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Rumen bacteria at work: Bioaugmentation strategies to enhance biogas production from cow manure

Short running head: Rumen bacteria at work

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Abstract

Aims: To investigate the effects of different bioaugmentation strategies for enhancing the biogas production from cow manure and evaluate microbial community patterns.

Methods and Results: Co-inoculation with cow rumen fluid and cow rumen-derived enriched microbial consortia were evaluated in anaerobic batch tests at 36 ^{o}C and 41 ^{o}C. Singular addition of both rumen fluid and enriched bioaugmentation culture had a promising enhancement on methane yields; however, the highest methane yield (311 mL CH_4/ g VS at 41 ^{o}C) was achieved when the anaerobic seed sludge was co-inoculated together with rumen fluid and enriched bioaugmentation culture. Bacterial community profiles were investigated by Ion PGM Platform and specific lignocellulolytic bacteria dynamics in batch tests were assessed by qPCR. The temperature had minor effects on the abundance of bacterial community; in which *Bacteroidetes* and *Firmicutes* were the most abundant phyla in all digesters. Furthermore, *Rikenellaceae*, *Clostridiaceae*, *Porphyromonadaceae*, *Bacteroidaceae* and *Ruminococcaceae* played a crucial role during the anaerobic degradation of cow manure. There was an important impact of *F. flavefaciens and R. albus* at the 41 °C, which in turn positively affected the methane production.

Conclusion: The degree of enhancement in biogas production can be upgraded by the co-inoculation of rumen-derived bioaugmentation culture with anaerobic seed sludge with high methanogenic activity.

Significance and Impact of the Study: A close look at the biotic interactions and their associations with abiotic factors might be valuable for evaluating rumen-related bioaugmentation applications.

Keywords: Anaerobic digestion; bioaugmentation; enrichment culture; lignocellulosic feedstock; cow rumen

Introduction

Anaerobic digestion (AD) is an environmental friendly technology which is increasingly implemented in the scope of waste management and clean energy production over the last decade (Romero-Güiza et al. 2016). Anaerobic conversion of feedstock is carried out by a diverse microbial consortia including chemolithoautotrophic and chemoheterotrophic bacteria and methanogens which act synergistically. As overall biogas production efficiency depends on activities of this complex microbial consortia (Azman et al. 2015).

A great quantity of animal manure is produced from animal farms and if it is not handled properly, it can cause environmental pollution and affect human health adversely (Holm-Nielsen et al. 2009). Thus, animal manure is one of the most widely used feedstock for anaerobic digesters. However, due to it is recalcitrant structure, hydrolysis is accepted as a rate-limiting step (Martin-Ryals et al. 2015). Thus, new strategies should be proposed to improve hydrolysis of lignocellulose-rich feedstock to enhance the economic feasibility of biogas plants.

Previous researches suggest that, microorganisms in rumen fluid are more efficient in lignocellulosic feedstock degradation thanks to higher cellulolytic activities than that of inocula from anaerobic digesters (Hu and Yu 2005; Yue et al. 2013). Moreover, hydrolytic microbial consortia can be derived from rumen fluid which is more efficient in volatile fatty acid production (Bayané and Guiot 2011) and can be applied to anaerobic digesters as a co-inoculum to enhance hydrolysis.

Investigating microbial community and dynamics of anaerobic digestion is an ongoing concern to improve the digestion efficiency. A combination of quantitative real-time polymerase chain reaction (qPCR) and multivariate analysis can shed a light on the functional contribution of different microbial groups on anaerobic digestion (Kim et al. 2010).

Ruminococcus flavefaciens, Ruminococcus albus and *Fibrobacter succinogenes* are considered as the three main cellulolytic bacterial species in the rumen (Koike and Kobayashi 2001; Nyonyo et al. 2014). *R. flavefacien and R. albus* are strictly anaerobic, gram positive bacteria and can ferment cellulosic and hemicellulosic compounds. *F. succinogenes* is considered as a dominant fibre-digesting bacterial species in the rumen environment and can digest fibres in a shorter time (Kobayashi et al. 2008) compared to the other two species. Monitoring these three species in anaerobic digesters can give an opinion about fibre digestion capacity.

In this study, we aimed to determine the most effective inoculation strategy for enhancing the biogas production from cow manure and establish the effects on the microbial community structure. In this scope, three different inocula (rumen fluid, digester sludge taken from full-scale plant and the enriched microorganisms responsible for the degradation of cellulosic material in the cow rumen) and their combinations were tested in the batch anaerobic digesters treating cow manure.

Materials and methods

Substrate and inocula

A 4-year-old healthy non-medicated cow was cared and handled in a barn of the Veterinary Faculty of Istanbul University, Turkey. Rumen fluid was obtained approximately 2 h after morning feeding by orogastric collection method and fresh samples brought to the laboratory immediately. Fresh cow manure was used as a feedstock in the digestion experiments and collected from the same cow. Seed sludge was taken from a successfully operated full-scale mesophilic anaerobic digester treating cattle manure in Bursa, Turkey. The cow manure and seed sludge samples were kept at 4 ^{o}C prior to use.

Enrichment studies

To cellulolytic microorganisms from rumen fluid was enriched by Bushnell Haas Medium (BHM) consisted of 0.0008 moles/L $MgSO_4 \cdot 7H_2O$, 0.0325 moles/L K_2HPO, 0.0125 moles/L NH_4NO_3, 0.0002 moles/L $FeCl_3 \cdot 6H_2O$, 0.0002 moles/L $CaCl_2$, under strictly anaerobic conditions (Lo et al. 2009). During enrichment studies, carboxymethyl cellulose (CMC) was used as the sole carbon source (0.04 moles/L). For the enrichment 120 mL glass bottles were used and filled with the medium up to 80 mL and inoculated with rumen fluid and incubated for 3 weeks at 37 °C. After 5 transfers, the cultures were used as inocula in the biochemical methane potential tests.

Biochemical methane potential tests (BMP)

BMP tests were used to assess the effects of three different inocula (cow rumen fluid, anaerobic sludge taken from a full-scale plant and the enriched microorganisms responsible for the degradation of cellulosic material in the cow rumen) and their combinations on methane production (Table 1). Physicochemical characteristics of cow manure were as follows: pH: 7.65 ± 0.4, TS: 15.1% ± 0.08, VS: 13.1% ± 0.08, sCOD: 13250 ± 315 mg/L, TKN: 1355 ± 105 mg/kg. Physicochemical characteristics of cow rumen fluid were as follows: pH: 6.80 ± 0.2, TS: 1.8% ± 0.02, VS: 1.44% ± 0.03, sCOD: 9420 ± 290 mg/L, TKN: 50 ± 4 mg/kg. Physicochemical characteristics of the anaerobic seed sludge were as follows: pH: 7.67 ± 0.5, TS: 9.6% ± 0.07, VS: 7.1% ± 0.05, sCOD: 6000 ± 120 mg/L. The digesters were loaded at an inoculum to substrate (I:S) ratio of 1:1, except for D1 which was operated only with cow manure. BMP tests were performed in 1 L glass digesters with an active volume of 800 mL and operated under mesophilic conditions (36 ^oC and 41 ^oC) and stirring rate of 100 rpm. The amount of rumen fluid added to the digesters were 40% of the total volume (v:v). The amount of enrichment cultures added to the digesters was approximately 1% of the microbial cell number of the seed sludge taken from the biogas plant (Sträuber et

al. 2015). A control digester containing only the seed sludge was also operated during the experiment and the background biogas production was subtracted from the experimental digesters.

Analytical parameters

Duplicate samples were taken from the digesters on days 0, 2, 5, 10, 20, 30 and 40 for pH and volatile fatty acids (VFAs) measurements. VFAs and biogas composition in the digesters were analyzed according to the procedure explained by Akyol et al (2016). Total solids (TS) and volatile solids (VS) were analyzed according to standard methods for influent and effluent slurry samples as day 0 and day 40, respectively. pH was measured using a HANNA HI 221 Microprocessor pH meter.

DNA extraction

Genomic DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, Germany) according to the manufacturer's instructions. After thawing and mixing triplicate samples, 500 µL sample was transferred to the Lysing Matrix E tubes included in the extraction kit. DNA concentration was determined using a NanoDrop 1000 photometer (Thermo Scientific, Germany), diluted to 25 ng/µL in DNase-free water, and stored at -20°C until further investigations.

Real-time PCR assay

The quantification of total archaea, total bacteria and three fibrolytic bacterial species, *Ruminococcus flavefaciens, Fibrobacter succinogenes,* and *Ruminococcus albus,* was carried out by the StepOnePlus^{TM} platform (Applied Biosystems, Life Technologies) with specific primers (Zhou et al 2011). The reaction mixture (20 µL) was composed of 10 µL SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 6 µL of milli-Q water, 1 µL

of 10 μM each primer, and 2 μL of extracted DNA. The amplification conditions were 95°C for 10 min for initial denaturation, and 45 cycles of 95°C for 15 s of denaturation and 60°C for 1 min of elongation and the real-time PCR assays were performed in triplicate for both the standards and each of the samples (StepOnePlus™ platform, Applied Biosystems, Life Technologies).

Prior to the real-time PCR analyses, gradient PCR were performed for optimization of the annealing temperature of the three primer sets for fibrolytic bacteria in which the lowest temperature was about 5 K below the T_m value of the primers. Reactions were carried out using a Techne-512 gradient thermal cycler under the following conditions: one cycle at 95°C for 10 min of initial denaturation, and 45 cycles of denaturation at 95°C for 45s, annealing ranging from 45°C to 55°C for 45 s and 72°C for 45 s of extension, and one cycle of final extension at 72°C for 5 min. Then, the PCR products were analysed by running on a 1% agarose gel containing ethidium bromide and visualised for a single specific band and the absence of primer dimer products.55°C was found as the optimum temperature both for *Ruminococcus flavefaciens and Fibrobacter succinogenes* primers. The optimum temperature was determined as 54°C for *Ruminococcus albus* primers.

5 sample-derived standards for total archaea, total bacteria, *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens*) were used for the real-time PCR analysis. Regular PCR was conducted for each primer set at determined annealing temperatures. After the confirmation of a single band of the correct size, the PCR products were purified using a PureLink® PCR purification kit (Life Technologies) and measured using NanoPhotometer® (Implen GmbH, Germany). Copy numbers were determined based on mass concentration and the length of the PCR product; serial dilutions were made with Tris-EDTA buffer and used as calibration standards for the real-time PCR.

Next generation sequencing

Bacterial community patterns were assessed by NGS-based metagenomics analysis applied using PGM™ Platform. 16S universal Eubacterial primers Bac515F (5'-GTGCCAGCMGCCGCGGTAA-3') and Bac806R (5'-GGACTACVSGGGTATCTAAT-3') primer sets were used for bacterial communities. First, a single-step 30-cycles PCR was performed for each sample by HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) (94 ^{o}C-3 min / 28 x 94 ^{o}C-30 sec / 53 ^{o}C-40 sec / 72 ^{o}C-1 min). All amplicons were diluted to the same concentration and purified using Agencourt Ampure beads kit (Agencourt Bioscience Corporation, MA, USA). The samples were then sequenced using the Ion PGM™ platform following the manufacturer's protocols. The Q25 sequence data obtained after the sequencing was analysed by the determined workflow. In the sequence data, short (<200bp) sequences without barcodes were removed from the data. Similarly, the data having incoherent base readings and including high homopolymeric region (> 6 bp) were removed from the analysis process.

Temporal analysis of fibrolytic bacterial species

The temporal change in total archaeal and bacterial abundances as well as fibrolytic species abundances were visualized with rank clocks (Collins et. al. 2008) using codyn package (Hallet et. al. 2016). Rank clocks shows the rank order of abundances over time in a circle, starting at 12 o'clock and continuing in a clockwise manner. In order to account for the directional changes in the overall system, the change in archaeal, bacterial and species abundances (A) relative to the control digester (D4) were calculated:

$$A_{relative} = \log(A_{bioaugmented} + 1) / \log(A_{control} + 1).$$

The differences between the species compositions of the digesters D1, D2, D3, D5, D6, D7 and that of the D4 (control) were determined at a corresponding time. Thus, principal response curves (PRC, a special case of redundancy analysis-RDA) of the communities in each digester were analysed (Van den Brink and Braak 1999) using vegan package (Oksanen et al. 2016). Curves demonstrate the deviations of community structure from the control digester, accounting for the directional changes in the system. Digesters at different temperatures analysed separately. Data was log(x+1) transformed previous to the analysis. Weights of the three species accompanied with the principal response curves were also shown. Higher species weight means that response of species follows the response curves, whereas the species with negative weight is the shows the opposite pattern. Zero or close to zero species weight means that species show either no response or the response is unrelated to the PRC pattern of the digesters. All analysis and visualizations are done in R version 3.4 (R Core Team, 2017).

Results

BMP experiments

The effect of bioaugmentation with rumen bacteria on methane production was evaluated under different strategies as summarized in Table 1. There was a major contribution of the addition of rumen fluid as well as enriched culture on the methane production both with and without the seed sludge (Fig. 1). The digesters containing only cow manure showed a weak digestion performance and stopped methane production after 10^{th} day at both temperatures. When compared, the addition of enrichment culture to cow manure achieved lower methane yields (D2_36 as 67 mL CH_4/ g VS and D2_41 as 90 mL CH_4/g VS) than the addition of rumen fluid to cow manure (D3_36 as 132 mL CH_4/ g VS and D3_41 as 183 mL CH_4/ g VS) at both temperatures. Furthermore, both configurations resulted in higher methane yields at

41 ^{o}C. Similarly, methane yield of the digester treating cow manure that was inoculated with the seed sludge (D4) were 125 mL CH_4/g VS and 168 mL CH_4/g VS at 36 ^{o}C and 41 ^{o}C, respectively. Nevertheless, when compared to the digester D4, the co-inoculation of enrichment culture to cow manure and the seed sludge increased the methane yield by 70% at 36 ^{o}C, (D5_36: 212 mL CH_4/g VS) and 36% at 41 ^{o}C (D5_41: 228 mL CH_4/g VS). The effect of the co-inoculation of cow rumen fluid to cow manure and the seed sludge (D6) seemed to differ greatly with respect to the temperature. The methane yield increased by 71% to 214 mL CH_4/g VS and 36% to 228 mL CH_4/g VS at 36 ^{o}C and 41 ^{o}C, respectively. The highest methane yield was achieved when the digester containing cow manure and the seed sludge was co-inoculated both with enrichment culture and rumen fluid (D7) as 261 mL CH_4/g VS and 311 mL CH_4/g VS at 36 ^{o}C and 41 ^{o}C, respectively. There was a clear difference in methane production due to the temperature conditions and the increase in methane yield was calculated as 109% and 85% at 36 ^{o}C and 41 ^{o}C, respectively. Although the highest methane yield was achieved for the digester D7 at 41 ^{o}C, the effect of bioaugmentation with the mixture of rumen fluid and enrichment culture was more apparent at 36 ^{o}C.

VFA production and system stability

Changes in VFA production as well as pH variations were investigated to interpret digestion stability with respect to different inoculation strategies. The accumulation of VFAs and/or the dominance of any VFA species during the anaerobic digestion processes can be successfully used to take a hint on the system performance (Franke-Whittle et al., 2014). VFA concentrations in the digesters produced at 36 ^{o}C and 41 ^{o}C are given in Fig. 2. In this particular study, comparatively higher concentrations of VFAs were recorded during the early days of the digestion than the threshold concentration stated in the literature (Siegert and Banks, 2005). In the digestion experiment operated at 36 ^{o}C, VFA concentrations tended to increase until 10th Day and VFA concentrations peaked on 10th Day in all digesters up to

8175 mg acetic acid/L and 8925 mg acetic acid/L in D6 and D7, respectively, except for D1 and D2. It was observed that VFA production continued to increase in D1 and D2 on 20th Day, which can be attributed to a possible sign of VFA inhibition. Thus, methanogenic activity was collapsed and biogas production stopped after 20th Day in D1 and D2 as mentioned earlier. However, the VFA production pattern in the digesters at 41 ^{o}C were quite different than that of 36 ^{o}C. VFA production in the digesters peaked on 10th Day except for D3, D4 and D5, in which highest VFA productions were recorded on 5th Day. Furthermore, the amount of VFAs in the digesters D6 and D7 on 10th Day were measured as 16735 mg acetic acid/L and 10985 mg acetic acid/L, respectively. Furthermore, the accumulation of VFAs during the first 10 days caused pH to decrease below 6.5 in the digesters D1, D2, D10 and D11 at 36 ^{o}C and D10 at 41 ^{o}C. Buffering capacity of cow manure due to excess alkalinity concentrations as well as successfully-further consumption of VFAs did not seem to generate any imbalances in the digesters excluding D1 and D2. The breakdown of the digesters D1 and D2 at 36 ^{o}C can be contributed to VFA inhibition and low pH that further subjected methanogenesis to substrate inhibition. Almost all of the VFAs were consumed in the other digesters and no accumulation was observed towards the end of the experiment. Remaining VFA concentrations in the digesters at the end of the digestion tests were below 80 mg acetic acid/L. The changing pattern of individual VFA species showed that acetic acid, propionic acid and butyric acid were the main VFA products.

Bacterial community pattern and dynamics

The bacterial community profiles of the rumen fluid and cow manure are shown in Fig 3. The bacterial community of the rumen fluid harboured 18 phyla, dominating with *Bacteroidetes* (56.8%), *Firmicutes* (22.4%) and *Proteobacteria* (5.4%). The manure sample was composed of 13 bacterial phyla. As in the rumen fluid, *Firmicutes* and *Bacteroidetes* were the most predominant phyla accounting for 64% and 16.8%, respectively. From the Krona charts, it

can be seen by far the great portions of the sequences were assigned to the class *Bacteroidia* and *Clostridia* which comprise strictly anaerobic bacteria that can degrade cellulolytic materials and other plant polymers (Ji et al. 2012). However, the shares of these classes show differences between rumen fluid and manure samples.

The bacterial community patterns of the batch cultures were screened for the 20th day of operation period in which biogas production reached a plateau. *Firmicutes* and *Bacteroidetes* were by far the most dominant phyla, presented more than 75% of the total reads in all digester samples (Fig. 4). However, their relative abundances in the digesters differed according to the operation temperature and digester compositions. As expected, *Clostridiaceae* and *Ruminococcaceae* whose members show high cellulolytic activity (Wang et al. 2013; Poszytek et al. 2017) were detected in all digesters (Fig. 5). Moreover, non-cellulolytic families *Rikenellaceae* and *Porphyromonadaceae* were also dominant in all digesters.

Whereas, an increase in the operation temperature resulted in an increase in the abundance of *Firmicutes* and *Proteobacteria* in the sole manure digester (non-inoculated, D1), there was a slight decrease in the abundance of *Bacteroidetes.* Although, the highest abundance of *Fibrobacteraceae* was detected in the D1_36 among all digesters, it became less dominant at 41°C (0.01%). Differently from all set-ups, the highest abundance of *Proteobacteria* was determined in the digester operated at 36°C, including the inoculation of only enrichment culture to the cow manure (21%, D2_36). However, the abundance went down due to the temperature increase. Moreover, *Clostridiaceae, Porphyromonadaceae* and *Marinilabiliaceae* became more abundant in the D2_41. There was a marked increase in the abundance of *Bacteroidetes* species in the digester inoculated with only rumen fluid, operated at 41°C (D3_41) than that of D3_36. Furthermore, 5°C increase in the operation temperature

resulted a slight rise in the abundance of *Porphyromonadaceae* in D3. The highest abundance of candidate family *Cloacimonetes* was observed in the D4_41.

Fig. 6A demonstrates relatively stable total archaea and bacteria abundances through the course of inocula, along with slight decrement in bacterial abundance at the end. There are also slight discrepancies from pattern (e. g. increment of archaeal abundance and return was also seen digesters D6 and D7). However, abundances of individual species were different in different digesters. Whereas *F. succinogenes* abundance was relatively stable in D2 and D5, it increased in D1 and decreased in D3, D6, D7. Moreover, while some of the digesters (e.g. D2 and D5) showed similar or lower species abundances relative to the control (D4) some digesters showed relatively higher species abundances through the time (e. g. D3, D6, D7). Fig. 6B shows a differential response of species abundance at 41 °C compared to the 36 °C. Although total archaea and bacteria abundances are similar to the control digester (D4), especially *R. flavefaciens* abundance was higher relative to the control and reached its maximum at the end of the experiment (i.e. in digesters D1, D2, D3, D5, D7). *R. albus* abundance increased initially and returned to the control abundance in digesters D1, D2, D3, D5. However, in digesters D6 and D7, it stayed higher through the time.

Discussion

Biogas production from cow manure is a common application in agricultural biogas plants; whereas, it is limited due to the poor biodegradability of cow manure because of its lignocellulosic content. Although anaerobic co-digestion of cow manure with other organic compounds has been in the spotlight in the recent years due to its high methane yield achievements (Akyol et al., 2016), mono-digestion of cow manure is still in use (Passos et al., 2017). Bioaugmentation with cellulolytic microorganisms is an alternative to increase methane production in such systems (Ozbayram et al. 2017; Tsapekos et al. 2017). The

methane yield from cow manure obtained in this study was comparable with the results in the literature. With the co-inoculation of rumen fluid and enrichment culture to the anaerobic seed sludge at different bioaugmentation strategies, the methane yield could be further increased by 36-109%. Similar results were reported, i.e., Deng and colleagues (2017) achieved 1.79-, 2.07- and 2.26-fold higher methane yields compared with the control digesters at different substrate loading rates of 3 g/d, 7 g/d and 14 g/d, respectively, when ruminal microbiota was co-inoculated with methanogenic sludge during the anaerobic digestion of rice straw. Similarly, the rice straw was pre-treated with the rumen fluid of cattle at 39 ^{o}C for 120 h in anaerobic conditions and methane yield increased by 82.6% and a technical digestion time was decreased of 40.0%, compared with the control (Zhang et al., 2016). In the study of Wall et al. (2015), the addition of rumen fluid of beef heifer during the biomethanation of grass silage facilitated a maximum methane yield of 371 mL CH_4/g VS with particle size less than 1 cm grass silage. In another study, highest methane yield (300 L CH_4/g VS) was achieved when rumen fluid and pig waste sludge was mixed as the inoculum during the anaerobic digestion of fique's bagasse (Quintero et al., 2012).

The variations in time of peak VFA production and concentrations are most probably due to the differences in operating temperatures and also the digester components. Deng and colleagues (2017) reported similar findings, in which acetic acid and propionic acid were stated as the major VFA products and varied slightly within 1.44-2.03 g/L and 1.13-1.61 g/L, respectively. The authors also highlighted that butyrate accumulation caused pH to drop below 6.24 and had a toxic effect on methanogens. In another recent study by Li et al (2017), different solid contents of corn stover as 3%, 5% and 10% (w/v), were pre-treated with rumen fluid to enhance its hydrolysis and acidification. The results showed that higher solid content of corn stover produced more VFAs; however, it was found out that VFAs accumulation occurred during the 10% (w/v) corn stover pre-treatment, leading to the higher VFAs

concentration for 5% (w/v) corn stover than that for 10% (w/v). In the study of Quintero et al. (2012), VFA inhibition was also reported during the fermentation assays when rumen fluid was sued as the inoculum. A VFA accumulation was observed during the process with rumen fluid (7440 mg/L) comparing with a consumption behaviour with rumen fluid and pig waste sludge which further reduced methane production.

The findings of the high-throughput sequencing data broadly support the work of other studies which showed that a major fraction of the total sequencing reads were composed of *Firmicutes* and *Bacteroidetes* in cow manure and rumen fluid (Shanks et al. 2011; Sun et al. 2015). At the family-level, by far the greatest share of the sequences of the rumen fluid assigned to *Prevotellaceae* sp. with an ability to excreate CMCase and xylanase to degrade lignocellulosic feedstock (Nyonyo et al. 2014). In contrast, *Ruminococcaceae* which includes representatives of cellulolytic bacteria (Wang et al. 2013) was accounted as a predominant family in the manure sample. *Rikenellaceae* species can use lactate, the members of *Porphyromonadaceae* can ferment various polysaccharides and the members of both families can produce fermentation products acetate and propionate (Sakamoto 2014a; Yi et al. 2014). Most probably, the enhancement in lignocellulose degradation as well as high concentrations of VFA production can be attributed to *Prevotellaceae* sp. and uncultered bacteria in the rumen fluid that increase the cellulolytic activity consequently.

The members of *Clostridiaceae, Porphyromonadaceae* and *Marinilabiliaceae* are characterized by having a fermentation ability of polysaccharides (Zhao et al. 2012; Sakamoto 2014b).Sun et al. (2015) found that the abundance of *Cloacimonetes* was determined in the similar range (6.0–14.5%) in the digesters treating cow manure as a sole substrate. *Clostridiaceae* and *Marinilabiliaceae* species became abundant in the digester co-inoculated with enrichment culture (D5_41) with respect to the temperature increase. Temperature change effected the bacterial communities in D6 and D7 on the same line. The

abundance of the species belonging to *Leptospiraceae* family, known to ferment wide variety of polysaccharides (Lory 2014), and *Rikenellaceae* increased in the D6 and D7 operated at 41°C. However, the bacteria belonging to *Syntrophomonadaceae* which compose of syntrophic acetogenic bacteria became less abundant.

Fig. S1 shows overall deviation in the community dynamics from the control digester. At 36 °C community structure in digesters inoculated with rumen fluid and/or enrichment culture were similar with control digester (D4) and *F. succinogenes* was the most important species in appearance of these patterns. On the contrary, *R. albus* had almost no impact in the differences of the digesters (Fig. S1A). First canonical axis explained 72% of the variation in the communities, 82% of it was explained by the digesters and time interaction 18% of the variation was explained by conditioned time course. At 41 °C community dynamics in the different digesters were critically different compared to the 36 °C (especially in D1, D2 and D7 at the end of the experiment; canonical coefficient >0.5, Fig. S1B). Differently, *R. albus* and *R. flavefaciens* had the most important impact in the community dynamics relative to the control at 41°C. First canonical axis explained 53% of the variation in the communities, 49% of it was explained by the digesters and time interaction 51% of the variation was explained by conditioned time course.

In summary, bioaugmentation strategies with cow rumen fluid as well as cow rumen-derived enriched microbial consortia with high hydrolytic activity can effectively increase the methane yield. The degree of enhancement can be upgraded by the co-inoculation of these two bioaugmentation approaches with anaerobic seed sludge having high methanogenic activity. Univariate bulk measures such as total abundances are not enough for mechanistically understanding of system performance. Thus, examining the multivariate responses of communities along with abiotic properties is crucial. A close look at the biotic interactions and their associations with abiotic factors might be valuable for evaluating

rumen-related bioaugmentation applications. Furthermore, methane yield can be further improved in support to bioaugmentation by assessing the impact of different operating conditions (i.e. I:S ratio, source of inoculum, digester configuration etc.).

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Digester components during the anaerobic digestion of cow manure at 36 ^{o}C and 41 ^{o}C.

Digester	Enrichment culture	Cow rumen fluid	Anaerobic seed sludge	Cow manure
D1				+
D2	+			+
D3		+		+
D4			+	+
D5	+		+	+
D6		+	+	+
D7	+	+	+	+

Figure Captions

Figure 1. Methane yields obtained during the batch experiments of anaerobic digestion treating manure at A) 36°C, B) 41°C (D1(▲), D2(●), D3 (x), D4(♦), D5(Δ), D6(○), D7(◊)).

Figure 2. VFA profiles obtained during the batch experiments of anaerobic digestion treating manure at A) 36°C, B) 41°C (Day 0(▨), Day 2(▤), Day 5(▥), Day 10(▦), Day 20(▤), Day 30(▩), Day 40(■))

Figure 3. The bacterial communities in phylum, class, order and family levels. A) Cow rumen fluid. B) Cow manure

Figure 4. Heatmap displaying the relative abundances of bacterial phyla with a proportion of at least 1% in at least one sample.

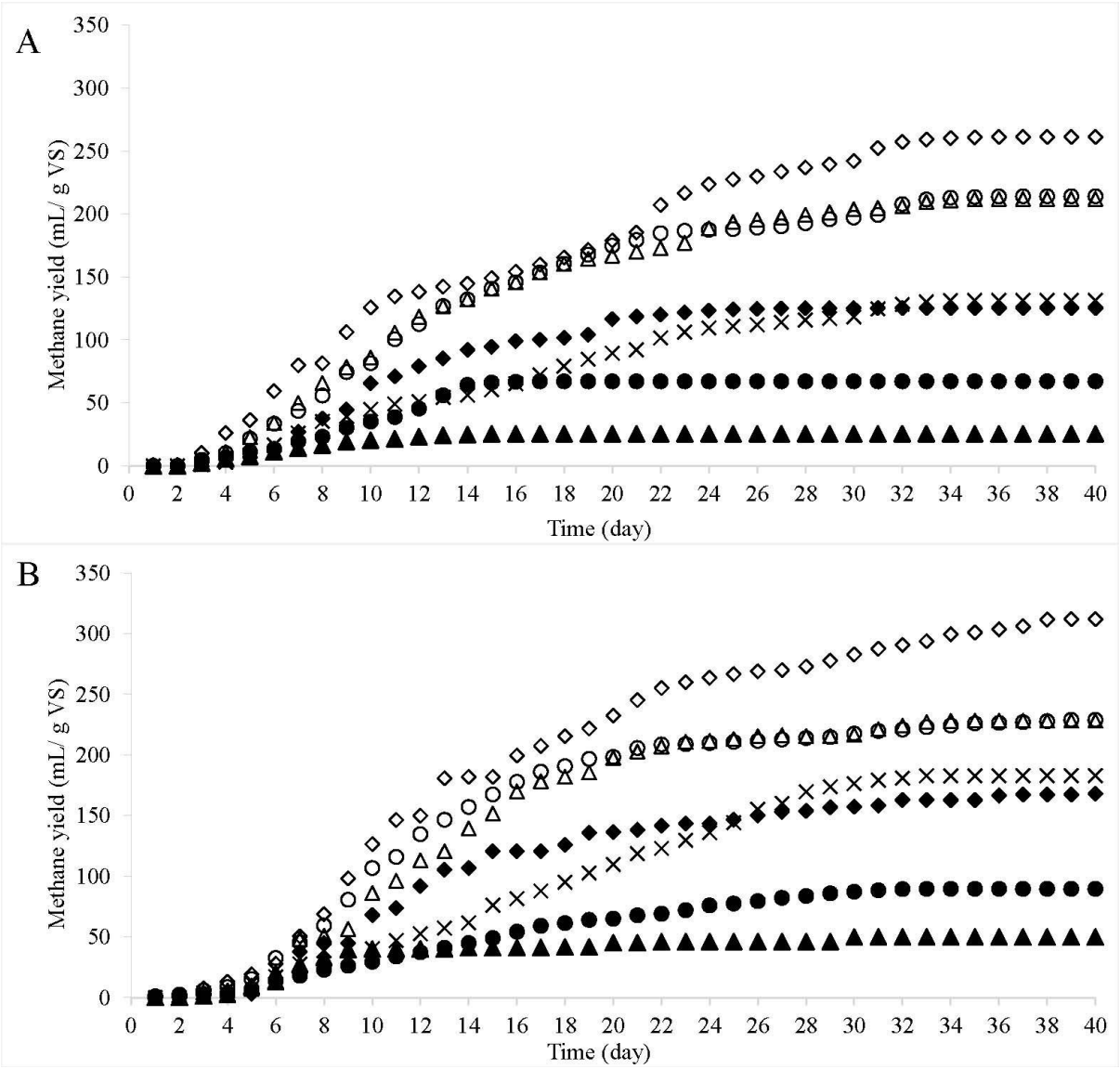
Figure 5. Heatmap displaying the relative abundances of bacterial families with a proportion of at least 1% in at least one sample.

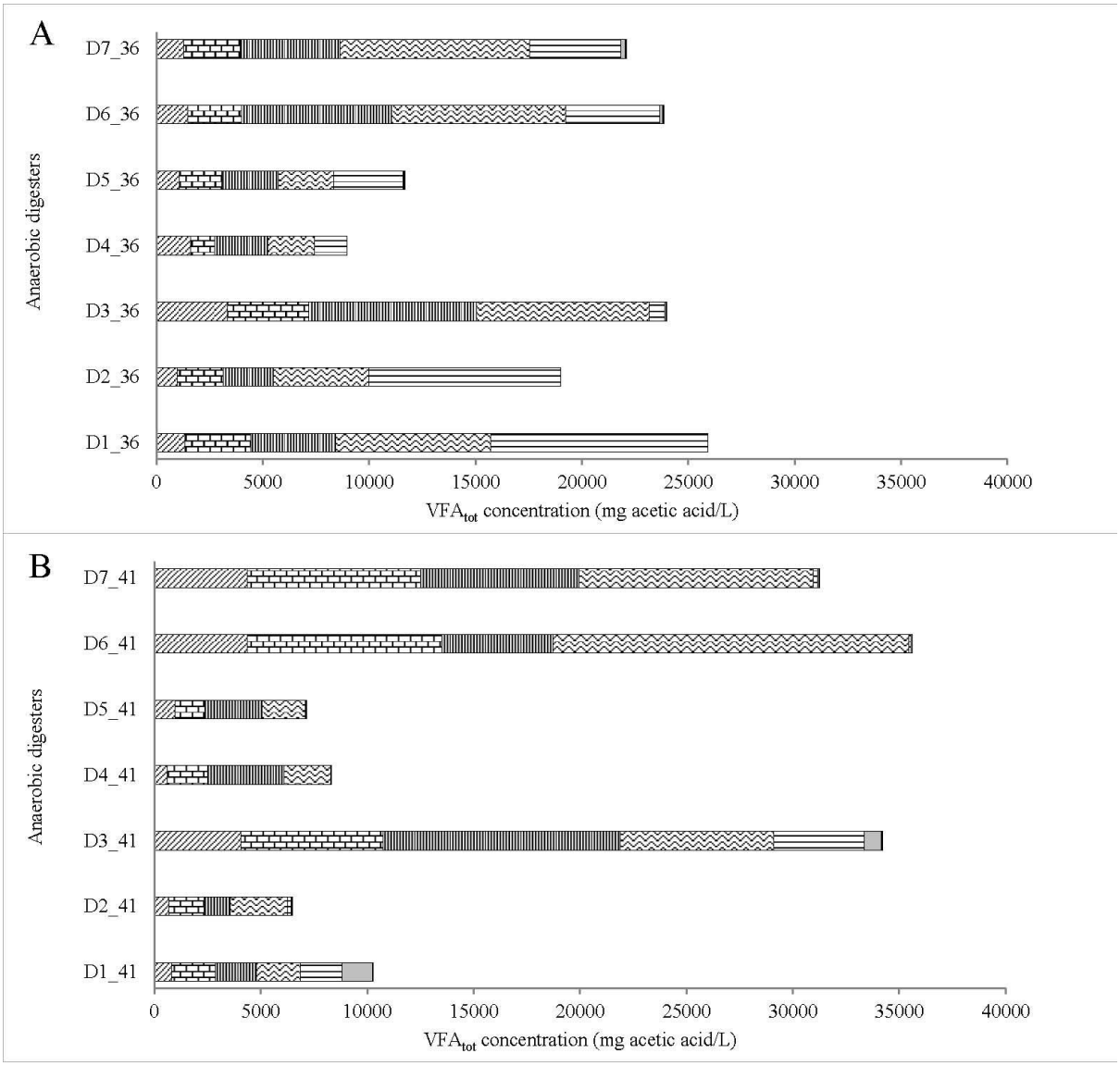
Figure 6. Rank clock plots of the total archaea (- - -), total bacteria (·····), *R. albus* (-----), *F. succinogenes* (——), *R. flavefaciens* (-·-·-) abundances relative to the control digester (D4) at A) 36°C and B) 41 °C. Vertical grey bars show the starting '12 o'clock' position on the rank clock. Series ends at the 12 o'clock position again unless there is no data after the day 20. 1 means no change in abundance relative to the control, over 1 is a positive and under 1 is a negative change. Area under 1 was shown with a grey shaded area.

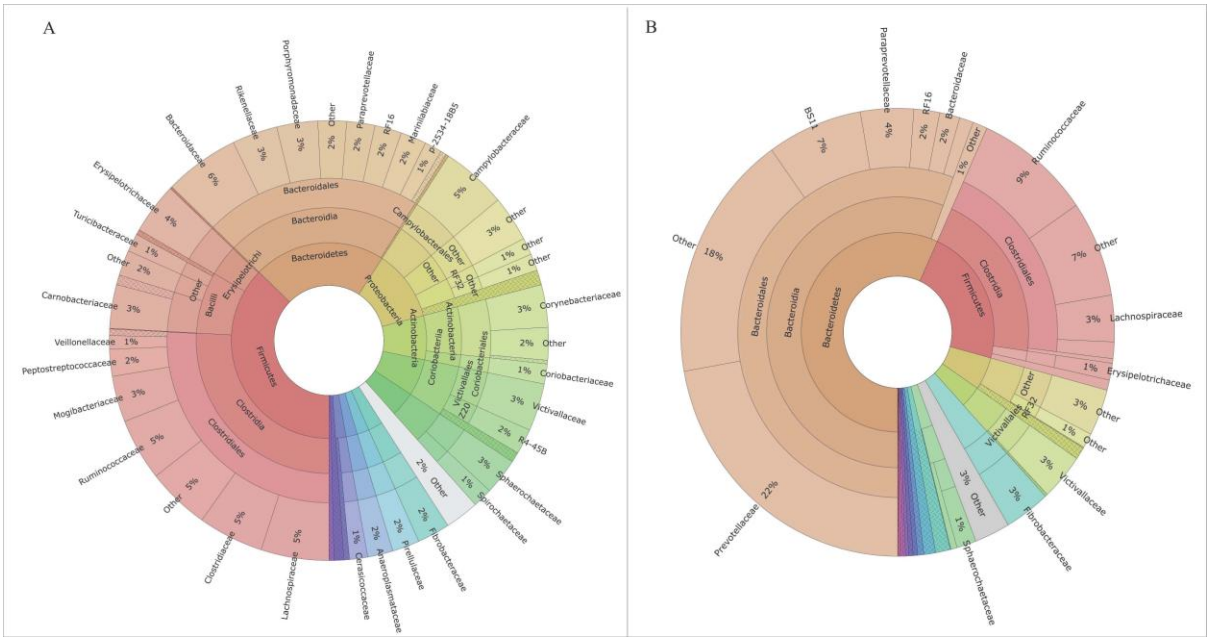
Supplementary Material Figure Captions

Figure S1. Principal response curves (PRC) with species weights for the digester data set at A)

36 °C and B) 41 °C and indicating the effects of every enrichment on the fibrolytic community structure in time relative to control digester







	D1_36	D2_36	D3_36	D4_36	D5_36	D6_36	D7_36	D1_41	D2_41	D3_41	D4_41	D5_41	D6_41	D7_41
Fibrobacteres	8.38	0.65	0.03	0.04	0.12	0.17	0.43	0.01	0.02	0.01	0.01	0.01	0.02	0.01
Actinobacteria	0.24	0.19	0.77	1.10	1.03	0.75	0.98	0.75	0.84	0.58	0.69	0.35	0.42	0.46
Synergistetes	0.18	0.68	0.69	0.77	0.86	0.89	0.94	3.35	3.74	2.73	1.96	2.38	2.20	0.87
Lentisphaerae	1.05	0.48	0.09	0.07	0.27	0.29	0.16	0.05	0.07	0.01	0.06	0.10	0.36	0.10
Tenericutes	0.25	3.64	0.06	0.08	0.30	0.06	0.05	0.06	0.02	0.01	0.03	0.02	0.06	0.39
Cloacimonetes	0.02	0.02	1.87	1.86	1.81	0.52	0.51	0.13	2.07	0.21	9.00	0.15	0.07	0.91
Firmicutes	35.36	33.02	42.15	37.69	34.79	51.24	48.18	45.71	46.49	39.26	43.88	48.12	40.29	42.03
Bacteroidetes	42.09	37.50	34.63	46.79	43.07	35.12	38.85	37.89	40.64	46.70	34.89	41.45	43.24	41.35
Acidobacteria	0.24	0.11	0.33	0.13	0.24	0.56	0.40	0.02	0.30	0.02	1.29	0.14	0.15	0.05
Spirochaetes	8.47	1.77	14.65	4.78	12.45	4.06	1.86	3.08	1.98	8.48	2.70	1.93	10.00	10.79
Chloroflexi	0.04	0.02	0.71	0.57	0.75	0.51	0.69	0.41	0.27	0.24	0.95	0.18	0.28	0.53
Proteobacteria	3.20	21.05	3.11	4.81	2.56	5.13	5.81	7.63	2.67	1.52	3.32	4.39	2.23	1.70

	D1_36	D2_36	D3_36	D4_36	D5_36	D6_36	D7_36	D1_41	D2_41	D3_41	D4_41	D5_41	D6_41	D7_41
Fibrobacteraceae	8.38	0.65	0.03	0.04	0.12	0.17	0.43	0.01	0.02	0.01	0.01	0.01	0.02	0.01
Cytophagaceae	4.56	1.95	0.97	0.85	1.26	1.80	1.30	1.29	1.14	0.43	0.48	1.03	2.78	1.38
Syntrophomonadaceae	0.07	0.08	4.03	1.34	1.70	5.93	6.66	2.92	3.32	1.33	3.14	2.80	1.49	2.08
Peptococcaceae	0.10	0.35	0.79	1.31	1.31	0.54	0.38	0.73	2.55	1.84	2.15	2.32	3.89	1.33
Pseudomonadaceae	0.48	7.87	0.14	0.58	0.18	0.15	0.80	0.15	0.20	0.11	0.16	0.39	0.05	0.05
Rikenellaceae	14.21	8.24	25.96	23.41	24.58	20.26	18.84	12.17	10.06	23.95	17.23	15.29	28.06	30.45
Bacillaceae	2.25	6.71	2.24	6.08	2.59	3.46	2.55	2.01	2.04	3.34	3.97	1.57	1.43	3.22
Clostridiaceae	17.66	11.88	20.00	14.25	15.88	22.88	21.22	20.80	18.66	23.14	19.10	25.13	19.00	19.14
Porphyromonadaceae	6.52	11.92	2.76	12.00	6.31	5.05	8.62	13.36	17.70	10.22	8.23	11.96	6.48	4.19
Erysipelotrichaceae	2.68	1.78	1.52	1.32	1.06	2.50	2.04	2.47	2.57	1.48	2.07	1.89	1.65	1.13
Campylobacteraceae	0.06	8.96	0.21	0.01	0.03	0.16	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.01
Alcaligenaceae	0.16	2.46	0.13	0.07	0.09	0.43	1.37	5.79	0.16	0.16	1.24	1.75	0.30	0.53
Sphingobacteriaceae	1.72	0.75	0.50	0.38	0.48	0.85	0.48	0.77	0.27	0.66	0.55	0.25	0.21	0.25
Cloacimonetes	0.02	0.02	1.87	1.86	1.81	0.52	0.51	0.13	2.07	0.21	9.00	0.15	0.07	0.91
Thermoanaerobacteraceae	0.05	0.02	0.24	0.29	0.30	0.71	0.12	1.50	0.47	0.11	0.56	0.30	0.08	0.23
Synergistaceae	0.18	0.68	0.69	0.77	0.86	0.89	0.94	3.35	3.74	2.73	1.96	2.38	2.20	0.87
Bacteroidaceae	3.38	8.58	2.53	8.89	8.34	2.07	7.14	8.20	9.44	2.90	7.68	5.72	2.53	3.47
Planococcaceae	2.80	0.06	0.26	0.02	0.07	1.00	0.06	0.02	0.03	0.31	0.03	0.02	0.04	0.03
Spirochaetaceae	8.40	1.71	5.88	2.52	8.85	4.02	1.76	1.83	1.48	5.93	1.09	1.47	2.69	6.25
Eubacteriaceae	0.96	1.16	1.45	0.67	1.14	1.94	1.91	1.14	0.85	0.60	0.71	1.30	1.90	0.66
Acidobacteriaceae	0.18	0.07	0.31	0.08	0.17	0.53	0.39	0.02	0.29	0.02	1.27	0.14	0.14	0.04
Lachnospiraceae	2.05	0.99	1.44	0.48	0.37	1.37	1.87	0.58	0.67	0.24	0.37	0.80	1.25	2.41
Prevotellaceae	7.35	0.17	0.48	0.03	0.04	1.16	0.54	0.29	0.51	1.10	0.06	0.24	1.86	0.58
Clostridiales family xi. incertae sedis	0.25	0.93	1.44	3.46	2.38	0.62	2.37	4.75	5.25	1.21	5.09	3.51	0.86	3.89
Ruminococcaceae	4.49	5.69	5.55	6.37	5.70	6.79	5.84	6.28	7.17	4.17	4.33	6.79	7.61	5.94
Marinilabiliaceae	4.08	5.78	1.29	0.95	1.07	3.60	1.77	1.76	1.44	7.38	0.44	6.87	1.29	0.97
Leptospiraceae	0.07	0.06	8.77	2.27	3.59	0.04	0.11	1.25	0.51	2.55	1.61	0.47	7.31	4.55
Acholeplasmataceae	0.18	3.64	0.05	0.06	0.24	0.04	0.03	0.05	0.02	0.01	0.03	0.02	0.03	0.39
Comamonadaceae	0.66	0.25	0.25	1.79	0.36	2.07	0.55	0.62	1.12	0.60	0.53	0.82	0.43	0.13