Identification of Propionate-Degrading Microbial Populations in Methanogenic Processes for Waste Treatment: *Methanosaeta* and *Methanoculleus*

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Abstract

Methanogenic processes have great potential in the sustainable treatment of organic wastes with the production of methane as a renewable source of energy. However, the broader application of methanogenic processes has been hindered by process instability frequently encountered during fluctuations in operational conditions. The accumulation of organic acids, particularly propionate, is considered to be an important cause of process instability. Therefore, in order to gain an understanding of microbial responses during process instability, it is imperative to identify microbial populations involved in the utilization of elevated levels of propionate. In this study, microbial community analysis showed that bacterial populations from the orders of *Syntrophobacterales* and *Clostridiales* were the primary syntrophic partners in anaerobic conversion of propionate. Archaeal populations associated with *Methanosaeta* and *Methanoculleus* dominated the propionate-degrading communities enriched in methanogenic batch bioreactors. The involvement of *Methanosaeta* and *Methanoculleus* in anaerobic conversion of propionate was further supported by the close correspondence between elevated propionate and increased population abundance of *Methanosaeta* and *Methanoculleus* in continuous anaerobic digesters treating animal waste. Subsequent testing using additional methanogenic batch bioreactors revealed that the dominance of *Methanosaeta* and *Methanoculleus* populations was linked to the anaerobic degradation of elevated levels of propionate and butyrate, but not the conversion of formate, acetate, or methanol into methane. The identification of microbial populations specifically linked to anaerobic conversion of elevated propionate in this study provided much needed insight for the understanding of microbial processes relevant to process stability in methanogenic waste treatment.

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Introduction

Methanogenic processes are vital in global elemental cycling, particularly given the relevance of methane as a potent greenhouse gas to climate change (Bae et al., 2018; Falkowski et al., 2008). As the end product of methanogenic conversion of organic matter, methane can also serve as a renewable source of energy. Therefore, methanogenic processes have been applied in the anaerobic treatment of diverse categories of organic waste with the simultaneous production of renewable energy, represented by anaerobic digestion as the most implemented technology (Hagos et al., 2017; Nallathambi Gunaseelan, 1997; Nasir et al., 2012). The broader adoption of methanogenic treatment processes, however, has been hindered by the difficulties in maintaining operational stability, especially during episodes of process perturbation (Chen et al., 2012; Yuan and Zhu, 2018).

Considerable efforts have been made to understand the underlying mechanisms of process instability in methanogenic treatment processes. Previous studies have shown that process perturbations, such as changes in operational conditions and organic loading rates, would result in process imbalance frequently characterized by pH fluctuations due to accumulation of organic acids (Ahring et al., 1995; Leitão et al., 2006; Li et al., 2014). Among the organic acids produced as intermediates during the anaerobic decomposition of organic materials, propionic acid, present as propionate at neutral pH conditions, is found to be the most persistent and inhibitory, likely due to its slower biodegradation kinetics and less favorable thermodynamics in anaerobic biotransformation into methane (Boe et al., 2010; Nielsen et al., 2007).

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The anaerobic degradation of propionate under methanogenic conditions requires syntrophic interactions between methanogens and fermentative bacteria partners (Schink, 1997). Fermentative bacteria are known to convert propionate into acetate and H_2 in two distinct pathways (Müller et al., 2010). The *Smithella* pathway, observed in *Smithella* spp., converts propionate to acetate and H_2 by dismutation followed by β-oxidation in the following reactions:

$$2CH_3CH_2COO^- \rightarrow CH_3CH_2CH_2COO^- + CH_3COO^- \quad (1)$$

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad (2)$$

The overall *Smithella* pathway has the following stoichiometry:

$$2CH_3CH_2COO^- + 2H_2O \rightarrow 3CH_3COO^- + H^+ + 2H_2 \quad (3)$$

The classic propionate oxidation pathway is found in all other known anaerobic propionate degraders as per the following stoichiometry:

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2 \quad (4)$$

In order for both pathways to be thermodynamically favorable, the products of propionate conversion, i.e. H_2 and acetate, need to be maintained at low levels, which can be achieved via the utilization of H_2 and acetate by methanogens in methanogenic processes. The overall stoichiometry of both anaerobic propionate oxidation pathways is identical following the consumption of H_2 and acetate by hydrogenotrophic and acetoclastic methanogenesis, respectively:

$$4CH_3CH_2COO^- + 2H_2O + 4H^+ \rightarrow 7CH_4 + 5CO_2 \quad (5)$$

Despite the large diversity of bacterial taxa known to participate in syntrophic propionate conversion, the bacterial partners specifically involved in this process have been consistently identified as populations from two bacterial orders — *Syntrophobacterales* and *Clostridiales* (Müller et al., 2010; Sieber et al., 2012). In contrast to the demonstrated specificity of these

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bacterial populations to anaerobic propionate conversion, much less is known about the methanogens that specifically partner with bacteria to convert the products of anaerobic propionate oxidation into methane. Given the thermodynamic necessity of maintaining adequately low H_2 partial pressure to enable anaerobic propionate oxidation, hydrogenotrophic methanogens, with the ability to consume H_2, have been the focus of previous studies (Li et al., 2018; Narihiro et al., 2015; Shigematsu et al., 2006; Stams et al., 1992; Wu et al., 1992). Diverse populations of methanogens have been found in methanogenic treatment processes. However, unlike the bacterial counterparts where lineages specifically linked to anaerobic propionate conversion have been identified, methanogens specifically involved in the degradation of propionate remain obscure.

With the objective of this study to identify methanogen populations specifically involved in the anaerobic conversion of propionate, methanogenic batch bioreactors were developed with elevated propionate as the sole substrate to enrich propionate-degrading microbial populations, which were profiled by 16S rRNA gene amplicon sequencing and clone library analysis. Subsequently, the roles of methanogen populations identified to specifically involved in anaerobic propionate conversion were further evaluated in continuous anaerobic digesters with episodes of elevated propionate. Findings from this study provide much needed insight into the understanding of microbial responses to elevated propionate during process perturbations in methanogenic waste treatment.

Materials and Methods

Development of methanogenic batch bioreactors

To enrich for methanogenic microbial populations specifically involved in the anaerobic conversion of propionate and other volatile organic acids (VFAs), methanogenic batch bioreactors were developed using one of the following compounds as the sole substrate: formate, acetate, propionate, butyrate, or methanol. All methanogenic batch bioreactors were set up using previously described protocols (Chen and He, 2015) and the same defined anaerobic medium prepared according to a previously described recipe (He and Sanford, 2002). The medium was autoclaved, cooled, and subsequently aliquoted into 160-mL serum bottles flushed with pure N_2 and sealed with butyl rubber stoppers and aluminum caps.

The first-generation methanogenic batch bioreactors were seeded with a 10 % (v/v) inoculum using the digestate from previously established continuous anaerobic digesters treating animal waste (Zhang et al., 2011) into 100-mL fresh anaerobic medium. Subsequent to inoculation, each batch bioreactor was fed with one of the following as the sole substrate: formate (50 mM), acetate (20 mM), propionate (20 mM), butyrate (20 mM), or methanol (30 mM). Additional feedings of the substrate at the same concentration were replenished when the substrate was depleted in the batch bioreactors. At the completion of 10 feedings of substrates, second-generation methanogenic batch bioreactors were established by transferring a 10% (v/v) inoculum from first-generation batch bioreactors into fresh medium followed by repeated feedings of the same substrate. At the conclusion of 10 feedings, the second-generation methanogenic batch bioreactors were sampled for microbial community analysis. All batch bioreactors were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 ºC with periodic monitoring of methane production and substrate concentration.

Configuration and operation of continuous anaerobic digesters treating animal waste

To identify methanogenic microbial populations specifically involved in anaerobic conversion of propionate, population dynamics of methanogens were monitored during two episodes of elevated propionate in previously established triplicate continuous anaerobic digesters established (Chen et al., 2012). Dairy wastewater was used as the regular feedstock for these continuous anaerobic digesters at an organic loading rate of 1.0 g volatile solids (VS)/L/day. The operating temperature was controlled at 35 °C with the hydraulic retention time maintained at 20 days throughout the study period. All continuous digesters exhibited stable operation prior to the two episodes of elevated propionate.

The first episode of elevated propionate was introduced into the anaerobic digesters with the direct addition of propionate without disrupting the regular feeding of dairy wastewater. To maintain propionate at elevated levels, a sodium propionate stock solution (5 M) was used to feed the triplicate continuous anaerobic digesters at a loading rate of 6.0 mmol/L/day for two days. Propionate levels in the anaerobic digesters were closely monitored and the propionate loading was increased to 9.0 mmol/L/day in order for the peak propionate level to reach 12.0 mM (Fig. 1). To replicate the microbial responses to elevated propionate, a 2nd episode of elevated propionate was introduced 10 days after the completion of the 1st episode of elevated propionate. Propionate was added at a loading rate of 9.0 mmol/L/day for 8 days, resulting in elevated propionate levels similar to those in the 1st episode of elevated propionate (Fig. 1). The loading rate of dairy wastewater remained unchanged at 1.0 g VS/L/day during the study period with or without the addition of extraneous propionate.

Process parameters of the continuous anaerobic digestions, including CH_4 production and VFA concentrations, were monitored as described previously (Chen et al., 2012). Biomass samples were taken from the continuous anaerobic digesters at five time points A-E as illustrated

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in Fig. 1. Samples were pelleted by centrifugation at 14,000× g for 15 min and preserved at −80 °C for further processing.

High-throughput sequencing of 16S rRNA gene amplicon library

The microbial communities in the methanogenic batch bioreactors with propionate as the sole substrate was analyzed by 16S rRNA gene amplicon library sequencing. First, whole community DNA was extracted from the biomass samples and purified as previously described (Zhang et al., 2009). DNA extracts from triplicate batch bioreactors were pooled for PCR amplification of the V4 region of the 16S rRNA gene with 515-F and 806-R Golay barcoded primers (Caporaso et al., 2012). PCR amplification and cleaning, amplicon quantification, and paired-end sequencing were completed with previously established protocols (Chen et al., 2017). All sequence reads were analyzed by the Mothur (v.1.35) platform following previously established procedures (Kozich et al., 2013).

After sequence quality processing including barcode and primer trimming, denoising, and chimera checking, operational taxonomic units (OTUs) were assigned by a 97% identity threshold using previously established procedures (Zhang and He, 2013) followed by assignment of taxonomic ranks with the RDP Classifier at the confidence threshold of 80% as previously described (Cole et al., 2003). Community compositions were estimated according to the taxonomic assignments of all valid sequences.

Clone library analysis of methanogenic batch bioreactors

The methanogen community in the propionate-amended methanogenic batch bioreactors was also characterized by clone library analysis following previously established protocols (Zhang

and He, 2013). Clone library analysis was performed to verify results from the high-throughput sequencing of 16S rRNA gene amplicon library and to obtain more complete 16S RNA gene sequences for more accurate taxonomic classification. Briefly, DNA extracts from the second-generation batch bioreactors were used for PCR amplification of the 16S rRNA genes with archaea-specific primers, Arch21F and Arch958R, as previously described (DeLong, 1992). Amplicons were subsequently purified and cloned into plasmid vectors following previously described procedures (Zhang et al., 2011). Approximately 20 cloned plasmid inserts were randomly selected for sequencing.

The resulting 16S rRNA gene sequences were checked for chimeric artifacts using the Chimera Check program in the Ribosomal Database Project II (Cole et al., 2003), with high quality sequences deposited at GenBank under the following accession numbers: KJ914860 — KJ914878. Subsequently, OTUs with 3% difference cutoff were assigned based on the average neighbor clustering algorithm. Phylogenetic analysis was conducted on representative 16S rRNA gene sequences of OTUs classified as *Methanosaeta* and *Methanoculleus* with closely related sequences in the NCBI GenBank database using MEGA 4.0 (Tamura et al., 2007).

Quantification of methanogen populations by real-time quantitative PCR (qPCR)

Microbial populations of interest were quantified by qPCR in the methanogenic batch bioreactors and continuous anaerobic digesters during two episodes of elevated propionate. All qPCR assays used the TaqMan chemistry following previously established protocols (Chen and He, 2016). The population-specific TaqMan primer/probe sets used in the qPCR assays included Mst702F-Mst753P-Mst862R (Yu et al., 2005), Mc274F-Mc361P-Mc477R (Chen et al., 2014), and Arc787F-Arc915P- Arc1059R (Yu et al., 2005), for the quantification of *Methanosaeta*,

Methanoculleus, and total archaea, respectively. DNA templates used as the standards for qPCR were partial 16S rRNA genes of representative methanogen populations cloned from the continuous anaerobic digesters in a previous study (Chen et al., 2014), including *Methanosaeta* (GenBank Accession No. JN052761) and *Methanoculleus* (GenBank Accession No. JN052756).

Analytical methods

Biogas production from the batch bioreactors or continuous anaerobic digesters was measured with a previously described water-displacement technique (Zhu et al., 2011). CH_4 content in biogas was determined with a Hewlett Packard 5890 Series II gas chromatograph equipped with a thermal conductivity detector and a Supelco packing column (60/80 Carbonxen®-1000; Sigma-Aldrich, St Louis, MO, USA) according to a previously described operating procedure (Chen and He, 2016). VFAs were quantified with an Agilent 1200 series High-Performance Liquid Chromatography (Agilent Technologies, Santa Clara, California, USA) equipped with a Bio-Rad Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, California, USA) as previously described (Chen and He, 2016).

Statistical analysis

To evaluate the response of specific methanogen populations to elevated propionate levels, the changes in the abundance of *Methanosaeta* as well as *Methanoculleus* during the two episodes of elevated propionate in the continuous anaerobic digesters were analyzed with the one-way analysis of variance (ANOVA) using qPCR results. Significant differences in the abundance of *Methanosaeta*/*Methanoculleus* between time points, i.e. time points A-E (Fig. 1), with or without elevated propionate were indicated by a probability value (p) less than 0.05 in ANOVA analysis.

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Post hoc comparisons were performed using Tukey's test to further identify the time points with significant difference in *Methanosaeta*/*Methanoculleus* abundance as indicated by a probability value less than 0.05. Additionally, correlations of the abundance between *Methanosaeta* and *Methanoculleus* in the continuous anaerobic digesters subjected to two episodes of elevated propionate were evaluated with the Pearson's correlation.

Results and Discussion

Propionate utilization in methanogenic batch bioreactors

High levels of propionate (20 mM) was used as the sole substrate to enrich methanogenic populations involved in anaerobic conversion of propionate. In the second-generation batch bioreactors amended with propionate, the utilization of propionate was accompanied with proportional increases in cumulative methane production (Supplementary Fig. S1). The methane yield from propionate averaged 1.61 mmol CH_4/mmol propionate, which was 92% of the theoretical methane yield according to the overall stoichiometry of anaerobic propionate conversion to methane (Eq. 5).

The agreement between experimental results and theoretical predictions on methane yields from propionate confirmed that the methanogenic populations enriched in the batch bioreactors were indeed involved in the syntrophic conversion of propionate. Therefore, these batch bioreactors were further studied to characterize the methanogen populations grown on propionate as the substrate.

Methanogen populations in methanogenic batch bioreactors

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The methanogen community in the propionate-amended batch bioreactors was profiled by 16S rRNA gene amplicon library sequencing. With archaeal sequences accounting for 40.1% of the overall microbial community, populations associated with *Methanosaeta* and *Methanoculleus* were found to dominate the archaeal community, with a combined relative abundance of 88% (Fig. 2a). Minor populations of methanogens included *Methanospirillum*, and *Methanobacterium*. The predominance of *Methanosaeta* and *Methanoculleus* suggested that *Methanosaeta* as acetoclastic methanogens likely utilized acetate as a product of propionate oxidation while *Methanoculleus* as hydrogenotrophic methanogens consumed H_2 to sufficiently low levels to sustain anaerobic biodegradation of elevated propionate.

To achieve more accurate phylogenetic classification of *Methanosaeta* and *Methanoculleus* populations identified by 16S rRNA gene amplicon library sequencing, the methanogen community was further characterized with clone library analysis in the propionate-amended batch bioreactors. As expected, *Methanosaeta* and *Methanoculleus* were again found to be the dominant methanogen populations (Fig. 2b). Combined, these two populations accounted for 95% of the archaeal community, which was consistent with the results from 16S rRNA gene amplicon library sequencing. The nearly exclusive dominance of *Methanosaeta* and *Methanoculleus* in the methanogen community further suggested the superior competitiveness and specific involvement of both methanogen populations in the conversion of elevated propionate.

Phylogenetic analysis of representative clones of 16S rRNA genes indicated that all clones of *Methanosaeta* were closely related to *Methanosaeta concilii* (Fig. 3), which has been identified as a primary acetoclastic methanogen population involved in the degradation of propionate in anaerobic digestion processes (Ban et al., 2013; Kim et al., 2015; Shigematsu et al., 2006; Zhang

et al., 2018). The majority of *Methanoculleus* clones had *Methanoculleus receptaculi* as the closest relative (Fig. 3). *M. receptaculi* is a strictly hydrogenotrophic methanogen (Cheng et al., 2008) and has been found to be important members of propionate-degrading consortia (Ahlert et al., 2016; Ban et al., 2013). These observations corroborated the potential competitiveness and specific involvement of *Methanosaeta* and *Methanoculleus* in anaerobic propionate conversion.

Bacterial populations in methanogenic batch bioreactors

Bacterial sequences accounted for 59.9% of the overall microbial community in the propionate-amended batch bioreactors as profiled by 16S rRNA gene amplicon library sequencing. Populations associated with *Syntrophobacter* and *Peptococcaceae* represented the majority of the bacterial community, with a combined relative abundance of 57% (Supplementary Fig. S2). It should be noted that *Syntrophobacter* and *Peptococcaceae* belong to the bacterial orders of *Syntrophobacterales* and *Clostridiales*, respectively, which are known to be involved in anaerobic propionate conversion as syntrophic partners (Müller et al., 2010; Sieber et al., 2012).

Other bacterial populations contributed more than 3% to the bacterial community participating in anaerobic propionate conversion included sequences classified as *Thermovirga* and *Thermotogaceae* (Supplementary Fig. S2), which have been frequently identified in methanogenic processes, however, with specific involvement in propionate conversion remained to be validated (Amin et al., 2021). Analysis of the bacterial populations in the propionate-amended batch bioreactors confirmed the abundance and importance of *Syntrophobacterales* and *Clostridiales* in syntrophic propionate degradation. However, further efforts are needed to understand the roles of other minor but significant bacterial populations such as *Thermovirga* in anaerobic propionate conversion.

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Propionate utilization in continuous anaerobic digesters treating animal wastewater

The continuous anaerobic digesters used animal wastewater as the substrate. As a result, the microbial community in the anaerobic digesters was much more complex than that in the batch bioreactors (Chen et al., 2012), providing ideal process conditions to further validate the competitiveness and specific involvement of *Methanosaeta* and *Methanoculleus* in anaerobic conversion of elevated propionate.

To identify methanogen populations specifically involved in the biodegradation of high concentrations of propionate, two episodes of elevated propionate was introduced to the continuous anaerobic digesters by the addition of extraneous propionate (Fig. 1). During both episodes of elevated propionate, the additions of propionate led to rapid increases in propionate concentration, peaking at about 12 mM (Fig. 4a). The concentration of propionate declined immediately following the cessation of propionate feeding, to levels before propionate addition. Corresponding to the increases in propionate concentration, methane production increased and subsequently declined when propionate concentration decreased (Fig. 4b), indicating the rapid conversion of propionate into methane. Notably, there was no indication of acetate accumulation in the anaerobic digesters in response to propionate addition (Fig. 4a), further demonstrating the effectiveness of the methanogen community in the utilization of intermediates from anaerobic propionate oxidation.

Dynamics of Methanosaeta and Methanoculleus in continuous anaerobic digesters in response to elevated propionate

Microbial community analysis of propionate-amended methanogenic batch bioreactors suggested the competitiveness and specific involvement of *Methanosaeta* and *Methanoculleus* in the biodegradation of high levels of propionate (Fig. 2a & 2b). It should be pointed out that in treatment processes using complex waste materials as the substrate, *Methanosaeta* and *Methanoculleus* might not be the dominant methanogens even with propionate accumulation, due to competitions from other methanogen populations that utilize various intermediates other than propionate. Thus, the continuous anaerobic digesters using animal wastewater as the substrate provided a relevant model process to simulate this scenario.

The specific responses of *Methanosaeta* and *Methanoculleus* populations to elevated propionate were monitored by qPCR in the continuous anaerobic digesters, where effective propionate conversion into methane was observed (Fig. 4). *Methanosaeta*- and *Methanoculleus*-specific qPCR assays showed that both methanogen populations responded positively to the addition of extraneous propionate. When the anaerobic digesters experienced the first episode of elevated propionate with an increase in propionate concentration from time point A to B (Fig. 1), the abundance of both *Methanosaeta* and *Methanoculleus* increased significantly (Fig. 5a & 5b). Afterwards, the pause in propionate addition at time point B (Fig. 1) and subsequent drop in the propionate concentration (Fig. 4a) led to the significant decline in the abundance of both *Methanosaeta* and *Methanoculleus* from time point B to C (Fig. 5a & 5b). The same population dynamics were again demonstrated during the second episode of elevated propionate from time point C to E (Fig. 5a & 5b), suggesting the specific involvement of *Methanosaeta* and *Methanoculleus* in the conversion of elevated propionate to methane.

Moreover, the population dynamics of *Methanosaeta* and *Methanoculleus* were correlated with a statistically significant correlation coefficient of 0.7, demonstrating the coordinated

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responses to elevated propionate between *Methanosaeta* and *Methanoculleus* in the continuous anaerobic digesters (Fig. 5c). The close coordination between *Methanosaeta* and *Methanoculleus* might explain the combined dominance of *Methanosaeta* and *Methanoculleus* in the propionate-degrading microbial communities observed in the methanogenic batch bioreactors with propionate as the sole substrate (Fig. 2).

Specificity of Methanosaeta and Methanoculleus to propionate conversion

The involvement of *Methanosaeta* and *Methanoculleus* in the conversion of propionate to methane was demonstrated by microbial community analysis of propionate-amended batch bioreactors by 16S rRNA gene sequencing and continuous anaerobic digesters by qPCR (Figs. 2 and 5). It remained to be verified whether the involvement of *Methanosaeta* and *Methanoculleus* was specific to propionate. Therefore, additional methanogenic batch bioreactors were set up using other organic compounds, including formate, acetate, butyrate, and methanol. The abundance of *Methanosaeta* and *Methanoculleus* was determined by qPCR and compared between batch bioreactors using different compounds as the sole substrate.

As expected, qPCR analysis revealed that *Methanosaeta* and *Methanoculleus* populations dominated in the propionate-amended batch bioreactors, accounting for a combined 87% of the archaeal community (Fig. 6), consistent with results from 16S rRNA gene amplicon library sequencing and clone library analysis (Fig. 2). Interestingly, the dominance of *Methanosaeta* and *Methanoculleus* was also found in the butyrate-amended batch bioreactors (Fig. 6). Previous studies have shown that anaerobic oxidation of butyrate uses β-oxidation to convert butyrate to acetate and H_2 (Schink, 1997) with the following stoichiometry:

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \qquad (6)$$

The products of anaerobic butyrate oxidation, i.e. acetate and H_2, were the same as those of propionate oxidation (Eq. 3 & 4). Thus, this similarity between the conversions of propionate and butyrate likely contributed to the dominance of *Methanosaeta* and *Methanoculleus* in both propionate- and butyrate-amended batch bioreactors. These observations pointed to the possibility that *Methanosaeta* and *Methanoculleus* were specifically linked to the anaerobic conversion of organic compounds with acetate and H_2 as the primary intermediate products.

It was observed that the relative abundance of acetoclastic *Methanosaeta* was higher in butyrate-amended batch bioreactors than that of the propionate-amended batch bioreactors (Fig. 6). In comparison, hydrogenotrophic *Methanoculleus* exhibited an opposite trend (Fig. 6). According to Eq. 6, anaerobic conversion of butyrate would set the molar ratio of acetate/H_2 in the product mix at 1.00. In contrast, anaerobic conversion of propionate following the classic pathway (Eq. 4) would yield an acetate/H_2 molar ratio of 0.33. The molar ratio of acetate/H_2 represents the distribution of substrates between acetoclastic methanogens (i.e. *Methanosaeta*) and hydrogenotrophic methanogens (i.e. *Methanoculleus*). Thus, the higher acetate/H_2 ratio from anaerobic conversion of butyrate than propionate is consistent with the greater relative abundance of *Methanosaeta* observed in butyrate- than propionate-amended batch bioreactors (Fig. 6).

It should be noted that anaerobic conversion of propionate could follow the *Smithella* pathway (Eq. 3), yielding an acetate/H_2 molar ratio of 1.50, which is higher than that of anaerobic butyrate conversion (Eq. 6). Thus, the prevalence of the *Smithella* pathway would have resulted in greater relative abundance of *Methanosaeta* in propionate- rather than butyrate-amended batch bioreactors, which however was not the case (Fig. 6). It could be deduced that the contribution of the *Smithella* pathway to anaerobic propionate conversion was insignificant in

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propionate-amended batch bioreactors, which is further corroborated by the lack of detection of *Smithella* in propionate-amended bioreactors (Fig. S2).

In the acetate-amended batch bioreactors, qPCR results showed that *Methanosaeta* represented more than 80% of the archaeal community, while the abundance of *Methanoculleus* was negligible (Fig. 6). These results were expected as acetate could be converted directly into methane by *Methanosaeta* populations alone as obligatory acetoclastic methanogens (Smith and Ingram-Smith, 2007). The absence of *Methanosaeta* and *Methanoculleus* was expected in the methanol-amended batch bioreactors, which was consistent with the inability to perform methylotrophic methanogenesis by either *Methanosaeta* or *Methanoculleus* (Maestrojuan et al., 1990; Smith and Ingram-Smith, 2007).

The absence of *Methanoculleus* in the formate-amended batch bioreactors (Fig. 6), however, was unexpected. With rare exceptions, the ability to utilize formate for methanogenesis has been found in almost all *Methanoculleus* species (Maestrojuan et al., 1990), including *M. receptaculi* (Cheng et al., 2008), with which many of the clones from the propionate-amended batch bioreactors were closely associated (Fig. 3). It is evident that *Methanoculleus* populations in the batch bioreactors were not able to compete for formate with other hydrogenotrophic methanogens in this study. Instead, it is possible that these *Methanoculleus* populations might be specifically competitive in the utilization of H_2 at very low partial pressure associated with the syntrophic oxidation of propionate or butyrate, as evidenced by the dominance of *Methanoculleus* as the hydrogenotrophic methanogens in propionate- and butyrate-amended batch bioreactors. Indeed, *Methanoculleus* populations have been shown to have high affinity to H_2 in previous studies (Hori et al., 2006; Sakai et al., 2009). Notably, *Methanosaeta* populations are also known to have high affinity to acetate and outcompete other acetoclastic methanogens at

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low acetate levels (Conklin et al., 2006). Thus, the involvement of *Methanosaeta* and *Methanoculleus* in the anaerobic conversion of propionate or butyrate could likely be attributed to the ability of these methanogens to utilize H_2 and acetate at low concentrations, a thermodynamic requirement for anaerobic oxidation of VFAs.

Conclusions

In this study, *Methanosaeta* and *Methanoculleus* were identified as the key acetoclastic and hydrogenotrophic methanogens, respectively, participating in the anaerobic degradation of propionate as a central intermediate in methanogenic processes. Bacterial populations from the orders of *Syntrophobacterales* and *Clostridiales* were confirmed as the primary syntrophic partners in anaerobic conversion of propionate utilizing the classic anaerobic propionate oxidation pathway. Findings from this study provide much needed insight into the understanding of microbial responses to elevated propionate, which is recognized as an inhibitory intermediate frequently contributing to process perturbations in methanogenic waste treatment. The identification of microbial populations specifically involved in anaerobic propionate degradation suggests the potential of exploiting microbial populations such as *Methanosaeta* and *Methanoculleus* in enhancing the stability of methanogenic treatment processes.

Author Contribution Statement QH and SC conceived and designed research. SC, YW, and HC conducted experiments. SC and CH analyzed data. QH and TCH wrote the manuscript. All authors read and approved the manuscript.

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Figure Captions:

FIG. 1 The addition of extraneous propionate to continuous anaerobic digesters during the two episodes of elevated propionate. Biomass sampling at 5 time points was shown by the arrows labeled with letters A to E. Feeding of dairy wastewater to the anaerobic digesters remained constant at 1.0 g VS/L/day throughput the study period.

FIG. 2 Composition of the archaeal community in methanogenic batch bioreactors with propionate as the sole substrate. The relative abundance of methanogen taxa is shown as percentage values determined by 16S rRNA gene amplicon library sequencing (a) and by clone library analysis of 16S rRNA genes (b).

FIG. 3 Neighbor-joining phylogenetic tree showing relationships of representative partial 16S rRNA gene sequences cloned from methanogenic batch bioreactors with propionate as the sole substrate to close relatives. Clones from this study are in bold. GenBank accession numbers of the 16S rRNA gene sequences are indicated in the parentheses. The numerical values at branch nodes indicate bootstrap values per 1,000 re-samplings. The scale bar represents the number of substitutions per sequence position.

FIG. 4 Performance of continuous anaerobic digesters during two episodes of elevated propionate: a) Concentrations of acetate and propionate; and b) methane production. Data are means of triplicate anaerobic digesters, with the error bars indicating the standard deviations.

FIG. 5 Abundance of a) *Methanosaeta* (Mst), b) *Methanoculleus* (Mc), and c) correlation between the abundance of *Methanosaeta* (Mst) and *Methanoculleus* (Mc) during two episodes of elevated propionate in the continuous anaerobic digesters. Time points A-E are illustrated in Fig. 1. The abundance of *Methanosaeta* and *Methanoculleus* was determined as the copies of population-specific 16S rRNA genes with qPCR. Data points were means of triplicates with the error bars showing standard deviations. Abundances of *Methanosaeta* or *Methanoculleus* (Mc) are not significantly different from each other at time points labeled with the same lowercase superscript letters (ANOVA, Tukey's test, $p < 0.05$).

FIG. 6 Abundance of *Methanosaeta* (Mst) and *Methanoculleus* (Mc) as a percentage of total archaea in methanogenic batch bioreactors amended by various organic acids or methanol as the sole substrate. Microbial abundance was determined as the copies of population-specific 16S rRNA genes with qPCR. Results were means of triplicates with the error bars showing standard deviations.

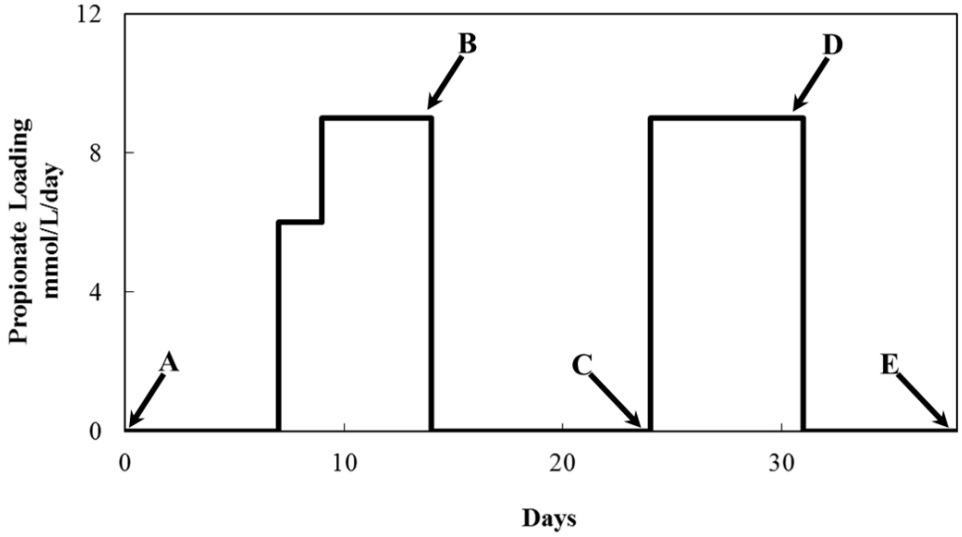

Fig. 1

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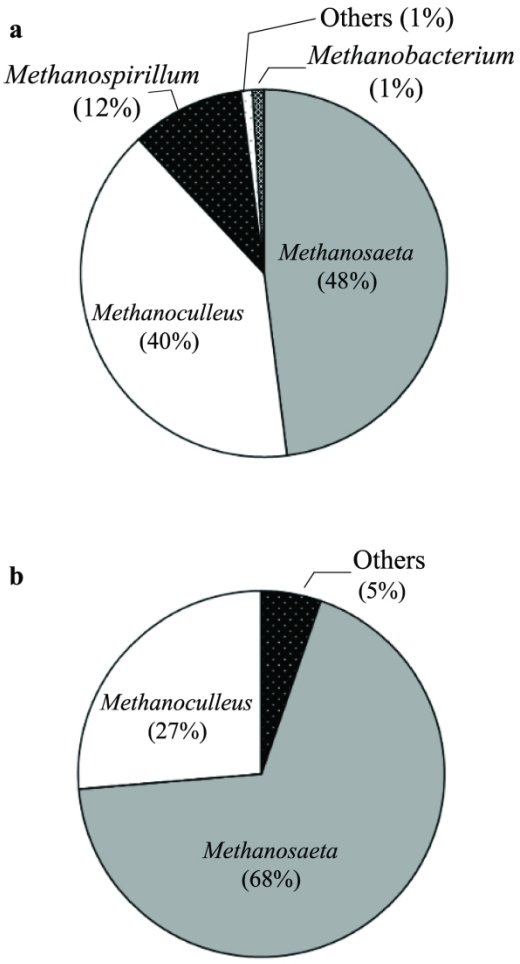

Fig. 2

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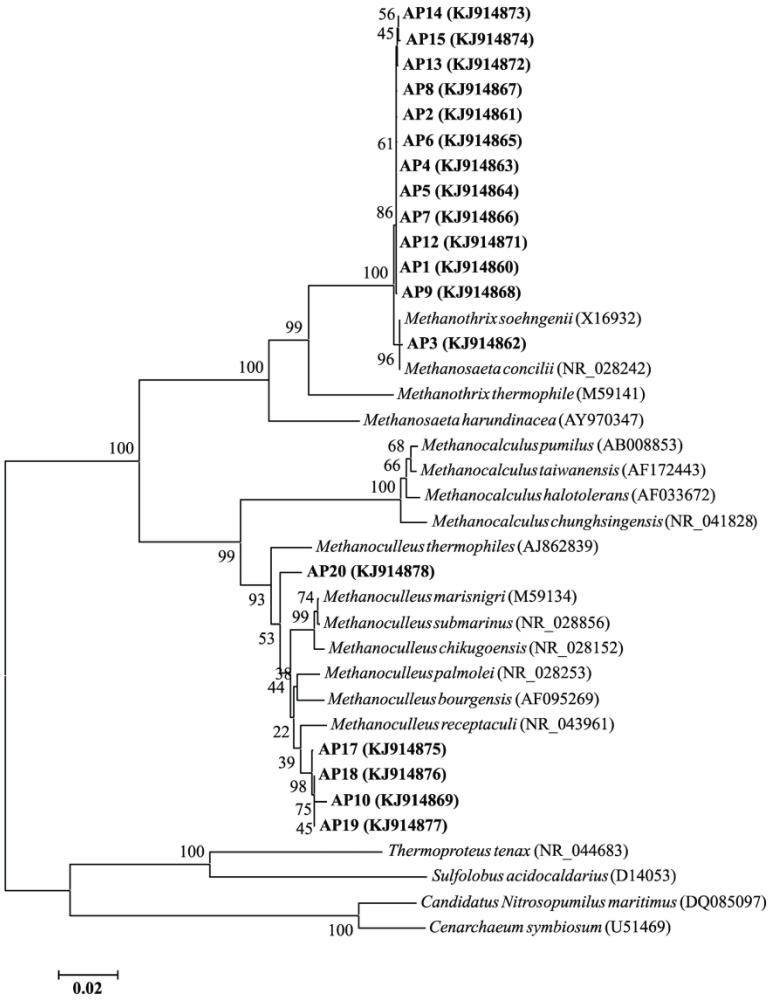

Fig. 3

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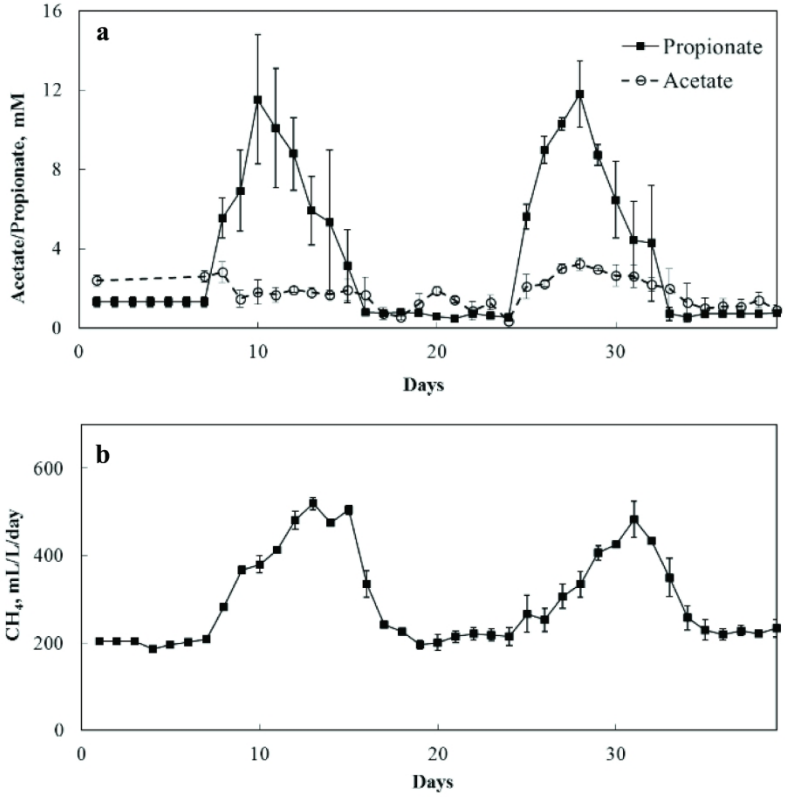

Fig. 4

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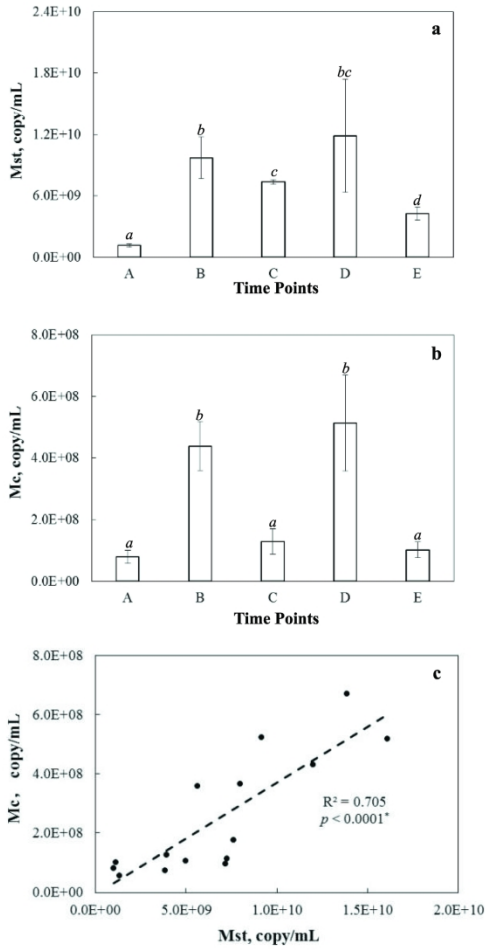

Fig. 5

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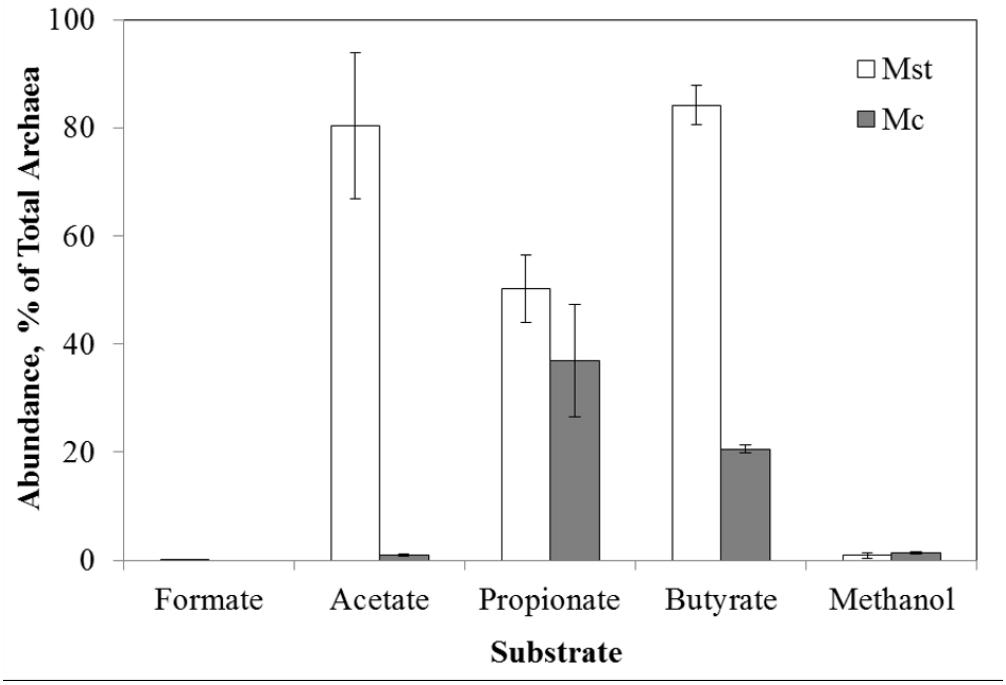

Fig. 6

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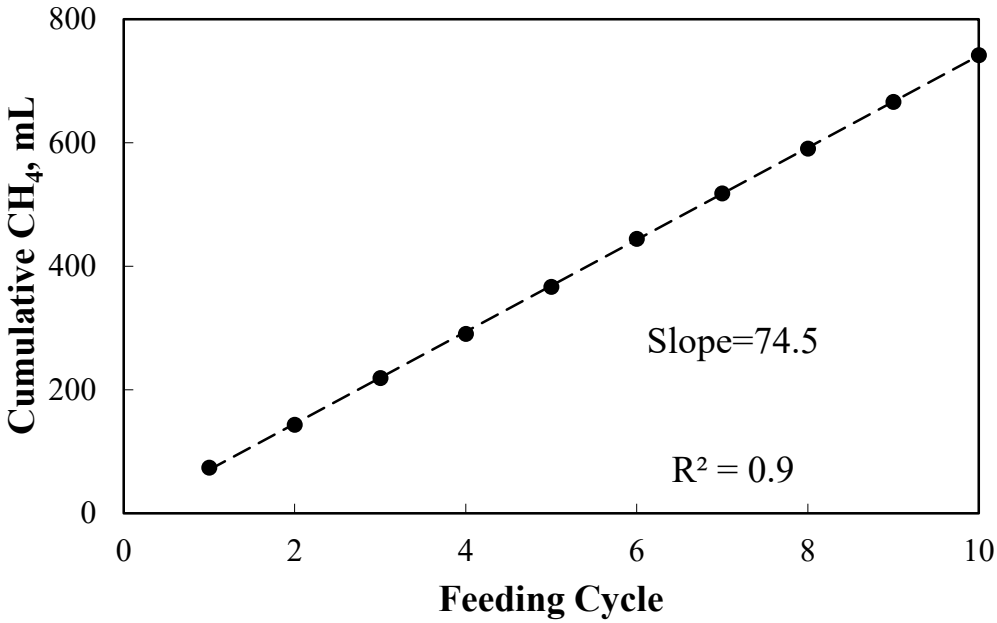

Fig. S1 Methane production in second-generation methanogenic batch bioreactors with propionate as the sole substrate. Propionate (20 mM) was fed after the depletion of propionate from the previous feeding. Data points were cumulative methane production from each feeding of propionate. The dashed line is the linear regression of cumulative methane production vs feedings.

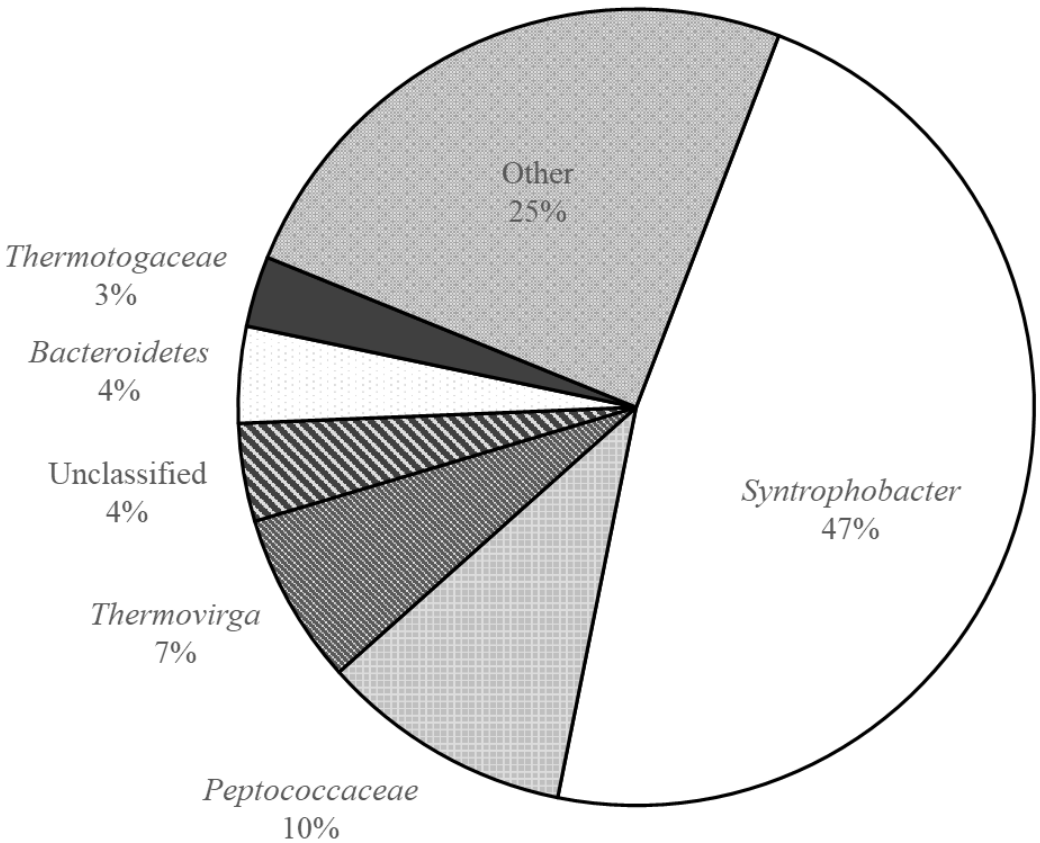

Fig. S2 Composition of the bacterial community in methanogenic batch bioreactors with propionate as the sole substrate. The relative abundance of bacterial taxa are shown as percentage values determined by 16S rRNA gene amplicon library sequencing. Shown are taxa with relative abundance greater than 3% at the genus level or the next taxonomic level when classification is possible