

**DRIVERS OF POPULATION SUCCESSION IN
ANAEROBIC COMMUNITIES OF METHANOGENIC
TREATMENT PROCESSES**

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Doctor of Philosophy

Degree

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ABSTRACT

Anaerobic digestion (AD) is a process in which complex organic matter is converted into methane and CO₂ with the activities of an anaerobic food web. Despite recent efforts to understanding the mechanisms of microbial community assembly, processes shaping the community structure in AD remain unresolved.

With this study, firstly, we investigated the anaerobic microbial community in the anaerobic treatment of dairy wastewater in triplicate anaerobic bioreactors over a 118-day period. The results showed successional trajectories of the microbial community during this period, with stochastic processes accounting for 66.5% -77.7% of community assembly. Subsequent network analysis indicated strong positive correlations between intra-taxa populations and negative correlations between inter-taxa populations, implying the importance of deterministic factors derived from internal dynamics. Secondly, in order to characterize the impact of deterministic processes on microbial community assembly, the anaerobic bioreactors were subjected to a change in substrate. As expected, microbial succession before and after the substrate shift was controlled mainly by deterministic processes (92.4%). For the archaeal community, *Methanosaeta* remained as the most abundant archaeal genus before and after substrate shift, while *Methanoregula* thrived after substrate shift. As for the bacteria, the top five abundant bacterial phyla before substrate shift were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Synergistetes* and *Actinobacteria*. Following the substrate shift, *Chloroflexi* and *Spirochaetes* outcompeted *Synergistetes* and *Actinobacteria* to join the top five abundant phyla, suggesting these populations might be metabolically responsive to the change in substrate. To evaluate community responses to other deterministic drivers, ammonia stress was tested in the AD bioreactors. Microbial community analysis revealed the increased contribution of deterministic processes with elevated levels of ammonia stress. As for the microbial responses, *Methanobacterium* and *Methanosarcina* were abundant methanogens in all bioreactors, suggesting the resistance of these populations to ammonia stress. Results of the bacterial compositions indicated that sequences of *Treponema* from the phylum *Spirochaetes* was inhibited when ammonia was added. After ammonia stress was relieved, the abundance of *Treponema* rebounded, indicating the lack of intolerance of this population toward ammonia stress and its competitiveness at low ammonia concentration. Additionally, to characterize the microbial response to osmotic stress, which is a common deterministic driver in AD, the impact of sodium stress was investigated in AD. Microbial composition analysis suggests that *Methanosarcina* and *Methanofollis* are methanogen populations tolerant to elevated sodium. As for the bacteria, the genus *Kosmotoga* in *Thermotogae* and genus *Aminobacterium* in phylum *Synergistetes* exhibited tolerance to sodium stress, while genus *Treponema* from phylum *Spirochaetes* were found to be sensitive to sodium. These observations again imply the importance of deterministic factors derived from internal dynamics. To identify the functional basis for the responses of anaerobic populations to deterministic drivers, metagenomics analysis was performed for the microbial communities in the anaerobic bioreactors with a focus on acetoclastic methanogenesis, which was a dominant pathway in anaerobic treatment. Metabolic pathways associated with acetate conversion was profiled in the obligate acetoclastic methanogen—*Methanosaeta*. The results supported the broad occurrence and importance of *Methanosaeta* populations in the methanogenic process.

Findings of this study will enhance the understanding of metabolic and ecological processes that shape methanogenic microbial communities, which could be used to develop models for the effective control and management of anaerobic conversion processes.

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CHAPTER I-INTRODUCTION AND LITERATURE REVIEW

1 Basic information of the anaerobic digestion process

Anaerobic digestion (AD) is a complex biochemical process in which organic matters are converted into biogas methane (CH_4) with the absence of oxygen^{1,2}. It has been widely applied in wastewater treatment and biogas production due to its potential advantages like lower operational costs, energy production from wastes et al. Studies of the anaerobic digestion originated from the early 20th century and remain to be the focus of many researchers. The AD process can be applied to treat various types of wastes, e.g., food waste³, sewage sludge, agriculture waste and dairy manure⁴. Among these substrates, dairy manure is an organic abundant waste and with poor management, it will produce ammonia and greenhouse gases; wastewater sludge is the semi-solid slurry produced from the municipal wastewater treatment processes and contains a variety of organic particles. Despite the widely application of the AD process in waste and biosolids treatments, our knowledge of the process is still limited due to the complexity of the process. The complexity of the AD process was summarized into three features: 1) microbiological aspects including anaerobic microbial compositions and interactions et al., operational aspects including the environmental conditions (e.g., T) and organic loading rates et al., and also chemical aspect including pH, nutrients and inhibitors et al. It is always considered as the “Animal in the darkroom” in the view of philosophical aspect² and our understanding of it is not comprehensive yet and needs to be updated from time to time. In the early stage of the AD study, the researches were focused on resolving the problems like poor practical and operational stability, high sensitivity to changes of environmental conditions and toxicants, long retention and start-up times, et al^{2,5}. Development of molecular techniques (16S rRNA and shotgun sequencing) facilitates the studies of microbial compositions and metabolic pathways in the AD process, which merits in broadening our knowledge of the process.

Chronologically, four steps are involved in the AD process and they are hydrolysis, acidogenesis and acetogenesis and methanogenesis^{6,7}. The process starts with the hydrolysis step in which insoluble organic matters (e.g., carbohydrates, fats, proteins) are broken down to soluble intermediates, which can be used by the acidogenic or acetogenic bacteria. In the acidogenesis step, sugars and amino acids produced in the first step are converted into organic acids, accompanying with CO_2 , H_2 and ammonia. In the acetogenesis, organic acids are degraded further into acetic acid, along with CO_2 , H_2 and ammonia. Finally, acetate and H_2 are converted into methane.

2 Microbial community assembly and its drivers in the AD process

Corresponding to the four chronological steps in the AD process, microbial organisms in the AD process can be divided into hydrolytic bacteria, acidogens, acetogens and methanogens. Microorganisms were considered as the core of the AD process due to their indispensable roles in the biochemical reactions⁸. Hydrolytic bacteria were phylogenetically diverse in the AD process and most of them were affiliated to phyla *Firmicutes* and *Bacteroidetes*. Compared with methanogens, they grow fast and are less sensitive to variations of the environmental conditions (e.g. pH and temperature)⁹. As for the acidogenic bacteria, most identified taxa were affiliated to phyla *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Proteobacteria*. With their activities, products of hydrolysis were converted into VFAs (acetate, propionate, butyrate et al.). Acetate produced by acidogenesis can be used by methanogens directly, while other intermediates like propionate, butyrate et al. need to be further degraded by syntrophic acetogens to form acetate, H_2/CO_2 . Syntrophic acetogens involved in different intermediates varied in the AD process. For propionate,

most commonly syntrophic acetogens reported were affiliated to genus *Pelotomaculum*, *Smithella* and *Syntrophobacter*¹⁰⁻¹². Acetogens affiliated to genera *Syntrophus* and *Syntrophomonas* played an important role in the oxidation of butyrate and the other fatty acids¹²⁻¹⁴. And for the methanogens, based on the substrates for methanogenesis, they can be classified into acetoclastic methanogens, hydrogenotrophic methanogen and methylotrophic methanogen¹⁵. Taxonomically, all the methanogens were classified into seven orders and they were *Methanobacteriales*, *Methanococcales*, *Methanopyrales*, *Methanosarcinales*, *Methanocellales*, *Methanopyrales* and *Methanomassiliicoccales*¹⁶⁻¹⁸. Microbial compositions in the AD process are affected by various factors (e.g. substrate types, operational conditions, inhibitors). With the bacterial compositions collecting from 28 studies, it was found that substrate type was driving the microbial variations in the reactors, distinct lineages of *Clostridia* and *Tenericutes* were unique to digesters treated with manure, while lineages of *Alphaproteobacteria*, *Betaproteobacteria* and *Chloroflexi* were unique to digesters treated with municipal wastes¹⁹.

Similar to the other ecological systems, microorganisms in the AD process do not exist in isolation but are through the microbial community assembly. Understanding how the community assemble and the drivers of the microbial assembly is the focus of microbial ecology. Currently, processes that influence the microbial assembly can be classified into two types: deterministic process based on niche-based theory and stochastic process based on neutral theory^{20, 21}. With niche-based theory, deterministic factors, i.e., environmental conditions, species traits, microbial interactions (cooperation, competition, mutualisms et al.) shape the community structure. On the contrary, researchers who hold the neutral theory believe that stochastic process, i.e., birth, death, extinction and speciation control the composition and diversity of the community. Debates of these two ecological theories have been continued for a long period and until recently, proportional roles of these two processes in shaping the microbial community were discussed and quantified with the application of molecular techniques and statistical analyses. With an analytical framework advancing ecological understanding developed, importance of community assembly processes, i.e., ecological drift, selection and dispersal were quantitatively estimated in the spatial turnover of bacterial community composition across two geologically distinct aquifer in southeastern Washington State²². Using null model analysis, it's indicated that in the groundwater microbial community, both deterministic and stochastic processes were of great importance in controlling the microbial assembly and succession, but their relative importance was related with time²⁰. Nevertheless, understanding of these mechanisms and importance of them in deciding the microbial community structure in the AD process is still limited. Moreover, within the AD process, interactions like mutualism, commensalism, competition et al. occur between the different taxa²³, which are critical to keep the functional stability of the microbial systems²⁴. Network analyses based on co-occurrence pattern was the common method for exploring the interactions within the microbial ecosystems^{25, 26}. Using a correlation-based statistical method, the co-occurrence/co-exclusive correlations within the bacterial community of a municipal wastewater treatment plant in a 5-year period were integrated, implying that the taxonomically related bacteria tend to be positively related (potential existence of the cooperative relationship et al.) and the taxonomically less related bacteria tend to be negatively related (potential existence of the competition relationship et al.)²⁷. Analysis of microbial compositions for the triplicate continuous anaerobic digesters with operational stability in two years showed a successional pattern of microbial interactions and identified *Clostridiales* as the keystone taxa in maintaining the stable performance and function²⁸. Disclosures of the ecological mechanisms controlling the microbial community

and the microbial interactions within the community will broaden our knowledge in ecological niches of taxa and help to dissect the food web in the AD process.

Among the drivers of the microbial assembly, substrate type is a complex feature and can forces variations of the microbial community due to the different content of organic matters or chemical parameters¹⁹. Furthermore, chemical feature (including pH, alkalinity, volatile fatty acid, inhibitors et al.) of the AD process is one of the main features that lead the complexity of the AD process by affecting the activities of the microbial organisms². Large varieties of inorganic (Ammonia, sulfate, metal ions et al.) and organic chemicals (long chain fatty acids and polar organics) were reported to be the inhibitors in the AD process. Among these chemicals, ammonia is accumulated in the degradation of nitrogen-rich substrate (proteins). Although ammonia is critical for the growth of bacteria, high level of ammonia may inhibit the AD process, especially for the methanogenesis²⁹. Several mechanisms for the ammonia inhibition were proposed and they were a variation in intracellular pH, inhibition of a specific enzyme reaction and increase of maintenance energy requirement³⁰. Inhibitory thresholds for ammonia in different studies varied. It's reported that the total ammonia of 1600 mg/L would lead to a 75% decrease of methanogenesis under mesophilic conditions³¹. While under thermophilic conditions, methane production decreased at the total ammonia concentration of 1000 mg/L³². Differences of the reported ammonia inhibitory levels might result from the different environmental conditions (pH and temperature). As there two species of ammonia (free ammonia and ammonium) exist in the aqueous environment, and percentages of these two species are decided by the pH and temperature. Among these two species, free ammonia is more toxic than ammonium. It's acknowledged that of the microbial communities involved in the AD process, methanogens are the most sensitive and the most likely to be influenced by excess ammonia^{32, 33}. Other cations like Na⁺ et al., are the micronutrients required for the bacterial growth. Proper levels of these ions support the microbial metabolism while excessive levels will cause the inhibition even failure of the AD process. It's reported that the optimal concentrations for Na⁺ and K⁺ in the AD process are 3.5~5.5 g/L and 2.4~4 g/L, respectively³⁴. High levels of salts have the potential of affecting the methanogenesis and acetogenesis in the AD process. Related researches will help unravelling the potential chemical drivers of microbial assembly and succession in the AD process.

3 Molecular techniques and bioinformatic tools applied in the microbial study

With the emerging of the next gene sequencing (NGS) platforms, e.g. 16S rRNA gene amplicons sequencing and metagenome shotgun sequencing, microbial researchers can investigate the microbiomes with high resolution and throughput³⁵. Containing both the highly conserved primer binding sites and the hypervariable regions, 16S rRNA gene amplicon sequencing was developed to be a usefully tool for microbial phylogenetic studies³⁶, especially for bacteria and archaea. And it is suggested to be a reliable molecular technique, because 16S rRNA sequences from distantly related lineages were implied to have the similar functionalities³⁷. Unlike the 16S rRNA amplicon sequencing, whole genome shotgun sequencing is designed for analysis of DNA sequences longer than 1000 base pairs. With shotgun sequencing, the target DNA will be broken into random fragments, after the fragments are sequenced, they will be reassembled based on their overlapping regions³⁸. Both 16S rRNA amplicon and metagenome shotgun sequencing have pros and cons, beyond taxonomy profiling, with metagenome shotgun sequencing, we can do additional analyses, e.g. metabolic function profiling, antibiotic resistance gene profiling. Nevertheless, when

composition analysis is the main purpose of the study, whole genome shotgun sequencing has the limited bacteria coverage, the high false positive and high cost et al.

Meanwhile, development of the NGS platforms stimulated lots of bioinformatic tool. For the 16S rRNA data, the most common and widely used bioinformatic tools are mothur³⁹, QIIME⁴⁰, QIIME2⁴¹ et al. Nowadays, with the optimizing sequencing analysis algorithm, the accuracy and taxonomy resolution of the 16S rRNA has been improved dramatically. Some researchers made comparisons between different bioinformatic pipelines and concluded that all the pipelines produced the similar results in the genus level³⁵. Among these pipelines, QIIME2 is a powerful and widely used one due to it is open-sources, easy to start and producing satisfying graphs et al. Likewise, lots of excellent tools are also developed for the shotgun data analysis, such as Metagenomic Phylogenetic Analysis (MetaPhlAn) approaches, Blast top hits et al. None of them is perfect in terms of utilization, precision and speed et al, and MetaPhlAn is much simpler than the other tools and require less for the computer³⁵.

4 Genomic analysis of genus *Methanosaeta*

Methanosaeta is reported to be the obligately acetoclastic methanogen. Strains of this genus have a higher affinity for acetate than that of *Methanosarcina*, which is the other methanogen that can use acetate for methane production. Hence, strains of *Methanosaeta* are favored when the acetate level is low. Nevertheless, unexpected competitiveness of *Methanosaeta* was found in the anaerobic treatment of animal wastewater with elevated acetate concentration⁴², indicating the potential genetic and physiological differences between strains competitive at high acetate level and those that are not. With the sequencing and analysis of genomes, genomics enables the function and structure analyses of entire genome. Genome of *Methanosaeta* was generated from a deep subsurface sedimentary environment from the metagenomic sequence data, suggesting its dominance and indispensable role in methane production and overall carbon cycle within the sedimentary environments⁴³. By comparing the three complete genomes of *Methanosaeta* (*Methanosaeta thermophila*, *Methanosaeta concilii* and *Methanosaeta harundinacea* 6Ac), an entire suite of genes and proteins involved in the methyl-group oxidation pathway were found⁴⁴, indicating that the methyl-group oxidation pathway could be used to produce reducing equivalents in the obligate acetoclastic methanogens - *Methanosaeta*.

CHAPTER II
ECOLOGICAL SUCCESSION OF MICROBIAL COMMUNITY
STRUCTURE IN ANAEROBIC TREATMENT OF DAIRY WASTEWATER

Abstract

Anaerobic digestion processes are characterized by the complexity of microbial populations that are required for proper process functions. To gain an understanding of the succession of microbial population and the ecological mechanisms (i.e., deterministic and stochastic process) that control the microbial assembly in the AD process, we investigated the anaerobic microbiomes in the anaerobic treatment of dairy wastewater in triplicates over a 118-day period. The treatment processes remained stable throughout the study period. However, the microbial populations involved in this process exhibited significant temporal dynamics. Results for the roles of the ecological processes suggested during the 118-day succession, stochastic processes are more dominant than the deterministic processes (roles of stochastic processes ranged from 66.5% to 77.7%). Nevertheless, over the 118-day, the role of stochastic processes decreased to 28.8%, indicating the important of deterministic processes, i.e. microbial interaction, in controlling the microbial community. Further population co-occurrence analyses based on spearman correlation revealed the top 5 strongest correlated pairs were all represented by populations of the same phylum, i.e. *Firmicutes*. In comparison, results from co-exclusion analyses indicated that strong negative correlations were more frequently observed between populations that had remote phylogenetic relationships. This observation is evident in the top 5 strongest negative correlations, i.e. ASV1020 (*Firmicutes*) and ASV1853 (*Chloroflexi*), ASV1853 (*Chloroflexi*) and ASV1019 (*Firmicutes*), ASV0266 (*Actinobacteria*) and ASV0014 (*Firmicutes*), ASV0355 (*Synergistetes*) and ASV0661 (*Firmicutes*), ASV1853 (*Chloroflexi*) and ASV1080 (*Proteobacteria*). Strong positive correlation relationships between intra-taxa populations and negative correlation relationships between inter-taxa populations imply both high levels of redundancy between closely related populations and competition between microbial populations with greater phylogenetic distance. The validity of these observations needs to be further investigated in other anaerobic treatment processes.

Keywords: Microbial interactions; co-occurrence/co-exclusive; anaerobic digestion; dairy manure

1 Introduction

Anaerobic digestion is a mature and widely applied technology to convert organic wastes into biogas with degradations of various microbiomes⁴⁵⁻⁴⁷. It's one of the most-energy-efficient microbial systems applied in both bioenergy production and industrial wastewater treatment⁴⁸. Substrates for anaerobic digestion are different types of biomass and waste including municipal wastewater sludge, dairy manure, chicken manure and sewage sludge et al⁵. Anaerobic digestion of these organic matters to biogas is a multi-steps process mediated by both bacterial and archaeal community. Generally, hydrolysis is the first and rate-limiting step. Polymers like carbohydrates, proteins and cellulose are hydrolyzed into simple sugars, fatty acids and amino acids et al by hydrolyzing bacteria. With acidogens and acetogens, these intermediates are converted into acetate and one-carbon compound directly or indirectly. Methanogenic archaea can degrade acetate and one-carbon compound into methane (CH₄) and carbon dioxide (CO₂)⁴⁹. The microbial communities among hydrolysis, acidogenesis, acetogenesis and methanogenesis are different in the process of anaerobic digestion. Almost 50 kinds of bacteria such as *Clostridium*, *Bacteroides*, *Proteobacteria*, *Pseudomonas*, *Bacillus* et al. are responsible for hydrolysis and acidogenesis. Methanogens like *Methanosaeta*, *Methanosarcina*, *Methanobacterium*, *Methanococcus* are the

main microorganisms for the final step of methane production⁸. The efficiency and stability of the anaerobic digestion process depends largely on the cooperated and syntrophic activities of these microbial communities⁵⁰.

Mechanisms that control the microbial community structure in the AD process include niche-based and neutral based theories^{20, 22}. To be specific, neutral based theory assumes that all species are ecological equivalent and stochastic processes like birth, death et al. control the composition of the local community. However, niche-based theory insists that deterministic processes, i.e. environmental conditions and microbial interaction, are important in mediating the microbial community. It's noteworthy that microbial community in the anaerobic digestion vary due to the different substrate types, operational conditions and microbial interactions. Great efforts have been made to investigate microbial dynamics due to different substrate types and operational conditions. Environmental factors, including operational conditions (e.g., temperature, organic loading rate), physicochemical variables (e.g., pH, volatile fatty acids, nutrients and inhibitors)), are known to influence the process stability and biogas production^{2, 51, 52}. Additionally, by comparing the microbiomes in 78 different digester samples with varied substrate type, it was found that substrate type was the most crucial factor that influenced the phylogenetic structures in the anaerobic digestion process¹⁹. Furthermore, network and multivariate analyses for the microbial communities from four anaerobic digesters suggested that deterministic factors, like microbial competition and niche differentiation were the main drivers of microbial community dynamics⁴⁸. Recent studies about microbial communities in the anaerobic digestion are focusing the microbial interaction processes (e.g., competition, syntropy) that are important for shaping microbial communities^{48, 53}. Nevertheless, ecological mechanisms of microbial community succession in the operational stable anaerobic digestion process remained to be deeply explored.

Due to the neglect of factors influences on microbial activities and function, our understanding about microbial community compositions and ecology in the anaerobic digestion are incomplete and likely biased with former cultured-based techniques⁵⁰. Development of molecular methods including high throughput 16S rRNA amplicon sequencing allows us exploring the microbial community compositions with great depth and accuracy²³. In this study, using a set of lab-scale anaerobic digesters treated with dairy manure as the model system, we investigated the microbial community fluctuations in a continuous anaerobic system in the operational stable state over a successive 118-day period with high-through 16S rRNA amplicon sequencing. Persistent key players in the anaerobic digesters were determined based on both presence and abundance. Both co-occurrence and co-exclusive networks based on correlation were constructed to dissect the microbial interactions in the microbial community in an operational stable anaerobic digestion system. Results of this study suggest that the engineered anaerobic digestion system is a microbially-mediated process and microbial interactions such as competition, syntrophic relationship are critical to maintain the functional stability of the anaerobic digesters.

2 Materials and Methods

2.1 Sample collection

Samples of this study were from triplicate control anaerobic digesters, which were set up prior to this study^{54, 55}. Substrate for the digesters were dairy manure collecting from the waste storage basin of a 600-head dairy farm in London County, Tennessee, USA. Over the past several decades, these three control digesters were maintained in stable state under the following

invariable conditions: at temperature 35 °C, a working volume of 3.6 L and a hydraulic retention time of 20 days. Samples for 7 time points (Day 1, Day 18, Day 32, Day 52, Day 69, Day 99 and Day 118) were collected in 120 days. Samples were centrifuged and the pellets were stored at -80 °C as soon as possible after taken. Sequencing data for samples were labeled as Day 1, Day 18, Day 32, Day 52, Day 69, Day 99 and Day 118.

2.2 DNA extraction, PCR amplification and sequencing

Total community DNA was extracted from all the samples with FastDNA spin kit with protocols similar to that in previous study ⁵⁶. After extraction, the conserved variable region V4 for bacterial and archaeal 16S rRNA was amplified with primer pairs 515F/806R. PCR conditions, methods of purification and quantification for amplicons were as previously described ⁵⁷. Sequencing for 16S rRNA was performed on an Illumina platform following the manual.

2.3 Sequencing and Statistical data analysis

Microbiome analysis package QIIME 2 was used to analyze raw sequencing data. Based on sequences quality reports, pipeline DADA2 was applied for quality filtering, low quality regions of the sequences were trimmed to get the table for the qualified amplicon sequence variants (ASVs). Furthermore, ASVs with count less than 5 were filtered and all the samples were rarefied to the same depth (the minimum depth among all the samples). After that, taxonomy was assigned to the sequences with q2-feature-classifier plugin. Pre-trained Naive Bayes classifier Greengenes 13_8 99% reference database was applied. Relative abundances of archaeal and bacterial genera were calculated and visualized.

Normalized stochasticity ratio based on specified taxonomic dissimilarity index and null model algorithm was calculated using the NST package in R studio. Occurrence frequency of each ASV was calculated based on its occurrence in all the samples. Average relative abundance of each ASV was also calculated. Relationship between the average abundance and occurrence frequency was analyzed. Both co-occurrence and co-exclusion networks were constructed based on the Spearman correlation analyses. Only strong (absolute spearman $r > 0.5$) and significant correlations ($p < 0.05$) were considered. All the other data analyses were performed in Microsoft Excel and Rstudio. Chord diagram of microbial composition was visualized using package circlize in Rstudio. Co-occurrence and co-exclusion networks were visualized in platform Gephi.

3 Results

3.1 Fluctuations of microbial composition in the anaerobic digesters over a 118-day period

The Abundant phyla in the community include *Firmicutes*, *Synergistetes*, *Verrucomicrobia*, *Bacteroidetes*, *Proteobacteria*, *Chloroflexi* and *Actinobacteria* and *Euryarchaeota*. At the beginning (from day 1 to day 69), *Firmicutes* was the most abundant phylum (relative abundance of which was from 24.82% to 38.73%), followed by phylum *Synergistetes* (relative abundance ranged from 10.82% to 16.51%). For day 99, relative abundance of *Synergistetes* increased to 17.59% and is greater than that of *Firmicutes* (17.35%). When it came to day 118, relative abundance of *Synergistetes* increased to be 21.65% and as for *Firmicutes*, the relative abundance of which had decreased to be 13.20%. Abundances for the other prevalent phyla fluctuated at the

same time. For *Verrucomicrobia*, the relative abundance increased from 6.91% in day 1 to 11.58% in day 32. After that, it decreased gradually to 9.44% in day 69 and then increased again to 12.42% in day 118. Similar situation occurred to phylum *Chloroflexi*, the relative abundance of which raised from 3.04% in day 1 to 7.21% in day 32. Lower abundance of *Chloroflexi* was found in microbial community in day 52 (4.30%). Then from day 69, abundance of *Chloroflexi* increased again and was 11.77% in day 118. For phylum *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, there were also fluctuations in relative abundances. However, the overall trends were different. Overall, the abundance of *Actinobacteria* increased from 1.55% in day 1 to 6.58% in day 118. On the contrary, relative abundance of *Bacteroidetes* (relative abundance in day 1 was 12.24% and in day 118 was 5.87%) and *Proteobacteria* (relative abundance in day 1 was 10.17% and in day 118 was 6.21%) decreased. For the archaeal phylum, *Euryarchaeota*, the relative abundance increased from 5.69% in day 1 to 7.87% in day 32 and then decreased to 4.09% in day 118 (Fig. 2-1).

For archaea, microbial community composition analyses in the genus level indicated that *Methanosaeta* was the most abundant archaeal genus in the anaerobic digesters over the successive 118-day period (Fig. 2-2). From day 1 to day 52, relative abundance of *Methanosaeta* remained stable and ranged from 61.84% to 65.71%. In day 69 and day 99, relative abundance of *Methanosaeta* dropped a little bit and were 48.77% and 46.42%, separately. In day 118, relative abundance of *Methanosaeta* increased to 53.35%. Additionally, there were two abundant hydrogenotrophic methanogens, *Methanospirillum* and *Methanobrevibacter*. Relative abundance of *Methanospirillum* in day 1 was 20.02% and decreased to 10.83% in day 118. As for *Methanobrevibacter*, relative abundance dropped from 8.13% in day 1 to 4.92% in day 18. Following the drop, relative abundance of *Methanobrevibacter* remained stable (~4.00%) for more than one month and was 4.29% in day 52. From day 52 to day 69, abundance of *Methanobrevibacter* thrived from 4.29% to 16.69%. After that, the abundance decreased gradually to 7.63% in day 118 (Fig. 2-2).

Bacterial community fluctuations were more complicated compared with that of archaea (Fig. 2-3). The top 5 abundant genera in day 1 were unclassified *Bacteroidales* (abundance: 8.04%, phylum: *Bacteroidetes*), *Clostridium* (abundance: 7.99%, phylum: *Firmicutes*), *Turcibacter* (abundance: 6.65%, phylum: *Firmicutes*), unclassified *Peptostreptococcaceae* (abundance: 6.18%, phylum: *Firmicutes*) and another *Peptostreptococcaceae* (abundance: 5.69%, phylum: *Firmicutes*). From day 18 to day 52, the top 5 genera in the bacterial community were the same, they were *WCHB1-41* (*Verrucomicrobia*), *Clostridium* (*Firmicutes*), an unclassified *Thermovirgaceae* (*Synergistetes*), an unclassified *Bacteroidales* (*Bacteroidetes*) and *HA73* (*Synergistetes*). Relative abundances of them in day 18 were 8.37%, 6.32%, 6.08%, 5.79% and 5.22%; in day 32, were 8.37%, 6.24%, 6.02%, 6.13% and 5.00%; in day 52, were 7.35%, 6.36%, 5.21%, 8.15% and 5.75%. When it came to day 69, *vadinCA02* substituted *Clostridium* (*Firmicutes*) and became one of the top 5 phyla and the top 5 abundant phyla in day 69 were *HA73* (abundance: 8.49%, phylum: *Synergistetes*), *WCHB1-41* (abundance: 6.47%, phylum: *Verrucomicrobia*), unclassified *Bacteroidales* (abundance: 6.21%, phylum: *Bacteroidetes*), an unclassified *Thermovirgaceae* (abundance: 4.62%, phylum: *Synergistetes*) and *vadinCA02* (abundance: 3.50%, phylum: *Synergistetes*). In day 99, the top 5 abundant phyla were *HA73* (abundance: 9.43%, phylum: *Synergistetes*), *WCHB1-41* (abundance: 7.78%, phylum: *Verrucomicrobia*), an unclassified *Thermovirgaceae* (abundance: 6.79%, phylum: *Synergistetes*), *R4-45B* (abundance: 6.06%, phylum: *Lentisphaerae*) and unclassified *Bacteroidales* (abundance: 4.89%, phylum: *Bacteroidetes*). At the end of the sample collection day 118, the top 5 abundant phyla were similar to those in day 99 and were *HA73* (abundance: 10.72%, phylum: *Synergistetes*), an unclassified

Thermovirgaceae (abundance: 10.40%, phylum: *Synergistetes*), *WCHB1-41* (abundance: 8.35%, phylum: *Verrucomicrobia*), *R4-45B* (abundance: 5.06%, phylum: *Lentisphaerae*) and *SHA-20* (abundance: 4.43%, phylum: *Chloroflexi*).

3.2 Roles of ecological mechanisms in microbial community succession

Stochastic ratios for each time interval ranged from 63.0% to 74.0%, indicating in the short time interval, stochastic processes are important in mediating the structure of the microbial community. However, when spreading the interval (from Day1 to Day118), roles of stochastic processes decreased to 29.1%, implying deterministic processes, i.e. microbial interaction, control the microbial community succession gradually over the long run (Table 2-1).

Core microbial composition in the anaerobic digestion was analyzed in temporal scale to investigate the successive populations. Ecological communities partition based on the occurrence frequency and abundance was applied to obtain the persistent, intermittent and transient groups in the anaerobic digestion process. And we classified the three ecological groups in the amplicon sequence variants (ASVs) level in order to get a finer knowledge of the microbial composition of anaerobic digesters treated with dairy manure. Referring to the previous study, ASVs that with occurrence frequency more than or equal to 80% of all the samples were classified as persistent ASVs, that with occurrence frequency greater than 20% but less than 80% were classified as intermittent ASVs, and that with occurrence frequency less than or equal to 20% were classified as transient ASVs. In our study, we found 2205 qualified ASVs in total. Among these ASVs, only 290 were persistent ASVs, accounting for 13% of the total ASVs. And the number of intermittent ASVs were 659, around 30% of the total ASVs. The remaining 1256 ASVs were all transient ASVs, accounting for more than half of the total ASVs (Fig. 2-4). In addition, the relationship between the average relative abundance and occurrence frequency of each ASV was fitted with the exponential function. A strong positive relationship was observed and R^2 for the function was 0.77, indicating the persistent ASVs were usually the abundant ones (Fig. 2-5). Total abundance of the persistent ASVs was 83.85%, which was dominant in the community. As for the intermittent and transient ASVs, the abundances were 13.38% and 2.77%, separately (Fig. 2-4). The results suggested that the persistent ASVs were generally abundant and important for functional stability in the anaerobic digestion system. Among the persistent ASVs, 26% of them were from phylum *Firmicutes*, 17% of them were from *Bacteroidetes*, 10% were from *Proteobacteria*, 8% were from *Verrucomicrobia*, numbers of persistent ASVs from *Synergistetes*, *Actinobacteria* and *Chloroflexi* were the same, accounting for 6% of the total persistent ASVs, respectively (Fig. 2-6). Taxa from these groups were reported to be involved in hydrolysis and syntrophic steps in the anaerobic digestion process. Besides, 4% of the persistent ASVs were from *Euryarchaeota*, which was the final participants in the biogas production process.

3.3 Core microbial groups in anaerobic community revealed by co-occurrence and co-exclusive analyses

In order to get to know the interactions between these persistent ASVs further, we selected the ASVs with average abundance greater than 0.5% and occurrence frequency 100% to analyze the co-occurrence (Fig.2-7) and co-exclusive relations (Fig. 2-8) between the ASVs. Only strong (absolute value of spearman r greater than 0.5) and significant ($p < 0.05$) correlations were shown in the figure. For both co-occurrence and co-exclusive networks, they were all colored by phylum. Size of each node on the left panel of the network is proportional to the abundance and size of each

node on the right panel is proportional to the number of connections. In the co-occurrence network, the top 5 strongest correlated groups were ASV0061 & ASV0661, ASV1020 & ASV0061, ASV1020 & ASV1019, ASV0061 & ASV1019 and ASV0661 & ASV1019. All of these ASVs were from phylum *Firmicutes*. Nevertheless, they have smaller degrees compared with members from *Planctomycetes*, *Synergistetes* and *Actinobacteria*. Co-occurrence may reflect the commonly preferred habitat associations or cooperative behaviors such as cross-feeding, syntrophic interactions and mutualistic interactions. Here in our study, observations of strong co-occurrence relations for ASVs in phylum *Firmicutes* were consistent with the previous finding that the closely related taxa have similar habitat associations. Results of co-exclusion analyses indicated that ASV1020 (*Firmicutes*) and ASV1853 (*Chloroflexi*), ASV1853 (*Chloroflexi*) and ASV1019 (*Firmicutes*), ASV0266 (*Actinobacteria*) and ASV0014 (*Firmicutes*), ASV0355 (*Synergistetes*) and ASV0661 (*Firmicutes*), ASV1853 (*Chloroflexi*) and ASV1080 (*Proteobacteria*) had the top 5 strongest negative correlation, which suggests the competition between these taxa.

4 Discussion

In our study, microbial community fluctuations in an engineered operational stable anaerobic digestion system over a successive 120 days were investigated. By comparing the community compositions from seven time points, both archaeal and bacterial community variations were assessed. Key taxa in the anaerobic digestion process treated with dairy manure were identified based on presence and abundance. Co-occurrence and co-exclusive networks analyses based on correlations suggest the importance of microbial interactions in keeping the functional stability of the anaerobic digestion process.

For the archaeal community, succession in the methanogens was observed. For all the microbial communities, *Methanosaeta* was the most abundant methanogens, followed by *Methanospirillum* and *Methanobrevibacter*. Even acetoclastic and hydrogenotrophic methanogenesis are the two major biogas production pathways in the anaerobic digestion process, acetoclastic methanogenesis is always considered to be the predominant methanogenesis pathway⁵⁸. *Methanosaeta*, together with *Methanosarcina*, are the only two genera of methanogens which are capable of converting acetate into methane. Unlike the versatile *Methanosarcina*, *Methanosaeta* can only use acetate as the substrate to produce methane. In our study, relative abundances of *Methanosaeta* ranged from 46.42% to 65.71%. While the relative abundances of *Methanosarcina* were much less than that of *Methanosaeta* and ranged from 0.45% to 1.20%. The results suggest that *Methanosaeta* is much more competitive than *Methanosarcina* in the operational stable anaerobic digesters treated with dairy manure, which is consistent with studies that in anaerobic digestion process with low acetate concentration, *Methanosaeta* would outcompete *Methanosarcina* and be the abundant acetoclastic methanogen due to its lower maximum specific growth rate ($\mu_{\max}$) and half saturation concentrations (K_s) for growth on acetate⁵⁸⁻⁶⁰. Although relative abundances of hydrogenotrophic methanogens were lower than that of acetoclastic methanogens, the presence of these hydrogenotrophic methanogens were critical to keep low partial pressure of hydrogen and the continuous degradation of volatile fatty acids (VFAs).

Corresponding to the steps in the anaerobic digestion process, bacterial community compositions in the process include hydrolyzers, acidogens and acetogens. With hydrolytic bacteria, complex compounds like carbohydrates, proteins and lipids can be converted into small molecular compounds like sugar, amino acid and peptides, which are the intermediates for the

microbiomes in the next groups of the food chain. After hydrolysis process, acidogens use the intermediates produced by hydrolyzers as electron acceptors to form products e.g., VFAs, alcohols, CO₂ and H₂. Among these products, acetate in VFAs, CO₂ and H₂ can be applied directly by methanogens and other products like higher VFAs are converted into acetate and H₂ by acetogens and be used by methanogens indirectly. At the beginning of this study (day1), the top 2 most abundant phyla were *Firmicutes* and *Bacteroidetes*, members of which are always found to be hydrolytic bacteria. From day18 to the end of the study, relative abundance of *Synergistetes* increased and became the top 2 most abundant phylum together with *Firmicutes*. It's worth mentioning that for the last two time points (day99 and day118), *Synergistetes* outcompeted *Firmicutes* and became the most abundant phyla in the bacterial community of the process. In order to investigate the compositional fluctuations of bacterial community in a finer taxonomic level, relative abundances of bacterial groups in the genus level were also calculated. At the beginning of the study, the top 5 abundant genera were all from *Firmicutes* and *Bacteroidetes*, members of which were recognized as hydrolyzers or acidogens. When it came to the end of the study, the top 5 abundant genera switched into genera affiliated with phyla *Synergistetes*, *Verrucomicrobia*, *Lentisphaerae* and *Chloroflexi*. Compared with members in *Firmicutes* and *Bacteroidetes*, Members of these phyla were usually found in the anaerobic digestion process with relative low abundance^{61,62}. However, *Verrucomicrobia* was found to be the predominant bacteria in the anaerobic digesters treated with sludge⁶³. Richness of these phyla in our study indicated the potential important roles of them in the hydrolysis, acidogenesis and acetogenesis processes.

Microbial community in the anaerobic digestion are diverse and the concept of microbial partition was introduced to help us with the exploration of the functional important groups^{64,65}. Based on the occurrence frequency in all the samples, persistent (≥80% of all samples), intermittent (20%-80%) and transient groups (≤20%) were divided²⁷. With the similar partition principles, we classified our high quality ASVs into three ecological categories. Although only 290 ASVs were classified to the persistent ASVs, the relative abundance of them took account of 76% of the whole community, which was supported by the positive relationship between mean abundance and the occurrence frequency. The results suggest that persistent ASVs in the community generally are the relatively abundant ones. Among the 290 persistent ASVs, 74 of them were affiliated with phylum *Firmicutes*, 49 of them were affiliated with phylum *Bacteroidetes*, 28 of them were affiliated with phylum *Proteobacteria*, 24 of them were affiliated with phylum *Verrucomicrobia* and 17 of them were affiliated with phylum *Synergistetes* et al. Based on the compositional analyses, these were the relatively abundant phyla in all the communities.

Since the anaerobic digestion is a highly microbes-mediated process, interactions between the groups in the community merits a lot to maintain the functional stability of the process. In our study, co-occurrence and co-exclusive analyses based on spearman correlation for the persistent ASVs were performed to explore further the microbial interactions. For the co-occurrence pattern, the strongest positive relationship was found between ASV0661 in genus *Turicibacter* and ASV0061 in genus *Clostridium*, both of which were affiliated with phylum *Firmicutes*. Similarly, taxa that with the top five strong positive relationships were all found to be affiliated with phylum *Firmicutes*, suggesting that co-occurring taxa are more taxonomically closely related. Co-occurrence relations between taxa indicate that they may have the similar ecological niches or have the mutualism interactions, for example, an exchange of metabolic intermediates⁶⁶⁻⁶⁸. Nevertheless, scenarios for co-exclusive patterns were on the contrary. The strongest negative relationship was found between ASV1020 belonging to an unknown genus from family *Peptostreptococcaceae* (affiliating with phylum *Firmicutes*) and ASV1853 belonging to an

unknown genus from order *CFB-26* (affiliating with phylum *Chloroflexi*). In addition, strong negative relationships were prevalent among taxa affiliated to different phyla, indicating the co-exclusive taxa are taxonomically distanced groups. Co-exclusive patterns revealed by the negative relationships might reflect the fierce competition between these taxa for limited carbon resources or other nutrients ⁶⁹.

5 Conclusions

With high throughput 16S rRNA sequencing, a temporal study of microbial community in the anaerobic digestion process treated with dairy manure was performed. During the stable successive 120 days, *Methanosaeta* was the most abundant methanogenic genus, suggesting acetoclastic methanogenesis was the most prevalent pathways for methane production in the lab-scale anaerobic digesters treated with dairy manure. Bacterial compositions fluctuations were much more complicated than that of archaea. To simplify, the top abundant genera affiliated with *Firmicutes* and *Bacteroidetes* in day1 switched into genera affiliated with *Synergistetes*, *Verrucomicrobia*, *Lentisphaerae* and *Chloroflexi* in day118. Strong and significant co-occurrence and co-exclusive patterns dissected the potential bacterial redundancy and microbial interactions existed in the microbial community, which are crucial to maintain the stability of the anaerobic digestion process.

Appendix A: Tables and Figures

Table 2-1 Taxonomic normalized stochasticity ratio based on specified dissimilarity index and null model algorithm for microbial community over a 118-day period

	Day1	Day18	Day32	Day52	Day69	Day99
Day18	0.78					
Day32	0.79	0.66				
Day52	0.78	0.75	0.70			
Day69	0.41	0.73	0.74	0.76		
Day99	0.31	0.61	0.67	0.63	0.72	
Day118	0.29	0.59	0.63	0.56	0.70	0.70

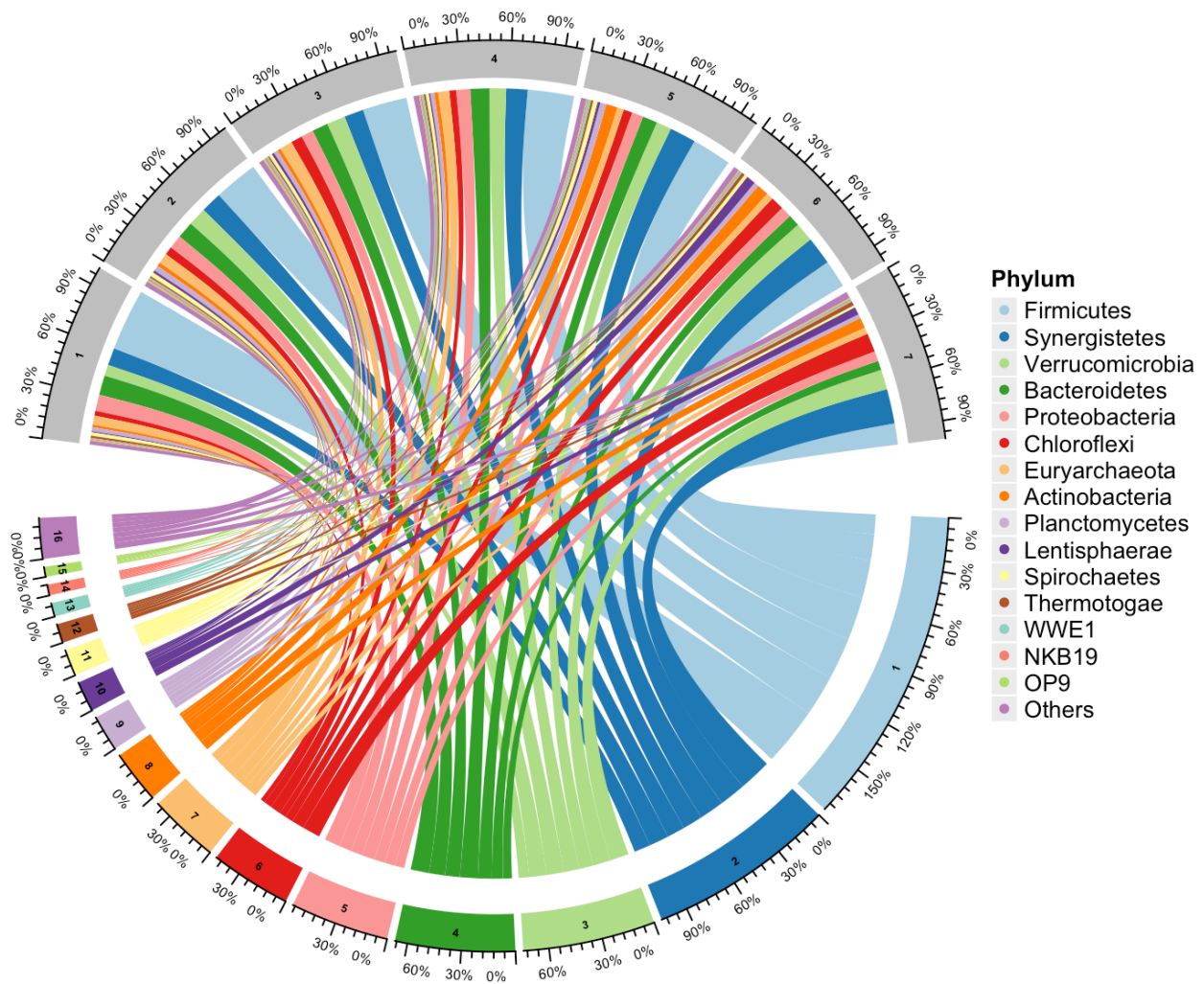


Fig. 2-1 Microbial community compositions in the anaerobic digesters over a successive 118-day period. Only the top 15 abundant phyla were shown in the figure. Minor groups were classified as others.

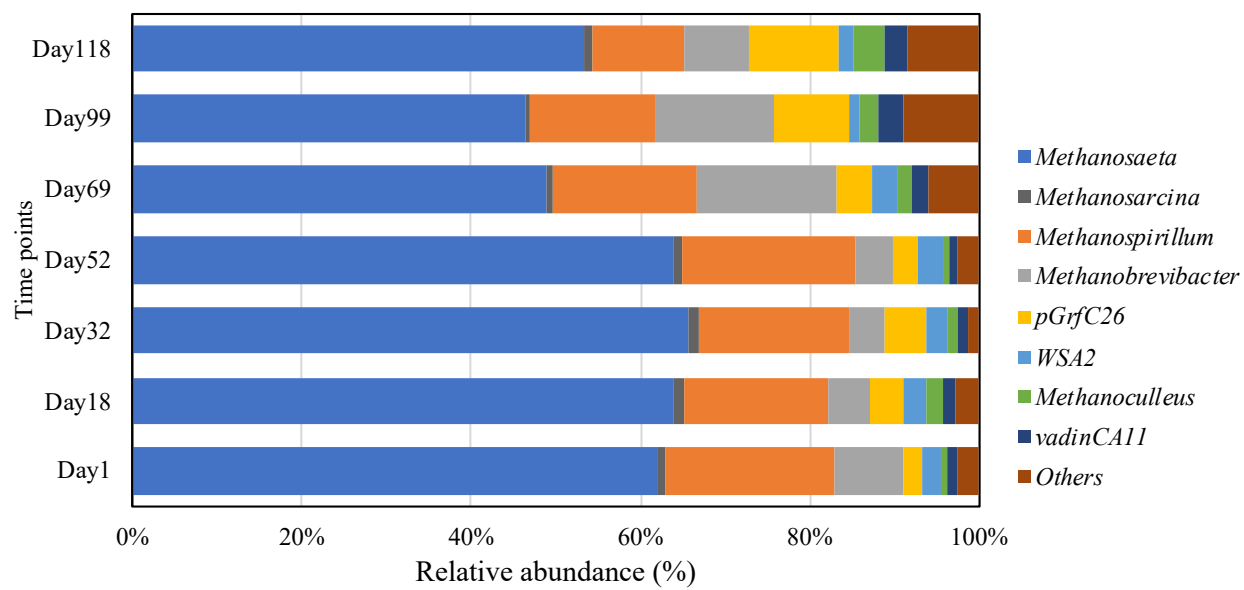


Fig. 2-2 Relative abundance of archaeal community in the anaerobic digestion process treated with dairy manure. Genera with low abundance in archaea in all communities were classified as others.

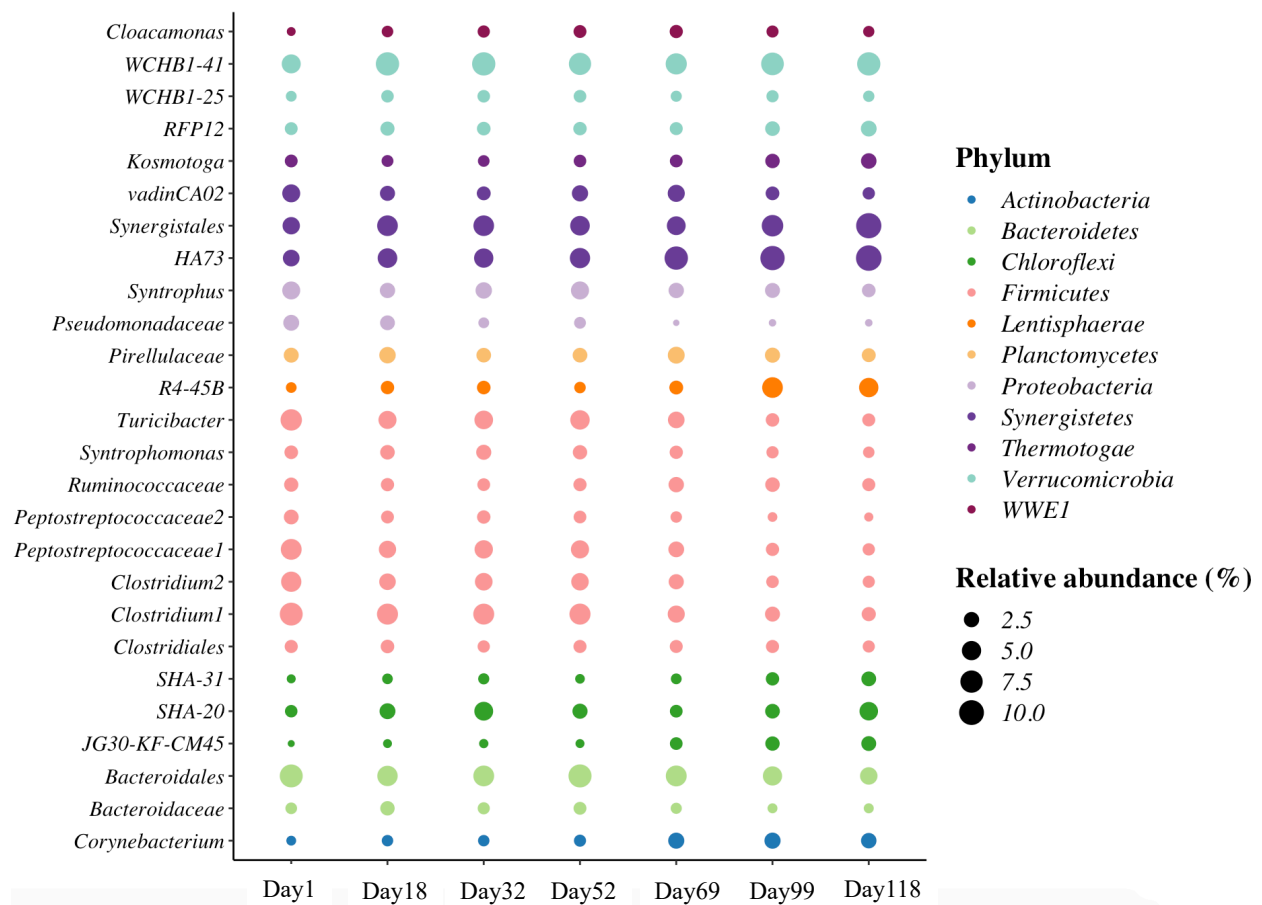


Fig. 2-3 Relative abundance of bacterial community in the anaerobic digestion process treated with dairy manure.

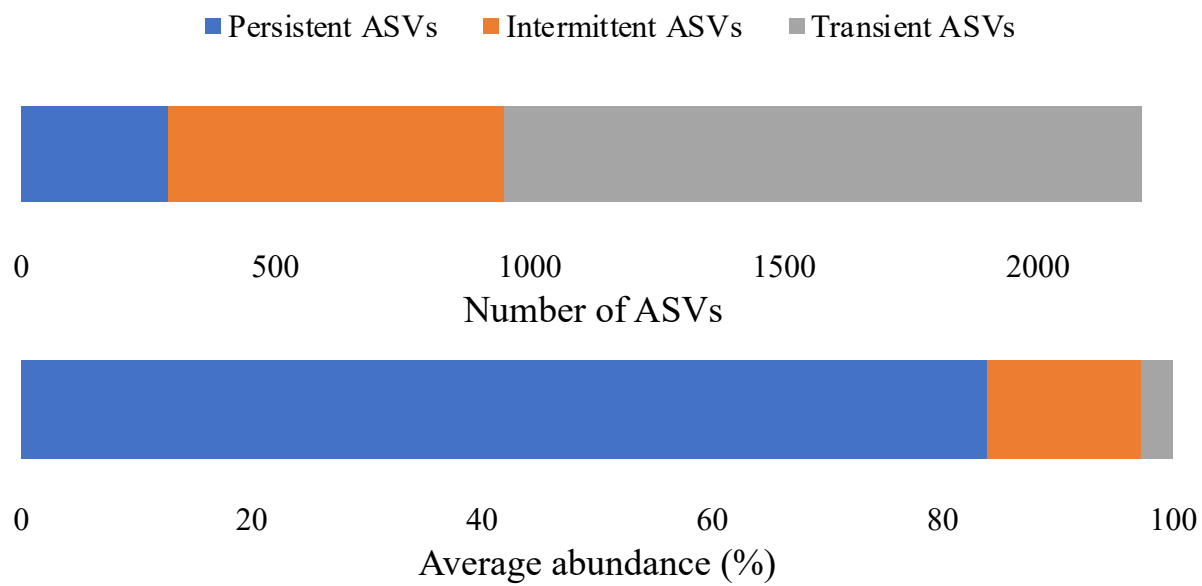


Fig. 2-4 Number of and abundance of amplicon sequence variants (ASV) in each ecological category.

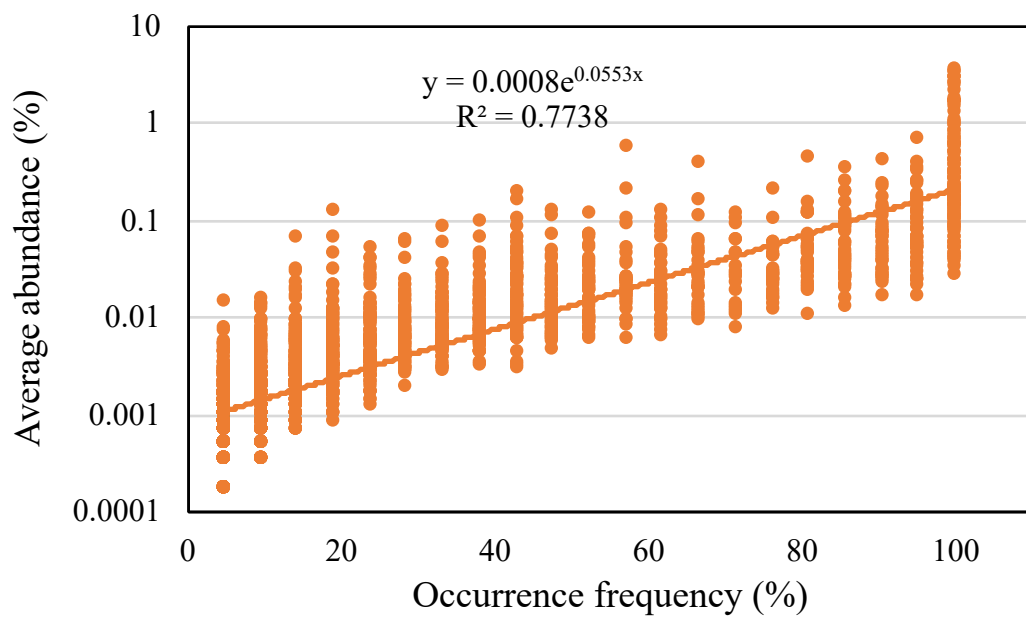


Fig. 2-5 The average abundance was shown to be related with the occurrence frequency.

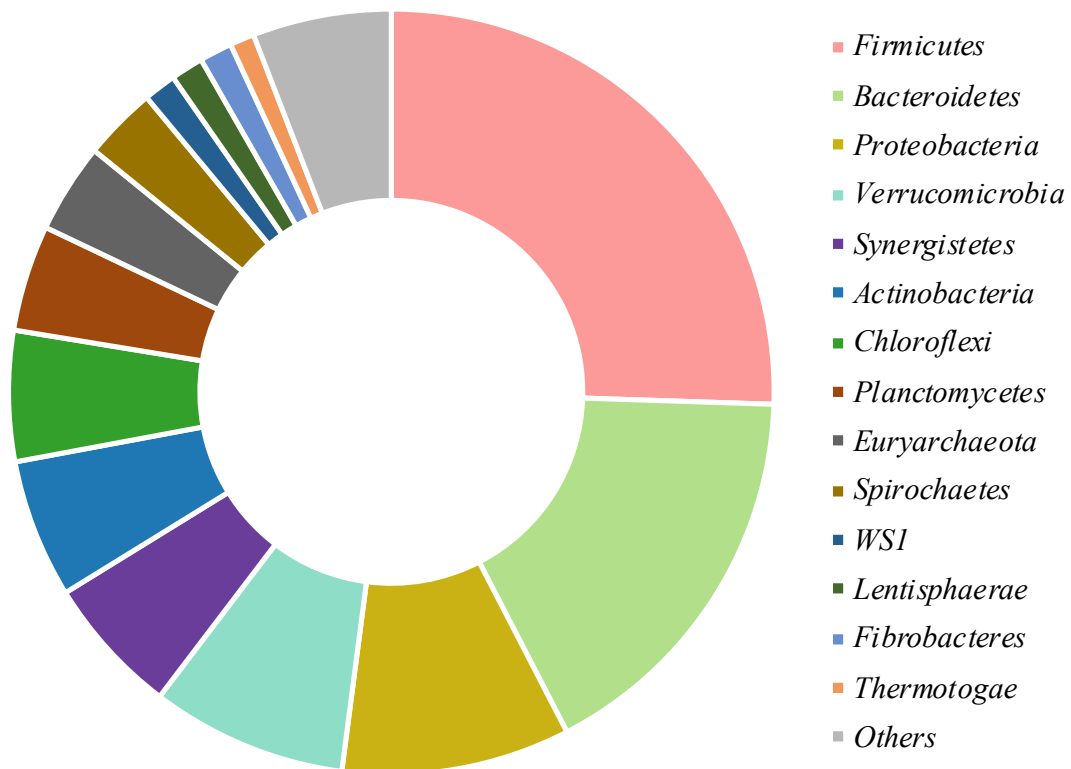


Fig. 2-6 Number of persistent ASVs in each phylum.

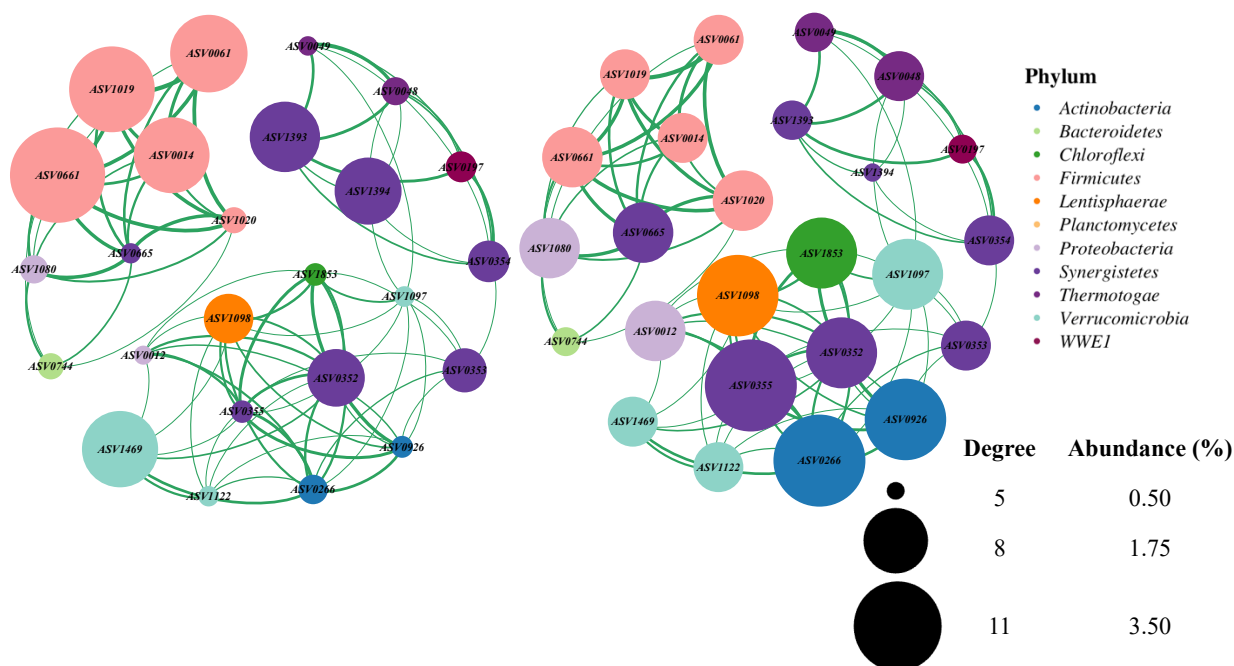


Fig. 2-7 Network of co-occurrence amplicon sequence variants (ASVs) based on correlation analyses. A connection stands for a strong ($r > 0.5$) and significant ($p < 0.05$) correlation. For each panel, ASVs were colored by phylum. Size of each node in the left panel is proportional to the abundance. And size of each node in the right panel is proportional to the number of connections (degree).

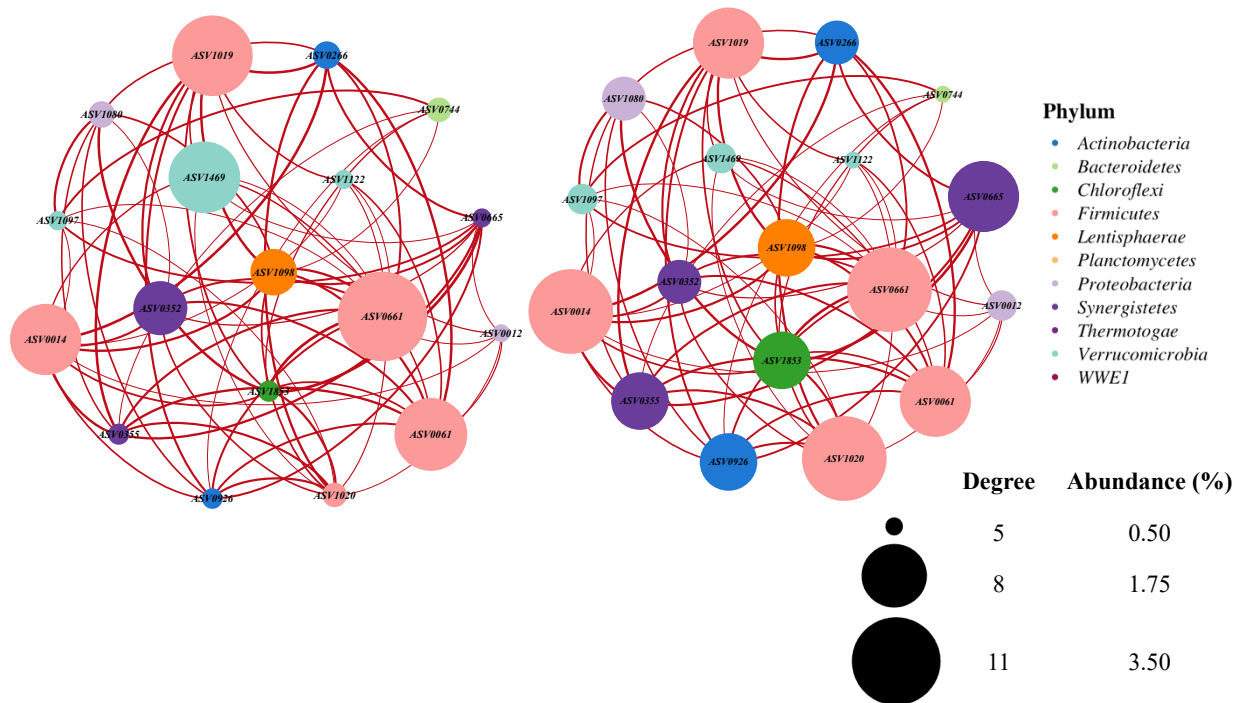


Fig. 2-8 Network of co-exclusion amplicon sequence variants (ASVs) based on correlation analyses. A connection stands for a strong (absolute spearman $r > 0.5$) and significant ($p < 0.05$) correlation. For each panel, ASVs were colored by phylum. Size of each node in the left panel is proportional to the abundance. And size of each node in the right panel is proportional to the number of connections (degree).

CHAPTER III
MICROBIAL TRANSITION IN AN ANAEROBIC TREATMENT
PROCESS WITH SUBSTRATE SHIFT

Abstract

Anaerobic digestion is a microbial process capable of both waste treatment and recovery of biogas as a renewable source of energy. In this study, responses of microbial community in the anaerobic digestion process with substrate shift, i.e. a shift from dairy manure to wastewater sludge were investigated. The results showed that *Methanosaeta* remained as the most abundant archaeal genus before and after substrate shift, indicating the important roles of acetoclastic methanogens in the continuous anaerobic digestion processes. Nevertheless, hydrogenotrophic methanogens like *Methanoculleus*, etc. in microbial community before the substrate shift were switched to *Methanoregula*, etc. after substrate shift. Additionally, non-methanogenic archaeal populations affiliated with phylum *Nanoarchaeota* and *Bathyarchaeota* were found to be relatively abundant before the substrate shift. As for the bacterial populations, *Firmicutes* and *Bacteroidetes* are found consistently as the two most abundant populations, the abundances before the substrate shift were 25.69 % and 23.04 %, after the substrate shift, they were 23.62 % and 21.36%. In addition, abundant bacterial populations before the substrate shift include *Proteobacteria*, *Synergistetes* and *Actinobacteria* with relative abundances averaging 10.85%, 6.38% and 4.52%, respectively. Following the substrate shift, other abundant bacterial phyla were *Proteobacteria*, *Chloroflexi* and *Spirochaetes* with relative abundance averaging 10.67%, 11.99% and 7.65%. Considering dairy manure is a nitrogen-rich substrate compared with wastewater sludge, further microbial community analysis for the anaerobic batch reactors with ammonia addition of 2g TAN/L to investigate the potential driving effects of ammonia in the methanogenic processes. The findings confirmed the relative tolerance of populations like *Methanoculleus*, *Actinobacteria* and *Synergistetes* in the methanogenic processes with slightly high level of ammonia. And populations from these phyla were found to be more abundant in the anaerobic digestion process treated with dairy manure than that with wastewater sludge. To the best of our knowledge, this study is exceptional in revealing the microbial community transition in one operating system, which decreases the uncertainties caused by different operating systems to a great extent. Given that ammonia is a common and important factor found in the anaerobic digestion processes, results of this study could have implications for indicating the existence of ammonia stress in the methanogenic treatment processes.

Keywords: Anaerobic digestion; dairy manure; wastewater sludge; microbial community; ammonia

1 Introduction

Anaerobic digestion (AD) is a process in which biodegradable complex polymers were converted into methane (CH₄) by activities of anaerobic microbiota ¹. Studies of anaerobic digestion process began last century and have become a hot topic due to the combined advantages of anaerobic digestion in both wastewater treatment and biofuel production ²⁻⁴. However, numerous researches on the scattered aspects of the AD process indicated its complexity, and still limited knowledge of this “black box” hinder the broader application of the AD process.

Four chronological steps occurred in the AD process, hydrolysis, acidogenesis, acetogenesis and methanogenesis, various taxa of microbial populations were involved in each step of AD or interact with each other to achieve good performance of the digesters. Hydrolysis is the first and considered as the rate-limiting step in the AD process, and in this step, complex lignin, cellulose, etc. can be degraded to small organic matters, which can be applied in the following

steps. Lots of bacteria were reported to be involved in the hydrolysis, for example, populations in phylum *Firmicutes* and *Bacteroidetes* were the most commonly found anaerobic hydrolytic bacteria in wastewater plant ⁵. In a study of full-scale anaerobic reactor, class *Clostridia* and family *Bacteroidaceae* were the dominant fermentative bacteria ⁶. In the acidogenesis and acetogenesis, monomeric intermediates produced in the first step are further converted into acetate, H₂, formate and CO₂, etc., which are utilized as substrates for the final methanogenic step. Among the four steps in the AD process, methanogenesis is the only step performed by archaeal populations. Three groups of methanogens are divided based on the substrate of methanogenesis, and they were acetoclastic, hydrogenotrophic and methylotrophic methanogens. Among these methanogens, *Methanosaeta* is the solely acetate-utilizing methanogen and has important role in the AD process ⁷⁻⁹.

Substrate, the nutritional source of anaerobic digestion, influence the digestion efficiency via shaping the community structures in the digesters. Besides, substrate is recognized to be the important factors affecting the performance and stability of AD process ¹⁰. By collecting sequencing results for a wide range of substrate types from literature, researchers have found that microbiomes clustered by substrate type, indicating different substrate type could drive variation of anaerobic microbiomes in reactors ¹¹. Among the substrates, dairy manure (DM) and wastewater sludge (WS) were applied most commonly. Generally, compositions of DM include fiber, cellulose, lignin et al ¹². Unlike DM, compositions in WS include the complex matter-peptidoglycans, which indicates that the hydrolysis step would be more difficult for anaerobic digestion process with WS as the substrate ^{13, 14}. Additionally, compared with wastewater sludge, dairy manure is characterized to be an organic/nitrogen-rich substrate as the animal waste, which may lead to the accumulation of ammonia in the methanogenic processes. Microbial variations to these two substrates are necessary to be identified. Even though there were some researchers worked on the variation of microbial communities in anaerobic digestion with different substrates ¹¹. Microbial responses to the substrate shift in single digester need to be revealed since the substrate for a single digester may vary in practice.

With this study, we aimed at investigating the microbial transition in triplicate mesophilic anaerobic digesters with substrate shift from DM to WS using next-generation sequencing of 16S rRNA gene amplicons. Microbial community analysis in the anaerobic batch reactors with ammonia addition of 2g TAN/L further confirmed the relative tolerance of populations like *Methanoculleus*, *Actinobacteria* and *Synergistetes*, etc. in anaerobic digestion process with slight high ammonia levels, such as the processes treated with animal waste. To the best of our knowledge, this study is exceptional in revealing the microbial community transition in one operating system, which decreases the uncertainties caused by different operating systems to a great extent. Given that ammonia is commonly found in the anaerobic digestion processes, results of this study could have implications for indicating the presence of ammonia stress in the methanogenic treatment processes.

2 Materials and Methods

2.1 Setup and operation of triplicate continuous anaerobic digesters

Triplicate mesophilic continuous anaerobic digesters with temperature controlled at 35 °C, a working volume of 3.6 L and a hydraulic retention time of 20 days were set up prior to this study ^{15, 16}. Substrate was shifted from dairy manure (DM) to wastewater sludge (WS) (day 0) to

investigate the microbial community transition in the anaerobic treatment process with substrate shift. Organic loading rate (OLR) was kept as 1.0 g volatile solids (VS)/L/day as before. All the digesters were exhibited stable operation with consistent pH and volatile fatty acid level. Effluents were sampled one year before (day -402) and after the substrate shift (day 346) to study the microbial responses to substrate change in these three digesters at stable state. Samples were centrifuged and stored at -80 °C as soon as possible after taken. Sequencing data for samples were labeled as day -402 and day 346.

2.2 Microbial responses to ammonia addition in batch reactors

To further investigate the microbial populations tolerant with relative high ammonia levels in the continuous anaerobic digesters, two groups (one control group, one treated group with ammonia addition of 2g TAN/L) of batch reactors were set up with a 10% (v/v) inoculum of the effluents from the continuous anaerobic digesters with the influents of the continuous anaerobic digesters as the substrate. The setup procedures were similar as previously described¹⁷. Triplicates were included in each group. Methane production was monitored periodically, and biomass samples were collected when there was no methane produced any more. After that, the samples were centrifuged and preserved at -80 °C for further microbial community analysis.

2.3 DNA extraction, PCR amplification and sequencing

Total community DNA was extracted from all the samples with FastDNA spin kit with protocols similar to that in previous study¹⁸. After extraction, the conserved variable region V4 for bacterial and archaeal 16S rRNA was amplified with primer pairs 515F/806R. PCR conditions, methods of purification and quantification for amplicons were as previously described¹⁹. Sequencing for 16S rRNA was performed on an Illumina platform following the manual.

2.4 Bioinformatics and Statistical analyses

Microbiome analysis package QIIME 2 was used to analyze raw sequencing data. Pipeline DADA2 was applied for quality filtering, low quality regions of the sequences were trimmed based on sequences quality reports. After that, closed-reference clustering was used to cluster sequences into ASVs using q2-vsearch. Taxonomy was assigned to the sequences by comparing the sequences to Silva 132 99% ASVs reference database. Archaeal and bacterial taxa profile in genus level with relatively abundant greater than 1.00% in at least one sample were visualized using Rstudio (<http://www.rstudio.com/>).

3 Results and discussion

3.1 Performance and microbial diversity of anaerobic digestion subjected to substrate shift

In order to investigate the microbial responses in anaerobic digestion with substrate shift, substrate for the triplicate anaerobic digesters fed with dilute dairy manure was switched to wastewater sludge. Both OLR (1 gVS/L/day) and HRT (20 day) were kept the same as before the substrate shift. Samples 402 days before the substrate shift and 346 days after the shift were collected for microbial community analysis (Fig. 3-1). Methane production over time illustrated

the stable performance before the shift and its restoration after the shift. Methane production at day -402 was 207 ± 9 mL/L/day, and the methane production dropped to 145 ± 5 mL/L/day 12 days after the substrate shift, indicating the potential microbial adaptation process to substrate shift. At day 346, the methane production returned to be 236 ± 66 mL/L/day (Fig. 3-1), implying the restored stable performance of the anaerobic digestion process.

Detrended correspondence analysis based on the amplicon sequence variants level showed the separation of microbial communities before and after the substrate shift (Fig. 3-2A). Within- and between-group beta diversity analysis further demonstrated the similar microbial composition within each group but varied microbial composition between these two groups (Fig. 3-2B), revealing the microbial transition in the anaerobic digestion followed the substrate shift, even with stable performance restored.

3.2 Responses of archaeal populations to the substrate shift

To investigate the archaeal responses in the anaerobic digestion subjected to substrate shift even with stable performance restored, the archaeal communities 402 days before and 346 days after the substrate shift were analyzed.

Prevalence of Methanosaeta populations before and after the substrate shift

Results of the archaeal populations showed that *Methanosaeta* was the consistently abundant methanogen before and after the substrate shift. The abundances of *Methanosaeta* before the substrate shift was 41.18 ± 10.00 % in the archaeal community at day -402 and become 35.42 ± 1.68 % at day 346. Abundances of *Methanosaeta* before and after the substrate shift didn't varied significantly, implying the important role of *Methanosaeta* in the methanogenic processes in this study (Fig. 3-3A).

In lots of natural and engineered processes, about 70% of the methane production is via acetoclastic pathway²⁰ and acetoclastic methanogenesis has been proved to be the dominant pathway for methane production²¹⁻²³. Only *Methanosaeta* and *Methanosarcina* could utilize acetate to produce methane in the methanogenic pathways. Unlike the versatile *Methanosarcina*, *Methanosaeta* is the obligately acetate-utilizing methanogen and tends to have lower maximum growth rate ($\mu_{\max}$) and half-saturation coefficient (K_s) than *Methanosarcina*²⁰, thus *Methanosaeta* are assumed to be abundant in the anaerobic digestion process with low acetate level and long hydraulic retention times. Prevalence of *Methanosaeta* populations in the anaerobic digestion subjected to substrate shift confirmed its important role in methanogenic process once again.

Variations of hydrogenotrophic methanogens following the substrate shift

Unlike the consistency of acetoclastic methanogens, hydrogenotrophic methanogens varied a lot in the anaerobic digestion process subjected to substrate shift. Hydrogenotrophic methanogens include *Methanospirillum*, *Methanobrevibacter* and *Methanoculleus* were significantly more abundant at day -402 than that at day 346, and the relative abundances of them in archaeal community at day -402 were 14.74 ± 5.08 %, 10.80 ± 4.17 % and 7.03 ± 2.03 %. However, at day 346, they were 8.05 ± 0.44 %, 0.36 ± 0.11 % and 0.79 ± 0.45 %. On the flip side, hydrogenotrophic methanogens include *Methanoregula*, *Methanobacterium* and *Methanolinea* thrived at day 346 after the substrate shift, with relative abundances of 25.82 ± 2.37 %, $8.36 \pm$

2.27 % and 8.26 ± 3.20 %. Nevertheless, relative abundances of them were less than 1.00% in the microbial community at day -402 (Fig. 3-3A).

Hydrogenotrophic methanogens are more diverse, widespread and could be found in all methanogenic orders except order *Methanomassiliicoccales*. It has been suggested as the ancestral form of methanogen and is responsible for 30% of the methane production^{20, 24}. Among the hydrogenotrophic methanogens mentioned above, *Methanobrevibacter* and *Methanobacterium* are affiliated with order *Methanobacteriales*, while *Methanospirillum*, *Methanoculleus*, *Methanoregula* and *Methanolinea* are affiliated with order *Methanomicrobiales*. Transition of hydrogenotrophic methanogens implied the functionally redundancy of populations in order *Methanobacteriales* and *Methanomicrobiales*.

Non-methanogenic archaeal population abundant before the substrate shift

In addition to methanogens, several non-methanogenic archaeal (NMA) populations were found to be abundant in the anaerobic digestion process fed with dairy manure at day -402. While at day 346, in the anaerobic digestion process with restored stable performance after substrate shift to wastewater sludge, very minor NMA populations were identified. At day -402, NMA populations identified were *Diapherotrites* from phylum *Nanoarchaeota* and two unclassified genera from *Bathyarchaeota*, with relative abundances 10.97 ± 3.08 %, 2.19 ± 0.79 % and 1.67 ± 0.59 %, respectively. While at day 346, relative abundance of *Diapherotrites* decreased to 2.35 ± 0.74 % and for the other two NMA populations, the relative abundances decreased to be less than 1.00% (Fig. 3-3B).

Non-methanogenic archaea are found globally distributed in many mesophilic environments. Some members of them are known to play important roles in ammonia oxidation²⁵ and to involve in protein mineralization²⁶ or aromatics degradation²⁷. However, functions of these NMA populations in the AD processes remain to be confirmed. Using NMA-specific qPCR, observations of minor abundant NMA populations in acetate, propionate, butyrate and methanol enriched cultures while merely no NMA populations in formate enriched cultures suggested the association between NMA and acetoclastic methanogens²⁸. In this study, discrepancy of the NMA populations abundances might be attributed to the differences in the feedstocks. It is possible that characteristics specific to wastewater sludge might be linked to the much lower abundance of NMA populations.

3.3 Impact of substrate shift on bacterial community structure

Bacterial community are much more diverse and complicated in the anaerobic digestion processes due to their crucial roles in hydrolysis, acidogenesis and acetogenesis steps. Compositions of bacteria were also analyzed to evaluate the microbial transitions in the AD process with substrate shift. The ratio of relative abundance of *Firmicutes* and *Bacteroidetes*, termed as F/B ratio hereafter, was also calculated as an indication of process stability.

Populations dynamics within abundant phylum Firmicutes and Bacteroidetes

The most abundant bacterial phyla at day -402 and day 346 were *Firmicutes* and *Bacteroidetes*. Ratios of F/B were 1.12 ± 0.16 at day -402 and 1.11 ± 0.15 at day 346 (Fig. 3-4A). Relative abundances of *Firmicutes* and *Bacteroidetes* at day -402 was 25.69 ± 3.55 % and 23.04

± 0.24 %, while at day 346, they were 23.62 ± 1.58 % and 21.36 ± 1.38 % (Fig. 3-4B). Together with F/B ratios, the results showed the overall stability of *Firmicutes* and *Bacteroidetes*, implying the functional stability of the methanogenic process. However, in the genus level, certain populations within phylum *Firmicutes* and *Bacteroidetes* had the balanced shift (Fig. 3-4C&D). For example, relative abundances of genus *Romboutsia*, *Turicibacter* and *Paeniclostridium* affiliated with *Firmicutes* at day -402 were 4.65 ± 0.84 %, 3.28 ± 0.61 % and 2.96 ± 0.91 % in the bacterial community, while at day 346, relative abundances of them were less than 1.00%. On the contrary, relative abundances of genus *Saccharofermentans* and *Ruminococcus 1* increased to be 2.88 ± 1.10 % and 2.44 ± 1.97 % at day 346 from less than 0.50% at day -402 (Fig. 3-4C). Similar scenarios were found in phylum *Bacteroidetes* (Fig. 3-4D).

Both *Firmicutes* and *Bacteroidetes* were identified to be among the most abundant bacteria phyla in the mesophilic anaerobic digestion process. By linking bacterial population and process performance through an episode of organic loading, F/B ratio was suggested to be responsive to process performance²⁹. Consistent with the previous study²⁹, F/B ratio greater than 1.00 indicated the process stability at day -402 and its restoration at day 346. As the two most abundant bacterial populations in the anaerobic digestion processes, members of these two phyla were reported to have diverse functions and be involved in hydrolysis, acidogenesis and acetogenesis^{30, 31}. Consistent overall *Firmicutes* and *Bacteroidetes* abundances at day -402 and day 346 with certain populations had balanced shift indicated the functional redundancy of these populations³².

Responses of other significant bacterial populations to substrate shift

Despite the two most abundant bacterial phyla, other significant phyla switched a lot (Fig. 3-5A). Among these phyla, relative abundances of *Proteobacteria* and *Cloacimonetes* didn't varied significantly, they were 10.84 ± 2.13 % at day -402 and 10.66 ± 1.39 % at day 346. While relative abundances of *Synergistetes*, *Actinobacteria* and *Verrucomicrobia* were significantly higher at day -402 than that at day 346, with values decreased from 6.38 ± 1.17 %, 4.52 ± 0.66 % and 3.93 ± 0.79 % to 2.43 ± 0.35 %, 2.09 ± 0.18 % and 2.21 ± 0.72 %. On the contrary, relative abundances of *Chloroflexi* and *Spirochaetes* were significantly less at day -402 than that at day 346, with values increased from 2.84 ± 0.47 % and 2.72 ± 1.01 % to 11.99 ± 1.03 % and 7.65 ± 1.90 % (Fig. 5A). Populations within these significant phyla were also transited followed the substrate shift. Uncultured genera from *Verrucomicrobia* and *Synergistetes* were more abundant at day -402, while *Treponema* from *Spirochaetes* and *Longilinea* and two uncultured genera from *Chloroflexi* thrived to be abundant genera at day 346 (Fig. 3-5B).

It's reported that both different substrates and operational conditions drive different bacterial diversity community^{11, 33, 34}. With the same operational conditions before and after the substrate shift in this study, varied bacterial populations at day -402 and day 346 might relate to the different substrate contents. Although it's not possible to infer functions of these important phyla in the methanogenic processes, abundant *Chloroflexi* identified in microbial community in the wastewater treatment plant indicated their important roles in the municipal sludge digesters³⁵. Based on the observation that acetate most stimulated the activities of *Spirochaetes*, presence of *Spirochaetal* communities in the methanogenic process suggested their roles in the acetate oxidation³⁶. Relatively abundant phyla, i.e. *Actinobacteria*, *Synergistetes* and *Verrucomicrobia* were also found to be widely distributed in the anaerobic digestion processes, implying their important roles in methane production^{37, 38}.

3.4 Varied populations that are potentially linked to ammonia in the anaerobic processes

As the animal waste, dairy manure contains relative higher level of nitrogen compared with that in wastewater sludge. According to the previous studies on anaerobic digestion fed with dairy manure, ammonia level in the digesters was around 2 g TAN/L¹⁵. Further microbial community in anaerobic batch reactors with ammonia addition of 2 g TAN/L was performed to link discrepancy of microbial community in anaerobic digesters fed with dairy manure and wastewater sludge to the tolerance of populations to ammonia.

No *Methanoculleus* in the control reactors was identified while relative abundance of 1.00 ± 0.41 % was identified in the microbial community with ammonia addition of 2 g TAN/L. The result was consistent with observation that *Methanoculleus* was significantly more abundant before the substrate shift, indicating the relative tolerant of *Methanoculleus* and ammonia might be the driver to the discrepancy of it in digesters fed with dairy manure and wastewater sludge. In addition, even no specific same genera in *Actinobacteria* and *Synergistetes* that were abundant in digesters fed with dairy manure were identified to be tolerant in reactors with ammonia addition. Relative abundances of some genera, i.e. *vadinCA02*, *unclassified Solirubrobacterales* and *Phycococcus*, increased with ammonia addition, indicating the tolerance of populations within these two phyla compared with other bacterial populations. On the flip side, relative abundance of *Treponema* from *Spirochaetes* that was abundant wastewater sludge decreased with the increased ammonia level, which might indicate their sensitive to ammonia. And the relative abundance of *Treponema* in the control reactors was 6.69 ± 3.87 % and decreased to 1.23 ± 1.48 % in reactors with ammonia addition of 2 g TAN/L (Fig. 3-6).

Methanoculleus has been reported to be prevalent in ammonia-rich engineered anaerobic processes and is an important hydrogenotrophic partner working with acetate-oxidizing bacteria in mesophilic environments³⁹, which is consistent with the observations in this study. It's suggested that *Methanoculleus* play as an important role in resistance of ammonia by enhancing syntrophic interactions⁴⁰. However, contrary results were found for *Treponema*, relative abundance of which was identified to increase with the increasing TAN level⁴⁰. However, the environment they dealt with is extreme and the ammonia level is as high as 10g TAN/L, different findings of genus *Treponema* dealing with ammonia suggested both the diversity within genus and the potential occurrence of adaption process.

4 Conclusions

Dairy manure was characterized as the substrate containing richer organic/nitrogen compared with wastewater sludge. One year after the substrate shift, the anaerobic digestion returned to be stable with similar methane production as before the shift, which might be linked to the unchanged abundances of methanogens, especially the acetoclastic methanogen *Methanosaeta*. Different levels of ammonia in the feeding substrates didn't lead to the varied methane production in stable stage, demonstrating the robust and versatile anaerobic community in the methanogenic processes. Nevertheless, compositions of the microbial community in the genus profile switched a lot following the substrate shift, which may relate to both the different compositions and physicochemical parameters of the substrates.

Appendix B: Tables and Figures

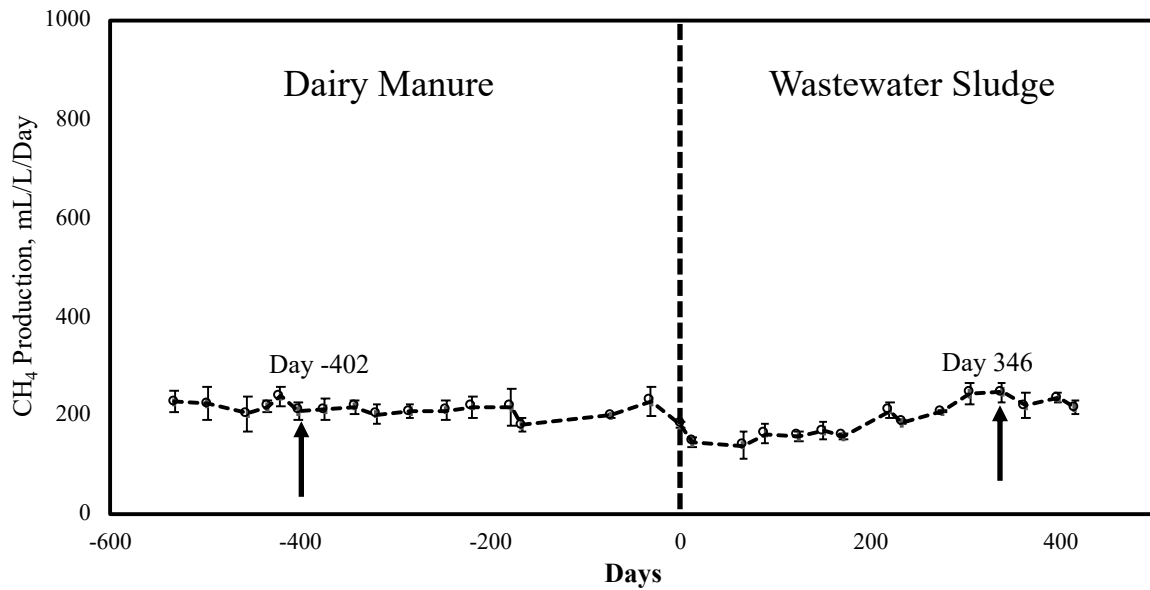


Fig. 3-1 Methane production (mL CH₄/L/Day) in the anaerobic digestion process. Data are means of triplicate digesters, with the error bars indicating the standard deviation. The vertical dashed line indicates substrate shift from dairy manure to wastewater sludge. Arrows indicate the time points for sample collection for microbial community analysis. Samples were labeled as Day -402 and Day 346.

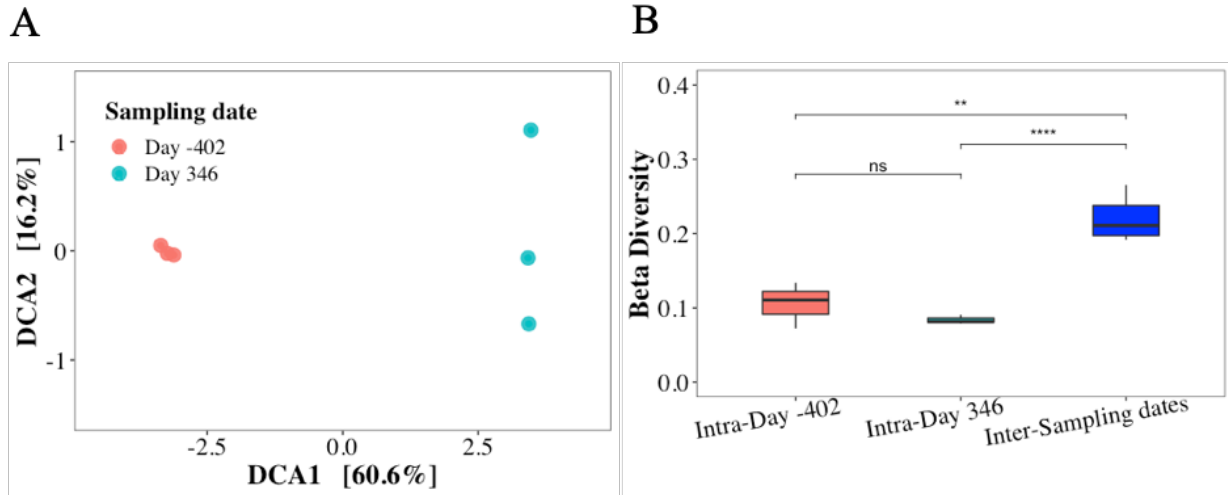


Fig. 3-2 A Detrended correspondence analysis based on amplicon sequence variants (ASV) level in the microbial communities before (DM) and after the substrate shift (WS) indicated the separation of communities in these two groups. B Intra and inter-group beta diversity calculated using weighted unifrac method (* $p < 0.05$; *** $p < 0.001$; unpaired one-tailed t test assuming equal variances) implied the similarity of communities in both groups.

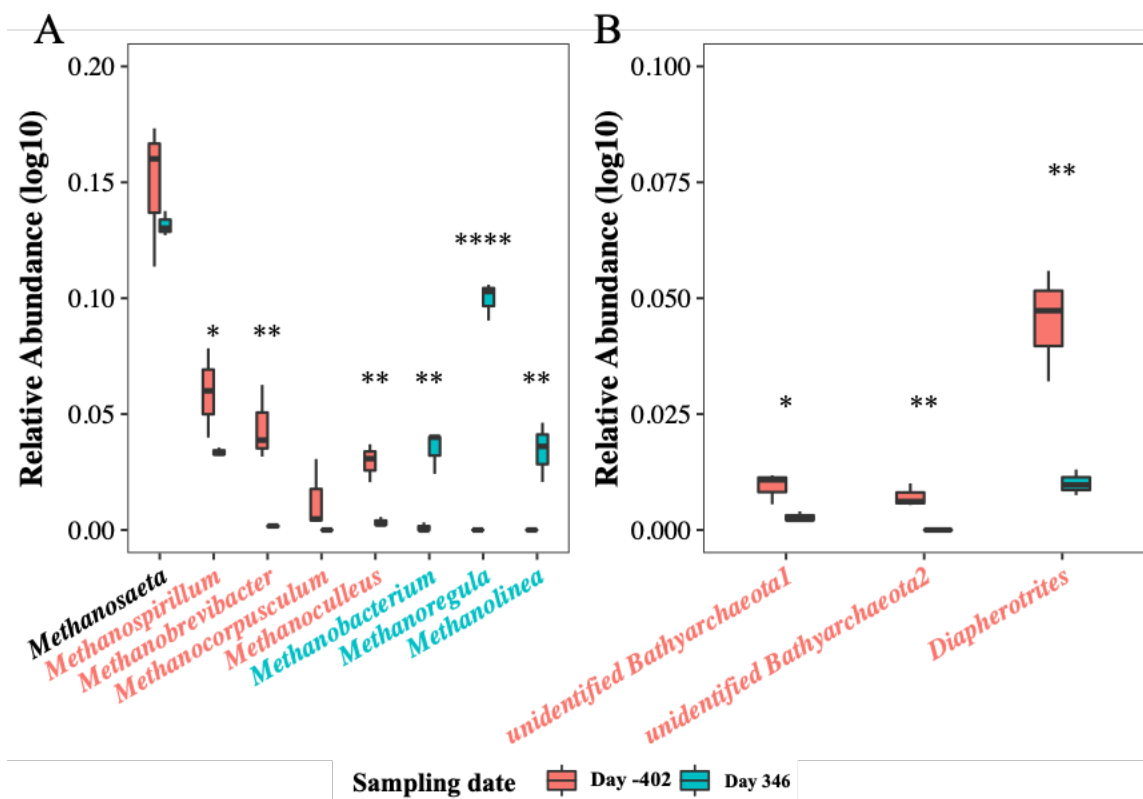


Fig. 3-3 **A** Abundant methanogens from phylum *Euryarchaeota* identified in the microbial communities before and after the substrate shift, indicating both the persistent prevalence of acetoclastic *Methanosaeta* and the alterations of some hydrogenotrophic methanogens. Methanogenic genera with relative abundance greater than 5.00% in archaeal community in at least one sample were involved. **B** Prevalence of non-methanogenic archaeal genera are identified in anaerobic digestion process treated with dairy manure. The top abundant genera were from phylum *Nanoarchaeota* and *Bathyarchaeota*. Non-methanogenic genera with relative abundance greater than 2.00% in archaeal community in at least one sample were involved were included. Genera with relative abundances significantly higher in community fed with dairy manure were indicated with red font, and genera with relative abundances significantly higher in community fed with wastewater sludge were indicated with blue font. Mean relative abundances of each methanogen before and after the substrate shift were compared using one-tailed unpaired t test assuming equal variances.

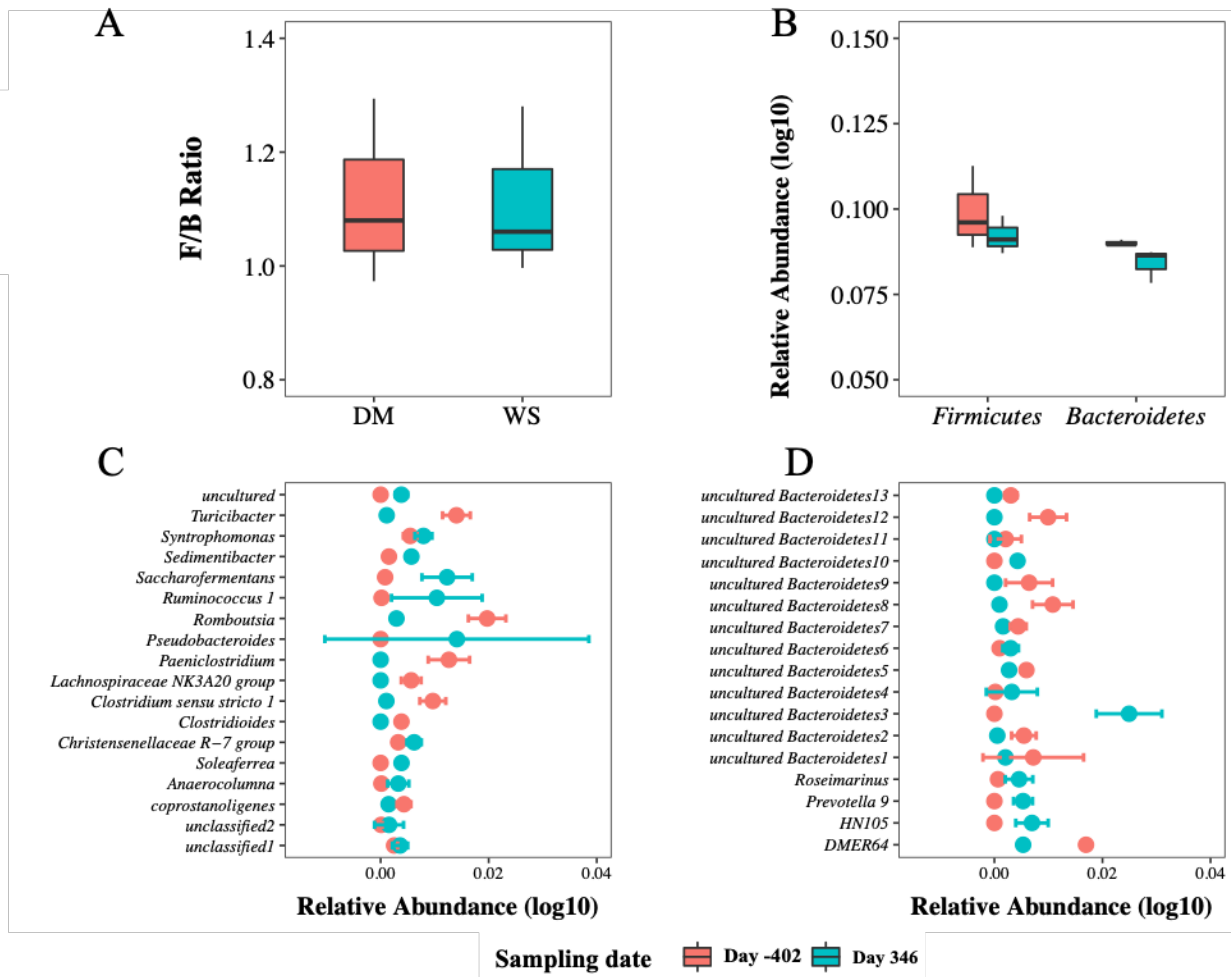


Fig. 3-4 **A** Comparisons of F/B ratios in the microbial community before and after the substrate shift using paired t-test indicated the functional stabilities of the methanogenic processes. **B** Overall relative abundances of *Firmicutes* and *Bacteroidetes* in the bacterial community before and after the substrate shift. **C** Microbial compositions in phylum *Firmicutes* in the genus profile. **D** Microbial compositions in phylum *Bacteroidetes* in the genus profile. revealed the functionally redundancy of bacterial populations identified in the methanogenic process before and after the substrate shift. Genus with relative abundance greater than 1.00% in at least one sample was involved.

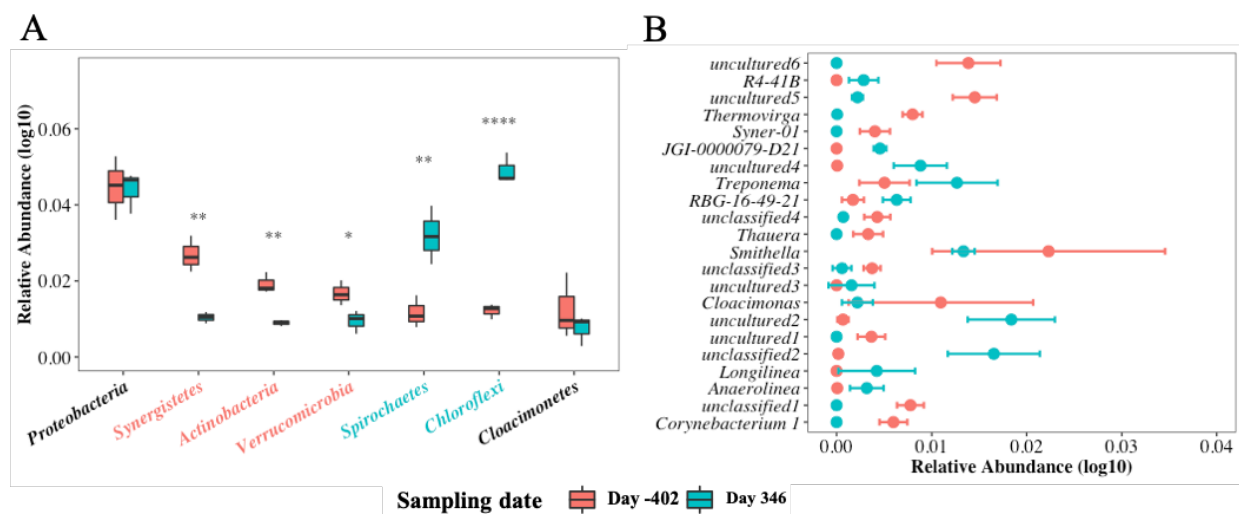


Fig. 3-5 **A** Other phyla with relative abundances greater than 5.00% in the bacterial community indicated the bacterial transition before and after the substrate shift. **B** Microbial genera with relative abundance greater than 1.00% in at least one sample in other abundant bacterial phyla. Phyla with relative abundances significantly higher in community fed with dairy manure were indicated with red font, and phyla with relative abundances significantly higher in community fed with wastewater sludge were indicated with blue font. Mean relative abundances of each methanogen before and after the substrate shift were compared using one-tailed unpaired t test assuming equal variances.

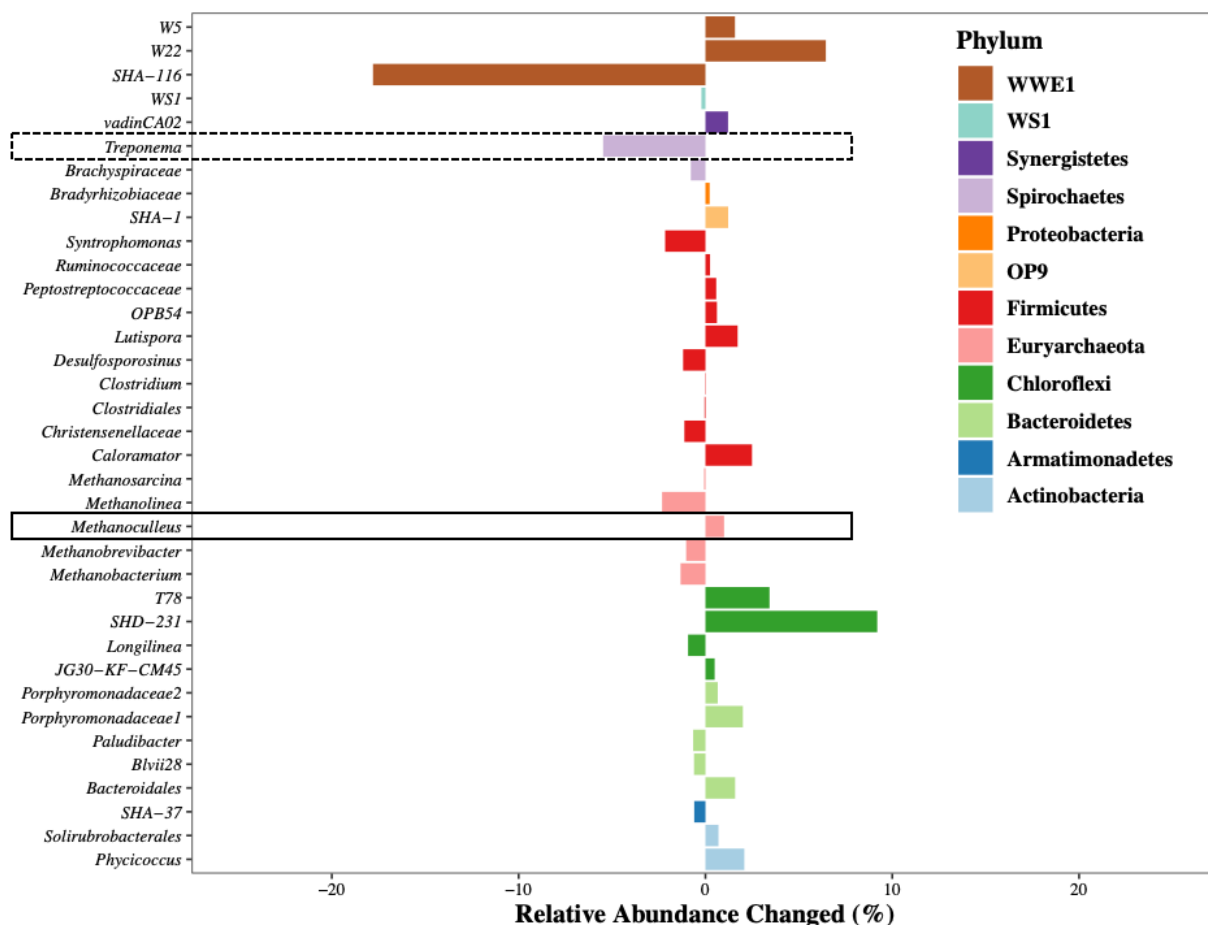


Fig. 3-6 Genera that are changed in the anaerobic batch reactors with ammonia addition of 2g TAN/L. Changed values were calculated as the averaged abundances in the reactors with ammonia addition of 2g TAN/L minus that in the control reactors. Genera in the dashed black square are identified to be more abundant in communities fed with dairy manure, and genera in the solid black square are identified to be more abundant in communities fed with wastewater sludge. Genera with relative abundance greater than 1.00% in at least one sample were included.

CHAPTER IV
DIFFERENTIATION OF RESISTANT METHANOGENS IN THE
ANAEROBIC BATCH REACTORS WITH AMMONIA ADDITION

Abstract

Ammonia concentration increases in the anaerobic digestion process treated with nitrogen-rich substrates, which will influence the microbial community and thus functional operation of the process. As the key players in the anaerobic digestion process, associations and functions of microbiomes in the process remained unclear due to its complexity in terms of functionality and community diversity. Microbiomes present in the anaerobic digestion were shaped by several factors, e.g. inhibitors like ammonia. In this study, two transfers of anaerobic reactors with ammonia addition were set up to investigate the microbial populations resistant or redundant to ammonia. With two transfers of anaerobic batch reactors, methanogens *Methanobacterium* and *Methanosarcina* were the relatively abundant methanogens in all reactors. Other methanogens like *Methanoculleus* was stimulated by ammonia addition of 2g TAN/L in both two transfers, *Methanospirillum* was stimulated by ammonia addition of 4g TAN/L in the first transfer and *Methanofollis* was thrived when ammonia was relieved in the second transfer. As for the bacterial compositions, genus *SHA-116* from phylum *WWE1* was abundant in the control reactors in both transfers and the relative abundances were 19.18% in control reactors in the first transfer and 14.85% in control reactors in the second transfer. Nevertheless, the relative abundances of *SHA-116* in the remaining reactors were all less than 1.40%. Another genus from phylum *WWE1*, *W22*, relative abundances of which increased a lot (relative abundance in reactors with ammonia addition of 2g TAN/L in the first transfer was 6.46%; relative abundance in reactors with ammonia addition of 4g TAN/L in the first transfer was 25.86% and relative abundance in reactors with ammonia addition of 2g TAN/L in the second transfer was 16.38%) when ammonia was added. The results indicated that when ammonia was added, genus *SHA-116* was disturbed and might be replaced by genus *W22*, which was affiliated with the same phylum *WWE1*, revealing the redundancy mechanism of microbial community to maintain its function. Moreover, relative abundances of genus *Treponema* from phylum *Spirochaetes* were all greater than 6.50%, when ammonia was added, the relative abundances decreased to be lower than 1.30%. After ammonia was relieved in the second transfer, the population rebounded and the relative abundance increased to 5.36%, implying the resilience of this group. Additionally, genera like *Porphyromonadaceae* (phylum *Bacteroidetes*) and *vadinCA02* (phylum *Synergistetes*) also thrived after the addition of ammonia. This study identified both the resistant archaeal and bacterial groups and microbial interactions were discussed based on their responses to ammonia addition. Results of this study supported the important roles of ecological mechanisms: resistance, resilience and functional redundancy of the anaerobic microbiome in maintaining their functionality.

Keywords: Ammonia-resistant microbiomes; microbial differentiation; anaerobic digestion; wastewater sludge

1 Introduction

Ammonia in the anaerobic digestion process is critical for both bacterial growth and neutralization of organic acids. On the one hand, it is the important nitrogen source for synthesis of nucleic acids, amino acids and thus proteins; on the other hand, ammonia can work as a base and help maintain neutral pH levels of the environment²⁹. Nonetheless, high levels of ammonia accumulated in the anaerobic digestion process fed with nitrogen-rich substrates^{33, 106} will influence to a great extent the functional performances and the compositions of the microbial populations, e.g. the methanogens¹⁰⁷. There are two species of ammonia existed in the aqueous

environment: ionized ammonia (NH_4^+) and free ammonia (NH_3). Proportions of these two species depend on both pH and temperature. Among these two species, NH_3 was suggested as the main reason of ammonia toxicity¹⁰⁸. Mechanisms of how ammonia affects microbial organisms in the anaerobic digestion process are still not clear. And mechanisms that were most widely accepted were: 1) the direct inhibition of cytosolic enzymes activities by NH_3 ; 2) variation of the intracellular pH; 3) depletion of intracellular potassium, et al.

Conflicting results about effects of ammonia on the archaeal community in the anaerobic digestion process were reported^{31, 108, 109}. Researches related to the effects of ammonia on methanogens were focused on the Some researches indicated that acetoclastic methanogens were more susceptible to ammonia compared with hydrogenotrophic methanogens, and a two-step syntrophic acetate oxidation (SAO) pathway would be preferred to produce methane from acetate¹⁰⁹. While others were on the contrary¹¹⁰. Additionally, by investigating the relationships between bacterial communities and environmental conditions, researchers found that *Clostridiales* and *Bacteroidales* were abundant in reactors with high levels of ammonia, suggesting the importance of ammonia in bacterial selection¹¹¹. Microbiomes that can deal with high ammonia level in the anaerobic digestion process still need to be further investigated. And the findings will expand the potential application of bioaugmentation with ammonia-tolerant microbial consortia in the anaerobic digestion process with high ammonia content. From the viewpoint of ecology, three ecological mechanisms (resistance, resilience and redundancy) are provided to maintain the microbial community function in the process of anaerobic digestion. A resistant population is the population that can deal with disturbance and the composition will not vary, a resilient population is the population that can rebound after the disturbance and a redundant population is the population that replaced the disturbed population without influencing the performance^{112, 113}. Within the microbial community in the anaerobic digestion process, hydrolytic bacteria are indicated as the abundant and redundant ones; syntrophic bacteria are usually the minority and resilient community⁵⁰. Compared with the diverse bacterial community, archaea are less diverse and more susceptible to disturbance. And among the methanogens, *Methanobacteriales* are regarded as resistant, *Methanosarcina* and *Methanomicrobiales* are regarded as resilient and redundant¹¹⁴. However, the impact of ammonia on these hydrogenotrophic methanogens were studied to a lower extent.

In this study, two transfers of anaerobic batch reactors were set up to investigate the resistance, resilience and redundancy of both the archaeal and bacterial community in the anaerobic digestion process with ammonia disturbance and relief. With the application of 16S rRNA high-throughput sequencing, we analyzed the microbial responses to two levels of ammonia addition in the first transfer and continuous ammonia addition and ammonia relief in the second transfer. Diversity based on richness and evenness were also dealt with to further reveal the influences of ammonia on microbial community in the anaerobic digestion process. Additionally, differentiation of abundant archaea was analyzed to validate that resistant taxa in the anaerobic digestion might exhibit a range of adaptation to the ammonia disturbance.

2 Materials and Methods

2.1 Set up two transfers of batch reactors

For the first transfer, two experimental groups with 2g TAN/L and 4g TAN/L were set up by adding 70 mL wastewater sludge (with the volatile solids as 20.0g VS/L) and 10 mL inoculum

from the continuous bench-scale anaerobic digesters (feedstock: wastewater sludge; Organic loading rate: 1.0 gVS/L/day) ⁵⁴ into 160-mL serum bottles flushed with pure N₂. Then seal the bottles with butyl rubber stoppers and aluminum caps. One control group was set up without adding ammonia accompanying with the experimental groups. All the groups were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 °C. After there was no more gas produced, the secondary batch of reactors for continuous ammonia addition and ammonia relief were established with similar method. Inoculum of the control group in the secondary transfer was from the control group in the first transfer. Inoculum of the continuous ammonia addition and ammonia relief were from the group of reactors with 2g TAN/L in the first transfer. Gas production and compositions were measured twice a week. At the end of each transfer, 1mL samples were collected and centrifuged at 14,000 g for 15 min, the supernatants were stored at -20 °C for volatile fatty acids analysis and the pellets were stored at -80 °C for further microbial community analysis.

2.2 Chemical Analysis

Biogas production was measured using a previously described water displacement method ¹¹⁵. Methane content in biogas was analyzed by a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen-1000; SigmaAldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 mL/min. GC analysis of methane used the following temperature scheme: oven 125 °C, injection port 150 °C and TCD detector 170 °C. Volatile fatty acids (VFA) in the aqueous phase were quantified using the Agilent Technologies 7890B GC system equipped with a flame ionization detector (FID) and a Agilent 122-3232 DB-FFAP column. Nitrogen was the column carrier gas at 2.4 ml/min. The following temperature scheme was used for VFA quantification: oven 120 °C, injection port 250 °C, and detector 250 °C. Total solid and volatile solid were measured with “2540 B&E” method and pH values at the end of each transfer were measured with a pH-meter.

2.3 Microbial community analysis

Total community DNA was extracted from all the samples with FastDNA spin kit with protocols similar to that in previous study ⁵⁶. After extraction, the conserved variable region V4 for bacterial and archaeal 16S rRNA was amplified with primer pairs 515F/806R. PCR conditions, methods of purification and quantification for amplicons were as previously described ⁵⁷. Sequencing for 16S rRNA was performed on an Illumina platform following the manual.

2.4 Sequencing and Statistical data analysis

Methods of processing sequencing dataset were same to the former chapters. One-way ANOVA and Tukey multiple pairwise-comparisons were performed between the average alpha diversity indices of groups in Rstudio. The 16S rRNA gene sequences for abundant methanogens were searched for homology using the BLAST program at the National Center for Biotechnology Information (NCBI), aligned with homologous sequences using Clustal Omega ¹¹⁶. Phylogenetic tree was constructed using neighbor-joining method (1000 bootstrap re-samplings) with MEGA 7.0 ¹¹⁷.

3 Results

3.1 Functional performances of the anaerobic batch reactors with ammonia addition/relief

In the first transfer, the average methane production of the control reactors was 331 ± 19 mL. With ammonia addition of 2g TAN/L, the average methane production was 332 ± 10 mL, and there is no significant difference compared with the average methane production in control reactors (Fig. 4-1). Nevertheless, with ammonia addition of 4g TAN/L, the average methane production decreased to 277 ± 2 mL. In addition, pH values at the end of the experiment in both control and 2g TAN/L reactors were similar (Control: 7.29 ± 0.02 ; 2g TAN/L: 7.28 ± 0.02). While in the reactors with 4g TAN/L ammonia addition, the pH value was 7.00 ± 0.04 , which was lower than that in control reactors, implying the potential of VFAs accumulation in the reactors with 4g TAN/L ammonia addition. Volatile fatty acids like acetate, propionate and butyrate were measured. And when it came the end of the experiment, butyrate in all reactors were not detected. Acetate levels in control reactors and reactors with ammonia addition of 2g TAN/L were around 0.4 mM, and in reactors with ammonia addition of 4g TAN/L, the acetate level was a little higher and was close to 1 mM. As for propionate, its concentrations in control reactors and reactors with ammonia addition of 2g TAN/L were close to zero, while in reactors with ammonia addition of 4g TAN/L, the propionate concentration was much higher and was almost 15 mM, indicating the accumulation of propionate in the anaerobic reactors when 4g TAN/L was added. Furthermore, in the second transfer, the average methane productions in three groups (Control, 2g TAN/L, no TAN addition) were close and were 329 ± 21 mL, 321 ± 25 mL and 331 ± 30 mL, respectively (Fig. 4-1). The pH values in these three groups were 7.34 ± 0.01 , 7.32 ± 0.06 and 7.22 ± 0.04 . Acetate levels in all the reactors in the second transfer were less than 1 mM and no propionate in most of the reactors were detected.

3.2 Responses of microbial community compositions

Relative abundances of archaea in the three groups (Control, 2g TAN/L, 4g TAN/L) in the first transfer were 18.68%, 14.33% and 10.69%, respectively. As for the relative abundances of archaea in the three groups (Control, 2g TAN/L, no TAN addition) in the second transfer, they were 18.40%, 7.70% and 18.33%. The most abundant archaeal genus in most reactors was *Methanobacterium* and *Methanosarcina*. In the first transfer, the relative abundances of *Methanobacterium* in the three groups (Control, 2g TAN/L, 4g TAN/L) were 9.12%, 7.78% and 6.48%, and for *Methanosarcina*, the relative abundances were 4.81%, 4.74% and 2.16%. In the second transfer, the relative abundances of *Methanobacterium* in the three groups (Control, 2g TAN/L, no TAN addition) were 11.09%, 2.18% and 12.41%, and for *Methanosarcina*, the relative abundances were 5.05%, 4.31% and 3.28%. Additionally, for the other minor archaeal taxa, relative abundance of *Methanospirillum* in reactors with ammonia addition of 4g TAN/L in the first transfer was 1.73%, while in other reactors, the relative abundances were less than 0.34%, implying the potential tolerance of *Methanospirillum* to ammonia at 4g TAN/L. Relative abundances of *Methanoculleus* in reactors with ammonia addition of 2g TAN/L in both first and second transfer were 1.00% and 0.74%, while in Control reactors, no *Methanoculleus* was identified, and in reactors with ammonia addition of 4g TAN/L in the first transfer and with no ammonia addition in the second transfer, the relative abundances of *Methanoculleus* were less than

0.06%. Besides, the relative abundances of *Methanofollis* in reactors with ammonia addition of 2g TAN/L in the first and second transfer were 0.07% and 0.09%, no *Methanofollis* was detected in control reactors and reactors with ammonia addition of 4g TAN/L. While in the reactors with no more ammonia addition in the second transfer, the relative abundance of *Methanofollis* was 1.60% (Fig. 4-2).

Top 4 abundant phyla in the bacterial communities were the same in all reactors and were *WWE1*, *Firmicutes*, *Chloroflexi* and *Bacteroidetes*. In the genus level, in the control reactors of both transfers, genus *SHA-116* from phylum *WWE1* was the most abundant genus and the relative abundances in the whole community in the two transfers were 19.18% and 14.85%, respectively. In the first transfer, the most abundant bacterial genus in reactors with ammonia addition of 2g TAN/L was genus *SHD-231* from phylum *Chloroflexi* and the relative abundance was 14.20%. And when it came to the reactors with ammonia addition of 4g TAN/L, the most abundant bacterial genus was genus *W22* from phylum *WWE1* and the relative abundance was 25.86%. It was worth mentioning that relative abundance of genus *W22* in the control reactors were 0.02%, which was much lower than that in the reactors with ammonia addition (relative abundance in reactors with ammonia addition of 2g TAN/L was 6.46%; relative abundance in reactors with ammonia addition of 4g TAN/L was 25.86%). Besides, relative abundance of an unclassified genus from family *Porphyromonadaceae* (phylum *Bacteroidetes*) increased as the levels of ammonia addition growing higher. Relative abundance of the unclassified genus from family *Porphyromonadaceae* in the control reactors was 0.26%, in the reactors with ammonia addition of 2g TAN/L was 2.26% and in the reactors with ammonia addition of 4g TAN/L was 12.97%. Results of bacterial composition in the first transfer indicated that genus *W22* and the unclassified genus from family *Porphyromonadaceae* (phylum *Bacteroidetes*) were the tolerant bacterial group in the anaerobic digestion process with ammonia addition. As for the bacterial compositions in the second transfer, the most abundant bacterial genus in the reactors with ammonia addition of 2g TAN/L was genus *W22* in phylum *WWE1* and the relative abundance was 16.38%. When there was no more ammonia addition in the second transfer, the most abundant bacterial genus switched to an unclassified genus in order *Bacteroidales* (phylum *Bacteroidetes*) and the relative abundance was 9.60%. Comparing all the bacterial communities in the two transfers together, we found that genus *Treponema* (phylum *Spirochaetes*) was among the top five abundant genera in reactors with no ammonia addition (control reactors in the first transfer, control reactors in the second transfer and reactors with no more ammonia addition in the second transfer), the relative abundances were 6.69%, 6.70% and 5.36, respectively. On the contrary, genus *W22* from phylum *WWE1* was among the top five abundant genera in reactors with ammonia addition (2g TAN/L in the first transfer, 4g TAN/L in the first transfer and 2g TAN/L in the second transfer), and the relative abundances were 6.46%, 25.86% and 16.38%. To be similar, relative abundances of genus *Porphyromonadaceae* from phylum *Bacteroidetes* also increased as ammonia was added and were 2.26% in the reactors with ammonia addition of 2g TAN/L in the first transfer, 12.97% in the reactors with ammonia addition of 4g TAN/L in the first transfer and 4.14% in the reactors with ammonia addition of 2g TAN/L in the second transfer. Last not the least, genus *vadinCA02* in phylum *Synergistetes* was only abundant in the reactors with ammonia addition of 2g TAN/L in the second transfer and the relative abundance was 6.03%. While in other reactors, the relative abundances were less than 1.50% (Fig. 4-3).

3.3 Differentiation of the abundant archaeal groups

For all the reactors in our study, the most abundant archaeal genera were *Methanobacterium* and *Methanosarcina*. In order to investigate whether there existed microbial differentiation in these two groups, distributions and relative abundances in the ASVs level for both *Methanobacterium* and *Methanosarcina* were analyzed. For *Methanobacterium*, 30 ASVs were identified, and among these 30 ASVs, only 8 ASVs (*ASV0010*, *ASV0002*, *ASV0001*, *ASV0005*, *ASV0018*, *ASV0012*, *ASV0019* and *ASV0007*) were abundant, relative abundances for the remaining ASVs were less than 5% in *Methanobacterium* in all communities. No obvious pattern was found for the distribution of ASVs in *Methanobacterium*. Among the 8 abundant ASVs, relative abundances of *ASV0001*, *ASV0002*, *ASV0005*, *ASV0007* and *ASV0010* were greater than 10.00% in at least one group. Ranges of relative abundances of *ASV0001*, *ASV0002*, *ASV0005*, *ASV0007* and *ASV0019* were 13.34% ~ 47.51%, 8.85% ~ 50.59%, 0.00% ~ 18.04%, 0.68% ~ 45.73% and 0.27% ~ 18.39%. Phylogenetic tree based on Neighbor-joining method was constructed for these 5 predominant *Methanobacterium* ASVs and 16s rRNA gene sequences for five referenced *Methanobacterium* strains to compare the evolutionary differences between *Methanobacterium* ASVs in this study and the known *Methanobacterium* strains. The results indicated considerable similarities between the *Methanobacterium* ASVs in this study and the known *Methanobacterium* strains (Fig. 4-4). Meanwhile, 8 ASVs (*ASV0003*, *ASV0004*, *ASV0008*, *ASV0009*, *ASV0016*, *ASV0017* and *ASV0030*) were identified to be affiliated with genus *Methanosarcina*. In the control reactors of both transfers, only *ASV0003*, *ASV0009* and *ASV0017* were identified among these 8 ASVs. And in reactors with ammonia addition of 4g TAN/L in the first transfer, three ASVs (*ASV0004*, *ASV0011* and *ASV0030*) were identified. In the remaining reactors (ammonia addition of 2g TAN/L in the first transfer, ammonia addition of 2g TAN/L in the second transfer and no ammonia addition in the second transfer), most of the ASVs were identified. According to phylogenetic analysis, *ASV0003*, which was the abundant ASV in the control reactors was found to be close to *Methanosarcina acetivorans* strain C2A and *ASV0004*, which was the abundant ASV in the reactors with ammonia addition, was close to *Methanosarcina flavescens* strain E03.2 (Fig. 4-5).

3.4 Diversity of microbial communities

Alpha diversity index Shannon was calculated in the ASVs level to evaluate the evenness and richness of the microbial communities. Shannon is an information statistic that consider both richness and evenness. For the communities in these two transfers, the values of Shannon index ranged from 3.75 to 4.49. The smallest Shannon index was found in communities with ammonia addition of 4g TAN/L in the first transfer and the greatest one was all found in communities with no ammonia addition in the second transfer. The results indicated that ammonia addition of 4g TAN/L would reduce the richness and evenness of the community. In addition, one-way ANOVA test was performed to compare whether the Shannon index of these groups differed significantly. And we obtained P-values as 0.0001, indicating there were significant differences for Shannon diversity between different groups. Tukey multiple pairwise-comparisons were performed further to compare the mean values of these estimators between different groups and the results were summarized in table 4-1. Moreover, dimensional NMDS analysis unweighted-Unifrac distance methods were applied to assess the microbial community differences. Compared with the communities with ammonia addition (2g TAN/L in the first transfer, 4g TAN/L in the first transfer and 2g TNA/L in the second transfer), communities from reactors with no ammonia addition

(control reactors in the first transfer, control reactors in the second transfer and reactors with no TAN in the second transfer) were close to each other for both distance methods (Fig. 4-6).

4 Discussion

Combined results of methane production, pH and VFAs levels in the reactors implied that ammonia addition of 4g TAN/L had a negative effect on both the functional performances and the microbial community in the anaerobic batch reactors. Researchers found that in an anaerobic digestion process treated with piggery wastes, 3g TAN/L of ammonia resulted into partial inhibition of the process¹¹⁸. Other studies focusing on the acclimation of archaea to high level of ammonia implied that the microbial community could tolerate ammonia up to 5g TAN/L in digested sewage sludge and 3g TAN/L in piggery manure^{119, 120}. Even though ammonia addition of 2g TAN/L has no significant influences on methane productions, both the archaeal and bacterial community compositions were affected. Relative abundances of archaea decreased to less than 8.00% in the reactors with ammonia addition of 2g TAN/L in the second transfer, implying the susceptibility of archaea to ammonia compared with bacteria. In the first transfer, relatively abundant hydrogenotrophic methanogens identified in the control group include *Methanobacterium*, *Methanosarcina*, *Methanolinea*, *Methanobrevibacter*, *Methanosaeta* et al. While in reactors with ammonia addition of 2g TAN/L, *Methanolinea* and *Methanobrevibacter* were substituted by *Methanoculleus*; and in reactors with ammonia addition of 4g TAN/L, were substituted by *Methanospirillum*, indicating the redundancy of groups from order *Methanomicrobials*. In the second transfer, in the reactors with ammonia addition of 2g TAN/L, the relative abundance of *Methanobacterium* decreased to 2.18% and *Methanosarcina* outcompeted *Methanobacterium* and became the most abundant hydrogenotrophic methanogen. In the reactors with no ammonia addition, total abundance of archaea recovered to be similar to the abundances in the control reactors, and relative abundance of *Methanobacterium* rebounded to be 12.41%. Besides, relative abundance of *Methanofollis*, which is not identified in the control reactors, increased to be 1.60%. Overall, for archaeal communities from reactors with ammonia addition in two transfers, *Methanobacterium* and *Methanosarcina* remained to be the relatively abundant hydrogenotrophic methanogens, which consistent with previous studies^{121, 122}, implying their resistance to ammonia. Furthermore, differentiation of analyses for *Methanobacterium* and *Methanosarcina* indicated that within the same genera, microorganisms might differentiate to adapt the environmental conditions.

It's worth noting that, the top abundant bacterial phyla in all reactors were *WWE1*, *Firmicutes*, *Chloroflexi* and *Bacteroidetes*. Members of *WWE1* were quite unique due to their remote genomes from current microbial genomes and lots of their genes were annotated with unknown functions¹²³. Proteome analysis for *Candidatus Cloacamonas* suggested their potential of being a hydrogen-producing syntrophy¹²⁴. Both *Firmicutes* and *Bacteroidetes* were frequently found to be abundant in the anaerobic digestion process. *Firmicutes* were known to be involving in hydrolyzing cellulose, lignin et al., and members in phylum *Bacteroidetes* were reported to be important fermenters and acidogens¹²⁵. As for *Chloroflexi*, it's reported to play an important role in cellulose and glucose degradation¹²⁶. Nevertheless, bacterial compositions in the genus level varied a lot. The abundances of *SHA-116* in phylum *WWE1* in the control reactors in two transfer were relatively high, and in reactors with ammonia addition, the relative abundances decreased to less than 1.00%. When ammonia was relieved in the second transfer, it didn't rebound back. Meanwhile, *W22* and *W5* from phylum *WWE1* in the reactors with ammonia addition thrived. And

SHA-116, *WW22* and *W5* were the genera affiliated to the same family *Cloacamonaceae*. Similar scenario was found for phylum *Bacteroidetes*, an unknown genus affiliated to order *Bacteroidales* was relatively abundant in control reactors. And when there was ammonia addition, an unknown genus affiliated to family *Porphyomonadaceae* thrived and relative abundance in reactors with ammonia addition of 4g TAN/L increased to 12.97%. For phylum Firmicutes, no obvious response pattern was observed similar to phyla *WWE1* and *Bacteroidetes*. However, relative abundances of *Syntrophomonas* were stable and ranged from 3.31% to 6.71% in all reactors, indicating the resilience of *Syntrophomonas* in the anaerobic digestion process with ammonia disturbance⁵⁰.

From the viewpoint of microbial diversity, there were no significant differences between Shannon indices of community from control reactors in the first and second transfers, implying the stability of community diversity over transfer for control reactors. In the first transfer, there was no significant difference between communities from control reactors and that from reactors with ammonia addition of 2g TAN/L (p value: 0.9993); difference between communities from control reactors and that from reactors with ammonia addition of 4g TAN/L was significant (p value: 0.0012), besides, difference between communities from reactors with ammonia addition of 2g TAN/L and that from reactors with ammonia addition of 4g TAN/L was also significant (p value: 0.0007), suggesting that ammonia addition decreased the microbial diversity of the anaerobic digestion process, only some of the microbiomes survived. In the second transfer, no significant difference between communities from control reactors and that from reactors with ammonia addition of 2g TAN/L was found (p value: 0.7730); however, significant differences were observed between communities from the control reactors and that from reactors with no ammonia addition (p value: 0.0042), indicating the microbial community thrived with ammonia alleviation in the second transfer. Additionally, microbial communities from control reactors in the two transfers and the reactors with no ammonia addition in the second transfer were quite close to each other compared with communities from reactors with ammonia addition, which was in agreement with former study that ammonia was a driving force for microbiome clustering¹²⁷.

5 Conclusions

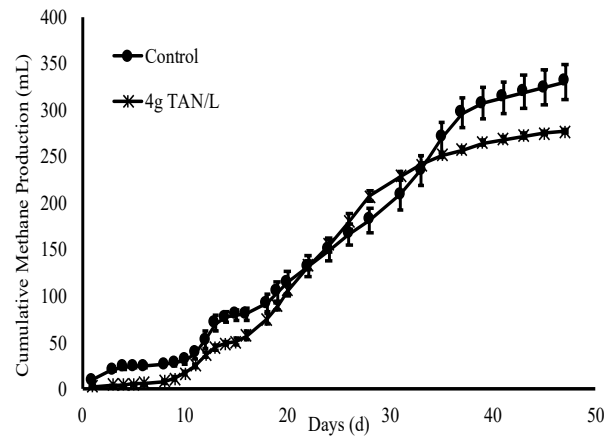
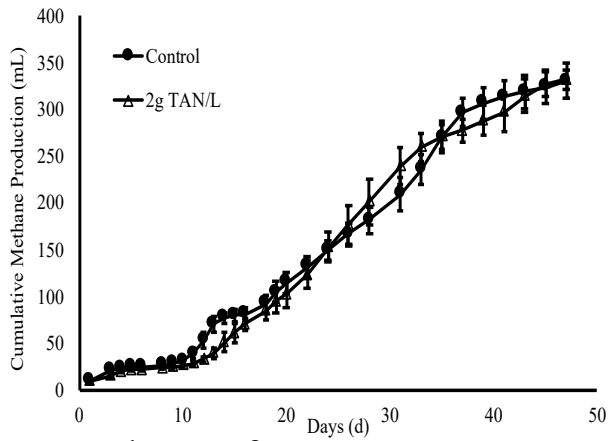
To sum up, with two transfers of anaerobic batch reactors, the microbial responses to ammonia addition and alleviation were studied. Both *Methanobacterium* and *Methanosarcina* were the resistant methanogens to the added ammonia; members of these two taxa differentiated to adapt to the elevated ammonia levels in the reactors. Methanogens, e.g. *Methanoculleus* and *Methanospirillum*, which were affiliated to order *Methanomicrobiales*, were thrived compared with others hydrogenotrophic methanogens, indicating their redundancy to the ammonia disturbance. As for the abundant bacterial groups (*WWE1*, *Firmicutes*, *Chloroflexi* and *Bacteroidetes*) in this study, they were functionally redundant.

Appendix C: Tables and Figures

Table 4-1 Results of Tukey multiple pairwise-comparisons for Shannon index

Comparison groups	P values
1st-Control VS 2nd-Control	0.0617
1st-Control VS 1st-2g TAN/L	0.9993
1st-Control VS 1st-4g TAN/L	0.0012
2nd-Control VS 2nd-2g TAN/L	0.7730
2nd-Control VS 2nd-No TAN	0.0375

First Transfer



Second Transfer

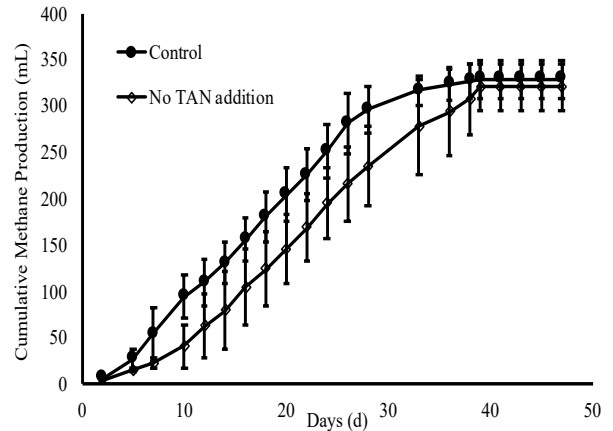
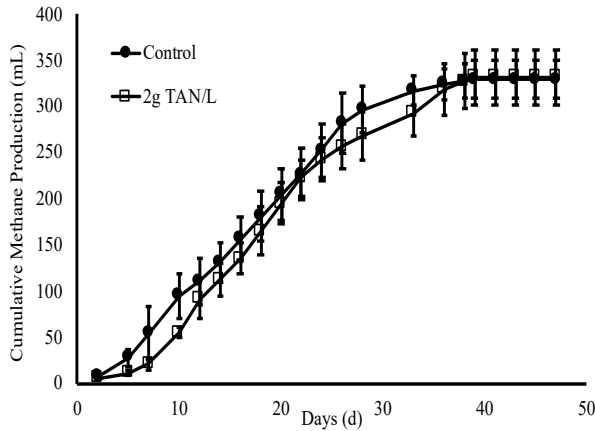


Fig. 4-1 Cumulative methane production in the anaerobic batch reactors with ammonia addition in two transfers.

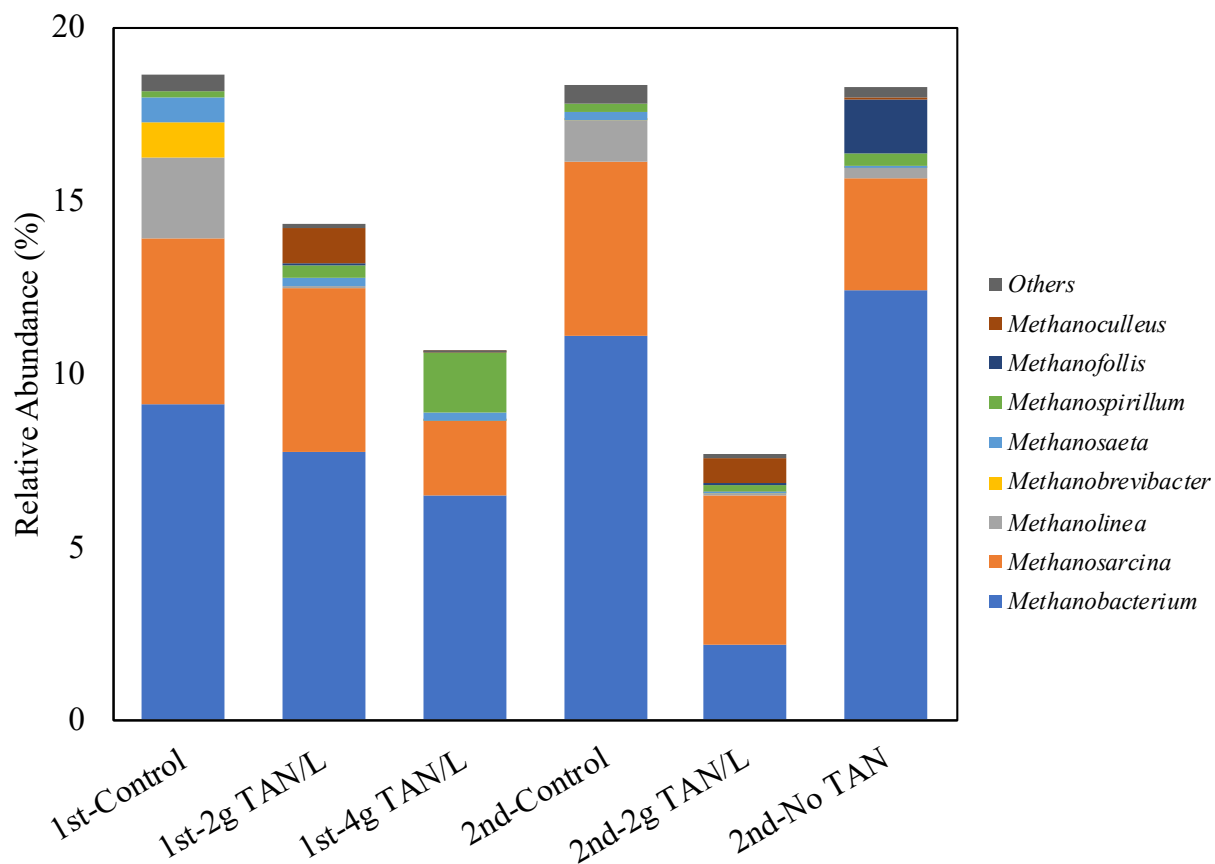


Fig. 4-2 Responses of hydrogenotrophic methanogens to ammonia addition in the anaerobic batch reactors in two transfers.

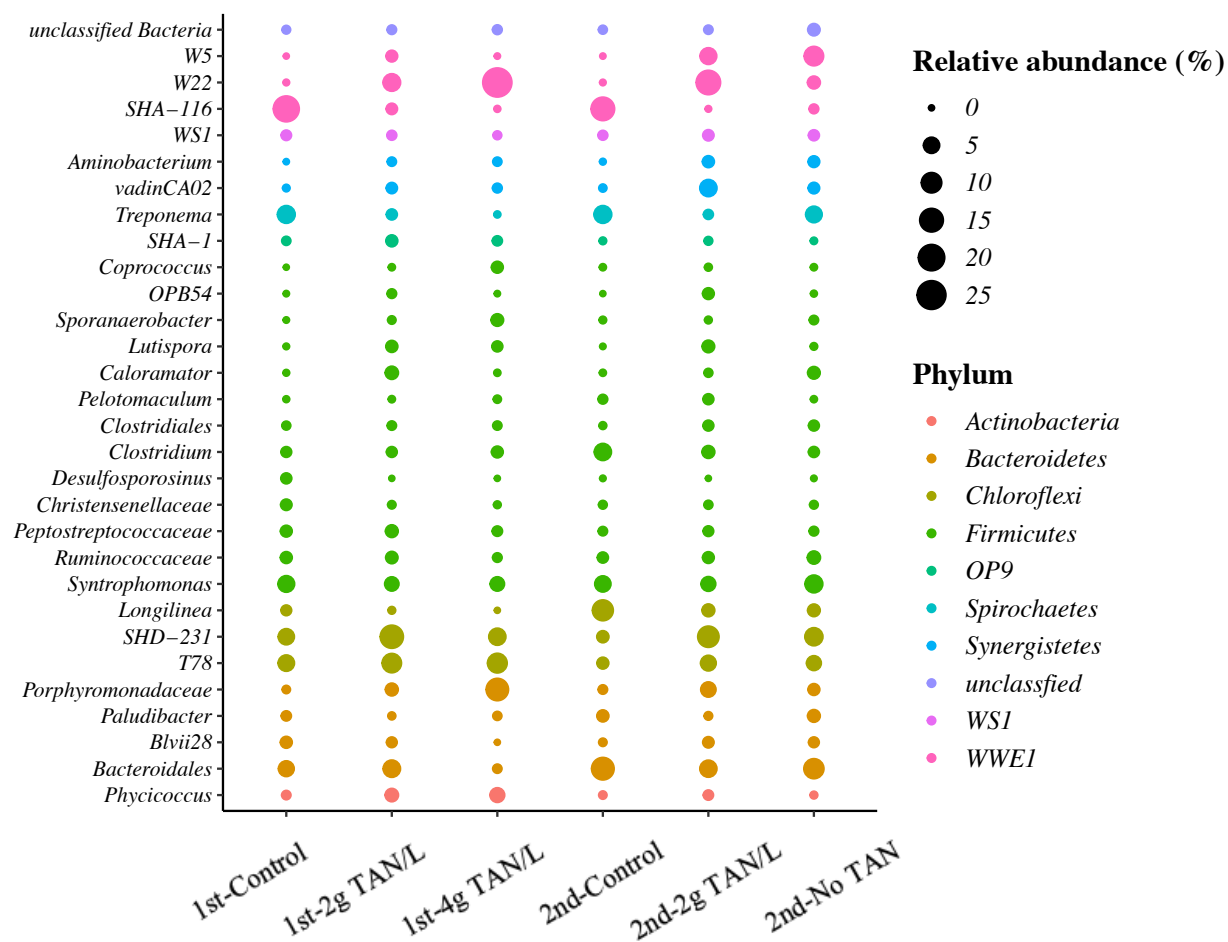


Fig. 4-3 Redundancy of bacterial communities in response to ammonia addition in the anaerobic batch reactors in two transfers.

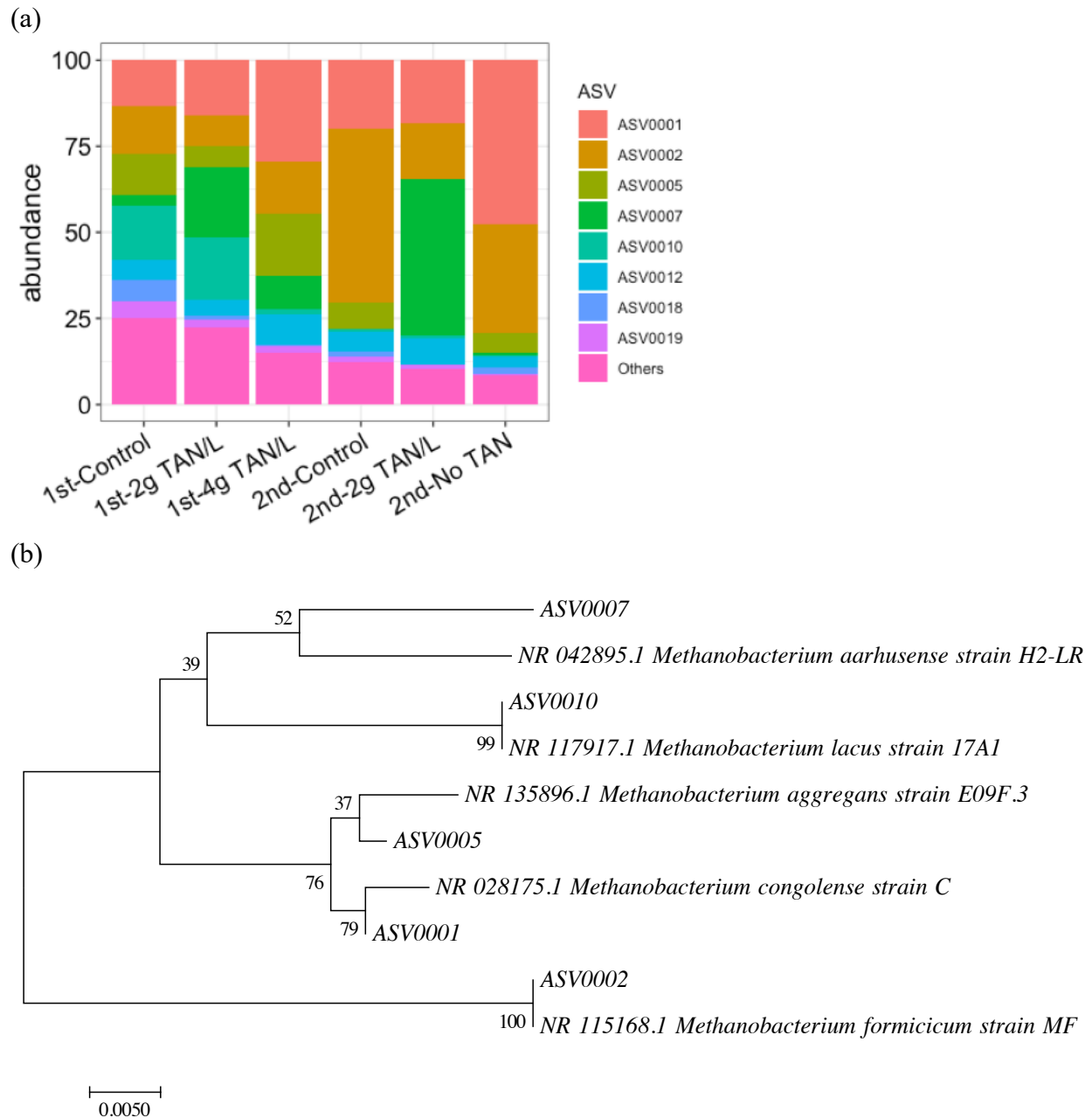


Fig. 4-4 Relative abundances (a) and phylogenetic differentiation (b) of amplicon sequences variants (ASVs) identified in genus *Methanobacterium*.

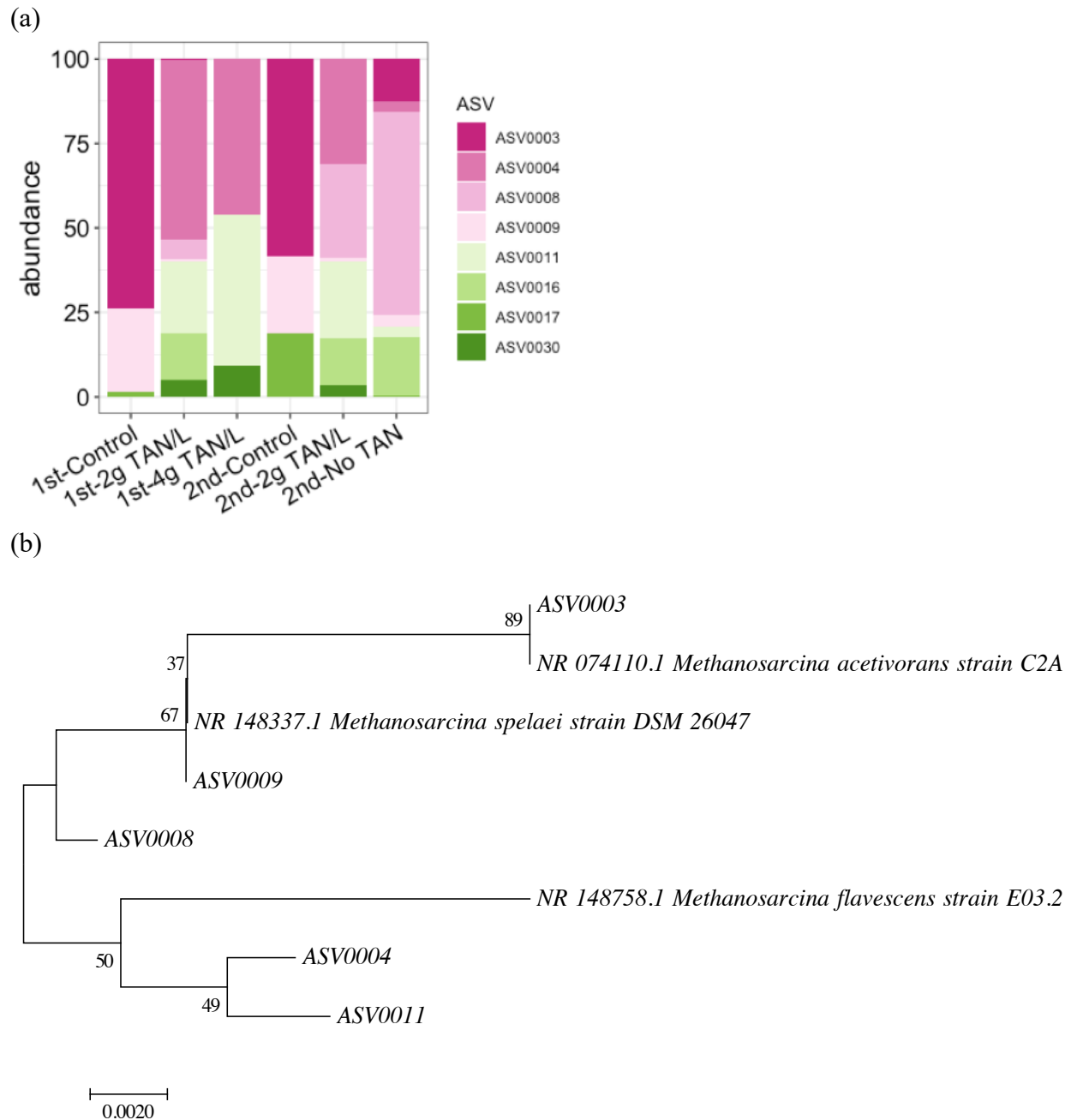


Fig. 4-5 Relative abundances (a) and phylogenetic differentiation (b) of amplicon sequences variants (ASVs) identified in genus *Methanosarcina*.

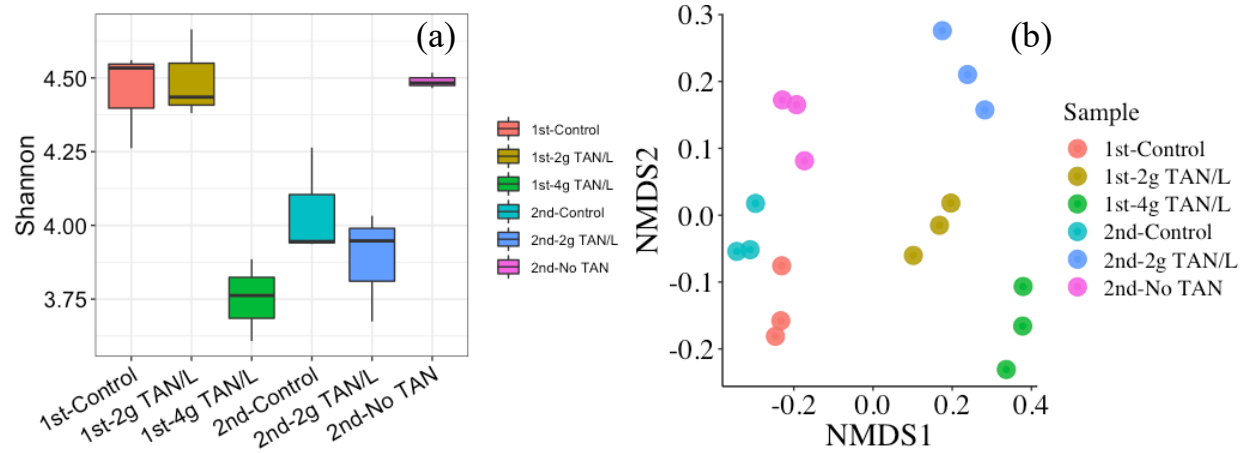


Fig. 4-6 Shannon index (a) and unweighted-unifrac distances (b) of microbial communities in the anaerobic batch reactors with ammonia addition in two transfers. Shannon indexes in (a) are means of triplicate digesters, with the error bars indicating the standard deviation.

CHAPTER V
IDENTIFICATION OF TOLERANT MICROBIAL POPULATIONS TO
ELEVATED SODIUM IN THE METHANOGENIC PROCESS

Abstract

It has been demonstrated that sodium plays a vital role in the energy-generating pathway of methanogenesis, however high levels of sodium in anaerobic digestion are reported to inhibit microbial growth and the methane production. In this study, two successive transfers of batch reactors were set up to identify the tolerant microbial populations to elevated sodium in the methanogenic process. In the first transfer, when sodium of 5g Na/L was added, no significant difference of methane production was found when compared with that in the control group; while in the reactors with sodium addition of 10g Na/L, 30% decrease of methane production was found. Methanogens affiliated to genera *Methanofollis*, *Methanospirillum* and *Methanoculleus* were found to be tolerant in the groups with elevated sodium levels. No significant differences were found in the methane productions among the three groups in the second transfer. However, predominance of *Methanosarcina* was found in the reactors with successive sodium addition of 5g Na/L, indicating the potential adaption of *Methanosarcina* to sodium in the methanogenic process. Phylogenetic analyses of the abundant ASV affiliated to tolerant *Methanosarcina* reveals it is closely related to *Methanosarcina mazei*, which has been demonstrated to be capable of adapting to NaCl concentrations of more than 30g /L. Taxonomic composition analyses of bacteria suggests that genus *Kosmotoga* in *Thermotogae* and genus *Aminobacterium* in phylum *Synergistetes* are relative tolerant to sodium, while genus *Treponema* from phylum *Spirochaetes* and this unclassified genus from order *Bacteroidales* are sensitive to sodium. Results of this study address that sodium is an important driver in shaping the microbial community and identify the functional taxa in the methanogenic process with sodium stress.

Keywords: Sodium, Microbial populations, Methanogenesis

1 Introduction

It has been testified that sodium plays an essential role in the bioenergetics of different kinds of bacteria since last century ¹. Methane production carried out by different methanogens from CO₂ + H₂, CH₃OH or CH₃COOH is proved to be strictly dependent on Na⁺ ^{2,3}. It's indicated that 230 ~ 350 mg/L of sodium is critical for the ATP formation and the oxidation of NADH in methanogens ⁴, implying the primary roles of sodium in bioenergetics. Additionally, it's demonstrated that the conversion of methyl-H₄MPT to methyl-CoM is coupled to a primary electrogenic sodium ion translocation, again revealed the important functions of sodium in the process of methanogenesis ⁵.

Nevertheless, elevated levels of sodium will slow down the growth of microorganism or even cause inhibition through dehydration. Sodium can enter the anaerobic digestion (AD) process by being added as the pH adjustment chemicals or released by the degradation of biomass ⁶. Even though cations (Na⁺, etc.) of salts in the solution must always be associated with anions, cations were found to determine the inhibition of salts to a large scale ⁷. It's demonstrated that with sodium level greater than 3.95 g Na/L in the anaerobic membrane bioreactor, methanogenesis was inhibited and methane production was decreased ⁸. Variable inhibition levels of sodium on the anaerobic digestion process were reported. With acetate as the substrate, sodium at a concentration of 10 g/L caused significant decrease of gas production and it was the second most toxic cation among the four cations (sodium, ammonium, calcium and magnesium) studied ⁷. Similar observations were also found in the study related to anaerobic co-digestion of de-sugared molasses with cow manure, 50% inhibition of methane yield occurred at sodium concentration of 11 g/L ⁹.

From the viewpoint of microbial composition, it's reported that a shift to the bacterial community with a lower richness and evenness occurred ¹⁰.

Unlike the archaeal community in the methanogenic process, bacterial communities are always considered to be more resistant and redundant to salt disturbances ¹¹. Halophiles can be found all over the bacterial phyla or even orders, and they vary widely in their physiological and metabolic properties ¹². As for the archaeal populations, halophilic microorganisms occur in three families: the *Halobacteriaceae*, the *Methanospirillaceae* and the *Methanosarcinacea*. Members of family *Halobacteriaceae* consists entirely of halophiles, while for family *Methanospirillaceae* and the *Methanosarcinacea*, both non-halophilic and organisms that can be adapted to hypersaline conditions are also involved ¹².

Within this chapter, using sodium as a stress, two successive anaerobic batch reactors were set up. Microbial community analyses were performed: 1) to find out methanogens that are tolerant to sodium; 2) to dissect the redundant bacterial community that can endure relative high level of sodium. Results of this study will guide how microbial community responses can be applied to predict the variations of environmental conditions.

2 Materials and Methods

2.1 Set up two transfers of batch reactors

For the first transfer, two experimental groups with 5g Na/L and 10g Na/L were set up by adding 70 mL wastewater sludge (with the volatile solids as 20.0g VS/L) and 10 mL inoculum from the continuous bench-scale anaerobic digesters (feedstock: wastewater sludge; Organic loading rate: 1.0 gVS/L/day) ¹³ into 160-mL serum bottles flushed with pure N₂. Then seal the bottles with butyl rubber stoppers and aluminum caps. One control group was set up without adding sodium accompanying with the experimental groups. All the groups were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 °C. After there was no more gas produced, the secondary batch of reactors for continuous sodium addition and sodium relief were established with similar method. Inoculum of the control group in the secondary transfer was from the control group in the first transfer. Inoculum of the continuous sodium addition and sodium relief were from the group of reactors with 2g TAN/L in the first transfer. Gas production and compositions were measured twice a week. At the end of each transfer, 1mL samples were collected and centrifuged at 14,000 g for 15 min, the supernatants were stored at -20 °C for volatile fatty acids analyses and the pellets were stored at -80 °C for further microbial community analyses.

2.2 Chemical Analysis

Biogas production was measured using a previously described water displacement method ¹⁴. Methane content in biogas was analyzed by a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen-1000; SigmaAldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 mL/min. GC analyses of methane used the following temperature scheme: oven 125 °C, injection port 150 °C and TCD detector 170 °C. Volatile fatty acids (VFA) in the aqueous phase were quantified using the Agilent Technologies 7890B GC system equipped with a flame ionization detector (FID) and a Agilent 122-3232 DB-FFAP column. Nitrogen was the column

carrier gas at 2.4 ml/min. The following temperature scheme was used for VFA quantification: oven 120 °C, injection port 250 °C, and detector 250 °C. Total solid and volatile solid were measured with “2540 B&E” method and pH values at the end of each transfer were measured with a pH-meter.

2.3 Microbial community analysis

Preparation of the 16S rRNA library, high throughput sequencing and the following data analyses were the same as previously described.

3 Results

3.1 Functional performance of the reactors with sodium addition over two successive transfers

In the first transfer, the average methane production of the control reactors was 341 ± 12 mL. With sodium addition of 5g Na/L, the average methane production was 333 ± 6 mL, and there is no significant difference compared with the average methane production in control reactors. Nevertheless, with sodium addition of 10g Na/L, the average methane production decreased to 234 ± 0 mL. Compared with the control reactors, the methane production was decreased by 30%. In addition, pH values at the end of the experiment in control reactors were 7.27 ± 0.05 , While in the reactors with sodium addition of 5g Na/L and 10g Na/L, the pH values were 7.13 ± 0.01 and 7.05 ± 0.01 , which was slight lower than that in control reactors. Additionally, in the second transfer, the average methane productions in three groups (2nd-Control, 2nd-5g Na/L, 2nd-no Na) were 356 ± 28 mL, 321 ± 8 mL and 330 ± 10 mL, respectively. No significant differences were found in the methane productions among the three groups in the second transfer. The pH values in these three groups were 7.25 ± 0.01 , 6.99 ± 0.01 and 7.24 ± 0.03 . Decreasing methane production in the reactors with 10g Na/L in the first transfer implied the inhibition of the sodium addition for methanogenesis.

3.2 Predominant of *Methanosarcina* with sodium addition in successive two transfers

Relative abundance of archaea in the whole community for group 1st-Control, 1st-5g Na/L, 1st-10g Na/L in the first transfer were 13.12 ± 4.07 %, 6.73 ± 1.55 % and 4.33 ± 0.30 % (Fig. 5-1), implying archaea might be more vulnerable compared with the bacterial community. In the second transfer, relative abundances of archaea for group 2nd-Control, 2nd-5g Na/L and 2nd-No Na were 15.37 ± 10.76 %, 6.40 ± 3.31 % and 6.32 ± 1.00 %. Abundant genera in the control group in first transfer (1st-Control) include *Methanobacterium* (relative abundance in Archaea is 42.77 ± 2.52 %), *Methanolinea* (relative abundance in Archaea is 26.04 ± 4.74 %) and *Methanosaeta* (relative abundance in Archaea is 12.49 ± 1.58 %). Mean values of relative abundances of the remaining archaeal genera in group 1st-Control are less than 5.00 %. In group 1st-5g Na/L, the most abundant genus in archaea is still *Methanobacterium* (relative abundance in Archaea is 32.71 ± 3.46 %), relative abundances of *Methanolinea* and *Methanosaeta* decreased to 3.69 ± 1.58 % and 2.51 ± 0.78 %. Meanwhile, relative abundances of *Methanofollis*, *Methanospirillum*, *Methanoculleus* and *Methanosarcina* increased to 15.96 ± 10.98 %, 21.53 ± 4.66 %, 12.45 ± 4.98 %

and 4.31 ± 3.27 %. However, in reactors with sodium level of 10g Na/L (1st-10g Na/L), the most abundant genus in Archaea turns to be *Methanofollis*, relative abundance of which is 55.19 ± 5.72 %, indicating the potential tolerance of *Methanofollis* to relative high levels of sodium. Additionally, relative abundance of *Methanosarcina* increased to 9.59 ± 8.21 %. Averaged relative abundances of other archaeal genera in group 1st-10g Na/L are less than 5.00 %. When it comes to the second transfer, in the control group (2nd-Control), *Methanobacterium* becomes much more predominant and the relative abundance of which is 86.13 ± 4.33 %. Relative abundances for all the other archaeal genera in group 2nd-Control are less than 5.00 %. Unexpectedly, in group 2nd-5g Na/L, with sodium addition of 5g Na/L in the second transfer, the most abundant methanogen is *Methanosarcina*, relative abundance of which is 65.04 ± 19.53 %, implying the competitiveness of *Methanosarcina* in the methanogenic process with sodium level of 5g /L. Following *Methanosarcina*, the abundant genera are *Methanofollis* (relative abundance in Archaea is 15.33 ± 10.91 %) and *Methanoculleus* (relative abundance in Archaea is 5.92 ± 5.16 %). In group with no more sodium addition in the second transfer (2nd-No Na), *Methanoculleus* becomes the most abundant one and relative abundance is 44.59 ± 16.93 %, followed by *Methanosaeta* (relative abundance in Archaea is 18.38 ± 15.99 %) and *Methanobacterium* (relative abundance in Archaea is 15.96 ± 8.56 %). Mean values of relative abundances of *Methanosarcina*, *Methanolinea* and *Methanofollis* are all less than 5.00 % (Fig. 5-2).

3.3 Phylogeny of the abundant ASVs affiliated to tolerant *Methanosarcina* and *Methanofollis*

Three ASVs (ASV0108, ASV0109 and ASV0117) were identified in group 2nd-5g Na/L. Among them, ASV0109 is predominant and percentages of it in *Methanosarcina* ranged from 84% to 100% in the triplicates in group 2nd-5g Na/L. Based on 16S rRNA, several strains (*Methanosarcina mazei* strain DSM 2053, *M. spelaei* strain MC-15, *M. barkeri* strain MS, etc.) in genus *Methnosarcina* were found to have 100% identities with ASV0109 using blastn in NCBI. Meanwhile, three ASVs (ASV0075, ASV0078 and ASV0080) were found in group 1st-10g Na/L. Percentages of ASV0075 in the triplicates ranged from 39.12% to 60.73%, for ASV0078, the range is 22.81% ~ 49.31%; for ASV0080, the range is 4.99% ~ 16.46%. Based on 16S rRNA sequences, these ASVs were found to share high identity with strains from *Methanofollis liminatans*, *M. ethanolicus* and *M. formosanus* (Fig. 5-3).

3.4 Stimulation of *Synergistetes* and *Thermotogae* in anaerobic reactors with sodium addition

Abundant bacterial phyla in the control group in both two transfers are consistent. The top six phyla in the control group in first and second transfers are *WWE1*, *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*, relative abundances of which in group 1st-Control are 17.89 ± 12.33 %, 15.97 ± 6.81 %, 13.17 ± 4.36 %, 12.58 ± 6.89 %, 12.25 ± 6.98 % and 11.70 ± 8.28 %. Relative abundances of the remaining phyla in group 1st-Control are less than 3.00 %; In group 2nd-Control, relative abundances of the top six phyla are 14.94 ± 2.29 %, 26.19 ± 6.85 %, 11.34 ± 0.75 %, 27.96 ± 3.33 %, 6.86 ± 1.48 % and 4.41 ± 0.13 %. Relative abundances of the remaining phyla in group 2nd-Control are less than 1.50%. In the reactors with sodium addition (1st-5g Na/L, 1st-10g Na/L and 2nd-5g Na/L), relative abundance of *Spirochaetes* decreased to be less than 1.00%, implying *Spirochaetes* might be sensitive taxa to sodium. When no sodium was added in the second transfer, relative abundance of *Spirochaetes* increased a little and is 2.92 ± 2.51 %. Additionally, relative abundances of phylum *WWE1* in group 1st-10g Na/L and 2nd-5g

Na/L decreased to be less than 0.10%, while in group 1st-5g Na/L, the relative abundance is $16.71 \pm 12.87\%$, indicating that phylum *WWEI* cannot endure either high level sodium or multi-transfers of low level sodium. Meanwhile, phylum *Actinobacterium*, *Synergistetes* and *Thermotogae* increased to be relatively abundant in the reactors with sodium addition. And the relative abundances of *Actinobacterium*, *Synergistetes* and *Thermotogae* in group 1st-5g Na/L are $4.86 \pm 2.39\%$, $14.60 \pm 5.56\%$ and $9.91 \pm 1.86\%$; in group 1st-10g Na/L are $5.52 \pm 1.23\%$, $3.34 \pm 0.29\%$ and $17.86 \pm 6.75\%$; in group 2nd-5g Na/L of the second transfer, are $7.07 \pm 1.02\%$, $6.03 \pm 2.32\%$ and $27.00 \pm 11.62\%$; in group 2nd-No Na of the second transfer, are $1.87 \pm 1.28\%$, $21.36 \pm 5.06\%$ and $10.63 \pm 17.59\%$. Phylum *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria* remained to be relatively abundant in all the groups. And the relative abundance of them in group 1st-5g Na/L are $14.39 \pm 3.73\%$, $9.65 \pm 3.93\%$, $11.93 \pm 2.84\%$ and $10.06 \pm 3.97\%$; in group 1st-10g Na/L are $33.29 \pm 1.98\%$, $12.53 \pm 2.41\%$, $8.12 \pm 2.78\%$ and $13.10 \pm 3.39\%$; in group 2nd-5g Na/L are $15.96 \pm 3.82\%$, $7.07 \pm 1.23\%$, $10.01 \pm 3.32\%$ and $19.31 \pm 3.55\%$; in group 2nd-No Na are $20.86 \pm 16.93\%$, $9.04 \pm 6.32\%$, $9.81 \pm 4.43\%$ and $9.16 \pm 3.66\%$ (Fig. 5-4).

Taxonomic analyses of bacterial community in genus level showed that genus in phyla *Thermotogae* that was stimulated a lot is affiliated to *Kosmotoga*, relative abundances of which is group 1st-5g Na/L, 1st-10g Na/L, 2nd-5g Na/L and 2nd-No Na are $3.33 \pm 1.82\%$, $17.65 \pm 6.77\%$, $26.95 \pm 11.65\%$ and $10.23 \pm 19.59\%$. However, relative abundances of genus *Kosmotoga* in two control groups (1st-Control and 2nd-Control) were close to 0.00%, implying the important function role of *Kosmotoga* in methanogenic process with sodium addition even with high level or successive transfers. Similar scenario was found for genus *Aminobacterium* in phylum *Synergistetes*. Relative abundance of genus *Aminobacterium* in 1st-Control and 2nd-Control are less than 0.30%, while in group 1st-5g Na/L, its abundance increases to be $14.19 \pm 5.50\%$, in group 1st-10g Na/L is $3.19 \pm 0.34\%$. In the second transfer, its abundance in group 2nd-5g Na/L is $5.87 \pm 2.39\%$, in group 2nd-No Na is $20.91 \pm 5.03\%$. Low abundances of *Aminobacterium* in group 1st-10g Na/L and 2nd-5g Na/L suggest that members of this genus might be susceptible to sodium addition. In addition, high abundance in group 2nd-No Na implies the functional redundancy of genus *Aminobacterium*. Meanwhile, Relative abundances of genus *Treponema* in 1st-Control, 2nd-Control and 2nd-No Na are $13.95 \pm 8.25\%$, $26.09 \pm 6.91\%$ and $2.85 \pm 2.47\%$, while in groups with sodium addition (1st-5g Na/L, 1st-10g Na/L, 2nd-5g Na/L), the relative abundances are less than 0.50%. Relative abundances of one unclassified genus from order Bacteroidales display the similar variation (In group 1st-Control is $10.05 \pm 6.06\%$; in 1st-5g Na/L is $3.30 \pm 0.64\%$, in group 1st-10g Na/L is $0.43 \pm 0.09\%$; in group 2nd-Control is $25.76 \pm 2.13\%$; in 2nd-5g Na/L is $0.14 \pm 0.05\%$ and in 2nd-No Na is $5.55 \pm 5.67\%$). The results indicate genus *Treponema* from phylum *Spirochaetes* and this unclassified genus from order *Bacteroidales* are sensitive genera to sodium.

4 Discussion

Methanofollis, which is the most abundant genus in the first transfer with sodium addition of 10g Na/L, is an obligately anaerobic methanogen from order *Methanomicrobiales*. It grows on H_2/CO_2 , formate, 2-propanol/ CO_2 and 2-butanol/ CO_2 . Six ASVs were identified in total within this genus, three of them (ASV0075, ASV0078 and ASV0080) were relatively abundant in the communities with sodium addition of 10g Na/L. Phylogenetic analyses based on 16S rRNA sequences indicated that they are phylogenetic close to *Methanofollis liminatans*¹⁵, *M. ethanolicus*¹⁶ and *M. formosanus*¹⁷ within genus *Methanofollis*. Optimal growth ranges of NaCl for members

of *Methanofollis* are relatively high, and the highest optimal NaCl level in *Methanofollis* species is 3% for *M. formosanus*¹⁷. Which might be the major reason enables them to be preferred and even abundant in the methanogenic process with elevated sodium.

As the versatile methanogen, members of *Methanosarcina* are believed to be diverse in morphology, physiology and functions. For example, *Methanosarcina acetivorans* is non-halophilic but can grow well in marine medium¹⁸; and predominance of *Methanosarcina* was also identified in anaerobic treatment of saline wastewater¹⁹. In this study, genus *Methanosarcina* is very minor population in the communities from control reactors in both transfer (averaged abundances of both control groups were less than 1.30%). With the increasing sodium level in the first transfer, relative abundance of *Methanosarcina* increased gradually (group 1st-5g Na/L: $4.31 \pm 3.27\%$; group 1st-10g Na/L: $9.58 \pm 8.21\%$), suggesting potential adaption process of *Methanosarcina* with salt stress. When it comes to the second transfer, with successive sodium addition of 5g Na/L, *Methanosarcina* thrived to the dominant methanogen in the community, relative abundances in the triplicates were all greater than 50.00%, implying *Methanosarcina* has been adapted to the environment with sodium of 5g Na/L and become the major methane producer. Within *Methanosarcina*, 11 ASVs were identified, most of them were very minor and distributed randomly among all the samples, except ASV0109, which is the most abundant ASV in communities from group 1st-10g Na/L and 2nd-5g Na/L. NCBI blast based on 16S rRNA sequences showed eight strains from *Methanosarcina* have 100.00% identity with ASV0109, and they are *M. mazei* strain DSM 2053²⁰, *M. spelaei* strain MC-15²¹, *M. spelaei* strain DSM 26047^{21, 22}, *M. barkeri* strain MS²³, *M. barkeri* strain DSM 800 (direct submission), *M. siciliae* strain T4/M²⁴, *M. horonobensis* HB-1=JCM 15518²⁵ and *M. soligelidi* strain SMA-21²⁶, which is also evident by the phylogenetic close relations between ASVs with *Methanosarcina* and the referenced partial sequences of 16S rRNA of *Methanosarcina* strains. It's worth mentioning that although *Methanosarcina* species listed above are not halophilic, they grow optimally in the environments with sodium of less than 4.6 g/L and pH around 7. Additionally, *Methanosarcina mazei* Göl, which was isolated from sewage sludge of wastewater treatment plant, was proved to grow well in marine salinities despite an increased lag phase²⁷. Research of genome wide expression profiling indicated that the ability of *M. mazei* Göl to adapt to high salinities through upregulations of various protective pathways including solute transport and biosynthesis, export of Na⁺, etc. and also pathways for modification of DNA and cell surface architecture^{28, 29}.

Abundant bacterial phyla in the control groups include *WWE1*, *Spirochaetes*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Proteobacteria*, suggesting their important roles in degrading complex organic matters in the methanogenic process. While in the reactors with sodium disturbance, relative abundance of *Spirochaetes* decreased to be less than 1.00%, even with sodium relief in the second transfer, the relative abundance of *Spirochaetes* didn't recover, revealing its sensitive to sodium disturbance. Genus that with relative abundance decreased a lot in *Spirochaetes* was *Treponema*, members of which are likely homoacetogens and play a key role in H₂ consumption and optimizing the performance of the anaerobic digestion system³⁰. And *Thermotogae* and *Synergistetes* were stimulated to be abundant, implying their quick response to the environmental variance and tolerance to elevated sodium in the methanogenic process³¹⁻³³. While in the second transfer, in the group of successive sodium addition of 5g/L, *Thermotogae* outcompeted the other phyla and became the top abundant phylum in the communities. Taxonomic composition analyses in the genus level showed that genus *Kosmotoga* was stimulated a lot. Although members of *Kosmotoga* reported were thermophilic, some of them, for example *Kosmotoga olearia*, can grow at temperature range of from 20 ~ 80 °C and saline level of 10~60

g NaCl/L ³². By comparing the transcriptomes of *K. olearia* grown at different temperatures, researchers found that it can adapt the mesophilic or even low temperature with strategies like increasing gene copy number by gene duplication and lateral acquisition ³⁴. As for *Synergistetes*, genus that was stimulated was *Aminobacterium*, members of which were reported to be the mesophilic amino acid degrading bacterium with high tolerance of sodium concentrations ^{33, 35}.

5 Conclusions

Abundant *Methanofollis* stimulated in the reactors with relative high sodium level in the first transfer revealed its higher tolerance to saline compared to other methanogens. When it comes to the second transfer, *Methanosarcina* outcompeted other methanogens and became the predominant methanogen in the reactors with successive sodium addition of 5g Na/L, indicating its adaption to the elevated sodium. Although bacterial communities are reported to more resistant than archaea, genus *Treponema* from phylum *Spirochaetes* and one unclassified *Bacteroidales* genus from phylum *Bacteroidetes* are showed to be sensitive to sodium addition. Meanwhile, *Kosmotoga* population from *Thermotogae* is tolerant to sodium and is thrived with sodium addition; genus *Aminobacterium* from phylum *Synergistetes* seems to be the redundant populations when there was a disturbance occurred in the methanogenic process.

Appendix D: Tables and Figures

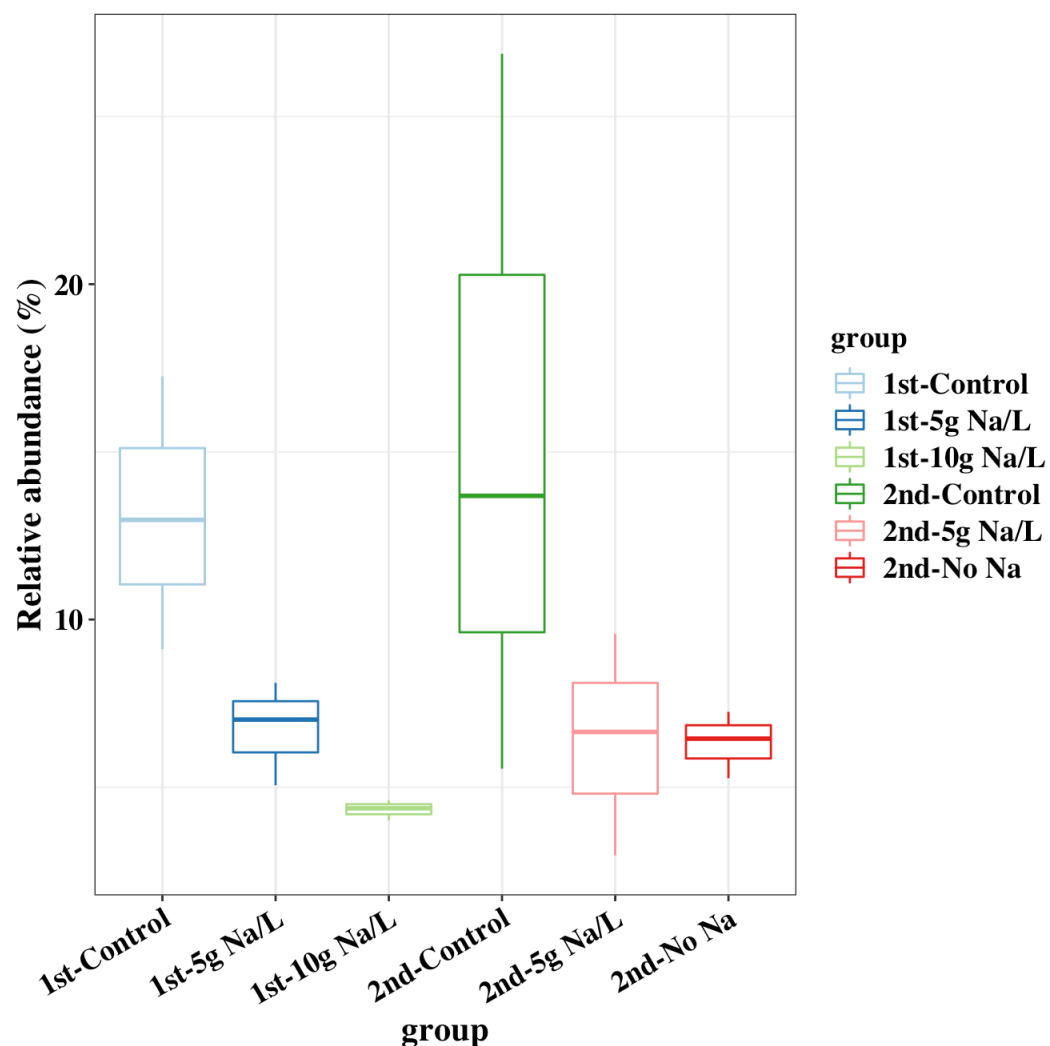


Fig. 5-1 Relative abundances of archaea in the whole microbial community in anaerobic batch reactors with two successive sodium reactors indicated that archaea are relative susceptible compared with the bacteria. The whisker for each box goes from the minimum value to the maximum value, while the box extends from quartile 1 to quartile 3 with a middle vertical line at the median value.

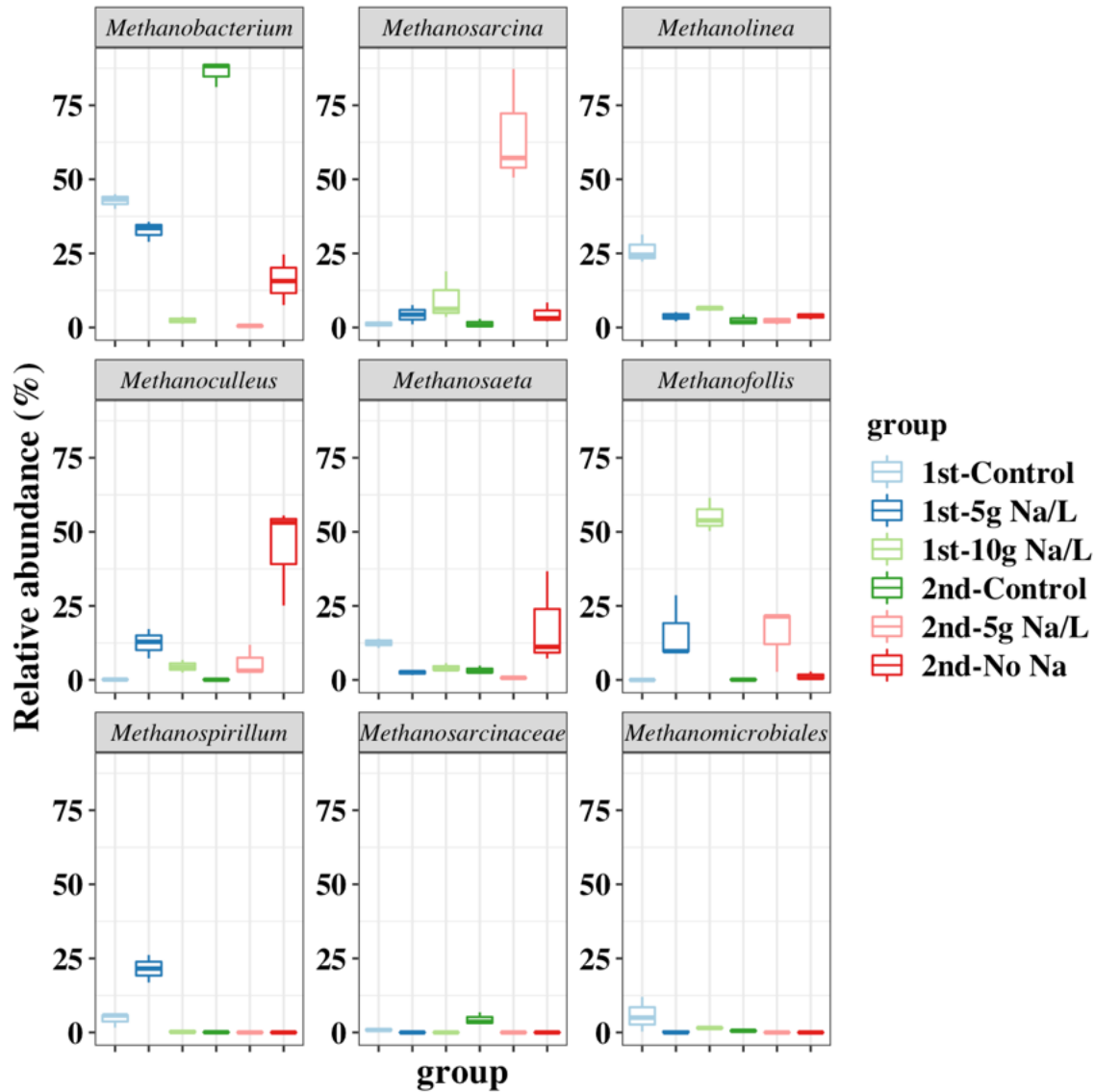


Fig. 5-2 Methanogens that are relatively abundant identified in the microbial community implied *Methanosarcina* and *Methanofollis* are tolerant methanogens in the methanogenic process with elevated sodium. The whisker for each box goes from the minimum value to the maximum value, while the box extends from quartile 1 to quartile 3 with a middle vertical line at the median value.

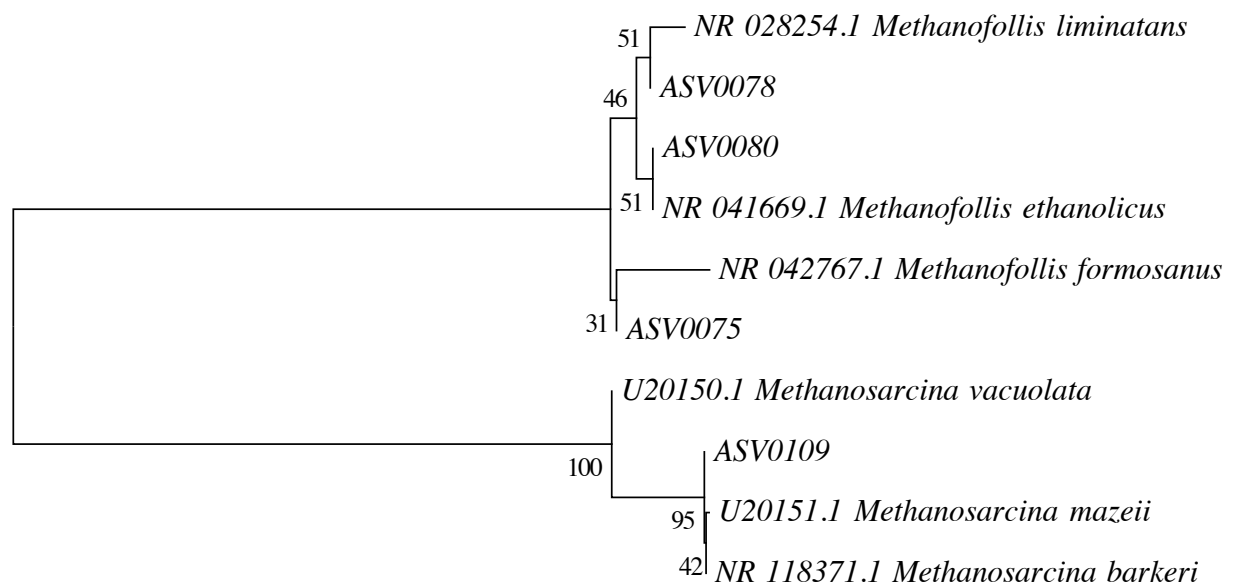


Fig. 5-3 16S rRNA-based phylogeny for abundant ASVs affiliated to tolerant *Methanosarcina* and *Methanofollis*.

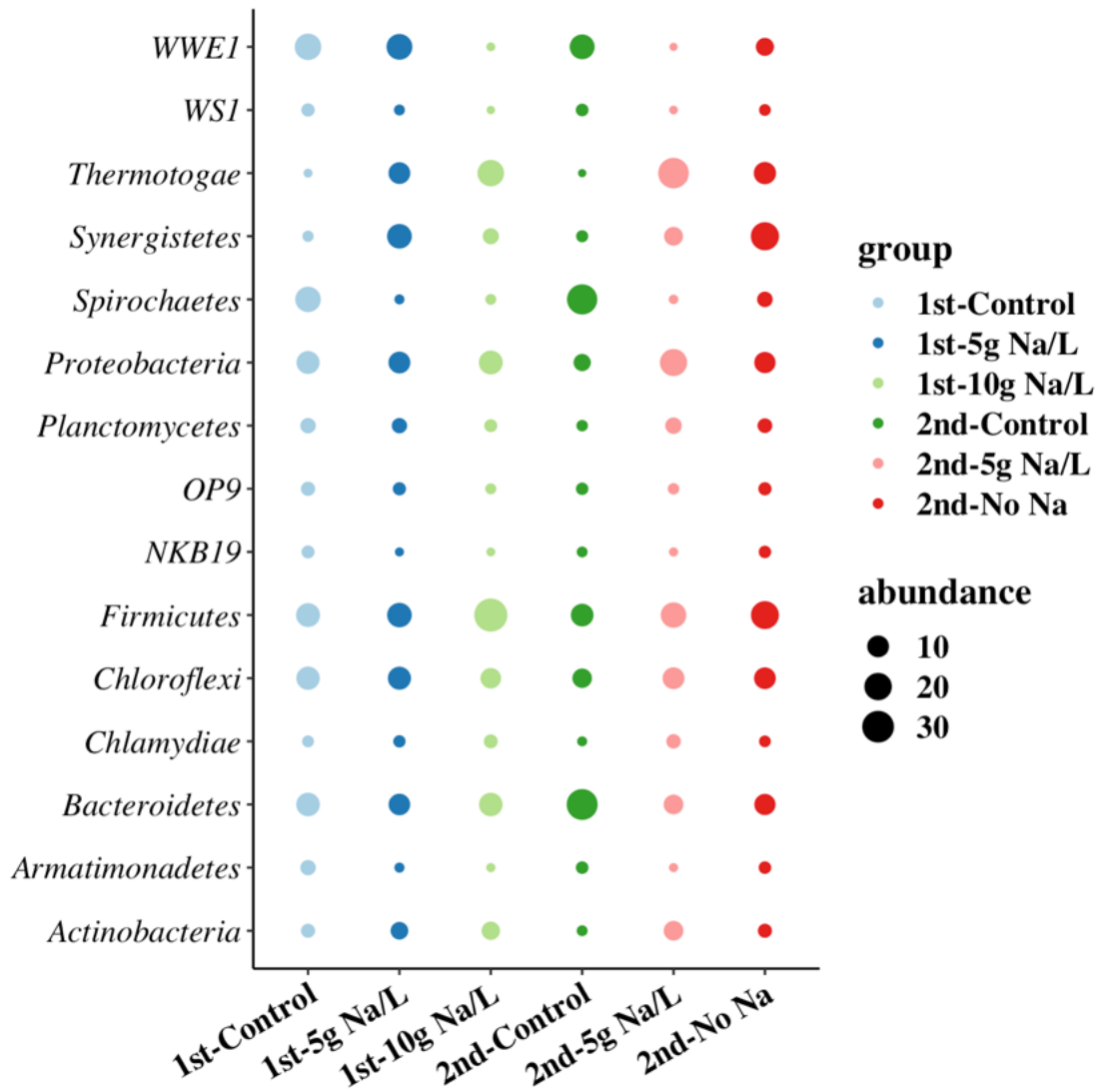


Fig. 5-4 Bacterial phyla composition in the anaerobic batch reactors with elevated sodium showed members of phylum *Thermotogae* and *Synergistetes* were stimulated by sodium addition while members of phylum *Spirochaetes* were inhibited.

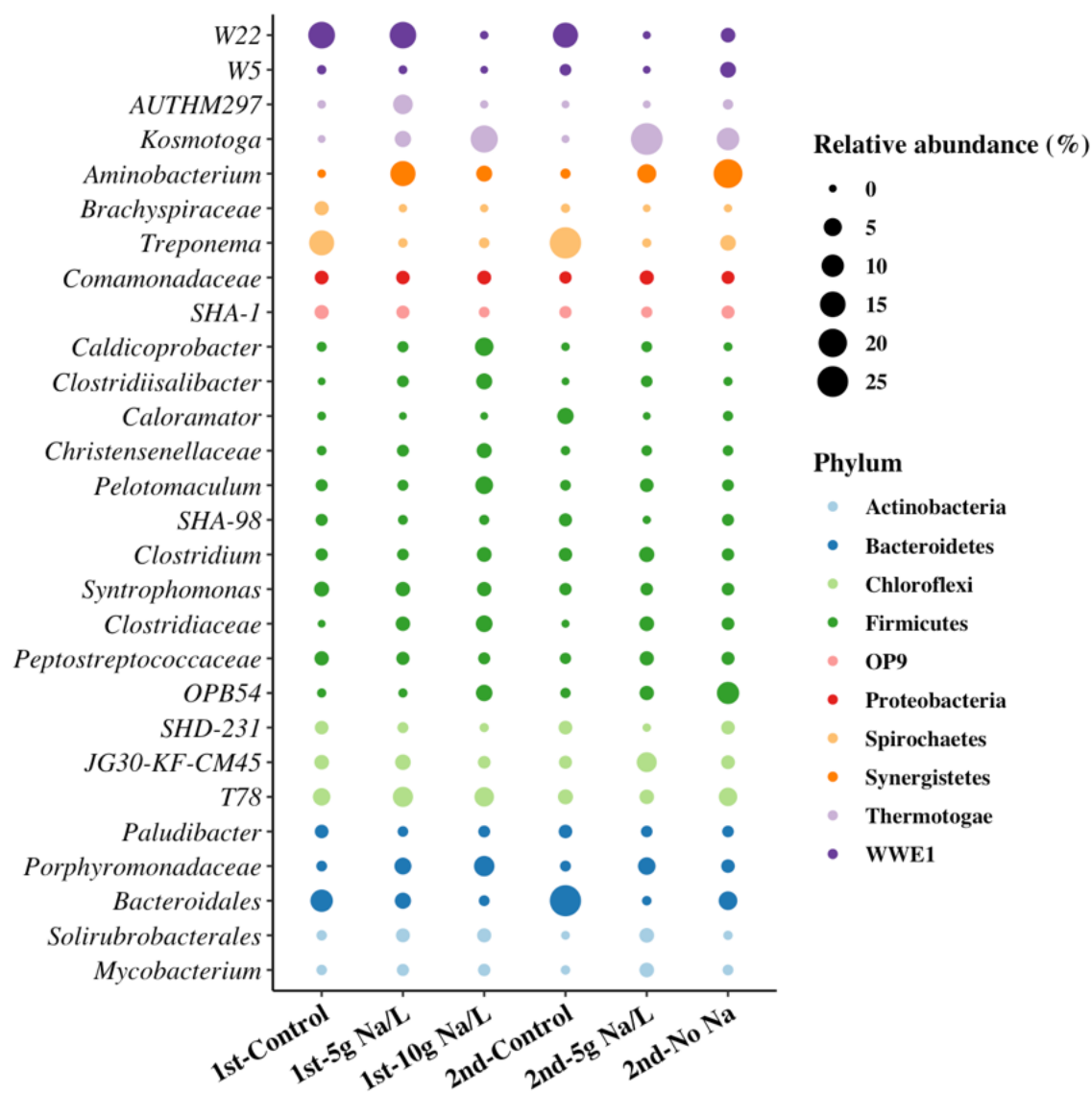


Fig. 5-5 Taxonomic composition of abundant bacteria dissected in a finer genus level showed that the tolerant genera bacteria were *Kosmotoga* affiliated to *Thermotogae* and *Aminobacterium* affiliated to *Synergistetes*, while genus *Treponema* from *Spirochaetes* and one unclassified genus from order *Bacteroidales* were susceptible to elevated sodium.

CHAPTER VI
CONSISTENCY OF FUNCTIONS ASSOCIATED WITH
METHANOGENESIS IN METHANOSAETA GENOMES RECOVERED
FROM A METHANOGENIC PROCESS

Abstract

Methanosarcina and *Methanosaeta* are the two methanogens that produce methane using acetate as the substrate. Unlike *Methanosarcina*, *Methanosaeta* was the obligately acetoclastic methanogen and was always found to be predominant in the methanogenic process. Meanwhile, differentiation within genus *Methanosaeta* was identified in the same methanogenic system. Existence of the functional discrepancy in acetate-related activities among these *Methanosaeta* genomes needs to be uncovered. In this study, four *Methanosaeta* genomes were recovered from the methanogenic process in our lab and the phylogenetic analysis of these recovered genomes indicated that Bin021 and Bin225 may affiliate with *M. concilii*, Bin025 and Bin123 were phylogenetically close to *M. harundinacea*. In detail, presences of genes associated with acetate transportation, acetoclastic methanogenesis and cobalamin biosynthesis were investigated to dissect potential differences of acetate-related functions in genomic level. Furthermore, orders and organizations of gene operons were also included. The results suggested that acetate transporter gene *ady2* was present in all the four recovered genomes and the two referenced genomes included in this study. Organizations of *ady2* in Bin021 and Bin225 were the same as that in *M. concilii*; while organization of *ady2* in Bin025 was the same as that in *M. harundinacea*, an unknown gene with a length of 272 bp was found to be inserted in Bin123. As for the acetoclastic methanogenesis pathway, most of the genes required were present in the recovered genomes, except some subunits of *cdh*, *mtr*, *hdr*, etc. Genomic organization analysis of these genes showed that the missing subunits were at the beginning or end of the respective contigs, implying the absences of them may due to the sequencing process. In addition, presence of the genes associated with cobalamin biosynthesis in all the *Methanosaeta* genomes was identical except in Bin021, in which, *cycG* might be lost due to sequencing process. Orders of genes (subunits of gene *cbi*) that were related to the corrin ring biosynthesis were the same among the *Methanosaeta* genomes included. High similarity of the presence and organizations for genes associated with acetate activities indicated the potential of functional consistency in methanogenesis for these *Methanosaeta* genomes. Results of this study provide the evidence that the consistent methanogenic functions of these *Methanosaeta* populations in the same system, which broadens our knowledge of the methanogenic populations in anaerobic food web in a finer genomic level.

Keywords: *Methanosaeta*; comparative genomics; methanogenesis; cobalamin biosynthesis

1 Introduction

As the obligate acetoclastic methanogen, strains from genus *Methanosaeta* can only use acetate as the source of energy. Due to the lower maximum specific growth rates ($\mu_{\max}$) and half saturation concentrations (K_s) for growth on acetate, *Methanosaeta* populations are usually considered to be more competitive and abundant in anaerobic digestion with low acetate level compared with the other acetoclastic methanogen *Methanosarcina*^{59, 60}. Nevertheless, dominance of *Methanosaeta* was found unexpected at elevated acetate level in an previous animal wastewater treatment⁴², and the observation was consistent in related studies^{132, 133}. Combined observations of *Methanosaeta* populations at both low and elevated acetate levels imply the importance of divergent growth, genetic and physiological differences within the *Methanosaeta* populations. Genomes sequences of methanogens help microbiologists answer many questions, while many others are inspired. For *Methanosaeta* species, there were three complete genomes published from

NCBI database so far, *Methanosaeta thermophila*, *Methanosaeta harundinacea*⁴⁴ and *Methanosaeta concilii*¹³⁴.

For acetate utilization in the *Methanosaeta*, a transport protein is necessary, since acetate (pKa=4.75 at 25 °C) is dissociated in the environmental conditions with neutral pH, which is usually the optimal conditions for the growth of *Methanosaeta* strains⁴⁴. As indicated in the previous study, the *Ady2* gene, which was identified as an active acetate transporter in *Saccharomyces cerevisiae*, was present in the published *Methanosaeta* genomes^{44, 135}. After entering into the *Methanosaeta* cell, acetate activation to acetyl-coenzyme A, which is the first step of acetoclastic methanogenesis, is believed to be the rate-limiting step^{136, 137}. And it's reported that the routes of acetate activation differed in *Methanosaeta* and *Methanosarcina* strains. In *Methanosaeta* strains, acetate activation is catalyzed by AMP-forming acetyl-CoA synthetase, while in *Methanosarcina*, the enzyme system is an acetate kinase/phosphotransacetylase system¹³⁸. It's indicated that same enzymes are utilized in the remaining steps in the acetoclastic methanogenesis for both *Methanosaeta* and *Methanosarcina*, the most characteristic and complex one is carbon monoxide dehydrogenase. Moreover, the final step of methanogenesis is the reduction of methyl-coenzyme M, which is catalyzed by methyl-coenzyme M methylreductase. In addition of processes like acetate transportation and methanogenesis, presence of cofactors, i.e. cobalamin, are important factors, which have potential influences on the utilization of acetate. Cobalamin (vitamin B12) is not only the essential cofactor of microbial growth, but also involved in the several points in the methanogenesis pathway. It's verified that methyl group from methanol to intermediate coenzyme M (HS-CoM) appears to be catalyzed by two enzymes, and one of them contains a cobalamin¹³⁹. In the pathway converting acetate to methane, the presence of cobalamin is thought to be related to the splitting of the C-C bond of acetate in *Methanosarcina thermophila*¹⁴⁰, the transfer of methyl group in *Methanosarcina barkeri*¹⁴¹, etc.

In order to uncover the differentiation of *Methanosaeta* population in the methanogenic process and the potential influences of differentiation on the microbial functions, four almost complete genomes of *Methanosaeta* were recovered from the continuous digesters and acetate enrichment in our lab with metagenomic high throughput sequencing. Phylogenetic and comparative genomic analyses were performed to reveal: 1) phylogenetic differentiation of genomes recovered and the referenced genomes; 2) consistency of functions associated with acetate activities among the *Methanosaeta* genomes. With these findings, we demonstrated that the consistency of functions of *Methanosaeta* in acetoclastic methanogenesis even with phylogenetic differentiation, which further broaden our knowledge of the functional role of *Methanosaeta* in the anaerobic food web in a finer genomic level.

2 Materials and Methods

2.1 Sample collection and metagenome shotgun sequencing

Samples of this study were from six anaerobic digesters, including triplicate control (labeled as C) and triplicate co-digestion digesters (labeled as D), which were set up prior to this study^{54, 55}. Substrate for the digesters were dairy manure collecting from the waste storage basin of a 600-head dairy farm in London County, Tennessee, USA. Over the past several decades, these three control digesters were operated under the following invariable conditions: at temperature 35 °C, a working volume of 3.6 L and a hydraulic retention time of 20 days. Samples were collected

and stored at -80 °C as soon as possible after taken. The DNA for metagenomic sequencing were extracted following the steps in the previous study ¹⁴².

2.2 Sequencing data analysis

More than 44Gb paired-end reads for each sample, with 1 billion raw reads in 6 samples representing two digesters respectively in FASTQ format are quality filtered in *Trimmomatic* v0.36 ¹⁴³ with sliding window size of 4, sliding window minimum quality of 15, minimum read length was set at 36, trailing minimum quality at 3, leading minimum quality at 3. As a result, 87% and 92% of the raw reads remained high-quality reads. (2) For achieving a better coverage, reads from 6 samples (duplicates in triplicates digesters) in type C and 6 samples in type D were respectively assembled. De novo co-assembly was performed respectively on two types of digester samples by *MEGAHIT* ¹⁴⁴, with a minimum k-mer varies from 1 to 127, maximum k-mer varies between 1 and 255, k-step between 1 and 28. For ensuring a reasonable length for open reading frames (ORF) prediction, all contigs with a length greater than 2000bp were remained, with 207,652 and 230,337 contigs in C and D respectively. For assembled contigs, N50 values are 7,204 and 7,715 for C and D; N75 values are 3,516 and 3,631 for C and D. Contigs length greater than 2000 bp reach the total length of more than 1 billion for each digestion type. GC% contents of the assembly are 51.19% and 50.68% respectively in C and D digester. For the complexity of the anaerobic animal waste digestion samples, the percentage of reads that could be assembled into long contigs suggest a relative higher assemble rate than other research. (3) ORFs were predicted from contigs using *Prodigal* ¹⁴⁵. (6) Taxonomy assignment of the ORFs was performed in *Kaiju* ¹⁴⁶. NCBI bacteria and archaea protein genomes were downloaded as the reference database. LCA (lowest common ancestors) algorithm was used in the taxonomy assignment. The running parameters were set with a minimum match length of 11 and a minimum match score of 65 under *mem* mode. Three draft genomes for *Methanosaeta* (Bin021 and Bin123 in C, Bin225 in D) were assembled from the metagenomic data for the six continuous anaerobic digesters. As an addition, another *Methanosaeta* genome (Bin025) recovered from acetate enrichment was also included.

2.3 Function annotation and phylogenetic analysis

Two referenced genomes of *Methanosaeta* (*Methanosaeta Concilii* and *Methanosaeta Harundinacea*) downloaded from NCBI database were also involved to illustrate the genomic differences between the recovered genomes and the referenced genomes. Firstly, app “NCBI Prok” in MiGA platform¹⁴⁷ (<http://microbial-genomes.org/>) was applied to obtain the protein sets for all the genomes. Secondly, the protein sets were searched against COG database (<https://www.ncbi.nlm.nih.gov/COG/>) to obtain the gene numbers in each functional category among the six genomes. In addition, functions were also annotated with GhostKOALA against Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.genome.jp/kegg/>) database for further functional pathway analysis. Thirdly, pangenomic and phylogenomic analyses were performed with anvi'o workflow in Meren lab ^{148, 149}. Program “anvi-pan-genome” was used to run the actual pangenomic analysis. In which, function “--use-ncbi-blast” was selected to use NCBI's blastp for amino acid sequence similarity search; the minbit score was set as default 0.5; MCL inflation parameter was set as 10 based on the suggestions for comparing very closely related genomes in the Meren Lab. Average nucleotide identity (ANI) scores compared with the two referenced genomes were also computed. After the analysis was done, program “anvi-display-pan”

was used to display the pangenome. Both ANI scores and KEGG annotations for the gene clusters were also visualized.

2.4 Characteristics of genes associated with acetate activities in *Methanosaeta*

Presence of gene (*ady2*) associated with acetate transporter was identified. Genes related to acetoclastic methanogenesis and cobalamin biosynthesis were identified based on referenced pathways in KEGG database. Organizations of genes involved in the acetate activities in the all the genomes were visualized using package “gggenes” in Rstudio.

3 Results

3.1 General features of *Methanosaeta* genomes

The lengths for Bin021, Bin025, Bin123 and Bin225 were 2.12 Mbp, 2.37 Mbp, 2.30 Mbp and 2.49 Mbp, respectively (Table 6-1). The completeness of these four recovered *Methanosaeta* genomes ranged from 92.30% to 99.35%, and the contamination was below 0.65% for them. Pangenomic analyses of *Methanosaeta* genomes were performed to explore the genomic differentiation among these *Methanosaeta* genomes using tools in anvi'o (Fig. 6-1). Values of ANI calculated against *M. concilii* using PyANI for Bin021, Bin025, Bin123 and Bin225 were 99.16%, 70.96%, 70.71%, 99.02%. Since a cutoff score of greater than 95% indicates the same species affiliation, Bin021 and Bin225 may affiliate with *M. concilii*. Similarly, ANI values calculated against *M. harundinacea* for Bin021, Bin025, Bin123 and Bin225 were 70.83%, 98.24%, 87.51%, 70.68%, indicating Bin025 may affiliate with *M. harundinacea*, while Bin123 was close to *M. harundinacea* when compared with *M. concilii*. In consistent, G+C contents for Bin021 (52.80%) and Bin225 (52.47%) were similar to that of *M. concilii* (51.03%); G+C contents for Bin025 (61.17%) and Bin123 (59.95%) were similar to that of *M. harundinacea* (60.60%). Numbers of proteins predicted for Bin025 (2,361) and Bin123 (2,311) were similar with that of *M. harundinacea* (2,371); however, numbers of proteins predicted for Bin021 (2,250) and Bin225 (2,598) were a little bit less than that of *M. concilii* (2,878). Coding densities for the recovered *Methanosaeta* genomes were 86.45% ~ 86.97%, greater than that of the referenced genomes (*M. concilii*: 83.59%; *M. harundinacea*: 85.95%), which may due to the incompleteness of the recovered genomes. Gene counts in each functional category compared against the COG database were summarized (Fig. 6-2). Besides functions unknown and general function prediction only, function categories that include lots of gene counts in the six *Methanosaeta* genomes were translation, ribosomal structure and biogenesis, coenzyme transport and metabolism and energy production and conversion, etc, which indicates they contain similar gene counts number in the basic functional categories as the mesophilic methanogen.

3.2 Genes involved in acetate activities in *Methanosaeta* genomes

There are two forms of acetate present in the liquid environment: undissociated and dissociated form. Percentages of these two forms are depending on both pH and temperature. Undissociated acetate can diffuse across the cytoplasmic membrane of bacteria freely¹⁵⁰. While for dissociate acetate, the dominant form in neutral environment, a transporter protein is required. Most *Methanosaeta* strains grow optimally in the environment with neutral pH, implying acetate

transporter is necessary. Gene *ady2* (K07034) and *fps1* (K00787) was identified to be involved in acetate transport based on the previous studies^{135, 151}. Gene *ady2* was found to be present in *M. concilii* and *M. harundinacea*⁴⁴, while *fps1* was not. Consistently, *ady2* was present and *fps1* was absent in all the four recovered genomes in this study. Organizations of *ady2* in Bin021 and Bin225 were the same as that in *M. concilii*; while organization of *ady2* in Bin025 was the same as that in *M. harundinacea*, an unknown gene with a length of 272 bp was found to be inserted in Bin123 (Fig. 6-3).

Simplified pathway for acetoclastic methanogenesis was extracted based on KEGG module M00357 (Fig. 6-4). Acetate activation is believed to be the first step of acetoclastic methanogenesis. Unlike *Methanosarcina* species, acetate activation is accomplished using AMP-forming acetyl-CoA synthetase (ACS) in *Methanosaeta*. Acetyl-CoA produced is converted to CH₃-H₄MPT with acetyl-CoA decarbonylase (*cdh*). The following steps are identical for three methanogenesis pathways, 1) methyl group in CH₃-H₄MPT is transferred to coenzyme M (CoM) and form methyl-CoM with enzymes of *mtr*; 2) CoB-S-S-CoM and methane are formed from methyl-CoM and CoB through enzymes of *mcr*; 3) CoM and CoB were reproduced from the reduction of CoM-S-S-CoB by the heterodisulfide reductase (i.e. *hdrABCDE*). Most of the genes required for acetoclastic methanogenesis pathways were present in the recovered genomes, except some subunits of gene *cdh*, *mtr*, *hdr*, etc. Genomic organization analysis of these genes showed that the missing subunits were at the beginning or end of the respective contigs, implying the absences of them may due to the sequencing process (Figs. 6-4~7). High similarity of the presence and organizations for genes associated with acetate activities indicated the potential of functional consistency for these *Methanosaeta* genomes.

3.3 Genes associated with biosynthesis of cobalamin in the *Methanosaeta* genomes

Even though methanogenesis is the final step for methane production, complicated reactors and factors influenced the process of methanogenesis. High levels of cobalamins found in *Methanosarcina barkeri* implied the important metabolic role of cobalamin in the methanogenic process¹⁵². Additionally, large numbers of B12-dependent methyltransferases including *mta*, *mtm*, *mtr*, etc., were present in methanogens, which are clearly important in several pathways for them¹⁵³. There are two ways for both bacteria and archaea to obtain cobalamin: 1) de nova pathway; 2) salvage pathway. It's demonstrated that only about half of the cobalamin-requiring bacteria have the complete genes encoding biosynthesis of cobalamin^{153, 154}. Pathways of cobalamin biosynthesis and its metabolic regulation were complex. A simplified anaerobic biosynthesis pathway was summarized as Fig. 8 based on KEGG database and relative reference¹⁵⁵. Presence of the genes associated with cobalamin biosynthesis in all the *Methanosaeta* genomes was identical except in Bin021, in which, *cycG* might be lost due to sequencing process (Table 6-3). Orders of genes (subunits of gene *cbi*) that were related to the corrin ring biosynthesis were also the same (Fig. 6-9).

4 Discussion

Genus *Methanosaeta*, previously described as *Methanothrix*, include the rod-shaped, flat-ended and sheathed methanogenic populations that are using acetate as the sole energy source¹⁵⁶. Type species in genus *Methanosaeta* include *M. thermophila* (*Methanothrix thermoacetophila*)¹⁵⁷, *M. concilii*¹⁵⁶ and *M. harundinacea*⁴⁴. Among them, *M. thermoacetophila* was found popular in

thermophilic condition while *M. concilii* and *M. harundinacea* were always in mesophilic environments. In this study, temperature of the anaerobic digestion process was around 35 °C, implying the existence of mesophilic methanogens in the process. In consistent with that, *Methanosaeta* genomes recovered from the anaerobic digestion process were closely related to *M. concilii* and *M. harundinacea*. Comparative genomic analyses of these three *Methanosaeta* species indicate that two mesophilic *Methanosaeta* (*M. concilii* and *M. harundinacea*) have similar genome size, coding density, similar gene numbers in each function category and exhibited considerable differences from that of *M. thermoacetophila*¹¹⁶. Consistent with the previous study, all the recovered genomes in this study, coding density of them are all greater than 80% and gene counts compared against COG database in each functional category showed the quite similar patterns. Besides unknown function and general function prediction only, functional categories with more gene counts were associated with translation, ribosomal structure and biogenesis; inorganic ion transport and metabolism; coenzyme transport and metabolism; energy production and conversion, etc.

Even though methanogenesis is the last step for methane production, our knowledge of methanogenesis process is still uncomplete for one reason or another, like the complex electron transfer, different formats of energy conservation in the associated microbial community. Genera *Methanosaeta* and *Methanosarcina* were always compared to reveal their differences in acetate-utilizing activities, the results indicated that *Methanosaeta* preferred to lower acetate level and they employ different enzymes to produce acetyl-CoA in the first step of methanogenesis^{158, 159}. Following study validated further that striking differences were discovered in electron transfer and energy conservation among these two acetate-utilizing methanogenic genera¹⁶⁰. Nevertheless, genomic comparison within genus *Methanosaeta*, especially the two mesophilic *Methanosaeta*, was still limited. It's suggested that nearly 50% of the predicted proteins in *M. concilii* are not shared with *M. thermophila*, indicating the potential gene activities like lateral gene transfer and gene duplication occurred in these genomes¹³⁴. Based on morphological, physiological and phylogenetic data, *M. harundinacea* was proposed to be novel specie in genus *Methanosaeta* which is slightly different from *M. concilii* in growth conditions¹⁶¹. Both species affiliated with *M. concilii* and *M. thermophila* were found in our anaerobic digestion process, implying their wide occurrence and important roles in the methanogenesis.

In this study, organizations of genes associated with acetoclastic methanogenesis were further identified with reference to gene database extracted from KEGG. From the perspective of gene presence and organization, no significant differences were found among these *Methanosaeta* genomes.

5 Conclusions

Among the four recovered genomes, Bin021 and Bin225 are affiliated with *M. concilii*, Bin025 and Bin123 were phylogenetically close to *M. harundianacea*. Presence and organizations of genes associated with acetate transporter, acetoclastic methanogenesis and relative cobalamin biosynthesis in the recovered genomes were also similar to that in the referenced *Methanosaeta* genomes.

Appendix E: Tables and Figures

Table 6-1 Summary of four *Methanosaeta* MAGs recovered from a methanogenic process and two related referenced genomes

	Bin021	Bin225	Bin025	Bin123	<i>Mst. concilii</i>	<i>Mst. harundinacea</i>
Contigs	159	169	80	78	2	2
% Completeness	92.30	99.35	99.34	98.68	100	100
% Contamination	0.00	0.65	0.00	0.00	0.00	0.00
Length (bp)	2,122,832	2,488,729	2,373,981	2,298,223	3,008,626	2,571,034
G+C content (%)	52.80	52.47	61.17	59.95	51.03	60.60
Predicted proteins	2250	2598	2361	2311	2878	2371
Coding density (%)	86.97	86.81	86.79	86.45	83.59	85.95

Table 6-2 Presence of genes associated with acetoclastic methanogenesis in *Methanosaeta* genomes (*Mst*: *Methanosaeta*; +: presence; --: absence)

Gene	Bin021	Bin225	Bin025	Bin123	<i>M. concilii</i>	<i>M. harundinacea</i>
acs	+	+	+	+	+	+
cdhC	+	+	--	--	+	+
cdhE	+	+	+	+	+	+
cdhD	+	+	+	+	+	+
mtrA	+	+	+	+	+	+
mtrB	+	+	+	+	+	+
mtrC	+	+	+	+	+	+
mtrD	+	+	+	+	+	+
mtrE	--	+	+	+	+	+
mtrF	+	+	+	+	+	+
mtrG	+	+	+	+	+	+
mtrH	+	+	+	+	+	+
mcrA	+	+	+	+	+	+
mcrB	+	+	+	+	+	+
mcrG	+	+	+	+	+	+
hdrA2	+	+	+	+	+	+
hdrB2	+	+	+	+	+	+
hdrC2	+	+	+	+	+	+
hdrD	+	+	--	--	+	+
hdrE	+	+	--	--	+	+
fdhA	+	--	+	+	+	+
fdhB	--	--	+	--	--	+

Table 6-3 Presence of genes associated with cobalamin synthesis in *Methanosaeta* genomes

Gene	Bin021	Bin225	Bin025	Bin123	<i>M. concilii</i>	<i>M. harundinacea</i>
hemB	+	+	+	+	+	+
hemC	+	+	+	+	+	+
hemD	+	+	+	+	+	+
cysG	--	+	+	+	+	+
cbiK	+	+	+	+	+	+
cbiX	--	--	--	--	--	--
cbiL	+	+	+	+	+	+
cbiH	+	+	+	+	+	+
cbiF	+	+	+	+	+	+
cbiG	+	+	+	+	+	+
cbiD	+	+	+	+	+	+
cbiJ	--	--	--	--	--	--
cbiT	+	+	+	+	+	+
cbiE	+	+	+	+	+	+
cbiC	+	+	+	+	+	+
cbiA	+	+	+	+	+	+
cbiP	+	+	+	+	+	+
cbiB	+	+	+	+	+	+
cobA(btuR)	--	--	--	--	--	--
cobU	--	--	--	--	--	--
cobS	+	+	+	+	+	+

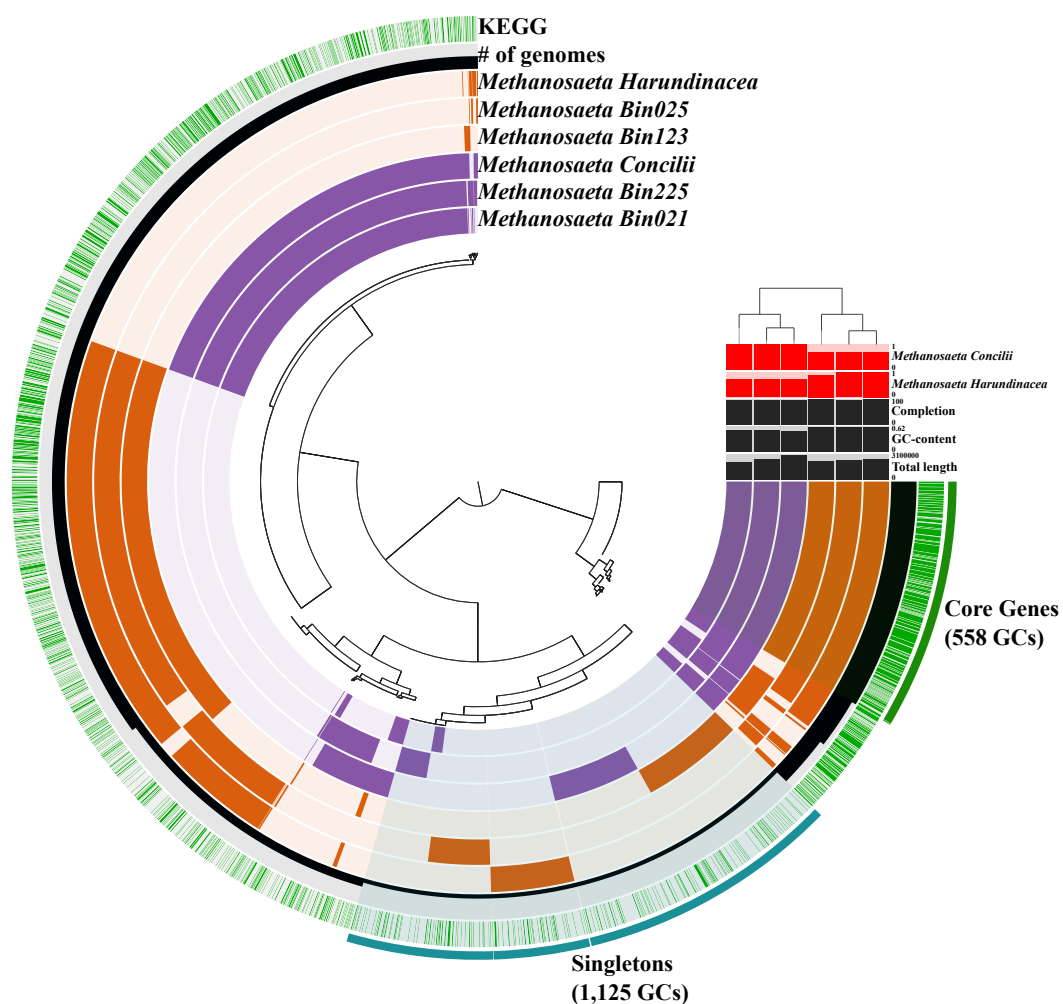


Fig. 6-1 Pangenome of *Methanosaeta* genomes generated using anvio. Each concentric circle represents a genome. Areas with low background opacity in each circle indicated the absence of a gene cluster in that genome. Red bar plots in the upper right depicted the ANI of each genome compared with the two referenced genomes.

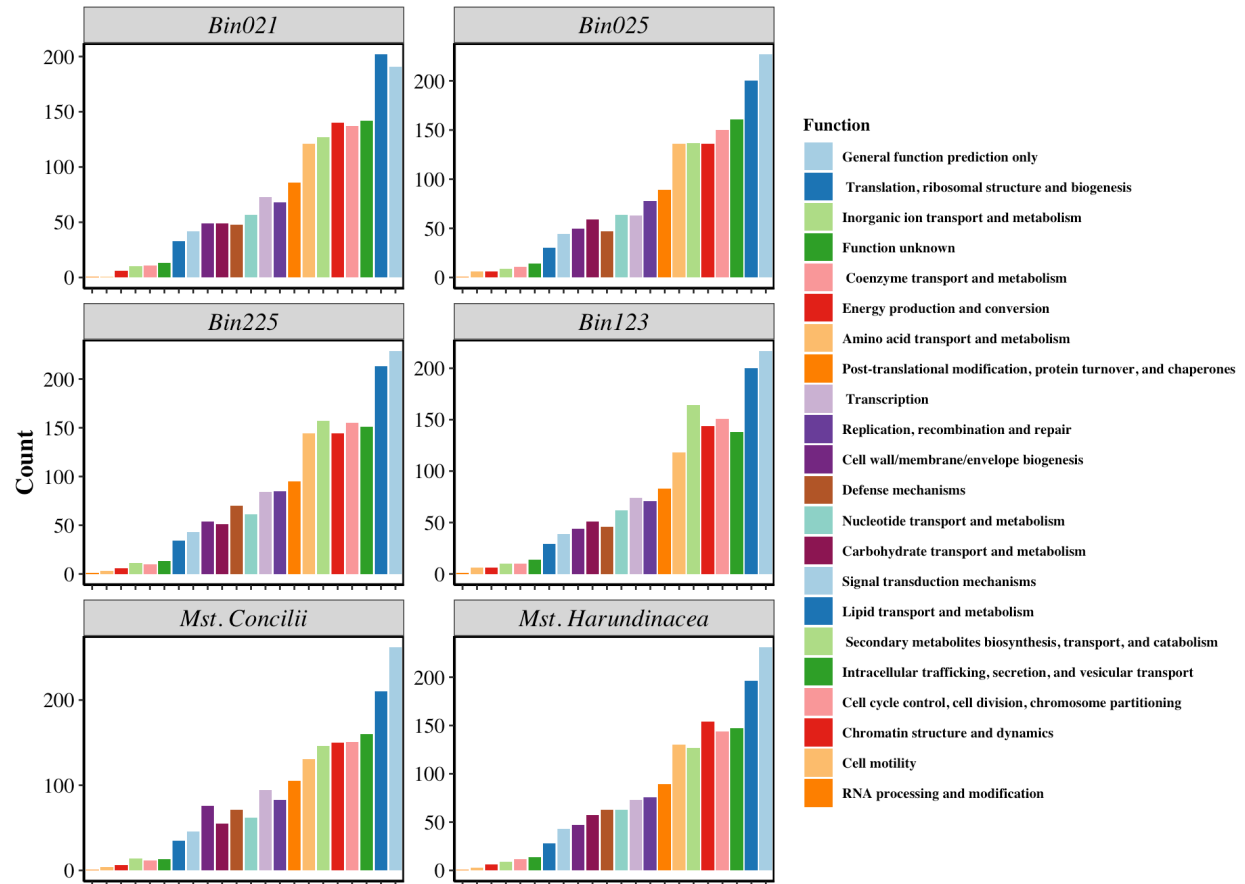


Fig. 6-2 Gene counts compared against with COG database in each functional category present in the genomes of *Methanosaeta*

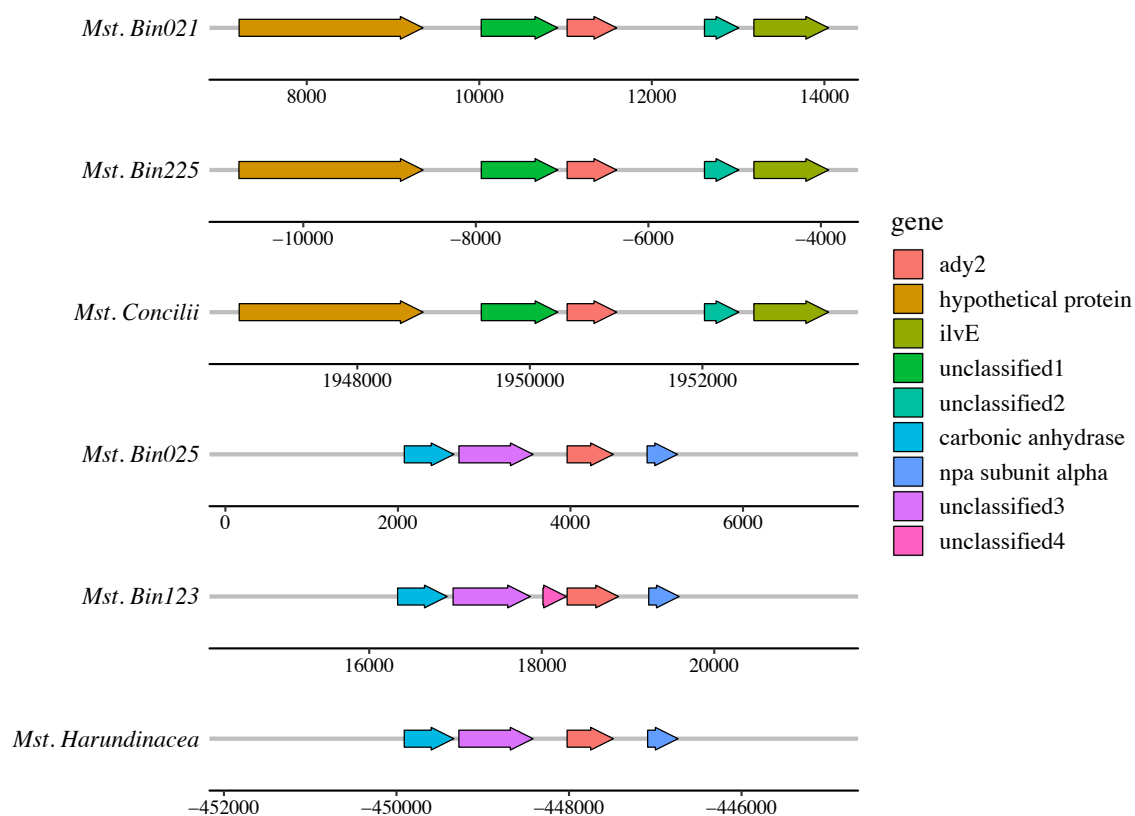


Fig. 6-3 Fragments of *Methanosaeta* genomes containing genes associated with acetate transporter Ady2 (*Mst.*: *Methanosaeta*)

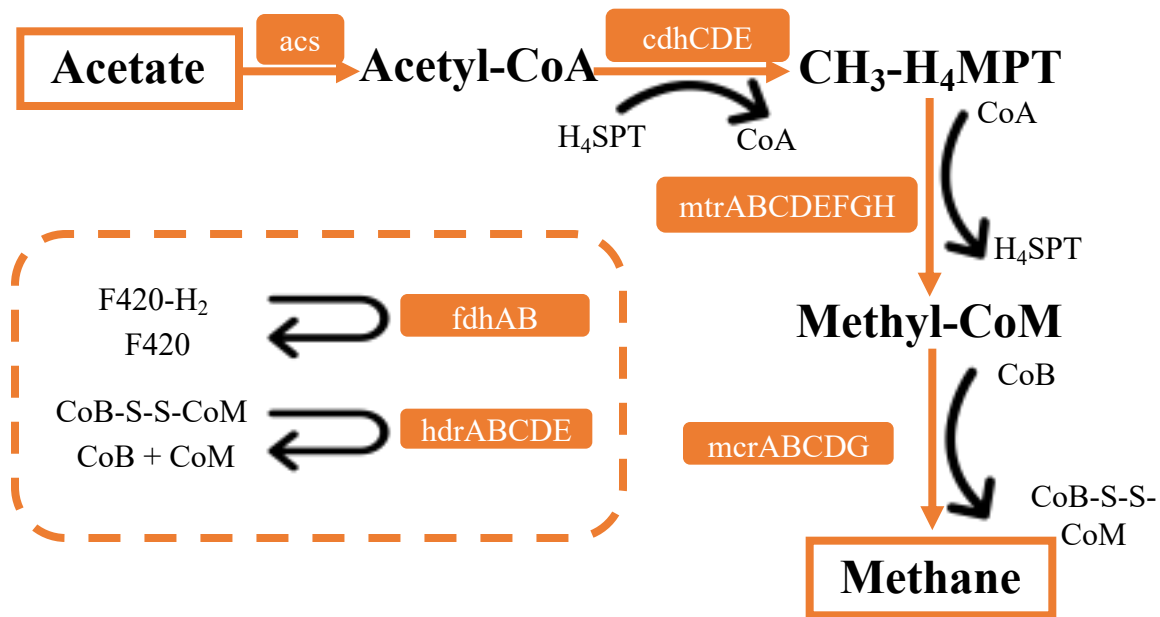


Fig. 6-4 Genes involved in acetoclastic methanogenesis in *Methanosaeta* genomes

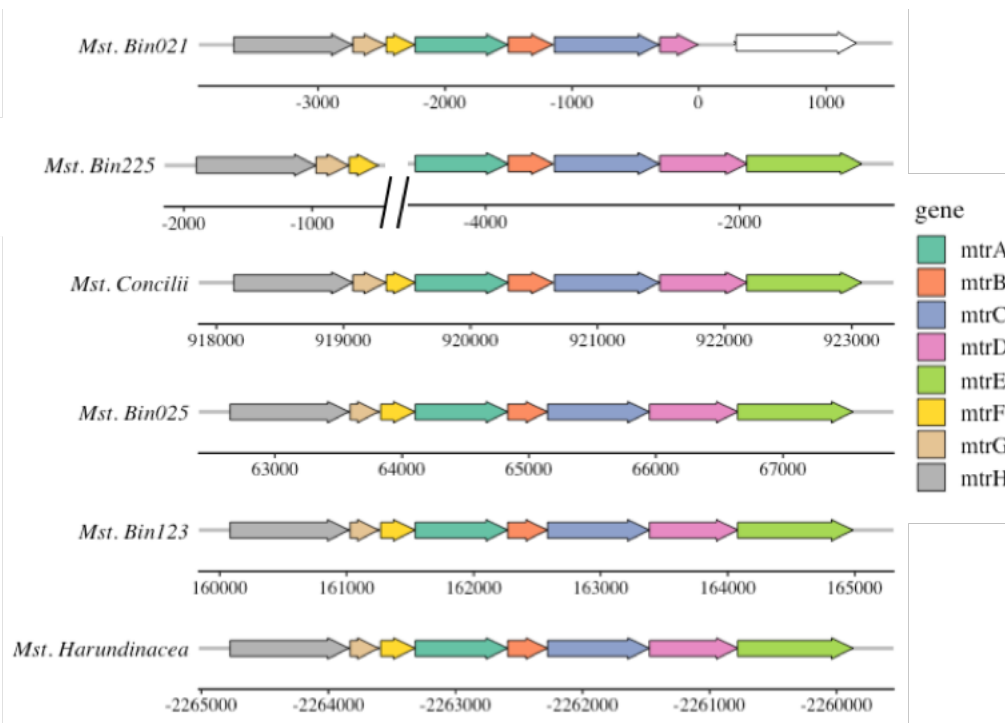


Fig. 6-5 Fragments of *Methanosaeta* genomes containing genes associated with mtr subunits, indicating the similar organizations of mtr genes. Missing of mtrE (blank arrow) in Bin021 is probably because it's at the beginning of the contig; Subunits of mtr in Bin225 are in two different contigs. (*Mst.*: *Methanosaeta*)

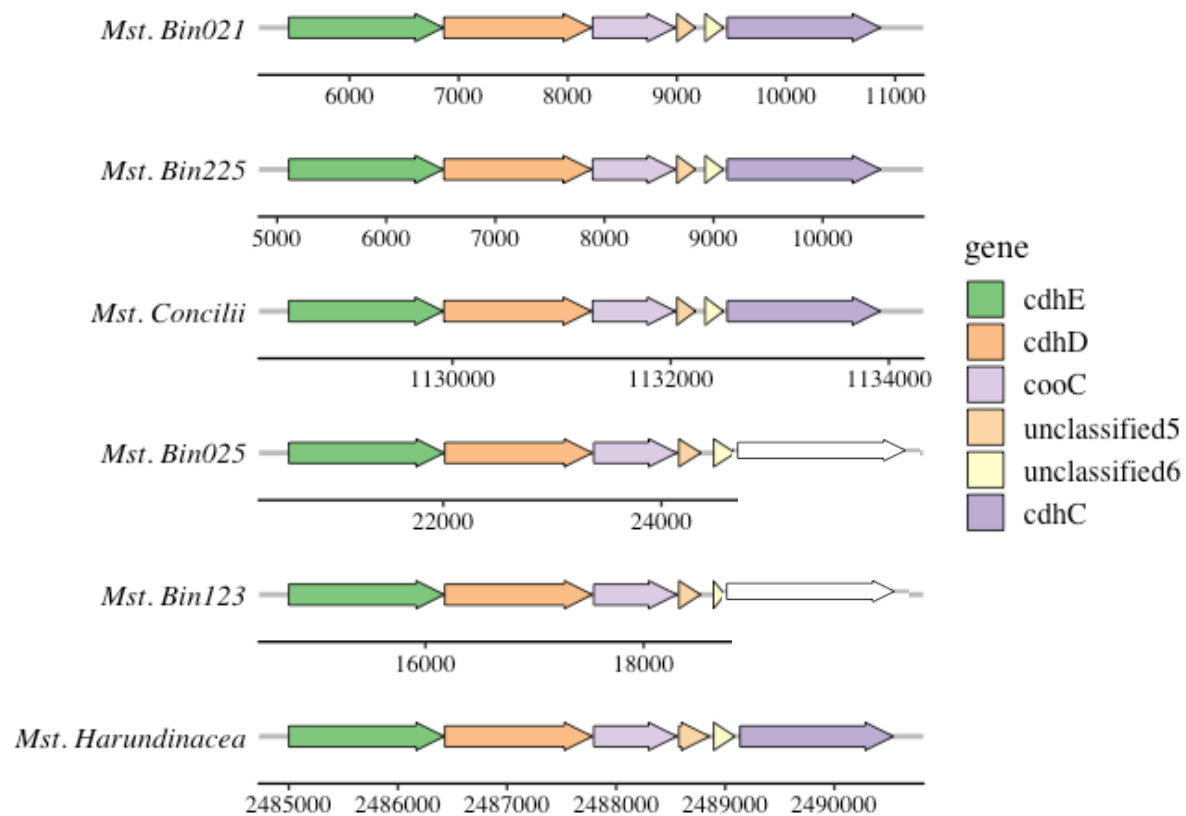


Fig. 6-6 Fragments of *Methanosaeta* genomes containing genes associated with cdh (*Mst.*: *Methanosaeta*)

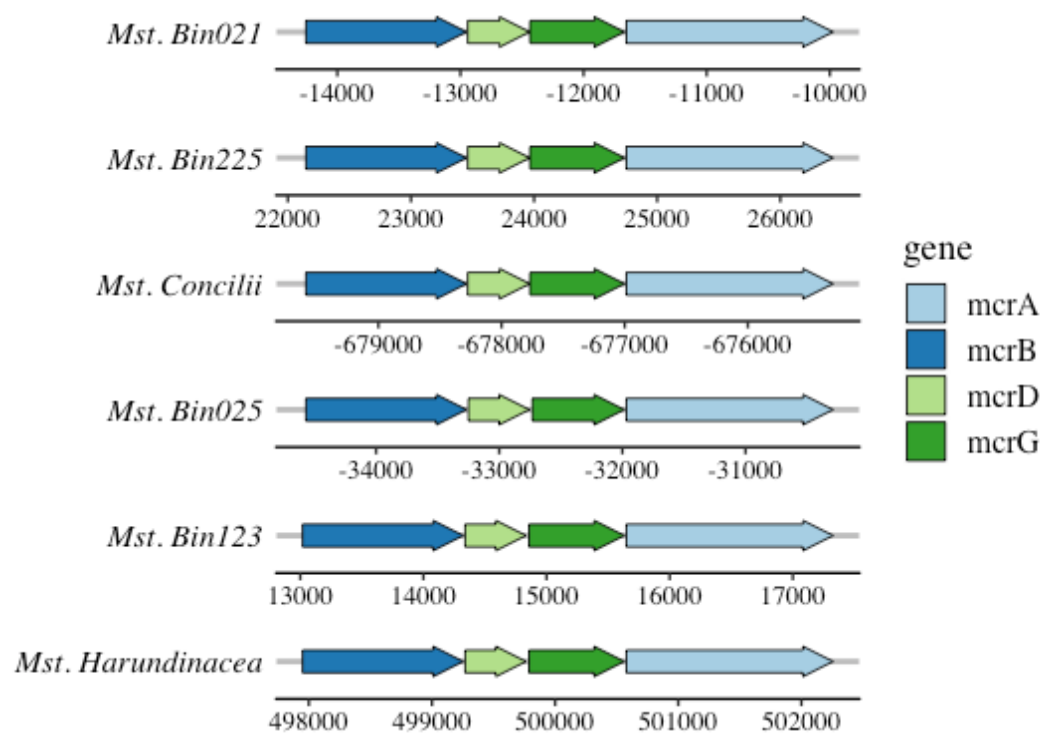


Fig. 6-7 Fragments of *Methanosaeta* genomes containing genes associated with mcr (*Mst.*: *Methanosaeta*)

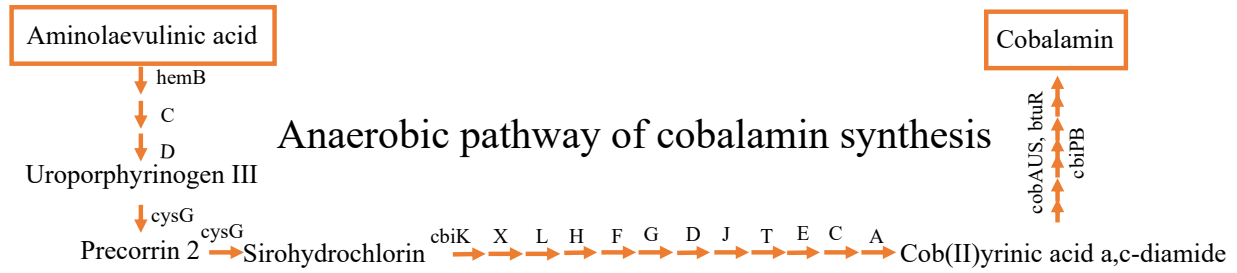


Fig. 6-8 Genes involved in anaerobic pathway of cobalamin synthesis

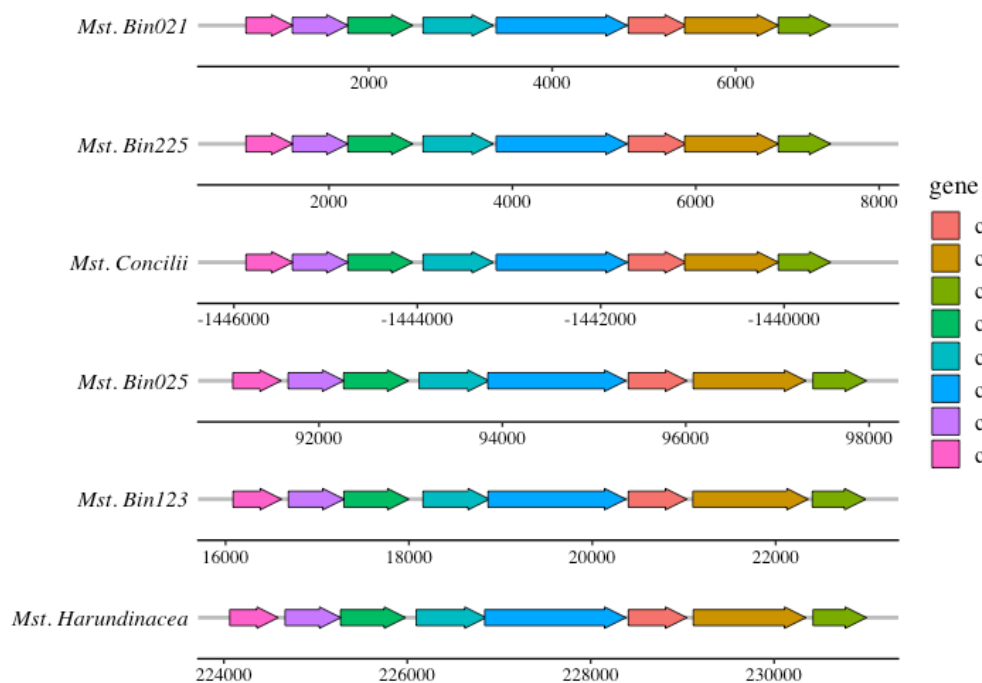


Fig. 6-9 Fragments of *Methanosaeta* genomes containing genes associated with corrin ring biosynthesis, imply there is no difference in the arrangement of genes in this operon (*Mst.*: *Methanosaeta*)

CHAPTER VII

CONCLUSIONS

In Chapter II, temporal study of microbial community in the anaerobic digestion process treated with dairy manure suggest that *Methanosaeta* was the most abundant methanogenic genus and the top abundant bacterial genera affiliated with *Firmicutes* and *Bacteroidetes* in day1 switched into genera affiliated with *Synergistetes*, *Verrucomicrobia*, *Lentisphaerae* and *Chloroflexi* in day118. Strong and significant co-occurrence and co-exclusive patterns dissected the potential bacterial redundancy and microbial interactions existed in the microbial community, which are crucial to maintain the stability of the anaerobic digestion process.

In order to characterize the impact of deterministic processes on microbial community assembly, the following three chapters (Chapter III ~ V) talk about the effects of both complex (Substrate, etc.) and simple (salts like ammonia, sodium, etc.) deterministic drivers in shaping the microbial structure in the methanogenic process. In Chapter III, influence of different substrate types on the methanogenic process was studied. For the archaeal community, *Methanosaeta* remained as the most abundant archaeal genus before and after substrate shift, and *Methanoregula* thrived after substrate shift. As for the bacteria, the top five abundant bacterial phyla before substrate shift are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Synergistetes* and *Actinobacteria*. Following the substrate shift, the top three bacterial phyla were consistent, and phyla *Chloroflexi* and *Spirochaetes* outcompeted *Synergistetes* and *Actinobacteria* and became the top five abundant phyla, suggesting these populations might be metabolically responsive to the change in substrate. To identify potentially common characteristics of deterministic drivers, ammonia stress was tested as another deterministic driver. Microbial community analysis revealed the increased contribution of deterministic process with elevated levels of ammonia stress in Chapter IV. As for the microbial responses, *Methanobacterium* and *Methanosarcina* were abundant in all reactors. Results of the bacterial compositions indicated that when ammonia was added, genus *SHA-116* was disturbed and might be replaced by genus *W22*, which was affiliated to the same phylum *WWE1*, again showing the significance of functional redundancy of bacterial populations to maintain functional stability. Moreover, genus *Treponema* from phylum *Spirochaetes* was inhibited when ammonia was added. After ammonia was relieved in the second transfer, the population rebounded implying the resilience of this group compared with other populations in this study. In addition, genus like *Porphyromonadaceae* (phylum *Bacteroidetes*) and *vadinCA02* (phylum *Synergistetes*) were also thrived as the addition of ammonia. Additionally, to testify the microbial responses to salt stress, which is a common deterministic driver, sodium stress was also dealt with in Chapter V. Microbial composition analysis suggests that *Methanosarcina* and *Methanofollis* are tolerant methanogens to elevated sodium. As for the bacteria, genus *Kosmotoga* in *Thermotogae* and genus *Aminobacterium* in phylum *Synergistetes* are relative tolerant to sodium, while genus *Treponema* from phylum *Spirochaetes* and this unclassified genus from order *Bacteroidales* are sensitive to sodium. These observations again imply the importance of deterministic factors derived from internal dynamics. In the Chapter VI, consistency of functions associated with acetate activities in the obligate methanogen-*Methanosaeta* was discussed. The results implied the wide occurrence and important roles of *Methanosaeta* in the methanogenic process.

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