw to soluble sugars for biofuel production. *PLoS ONE* ***2022,*** 17, e0262304.
55. Zhu, X.; Campanaro, S.; Treu, L.; Seshadri, R.; Ivanova, N.; Kougias, P.G; Kyrpides, N.; Angelidaki, I. Metabolic dependencies govern microbial syntrophies during methanogenesis in an anaerobic digestion ecosystem. *Microbiome* **2020,** *8*, 22.
56. Mortazavi, A.; Williams, B.A.; McCue, K.; Schaeffer, L.; Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **2008,** *5,* 621.
57. De Bok, F.A.; Plugge, C.M.; Stams, A.J. Interspecies electron transfer in methanogenic propionate degrading consortia. *Water Res.* **2004,** *38,* 1368–1375.
58. Jiang, Y.; Wu, R.; Zhou, J.; He, A.; Xu, J.; Xin, F.; Zhang, W.; Ma, J.; Jiang, M.; Dong, W. Recent advances of biofuels and biochemicals production from sustainable resources using co-cultivation systems. *Biotechnol. Biofuels* **2019,** *12,* 155.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 May 2023 doi:10.20944/preprints202305.0930.v1

59. Sasaki, D.; Morita, M.; Sasaki, K.; Watanabe, A.; Ohmura, N. Acceleration of cellulose degradation and shift of product via methanogenic co-culture of a cellulolytic bacterium with a hydrogenotrophic methanogen. *J. Biosci. Bioeng.* **2012,** *114*, 435–439.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.